

VH3 BINDING SEPARATION MATRIX AND METHODS FOR USING THE SAME

TECHNICAL FIELD

The present invention relates to the field of separation of biomolecules. More specifically, it relates
5 to a separation matrix for affinity chromatography and separation of biomolecules based on the
presence of a VH3 chain, such as immunoglobulins and immunoglobulin fractions. The invention also
relates to methods of using said separation matrix.

BACKGROUND

Immunoglobulins and immunoglobulin fragments represent the most prevalent biopharmaceutical
10 products in either manufacture or development worldwide. The high commercial demand for, and
hence value of, this particular therapeutic market has led to the emphasis being placed on
pharmaceutical companies to maximize the productivity of their respective manufacturing processes
whilst controlling the associated costs.

Affinity chromatography is used in most cases, as one of the key steps in the purification of these
15 immunoglobulin molecules, such as monoclonal or polyclonal antibodies, or fragments thereof. A
particularly interesting class of affinity reagents is proteins capable of specific binding to invariable
parts of an immunoglobulin molecule, such interaction being independent of the antigen-binding
specificity of the antibody. Such reagents can be widely used for affinity chromatography recovery of
immunoglobulins from different samples, such as but not limited to serum or plasma preparations or
20 cell culture derived feed stocks. An example of such a protein is staphylococcal protein A (SpA),
containing domains capable of binding to the Fc and Fab portions of IgG immunoglobulins from
different species. These domains are commonly denoted as the E-, D-, A-, B- and C-domains.

SpA-based proteins have, due to their high affinity and selectivity, found a widespread use in the field
of biotechnology, e.g. as ligands in affinity chromatography for capture and purification of antibodies
25 as well as for detection or quantification. At present, SpA-based affinity medium is probably the most
widely used affinity medium for isolation of monoclonal antibodies and their fragments from
different samples including industrial cell culture supernatants. Accordingly, various matrices
comprising protein A or protein A-derived ligands are commercially available, for example, in the
form of MabSelect™ SuRe, MabSelect™ SuRe LX, MabSelect Prisma™ and HiScreen Fibro™ Prisma
30 from Cytiva™, Uppsala, Sweden. Certain Protein A and Protein A-derived ligands have binding affinity
for both the Fc part of an antibody and for some VH domains of antibodies, in particular VH3. As a

result, co-purification of product-related impurities such as half-antibodies and truncated variants may occur and require elution schemes which are complex and/or not sufficiently mild. Additionally, the purification of bispecific antibodies, such as emicizumab, or fragments thereof requires complex elution schemes to ensure that only correctly paired bispecific antibodies or fragments thereof are
5 obtained instead of a mixture of correctly and incorrectly paired antibodies and fragments. Thus, there is an unmet need in the field for simplified and reliable isolation methods for antibodies and fragments thereof.

SUMMARY OF THE INVENTION

10 It is an objective of the present disclosure to provide a VH3-binding affinity separation matrix which exhibits desirable alkali clean stability properties.

It is further an objective of the present disclosure to provide a VH3-binding affinity separation matrix that exhibits no or significantly reduced binding to the Fc region of immunoglobulins.

The above objectives have been attained by providing a separation matrix for purification of
15 antibodies or antibody fragments comprising at least one VH3 chain, said separation matrix comprising a VH3 binding ligand coupled to a porous support, wherein said VH3 binding ligand comprises at least one VH3 binding polypeptide having a significantly reduced or abolished binding affinity for a Fc fragment, and wherein the porous support is in beaded or particle form.

The VH3 binding ligand may preferably comprise at least one VH3 binding polypeptide based on any
20 of the native Protein A domains A_{wt} (SEQ ID NO 92), B_{wt} (SEQ ID NO 91), C_{wt} (SEQ ID NO 90), D_{wt} (SEQ ID NO 93), E_{wt} (SEQ ID NO 94), or the engineered Protein A domains Z_{wt} (SEQ ID NO 89), Z_{var} (SEQ ID NO 88) or Z_{var2} (SEQ ID NO 95),

wherein the amino acid at the position corresponding to position 9 in SEQ ID NO 89 is selected from Q, Y and A, the amino acid at the position corresponding to position 10 in SEQ ID NO 89 is selected
25 from Q and Y, the amino acid at the position corresponding to position 11 in SEQ ID NO 89 is selected from T, E and R, the amino acid at the position corresponding to position 13 in SEQ ID NO 89 is selected from L, E, R, A and Q, , the amino acid at the position corresponding to position 14 in SEQ ID NO 89 is selected from L, E, R, A, Q and W, the amino acid at the position corresponding to position 17 in SEQ ID NO 89 is selected from A, H and L, the amino acid at the position corresponding to
30 position 18 in SEQ ID NO 89 is selected from R, L and H, the amino acid at the position corresponding to position 26 in SEQ ID NO 89 is selected from Q and S, the amino acid at the position corresponding

to position 28 in SEQ ID NO 89 is selected from N and A, and the amino acid at the position corresponding to position 29 in SEQ ID NO 89 is selected from A and G.

The porous support may comprise polymer particles having a Dry solids weight (D_w) of 50-200 mg/ml, a volume-weighted median diameter (D_{50v}) of 30-100 μm . The polymer particles may be cross-linked.

The separation matrix may comprises at least 12 mg/ml VH3 binding ligands, such as at least 14 mg/ml, such as at least 14.5 mg/ml, at least 15 mg/ml, at least 15.5 mg/ml, at least 16 mg/ml, at least 16.5 mg/ml, at least 17 mg/ml, at least 17.5 mg/ml, at least 18 mg/ml, at least 18.5 mg/ml, at least 19 mg/ml, at least 19.5 mg/ml, at least 20 mg/ml, at least 20.5 mg/ml, at least 21 mg/ml, at least 21.5 mg/ml, or at least 22 mg/ml VH3 binding ligands.

The porous support may have a D_w of 50-150 mg/ml, 50-120 mg/ml, 50-100 mg/ml, 50-90 mg/ml, 60-80 mg/ml, or 60-75 mg/ml, such as at least 63 mg/ml, or at least 65 mg/ml, or at least 70 mg/ml.

The porous support may have a volume-weighted median diameter (D_{50v}) of 35-90 μm , 40-80 μm , 50-70 μm , 55-70 μm , 55-67 μm , 58-70 μm , or 58-67 μm , such as at least 60 μm , or at least 62 μm .

The porous support may have a K_d value, measured by inverse size exclusion chromatography with dextran of Mw 110 kDa as a probe molecule, of 0.6-0.95, such as a K_d value of 0.7-0.9, or a K_d value of 0.6-0.8, such as a K_d value of about 0,67, or a K_d value of about 0,72, or a K_d value of about 0,75.

In a variant of the separation matrix according to the above, the amino acids at positions 9/10/11 are QQT, QYT, YQT, AQE, AQR or AYR; preferably QQT, QYT, AQE, AQR or AYR; more preferably AQE, AQR or AYR; most preferably AQR or AYR. In a variant of the separation matrix according to the above, the amino acids at positions 17/18 are AR, HL or LH; preferably HL or AR. In a variant of the separation matrix according to the above, the amino acids at positions 28/29 are NA, NG, AA or AG. In a variant of the separation matrix according to the above, independently of each other, the amino acids at positions 9/10/11 are AQE, AQR or AYR; and the amino acids at positions 13/14 are LA, AA, AE, AL, AQ, AR, EA, EE, EL, EQ, ER, LA, LE, LL, LQ, LR, QA, QE, QL, QQ, QR, RA, RE, RL, RQ, RR or LW; and the amino acids at positions 17/18 are LH or AR. In a variant of the separation matrix according to the above, the amino acids at positions 9/10/11/13/14/17/18 are QQTLALH, QYTLALH, YQTLALH, QQTLAAR, AQELALH, AYRLALH, AYRLWLH, AYRLWAR, AYRLAHL and AYRLWHL AQRLALH, AYRLAAR. In a variant of the separation matrix according to the above, the amino acids at positions 26/28/29 are QNG, QAA, QAG, QNA, or SAG; preferably QNG, QNA, SAG or QAG. In a variant of the separation matrix according to the above, the VH3 binding polypeptide is selected from SEQ ID NO:1 – SEQ ID

NO:87 and SEQ ID NO: 99- SEQ ID NO:107, SEQ ID NO:111-SEQ ID NO:112, SEQ ID NO: 114-SEQ ID NO: 120, SEQ ID NO: 124-SEQ ID NO: 125 and SEQ ID NO:127-SEQ ID NO:144.

The VH3 binding ligand may comprise multimers of the polypeptide, said multimers comprising at least two polypeptides. According to one variant, the polypeptides are chosen from the group consisting of SEQ ID NO:1 – SEQ ID NO:87, SEQ ID NO: 99- SEQ ID NO:107, SEQ ID NO:111-SEQ ID NO:112, SEQ ID NO: 114-SEQ ID NO: 120, SEQ ID NO: 124-SEQ ID NO: 125 and SEQ ID NO:127-SEQ ID NO:144. The multimers may be homodimers or heterodimers. The multimer may be a dimer, a trimer, a tetramer, a pentamer, a hexamer, a heptamer, an octamer or a nonamer. In one variant of the separation matrix according to the above, the VH3-binding ligand comprises any one of the polypeptides according to SEQ ID NO 96, SEQ ID NO 97 or SEQ ID NO 98.

The ligand may comprise a coupling element, said coupling element being one or more cysteine residues, one or more lysine residues, or one or more histidine residues at the C-terminal end of the ligand. The ligand preferably comprises one or more cysteine residues at the C-terminal end of the ligand.

Also provided by the present disclosure is a method for isolation of antibodies or antibody fragments comprising at least one VH3 chain, comprising the steps of:

- a) contacting a liquid sample comprising a VH3 chain-containing antibody or antibody fragment with a separation matrix;
- b) washing said separation matrix with one or a combination of several washing liquids;
- c) eluting the VH3 chain-containing antibody or antibody fragment from the separation matrix with an elution liquid; and
- d) cleaning the separation matrix with a cleaning liquid;

wherein the separation matrix is according to the above.

With the method for isolation disclosed above, an IgG capacity after 24 h incubation in 0.5 M NaOH at 22 +/- 2 °C may be at least 90% of the IgG capacity before the incubation, such as at least 91%, or at least 92%, or at least 93%. With the method for isolation disclosed above, an VHH capacity after 24 h incubation in 0.5 M NaOH at 22 +/- 2 °C may be at least 90% of the VHH capacity before the incubation, such as at least 91%, or at least 92%, or at least 93%. With the method for isolation disclosed above, an IgG capacity after 50 h incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 80% of the IgG capacity before the incubation, such as at least 81%, or at least 82%, or at least 83%, or at least 84%.

Additionally, the present disclosure provides for a method for separation of bispecific antibodies or antibody fragments comprising one VH3 chain from variants of the antibody or antibody fragment comprising two VH3 chains or no VH3 chain, comprising the steps of:

- a) contacting a liquid sample comprising said bispecific antibodies with a separation matrix,
- 5 b) washing said separation matrix with one or a combination of several washing liquids,
- c) eluting said bispecific antibody from the separation matrix with an elution liquid and at a decreasing pH and
- d) cleaning the separation matrix with a cleaning liquid,

wherein the separation matrix is according to the above.

- 10 With the method for separation disclosed above, an IgG capacity after 24 h incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 90% of the IgG capacity before the incubation, such as at least 91%, or at least 92%, or at least 93%. With the method for separation disclosed above, an IgG capacity after 50 h incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 80% of the IgG capacity before the incubation, such as at least 81%, or at least 82%, or at least 83%, or at least 84%.

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FIGURES

- Fig. 1A shows Biacore analysis of inventive VH3 binding polypeptides (SEQ ID NO:1 to 4) using 5000 nM trastuzumab Fc fragment as analyte in comparison to an alkali clean stabile positive control that binds both Fc and Fab fragments of trastuzumab (SEQ ID NO:88) and a non-Fc-binding control (SEQ
- 20 ID NO:180). For the purpose of clear representation, results of the Biacore analysis are shown in two graphs in Fig. 1A, one including and one excluding data corresponding to SEQ ID NO:88.

