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(54) Title: FUSION PROTEINS FOR OPHTHALMOLOGY WITH INCREASED EYE RETENTION

(57) Abstract: The combination of a first binding site specifically binding to a target associated with an eye disease and a second binding site specifically binding to a target influencing the retention in the eye a multispecific binder provides for improved intravitreal retention compared to a monospecific binder. The second binding site specifically binds to a compound/molecules found in the extracellular matrix (ECM) in vitreous humor/retina. This compound of the extracellular matrix has to be present in amounts allowing a sufficient loading/dose of the drug to be bound. It has been found that collagen, especially collagen II, is a suitable compound in the ECM in the vitreous humor for this purpose. Thus, herein is reported a multispecific binder comprising a first binding site specifically binding to a therapeutic ocular target, and a second binding site specifically binding to collagen II.



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### **Fusion proteins for ophthalmology with increased eye retention**

The current invention is in the field of ophthalmologic diseases and their treatment. Herein are reported fusion proteins, i.e. multifunctional binders, for intraocular/intravitreal application that are suitable for the treatment of ophthalmologic diseases. Due to their multifunctionality the fusion protein can bind to an eye retention target and a therapeutic target.

#### **Background of the Invention**

One of the factors resulting in the clearance of therapeutic molecules from the eye is diffusion. The diffusive properties of a therapeutic molecule are mainly determined by its size eventually in combination with Fc-receptor binding. After clearance from the eye the therapeutic molecule can be found in the systemic circulation.

Kleinberg, T.T. et al. (Surv. Ophthalmol. 56 (2011) 300-323) provided a review of vitreous substitutes. Permanent vitreous replacement has been attempted with collagen, hyaluronic acid, hydroxypropyl methyl cellulose, and natural hydrogel polymers. None, however, have proven to be clinically viable.

Favara, D.M. and Harris, A.L. (EMBO Mol. Med. 6 (2014) 577-579) disclosed a VEGF sticky-trap as non-systemically acting angiogenesis inhibitor with local inhibition of angiogenesis without detectable systemic side effects. The VEGF sticky-trap is a dimer of a polypeptide comprising VEGF receptor 1 domain 2, VEGF receptor 2 domain 3, a CH3 domain and heparin-binding domain (parts from exons 6, 7 and 8).

Ponsioen, T. L., et al. (Invest. Ophthal. Vis. Sci. 49 (2008) 4089-4095) disclosed the collagen distribution in the human vitreoretinal interface. Retinectomy samples expressed mRNA of all tested collagen types.

WO 2008/135734 discloses a composition comprising an antibody or fragment thereof against oxidized collagen II in which the antibody or fragment is conjugated to a pharmaceutically active moiety.

Uysal, H., et al. (Mol. Immunol. 45 (2008) 2196-2204) disclosed the crystal structure of the pathogenic collagen type II-specific monoclonal antibody CIIC1 Fab.

Nandakumar, K-S., et al. (Eur. J. Immunol. 33 (2003) 2269-2277) disclosed the induction of arthritis by single monoclonal IgG anti-collagen type II antibodies and enhancement of arthritis in mice lacking inhibitory FcγRIIb.

5 Xu, Y., et al. (Mol. Immunol. 41 (2004) 411-419) disclosed that two monoclonal antibodies to precisely the same epitope of type II collagen select non-cross-reactive phage clones by phage display.

WO 2012/047583 discloses antibodies binding human collagen II.

### **Summary of the Invention**

The current invention is directed to anti-human collagen II antibodies.

10 Disclosed herein is an anti-human collagen II antibody comprising the six CDRs determined according to Kabat of / as in

- a) SEQ ID NO: 09 and SEQ ID NO: 10, or
- b) SEQ ID NO: 12 and SEQ ID NO: 13, or
- c) SEQ ID NO: 15 and SEQ ID NO: 16.

15 In one embodiment the antibody comprises a heavy chain variable domain and a light chain variable domain of

- a) SEQ ID NO: 09 and SEQ ID NO: 10, or
- b) SEQ ID NO: 12 and SEQ ID NO: 13, or
- c) SEQ ID NO: 15 and SEQ ID NO: 16.

20 In one embodiment the antibody is a scFv.

Herein is disclosed as an aspect an antibody binding to the same epitope as an antibody comprising a heavy chain variable domain and a light chain variable domain of

- a) SEQ ID NO: 09 and SEQ ID NO: 10, or
- 25 b) SEQ ID NO: 12 and SEQ ID NO: 13, or
- c) SEQ ID NO: 15 and SEQ ID NO: 16.

Herein is disclosed as an aspect a pharmaceutical formulation comprising the antibody as disclosed herein and optionally a pharmaceutically acceptable excipient.

5 In one embodiment the pharmaceutical formulation is for use in the treatment of ocular vascular diseases.

Herein is disclosed as an aspect the antibody as disclosed herein for use as a medicament.

In one embodiment the use is for the treatment of ocular vascular diseases.

10 Herein disclosed as an aspect is the use of the antibody as disclosed herein in the manufacture of a medicament.

In one embodiment the use is for the manufacture of a medicament for the treatment of ocular vascular disease.

Herein is disclosed as an aspect the antibody as disclosed herein for use in the treatment of ocular vascular disease.

15 Herein is disclosed as an aspect a method of treatment of a patient suffering from an ocular vascular disease by administering an antibody as disclosed herein to a patient in the need of such treatment.

The current invention reports fusion proteins having at least two binding sites, whereof one specifically binds to collagen II.

20 It has been found that by combining a first binding site that specifically binds to a target associated with an eye disease and a second binding site that specifically binds to a target influencing the retention in the eye (eye retention target) a multispecific binder can be provided with improved intravitreal retention compared to a molecule not having the binding specificity to the eye retention target. The  
25 second binding site specifically binds to a compound or molecule found in the extracellular matrix (ECM) in vitreous humor or the retina. This compound of the extracellular matrix has to be present in amounts allowing a sufficient loading and thereby dosing of the multispecific binder. It has been found that collagen, especially collagen II, is a suitable compound in the ECM in the vitreous humor for  
30 this purpose.

Such a multispecific binder can be produced recombinantly as (recombinant) fusion protein.

Thus, disclosed herein as an aspect is a fusion protein comprising

- a first binding site specifically binding to a first antigen, and
- 5       - a second binding site specifically binding to a compound present in the extracellular matrix of the vitreous humor.

In one embodiment the compound present in the extracellular matrix of the vitreous humor is a collagen. In one embodiment the collagen is collagen II.

In one embodiment the first antigen is related to an ocular vascular disease.

10       Also disclosed herein as an aspect is a fusion protein comprising

- a first binding site specifically binding to a first antigen,
- and
- a second binding site specifically binding to collagen II.

In one embodiment the fusion protein comprises

- 15       - a first binding site specifically binding to a first antigen,
- a second binding site specifically binding to collagen II, and
- a third binding site specifically binding to a second antigen.

In one embodiment of all aspects as disclosed herein collagen II is human collagen II. In one embodiment human collagen II has the amino acid sequence of SEQ ID  
20       NO: 17 or 18 or 19.

In one embodiment of all aspects as disclosed herein each of the binding sites is selected independently of each other from the group consisting of antibody binding sites, antibody fragments, anticalin, DARPIN, receptor ligand or binding fragment thereof, receptor or binding fragment thereof, and tetranectin domain.

25       In one embodiment of all aspects as disclosed herein each of the binding sites is independently of each other an antibody binding site or an antibody fragment. In

one embodiment each of the binding sites is a pair of an antibody heavy chain variable domain and an antibody light chain variable domain.

5 In one embodiment of all aspects as disclosed herein the first binding site is comprised in a first polypeptide and the second binding site is comprised in a second polypeptide, wherein the first polypeptide is conjugated or fused to the second polypeptide either directly or via a peptidic linker or via a disulfide bond.

10 In one embodiment of all aspects as disclosed herein the first binding site is comprised in a first polypeptide, the second binding site is comprised in a second polypeptide and the third binding site is comprised in a third polypeptide, wherein the first polypeptide and the third polypeptide form an antibody or antibody fragment and the antibody or antibody fragment is conjugated to the second polypeptide either directly or via a peptidic linker or a disulfide bond.

15 In one embodiment of all aspects as disclosed herein the first polypeptide, the second polypeptide and the third polypeptide are selected independently of each other from the group consisting of scFv, dsscFv, Fab, dsFab, CrossFab, monobody, and VHH (sc=single chain, ds=disulfide-stabilized). In one embodiment one of the polypeptides is a Fab or a dsFab and the other polypeptide is a scFv or dsscFv and the polypeptides are conjugated via a peptidic linker. In one embodiment two of the polypeptides are Fabs or dsFabs and the other polypeptide is a scFv or dsscFv and  
20 the polypeptides are conjugated via a peptidic linker.

In one embodiment of all aspects as disclosed herein the fusion protein comprises

- as first binding site a Fab specifically binding to a first antigen,
- as second binding site a scFv specifically binding to collagen II, and
- a peptidic linker,

25 wherein the Fab is conjugated by a peptide bond at one of its C-termini to the N-terminus of the peptidic linker and the scFv is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.

In one embodiment of all aspects as disclosed herein the fusion protein comprises

- a first binding site specifically binding to a first antigen,
- 30 - as second binding site a scFv specifically binding to collagen II,

- 6 -

- a third binding site specifically binding to a second antigen, and
- a peptidic linker,

5 wherein the combined first and third binding sites are conjugated by a peptide bond at their C-terminus to the N-terminus of the peptidic linker and the scFv is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.

In one embodiment of all aspects as disclosed herein the combined first and third binding sites are within/are at least a F(ab')<sub>2</sub> or a diabody or a BITE or a tandAb or a DART.

10 In one embodiment of all aspects as disclosed herein the first antigen and/or the second antigen is a therapeutic ocular target / is related to an ocular vascular disease.

15 In one embodiment of all aspects as disclosed herein the first antigen and/or the second antigen are independently of each other selected from the group consisting of ANG2, VEGF, PDGF-B, and IL-1beta.

In one embodiment of all aspects as disclosed herein the first antigen and/or the second antigen are different antigens selected from the group consisting of ANG2, VEGF, PDGF-B, and IL-1beta.

In one embodiment the scFv specifically binding to collagen II comprises

- 20
- a) a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 09 and a light chain variable domain of SEQ ID NO: 10, or
  - b) a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain of SEQ ID NO: 13, or
  - c) a heavy chain variable domain with the amino acid sequence of SEQ ID
- 25
- NO: 15 and a light chain variable domain of SEQ ID NO: 16.

In one embodiment of all aspects as disclosed herein the scFv specifically binding to collagen II comprises a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain of SEQ ID NO: 13.

In one embodiment of all aspects as disclosed herein the scFv specifically binding to collagen II has the amino acid sequence of SEQ ID NO: 11 or SEQ ID NO: 14 or SEQ ID NO: 17.

5 In one embodiment of all aspects as disclosed herein the scFv specifically binding to collagen II has the amino acid sequence of SEQ ID NO: 14.

In one embodiment of all aspects as disclosed herein the first binding site and the third binding site are Fabs.

In one embodiment of all aspects as disclosed herein the fusion protein comprises

- a Fab specifically binding to ANG2, VEGF, PDGF-B, or IL-1beta,
- 10 - a scFv specifically binding to collagen II comprising a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain of SEQ ID NO: 13, and
- a peptidic linker,

15 whereby the Fab is conjugated by a peptide bond at one of its C-termini to the N-terminus of the peptidic linker and the scFv is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.

In one embodiment of all aspects as disclosed herein the fusion protein as a molecular weight of less than 75 kDa.

20 In one embodiment of all aspects as disclosed herein the fusion protein is devoid of an antibody Fc-region.

Herein is disclosed as an aspect a pharmaceutical formulation comprising the fusion protein as disclosed herein and optionally a pharmaceutically acceptable excipient.

25 In one embodiment the pharmaceutical formulation is for use in the treatment of ocular vascular diseases.

Herein is disclosed as an aspect the fusion protein as disclosed herein for use as a medicament.

In one embodiment the use is for the treatment of ocular vascular diseases.



Herein disclosed as an aspect is the use of the fusion protein as disclosed herein in the manufacture of a medicament.

In one embodiment the use is for the manufacture of a medicament for the treatment of ocular vascular disease.

5 Herein is disclosed as an aspect the fusion protein as disclosed herein for use in the treatment of ocular vascular disease.

Herein is disclosed as an aspect a method of treatment of a patient suffering from ocular vascular diseases by administering the fusion protein as disclosed herein to a patient in the need of such treatment.

10 **Detailed Description of the Invention**

General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991).

15 As used herein, the amino acid positions of all constant regions and domains of the heavy and light chain are numbered according to the Kabat numbering system described in Kabat, et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) and is referred to as “numbering according to Kabat” herein. Specifically, the Kabat  
20 numbering system (see pages 647-660) of Kabat, et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) is used for the light chain constant domain CL of kappa and lambda isotype, and the Kabat EU index numbering system (see pages  
25 661-723) is used for the constant heavy chain domains (CH1, Hinge, CH2 and CH3, which is herein further clarified by referring to “numbering according to Kabat EU index” in this case).

Useful methods and techniques for carrying out the current invention are described in e.g. Ausubel, F.M. (ed.), Current Protocols in Molecular Biology, Volumes I to  
30 III (1997); Glover, N.D., and Hames, B.D., ed., DNA Cloning: A Practical Approach, Volumes I and II (1985), Oxford University Press; Freshney, R.I. (ed.), Animal Cell Culture – a practical approach, IRL Press Limited (1986); Watson, J.D., et al., Recombinant DNA, Second Edition, CHSL Press (1992); Winnacker,

E.L., From Genes to Clones; N.Y., VCH Publishers (1987); Celis, J., ed., Cell Biology, Second Edition, Academic Press (1998); Freshney, R.I., Culture of Animal Cells: A Manual of Basic Technique, second edition, Alan R. Liss, Inc., N.Y. (1987).

5 The use of recombinant DNA technology enables the generation of derivatives of a nucleic acid. Such derivatives can, for example, be modified in individual or several nucleotide positions by substitution, alteration, exchange, deletion or insertion. The modification or derivatization can, for example, be carried out by means of site directed mutagenesis. Such modifications can easily be carried out by  
10 a person skilled in the art (see e.g. Sambrook, J., et al., Molecular Cloning: A laboratory manual (1999) Cold Spring Harbor Laboratory Press, New York, USA; Hames, B.D., and Higgins, S.G., Nucleic acid hybridization – a practical approach (1985) IRL Press, Oxford, England).

#### I. DEFINITIONS

15 It is herewith expressly stated that the term “comprising” as used herein comprises the term “consisting of”. Thus, all aspects and embodiments that contain the term “comprising” are likewise disclosed with the term “consisting of”.

The term “about” denotes a range of +/- 20 % of the thereafter following numerical value. In one embodiment the term about denotes a range of +/- 10 % of the  
20 thereafter following numerical value. In one embodiment the term about denotes a range of +/- 5 % of the thereafter following numerical value.

The term "(intact) antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies.

25 The term "(intact) antibody" refers to immunoglobulin molecules with varying structures. Intact IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly,  
30 from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types,

called kappa ( $\kappa$ ) and lambda ( $\lambda$ ), based on the amino acid sequence of its constant domain.

5 The term "antibody fragment" denotes a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')<sub>2</sub>; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

10 The terms "antibody binding site" denotes the amino acid residues of an antibody that are responsible for antigen binding. Generally this is a pair of an antibody heavy chain variable domain and light chain variable domain. The antigen-binding site of an antibody comprises amino acid residues from the "hypervariable regions" or "HVRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chain variable domains of an antibody comprise from N- to C-terminus the regions FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4 (immunoglobulin framework). Especially, the CDR3 region of the heavy chain is the region, which contributes most to antigen binding and defines the antibody.

20 The term "binding (to an antigen)" denotes the binding of an antibody to its antigen in an in vitro assay, in one embodiment in a binding assay in which the antibody is bound to a surface and binding of the antigen to the antibody is measured by Surface Plasmon Resonance (SPR). Binding means a binding affinity ( $K_D$ ) of about  $10^{-7}$  M or less, in some embodiments of  $10^{-13}$  to  $10^{-8}$  M.

25 Binding can be investigated by a BIAcore assay (GE Healthcare Biosensor AB, Uppsala, Sweden). The affinity of the binding is defined by the terms  $k_a$  (rate constant for the association of the antibody from the antibody/antigen complex),  $k_d$  (dissociation constant), and  $K_D(k_d/k_a)$ .

The term „binding site“ denotes any proteinaceous entity that shows binding specificity to a target.

30 The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgA<sub>1</sub>, and IgA<sub>2</sub>. The heavy chain constant

domains that correspond to the different classes of immunoglobulins are called  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$ , respectively.

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

The terms "host cell", "host cell line", and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

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

The term "hypervariable region" or "HVR", as used herein, refers to each of the regions of an antibody variable domain which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and form structurally defined loops ("hypervariable loops"), and/or contain the antigen-contacting residues ("antigen contacts"). Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs as denoted herein include

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

5 (b) CDRs occurring at amino acid residues 24-34 (L1), 50-56 (L2), 89-97 (L3), 31-35b (H1), 50-65 (H2), and 95-102 (H3) (Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, 5th ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), NIH Publication 91-3242.);

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

15 (d) combinations of (a), (b), and/or (c), including HVR amino acid residues 46-56 (L2), 47-56 (L2), 48-56 (L2), 49-56 (L2), 26-35 (H1), 26-35b (H1), 49-65 (H2), 93-102 (H3), and 94-102 (H3).

20 In one embodiment, HVR residues comprise those residues identified elsewhere in the specification as being CDR residues.

Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to the Kabat EU index numbering system (Kabat et al., *supra*).

25 A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. In certain embodiments, a human antibody is derived from a non-human transgenic mammal, for example a mouse, a rat, or a rabbit. In certain embodiments, a human antibody is derived from a hybridoma cell line. In certain embodiments, a human antibody is derived from a (phage) display library. In certain embodiments, a human antibody is derived from a human B-cell.

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

An "isolated" antibody is one that has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than

95 % or 99 % purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., size-exclusion chromatography or ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman, S. et al., J. Chrom. B 848 (2007) 79-87.

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An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

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

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The term "ocular vascular disease" includes, but is not limited to intraocular neovascular syndromes such as diabetic retinopathy, diabetic macular edema, retinopathy of prematurity, neovascular glaucoma, retinal vein occlusions, central retinal vein occlusions, macular degeneration, age-related macular degeneration, retinitis pigmentosa, retinal angiomatous proliferation, macular telangiectasia, ischemic retinopathy, iris neovascularization, intraocular neovascularization, corneal neovascularization, retinal neovascularization, choroidal neovascularization, and retinal degeneration (see e.g. Garner, A., Vascular diseases,

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In: Pathobiology of ocular disease, A dynamic approach, Garner, A., and Klintworth, G.K., (eds.), 2nd edition, Marcel Dekker, New York (1994), pp. 1625-1710).

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

10 A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

15 The term "peptidic linker" as used herein denotes a peptide with amino acid sequences, which is in one embodiment of synthetic origin. A "peptidic linker" represents a linear chain of amino acid residues. This linear chain of amino acid residues has a length of 1 to 30 residues.

20 In one embodiment the peptidic linker is rich in glycine, glutamine, and/or serine residues. In one embodiment, these residues are arranged e.g. in small repetitive units of up to five amino acids, such as GS (SEQ ID NO: 21), GGS (SEQ ID NO: 22), GGGs (SEQ ID NO: 23), and GGGGS (SEQ ID NO: 24). The small repetitive unit may be repeated for one to five times. At the amino- and/or carboxyl-terminal ends of the multimeric unit up to six additional arbitrary, naturally occurring amino acids may be added.

25 The peptidic linker is in one embodiment a peptide with an amino acid sequence with a length of up to 30 amino acid residues, in one embodiment with a length of 5 to 20 amino acid residues. In one embodiment the peptidic linker is  $(GxS)_n$  with  $G = \text{glycine}$ ,  $S = \text{serine}$ ,  $(x = 3, n = 2, 3, 4 \text{ or } 5)$  or  $(x = 4 \text{ and } n = 2, 3, \text{ or } 4)$ , in one embodiment with  $x = 3, n = 2$ , in one embodiment with  $x = 4, n = 2$ . This peptidic linker may nevertheless comprise additional glycine and/or serine residues at one or both of its termini.

30 Other synthetic peptidic linkers are composed of a single amino acid, which is repeated between 10 to 20 times and may comprise at the amino- and/or carboxyl-terminal end up to six additional arbitrary, naturally occurring amino acids.

Besides synthetic GS-rich peptidic linkers also naturally occurring peptidic linker such as IgG hinge, linker of human P-glycoprotein, C-terminal linker of human replicatin protein A, linker of the parathyroid hormone-related protein, can be used.

5 All peptidic linkers can be encoded by a nucleic acid molecule and therefore can be recombinantly expressed. As the linkers are themselves peptides, the polypeptide connected by the linker are connected to the linker via a peptide bond that is formed between two amino acids.

10 The term "recombinant" or „recombinantly produced“ denotes polypeptides that are prepared, expressed, created or isolated by recombinant means. This includes polypeptides isolated from a host cell such as a NS0 or CHO cell or from an animal (e.g. a mouse) that is transgenic or polypeptides expressed using a recombinant expression vector transfected into a host cell.

15 As used herein, “treatment” (and grammatical variations thereof such as “treat” or “treating”) refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or  
20 palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies or Fc-region fusion polypeptides as reported herein are used to delay development of a disease or to slow the progression of a disease.

25 The term “valent” as used within the current application denotes the presence of a specified number of binding sites in a (antibody) molecule. As such, the terms “bivalent”, “tetravalent”, and “hexavalent” denote the presence of two binding site, four binding sites, and six binding sites, respectively, in a (antibody) molecule. The bispecific antibodies as reported herein are in one preferred embodiment “bivalent”.

30 The term “variable region” or “variable domain” refer to the domain of an antibody heavy or light chain that is involved in binding of the antibody to its antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of an antibody generally have similar structures, with each domain comprising four framework regions (FRs) and three hypervariable regions (HVRs) (see, e.g., Kindt, T.J. et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., N.Y. (2007), page



91). A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano, S. et al., *J. Immunol.* 150 (1993) 880-887; Clackson, T. et al., *Nature* 352 (1991) 624-628).

The term "vector", as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors".

The term "therapeutic ocular target" denotes a molecule involved in an ocular vascular disease.

The term "diabody" denotes a non-covalent dimer of single chain Fv (scFv) fragment that consists of the heavy chain variable (VH) and light chain variable (VL) regions connected by a small peptide linker. Common linkers in scFvs have 14–15 amino acid residues and are between the N- and C-termini of the variable domains. However, using linkers of 3–12 amino acid residues in length will result in the formation of a diabody.

The term "Tandem scFv (taFv)" denotes a molecule wherein two scFv molecules are conjugated through a short linker.

The term "miniantibody or minibody" denotes a bivalent (or bispecific) (scFv)<sub>2</sub> produced by association of two scFv molecules through two modified dimerization domains.

The term "tandAb" denotes a tetravalent, bispecific antibody format that consists of two binding sites for each antigen. It consists only of variable immunoglobulin-domains, that are connected by linker.

The term "BiTE" denotes a bi-specific T-cell engager (BiTEs). These are a class of artificial bispecific monoclonal antibodies that direct a host's immune system, more specifically the T-cells' cytotoxic activity, against cancer cells. BiTEs are fusion proteins consisting of two single-chain variable fragments (scFvs) of different

antibodies, or amino acid sequences from four different genes, on a single peptide chain of about 55 kilodaltons. One of the scFvs binds to T-cells via the CD3 receptor, and the other to a tumor cell via a tumor specific molecule.

5 The term “DART” denotes a molecule consisting of two engineered Fv fragments which have their own VH exchanged with the other one. In detail, the Fv1 comprises a VH from antibody A and a VL from antibody B, while the Fv2 comprises a VH from Ab-B and VL from Ab-A. This inter-exchange of Fv domains releases variant fragments from the conformational constraint by the short linking peptide.

10 “Collagen” is the main structural protein in the extracellular space in the various connective tissues in animal bodies. As the main component of connective tissue, it is the most abundant protein in mammals making up from 25% to 35% of the whole-body protein content. Depending upon the degree of mineralization, collagen tissues may be rigid (bone), compliant (tendon), or have a gradient from  
15 rigid to compliant (cartilage). Collagen, in the form of elongated fibrils, is mostly found in fibrous tissues such as tendons, ligaments and skin. It is also abundant in corneas, cartilage, bones, blood vessels, the gut, intervertebral discs and the dentin in teeth. In muscle tissue, it serves as a major component of the endomysium. Collagen constitutes one to two percent of muscle tissue, and accounts for 6% of  
20 the weight of strong, tendinous muscles. The fibroblast is the most common cell that creates collagen.

“Type II collagen” is the basis for articular cartilage and hyaline cartilage. It makes up 50% of all protein in cartilage and 85-90% of collagen of articular cartilage. Type II collagen does form fibrils. This fibrillar network of collagen allows  
25 cartilage to entrap the proteoglycan aggregate as well as provide tensile strength to the tissue. Type II collagen is found in cartilage and the vitreous humor of the eye.

## II. THE VITREOUS HUMOR/BODY

The matrix that fills most space in the eye is denoted as vitreous humor/body.

30 The human vitreous humor is a clear aqueous solution, which fills the posterior compartment of the eye, located between the lens and the retina. It occupies about 80% of the volume of the eyeball and comprises 99% water but has a gel-like structure at birth due to a network of collagen fibrils and large molecules of hyaluronic acid. Its volume is about 4-5 ml (Beauthier, J.P., (2008) In: De Boeck

Université [Ed]. *Traité de médecine légale*. Bruxelles: 715–725). Vitreous humor contains several low molecular weight solutes including inorganic salts, sugars and ascorbic acid. The total concentration of protein in human vitreous is approximately 1200 µg/ml, of which collagen accounts for 180 µg/ml (see e.g. Aretz, S., et al., *Prot. Sci.* 11 (2013) 22; Theocharis, A.D., et al., *Biochim.* 84 (2002) 1237–1243). An average protein concentration of the healthy vitreous humor of 0.5 mg/mL, consisting largely of albumin (60–70%) is reported by Angi, M., et al. (Hindawi Publishing Corporation, *Mediators of Inflammation*, Volume 2012, Article ID 148039). Further it is reported therein that components of the vitreous humor are globulins, coagulation proteins, complement factors, and low-molecular-weight proteins (Ulrich, J.N., et al., *Clin. Exp. Ophthalmol.* 36 (2008) 431–436). The ciliary body provides a constant fluid exchange by diffusion, ultrafiltration, and active transport of aqueous fluid into the posterior segment (Bishop, P.N., *Eye*, 16 (2002) 454–460). Proteins may accumulate in the vitreous by local secretion (e.g., glycoprotein), filtration from blood (e.g., albumin), or diffusion from the surrounding tissues (Wu, C.W., *Am. J. Ophthalmol.*, 137 (2004) 655–661). Because of the close contact between the vitreous and the inner retina, physiological and pathological conditions of the retina affect both the proteome and the biochemical properties of the vitreous humor.

### III. MULTIFUNCTIONAL BINDERS FOR OPHTHALMOLOGY WITH INCREASED EYE RETENTION

It has been found that by the combination of a first binding site specifically binding to a target associated with an eye disease and a second binding site specifically binding to a target influencing the retention in the eye a multispecific binder can be provided with improved intravitreal retention compared to a monospecific binder. The second binding site specifically binds to a compound/molecules found in the extracellular matrix (ECM) in vitreous humor/retina. This compound of the extracellular matrix has to be present in amounts allowing a sufficient loading/dose of the drug to be bound. It has been found that collagen, especially collagen II, is a suitable compound in the ECM in the vitreous humor for this purpose.

With a long intravitreal half-life less frequent injections are required, with a short half-life in the systemic circulation a low system exposure can be effected, and with the combination of both an increased efficacy and reduced side effects are expected.

A long intravitreal half-life can be achieved by

- high molecular weight (IgGs, addition of e.g. PEG to smaller formats such as diabodies, Fabs etc.),
- 5 - high affinity and avidity to retention target (lower efficient drug concentration results in less frequent dosing),
- high thermal stability at 37°C,
- decreasing diffusion of the molecule across vitreous humor and blood retina barrier (BRB),
- optimal charge or pI.

10 Rapid systemic clearance can be achieved by

- engineering the Fc-region for reduced FcRn binding,
- low(er) molecular weight (Fab, Diabody, DARPINs),
- low administered doses (dose also depends on affinity).

15 The aim of the current invention is to provide a long lasting drug for application into the eye. This reduces the number of application required and likewise the time between the single applications. This can be achieved on the one hand by increasing the does administered at each application or on the other hand by increasing the half-life and durability of the drug in the eye after application.

20 The invention relates in general to a multispecific binder (i.e. a recombinant fusion protein) comprising

- a first binding site specifically binding to a therapeutic ocular target,
- and
- a second binding site specifically binding to collagen II.

25 In one embodiment each of the binding sites is selected independently of each other from the group consisting of an antibody binding site, an anticalin, a DARPIN, a receptor ligand or binding fragment thereof, a receptor or binding fragment thereof, a tetranectin domain.

In one embodiment each of the binding sites is an antibody binding site. In one embodiment each of the binding sites is a (cognate) pair of an antibody heavy chain variable domain and an antibody light chain variable domain.

5 In one embodiment the first binding site is comprised in a first domain and the second binding site is comprised in a second domain and the first domain is conjugated to the second domain either directly or via a peptidic linker. In one embodiment the first domain and the second domain are selected independently of each other from the group consisting of scFv, dsscFv, Fab, dsFab, CrossFab, monobody, and VHH (sc=single chain, ds=disulfide-stabilized). In one  
10 embodiment one of the domains is a Fab or a dsFab and the other domain is a scFv or dsscFv and the domains are conjugated via a peptidic linker.

In one embodiment the multispecific binder is selected from the group consisting of tandem-Fv, diabody, single-chain diabody, disulfide-stabilized diabody, DART, scFv<sub>2</sub>, Fab-scFv, minibody.

15 Herein is disclosed a multispecific binder (i.e. a recombinant fusion protein) comprising

- a Fab or scFv comprising a first binding site specifically binding to a therapeutic ocular target,
- a scFv specifically binding to collagen II, and
- 20 - a peptidic linker,

whereby the Fab or the scFv comprising the first binding site is conjugated by a peptide bond at one of its C-termini to the N-terminus of the peptidic linker and the scFv specifically binding to collagene II is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.

25 In one embodiment the therapeutic ocular target is selected from the group consisting of ANG2, VEGF, PDGF-B, IL-1beta.

In one embodiment the multispecific binder is a bispecific binder comprising

- a Fab specifically binding to ANG2, VEGF, PDGF-B, or IL-1beta,
- a scFv specifically binding to collagen II, and

- a peptidic linker,

whereby the Fab is conjugated by a peptide bond at one of its C-termini to the N-terminus of the peptidic linker and the scFv is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.

5 In one embodiment the multispecific binder is a trispecific binder comprising

- a first binding site specifically binding to ANG2, VEGF, PDGF-B, or IL-1beta,
- a second binding site specifically binding to ANG2, VEGF, PDGF-B, or IL-1beta,

10 - a scFv specifically binding to collagen II, and

- a peptidic linker,

whereby the combined first and second binding site are conjugated by a peptide bond at their C-terminus to the N-terminus of the peptidic linker and the scFv is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.

15

In one embodiment the scFv specifically binding to collagen II comprises

- a) a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 09 and a light chain variable domain of SEQ ID NO: 10, or
- b) a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain of SEQ ID NO: 13, or
- c) a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 15 and a light chain variable domain of SEQ ID NO: 16.