Fig. 1B shows Biacore analysis of inventive VH3 binding polypeptides (SEQ ID NO:1 to 4) using 5000 nM trastuzumab Fab fragment as analyte in comparison to a non-Fc-binding control (SEQ ID NO:180).

- Fig. 2A shows Biacore analysis of inventive VH3 binding polypeptides (SEQ ID NO:5 to 12) using 5000
- 25 nM trastuzumab Fc fragment as analyte in comparison to an alkali clean stabile positive control that binds both Fc and Fab fragments of trastuzumab (SEQ ID NO:88). For the purpose of clear representation, results of the Biacore analysis are shown in two graphs in Fig. 2A, one including and one excluding data corresponding to SEQ ID NO:88.

- Fig. 2B shows Biacore analysis of inventive VH3 binding polypeptides (SEQ ID NO:5 to 12) using 625
- 30 nM trastuzumab as analyte in comparison to an alkali clean stabile positive control that binds both Fc

and Fab fragments of trastuzumab (SEQ ID NO:88). For the purpose of clear representation, results of the Biacore analysis are shown in two graphs in Fig. 2B, one including and one excluding data corresponding to SEQ ID NO:88.

Fig. 3A shows Biacore analysis of inventive VH3 binding polypeptides (SEQ ID NO:13 to 15) using 5000 nM trastuzumab Fc fragment as analyte in comparison to an alkali clean stabile positive control that binds both Fc and Fab fragments of trastuzumab (SEQ ID NO:88). For the purpose of clear representation, results of the Biacore analysis are shown in two graphs in Fig. 3A, one including and one excluding data corresponding to SEQ ID NO:88.

Fig. 3B shows Biacore analysis of inventive VH3 binding polypeptides (SEQ ID NO:10 and SEQ ID NO:13 to 15) using 2500 nM trastuzumab Fab fragment as analyte in comparison to an alkali clean stabile positive control that binds both Fc and Fab fragments of trastuzumab (SEQ ID NO:88) and a non-Fc-binding control that is not alkali clean stabile (Negative CTRL).

Fig. 4 shows an overlay of chromatograms for Pembrolizumab, Guselkumab, Cetuximab and Rituximab on a prototype separation matrix according to the present disclosure.

Fig. 5A shows a comparison of the Dynamic Binding Capacity for Trastuzumab at 6min RT for the prototype (denoted VH3), using MabSelect Prisma™ (Cytiva™), Praesto® Jetted A50 (Purolite) and JSR Amsphere™ A3 (JSR Life Sciences).

Fig. 5B shows a comparison of the Dynamic Binding Capacity for the Fab fragment of Trastuzumab at 6min RT for the prototype (denoted VH3), using MabSelect Prisma™ (Cytiva™), Praesto® Jetted A50 (Purolite) and JSR Amsphere™ A3 (JSR Life Sciences).

Fig. 5C shows a comparison of the Dynamic Binding Capacity for the VHH-EgA1 at 6min RT for the prototype (denoted VH3), using MabSelect Prisma™ (Cytiva™), Praesto® Jetted A50 (Purolite) and JSR Amsphere™ A3 (JSR Life Sciences).

Fig. 6 shows the possible pairings of VH chains occurring in a production of bispecific antibodies. Fig 6A shows a heterodimeric, bispecific antibody with one VH1 chain and one VH3 chain Fig. 6B shows a homodimeric antibody with two VH1 chains. Fig. 6C shows a homodimeric antibody with two VH3 chains.

Fig. 7 shows a chromatogram for a bispecific antibody, Herceptin® (Emicizuma) on a prototype separation matrix according to the present disclosure with impaired Fc binding.

Fig. 8A shows the Dynamic Binding Capacity of a VH3 prototype resin for Trastuzumab and VHH over 100 cycles of CIP at 0.5M NaOH.

Fig. 8B shows the Dynamic Binding Capacity of a VH3 prototype resin for Trastuzumab over 200 cycles of CIP at 0.5 M NaOH.

DEFINITIONS

- 5 The terms “antibody” and “immunoglobulin” (abbreviated Ig) may be used interchangeably herein and refers to an antigen-binding protein having a basic four-polypeptide chain structure consisting of two heavy (H) chains and two light (L) chains, said chains being stabilized by interchain or intrachain disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region (CH). The CH region may comprise three different domains,
- 10 CH1, CH2 and CH3. The VH region may comprise three different domains, VH1, VH2 and VH3. Each light chain is comprised of a light chain variable region (VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. There are two types of light chain in humans, kappa chain and lambda chain. The term is to be understood to include any antibody, including but not limited to monoclonal antibodies, bi-specific antibodies, and multi-specific
- 15 antibodies, as well as fragments of antibodies, fusion proteins comprising antibodies or antibody fragments and conjugates comprising antibodies or antibody fragments, such as Antibody-Drug Conjugates (ADC).

The term “mAb” stands for monoclonal antibody.

- The term “Fc region” refers to a C-terminal region of an IgG antibody, in particular, the C-terminal
- 20 region of the heavy chain(s) of said IgG antibody. The term “Fc binding” refers to the capability to bind to said region.

The term “Fab” or “Fab fragment” refers to the fragment antigen-binding region and includes both a constant domain and the variable domains of both the heavy and light chains. Fab are often monovalent having one antigen-binding site.

- 25 The term “Fab’” relates to monovalent Fab’ fragments having a free sulfhydryl group useful for conjugation to other molecules.

The term “(Fab’)2” relates to divalent fragments having two antigen-binding regions that are linked by disulfide bonds.

The term “(Fab2)” relates to bispecific Fab dimers. The term (Fab3) relates to trispecific Fab trimers.

- 30 The term “Fv fragment” refers to the fragment variable region and contains only the two variable domains, VH and VL. The VH and VL are held together in Fv fragments by non-covalent interactions.

The term “F(ab) fragment” refers to a fragment having one antigen-binding site.

The term “ScFv fragment” relates to Fv type fragments consisting of the VH and VL domains linked by an engineered flexible linker peptide.

5 The term “Bivalent diabody” refers to a fragment having two scFv fragments. The terms multivalent “triabody”/“tetraabody” refers to fragments having three or four scFv fragments, respectively.

The term “Minibodies” refers to scFv-CH3 fusion proteins assembled into bivalent dimers.

The term “sdAb fragment”, also known as “nanobody” refers to a single domain antibody, exclusively composed by heavy chain homodimers lacking light chains having camelid origin.

10 The term “VHH-fragment” or “VHH” relates to the Fab portion of a sdAb fragment consisting of a single monomeric variable heavy chain.

The term “bispecific antibody” stands for an antibody that can bind to two different types of antigen or two different epitopes on the same antigen. Likewise, a tri-specific antibody stands for an antibody that can bind to three different types of antigen or three different epitopes on the same antigen. The term “multi-specific antibody” stands for an antibody that can bind more than two
15 different types of antigen or more than two different epitopes on the same antigen. A bi-specific or multi-specific antibody is a heterodimer, with differing variable regions accounting for the bi- or multi-specificity, as opposed to a mAb which is a homodimer. The bi-specificity or multi-specificity may be due to the variable light chains or the variable heavy chains.

20 The terms “VH-binding polypeptide”, “VH-binding agent” and “VH-binding protein” mean a polypeptide, molecule, or protein, respectively, capable of binding to the variable heavy chain (VH) of an antibody, such as the Fab Portion. Such a polypeptide or protein includes, but is not limited to, e.g. Protein A and Protein G, or any fragment or fusion protein thereof that has maintained said binding property.

25 The term “liquid sample” or “sample” as used herein, refers to a liquid containing at least one target substance which is sought to be purified from other substances also present. Liquid samples can, for example, be aqueous solutions, organic solvent systems, or aqueous/organic solvent mixtures or solutions. The source liquids are often complex mixtures or solutions containing many biological molecules (such as proteins, antibodies, hormones, and viruses), small molecules (such as salts, sugars, lipids, etc.) and even particulate matter. While a typical source liquid of biological origin may
30 begin as an aqueous solution or suspension, it may also contain organic solvents used in earlier separation steps such as solvent precipitations, extractions, and the like. Examples of liquid samples that may contain valuable biological substances amenable to the purification by various embodiments of the present invention include, but are not limited to, a culture supernatant from a

bioreactor, a homogenized cell suspension, plasma, plasma fractions, and milk. The liquid sample or sample is often clarified before application to a chromatography resin.

A "buffer" is a substance which, by its presence in solution, increases the amount of acid or alkali that must be added to cause unit change in pH. A buffered solution resists changes in pH by the action of its acid-base conjugate components. Buffered solutions for use with biological reagents are generally capable of maintaining a constant concentration of hydrogen ions such that the pH of the solution is within a physiological range. The term "physiological pH" refers to the pH of mammalian blood (i.e., 7.38 or about 7.4). Thus, a physiologic pH range is from about 7.2 to 7.6. Traditional buffer components include, but are not limited to, organic and inorganic salts, acids and bases.

Exemplary buffers for use in purification of biological molecules (e.g., protein molecules) include the zwitterionic or "Good" Buffers, see e.g., Good et al. (1966) *Biochemistry* 5:467 and Good and Izawa (1972) *Methods Enzymol.* 24:62.

"Washing liquid", "wash buffer" or "column wash" as used herein all refer to the liquid used to carry away impurities from the chromatography resin to which is bound the target substance. More than one wash liquid can be employed sequentially, e.g., with the successive wash liquids having varying properties such as pH, conductivity, solvent concentration, etc., designed to dissociate and remove varying types of impurities that are non-specifically associated with the chromatography resin.

The term "equilibration buffer" refers in the present disclosure to a buffer used to prepare the affinity matrix, with bound target protein, for the elution, or for loading of the target molecule.

Equilibration buffer may also be used for wash of the affinity matrix with bound target protein.

"Elution liquid" or "elution buffer", which are used interchangeably herein, refers herein to the liquid that is used to dissociate the target substance from the chromatography resin, thereby eluting the binding region-containing protein from the immobilized binding agent, after it has been washed with one or more wash liquids. The elution liquid acts to dissociate the target substance without denaturing it irreversibly. Typical elution liquids are well known in the chromatography art and may have a different pH (typically lower pH), higher concentrations of salts, free affinity ligands or analogs, or other substances that promote dissociation of the target substance from the chromatography resin. "Elution conditions" refers to process conditions imposed on the target substance-bound chromatography resin that dissociate the target substance from the chromatography resin, such as the contacting of the target substance-bound chromatography resin with an elution liquid or elution buffer to produce such dissociation.

Preferably the elution buffer has a low pH and thereby disrupts interactions between separation matrix and the protein of interest. Typically, the low pH elution buffer has a pH in the range from about 2 to about 5, such as in the range from about 3 to about 4. Examples of buffers that will control the pH within this range include glycine, phosphate, acetate, and citrate buffers, as well as combinations of these. Commonly used buffers are citrate and acetate buffers, most preferably sodium citrate or sodium acetate buffers.

DETAILED DESCRIPTION OF THE INVENTION

The inventors had as an objective to develop an affinity separation matrix comprising VH3-binding ligands, wherein said affinity separation matrix is alkali resistant and wherein the ligands have no or significantly reduced binding to the Fc region of immunoglobulins. It was a further objective to develop a VH3 binding affinity separation matrix with a satisfactory efficiency in binding capacity and flow characteristics when used in chromatography. It was an additional objective to develop a VH3 binding affinity separation matrix that allows for an elution of a biological target molecule at a milder pH than existing separation matrices.

In a first aspect, the inventors have attained the objective above by developing a separation matrix for purification of antibodies or antibody fragments comprising at least one VH3 chain, said separation matrix comprising a VH3 binding ligand coupled to a porous support, wherein said VH3 binding ligand comprises at least one VH3 binding polypeptide having a significantly reduced or abolished binding affinity for a Fc fragment, and wherein the porous support is in beaded or particle form.

Preferably, the VH3 binding ligand comprises a VH3 binding polypeptide based on any of the native Protein A domains A_{wt} (SEQ ID NO 192), B_{wt} (SEQ ID NO 191), C_{wt} (SEQ ID NO 190), D_{wt} (SEQ ID NO 193), E_{wt} (SEQ ID NO 194), or the engineered Protein A domains Z_{wt} (SEQ ID NO 89), Z_{var} (SEQ ID NO 88) or Z_{var2} (SEQ ID NO 195).

SEQ ID NO 88 - Z_{var}

VDAKFDKEAQEAFYEILHLPNLTEEQRNAFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 89 – Z_{wt}

VDNKFNKEQQNAFYELHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 90 – C domain (wt)

ADNKFNKEQQNAFYEILHLPNLTEEQRNGFIQSLKDDPSVSKEILAEAKKLNDAAQAPK

SEQ ID NO 91 – B domain (wt)

ADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAAQAPK

5 SEQ ID NO 92 – A domain (wt)

ADNNFNKEQQNAFYEILNMPNLNEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPK

SEQ ID NO 93 – D domain (wt)

ADAQQNKFNKDQQSAFYEILNMPNLNEEQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPK

SEQ ID NO 94 – E domain (wt)**10** AQQNAFYQVLNMPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLNDSSQAPK**SEQ ID NO 95 – Z_{var2}**

VDAKFDKEQQNAFYEILHLPNLTEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAAQAPK

In the VH3 binding polypeptide based on any one of the above-mentioned domains, the amino acid at the position corresponding to position 9 in SEQ ID NO 89 is selected from Q, Y and A, the amino acid at the position corresponding to position 10 in SEQ ID NO 89 is selected from Q and Y, the amino acid at the position corresponding to position 11 in SEQ ID NO 89 is selected from T, E and R, the amino acid at the position corresponding to position 13 in SEQ ID NO 89 is selected from L, E, R, A and Q, the amino acid at the position corresponding to position 14 in SEQ ID NO 89 is selected from L, E, R, A, Q and W, the amino acid at the position corresponding to position 17 in SEQ ID NO 89 is selected from A, H and L, the amino acid at the position corresponding to position 18 in SEQ ID NO 89 is selected from R, L and H, the amino acid at the position corresponding to position 26 in SEQ ID NO 89 is selected from Q and S, the amino acid at the position corresponding to position 28 in SEQ ID NO 89 is selected from N and A, and the amino acid at the position corresponding to position 29 in SEQ ID NO 89 is selected from and G.

In one variant of the first aspect, the amino acids at positions 9/10/11 may preferably be QQT, QYT, YQT, AQE, AQR or AYR; more preferably QQT, QYT, AQE, AQR or AYR; more preferably AQE, AQR or AYR; most preferably AQR or AYR.

In one variant of the first aspect, the amino acids at positions 17/18 may preferably be AR, HL or LH; preferably HL or AR.

In one variant of the first aspect, the amino acids at positions 28/29 may be NA, NG, AA or AG.

In yet one variant of the first aspect, independently of each other, the amino acids at positions
 5 9/10/11 are AQE, AQR or AYR; and the amino acids at positions 13/14 are LA, AA, AE, AL, AQ, AR, EA, EE, EL, EQ, ER, LA, LE, LL, LQ, LR, QA, QE, QL, QQ, QR, RA, RE, RL, RQ, RR or LW; and the amino acids at positions 17/18 are LH or AR.

In yet one variant of the first aspect the amino acids at positions 9/10/11/13/14/17/18 are QQTLALH, QYTLALH, YQTLALH, QQTLAAR, AQELALH, AYRLALH, AYRLWLH, AYRLWAR, AYRLAHL and AYRLWHL
 10 AQRLALH, AYRLAAR.

In one variant of the first aspect the amino acids at positions 26/28/29 are QNG, QAA, QAG, QNA, or SAG; preferably QNG, QNA, SAG or QAG.

The remaining positions in such a functional VH3 binding polypeptide may be varied as long as the three-dimensional structure is not altered as compared to that of the Z_{wt} domain (SEQ ID NO 89),
 15 and as long as it at least retains VH3 binding capacity, the binding to the Fc region of immunoglobulins is deleted or significantly reduced, and it is alkali-stabilized as compared to the Z_{wt} domain (SEQ ID NO 89). The variation may be conservative amino acid substitutions for an amino acid with a similar or identical charge, hydrophobicity, etc., and the skilled person is able to determine what such a variation of an amino acid may be.

20 Preferably, the VH3-binding ligands comprise at least one polypeptide selected from SEQ ID NO:1 – SEQ ID NO:87 and SEQ ID NO: 99- SEQ ID NO:107, SEQ ID NO:111-SEQ ID NO:112, SEQ ID NO: 114-SEQ ID NO: 120, SEQ ID NO: 124-SEQ ID NO: 125 and SEQ ID NO:127-SEQ ID NO:144 .

SEQ ID NO 1 - Z_{var}(A9Q,E11T,F13L,Y14A)

VDAKFDKEQQTALAEILHLPNLTEEQRNAFIQSLKDDPSVSKAILAEAKKLNDQAQPK

25 **SEQ ID NO 2 - Z_{var}(A9Q,Q10Y,E11T,F13L,Y14A)**

VDAKFDKEQYTALAEILHLPNLTEEQRNAFIQSLKDDPSVSKAILAEAKKLNDQAQPK

SEQ ID NO 3 - Z_{var}(A9Y,E11T,F13L,Y14A)

VDAKFDKEYQTALAEILHLPNLTEEQRNAFIQSLKDDPSVSKAILAEAKKLNDQAQPK

SEQ ID NO 4 - Z_{var}(A9Q,E11T,F13L,Y14A,L17A,H18R)

SEQ ID NO VDAKFDKEQQTALAEIARLPNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 5- Z_{var}(F13L,Y14A)

VDAKFDKEAQEALAEILHLPNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

5 **SEQ ID NO 6- Z_{var}(E11R,F13L,Y14A)**

VDAKFDKEAQRALAEILHLPNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 7- Z_{var}(Q10Y,E11R,F13L,Y14A)

VDAKFDKEAYRALAEILHLPNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 8- Z_{var}(Q10Y,E11R,F13L,Y14W)

10 VDAKFDKEAYRALWEILHLPNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 9- Z_{var}(Q10Y,E11R,F13L,Y14W,L17A,H18R)

VDAKFDKEAYRALWEIARLPNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 10- Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R)

VDAKFDKEAYRALAEIARLPNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

15 **SEQ ID NO 11- Z_{var}(Q10Y,E11R,F13L,Y14A,L17L,H18L)**

VDAKFDKEAYRALAEIHLPLNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 12- Z_{var}(Q10Y,E11R,F13L,Y14W,L17H,H18L)

VDAKFDKEAYRALWEIHLPLNLTEEQRNAFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 13- Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R,A29G)

20 VDAKFDKEAYRALAEIARLPNLTEEQRNGFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 14- Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A)

VDAKFDKEAYRALAEIARLPNLTEEQRAAFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 15- Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A,A29G)

VDAKFDKEAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKILAEAKKLNDAPK

SEQ ID NO 16 - Z_{var}(Q10Y,E11R,F13A,Y14A,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAAAEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 17 - Z_{var}(Q10Y,E11R,F13A,Y14E,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAAEEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

5 SEQ ID NO 18 - Z_{var}(Q10Y,E11R,F13A,Y14L,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAALEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 19 - Z_{var}(Q10Y,E11R,F13A,Y14Q,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAAQEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 20 - Z_{var}(Q10Y,E11R,F13A,Y14R,L17A,H18R,N28A,A29G)

10 VDAKFDKEAYRAAREIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 21 - Z_{var}(Q10Y,E11R,F13E,Y14A,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAEAEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 22 - Z_{var}(Q10Y,E11R,F13E,Y14E,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAEEEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

15 SEQ ID NO 23 - Z_{var}(Q10Y,E11R,F13E,Y14L,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAELEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 24 - Z_{var}(Q10Y,E11R,F13E,Y14Q,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAEQEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 25 - Z_{var}(Q10Y,E11R,F13E,Y14R,L17A,H18R,N28A,A29G)

20 VDAKFDKEAYRAEREIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 26 - Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A,A29G)

VDAKFDKEAYRALAEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 27 - Z_{var}(Q10Y,E11R,F13L,Y14E,L17A,H18R,N28A,A29G)

VDAKFDKEAYRALEEIRLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPK

SEQ ID NO 28 - Z_{var}(Q10Y,E11R,F13L,Y14L,L17A,H18R,N28A,A29G)

VDAKFDKEAYRALLEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 29 - Z_{var}(Q10Y,E11R,F13L,Y14Q,L17A,H18R,N28A,A29G)

VDAKFDKEAYRALQEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

5 SEQ ID NO 30 - Z_{var}(Q10Y,E11R,F13L,Y14R,L17A,H18R,N28A,A29G)

VDAKFDKEAYRALREIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 31 - Z_{var}(Q10Y,E11R,F13Q,Y14A,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAQAEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 32 - Z_{var}(Q10Y,E11R,F13Q,Y14E,L17A,H18R,N28A,A29G)

10 VDAKFDKEAYRAQEEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 33 - Z_{var}(Q10Y,E11R,F13Q,Y14L,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAQLEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 34 - Z_{var}(Q10Y,E11R,F13Q,Y14Q,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAQQEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

15 SEQ ID NO 35 - Z_{var}(Q10Y,E11R,F13Q,Y14R,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAQREIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 36 - Z_{var}(Q10Y,E11R,F13R,Y14A,L17A,H18R,N28A,A29G)

VDAKFDKEAYRAREIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 37 - Z_{var}(Q10Y,E11R,F13R,Y14E,L17A,H18R,N28A,A29G)

20 VDAKFDKEAYRAREEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 38- Z_{var}(Q10Y,E11R,F13R,Y14L,L17A,H18R,N28A,A29G)

VDAKFDKEAYRARLEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 39- Z_{var}(Q10Y,E11R,F13R,Y14Q,L17A,H18R,N28A,A29G)

VDAKFDKEAYRARQEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAPK

SEQ ID NO 40- Z_{var}(Q10Y,E11R,F13R,Y14R,L17A,H18R,N28A,A29G)

VDAKFDKEAYRARREIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDQAQPK

SEQ ID NO 41- Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R,Q26S,N28A,A29G)

VDAKFDKEAYRALAEIARLPNLTEESRAGFIQSLKDDPSVSKAILAEAKKLNDQAQPK

5 **SEQ ID NO 42 – Z_{wt}(A9Q,E11T,F13L,Y14A)**

VDNKFNKEQQTALAEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 43 - Z_{wt}(A9Q,Q10Y,E11T,F13L,Y14A)

VDNKFNKEQYTALAEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 44 - Z_{wt}(A9Y,E11T,F13L,Y14A)

10 VDNKFNKEYQTALAEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 45 - Z_{wt}(A9Q,E11T,F13L,Y14A,L17A,H18R)

VDNKFNKEQQTALAEIARLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 46 - Z_{wt}(F13L,Y14A)

VDNKFNKEAQEALAEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

15 **SEQ ID NO 47 - Z_{wt}(E11R,F13L,Y14A)**

VDNKFNKEAQRALAEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 48 - Z_{wt}(Q10Y,E11R,F13L,Y14A)

VDNKFNKEAYRALAEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 49 - Z_{wt}(Q10Y,E11R,F13L,Y14W)

20 VDNKFNKEAYRALWEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 50 - Z_{wt}(Q10Y,E11R,F13L,Y14W,L17A,H18R)

VDNKFNKEAYRALWEIARLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 51 - Z_{wt}(Q10Y,E11R,F13L,Y14A,L17A,H18R)

VDNKFNKEAYRALAEIARLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQPK

25

SEQ ID NO 52 - Z_{wt}(Q10Y,E11R,F13L,Y14A,L17L,H18L)

VDNKFNKEAYRALAEIHLLPNLNNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 53 - Z_{wt}(Q10Y,E11R,F13L,Y14W,L17H,H18L)

VDNKFNKEAYRALWEIHLLPNLNNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAPK

5 **SEQ ID NO 54 - Z_{wt}(Q10Y,E11R,F13L,Y14A,L17A,H18R,A29G)**

VDNKFNKEAYRALAEIARLPNLNNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 55 - Z_{wt}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A)