20

In one embodiment the scFv specifically binding to collagen II comprises a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain of SEQ ID NO: 13.

25

In one embodiment the scFv specifically binding to collagen II has the amino acid sequence of SEQ ID NO: 11 or SEQ ID NO: 14 or SEQ ID NO: 17.

In one embodiment the scFv specifically binding to collagen II has the amino acid sequence of SEQ ID NO: 14.

In one embodiment the multispecific binder is a bispecific binder comprising

- a Fab specifically binding to ANG2, VEGF, PDGF-B, or IL-1beta,
- 5 - a scFv specifically binding to collagen II comprising a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain of SEQ ID NO: 13, and
- a peptidic linker,

10 whereby the Fab is conjugated by a peptide bond at one of its C-termini to the N-terminus of the peptidic linker and the scFv is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.

The intravitreal half-life and durability of a drug can be increased by different means, such as amongst others for example an increase of the hydrodynamic radius of the drug (thereby slowing down the diffusion from the eye), a high affinity of the drug to its target (thereby reducing the dissociation of drug-target complexes), a high (thermal) degradation stability in the eye, and a high injectable dose).

20 The main factors thought to influencing durability are the dose (an increase of the applicable dose adds positively to durability), the half-life (an increase in half-life adds positively to durability) and the affinity to the target (represented by  $K_D$ ) (an increase in affinity adds positively to durability).

25 After intravitreal application a large amount of drug has to be bound by the compound of the ECM in the vitreous humor selected for retention of the drug in the eye. The binding kinetic to said compound must allow a sufficient remaining diffusion of drug into retina/choroidea to maintain minimum effective dose (aim: drug concentration above minimum effective dose as long as possible after the intravitreal application).

30 The „diffusion rate“ (dependent on  $k_{on}/k_{off}$  towards the compound of the ECM selected for retention of the drug in the eye and on the capacity of the depot in the vitreous humor) has to be equal or slightly higher than elimination rate into systemic circulation.

The „capacity” (= depot size) of the compound of the ECM selected for retention of the drug in the eye has to be high enough. The capacity is dependent on the amount/accessibility of the compound of the ECM selected for retention of the drug in the eye, the number of binding sites thereof, and on the turnover thereof.

5 The binder-ECM compound-interaction should reduce the diffusion constant and thereby the clearance from the eye of the conjugate. A reduced diffusion rate in vitreous humor is a prerequisite for increased/improved eye retention. The diffusion constant of fluorescently labeled proteins in complex solutions can be determined by Fluorescence Correlation Spectroscopy (FCS; i.e. DLS using  
10 fluorescence).

Parameters as concentration, diffusion coefficients and MW can be determined directly from the measurement. The testing of the diffusivity can be performed in an artificial test solution (comprising the compound of the ECM selected for retention of the drug) „representing“ the composition of vitreous fluid or directly in  
15 vitreous fluid of minipigs.

Fluorescence Correlation Spectroscopy (FCS) analyzes the stochastic movement of fluorescently labeled molecules in an open microscopic volume element irradiated by a focused laser beam. FCS has been successfully applied for the study of molecular interactions in solution. One binding partner is labeled with a  
20 fluorophore and incubated with the designated interactor. Upon binding, the MW and hence the diffusional mobility of the labeled complex is altered, which can be quantified by FCS. Titration of the labeled ligand over a constant concentration of binding partner allow determining the affinity of the interaction. Time resolved measurements will give rise to the corresponding rate constants. Thus, a sufficient  
25 shift in complex size FCS can be used for the determination of dissociation- and rate constants.

Brownian motion drives the diffusion of fluorescence-labeled molecules through the illuminated detection volume. The photons emitted while passing through the volume element are recorded on ultra-sensitive avalanche photo detectors (APD).  
30 The fluctuations are analyzed by treating the recorded photon counts with a mathematical method called autocorrelation and fitting the deduced autocorrelation function to an appropriate biophysical model.



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$$J = -D \cdot dc/dx$$

$$dc/dt = D \cdot (d^2c)/(dx^2)$$

J: diffusion flux

D: diffusion constant

5 c: concentration

x: distance

t: time

10 Compound of the ECM that can be selected for retention of a drug in the eye are potentially insoluble proteins found in the vitreous humor/body, such as, for example, collagen (type II, IX, V/XI, IV etc.), hyaluronic acid (forms structures together with collagen), chondroitin sulfate, and heparin sulfate.

15 The current invention is directed to a (at least) bispecific binder comprising a first binding site specifically binding to a target for exerting a therapeutic effect and a second binding site specifically binding to a compound of the ECM selected for retention of the (at least) bispecific binder in the eye.

An exemplary binder according to the invention is an anti-digoxigenin binder combined with a second binding specificity directed against a compound of the ECM selected for retention of the drug in the eye.

Different constructs have been tested in vitro and in vivo:

20 as reference:

- the anti-digoxigenin antibody Fab (denoted as FAB in the following),
- the anti-digoxigenin antibody Fab conjugated to a PEG residue of 20 kDa (denoted as FAB-PEG in the following),

as bispecific binder/fusion protein:

- 25
- the anti-digoxigenin antibody Fab conjugated to a heparin-binding domain (human VEGF fragment comprising residues 111-165; denoted as FAB-HBD in the following),
  - the anti-digoxigenin antibody Fab conjugated to three different anti-collagen II antibody scFv (denoted as FAB-COLL-I (SEQ ID NO: 9 (VH)),

10 (VL) and 11 (scFv)), FAB-COLL-II (SEQ ID NO: 12 (VH), 13 (VL) and 14 (scFv)), FAB-COLL-III (SEQ ID NO: 15 (VH), 16 (VL) and 17 (scFv) in the following, which differ in the binding kinetic).

5 In a minipig study the concentration of the different constructs was determined in vitreous, retina and choroid at 168, 336 and 672 h after intravitreal injection (d0) of a 500 nM solution of the respective construct.

In the vitreous the following time-dependent concentrations have been determined:

	168 h (pmol/g)	336 h (pmol/g)	672 h (pmol/g)
FAB	82.5	53.6	6.7
FAB-PEG	128.6	93.3	32.5
FAB-HBD	45.2	10.3	2.1
FAB-COLL-I	165.6	59.2	10.4
FAB-COLL-II	171.0	58.5	19.6
FAB-COLL-III	149.3	62.0	11.1

In the retina the following time-dependent concentrations have been determined:

	168 h (pmol/g)	336 h (pmol/g)	672 h (pmol/g)
FAB	85.9	17.6	5.6
FAB-PEG	50.3	43.6	5.4
FAB-HBD	72.5	12.6	1.1
FAB-COLL-I	78.2	52.6	6.7
FAB-COLL-II	101.3	67.7	13.6
FAB-COLL-III	68.2	41.6	6.6

In the choroid the following time-dependent concentrations have been determined:

	168 h (pmol/g)	336 h (pmol/g)	672 h (pmol/g)
FAB	29.6	21.4	2.0
FAB-PEG	54.8	34.4	23.9
FAB-HBD	60.2	13.1	1.6
FAB-COLL-I	64.2	51.7	5.4
FAB-COLL-II	68.2	41.6	6.6
FAB-COLL-III	129.5	37.8	7.2

10 The different collagen scFvs have the following in vitro characteristics:

The half-life of the different constructs in the different compartments (tissues) of the eye is shown in Figure 1.

The exposure of different compartments (tissues) of the eye with respect to the different constructs is shown in Figure 2.

The characteristic parameters the constructs were determined in vivo in minipig and in vitro using BIAcore as well as in an artificial diffusion test solution. The data is shown in the following table.

5

	K <sub>D</sub> (nM) porcine / human collagen II	diffusion rate FCS (increase in PBS comprising ECM compound at equimolar concentration compared to PBS alone)	diffusion rate FCS (increase in vitreous fluid compared to PBS)
FAB	n.a.	100 %	100 %
FAB-PEG	n.a.	+ 65-100 %	+ 70-100 %
FAB-HBD (40 nM)	n.a.	+ 25 %	+ 35 %
FAB-COLL-I (2 nM)	56 / 30	+ 180 %	+ 35-130 %
FAB-COLL-II (8 nM)	50 / 15	+ 260 %	+ 140-310 %
FAB-COLL-III (8 nM)	342 / 180	+ 40 %	+ 30-85 %

	concentration (nM)	diffusion time vitreous fluid (micro-sec)	diffusion time PBS (micro-sec)
FAB	8	270	267
FAB-COLL-I	2	632	477
FAB-COLL-II	8	1113	347
FAB-COLL-III	8	497	390

For FAB-COLL-II a 3.2 times increased diffusion time (i.e. a reduced diffusion) has been found in VF, and a 2.7 times increased diffusion time in PBS supplemented with collagen (same FAB-COLL-II concentration).

	t <sub>1/2</sub> vitreous (h)	C <sub>0</sub> estimate (nM)
FAB	135	196
FAB-PEG	249	205
FAB-HBD (40 nM)	118	121
FAB-COLL-I (8 nM)	125	421

	t <sub>1/2</sub> vitreous (h)	C0 estimate (nM)
FAB-COLL-II (2 nM)	169	341
FAB-COLL-III (8 nM)	134	355

The multispecific binder/fusion protein as disclosed herein

- supports long intravitreal half-life and short systemic half-life to allow for infrequent dosing and to minimize/rule out systemic toxic effects,
- 5 - has vitreous body retention resulting in slower release from the eye, low systemic C<sub>max</sub> and less systemic toxicity,
- has increased affinity to the selected ECM compounds leading to lower efficient drug concentration, which may result in less frequent dosing,
- 10 - has a specific vitreous body retention moiety leading to long intravitreal half-life,
- has a low molecular weight format combining a vitreous body retention moiety to compensate fast diffusion across vitreous body and blood retinal barrier,
- is a low molecular weight format most feasible for use in eye device,
- 15 - by the addition of a third specificity may lead to even higher efficacy,
- when comprising an Fc-region is a high MW format with shortened systemic half-life due to 'silent' Fc part, which does not bind to FcRn.

In one aspect, the invention provides isolated antibodies that bind to human collagen II.

- 20 In certain embodiments, the anti-human collagen II antibody has a diffusion time in vitreous fluid of minipigs in micro-seconds at 8 nM concentration of more than 750, in one embodiment of more than 1000.

In certain embodiments, the anti-human collagen II antibody also specifically binds to porcine collagen II.

In certain embodiments, the anti-human collagen II antibody has a  $K_D$  value for binding to porcine collagen II of less than 400 nM at a concentration of 8 nM. In one embodiment the  $K_D$  is less than 100 nM.

5 In certain embodiments, the anti-human collagen II antibody has a  $K_D$  value for human collagen II of less than 200 nM. In one embodiment the  $K_D$  is less than 50 nM.

In certain embodiments, the anti-human collagen II antibody has a half-life in the minipig vitreous of more than 150 hours.

10 In certain embodiments, the anti-human collagen II antibody has an estimated  $C_0$  in minipig of more than 200 nM. In one embodiment the  $C_0$  is more than 300 nM.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VH HVR determined according to Kabat of SEQ ID NO: 09.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VH HVR determined according to Kabat of SEQ ID NO: 12.

15 In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VH HVR determined according to Kabat of SEQ ID NO: 15.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VL HVR determined according to Kabat of SEQ ID NO: 10.

20 In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VL HVR determined according to Kabat of SEQ ID NO: 13.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VL HVR determined according to Kabat of SEQ ID NO: 16.

25 In another aspect, an antibody of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences determined according to Kabat of SEQ ID NO: 09; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences determined according to Kabat of SEQ ID NO: 10.

30 In another aspect, an antibody of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences determined according to Kabat of SEQ ID NO: 12; and (b) a VL domain comprising at least

one, at least two, or all three VL HVR sequences determined according to Kabat of SEQ ID NO: 13.

5 In another aspect, an antibody of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences determined according to Kabat of SEQ ID NO: 15; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences determined according to Kabat of SEQ ID NO: 16.

10 In any of the above embodiments, an anti-human collagen II antibody is humanized. In one embodiment, an anti-human collagen II antibody comprises HVRs as in any of the above embodiments, and further comprises an acceptor human framework, e.g. a human immunoglobulin framework or a human consensus framework.

15 In another aspect, an anti-human collagen II antibody comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 09. In certain embodiments, a VH sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-human collagen II antibody comprising that  
20 sequence retains the ability to bind to human collagen II. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 09. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-human collagen II antibody comprises the VH sequence in SEQ ID NO: 09, including post-  
25 translational modifications of that sequence.

30 In another aspect, an anti-human collagen II antibody comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 12. In certain embodiments, a VH sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-human collagen II antibody comprising that sequence retains the ability to bind to human collagen II. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ

ID NO: 12. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-human collagen II antibody comprises the VH sequence in SEQ ID NO: 12, including post-translational modifications of that sequence.

5 In another aspect, an anti-human collagen II antibody comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 15. In certain embodiments, a VH sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains  
10 substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-human collagen II antibody comprising that sequence retains the ability to bind to human collagen II. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 15. In certain embodiments, substitutions, insertions, or deletions occur in  
15 regions outside the HVRs (i.e., in the FRs). Optionally, the anti-human collagen II antibody comprises the VH sequence in SEQ ID NO: 15, including post-translational modifications of that sequence.

In another aspect, an anti-human collagen II antibody is provided, wherein the antibody comprises a light chain variable domain (VL) having at least 90%, 91%,  
20 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 10. In certain embodiments, a VL sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-human collagen II antibody comprising that sequence retains the ability to bind to human collagen II. In certain  
25 embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 10. In certain embodiments, the substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-human collagen II antibody comprises the VL sequence in SEQ ID NO: 10, including post-translational modifications of that sequence.  
30

In another aspect, an anti-human collagen II antibody is provided, wherein the antibody comprises a light chain variable domain (VL) having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 13. In certain embodiments, a VL sequence  
35 having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity

contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-human collagen II antibody comprising that sequence retains the ability to bind to human collagen II. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 13. In certain embodiments, the substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-human collagen II antibody comprises the VL sequence in SEQ ID NO: 13, including post-translational modifications of that sequence.

In another aspect, an anti-human collagen II antibody is provided, wherein the antibody comprises a light chain variable domain (VL) having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 16. In certain embodiments, a VL sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-human collagen II antibody comprising that sequence retains the ability to bind to human collagen II. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 16. In certain embodiments, the substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-human collagen II antibody comprises the VL sequence in SEQ ID NO: 16, including post-translational modifications of that sequence.

In another aspect, an anti-human collagen II antibody is provided, wherein the antibody comprises a VH as in any of the embodiments provided above, and a VL as in any of the embodiments provided above. In one embodiment, the antibody comprises the VH and VL sequences in SEQ ID NO: 09 and SEQ ID NO: 10, respectively, including post-translational modifications of those sequences.

In another aspect, an anti-human collagen II antibody is provided, wherein the antibody comprises a VH as in any of the embodiments provided above, and a VL as in any of the embodiments provided above. In one embodiment, the antibody comprises the VH and VL sequences in SEQ ID NO: 12 and SEQ ID NO: 13, respectively, including post-translational modifications of those sequences.

In another aspect, an anti-human collagen II antibody is provided, wherein the antibody comprises a VH as in any of the embodiments provided above, and a VL as in any of the embodiments provided above. In one embodiment, the antibody



comprises the VH and VL sequences in SEQ ID NO: 15 and SEQ ID NO: 16, respectively, including post-translational modifications of those sequences.

In a further aspect, the invention provides an antibody that binds to the same epitope as an anti-human collagen II antibody provided herein.

5 In a further aspect of the invention, an anti-human collagen II antibody according to any of the above embodiments is a monoclonal antibody, including a chimeric, humanized or human antibody. In one embodiment, an anti-human collagen II antibody is an antibody fragment, e.g., a Fv, Fab, Fab', scFv, diabody, or F(ab')<sub>2</sub> fragment.