VDNKFNKEAYRALAEIARLPNLNNEEQRAAFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 56 - Z_{wt}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A,A29G)

10 VDNKFNKEAYRALAEIARLPNLNNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 57 - Z_{wt}(Q10Y,E11R,F13A,Y14A,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAAAEIARLPNLNNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 58 - Z_{wt}(Q10Y,E11R,F13A,Y14E,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAAEEIARLPNLNNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

15 **SEQ ID NO 59 - Z_{wt}(Q10Y,E11R,F13A,Y14L,L17A,H18R,N28A,A29G)**

VDNKFNKEAYRAALEIARLPNLNNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 60 - Z_{wt}(Q10Y,E11R,F13A,Y14Q,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAAQEIARLPNLNNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 61 - Z_{wt}(Q10Y,E11R,F13A,Y14R,L17A,H18R,N28A,A29G)

20 VDNKFNKEAYRAAREIARLPNLNNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 62 - Z_{wt}(Q10Y,E11R,F13E,Y14A,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAAEIARLPNLNNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 63 - Z_{wt}(Q10Y,E11R,F13E,Y14E,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAEEEIARLPNLNNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 64 - Z_{wt}(Q10Y,E11R,F13E,Y14L,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAELEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 65 - Z_{wt}(Q10Y,E11R,F13E,Y14Q,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAEQEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

5 **SEQ ID NO 66 - Z_{wt}(Q10Y,E11R,F13E,Y14R,L17A,H18R,N28A,A29G)**

VDNKFNKEAYRAEREIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 67 - Z_{wt}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A,A29G)

VDNKFNKEAYRALAEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 68 - Z_{wt}(Q10Y,E11R,F13L,Y14E,L17A,H18R,N28A,A29G)

10 VDNKFNKEAYRALEEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 69 - Z_{wt}(Q10Y,E11R,F13L,Y14L,L17A,H18R,N28A,A29G)

VDNKFNKEAYRALLEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 70 - Z_{wt}(Q10Y,E11R,F13L,Y14Q,L17A,H18R,N28A,A29G)

VDNKFNKEAYRALQEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

15 **SEQ ID NO 71 - Z_{wt}(Q10Y,E11R,F13L,Y14R,L17A,H18R,N28A,A29G)**

VDNKFNKEAYRALREIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 72 - Z_{wt}(Q10Y,E11R,F13Q,Y14A,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAQAEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 73 - Z_{wt}(Q10Y,E11R,F13Q,Y14E,L17A,H18R,N28A,A29G)

20 VDNKFNKEAYRAQEEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 74 - Z_{wt}(Q10Y,E11R,F13Q,Y14L,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAQLEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 75 - Z_{wt}(Q10Y,E11R,F13Q,Y14Q,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAQQEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 76 - Z_{wt}(Q10Y,E11R,F13Q,Y14R,L17A,H18R,N28A,A29G)

VDNKFNKEAYRAQREIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 77 - Z_{wt}(Q10Y,E11R,F13R,Y14A,L17A,H18R,N28A,A29G)

VDNKFNKEAYRARAIEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

5 **SEQ ID NO 78 - Z_{wt}(Q10Y,E11R,F13R,Y14E,L17A,H18R,N28A,A29G)**

VDNKFNKEAYRAREEIEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 79 - Z_{wt}(Q10Y,E11R,F13R,Y14L,L17A,H18R,N28A,A29G)

VDNKFNKEAYRARLEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 80 - Z_{wt}(Q10Y,E11R,F13R,Y14Q,L17A,H18R,N28A,A29G)

10 VDNKFNKEAYRARQEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 81 - Z_{wt}(Q10Y,E11R,F13R,Y14R,L17A,H18R,N28A,A29G)

VDNKFNKEAYRARREIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 82 - Z_{wt}(Q10Y,E11R,F13L,Y14A,L17A,H18R,Q26S,N28A,A29G)

VDNKFNKEAYRALAEIARLPNLNEESRAGFIQSLKDDPSQSKAILAEAKKLNDAPK

15 **SEQ ID NO 83 - C(Q9A,Q10Y,F13L,Y14A,L17A,H18R,N28A)**

ADNKFNKEAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKEILAEAKKLNDAPK

SEQ ID NO 84 - B(Q9A,Q10Y,F13L,Y14A,L17A,H18R,N28A)

ADNKFNKEAYRALAEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAPK

SEQ ID NO 85 - A(Q10A,Q11Y,F14L,Y15A,L18A,H19R,N29A)

20 ADNNFNKEAYRALAEIARMPNLNEEQRAGFIQSLKDDPSQSANLLSEAKKLNESQPK

SEQ ID NO 86 - D(Q12A,Q13Y,F16L,Y17A,L20A,N21R,N31A)

ADAQQNKFNDAYRALAEIARMPNLNEEQRAGFIQSLKDDPSQSTNVLGEAKKLNESQPK

SEQ ID NO 87 - E(Q2A,Q3Y,F6L,Y7A,L10A,N11R,N21A)

AAYRALAQVARMPNLNADQRAGFIQSLKDDPSQSANVLGEAQKLNDSQPK

SEQ ID NO 99 – E(Q2A,Q3Y,N4R,F6L,Y7A,L10A,N11R,N21A)

AQHDEAAYRALAQVARM PN LNADQRAGFIQSLKDDPSQSANVLGEAQKL NDSQAPK

SEQ ID NO 100 - D(Q12A,Q13Y,S14R,F16L,Y17A,L20A,N21R,N31A)

ADAQQNKFNKDAYRALAEIARMPN LN NEEQRAGFIQSLKDDPSQSTNVLGEAKKL N ESQAPK

5 **SEQ ID NO 101 - A(Q9A,Q10Y,N11R,F13L,Y14A,L17A,H18R,N28A)**

ADNNFNKEAYRALAEIARMPN LN NEEQRAGFIQSLKDDPSQSANLLAEAKKL N ESQAPK

SEQ ID NO 102 - B(Q9A,Q10Y,N11R,F13L,Y14A,L17A,H18R,N28A)

ADNKFNKEAYRALAEIARLPN LN NEEQRAGFIQSLKDDPSQSANLLAEAKKL N DAQAPK

SEQ ID NO 103 - C(Q9A,Q10Y,N11R F13L,Y14A,L17A,H18R,N28A)

10 ADNKFNKEAYRALAEIARLPN L TEEQRAGFIQSLKDDPSVSKEILAEAKKL N DAQAPK

SEQ ID NO 104 – Z_{wt}(Q9A,Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A,A29G)

VDNKFNKEAYRALAEIARLPN LN NEEQRAGFIQSLKDDPSQSANLLAEAKKL N DAQAPK

SEQ ID NO 105 – Z_{v_{ar2}}(Q9A,Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A,A29G)

VDAKFDKEAYRALAEIARLPN L TEEQRAGFIQSLKDDPSQSANLLAEAKKL N DAQAPK

15 **SEQ ID NO 106 – C(A1I,D2A,N3A,K4Q,F5H,N6D,E8D,Q9A,Q10Y,N11R,F13L,Y14A,L17A,
H18R,N28A,K35R,K42L)**

IAAQHDKDAYRALAEIARLPN L TEEQRAGFIQSLRDDPSVSLEILAEAKKL N DAQAPK

**SEQ ID NO 107 - C(A1I,D2A,N3A,K4Q,F5H,N6D,Q9A,Q10Y,N11R,F13L,Y14A,L17A,H18R,E25D,N28A,
K35R,K42L,A46G)**

20 IAAQHDKEAYRALAEIARLPN L TEDQRAGFIQSLRDDPSVSLEILGEAKKL N DAQAPK

SEQ ID NO 111 - C(A1V,Q9A,Q10Y,N11R,F13L,Y14A,L17A,H18R,N28A,A46K)

VDNKFNKEAYRALAEIARLPN L TEEQRAGFIQSLKDDPSVSKEILKEAKKL N DAQAPK

**SEQ ID NO 112 - A(K7R,Q9A,Q10Y,N11R,F13L,Y14A,E15H,L17A,H18R,M19V,N28A,K35R,A46S,
K49R,K50R,K58G)**

25 VDAKFNKEAYRALAEIARLPN L TEEQRAGFIQSLRDDPSQSANLLSEAKRL N ESQAPG

SEQ ID NO 114 - A(Q10A,Q11Y,N12R,F14L,Y15A,L18A,H19R,Q27S,N29A)

ADNNFNKEAYRALAEIARMPNLNEESRAGFIQSLKDDPSQSANLLAEAKKLNESQAPK

SEQ ID NO 115 - B(Q9A,Q10Y,N11R,F13L,Y14A,L17A,H18R,Q26S,N28A)

ADNKFNKEAYRALAEIARLPNLNEESRAGFIQSLKDDPSQSANLLAEAKKLNDQAAPK

5 **SEQ ID NO 116 - C(Q9A,Q10Y,N11R F13L,Y14A,L17A,H18R,Q26S,N28A)**

ADNKFNKEAYRALAEIARLPNLTEESRAGFIQSLKDDPSVSKEILAEAKKLNDQAAPK

SEQ ID NO 117 - Zwt(Q9A,Q10Y,E11R,F13L,Y14A,L17A,H18R,Q26S,N28A,A29G)

VDNKFNKEAYRALAEIARLPNLNEESRAGFIQSLKDDPSQSANLLAEAKKLNDQAAPK

SEQ ID NO 118 - Zvar2(Q9A,Q10Y,E11R,F13L,Y14A,L17A,H18R,Q26S,N28A,A29G)

10 VDAKFDKEAYRALAEIARLPNLTEESRAGFIQSLKDDPSQSANLLAEAKKLNDQAAPK

**SEQ ID NO 119 - C(A1I,D2A,N3A,K4Q,F5H,N6D,E8D,Q9A,Q10Y,N11R,F13L,Y14A,L17A,
H18R,Q26S,N28A,K35R,K42L)**

IAAQHDKDAYRALAEIARLPNLTEESRAGFIQSLRDDPSVSLEILAEAKKLNDQAAPK

SEQ ID NO 120- C(A1I,D2A,N3A,K4Q,F5H,N6D,Q9A,Q10Y,N11R,F13L,Y14A,L17A,H18R,

15 **E25D,Q26S,N28A, K35R,K42L,A46G)**

IAAQHDKEAYRALAEIARLPNLTEDSRAGFIQSLRDDPSVSLEILGEAKKLNDQAAPK

SEQ ID NO 124 - C(A1V,Q9A,Q10Y,N11R,F13L,Y14A,L17A,H18R,Q26S,N28A,A46K)

VDNKFNKEAYRALAEIARLPNLTEESRAGFIQSLKDDPSVSKEILKEAKKLNDQAAPK

SEQ ID NO 125 - A(K7R,Q9A,Q10Y,N11R,F13L,Y14A,E15H,L17A,H18R,M19V,Q26S,N28A,K35R,A46S,

20 **K49R,K50R,K58G)**

VDAKFNKEAYRALAEIARLPNLTEESRAGFIQSLRDDPSQSANLLSEAKRLNESQAPG

SEQ ID NO 127 - E(Q2A,Q3Y,N4E,F6R,Y7A,L10A,N11R,N21A)

AQHDEAAAYEARAQVARMPNLNADQRAGFIQSLKDDPSQSANVLGEAQKLNDQAPK

SEQ ID NO 128 - B(Q9A,Q10Y,N11E,F13R,Y14A,L17A,H18R,N28A)

25 ADNKFNKEAYEARAEIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDQAAPK

SEQ ID NO 129 - C(Q9A,Q10Y,N11E,F13R,Y14A,L17A,H18R,N28A)

ADNKFNKEAYEARAEIARLPNLTEEQRAGFIQSLKDDPSVSKEILAEAKKLNDQAQPK

SEQ ID NO 130 - E(Q2A,Q3Y,N4E,F6R,Y7A,L10A,N11L,N21A)