10 In a further aspect of the invention, an anti-human collagen II antibody according to any of the above embodiments is a monoclonal antibody scFv fragment or Fab. In one embodiment the scFv fragment has an amino acid sequence of SEQ ID NO: 11. In one embodiment the scFv fragment has an amino acid sequence of SEQ ID NO: 14. In one embodiment the scFv fragment has an amino acid sequence of SEQ ID NO: 17.

#### IV. PRODUCTION

The multispecific binder/fusion protein as disclosed herein is produced by recombinant means. Thus, one aspect as reported herein is a nucleic acid encoding the multispecific binder as reported herein and a further aspect is a cell comprising  
20 the nucleic acid encoding a multispecific binder as reported herein. Methods for recombinant production are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the multispecific binder and usually purification to a pharmaceutically acceptable purity. For the expression of the multispecific binder as aforementioned in a host  
25 cell, nucleic acids encoding the respective chains are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, PER.C6 cells, yeast, or E.coli cells, and the multispecific binder is recovered from the cells (cultivation supernatant or cells after lysis). General methods for  
30 recombinant production of antibodies are well-known in the state of the art and described, for example, in the review articles of Makrides, S.C., Protein Expr. Purif. 17 (1999) 183-202, Geisse, S., et al., Protein Expr. Purif. 8 (1996) 271-282, Kaufman, R.J., Mol. Biotechnol. 16 (2000) 151-160, and Werner, R.G., Drug Res. 48 (1998) 870-880.

Antibodies may be produced using recombinant methods and formulations, e.g., as described in US 4,816,567.

In one embodiment, isolated nucleic acid(s) encoding a multispecific binder as described herein is(are) provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the multispecific binder. In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the multispecific binder and an amino acid sequence comprising the VH of the multispecific binder, or (2) a first vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the multispecific binder and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the multispecific binder. In one embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp20 cell). In one embodiment, a method of making a multispecific binder as reported herein is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the multispecific binder, as provided above, under conditions suitable for expression of the multispecific binder, and optionally recovering the multispecific binder from the host cell (or host cell culture medium).

Accordingly one aspect as reported herein is a method for the preparation of a multispecific binder as reported herein, comprising the steps of

- a) transforming a host cell with vectors comprising nucleic acid molecules encoding the multispecific binder,
- b) culturing the host cell under conditions that allow synthesis of the multispecific binder, and
- c) recovering the multispecific binder from the culture.

In one embodiment the recovering step under c) includes the use of a light chain constant domain specific capture reagent (which e.g. specific for the kappa or the lambda constant light chain, depending on whether a kappa or a lambda light chain is contained in the bispecific antibody). In one embodiment this light chain specific capture reagent is used in in a bind-and-elute-mode. Examples of such light chain constant domain specific capture reagents are e.g. KappaSelect™ and LambdaFabSelect™ (available from GE Healthcare/BAC), which are based on a

highly rigid agarose base matrix that allows high flow rates and low back pressure at large scale. These materials contain a ligand that binds to the constant region of the kappa or the lambda light chain, respectively (i.e. fragments lacking the constant region of the light chain will not bind). Both are therefore capable of binding other target molecules containing the constant region of the light chain, for example, IgG, IgA and IgM. The ligands are attached to the matrix via a long hydrophilic spacer arm to make them easily available for binding to the target molecule. They are based on a single-chain antibody fragment that is screened for either human Ig kappa or lambda.

The multispecific binders are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, affinity chromatography (protein A-Sepharose, or KappaSelect<sup>TM</sup>, LambdaFabSelect<sup>TM</sup>), hydroxylapatite chromatography, gel electrophoresis, or dialysis.

DNA and RNA encoding monoclonal antibodies is readily isolated and sequenced using conventional procedures. B-cells or hybridoma cells can serve as a source of such DNA and RNA. Once isolated, the DNA may be inserted into expression vectors, which are then transfected into host cells such as HEK 293 cells, CHO cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of recombinant monoclonal antibodies in the host cells.

Purification of multispecific binder is performed in order to eliminate cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art (see e.g. Ausubel, F., et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987)). Different methods are well established and widespread used for protein purification, such as affinity chromatography (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and

electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M.A., Appl. Biochem. Biotech. 75 (1998) 93-102).

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

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for multispecific binder-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been “humanized”, resulting in the production of a multispecific binder with a partially or fully human glycosylation pattern. See Gerngross, T.U., Nat. Biotech. 22 (2004) 1409-1414; and Li, H. et al., Nat. Biotech. 24 (2006) 210-215.

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

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

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (HEK293 or 293 cells as described, e.g., in Graham, F.L., et al., J. Gen Virol. 36 (1977) 59-74); baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, J.P., Biol. Reprod. 23 (1980) 243-252); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK); buffalo rat liver cells (BRL 3A); human lung cells (W138); human

liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather, J.P., et al., *Annals N.Y. Acad. Sci.* 383 (1982) 44-68; MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR<sup>-</sup> CHO cells (Urlaub, G., et al., *Proc. Natl. Acad. Sci. USA* 77 (1980) 4216-4220); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki, P. and Wu, A.M., *Methods in Molecular Biology*, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2004), pp. 255-268.

## 10 V. PHARMACEUTICAL FORMULATION

The multispecific binder/fusion proteins as disclosed herein may have a valuable efficacy/safety profile and may provide benefits for a patient in the need of the respective therapy.

15 In one aspect, a multispecific binder as reported herein for use as a medicament is provided.

In a further aspect, the invention provides for the use of a multispecific binder in the manufacture or preparation of a medicament. An “individual” according to any embodiments may be a human.

20 In a further aspect, the invention provides pharmaceutical formulations comprising any of the multispecific binder provided herein, e.g., for use in any of the herein outlined therapeutic methods. In one embodiment, a pharmaceutical formulation comprises any of the multispecific binder provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical formulation comprises any of the multispecific binder provided herein and at least one additional  
25 therapeutic agent.

One aspect as reported herein is a pharmaceutical formulation comprising a multispecific binder as reported herein.

30 Pharmaceutical formulations of a multispecific binder as described herein are prepared by mixing such multispecific binder having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's *Pharmaceutical Sciences*, 16th edition, Osol, A. (ed.) (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers

are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyl dimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as poly(vinylpyrrolidone); amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rhuPH20 (HYLENEX<sup>®</sup>, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rhuPH20, are described in US 2005/0260186 and US 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

Exemplary lyophilized antibody formulations are described in US 6,267,958. Aqueous antibody formulations include those described in US 6,171,586 and WO 2006/044908, the latter formulations including a histidine-acetate buffer.

The formulation herein may also contain more than one active ingredients as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended.

The formulations to be used for *in vivo* administration are generally sterile. Sterility may be readily accomplished, *e.g.*, by filtration through sterile filtration membranes.

Another aspect as reported herein is the use of a multispecific binder as reported herein for the manufacture of a pharmaceutical formulation. A further aspect as reported herein is a method for the manufacture of a pharmaceutical formulation comprising a multispecific binder as reported herein. In another aspect, a formulation is provided, e.g. a pharmaceutical formulation, containing a multispecific binder as reported herein, formulated together with a pharmaceutical carrier.

Many possible modes of delivery can be used, including, but not limited to intraocular application or topical application. In one embodiment the application is intraocular and includes, but it's not limited to, subconjunctival injection, intracanalicular injection, injection into the anterior chamber via the temporai limbus, intrastromal injection, intracorneal injection, subretinal injection, aqueous humor injection, subtenon injection or sustained delivery device, intravitreal injection (e.g., front, mid or back vitreal injection). In one embodiment the application is topical and includes, but it's not limited to eye drops to the cornea.

In one embodiment the multispecific binder or pharmaceutical formulation as reported herein is administered via intravitreal application, e.g. via intravitreal injection. This can be performed in accordance with standard procedures known in the art (see, e.g., Ritter et al., *J. Clin. Invest.* 116 (2006) 3266-3276, Russelakis-Carneiro et al., *Neuropathol. Appl. Neurobiol.* 25 (1999) 196-206, and Wray et al., *Arch. Neurol.* 33 (1976) 183-185).

In some embodiments, therapeutic kits are provided that contain one or more doses of a multispecific binder present in a pharmaceutical formulation described herein, a suitable device for intravitreal injection of the pharmaceutical formulation, and an instruction detailing suitable subjects and protocols for carrying out the injection. In these embodiments, the formulations are typically administered to the subject in need of treatment via intravitreal injection. This can be performed in accordance with standard procedures known in the art (see, e.g., Ritter et al., *J. Clin. Invest.* 116 (2006) 3266-3276, Russelakis-Carneiro et al., *Neuropathol. Appl. Neurobiol.* 25 (1999) 196-206, and Wray et al., *Arch. Neurol.* 33 (1976) 183-185).

The formulations may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben,

chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the formulations. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents, which delay absorption such as aluminum monostearate and gelatin.

5

Regardless of the route of administration selected, the multispecific binder as reported herein, which may be used in a suitable hydrated form, and/or the pharmaceutical formulations as reported herein, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

10

Actual dosage levels of the active ingredients in the pharmaceutical formulations as reported herein may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, formulation, and mode of administration, without being toxic to the patient.

15

The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular formulations employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular formulations employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

20

The formulation must be sterile and fluid to the extent that the formulation is deliverable by syringe. In addition to water, the carrier preferably is an isotonic buffered saline solution.

25

Proper fluidity can be maintained, for example, by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the formulation.

30

The formulation can comprise an ophthalmic depot formulation comprising an active agent for subconjunctival administration. The ophthalmic depot formulation comprises microparticles of essentially pure active agent, e.g., the multispecific binder as reported herein. The microparticles comprising the multispecific binder as reported herein can be embedded in a biocompatible pharmaceutically acceptable polymer or a lipid-encapsulating agent. The depot formulations may be adapted to release all of substantially all the active material over an extended



5 period of time. The polymer or lipid matrix, if present, may be adapted to degrade sufficiently to be transported from the site of administration after release of all or substantially all of the active agent. The depot formulation can be liquid formulation, comprising a pharmaceutical acceptable polymer and a dissolved or dispersed active agent. Upon injection, the polymer forms a depot at the injections site, e.g. by gelifying or precipitating.

Another aspect as reported herein is the multispecific binder as reported herein for use in the treatment of ocular vascular diseases.

10 Another aspect as reported herein is the pharmaceutical formulation as reported herein for use in the treatment of ocular vascular diseases.

Another aspect as reported herein is the use of a multispecific binder as reported herein for the manufacture of a medicament for the treatment of ocular vascular disease.

15 Another aspect as reported herein is method of treatment of patient suffering from ocular vascular diseases by administering a multispecific binder as reported herein to a patient in the need of such treatment.

## VI. THERAPEUTIC METHODS

Any of the multispecific binder/fusion proteins disclosed herein may be used in therapeutic methods.

20 In certain embodiments, a multispecific binder for use in a method of treatment is provided. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below. An “individual” according to any of the above embodiments is in one preferred embodiment a human.

25 In certain embodiments, a multispecific binder for use in a method of treatment is provided. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below. An “individual” according to any of the embodiments is in one preferred embodiment a human.

30 Multispecific binder as reported herein would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for

consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The multispecific binder need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of multispecific binder present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

For the prevention or treatment of disease, the appropriate dosage of a multispecific binder as reported herein (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the type of multispecific binder, the severity and course of the disease, whether the multispecific binder is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the multispecific binder, and the discretion of the attending physician. The multispecific binder is suitably administered to the patient at one time or over a series of treatments. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. The progress of this therapy is easily monitored by conventional techniques and assays.

## VII. ARTICLES OF MANUFACTURE

In another aspect as reported herein, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a formulation, which is by itself or combined with another formulation effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the

container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the formulation is a multispecific binder as reported herein. The label or package insert indicates that the formulation is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a formulation contained therein, wherein the formulation comprises a multispecific binder as reported herein; and (b) a second container with a formulation contained therein, wherein the formulation comprises a further therapeutic agent. The article of manufacture in this embodiment as reported herein may further comprise a package insert indicating that the formulations can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically acceptable buffer, such as bacteriostatic water for injection (BWFI) or phosphate-buffered saline. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

#### VIII. MODIFICATIONS

In a further aspect, a multispecific binder according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-5 below:

##### 20 **1. Antibody Affinity**

In certain embodiments, the multispecific binder provided herein has an equilibrium dissociation constant ( $K_D$ ) of  $\leq 100$  nM (e.g.  $10^{-7}$  M or less, e.g. from  $10^{-7}$  M to  $10^{-13}$ ) for any of its targets.

In one embodiment,  $K_D$  is measured using a BIACORE<sup>®</sup> surface plasmon resonance assay. For example, an assay using a BIACORE<sup>®</sup>-2000 or a BIACORE<sup>®</sup>-3000 (GE Healthcare Inc., Piscataway, NJ) is performed at 25 °C with immobilized antigen CM5 chips at ~10 response units (RU). In one embodiment, carboxymethylated dextran biosensor chips (CM5, GE Healthcare Inc.) are activated with *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 µg/mL (~0.2 µM) before injection at a flow rate of 5 µL/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block non-reacted groups. For kinetics measurements,

two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05 % polysorbate 20 (TWEEN-20<sup>TM</sup>) surfactant (PBST) at 25 °C at a flow rate of approximately 25 µL/min. Association rates ( $k_{on}$ ) and dissociation rates ( $k_{off}$ ) are calculated using a simple one-to-one Langmuir binding model (BIAcore<sup>®</sup> Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant ( $K_D$ ) is calculated as the ratio  $k_{off}/k_{on}$  (see, e.g., Chen, Y. et al., J. Mol. Biol. 293 (1999) 865-881). If the on-rate exceeds  $10^6 \text{ M}^{-1} \text{ s}^{-1}$  by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25 °C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophotometer (Aviv Instruments) or a 8000-series SLM-AMINCO<sup>TM</sup> spectrophotometer (ThermoSpectronic) with a stirred cuvette.

## 2. Chimeric and Humanized Binding Sites

In certain embodiments, a multispecific binder provided herein comprises an antibody binding site of a chimeric or humanized antibody.

Certain chimeric antibodies are described, e.g., in US 4,816,567; and Morrison, S.L., et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a “class switched” antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

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

are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.

5 Humanized antibodies and methods of making them are reviewed, e.g., in Almagro, J.C. and Fransson, J., *Front. Biosci.* 13 (2008) 1619-1633, and are further described, e.g., in Riechmann, I., et al., *Nature* 332 (1988) 323-329; Queen, C., et al., *Proc. Natl. Acad. Sci. USA* 86 (1989) 10029-10033; US 5,821,337, US 7,527,791, US 6,982,321, and US 7,087,409; Kashmiri, S.V., et al., *Methods* 36 (2005) 25-34 (describing specificity determining region (SDR) grafting); Padlan, 10 E.A., *Mol. Immunol.* 28 (1991) 489-498 (describing "resurfacing"); Dall'Acqua, W.F. et al., *Methods* 36 (2005) 43-60 (describing "FR shuffling"); Osbourn, J. et al., *Methods* 36 (2005) 61-68; and Klimka, A. et al., *Br. J. Cancer* 83 (2000) 252-260 (describing the "guided selection" approach to FR shuffling).

15 Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (see, e.g., Sims, M.J., et al., *J. Immunol.* 151 (1993) 2296-2308; framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter, P., et al., *Proc. Natl. Acad. Sci. USA* 89 (1992) 4285-4289; and Presta, L.G., et al., *J. Immunol.* 151 (1993) 2623-2632); 20 human mature (somatically mutated) framework regions or human germline framework regions (see, e.g., Almagro, J.C. and Fransson, J., *Front. Biosci.* 13 (2008) 1619-1633); and framework regions derived from screening FR libraries (see, e.g., Baca, M. et al., *J. Biol. Chem.* 272 (1997) 10678-10684 and Rosok, M.J. et al., *J. Biol. Chem.* 271 (1996) 22611-22618).

### 25 **3. Human Antibody Binding Sites**

In certain embodiments, a multispecific binder provided herein comprises an antibody binding site of a human antibody.

Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk, M.A. and van de Winkel, 30 J.G., *Curr. Opin. Pharmacol.* 5 (2001) 368-374 and Lonberg, N., *Curr. Opin. Immunol.* 20 (2008) 450-459.

Human antibodies maybe prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact

antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, N., *Nat. Biotech.* 23 (2005) 1117-1125. See also, e.g., US 6,075,181 and US 6,150,584 describing XENOMOUSE™ technology; US 5,770,429 describing HUMAB® technology; US 7,041,870 describing K-MOUSE® technology, and US 2007/0061900, describing VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described (see, e.g., Kozbor, D., *J. Immunol.* 133 (1984) 3001-3005; Brodeur, B.R., et al., *Monoclonal Antibody Production Techniques and Applications*, Marcel Dekker, Inc., New York (1987), pp. 51-63; and Boerner, P., et al., *J. Immunol.* 147 (1991) 86-95). Human antibodies generated via human B-cell hybridoma technology are also described in Li, J. et al., *Proc. Natl. Acad. Sci. USA* 103 (2006) 3557-3562. Additional methods include those described, for example, in US 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, J., *Xiandai Mianyixue* 26 (2006) 265-268 (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers, H.P. and Brandlein, S., *Histology and Histopathology* 20 (2005) 927-937 and Vollmers, H.P. and Brandlein, S., *Methods and Findings in Experimental and Clinical Pharmacology* 27 (2005) 185-191.

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

#### 4. Library-Derived Antibody Binding Sites

Multispecific binder as reported herein may comprise an antibody binding site of an antibody isolated by screening combinatorial libraries for antibodies with the desired activity or activities.

5 For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are reviewed, e.g., in Hoogenboom, H.R. et al., *Methods in Molecular Biology* 178 (2001) 1-37 and further described, e.g., in the McCafferty, J. et al., *Nature* 348 (1990) 552-554; Clackson, T. et al., *Nature* 352  
10 (1991) 624-628; Marks, J.D. et al., *J. Mol. Biol.* 222 (1992) 581-597; Marks, J.D. and Bradbury, A., *Methods in Molecular Biology* 248 (2003) 161-175; Sidhu, S.S. et al., *J. Mol. Biol.* 338 (2004) 299-310; Lee, C.V. et al., *J. Mol. Biol.* 340 (2004) 1073-1093; Fellouse, F.A., *Proc. Natl. Acad. Sci. USA* 101 (2004) 12467-12472; and Lee, C.V. et al., *J. Immunol. Methods* 284 (2004) 119-132.

15 In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter, G., et al., *Ann. Rev. Immunol.* 12 (1994) 433-455. Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments.  
20 Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antibodies to a wide range of non-self and also self-antigens without any immunization as described by Griffiths, A.D., et al., *EMBO J.* 12 (1993) 725-734.  
25 Finally, naive libraries can also be made synthetically by cloning non-rearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom, H.R. and Winter, G., *J. Mol. Biol.* 227 (1992) 381-388. Patent publications describing human antibody phage  
30 libraries include, for example: US 5,750,373, and US 2005/0079574, US 2005/0119455, US 2005/0266000, US 2007/0117126, US 2007/0160598, US 2007/0237764, US 2007/0292936, and US 2009/0002360.

Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.

## 5. Multispecific Binder Variants

In certain embodiments, amino acid sequence variants of the multispecific binder provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the multispecific binder.

5 Amino acid sequence variants of a multispecific binder may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the multispecific binder, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the multispecific binder. Any combination of

10 deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.

### a) Substitution, Insertion, and Deletion Variants

In certain embodiments, multispecific binder variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in the Table below under the heading of "preferred substitutions". More substantial changes are provided in the following Table under the heading of "exemplary substitutions", and as further described below in reference to amino acid side chain classes. Amino

15 acid substitutions may be introduced into a multispecific binder of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

20

**TABLE.**

<b>Original Residue</b>	<b>Exemplary Substitutions</b>	<b>Preferred Substitutions</b>
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp



<b>Original Residue</b>	<b>Exemplary Substitutions</b>	<b>Preferred Substitutions</b>
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

- (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- (3) acidic: Asp, Glu;
- 5 (4) basic: His, Lys, Arg;
- (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

- 10 One type of substitutional variant involves substituting one or more hypervariable region residues of a parent multispecific binder (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (*e.g.*, improvements) in certain biological properties (*e.g.*, increased affinity, reduced immunogenicity) relative to the parent antibody multispecific binder and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured
- 15

multispecific binder, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant multispecific binder displayed on phage and screened for a particular biological activity (e.g. binding affinity).

5

Alterations (e.g., substitutions) may be made in HVRs, e.g., to improve multispecific binder affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g., Chowdhury, P.S., *Methods Mol. Biol.* 207 (2008) 179-196), and/or residues that contact antigen, with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom, H.R. et al. in *Methods in Molecular Biology* 178 (2002) 1-37. In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any multispecific binder variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

10

15

20

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the multispecific binder to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may, for example, be outside of antigen contacting residues in the HVRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

25

30

A useful method for identification of residues or regions of a multispecific binder that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham, B.C. and Wells, J.A., *Science* 244 (1989) 1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively

35

charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the multispecific binder with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-multispecific binder complex to identify contact points between the multispecific binder and antigen can be used. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include a multispecific binder with an N-terminal methionyl residue. Other insertional variants of the multispecific binder molecule include the fusion to the N- or C-terminus of the multispecific binder to an enzyme (e.g. for ADEPT) or a polypeptide.

The following examples, sequences and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

## **Description of the Figures**

**Figure 1** Half-life of the different constructs in the different compartments of the eye; 1: FAB-PEG, 2: FAB-HBD, 3: FAB, 4: FAB-COLL-I, 5: FAB-COLL-II, 6: FAB-COLL-III; upper bar: vitreous, middle bar: retina, lower bar: choroid.

**Figure 2** Exposure of different compartments (tissues) of the eye to the different constructs; 1: FAB-COLL-I, 2: FAB-COLL-II, 3: FAB-COLL-III, 4: FAB, 5: FAB-HBD, 6: FAB-PEG; left bar: vitreous, middle bar: retina, right bar: choroid.

## **Materials and Methods**

### **Recombinant DNA techniques**

Standard methods were used to manipulate DNA as described in Sambrook, J. et al., Molecular Cloning: A laboratory manual; Cold Spring Harbor Laboratory

Press, Cold Spring Harbor, New York (1989). The molecular biological reagents were used according to the manufacturer's instructions.

#### Gene synthesis

5 Desired gene segments were ordered according to given specifications at Genart (Regensburg, Germany).

#### DNA sequence determination

DNA sequences were determined by double strand sequencing performed at MediGenomix GmbH (Martinsried, Germany) or SequiServe GmbH (Vaterstetten, Germany).

#### 10 DNA and protein sequence analysis and sequence data management

The GCG's (Genetics Computer Group, Madison, Wisconsin) software package version 10.2 and Infomax's Vector NT1 Advance suite version 8.0 was used for sequence creation, mapping, analysis, annotation and illustration.

#### Expression vectors

15 For the expression of the described antibodies expression plasmids for transient expression (e.g. in HEK293-F cells) based either on a cDNA organization with or without a CMV-Intron A promoter or on a genomic organization with a CMV promoter were used.

20 The transcription unit of the antibody gene was composed of the following elements:

- unique restriction site(s) at the 5' end,
- the immediate early enhancer and promoter from the human cytomegalovirus,
- in the case of the cDNA organization the Intron A sequence,
- 25 - a 5'-untranslated region of a human immunoglobulin gene,
- a nucleic acid encoding an immunoglobulin heavy chain signal sequence,
- a nucleic acid encoding the human antibody chain (wild-type or with domain exchange) either as cDNA or in genomic organization with the
- 30 immunoglobulin exon-intron organization,
- a 3' non-translated region with a polyadenylation signal sequence, and

- unique restriction site(s) at the 3' end.

Beside the antibody expression cassette the plasmids contained:

- an origin of replication which allows replication of this plasmid in *E. coli*,
- 5 - a  $\beta$ -lactamase gene which confers ampicillin resistance in *E. coli*, and
- the dihydrofolate reductase gene from *Mus musculus* as a selectable marker in eukaryotic cells.

10 The nucleic acids encoding the antibody chains were generated by PCR and/or gene synthesis and assembled by known recombinant methods and techniques by connection of the according nucleic acid segments e.g. using unique restriction sites in the respective vectors. The subcloned nucleic acid sequences were verified by DNA sequencing. For transient transfections larger quantities of the plasmids were prepared by plasmid preparation from transformed *E. coli* cultures (Nucleobond AX, Macherey-Nagel).

#### 15 Cell culture techniques

Standard cell culture techniques were used as described in Current Protocols in Cell Biology (2000), Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J. and Yamada, K.M. (eds.), John Wiley & Sons, Inc.

20 The bispecific antibodies were expressed by transient co-transfection of the respective expression plasmids in HEK293-F cells growing in suspension as described below.

#### Example 1

##### **Expression and Purification**

##### Transient transfections in HEK293-F system

25 The fusion constructs were generated by transient transfection with the respective plasmids using the HEK293-F system (Invitrogen) according to the manufacturer's instruction. Briefly, HEK293-F cells (Invitrogen) growing in suspension either in a shake flask or in a stirred fermenter in serum-free FreeStyle™ 293 expression medium (Invitrogen) were transfected with a mix of the respective expression plasmids and 293fectin™ or fectin (Invitrogen). For 2 L shake flask (Corning) 30 HEK293-F cells were seeded at a density of  $1 \cdot 10^6$  cells/mL in 600 mL and incubated at 120 rpm, 8 % CO<sub>2</sub>. The day after the cells were transfected at a cell

density of approx.  $1.5 \times 10^6$  cells/mL with approx. 42 mL of a mixture of A) 20 mL Opti-MEM (Invitrogen) with 600  $\mu$ g total plasmid DNA (1  $\mu$ g/mL) encoding the heavy or modified heavy chain, respectively and the corresponding light chain in an equimolar ratio and B) 20 ml Opti-MEM with 1.2 mL 293 fectin or fectin (2  $\mu$ L/mL). According to the glucose consumption glucose solution was added during the course of the fermentation. The supernatant containing the secreted antibody was harvested after 5-10 days and antibodies were either directly purified from the supernatant or the supernatant was frozen and stored.

### Purification

The polypeptide-containing culture supernatants were filtered and purified by two chromatographic steps. The antibodies were captured by affinity chromatography using HiTrap KappaSelect (GE Healthcare) equilibrated with PBS (1 mM  $\text{KH}_2\text{PO}_4$ , 10 mM  $\text{Na}_2\text{HPO}_4$ , 137 mM NaCl, 2.7 mM KCl), pH 7.4. Unbound proteins were removed by washing with equilibration buffer, and the fusion polypeptide was recovered with 100 mM citrate buffer, pH 2.9, and immediately after elution neutralized to pH 6.0 with 1 M Tris-base, pH 9.0. Size exclusion chromatography on HiLoad 26/60 Superdex 75<sup>TM</sup> (GE Healthcare) was used as second purification step. The size exclusion chromatography was performed in 20 mM histidine buffer, 0.14 M NaCl, pH 6.0. The polypeptide containing solutions were concentrated with an Ultra free -CL centrifugal filter unit equipped with a Biomax-SK membrane (Millipore, Billerica, MA) and stored at -80 °C.

The protein concentrations of the polypeptides were determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence.

Purity and integrity of the polypeptides molecules were analyzed by CE-SDS using a LabChip GX II (PerkinElmer) with Protein Express Chip and HT Protein Express Reagents Kit.

Aggregate content was determined by high-performance SEC using a Biosuite High Resolution SEC, 250 Å, 5  $\mu$ m analytical size-exclusion column (Waters GmbH) using 200 mM  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , 250 mM KCl, pH 7.0 as running buffer.

The integrity of the amino acid backbone of reduced polypeptides was verified by Nano Electrospray QTOF mass spectrometry after removal of N-glycans by

enzymatic treatment with a combination of neuraminidase, O-glycanase and peptide-N-glycosidase F (Roche Applied Science).

### **Example 2**

#### **Binding to human and porcine collagen II**

5 Binding kinetics of anti-collagen antibodies to human Collage type II (Millipore CC052) and porcine Collagen type II (USBiological C7510-31) was investigated by surface plasmon resonance using a BIAcore T200 instrument (GE Healthcare). All experiments were performed at 25°C using HBS-P (10 mM His, 140 mM NaCl, 0.05% Tween 20 pH 7.4) as running and dilution buffer. Collagen type II was  
10 immobilized on a Series S CM5 Sensor Chip (GE Healthcare) using standard amine coupling chemistry. Anti-Collagen antibodies were injected for 180 s with concentrations from 1.23 up to 900 nM (1:3 dilution series) onto the surface (association phase). The dissociation phase was monitored for 600 sec by washing with running buffer. The surface was regenerated by injecting 0.85% H<sub>3</sub>PO<sub>4</sub> for 60  
15 sec. Bulk refractive index differences were corrected by subtracting the response obtained from a mock surface. Blank injections were subtracted (double referencing). The derived curves were fitted to a 1:1 Langmuir binding model using the BIAevaluation software.

### **Example 3**

#### **20 Minipig pharmacokinetic study**

Female minipigs, 7-8 kg each, were administered 1.25 nmol of each drug by IVT injection. The aimed initial concentration was 500 nM in the eye for each molecule. Vitreous, retina and choroid samples were collected at three termination time points 168, 336 and 672 hours after application.

### **Example 4**

#### **Pharmacokinetic parameter determination**

Minipig serum, aqueous humor, vitreous humor and ocular tissue (retina, choroid, sclera, iris, lens, ciliary body) were analyzed with an ECLIA method using an ELECSYS instrument (Roche Diagnostics GmbH).

30 Briefly, test sample (calibrator, quality control or study sample), first detection antibody *mAb<H-Fab(kappa)>M-1.7.10-IgG-Bi*, second detection antibody *mAb<H-Fab(CH1)>M-1.19.31-IgG-Ru*, and SA-beads are added stepwise to a

detection vessel and incubated for 9 minutes in each step. Finally, the SA-beads-bound complex is detected by a measuring cell, which numbers the counts of SA-beads in repeat. The counts are proportional to the analyte concentration in the test sample.

5 Bi=biotin, Ru=ruthenium label, SA=streptavidin

Prior to analysis, vitreous humor and ocular tissue samples were mechanically lysed in tissue extraction buffer (10 mM Tris, 137 mM NaCl, 1% Triton, 10% Glycerin) containing protease inhibitors using the Magana Lyser Homogenisator (Roche Diagnostics GmbH).

10 The assay calibration range for the three collagen binder conjugates FAB-COLL-I, -II, and -III was between 4.92 ng/mL and 3000 ng/mL (assay concentration).

Serum samples were diluted 1:10 to 1:20 to obtain valid results. Standard curve, quality control and sample dilutions were done in assay buffer incl. minipig serum resulting in 10% matrix concentration. Experimental serum samples below 49.2  
15 ng/mL were annotated as "BLQ".

Aqueous humor, vitreous humor and ocular tissue samples were measured undiluted and diluted up to 1:50 to obtain valid results. Standard curve, quality control and sample dilutions were done in assay buffer without matrix. Experimental aqueous humor, vitreous humor and ocular tissue samples below  
20 4.92 ng/mL were annotated as "BLQ".

### **Example 5**

#### **Diffusion parameter determination**

The test solutions - vitreous fluid of minipigs – was stored at -80°C.

25 Dig-3-cme-eda-Cy5 was dissolved in DMF and adjusted to 1 mM Dig-Cy5 in 30% DMF/dilution buffer). A working stock was prepared as a 50 µM Dig-Cy5 solution in PBS/0.2%BSA/1.5% DMF. PBS was purchased at LONZA (#17-516F), pH 7.3 – pH 7.5 and was supplemented with 0.2% BSA (fraction V). Measurements are done in 384-well glass bottom assay plates (MMI, #60200).

30 One sample was thawed on ice. The fluid is highly viscous and transparent. The sample was cautiously pipetted up and down ten times with a cropped 1000 µL tip.



It does foam mildly. Aliquots of 100 $\mu$ l (using a cropped 200 $\mu$ l tip) are frozen on dry ice and stored at -80°C.

5 The other samples were thawed and liquefied alike. The bulk amount of all three samples is pooled, aliquoted and stored at -80°C with sample name "all". Some original aliquots are stored as reference sample.

10 FCS measurements were performed with a ConfoCor2 FCS unit connected to an Axiovert 100M equipped with a C-Apochromat 40x N.A. 1.2 water immersion lens (Carl Zeiss, Jena, Germany). At this instrument Cy5 was excited with a 633 helium-neon laser. The red fluorescence emitted by Cy5 was detected with an LP 650 long pass filter. Measurements were performed typically with acquisition settings of 10 times for 10 seconds. The fluorescence fluctuations were auto-correlated with appropriate fitting formalisms. Data analysis allows determining the brightness, behavior and diffusion time of fluorescent particles in homogenous solution.

15

**Patent Claims**

1. An anti-human collagen II antibody comprising the CDRs determined according to Kabat of
  - a) SEQ ID NO: 09 and SEQ ID NO: 10, or
  - 5 b) SEQ ID NO: 12 and SEQ ID NO: 13, or
  - c) SEQ ID NO: 15 and SEQ ID NO: 16.
2. The antibody according to claim 1 comprising a heavy chain variable domain and a light chain variable domain of
  - a) SEQ ID NO: 09 and SEQ ID NO: 10, or
  - 10 b) SEQ ID NO: 12 and SEQ ID NO: 13, or
  - c) SEQ ID NO: 15 and SEQ ID NO: 16.
3. The antibody according to any one of claims 1 to 2, wherein the antibody is a scFv.
4. A pharmaceutical formulation comprising the antibody according to any one of claims 1 to 3 and optionally a pharmaceutically acceptable excipient.  
15
5. The formulation according to claim 4, wherein the pharmaceutical formulation is for use in the treatment of ocular vascular diseases.
6. The antibody according to any one of claims 1 to 3 for use as a medicament.
7. The antibody according to claim 6, wherein the use is for the treatment of ocular vascular diseases.  
20
8. The use of the antibody according to any one of claims 1 to 3 in the manufacture of a medicament.
9. The use according to claim 8, wherein the use is for the manufacture of a medicament for the treatment of ocular vascular disease.
- 25 10. The antibody according to any one of claims 1 to 3 for use in the treatment of ocular vascular disease.

11. A method of treatment of a patient suffering from ocular vascular diseases by administering the antibody according to any one of claims 1 to 3 to a patient in the need of such treatment.
12. A fusion protein comprising
  - 5 - a first binding site specifically binding to a first antigen,
  - a second binding site specifically binding to collagen II.
13. The fusion protein according to claim 12, wherein
  - the first binding site is a Fab specifically binding to a first antigen,
  - the second binding site is a scFv specifically binding to collagen II, and

10 wherein the Fab is conjugated by a peptide bond at one of its C-termini to the N-terminus of the peptidic linker and the scFv is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.
14. The fusion protein according to any one of claims 12 to 13, further comprising a third binding site specifically binding to a second antigen,

15 wherein

  - the third binding site is a scFv specifically binding to collagen II,

whereby the first and third binding sites are selected from the group consisting of F(ab')<sub>2</sub>, diabody, BITE, tandAb, and DART and the first and third binding site is conjugated by a peptide bond at their C-terminus to the N-

20 terminus of the peptidic linker and the scFv is conjugated by a peptide bond at its N-terminus to the C-terminus of the peptidic linker.
15. The fusion protein according to any one of claims 12 to 14, wherein the first antigen and/or the second antigen is a therapeutic ocular target.
16. The fusion protein according to any one of claims 12 to 15, wherein the first antigen and/or the second antigen are independently of each other selected  

25 from the group consisting of ANG2, VEGF, PDGF-B, and IL-1beta.

17. The fusion protein according to any one of claims 12 to 15, wherein the first antigen and/or the second antigen are different antigens selected from the group consisting of ANG2, VEGF, PDGF-B, and IL-1beta.
18. The fusion protein according to any one of claims 12 to 17, wherein the scFv specifically binding to collagen II comprises
- 5 a) a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 09 and a light chain variable domain of SEQ ID NO: 10, or
- b) a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain of SEQ ID NO: 13, or
- 10 c) a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 15 and a light chain variable domain of SEQ ID NO: 16.
19. The fusion protein according to claim 18, wherein the scFv specifically binding to collagen II comprises a heavy chain variable domain with the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain of
- 15 SEQ ID NO: 13.
20. The fusion protein according to any one of claims 12 to 19, wherein the scFv specifically binding to collagen II has the amino acid sequence of SEQ ID NO: 11 or SEQ ID NO: 14 or SEQ ID NO: 17.
21. The fusion protein according to claim 20, wherein the scFv specifically binding to collagen II has the amino acid sequence of SEQ ID NO: 14.
- 20
22. The fusion protein according to any one of claims 14 to 21, wherein the first binding site and the third binding site are Fabs.
23. A pharmaceutical formulation comprising the fusion protein according to any one of claims 12 to 22 and optionally a pharmaceutically acceptable excipient.
- 25
24. The formulation according to claim 23, wherein the pharmaceutical formulation is for use in the treatment of ocular vascular diseases.
25. The fusion protein according to any one of claims 12 to 22 for use as a medicament.

- 60 -

26. The fusion protein according to claim 25, wherein the use is for the treatment of ocular vascular diseases.
27. The use of the fusion protein according to any one of claims 12 to 22 in the manufacture of a medicament.
- 5 28. The use according to claim 27, wherein the use is for the manufacture of a medicament for the treatment of ocular vascular disease.
29. The fusion protein according to any one of claims 12 to 22 for use in the treatment of ocular vascular disease.
- 10 30. A method of treatment of a patient suffering from ocular vascular diseases by administering the fusion protein according to any one of claims 12 to 22 to a patient in the need of such treatment.

Figure 1

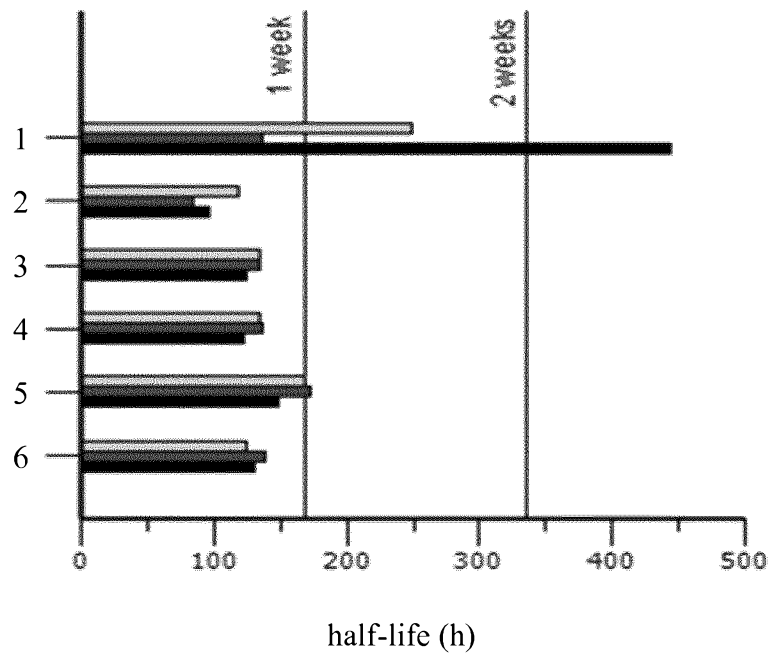
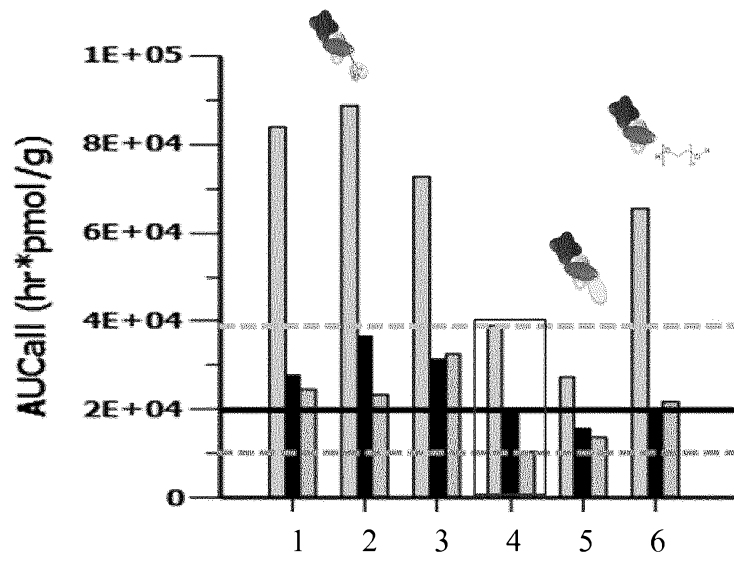


Figure 2



eolf-seq1 (2)  
SEQUENCE LISTING

<110> F. Hoffmann-La Roche AG  
<120> Fusion proteins for ophthalmology with increased eye retention  
<130> P33669-WO (ASK)  
<150> EP16173166.6  
<151> 2016-06-06  
<160> 24  
<170> PatentIn version 3.5  
<210> 1  
<211> 228  
<212> PRT  
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Ala Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
35 40 45  
Ser Ser Ile Asn Ile Gly Ala Thr Tyr Ile Tyr Tyr Ala Asp Ser Val  
50 55 60  
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  
65 70 75 80  
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
85 90 95  
Ala Arg Pro Gly Ser Pro Tyr Glu Tyr Asp Lys Ala Tyr Tyr Ser Met  
100 105 110  
Ala Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr  
115 120 125  
Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser  
130 135 140  
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
145 150 155 160



eof-seq1 (2)

Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His  
165 170 175

Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser  
180 185 190

Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys  
195 200 205

Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu  
210 215 220

Pro Lys Ser Cys  
225

<210> 2  
<211> 214  
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<400> 2

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Lys Asn Tyr  
20 25 30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
35 40 45

Tyr Tyr Ser Ser Thr Leu Leu Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ser Ile Thr Leu Pro Pro  
85 90 95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala  
100 105 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
115 120 125

eof-seq1 (2)

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
 130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
 145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
 165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
 180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
 195 200 205

Phe Asn Arg Gly Glu Cys  
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<210> 3

<211> 293

<212> PRT

<213> Artificial sequence

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<223> anti-digoxigenin Fab + HBD HC

<400> 3

Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Gly  
 1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr  
 20 25 30

Ala Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
 35 40 45

Ser Ser Ile Asn Ile Gly Ala Thr Tyr Ile Tyr Tyr Ala Asp Ser Val  
 50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  
 65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95

Ala Arg Pro Gly Ser Pro Tyr Glu Tyr Asp Lys Ala Tyr Tyr Ser Met  
 100 105 110

eo1f-seq1 (2)

Ala Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr  
115 120 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser  
130 135 140

Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
145 150 155 160

Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His  
165 170 175

Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser  
180 185 190

Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys  
195 200 205

Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu  
210 215 220

Pro Lys Ser Cys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ala Arg  
225 230 235 240

Gln Glu Asn Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe  
245 250 255

Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser  
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Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys  
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Asp Lys Pro Arg Arg  
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- <210> 4
- <211> 488
- <212> PRT
- <213> Artificial Sequence

- <220>
- <223> anti-digoxigenin Fab + anti-collagen II scFV I HC

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr  
Page 4

## eolf-seql (2)

20

25

30

Ala Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
 35 40 45

Ser Ser Ile Asn Ile Gly Ala Thr Tyr Ile Tyr Tyr Ala Asp Ser Val  
 50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  
 65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95

Ala Arg Pro Gly Ser Pro Tyr Glu Tyr Asp Lys Ala Tyr Tyr Ser Met  
 100 105 110

Ala Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr  
 115 120 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser  
 130 135 140

Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
 145 150 155 160

Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His  
 165 170 175

Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser  
 180 185 190

Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys  
 195 200 205

Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu  
 210 215 220

Pro Lys Ser Cys Gly Gly Gly Gly Ser Gly Gly Gly Gly ser Glu Ile  
 225 230 235 240

Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly Glu Arg  
 245 250 255

Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Arg Asp Phe Leu Ala  
 260 265 270

eo1f-seq1 (2)

Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile Tyr Gly  
 275 280 285

Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly  
 290 300

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro Glu Asp  
 305 310 315 320

Phe Ala Val Tyr Tyr Cys Gln Gln Arg Gly Ser Ala Pro Leu Thr Phe  
 325 330 335

Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly  
 340 345 350

Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln Val Gln  
 355 360 365

Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser Val Lys  
 370 375 380

Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr Ala Ile Ser  
 385 390 395 400

Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly Gly Ile  
 405 410 415

Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe Gln Gly Arg  
 420 425 430

Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr Met Glu Leu  
 435 440 445

Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Asp  
 450 455 460

Pro Asn Gly Asn Ile Val Leu Ser Glu Tyr Phe Asp Tyr Trp Gly Gln  
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Gly Thr Leu Val Thr Val Ser Ser  
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<211> 484

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<213> Artificial Sequence

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<223> anti-digoxigenin Fab + anti-collagen II scFV II HC

eof-seq1 (2)

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 1 5 10 15  
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr  
 20 25 30  
 Ala Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
 35 40 45  
 Ser Ser Ile Asn Ile Gly Ala Thr Tyr Ile Tyr Tyr Ala Asp Ser Val  
 50 55 60  
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  
 65 70 75 80  
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95  
 Ala Arg Pro Gly Ser Pro Tyr Glu Tyr Asp Lys Ala Tyr Tyr Ser Met  
 100 105 110  
 Ala Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr  
 115 120 125  
 Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser  
 130 135 140  
 Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
 145 150 155 160  
 Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His  
 165 170 175  
 Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser  
 180 185 190  
 Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys  
 195 200 205  
 Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu  
 210 215 220  
 Pro Lys Ser Cys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu Ile  
 225 230 235 240

eof-seq1 (2)

Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly Glu Arg  
 245 250 255

Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Asp Asp Trp Leu Ala  
 260 265 270

Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile Tyr Asp  
 275 280 285

Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly  
 290 295 300

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro Glu Asp  
 305 310 315 320

Phe Ala Val Tyr Tyr Cys Gln Gln Gly Asp Thr Ala Pro Ile Thr Phe  
 325 330 335

Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly  
 340 345 350

Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln Val Gln  
 355 360 365

Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser Val Lys  
 370 375 380

Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr Ala Ile Ser  
 385 390 395 400

Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly Gly Ile  
 405 410 415

Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe Gln Gly Arg  
 420 425 430

Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr Met Glu Leu  
 435 440 445

Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Asn  
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Ile Val Gly Asp Tyr Leu Leu Asp Tyr Trp Gly Gln Gly Thr Leu Val  
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Thr Val Ser Ser

eof-seq1 (2)

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 20 25 30  
 Ala Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
 35 40 45  
 Ser Ser Ile Asn Ile Gly Ala Thr Tyr Ile Tyr Tyr Ala Asp Ser Val  
 50 55 60  
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  
 65 70 75 80  
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95  
 Ala Arg Pro Gly Ser Pro Tyr Glu Tyr Asp Lys Ala Tyr Tyr Ser Met  
 100 105 110  
 Ala Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr  
 115 120 125  
 Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser  
 130 135 140  
 Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu  
 145 150 155 160  
 Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His  
 165 170 175  
 Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser  
 180 185 190  
 Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys  
 195 200 205



eolf-seq1 (2)

Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu  
 210 215 220

Pro Lys Ser Cys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu Ile  
 225 230 235 240

Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly Glu Arg  
 245 250 255

Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ala Asp Trp Leu Ala  
 260 265 270

Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile Tyr Asp  
 275 280 285

Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly  
 290 295 300

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro Glu Asp  
 305 310 315 320

Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Asp Gly Trp Pro Ile Thr Phe  
 325 330 335

Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly  
 340 345 350

Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln Val Gln  
 355 360 365

Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser Val Lys  
 370 375 380

Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr Ala Ile Ser  
 385 390 395 400

Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly Gly Ile  
 405 410 415

Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe Gln Gly Arg  
 420 425 430

Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr Met Glu Leu  
 435 440 445

Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg His  
 450 455 460

e0lf-seq1 (2)

Leu Tyr Tyr Met Leu Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val  
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Ser Ser

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<223> peptidic linker

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<223> peptidic linker

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Gly Gly Gly Ser  
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1 5 10 15

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20 25 30

Ala Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met  
35 40 45

eolf-seq1 (2)  
Gly Gly Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe  
50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr  
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys  
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Ala Arg Asp Pro Asn Gly Asn Ile Val Leu Ser Glu Tyr Phe Asp Tyr  
100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser  
115 120

<210> 10  
<211> 107  
<212> PRT  
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Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly  
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Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Arg Asp Phe  
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
35 40 45

Tyr Gly Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Gly Ser Ala Pro Leu  
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Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys  
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<220>

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20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
35 40 45

Tyr Gly Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Gly Ser Ala Pro Leu  
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly Ser  
100 105 110

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln  
115 120 125

Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser  
130 135 140

Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr Ala  
145 150 155 160

Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly  
165 170 175

Gly Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe Gln  
180 185 190

Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr Met  
195 200 205

Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala  
210 215 220

Arg Asp Pro Asn Gly Asn Ile Val Leu Ser Glu Tyr Phe Asp Tyr Trp  
225 230 235 240

eolf-seq1 (2)

Gly Gln Gly Thr Leu Val Thr Val Ser Ser  
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Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr  
20 25 30

Ala Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met  
35 40 45

Gly Gly Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe  
50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr  
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys  
85 90 95

Ala Arg Asn Ile Val Gly Asp Tyr Leu Leu Asp Tyr Trp Gly Gln Gly  
100 105 110

Thr Leu Val Thr Val Ser Ser  
115

<210> 13  
<211> 107  
<212> PRT  
<213> Artificial sequence

<220>  
<223> anti-collagen II scFV II VL

<400> 13

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly  
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Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Asp Asp Trp

eo1f-seq1 (2)

20

25

30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
 35 40 45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
 50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
 65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Gly Asp Thr Ala Pro Ile  
 85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys  
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<210> 14

<211> 246

<212> PRT

<213> Artificial Sequence

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<223> anti-collagen II scFV II scFv

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 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Asp Asp Trp  
 20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
 35 40 45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
 50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
 65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Gly Asp Thr Ala Pro Ile  
 85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly Ser  
 100 105 110

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln  
 115 120 125

eof-seq1 (2)

Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser  
 130 135 140

Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr Ala  
 145 150 155 160

Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly  
 165 170 175

Gly Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe Gln  
 180 185 190

Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr Met  
 195 200 205

Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala  
 210 215 220

Arg Asn Ile Val Gly Asp Tyr Leu Leu Asp Tyr Trp Gly Gln Gly Thr  
 225 230 235 240

Leu Val Thr Val Ser Ser  
 245

<210> 15  
 <211> 115  
 <212> PRT  
 <213> Artificial sequence

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 <223> anti-collagen II scFV III VH

<400> 15

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 1 5 10 15

Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr Ala Ile  
 20 25 30

Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly Gly  
 35 40 45

Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe Gln Gly  
 50 55 60

Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr Met Glu  
 65 70 75 80

e0lf-seq1 (2)

Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg  
85 90 95

His Leu Tyr Tyr Met Leu Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr  
100 105 110

Val Ser Ser  
115

<210> 16  
<211> 107  
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20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
35 40 45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Asp Gly Trp Pro Ile  
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys  
100 105

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<220>  
<223> anti-collagen II scFV III scFV

<400> 17

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eof-seq1 (2)

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                   20                                  25                                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile  
           35                                  40                                  45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly  
   50                                  55                                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro  
   65                                  70                                  75                                  80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Asp Gly Trp Pro Ile  
                   85                                  90                                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly Ser  
           100                                  105                                  110

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln  
   115                                  120                                  125

Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser  
   130                                  135                                  140

Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr Ala  
   145                                  150                                  155                                  160

Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly  
           165                                  170

Gly Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe Gln  
           180                                  185                                  190

Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr Met  
   195                                  200                                  205

Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala  
   210                                  215                                  220

Arg His Leu Tyr Tyr Met Leu Asp Tyr Trp Gly Gln Gly Thr Leu Val  
   225                                  230                                  235                                  240

Thr Val Ser Ser

eof-seq1 (2)

<211> 1418

<212> PRT

<213> Homo sapiens

<400> 18

Met Ile Arg Leu Gly Ala Pro Gln Thr Leu Val Leu Leu Thr Leu Leu  
1 5 10 15

Val Ala Ala Val Leu Arg Cys Gln Gly Gln Asp Val Arg Gln Pro Gly  
20 25 30

Pro Lys Gly Gln Lys Gly Glu Pro Gly Asp Ile Lys Asp Ile Val Gly  
35 40 45

Pro Lys Gly Pro Pro Gly Pro Gln Gly Pro Ala Gly Glu Gln Gly Pro  
50 55 60

Arg Gly Asp Arg Gly Asp Lys Gly Glu Lys Gly Ala Pro Gly Pro Arg  
65 70 75 80

Gly Arg Asp Gly Glu Pro Gly Thr Pro Gly Asn Pro Gly Pro Pro Gly  
85 90 95

Pro Pro Gly Pro Pro Gly Pro Pro Gly Leu Gly Gly Asn Phe Ala Ala  
100 105 110

Gln Met Ala Gly Gly Phe Asp Glu Lys Ala Gly Gly Ala Gln Leu Gly  
115 120 125

Val Met Gln Gly Pro Met Gly Pro Met Gly Pro Arg Gly Pro Pro Gly  
130 135 140

Pro Ala Gly Ala Pro Gly Pro Gln Gly Phe Gln Gly Asn Pro Gly Glu  
145 150 155 160

Pro Gly Glu Pro Gly Val Ser Gly Pro Met Gly Pro Arg Gly Pro Pro  
165 170 175

Gly Pro Pro Gly Lys Pro Gly Asp Asp Gly Glu Ala Gly Lys Pro Gly  
180 185 190

Lys Ala Gly Glu Arg Gly Pro Pro Gly Pro Gln Gly Ala Arg Gly Phe  
195 200 205

Pro Gly Thr Pro Gly Leu Pro Gly Val Lys Gly His Arg Gly Tyr Pro  
210 215 220

Gly Leu Asp Gly Ala Lys Gly Glu Ala Gly Ala Pro Gly Val Lys Gly



eo1f-seq1 (2)

Arg Pro Gly Glu Pro Gly Leu Pro Gly Ala Arg Gly Leu Thr Gly Arg  
 485 490 495

Pro Gly Asp Ala Gly Pro Gln Gly Lys Val Gly Pro Ser Gly Ala Pro  
 500 505 510

Gly Glu Asp Gly Arg Pro Gly Pro Pro Gly Pro Gln Gly Ala Arg Gly  
 515 520 525

Gln Pro Gly Val Met Gly Phe Pro Gly Pro Lys Gly Ala Asn Gly Glu  
 530 535 540

Pro Gly Lys Ala Gly Glu Lys Gly Leu Pro Gly Ala Pro Gly Leu Arg  
 545 550 555 560

Gly Leu Pro Gly Lys Asp Gly Glu Thr Gly Ala Ala Gly Pro Pro Gly  
 565 570 575

Pro Ala Gly Pro Ala Gly Glu Arg Gly Glu Gln Gly Ala Pro Gly Pro  
 580 585 590

Ser Gly Phe Gln Gly Leu Pro Gly Pro Pro Gly Pro Pro Gly Glu Gly  
 595 600 605

Gly Lys Pro Gly Asp Gln Gly Val Pro Gly Glu Ala Gly Ala Pro Gly  
 610 615 620

Leu Val Gly Pro Arg Gly Glu Arg Gly Phe Pro Gly Glu Arg Gly Ser  
 625 630 635 640

Pro Gly Ala Gln Gly Leu Gln Gly Pro Arg Gly Leu Pro Gly Thr Pro  
 645 650 655

Gly Thr Asp Gly Pro Lys Gly Ala Ser Gly Pro Ala Gly Pro Pro Gly  
 660 665 670

Ala Gln Gly Pro Pro Gly Leu Gln Gly Met Pro Gly Glu Arg Gly Ala  
 675 680 685

Ala Gly Ile Ala Gly Pro Lys Gly Asp Arg Gly Asp Val Gly Glu Lys  
 690 695 700

Gly Pro Glu Gly Ala Pro Gly Lys Asp Gly Gly Arg Gly Leu Thr Gly  
 705 710 715 720

Pro Ile Gly Pro Pro Gly Pro Ala Gly Ala Asn Gly Glu Lys Gly Glu  
 725 730 735

eof-seq1 (2)

Val Gly Pro Pro Gly Pro Ala Gly Ser Ala Gly Ala Arg Gly Ala Pro  
740 745 750

Gly Glu Arg Gly Glu Thr Gly Pro Pro Gly Pro Ala Gly Phe Ala Gly  
755 760 765

Pro Pro Gly Ala Asp Gly Gln Pro Gly Ala Lys Gly Glu Gln Gly Glu  
770 775 780

Ala Gly Gln Lys Gly Asp Ala Gly Ala Pro Gly Pro Gln Gly Pro Ser  
785 790 795 800

Gly Ala Pro Gly Pro Gln Gly Pro Thr Gly Val Thr Gly Pro Lys Gly  
805 810 815

Ala Arg Gly Ala Gln Gly Pro Pro Gly Ala Thr Gly Phe Pro Gly Ala  
820 825 830

Ala Gly Arg Val Gly Pro Pro Gly Ser Asn Gly Asn Pro Gly Pro Pro  
835 840 845

Gly Pro Pro Gly Pro Ser Gly Lys Asp Gly Pro Lys Gly Ala Arg Gly  
850 855 860

Asp Ser Gly Pro Pro Gly Arg Ala Gly Glu Pro Gly Leu Gln Gly Pro  
865 870 875 880

Ala Gly Pro Pro Gly Glu Lys Gly Glu Pro Gly Asp Asp Gly Pro Ser  
885 890 895

Gly Ala Glu Gly Pro Pro Gly Pro Gln Gly Leu Ala Gly Gln Arg Gly  
900 905 910

Ile Val Gly Leu Pro Gly Gln Arg Gly Glu Arg Gly Phe Pro Gly Leu  
915 920 925

Pro Gly Pro Ser Gly Glu Pro Gly Lys Gln Gly Ala Pro Gly Ala Ser  
930 935 940

Gly Asp Arg Gly Pro Pro Gly Pro Val Gly Pro Pro Gly Leu Thr Gly  
945 950 955 960

Pro Ala Gly Glu Pro Gly Arg Glu Gly Ser Pro Gly Ala Asp Gly Pro  
965 970 975

Pro Gly Arg Asp Gly Ala Ala Gly Val Lys Gly Asp Arg Gly Glu Thr  
980 985 990

eolf-seq1 (2)

Gly Ala Val Gly Ala Pro Gly Ala Pro Gly Pro Pro Gly Ser Pro Gly  
 995 1000 1005

Pro Ala Gly Pro Thr Gly Lys Gln Gly Asp Arg Gly Glu Ala Gly  
 1010 1015 1020

Ala Gln Gly Pro Met Gly Pro Ser Gly Pro Ala Gly Ala Arg Gly  
 1025 1030 1035

Ile Gln Gly Pro Gln Gly Pro Arg Gly Asp Lys Gly Glu Ala Gly  
 1040 1045 1050

Glu Pro Gly Glu Arg Gly Leu Lys Gly His Arg Gly Phe Thr Gly  
 1055 1060 1065

Leu Gln Gly Leu Pro Gly Pro Pro Gly Pro Ser Gly Asp Gln Gly  
 1070 1075 1080

Ala Ser Gly Pro Ala Gly Pro Ser Gly Pro Arg Gly Pro Pro Gly  
 1085 1090 1095

Pro Val Gly Pro Ser Gly Lys Asp Gly Ala Asn Gly Ile Pro Gly  
 1100 1105 1110

Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser Gly Glu Thr Gly  
 1115 1120 1125

Pro Ala Gly Pro Pro Gly Asn Pro Gly Pro Pro Gly Pro Pro Gly  
 1130 1135 1140

Pro Pro Gly Pro Gly Ile Asp Met Ser Ala Phe Ala Gly Leu Gly  
 1145 1150 1155

Pro Arg Glu Lys Gly Pro Asp Pro Leu Gln Tyr Met Arg Ala Asp  
 1160 1165 1170

Gln Ala Ala Gly Gly Leu Arg Gln His Asp Ala Glu Val Asp Ala  
 1175 1180 1185

Thr Leu Lys Ser Leu Asn Asn Gln Ile Glu Ser Ile Arg Ser Pro  
 1190 1195 1200

Glu Gly Ser Arg Lys Asn Pro Ala Arg Thr Cys Arg Asp Leu Lys  
 1205 1210 1215

Leu Cys His Pro Glu Trp Lys Ser Gly Asp Tyr Trp Ile Asp Pro

eof-seq1 (2)

1220 1225 1230

Asn Gln Gly Cys Thr Leu Asp Ala Met Lys Val Phe Cys Asn Met  
 1235 1240 1245

Glu Thr Gly Glu Thr Cys Val Tyr Pro Asn Pro Ala Asn Val Pro  
 1250 1255 1260

Lys Lys Asn Trp Trp Ser Ser Lys Ser Lys Glu Lys Lys His Ile  
 1265 1270 1275

Trp Phe Gly Glu Thr Ile Asn Gly Gly Phe His Phe Ser Tyr Gly  
 1280 1285 1290

Asp Asp Asn Leu Ala Pro Asn Thr Ala Asn Val Gln Met Thr Phe  
 1295 1300 1305

Leu Arg Leu Leu Ser Thr Glu Gly Ser Gln Asn Ile Thr Tyr His  
 1310 1315 1320

Cys Lys Asn Ser Ile Ala Tyr Leu Asp Glu Ala Ala Gly Asn Leu  
 1325 1330 1335

Lys Lys Ala Leu Leu Ile Gln Gly Ser Asn Asp Val Glu Ile Arg  
 1340 1345 1350

Ala Glu Gly Asn Ser Arg Phe Thr Tyr Thr Ala Leu Lys Asp Gly  
 1355 1360 1365

Cys Thr Lys His Thr Gly Lys Trp Gly Lys Thr Val Ile Glu Tyr  
 1370 1375 1380

Arg Ser Gln Lys Thr Ser Arg Leu Pro Ile Ile Asp Ile Ala Pro  
 1385 1390 1395

Met Asp Ile Gly Gly Pro Glu Gln Glu Phe Gly Val Asp Ile Gly  
 1400 1405 1410

Pro Val Cys Phe Leu  
 1415

<210> 19  
 <211> 1487  
 <212> PRT  
 <213> Homo sapiens

<400> 19

Met Ile Arg Leu Gly Ala Pro Gln Thr Leu Val Leu Leu Thr Leu Leu

eolf-seq1 (2)