AQHDEAA YEARAQVALMPNLNADQRAGFIQSLKDDPSQSANVLGEAQKLNDSQAPK

5 **SEQ ID NO 131 - B(Q9A,Q10Y,N11E,F13R,Y14A,L17A,H18L,N28A)**

ADNKFNKEAYEARAEIALLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 132 - C(Q9A,Q10Y,N11E,F13R,Y14A,L17A,H18L,N28A)

ADNKFNKEAYEARAEIALLPNLTEEQRAGFIQSLKDDPSVSKEILAEAKKLNDQAQPK

SEQ ID NO 133 - E(Q2A,N4R,F6R,Y7A,N11R,N21A)

10 AQHDEAAQRARAQVLRMPNLNADQRAGFIQSLKDDPSQSANVLGEAQKLNDSQAPK

SEQ ID NO 134 - B(Q9A,N11R,F13R,Y14A,H18R,N28A)

ADNKFNKEAQRARAEILRPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 135 - C(Q9A,N11R,F13R,Y14A,H18R,N28A)

ADNKFNKEAQRARAEILRPNLTEEQRAGFIQSLKDDPSVSKEILAEAKKLNDQAQPK

15 **SEQ ID NO 136 - E(Q2A,Q3Y,N4E,F6L,Y7R,L10A,N11R,N21A)**

AQHDEAA YEALRQVARM PNLNADQRAGFIQSLKDDPSQSANVLGEAQKLNDSQAPK

SEQ ID NO 137 - B(Q9A,Q10Y,N11E,F13L,Y14R,L17A,H18R,N28A)

ADNKFNKEAYEALREIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 138 - C(Q9A,Q10Y,N11E,F13L,Y14R,L17A,H18R,N28A)

20 ADNKFNKEAYEALREIARLPNLTEEQRAGFIQSLKDDPSVSKEILAEAKKLNDQAQPK

SEQ ID NO 139 - E(Q2A,Q3Y,N4E,F6A,Y7R,L10A,N11R,N21A)

AQHDEAA YEAA RQVARM PNLNADQRAGFIQSLKDDPSQSANVLGEAQKLNDSQAPK

SEQ ID NO 140 - B(Q9A,Q10Y,N11E,F13A,Y14R,L17A,H18R,N28A)

ADNKFNKEAYEAA REIARLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDQAQPK

SEQ ID NO 141 - C(Q9A,Q10Y,N11E,F13A,Y14R,L17A,H18R,N28A)

ADNKFNKEAYEAAREIARLPNLTEEQRAGFIQSLKDDPSVSKEILAEAKKLNDAAQAPK

SEQ ID NO 142 - E(Q2Y,N4R,F6L,Y7A,L10A,N11L,N21A)

AQHDEAYQRALAQVALMPNLNADQRAGFIQSLKDDPSQSANVLGEAQKLNDSSQAPK

5 SEQ ID NO 143 - B(Q9Y,N11R,F13L,Y14A,L17A,H18L,N28A)

ADNKFNKEYQRALAEIALLPNLNEEQRAGFIQSLKDDPSQSANLLAEAKKLNDAAQAPK

SEQ ID NO 144 - C(Q9Y,N11R,F13L,Y14A,L17A,H18L,N28A)

ADNKFNKEYQRALAEIALLPNLTEEQRAGFIQSLKDDPSVSKEILAEAKKLNDAAQAPK

- 10 The VH3 binding ligand may be a multimer of any one of the VH3 binding polypeptides according to the above. The VH3 binding ligand may be a homodimeric multimer of a VH3 binding polypeptide according to the above. The VH3 binding ligand may be a heterodimeric multimer of at least two of the VH3 binding polypeptides according to the above. The multimer may comprise two, three, four, five, six, seven, eight or nine VH3 binding polypeptides. In an alternative language, the multimers
- 15 may be a dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer or a nonamer. Preferably, the ligands comprise four, five, six or seven alkali-stabilized VH3 binding polypeptides, such as five or six alkali-stabilized VH3 binding polypeptides.

Preferably the multimer is according to one of the following sequences SEQ ID NO 96, SEQ ID NO 97 or SEQ ID NO 98.

20 SEQ ID NO 96 - Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A)₄

AQYEDGKQYTDTVDAKFDKEAYRALAEIARLPNLTEEQRAAFIQSLKDDPSVSKAILAEAKKLNDAAQAPKVDKFDK
EAYRALAEIARLPNLTEEQRAAFIQSLKDDPSVSKAILAEAKKLNDAAQAPKVDKFDKEAYRALAEIARLPNLTEEQR
AAFIQSLKDDPSVSKAILAEAKKLNDAAQAPKVDKFDKEAYRALAEIARLPNLTEEQRAAFIQSLKDDPSVSKILAEA
KKLNDAAQAPKHHHHHC

25 SEQ ID NO 97 - Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A,A29G)₄

AQYEDGKQYTDTVDAKFDKEAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPKVDKFDK
EAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKAILAEAKKLNDAAQAPKVDKFDKEAYRALAEIARLPNLTEEQR
AGFIQSLKDDPSVSKILAEAKKLNDAAQAPKVDKFDKEAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKILAEA
KKLNDAAQAPKHHHHHC

SEQ ID NO 98 - Z_{var}(Q10Y,E11R,F13L,Y14A,L17A,H18R,N28A,A29G)₆

AQYEDGKQYTDTVDAKFDKEAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKILAEAKKLNDAAQAPKVDKFDK
 EAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKILAEAKKLNDAAQAPKVDKFDKFDKEAYRALAEIARLPNLTEEQR
 AGFIQSLKDDPSVSKILAEAKKLNDAAQAPKVDKFDKFDKEAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKILAEA
 5 KKLNDAAQAPKVDKFDKFDKEAYRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKILAEAKKLNDAAQAPKVDKFDKFDKEA
 YRALAEIARLPNLTEEQRAGFIQSLKDDPSVSKILAEAKKLNDAAQAPKHHHHHHC

The multimer may further comprise a linker, spacer, or additional amino acid(s) N-terminally, C-terminally or between two monomeric sequences in the multimer. The additional amino acid(s) may for instance originate from the cloning process of the ligand, polypeptide or multimer or constitute a
 10 residue from a cleaved off signaling sequence.

The amount of coupled ligand can be controlled by the concentration of ligand used in the coupling process, by the coupling conditions used and/or by the pore structure of the support used. As a general rule the absolute binding capacity of the matrix increases with the amount of coupled ligand, at least up to a point where the pores become significantly constricted by the coupled ligand. The
 15 relative binding capacity per mg coupled ligand will decrease at high coupling levels, resulting in a cost-benefit optimum within the ranges specified herein.

The separation matrix disclosed herein preferably comprises at least 12 mg/ml VH3 binding ligands, such as at least 14 mg/ml, such as at least 14.5 mg/ml, at least 15 mg/ml, at least 15.5 mg/ml, at least 16 mg/ml, at least 16.5 mg/ml, at least 17 mg/ml, at least 17.5 mg/ml, at least 18 mg/ml, at
 20 least 18.5 mg/ml, at least 19 mg/ml, at least 19.5 mg/ml, at least 20 mg/ml, at least 20.5 mg/ml, at least 21 mg/ml, at least 21.5 mg/ml, or at least 22 mg/ml VH3 binding ligands.

The ligand may comprise a coupling element, said coupling element being one or more amino acid residues at the C-terminal or N-terminal end of the ligand, preferably at the C-terminal end. Preferably the coupling element is one or more cysteine residues, one or more lysine residues, or
 25 one or more histidine residues. Preferably, the ligand may comprise one or more cysteine residues at the C-terminal end of the ligand.

The coupling element(s) may be directly linked to the C- or N-terminus, or it/they may be linked via a linker comprising up to 15 amino acids, such as 1-5, 1-10 or 5-10 amino acids. This stretch should preferably also be sufficiently stable in alkaline environments so as to not impair the properties of
 30 the mutated ligand. For this purpose, it is advantageous if the stretch does not contain asparagine. It can additionally be advantageous if the stretch does not contain glutamine. An advantage of having a C- or N-terminal cysteine is that endpoint coupling of the ligand can be achieved through reaction of

the cysteine thiol with an electrophilic group on a support, as described below. This provides excellent mobility of the coupled ligand which is important for the binding capacity.

The ligand may be coupled to the support via thioether bonds. Methods for performing such coupling are well-known in this field and easily performed by the skilled person in this field using standard techniques and equipment. Thioether bonds are flexible and stable and generally suited for use in affinity chromatography. In particular when the thioether bond is via a terminal or near-terminal cysteine residue on the ligand, the mobility of the coupled ligand is enhanced which provides improved binding capacity and binding kinetics. In some embodiments the ligand is coupled via a C-terminal cysteine. This allows for efficient coupling of the cysteine thiol to electrophilic groups, e.g. epoxide groups, halohydrin groups etc. on a support, resulting in a thioether bridge coupling. The ligand can e.g. be coupled via single-point attachment, e.g. via a single cysteine or by multipoint attachment, such as randomized multipoint attachment, or directed multipoint attachment, using e.g. a plurality of lysines or other coupling groups near a terminus of the ligand.

The solid support of the matrix according to the invention can be of any suitable well-known kind. A conventional affinity separation matrix is often of organic nature and based on polymers that expose a hydrophilic surface to the aqueous media used, i.e. expose hydroxy (-OH), carboxy (-COOH), carboxamido (-CONH₂, possibly in N- substituted forms), amino (-NH₂, possibly in substituted form), oligo- or polyethylenoxy groups on their external and, if present, also on internal surfaces.

The solid support is porous. The porosity can be expressed as a K_{av} or K_d value (the fraction of the pore volume available to a probe molecule of a particular size) measured by inverse size exclusion chromatography, e.g. according to the methods described in Gel Filtration Principles and Methods, Pharmacia LKB Biotechnology 1991, pp 6-15 13. By definition, both K_d and K_{av} values always lie within the range 0-1. The porous support may have a K_d value of 0.6-0.95, such as a K_d value of 0.7-0.90, or a K_d value of 0.6-0.8, as measured with dextran of Mw 110 kDa as a probe molecule. The porous support may preferably have a K_d value of about 0.67. The porous support may preferably have a K_d value of about 0.72. The porous support may preferably have a K_d value of about 0.75.

The higher the K_d value is, the larger the pores in the porous support, and the larger the fraction of the inner volume of the beads that is accessible to a solute molecule, such as biomolecules, such as immunoglobulins. The skilled person is aware of this, and able to calculate the K_d of a porous support as described above. With the larger pores mentioned as preferred above, a larger amount of ligands are coupled to the porous support. Furthermore, with larger pores, such as mentioned above, a VH3 chain-containing protein may access the ligands also within the pores. Thus, a larger binding capacity is achieved.

Multimeric ligands, such as a pentamer or a hexamer gives higher DBC compared to lower multimeric ligands such as tetramer ligands or lower. This effect is especially true on solid supports with high K_d values of 0.7-0.9.

For smaller target molecules, such as VHH or ScFV, ligand density has a high impact on the binding capacity, due to high accessibility to the ligand. The size of smaller target molecules makes it easier for these target molecules to penetrate the entire pore volume of the porous support, due to less steric hindrance, and to reach the majority of the immobilized ligands. Thus there is a higher accessibility to the ligands for smaller target molecules. Consequently, a high ligand density may be beneficial for smaller target molecules. However, for larger target molecules the upper limit for a beneficial ligand density will be lower than for a smaller target molecule, as the ligand density will confer steric hindrance for the larger target molecule to access all the ligands in the pore volume.

The support may comprise a polyhydroxy polymer, such as a polysaccharide. Examples of polysaccharides include e.g. dextran, starch, cellulose, pullulan, agar, agarose etc. Polysaccharides are inherently hydrophilic with low degrees of nonspecific interactions, they provide a high content of reactive (activatable) hydroxyl groups, and they are generally stable towards alkaline cleaning solutions used in bioprocessing.

The support may comprise agar or agarose. The supports or base matrices used for the present invention can easily be prepared according to standard methods, such as inverse suspension gelation (S Hjerten: Biochim Biophys Acta 79(2), 393-398 (1964)). Alternatively, the base matrices are commercially available products, such as crosslinked agarose beads sold under the name of SEPHAROSE™ FF (Cytiva™). In an embodiment, which is especially advantageous for large-scale separations, the support has been adapted to increase its rigidity using the methods described in US6602990 or US7396467, which are hereby incorporated by reference in their entirety, and hence renders the matrix more suitable for high flow rates.

The support, such as a polysaccharide or agarose support, may preferably be crosslinked, such as with hydroxyalkyl ether crosslinks. Crosslinker reagents producing such crosslinks can be e.g. epihalohydrins like epichlorohydrin, diepoxides like butanediol diglycidyl ether, allylating reagents like allyl halides or allyl glycidyl ether. Crosslinking is beneficial for the rigidity of the support and improves the chemical stability. Hydroxyalkyl ether crosslinks are alkali stable and do not cause significant nonspecific adsorption.

Alternatively, the solid support may be based on synthetic polymers, such as polyvinyl alcohol, polyhydroxyalkyl acrylates, polyhydroxyalkyl methacrylates, polyacrylamides, polymethacrylamides

etc. In case of hydrophobic polymers, such as matrices based on divinyl and monovinyl-substituted benzenes, the surface of the matrix is often hydrophilised to expose hydrophilic groups as defined above to a surrounding aqueous liquid. Such polymers are easily produced according to standard methods, see e.g. "Styrene based polymer supports developed by suspension polymerization" (R Arshady: Chimica e L'Industria 70(9), 70-75 (1988)). Alternatively, a commercially available product, such as SOURCE™ (Cytiva™) is used. In another alternative, the solid support according to the invention comprises a support of inorganic nature, e.g. silica, zirconium oxide etc. In another alternative, the support particles are magnetic. One example of such support particles is polysaccharide or synthetic polymer beads comprising e.g. magnetite particles, such that the beads can be used in magnetic batch separations.

The separation matrix is in beaded or particle form. Matrices in beaded or particle form can be used as a packed bed or in a suspended form. Suspended forms include those known as expanded beds and pure suspensions, in which the particles or beads are free to move. In case of packed bed and expanded beds, the separation procedure commonly follows conventional chromatography with a concentration gradient. In case of pure suspension, batch-wise mode will be used. Preferably, the separation matrix according to the above is packed in a chromatography column.

A separation matrix designed for large scale chromatography and bioprocess may, in general, have a volume-weighted median diameter (D50V) typically from 30µm up to 100µm and a Dry solids weight (Dw) from 50mg/mL to 200mg/mL.

The flow rate should preferably be about 250-500 cm/h in large scale columns with a 20 cm bed height at a back pressure of <3 bar and with a bed volume of >3L. The skilled person within the technical field is able to calculate the flow rate in a column of another size, height and volume, and subsequently able to adjust the settings accordingly.

The porous support in a beaded or particle form according to the present disclosure may have a Dw of 50-200 mg/ml, such as 50-150 mg/ml, 50-120 mg/ml, 50-100 mg/ml, 50-90 mg/ml, 60-80 mg/ml, or 60-75 mg/ml. Preferably, the Dw is of at least 63 mg/ml, or at least 65 mg/ml. The Dw may be at least of 70 mg/ml.

The porous support in a beaded or particle form may have a D50v of 30-100. According to the present disclosure, the D50v is preferably 35-90 µm, 40-80 µm, or 50-70 µm, such as 55-70 µm, 55-67 µm, 58-70 µm, or 58-67 µm. The D50v may for instance be at least 60 µm, or at least 62 µm.

The separation matrix disclosed above has a selectivity for VH3 chains and does not bind to an VH1 chain or VH2 chain, as shown below in Example 3 and Fig. 4. This example also shows that the

separation matrix disclosed above does not bind to an Fc region. Thus, it can be used in a method for isolation of any antibody or antibody fragments comprising at least one VH3 chain. The antibody or antibody fragment may be selected from a non-exhaustive list of monoclonal antibody, a bispecific antibody, a trispecific antibody, Fab fragments, Fab' fragments, (Fab')₂ fragments, (Fab₂) fragments, (Fab₃) fragments, Fv fragments, ScFv fragments, Bivalent diabodies, Multivalent triabodies, multivalent tetrabodies, minibodies, sdAb fragments, VHH-fragments and any other protein or polypeptide comprising a VH3 chain. The skilled person is aware that there is large variation for design of Antibodies, Antibody fragments and fusion proteins that comprise a VH3 chains. See i.e. Ulrich Brinkmann & Roland E. Kontermann (2017) "The making of bispecific antibodies", mAbs, 9:2, 182-212, DOI: 10.1080/19420862.2016.1268307.

The above-mentioned method for isolation of any antibody or antibody fragments comprising at least one VH3 chain comprises the steps of:

- a) contacting a liquid sample comprising a VH3 chain-containing antibody or antibody fragment with a separation matrix according to the above;
- b) washing said separation matrix with one or a combination of several washing liquids;
- c) eluting the VH3 chain-containing antibody or antibody fragment from the separation matrix with an elution liquid; and
- d) cleaning the separation matrix with a cleaning liquid.

The elution may be performed by using any suitable elution liquid used for elution from a Protein A separation matrix. The elution liquid can e.g. be a solution or buffer with pH 4 or lower, such as pH 2.5 - 4 or 2.8 - 3.5.

Due to the above-mentioned selectivity for VH3, the separation matrix may further be used in a method for separation of bispecific antibodies comprising one VH3 chain from variants of the antibody or antibody fragment comprising two VH3 chains or no VH3 chain. Said method comprises the steps of:

- a) contacting a liquid sample comprising a bispecific antibody with a separation matrix;
- b) washing said separation matrix with one or a combination of several washing liquids;
- c) eluting said bispecific antibody from the separation matrix with an elution liquid and at a decreasing pH; and
- d) cleaning the separation matrix with a cleaning liquid.

This method above may also be used for separation of trispecific antibodies.

The pH may be decreased by using a pH gradient. Alternatively, the pH may be decreased in a stepwise manner, similar to a gradient, using buffer solutions of different pH. The pH range during elution may be from about 5.5 to about 2, such as from about 5 to about 2, from about 4.5 to about 2, or from about 4 to about 2. Apart from the pH gradient, the elution liquid in the separation method is as disclosed above for the separation of any antibody or antibody fragment comprising at least one VH3 chain.

The decreasing of the pH may be performed by adding an elution buffer with a lower pH than a previous washing buffer or equilibration buffer. By gradually increasing the amount of elution buffer in relation to an equilibration or washing buffer, a decreasing pH is achieved, and elution will occur. The equilibration buffer should preferably have a pH of about 5.8-6 in order to prevent any undesired dissociation of a target molecule. However, it may be as low as possible as long as it does not cause a pre-mature dissociation of the target molecule, or antibody, from the separation matrix.

This method enables to separate bispecific antibodies or antibody fragments from monospecific antibodies or antibody fragments, or mismatched antibodies or antibody fragments, based on the presence of a VH3 chain. A monoclonal monospecific antibody has two identical VH chains. A bispecific antibody can be designed to comprise two different VH chains. Fig. 7 illustrates results of the method disclosed above. A bispecific or monospecific antibody or antibody fragment with no VH3 chain will not bind to the separation matrix and therefore flow through the separation matrix. A bispecific antibody or antibody fragment with one VH3 chain will bind to the separation matrix, and a bispecific or monospecific antibody or antibody fragment with two VH3 chains will bind even harder to the separation matrix. Thus, upon elution, the bispecific antibody with one VH3 chain will dissociate from the separation matrix at a higher pH and earlier than the antibody with two VH3 chains.

In the examples herein, an equilibration or washing buffer comprising the same salt or acid as the elution buffer is used. This is to ensure a linear gradient in the examples and to better visualize the advantage of the elution conditions. However, the skilled person will realize that any commonly used equilibration or washing buffer may be used.

Thus, the elution conditions mentioned above offer a clear separation of the homodimers and heterodimers of the antibody on a VH3 binding separation matrix (See fig. 7). Peak 1, at the highest elution pH, corresponds to the heterodimeric bsAb having one VH3 class chain and one VH chain of either VH1 class or VH2 class, which is the bsAB that is the target for purification and separation. This

has been confirmed by LC-MS data (not shown). Peak 2, at a lower pH, corresponds to a homodimeric species having two VH3 class chains, also having been confirmed by LC-MS data (not shown).

5 The Dynamic Binding Capacity (DBC) for the separation matrix according to the above is shown in Example 4, Figs 5A-5C. Here it can be observed that a prototype of the separation matrix according to the above has a higher DBC for Fab fragment and VHH fragment, compared to other Protein A based separation matrices, and is equivalent in DBC for a mAb comprising a VH3 chain.

10 The performance of the separation matrix according to the above is shown in Example 6. Here it is shown that the purification performance of a prototype VH3 binding separation matrix is equivalent to the commercially available MabSelect PrismA™.

15 The separation matrix according to the above has an excellent alkaline stability. The IgG capacity after 100 cycles, corresponding to 24 h incubation, in 0.5 M NaOH at 22 +/- 2 °C is at least 90% of the IgG capacity before the incubation. The VHH capacity after 100 cycles, corresponding to 24 h incubation in 0.5 M NaOH at 22 +/- 2 °C is also at least 90% of the VHH capacity before the incubation. The capacity for both IgG and VHH after 24 h incubation in 0.5 M NaOH is preferably at least 91%, or at least 92%, or at least 93%.

20 The IgG capacity for the prototype after 200 CIP cycles, corresponding to 50 h incubation, in 0.5 M NaOH at 22 +/- 2 °C is at least 80% of the IgG capacity before any incubation. The IgG capacity after 50 h incubation in 0.5 M NaOH is preferably at least 81%, or at least 82%, or at least 83%, or at least 84%.

These alkaline stability studies above show that the lifetime for the separation matrix according to the above is excellent and provides for an advantageous process economy when using said separation matrix.

25 While the invention has been described with reference to various exemplary aspects and embodiments, it will be understood by those skilled in the art that various changes may be made, and equivalents may be substituted for elements thereof without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or molecule to the teachings of the invention without departing from the essential scope thereof. Therefore, it is intended that the invention not be limited to any particular embodiment contemplated, but that the invention will include all embodiments falling within the scope of the appended claims.

30

EXAMPLES

The Examples below are intended to be illustrative of the effects and advantages of the separation matrix disclosed. They are not meant to in any way limit the scope of the invention to the exemplified embodiments.

Example 1 – VH3 binding and Fc binding properties**1A: Biacore analysis of binding affinity for Fc and Fab fragments of trastuzumab**

To assess binding to Fc (SEQ ID NO:148) and Fab (SEQ ID NO:149 and 150), VH3 binding polypeptides (SEQ ID NO:1 to 4) were immobilized on a Biacore CM5 chip. Trastuzumab Fc (5000 nM) and trastuzumab Fab (5000 nM) fragments were used as analyte.

Biacore binding analysis:

Materials and equipment used were as follows: CM5 sensor chips (Cytiva™), Biacore™ NHS coupling kit (Cytiva™), VH3 binding polypeptides (SEQ ID NO:1 to 4); Biacore™ 8K+ (Cytiva™); Fab (prepared from trastuzumab in-house), Fc from trastuzumab.

- 15 Immobilization was performed using a standard method in Biacore software with coupling of VH3 binding polypeptide variants in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1). VH3 binding polypeptide variants were diluted in an appropriate pH (based on pH-scouting) in acetate buffer at a concentration ranging from 10-30 µg/ml. The immobilization levels did to some degree vary between different polypeptide variants (approx. 600-1300 Ru).
- 20 In each run the polypeptide was immobilized in FC2. Multiple chips were used until all candidates were tested.

Biacore™ method for binding analysis:

Running buffer: PBS-P+

Flow rate: 10 µl/min

- 25 Sample injection: 600 s/10 min over both Flow Cells (FC1 and FC2)

Dissociation time: 600 s/10 min

Regeneration: 10 mM Glycin-HCl pH 1.5, 30 µl/min, 2x30 s

Injectons of analyte (Fab or Fc fragment of trastuzumab) for each channel (cycles) were as follows: buffer, 156 nM, 313 nM, 625 nM, 1250 nM, 2500 nM, 5000 nM.