1		5		10		15									
Val	Ala	Ala	Val	Leu	Arg	Cys	Gln	Gly	Gln	Asp	Val	Gln	Glu	Ala	Gly
		20						25					30		
Ser	Cys	Val	Gln	Asp	Gly	Gln	Arg	Tyr	Asn	Asp	Lys	Asp	Val	Trp	Lys
		35					40					45			
Pro	Glu	Pro	Cys	Arg	Ile	Cys	Val	Cys	Asp	Thr	Gly	Thr	Val	Leu	Cys
	50					55					60				
Asp	Asp	Ile	Ile	Cys	Glu	Asp	Val	Lys	Asp	Cys	Leu	Ser	Pro	Glu	Ile
65					70					75					80
Pro	Phe	Gly	Glu	Cys	Cys	Pro	Ile	Cys	Pro	Thr	Asp	Leu	Ala	Thr	Ala
				85					90					95	
Ser	Gly	Gln	Pro	Gly	Pro	Lys	Gly	Gln	Lys	Gly	Glu	Pro	Gly	Asp	Ile
			100					105					110		
Lys	Asp	Ile	Val	Gly	Pro	Lys	Gly	Pro	Pro	Gly	Pro	Gln	Gly	Pro	Ala
		115					120					125			
Gly	Glu	Gln	Gly	Pro	Arg	Gly	Asp	Arg	Gly	Asp	Lys	Gly	Glu	Lys	Gly
	130					135					140				
Ala	Pro	Gly	Pro	Arg	Gly	Arg	Asp	Gly	Glu	Pro	Gly	Thr	Pro	Gly	Asn
145					150					155					160
Pro	Gly	Pro	Pro	Gly	Pro	Pro	Gly	Pro	Pro	Gly	Pro	Pro	Gly	Leu	Gly
				165					170					175	
Gly	Asn	Phe	Ala	Ala	Gln	Met	Ala	Gly	Gly	Phe	Asp	Glu	Lys	Ala	Gly
			180					185					190		
Gly	Ala	Gln	Leu	Gly	Val	Met	Gln	Gly	Pro	Met	Gly	Pro	Met	Gly	Pro
		195					200					205			
Arg	Gly	Pro	Pro	Gly	Pro	Ala	Gly	Ala	Pro	Gly	Pro	Gln	Gly	Phe	Gln
	210					215					220				
Gly	Asn	Pro	Gly	Glu	Pro	Gly	Glu	Pro	Gly	Val	Ser	Gly	Pro	Met	Gly
225					230					235					240
Pro	Arg	Gly	Pro	Pro	Gly	Pro	Pro	Gly	Lys	Pro	Gly	Asp	Asp	Gly	Glu
				245					250					255	



eof-seq1 (2)

Ala Gly Lys Pro Gly Lys Ala Gly Glu Arg Gly Pro Pro Gly Pro Gln  
 260 265 270

Gly Ala Arg Gly Phe Pro Gly Thr Pro Gly Leu Pro Gly Val Lys Gly  
 275 280 285

His Arg Gly Tyr Pro Gly Leu Asp Gly Ala Lys Gly Glu Ala Gly Ala  
 290 295 300

Pro Gly Val Lys Gly Glu Ser Gly Ser Pro Gly Glu Asn Gly Ser Pro  
 305 310 315 320

Gly Pro Met Gly Pro Arg Gly Leu Pro Gly Glu Arg Gly Arg Thr Gly  
 325 330 335

Pro Ala Gly Ala Ala Gly Ala Arg Gly Asn Asp Gly Gln Pro Gly Pro  
 340 345 350

Ala Gly Pro Pro Gly Pro Val Gly Pro Ala Gly Gly Pro Gly Phe Pro  
 355 360 365

Gly Ala Pro Gly Ala Lys Gly Glu Ala Gly Pro Thr Gly Ala Arg Gly  
 370 375 380

Pro Glu Gly Ala Gln Gly Pro Arg Gly Glu Pro Gly Thr Pro Gly Ser  
 385 390 395 400

Pro Gly Pro Ala Gly Ala Ser Gly Asn Pro Gly Thr Asp Gly Ile Pro  
 405 410 415

Gly Ala Lys Gly Ser Ala Gly Ala Pro Gly Ile Ala Gly Ala Pro Gly  
 420 425 430

Phe Pro Gly Pro Arg Gly Pro Pro Gly Pro Gln Gly Ala Thr Gly Pro  
 435 440 445

Leu Gly Pro Lys Gly Gln Thr Gly Glu Pro Gly Ile Ala Gly Phe Lys  
 450 455 460

Gly Glu Gln Gly Pro Lys Gly Glu Pro Gly Pro Ala Gly Pro Gln Gly  
 465 470 475 480

Ala Pro Gly Pro Ala Gly Glu Glu Gly Lys Arg Gly Ala Arg Gly Glu  
 485 490 495

Pro Gly Gly Val Gly Pro Ile Gly Pro Pro Gly Glu Arg Gly Ala Pro  
 500 505 510

eof-seq1 (2)

Gly Asn Arg Gly Phe Pro Gly Gln Asp Gly Leu Ala Gly Pro Lys Gly  
515 520 525

Ala Pro Gly Glu Arg Gly Pro Ser Gly Leu Ala Gly Pro Lys Gly Ala  
530 535 540

Asn Gly Asp Pro Gly Arg Pro Gly Glu Pro Gly Leu Pro Gly Ala Arg  
545 550 555 560

Gly Leu Thr Gly Arg Pro Gly Asp Ala Gly Pro Gln Gly Lys Val Gly  
565 570 575

Pro Ser Gly Ala Pro Gly Glu Asp Gly Arg Pro Gly Pro Pro Gly Pro  
580 585 590

Gln Gly Ala Arg Gly Gln Pro Gly Val Met Gly Phe Pro Gly Pro Lys  
595 600 605

Gly Ala Asn Gly Glu Pro Gly Lys Ala Gly Glu Lys Gly Leu Pro Gly  
610 615 620

Ala Pro Gly Leu Arg Gly Leu Pro Gly Lys Asp Gly Glu Thr Gly Ala  
625 630 635 640

Ala Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Glu Arg Gly Glu Gln  
645 650 655

Gly Ala Pro Gly Pro Ser Gly Phe Gln Gly Leu Pro Gly Pro Pro Gly  
660 665 670

Pro Pro Gly Glu Gly Gly Lys Pro Gly Asp Gln Gly Val Pro Gly Glu  
675 680 685

Ala Gly Ala Pro Gly Leu Val Gly Pro Arg Gly Glu Arg Gly Phe Pro  
690 695 700

Gly Glu Arg Gly Ser Pro Gly Ala Gln Gly Leu Gln Gly Pro Arg Gly  
705 710 715 720

Leu Pro Gly Thr Pro Gly Thr Asp Gly Pro Lys Gly Ala Ser Gly Pro  
725 730 735

Ala Gly Pro Pro Gly Ala Gln Gly Pro Pro Gly Leu Gln Gly Met Pro  
740 745 750

Gly Glu Arg Gly Ala Ala Gly Ile Ala Gly Pro Lys Gly Asp Arg Gly  
755 760 765

eof-seq1 (2)

Asp Val Gly Glu Lys Gly Pro Glu Gly Ala Pro Gly Lys Asp Gly Gly  
770 775 780

Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro Ala Gly Ala Asn  
785 790 795 800

Gly Glu Lys Gly Glu Val Gly Pro Pro Gly Pro Ala Gly Ser Ala Gly  
805 810 815

Ala Arg Gly Ala Pro Gly Glu Arg Gly Glu Thr Gly Pro Pro Gly Pro  
820 825 830

Ala Gly Phe Ala Gly Pro Pro Gly Ala Asp Gly Gln Pro Gly Ala Lys  
835 840 845

Gly Glu Gln Gly Glu Ala Gly Gln Lys Gly Asp Ala Gly Ala Pro Gly  
850 855 860

Pro Gln Gly Pro Ser Gly Ala Pro Gly Pro Gln Gly Pro Thr Gly Val  
865 870 875 880

Thr Gly Pro Lys Gly Ala Arg Gly Ala Gln Gly Pro Pro Gly Ala Thr  
885 890 895

Gly Phe Pro Gly Ala Ala Gly Arg Val Gly Pro Pro Gly Ser Asn Gly  
900 905 910

Asn Pro Gly Pro Pro Gly Pro Pro Gly Pro Ser Gly Lys Asp Gly Pro  
915 920 925

Lys Gly Ala Arg Gly Asp Ser Gly Pro Pro Gly Arg Ala Gly Glu Pro  
930 935 940

Gly Leu Gln Gly Pro Ala Gly Pro Pro Gly Glu Lys Gly Glu Pro Gly  
945 950 955 960

Asp Asp Gly Pro Ser Gly Ala Glu Gly Pro Pro Gly Pro Gln Gly Leu  
965 970 975

Ala Gly Gln Arg Gly Ile Val Gly Leu Pro Gly Gln Arg Gly Glu Arg  
980 985 990

Gly Phe Pro Gly Leu Pro Gly Pro Ser Gly Glu Pro Gly Lys Gln Gly  
995 1000 1005

Ala Pro Gly Ala Ser Gly Asp Arg Gly Pro Pro Gly Pro Val Gly

eof-seq1 (2)

1010						1015									1020
Pro	Pro	Gly	Leu	Thr	Gly	Pro	Ala	Gly	Glu	Pro	Gly	Arg	Glu	Gly	
1025						1030					1035				
Ser	Pro	Gly	Ala	Asp	Gly	Pro	Pro	Gly	Arg	Asp	Gly	Ala	Ala	Gly	
1040						1045					1050				
Val	Lys	Gly	Asp	Arg	Gly	Glu	Thr	Gly	Ala	Val	Gly	Ala	Pro	Gly	
1055						1060					1065				
Ala	Pro	Gly	Pro	Pro	Gly	Ser	Pro	Gly	Pro	Ala	Gly	Pro	Thr	Gly	
1070						1075					1080				
Lys	Gln	Gly	Asp	Arg	Gly	Glu	Ala	Gly	Ala	Gln	Gly	Pro	Met	Gly	
1085						1090					1095				
Pro	Ser	Gly	Pro	Ala	Gly	Ala	Arg	Gly	Ile	Gln	Gly	Pro	Gln	Gly	
1100						1105					1110				
Pro	Arg	Gly	Asp	Lys	Gly	Glu	Ala	Gly	Glu	Pro	Gly	Glu	Arg	Gly	
1115						1120					1125				
Leu	Lys	Gly	His	Arg	Gly	Phe	Thr	Gly	Leu	Gln	Gly	Leu	Pro	Gly	
1130						1135					1140				
Pro	Pro	Gly	Pro	Ser	Gly	Asp	Gln	Gly	Ala	Ser	Gly	Pro	Ala	Gly	
1145						1150					1155				
Pro	Ser	Gly	Pro	Arg	Gly	Pro	Pro	Gly	Pro	Val	Gly	Pro	Ser	Gly	
1160						1165					1170				
Lys	Asp	Gly	Ala	Asn	Gly	Ile	Pro	Gly	Pro	Ile	Gly	Pro	Pro	Gly	
1175						1180					1185				
Pro	Arg	Gly	Arg	Ser	Gly	Glu	Thr	Gly	Pro	Ala	Gly	Pro	Pro	Gly	
1190						1195					1200				
Asn	Pro	Gly	Pro	Pro	Gly	Pro	Pro	Gly	Pro	Pro	Gly	Pro	Gly	Ile	
1205						1210					1215				
Asp	Met	Ser	Ala	Phe	Ala	Gly	Leu	Gly	Pro	Arg	Glu	Lys	Gly	Pro	
1220						1225					1230				
Asp	Pro	Leu	Gln	Tyr	Met	Arg	Ala	Asp	Gln	Ala	Ala	Gly	Gly	Leu	
1235						1240					1245				

eolf-seq1 (2)

Arg Gln His Asp Ala Glu Val Asp Ala Thr Leu Lys Ser Leu Asn  
 1250 1255 1260

Asn Gln Ile Glu Ser Ile Arg Ser Pro Glu Gly Ser Arg Lys Asn  
 1265 1270 1275

Pro Ala Arg Thr Cys Arg Asp Leu Lys Leu Cys His Pro Glu Trp  
 1280 1285 1290

Lys Ser Gly Asp Tyr Trp Ile Asp Pro Asn Gln Gly Cys Thr Leu  
 1295 1300 1305

Asp Ala Met Lys Val Phe Cys Asn Met Glu Thr Gly Glu Thr Cys  
 1310 1315 1320

Val Tyr Pro Asn Pro Ala Asn Val Pro Lys Lys Asn Trp Trp Ser  
 1325 1330 1335

Ser Lys Ser Lys Glu Lys Lys His Ile Trp Phe Gly Glu Thr Ile  
 1340 1345 1350

Asn Gly Gly Phe His Phe Ser Tyr Gly Asp Asp Asn Leu Ala Pro  
 1355 1360 1365

Asn Thr Ala Asn Val Gln Met Thr Phe Leu Arg Leu Leu Ser Thr  
 1370 1375 1380

Glu Gly Ser Gln Asn Ile Thr Tyr His Cys Lys Asn Ser Ile Ala  
 1385 1390 1395

Tyr Leu Asp Glu Ala Ala Gly Asn Leu Lys Lys Ala Leu Leu Ile  
 1400 1405 1410

Gln Gly Ser Asn Asp Val Glu Ile Arg Ala Glu Gly Asn Ser Arg  
 1415 1420 1425

Phe Thr Tyr Thr Ala Leu Lys Asp Gly Cys Thr Lys His Thr Gly  
 1430 1435 1440

Lys Trp Gly Lys Thr Val Ile Glu Tyr Arg Ser Gln Lys Thr Ser  
 1445 1450 1455

Arg Leu Pro Ile Ile Asp Ile Ala Pro Met Asp Ile Gly Gly Pro  
 1460 1465 1470

Glu Gln Glu Phe Gly Val Asp Ile Gly Pro Val Cys Phe Leu  
 1475 1480 1485

eo1f-seq1 (2)

<210> 20  
 <211> 268  
 <212> PRT  
 <213> Homo sapiens

<400> 20

Met Ser Ala Phe Ala Gly Leu Gly Pro Arg Glu Lys Gly Pro Asp Pro  
 1 5 10 15

Leu Gln Tyr Met Arg Ala Asp Gln Ala Ala Gly Gly Leu Arg Gln His  
 20 25 30

Asp Ala Glu Val Asp Ala Thr Leu Lys Ser Leu Asn Asn Gln Ile Glu  
 35 40 45

Ser Ile Arg Ser Pro Glu Gly Ser Arg Lys Asn Pro Ala Arg Thr Cys  
 50 55 60

Arg Asp Leu Lys Leu Cys His Pro Glu Trp Lys Ser Gly Asp Tyr Trp  
 65 70 75 80

Ile Asp Pro Asn Gln Gly Cys Thr Leu Asp Ala Met Lys Val Phe Cys  
 85 90 95

Asn Met Glu Thr Gly Glu Thr Cys Val Tyr Pro Asn Pro Ala Asn Val  
 100 105 110

Pro Lys Lys Asn Trp Trp Ser Ser Lys Ser Lys Glu Lys Lys His Ile  
 115 120 125

Trp Phe Gly Glu Thr Ile Asn Gly Gly Phe His Phe Ser Tyr Gly Asp  
 130 135 140

Asp Asn Leu Ala Pro Asn Thr Ala Asn Val Gln Met Thr Phe Leu Arg  
 145 150 155 160

Leu Leu Ser Thr Glu Gly Ser Gln Asn Ile Thr Tyr His Cys Lys Asn  
 165 170 175

Ser Ile Ala Tyr Leu Asp Glu Ala Ala Gly Asn Leu Lys Lys Ala Leu  
 180 185 190

Leu Ile Gln Gly Ser Asn Asp Val Glu Ile Arg Ala Glu Gly Asn Ser  
 195 200 205

Arg Phe Thr Tyr Thr Ala Leu Lys Asp Gly Cys Thr Lys His Thr Gly  
 210 215 220

eolf-seq1 (2)

Lys Trp Gly Lys Thr Val Ile Glu Tyr Arg Ser Gln Lys Thr Ser Arg  
225 230 235 240

Leu Pro Ile Ile Asp Ile Ala Pro Met Asp Ile Gly Gly Pro Glu Gln  
245 250 255

Glu Phe Gly Val Asp Ile Gly Pro Val Cys Phe Leu  
260 265

<210> 21  
<211> 2  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> linker monomer

<400> 21

Gly Ser  
1

<210> 22  
<211> 3  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> linker monomer

<400> 22

Gly Gly Ser  
1

<210> 23  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> linker monomer

<400> 23

Gly Gly Gly Ser  
1

<210> 24  
<211> 5  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> linker monomer

eof-seq1 (2)

<400> 24

Gly Gly Gly Gly Ser  
1 5