All sensorgrams were generated as reference subtracted and the responses as the difference between baseline before injection and signal just before end of injection.

Results

The response levels of the trastuzumab Fc and Fab interactions of the VH3 binding polypeptide variants are depicted in Fig. 1A and Fig. 1B. The data show that the VH3 binding polypeptide variants SEQ ID NO:1- 4 exhibit a significantly reduced or abolished binding affinity for the Fc fragment of trastuzumab while retaining binding to the Fab fragment of trastuzumab. The positive reference, SEQ ID NO:88 shows binding to the Fc fragment of trastuzumab with 2000 RU at injection. The non-Fc-binding control (SEQ ID NO:147) shows no interaction with Fc (0 RU at injection), and weak binding to Fab with ~100 RU at injection.

1B: Biacore analysis of binding affinity for Fc fragment of trastuzumab and trastuzumab

To assess binding to the Fc fragment of trastuzumab and trastuzumab, the VH3 binding polypeptides were immobilized on a Biacore CM5 chip. Trastuzumab Fc fragment (5000 nM) and trastuzumab (625 nM) were used as analyte.

Biacore binding analysis:

Materials and equipment used were as follows: CM5 sensor chips (Cytiva™), Biacore™ NHS coupling kit (Cytiva™), VH3 binding polypeptides (SEQ ID NO:5 to 12); Biacore™ 8K+ (Cytiva™); Fc from trastuzumab (SEQ ID NO:148); trastuzumab (SEQ ID NO:145 and 146).

Immobilization was performed using a standard method in Biacore software with coupling of VH3 binding polypeptide variants in FC2 and activation/inactivation in FC1. VH3 binding polypeptide variants were diluted in an appropriate pH (based on pH-scouting) in acetate buffer at a concentration ranging from 10-30 µg/ml. The immobilization levels did to some degree vary between different polypeptide variants (approx. 600-1300 Ru).

In each run the polypeptide was immobilized in FC2. Multiple chips were used until all candidates were tested.

Biacore method for binding analysis:

Running buffer: PBS-P+

Flow rate: 10 µl/min

Sample injection: 600 s/10 min over both Flow Cells (FC1 and FC2)

Dissociation time: 600 s/10 min

Regeneration: 10 mM Glycin-HCl pH 1.5, 30 µl/min, 2x30 s

Injections of analyte (trastuzumab or Fc fragment of trastuzumab) for each channel (cycles) were as follows: buffer, 156 nM, 313 nM, 625 nM, 1250 nM, 2500 nM, 5000 nM.

All sensorgrams were generated as reference subtracted and the responses as the difference between baseline before injection and signal just before end of injection.

5 *Results*

The response levels of the Fc fragment of trastuzumab and the trastuzumab interactions with the VH3 binding polypeptide variants (SEQ ID NO:5 to 12) are depicted in Fig. 2A and Fig. 2B. The data show that the novel VH3 binding polypeptide variants (SEQ ID NO:5 to 12) exhibit a significantly reduced or abolished binding affinity for Fc fragment of trastuzumab while retaining binding to
10 trastuzumab. The positive reference (SEQ ID NO:88) shows binding to both Fc and Trastuzumab with >2000 RU and >6000 RU at injection, respectively.

1C: Biacore analysis of binding affinity for Fc and Fab fragments of trastuzumab and trastuzumab

To assess binding to trastuzumab and to the Fc and Fab fragments of trastuzumab, the VH3 binding
15 polypeptides (SEQ ID NO:13 to 15) were immobilized on a Biacore™ CM5 chip. Trastuzumab Fc fragment (5000 nM), trastuzumab Fab fragment (2500 nM) and trastuzumab (625 nM) were used as analyte.

Biacore binding analysis:

Materials and equipment used were as follows: CM5 sensor chips (Cytiva™), Biacore™ NHS coupling
20 kit (Cytiva™), VH3 binding polypeptides (SEQ ID NO:13 to 15); Biacore™ 8K+ (Cytiva™); Fab (prepared from trastuzumab in-house), Fc from trastuzumab, trastuzumab.

Immobilization was performed using a standard method in Biacore software with coupling of VH3 binding polypeptide variants in FC2 and activation/inactivation in FC1. VH3 binding polypeptide variants were diluted in an appropriate pH (based on pH-scouting) in acetate buffer at a
25 concentration ranging from 10-30 µg/ml. The immobilization levels did to some degree vary between different polypeptide variants (33 approx. 600-1300 Ru).

In each run the polypeptide was immobilized in FC2. Multiple chips were used until all candidates were tested.

Biacore method for binding analysis:

30 Running buffer: PBS-P+

Flow rate: 10 µl/min

Sample injection: 600 s/10 min over both Flow Cells (FC1 and FC2)

Dissociation time: 600 s/10 min

Regeneration: 10 mM Glycin-HCl pH 1.5, 30 µl/min, 2x30 s

Injectations of analyte (Fab or Fc fragment of trastuzumab or trastuzumab) for each channel (cycles)

5 were as follows: buffer, 156 nM, 313 nM, 625 nM, 1250 nM, 2500 nM, 5000 nM.

All sensorgrams were generated as reference subtracted and the responses as the difference between baseline before injection and signal just before end of injection.

Results

The response levels of the trastuzumab Fc, trastuzumab Fab and trastuzumab interactions
10 with the VH3 binding polypeptide variants (SEQ ID NO:13 to 15) are depicted in Fig. 3A, Fig. 3B and Fig. 3C. The results show that the VH3 binding polypeptide variants (SEQ ID NO:13-15) exhibit a significantly reduced or abolished binding affinity for the Fc fragment of trastuzumab while retaining binding to trastuzumab and to the Fab fragment of trastuzumab. The alkali clean stable positive control that binds both Fc and Fab fragments of trastuzumab (SEQ ID NO:88) shows binding to
15 trastuzumab Fc, trastuzumab Fab and trastuzumab with >2000 RU, >300 RU and >6000 RU at injection, respectively.

The three additional VH3 binding polypeptides SEQ ID NO:13 to 15 show improved binding to trastuzumab and trastuzumab Fab in comparison to the VH3 binding polypeptide (SEQ ID NO:10) tested in Example 1B. SEQ ID NO:13 and SEQ ID NO:15 show improved binding to both Fab and
20 trastuzumab in comparison to the non-Fc-binding control that is not alkali clean stable (Negative CTRL). SEQ ID NO:13 and SEQ ID NO:15 show improved binding to Fab in comparison to the alkali clean stable positive control SEQ ID NO:88 that binds both Fc and Fab fragments. SEQ ID NO:13 and SEQ ID NO:15 show comparable trastuzumab response values to that of the positive control SEQ ID NO:88.

25 Consequently, the above shows that sequences according to the present disclosure has a VH3 binding activity, while the Fc binding activity is significantly reduced or deleted.

1D: Biacore analysis of binding of Fc and Fab fragments of trastuzumab for SEQ ID NO: 89-95, SEQ ID NO 99-144

Materials and equipment used were as follows: CM5 sensor chips (Cytiva™), Biacore™ NHS coupling
30 kit (Cytiva™), the polypeptides (SEQ ID NO:89 – SEQ ID NO: 94 and SEQ ID NO:185-SEQ ID NO:239); Biacore™ 8K+ (Cytiva™); Fab-fragment and Fc-fragment (prepared from trastuzumab in-house).

- Immobilization was performed using a standard method in Biacore software with coupling of polypeptide variants in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1). The polypeptide variants used for immobilization were diluted in acetate-buffer (Cytiva™) with a pH value > 1 unit below the pI of the polypeptide at a concentration ranging from 10-50 µg/ml. The
- 5 immobilization levels did to a certain degree vary between different polypeptide variants (approx. 700-2000 Ru, see Table 16).

In each run the polypeptide was immobilized in FC2. Multiple chips were used until all candidates were tested.

Biacore™ method for binding analysis:

- 10 Running buffer: PBS-P+
- Flow rate: 10 µl/min
- Sample injection: 600 s/10 min over both Flow Cells (FC1 and FC2)
- Dissociation time: 600 s/10 min
- Regeneration: 10 mM Glycin-HCl pH 1.5, 30 µl/min, 2x30 s
- 15 Injections of analytes Fab and Fc fragment of trastuzumab at 500 nM concentration respectively in running buffer.

All sensorgrams were generated as reference subtracted and the responses as the difference between baseline before injection and signal just before end of injection.

Results

- 20 The response levels of the trastuzumab Fc and Fab interactions of the polypeptide variants in the sensorgrams have been translated and summarized in Table 1 (levels rounded to the nearest multiple of 50).

Table 1. Binding of Fc and Fab fragments of trastuzumab for SEQ ID NO: 89-95, SEQ ID NO 99-144

SEQ ID NO	Immobilization level (Ru)	Fc binding (Ru)	Fab binding (Ru)
SEQ ID NO:94	783	1650	250
SEQ ID NO:93	861	1850	200
SEQ ID NO:92	802	1700	500
SEQ ID NO:91	986	2200	750
SEQ ID NO:90	1098	2100	2300

SEQ ID NO:89	892	1750	0
SEQ ID NO:95	711	1500	0
SEQ ID NO:99	1257	50	250
SEQ ID NO:100	1360	0	100
SEQ ID NO:101	1342	0	2400
SEQ ID NO:102	1363	0	550
SEQ ID NO:103	1333	0	600
SEQ ID NO:104	1145	0	550
SEQ ID NO: 105	1372	0	150
SEQ ID NO:106	1366	0	2550
SEQ ID NO:107	1354	0	3050
SEQ ID NO:108	1383	0	0
SEQ ID NO:109	1449	0	0
SEQ ID NO:110	1144	0	0
SEQ ID NO:111	1461	0	2500
SEQ ID NO:112	1090	0	150
SEQ ID NO:113	1987	0	0
SEQ ID NO:114	1174	0	100
SEQ ID NO:115	1213	0	150
SEQ ID NO:116	1226	0	2250
SEQ ID NO:117	1244	0	150
SEQ ID NO:118	1158	0	250
SEQ ID NO:119	1243	0	2650
SEQ ID NO:120	1231	0	3550
SEQ ID NO:121	1377	0	0
SEQ ID NO:122	1505	0	0

SEQ ID NO:123	1592	0	0
SEQ ID NO:124	1698	0	3100
SEQ ID NO:125	1293	0	200
SEQ ID NO:126	1343	0	50
SEQ ID NO:127	1223	0	350
SEQ ID NO:128	1227	0	1350
SEQ ID NO:129	1311	0	3100
SEQ ID NO:130	1210	50	350
SEQ ID NO:131	1058	0	1250
SEQ ID NO:132	1210	0	2600
SEQ ID NO:133	1179	0	300
SEQ ID NO:134	1401	0	1600
SEQ ID NO:135	1424	0	3400
SEQ ID NO:136	1971	0	650
SEQ ID NO:137	1227	0	500
SEQ ID NO:138	1333	0	2150
SEQ ID NO:139	1866	0	500
SEQ ID NO:140	1421	0	1200
SEQ ID NO:141	1361	0	2650
SEQ ID NO:142	1096	0	150
SEQ ID NO:143	1057	0	850
SEQ ID NO:144	1111	0	2200

Of the tested sequences above according to the present disclosure (SEQ ID NO. 99-144), only 10 did not exhibit a sufficient VH3 binding to be considered an VH3 binding polypeptide. A R_u value of 50 is considered to not be binding to the fragment in question. However, all the sequences according to the present disclosure has a deleted or substantially reduced Fc binding.

1E. Test of VH3 binding multimers by chromatography

A subset of alkali clean stabile VH3 binding polypeptides with abolished Fc binding (SEQ ID NO:14 and 15) were chosen for further studies on column. The further studies included multimerization of the polypeptides to tetramers and hexamers and expression of said multimers in *E. coli*. On these multimeric VH3 binding polypeptides (SEQ ID NO:96-98), a primary characterization was performed, wherein the ligands were evaluated for dynamic binding capacity (DBC), pH elution and alkali clean stability.

The effect of A29G mutation on tetramers of VH3 binding polypeptides (SEQ ID NO:96 and 97) was investigated for DBC and elution pH in a pH gradient using trastuzumab. A hexamer of a VH3 binding polypeptide (SEQ ID NO:98) was tested for DBC and elution pH in a pH gradient using trastuzumab and different target molecules. Moreover, alkali clean stability of SEQ ID NO:98 was tested by running 4h incubations with 0.5 M NaOH followed by DBC measurements. A hexamer (SEQ ID NO: 151) of an alkali clean stabile polypeptide that binds to both the Fc and Fab fragments of trastuzumab (SEQ ID NO: 88) was used as control.

15 *DBC measurement*

DBC measurements were made in Tricorn™ 5/50 columns. The column was equilibrated with PBS buffer. Trastuzumab, trastuzumab Fab fragment or nanobody was loaded to the columns via the sample pump at desired flow rate typically 0.166 mL/min to achieve 6 min residence time (depending on the bed height of the column) until the UV signal of approx. 20% of maximum was reached. The column was then washed with PBS buffer at flow rate 1 mL/min. The protein was eluted with elution buffer (50 mM sodium citrate pH 2.5) at flow rate 1 mL/min. The column was cleaned with 0.1 M NaOH, at a flow rate of 0.166 mL/min for 15 min followed by re-equilibration with PBS buffer.

pH-elution using a gradient

25 pH elution measurements were made in Tricorn™ 5/50 columns. The column was equilibrated with PBS buffer. Approximately 10 mg Trastuzumab, Trastuzumab Fab fragment or nanobody was loaded to the columns via the sample pump at desired flow rate typically 0.166 mL/min to achieve 6 min residence time. The column was then washed with PBS buffer at flow rate 1 mL/min. The protein was eluted with an elution gradient from 50 mM sodium citrate pH 6.5 to 50 mM sodium citrate pH 2.5 at flow rate 1 mL/min. The column was cleaned with 0.1 M NaOH, at a flow rate of 0.166 mL/min for 15 min followed by re-equilibration with PBS buffer. The elution pH is determined as the apex of the elution peak.

Accelerated NaOH studies

Accelerated NaOH studies were performed by running a start DBC run to measure resin capacity at a set residence time followed by a fill up of the column with 0.5 M NaOH. When the column was completely filled, the flow was stopped, and the column was incubated in NaOH for 4 h followed by re-equilibration with PBS and a new DBC measurement. This iteration was continued until the column had been incubated for a total of 24 h in 0.5 M NaOH.

Results

Evaluation VH3 binding polypeptide tetramers by chromatography

Tetrameric variants (SEQ ID NO:96 and SEQ ID NO:97) were evaluated with DBC measurements and pH elution. The results are summarized in Table 2.

Table 2. Reference resin is SEQ ID NO:151. The two test resins SEQ ID NO:96 and SEQ ID NO:97 exhibit only VH3 interaction. DBC was measured with trastuzumab at 6 min residence time (RT) and 10 % break-through. The pH elution is measured with approximately 10 mg trastuzumab. Ligand density (LD) is shown.

Multimeric variant	SEQ ID NO:	LD (mg/mL)	DBC trastuzumab (mg/mL)	Elution pH
Reference	151	16.1	63.8	3.75
VH3_1	96	14.2	32.7	4.15
VH3_2	97	15.8	29.0	3.75

Evaluation VH3 binding polypeptide hexamers by chromatography

The hexameric variant (SEQ ID NO:98) coupled resin was packed in columns and initially tested for dynamic binding capacity (DBC) for trastuzumab, trastuzumab Fab and Variable Heavy Heavy fragment (VHH). The results are summarized in Table 3.

Table 3. Results of dynamic binding capacity for SEQ ID NO:98 in comparison with SEQ ID NO:151 on 60 µm agarose beads. Ligand density (LD) and DBC is shown.

Multimeric variant	SEQ ID NO	LD (mg/mL)	DBC mAb(g/L)	DBC Fab (g/L)	DBC VHH (g/L)
Reference	181	16.1	58	24	23
VH_3	98	18.3	57	45	33

Analysis of alkali clean stability on column

An accelerated NaOH study was made to test alkali clean stability of VH3_3 (SEQ ID NO:98) on beads. SEQ ID NO:151 was used as reference. In this study, DBC was measured at 6 min RT followed by 4 h incubations in 0.5 M NaOH with DBC measurements between each incubation. The resin was incubated in a total of 24 h. Results from the alkali clean stability study is shown in Table 4.

Table 4. Summary of the DBC values for SEQ ID NO:98 in comparison with SEQ ID NO:151 at start and after 4h incubation with 0.5 M NaOH

Time	Reference (g/L)	Reference Relative (%)	VH_3 (g/L)	VH_3 Relative (%)
Start	66	100	55	100
4h	66	100	55	100
8 h	65	98	54	98
12 h	65	98	54	98
16 h	65	98	54	98
20 h	66	100	52	95
24 h	65	98	52	95

10 *Conclusions*

Column studies show that the single A29G mutation of SEQ ID NO:96 to SEQ ID NO:97 causes the elution pH to decrease thus indicating a higher affinity. Moreover, the increased affinity of SEQ No:98, compared to a reference, leads to an increased capacity for a single 1:1 interaction such as

Fab, VHH or bispecific antibody (Table 3). Surprisingly, these mutations could be made on the backbone without significantly lowering the alkali clean stability of the protein (Table 4).

Example 2 - Separation matrix

- 5 For the prototype tested, a ligand comprising polypeptides according to SEQ ID NO:15, was used. Hexamers of SEQ ID NO:15, corresponding to SEQ ID NO: 98 were expressed and purified by conventional means known to the skilled person.

The purified hexamer SEQ ID NO: 98 was immobilized on agarose beads as a base matrix according to the exemplary method below.

10 *Activation*

The base matrix used was rigid cross-linked agarose beads with the indicated volume-weighted median diameter, prepared according to the methods of US6602990 and with the indicated pore size corresponding to an inverse gel filtration chromatography K_{av} value of 0.70 for dextran of Mw 110 kDa, according to the methods described in Gel Filtration Principles and Methods, Pharmacia LKB

- 15 Biotechnology 1991, pp 6-13.

25 mL (g) of drained base matrix, 10.0 mL distilled water and 2.02 g NaOH (s) was mixed in a 100 mL flask with mechanical stirring for 10 min at 25°C. 4.0 mL of epichlorohydrin was added and the reaction progressed for 2 hours. The activated gel was washed with 10 gel sediment volumes (GV) of water.

20 *Coupling*

To a protein water solution, sodium carbonate (0,01 M), sodium bicarbonate (0,1 M), sodium chloride (0,15 M) and EDTA disodium salt (1 mmol) were added and when all had dissolved, Dithiothreitol (DTT, 0,1 M) was added. The pH was adjusted to above pH 8.0. The reaction mixture was placed on a shaking table (23 °C, 500 rpm) and left to reduce for 2 hours.

- 25 PD10 prepacked gel filtration columns (Cytiva™) were used to desalt the protein. The columns were equilibrated with desalting solution (0.15 NaCl, 1 mM EDTA) prior to loading the protein (max 2.5 mL). The eluted fractions were collected and combined.

The protein concentration of the desalted solution was determined by UV absorbance at 276 nm with a protein extinction coefficient of 1.0.

The activated gel was washed with 5 GV 0,1M Trisaminomethane (Tris) buffer pH 8.4. 15 mL gel, 20 mg ligand/mL gel ((11.7 mL), 3.3 mL Tris buffer and 7.0 g sodium sulfate were mixed in a 50mL flask and stirred at 33°C for 4 h.

- 5 After immobilization, the gel was washed with 3x1 GV distilled water. The gel and 1 GV (0.1 M phosphate/1 mM EDTA/7.5% Thioglycerol pH 8.5) were mixed and the flasks were left stirring at room temperature for 15-20h. The gels were then washed 3 times alternately with 3x1GV 0.5 M HAc and 3x1GV 0.1 M TRIS/0.15 M NaCl pH 8.5 and then with 10x1GV mL distilled water. The gel was conditioned in 20% EtOH in a 50% slurry.

Determination of ligand density through amino acid analysis, AAA

- 10 Prototypes were dried, and the dry weight determined. The skilled person is aware of known general methods for performing such a procedure. Thereafter, the prototypes were sent dried for amino acid analysis. With the corresponding dry weights and an excel calculation sheet containing information of the protein size and all data on the primary amino sequence of the protein the ligand densities could be derived in mg ligand/mL resin.

15

Example 3 - Selectivity of VH binding

To test whether the ligands according to the present disclosure have any affinity for VH1 and VH2 class of antibody, three VH1 class of antibodies and one VH2 class of antibody were tested on the prototype. The antibodies of VH1 class tested were Rituximab, Guselkumab, and Pembrolizumab.

- 20 The VH2 class of antibody was Cetuximab. The properties of these four antibodies are listed below in Table 5.

Table 5. Molecules used for selectivity study of the prototype.

Molecule	VHH class	Other properties	Source	Comments
Rituximab	VH1	Chimeric (murine/human); IgG1	In-house	Biosimilar
Guselkumab	VH1	Human; IgG1	Apoteket/Janssen	Tremfya®
Pembrolizumab	VH1	Humanized (from mouse); IgG4	Apoteket/MSD	Keytruda®
Cetuximab	VH2	Chimeric (mouse/human); IgG1	In-house	Biosimilar

The prototype was packed in 1 mL column, and 5 mg of antibody was loaded onto the column using an ÄKTA pure™ 25 chromatography systems and the UNICORN™ method according to Table 6.

Table 6. UNICORN™ method used for specificity test study. (CV = Column Volume)

Step	Inlet	Buffer	Length (CV)	Flow rate (mL/min)	Comments
Equilibration	A1	20 mM Na-phosphate, 150 mM NaCl, pH 7.2	5	0.5	
Sample application	N/A	Superloop, 2.5 mL	varied	0.166	Collect FT
Column wash 1	A1	20 mM Na-phosphate, 150 mM NaCl, pH 7.2	5	0.25	
Column wash 2	A2	50 mM Na-citrate, pH 6.0	5	0.25	
Elution	B1	50 mM Na-citrate, pH 3	5	0.25	Collect Elution
Column strip	A4	100 mM acetic acid	2	0.25	
Column CIP	A3	0.5 M NaOH	3	0.20	Up flow
Re-equilibration 1	A1	20 mM Na-phosphate, 150 mM NaCl, pH 7.2	2	0.20	Up flow
Re-equilibration 2	A1	20 mM Na-phosphate, 150 mM NaCl, pH 7.2	2	0.5	Up flow

- 5 Flow-through was collected and antibody concentration was determined. The results are shown in Fig. 4, where it is shown that none of the antibodies in Table 6 bound to the VH3 binding prototype. Thus, it is shown that the VH3 binding prototype does not bind to VH1 and VH2 class of antibodies. Furthermore, this also demonstrated that the VH3 binding prototype has no affinity towards the Fc fragment or a light chain of an antibody.

10

Example 4 – Dynamic Binding Capacity

Dynamic binding capacity (DBC) was tested for the prototype using the following test molecules listed in Table 7.

Table 7. List of the test molecules used for DBC evaluation.

Molecule	MW (kDa)	Source	Comments
Trastuzumab	145.4	In-house	Biosimilar to Herceptin® (Roche)
Fab-fragment of Trastuzumab	48.6	In-house	Cleaved in-house from Trastuzumab*
VHH-EgA1 (VHH)	15.7	In-house	Single domain (VH)/Camelid VH domain

* For instance according to Larsson, Lars-Inge (September 1988). Immunocytochemistry: Theory and practice. Crc Press. p. 1. ISBN 0-8493-6078-1.

The prototype (denoted VH3) was tested using MabSelect PrismA™ (Cytiva™), Praesto® Jetted A50 (PuroLITE) and JSR Amsphere™ A3 (JSR Life Sciences) as references.

The resins were packed into Tricorn™ 5/50 column, 1 ml. An ÄKTA pure™ 25 chromatography system was used for DBC experiments. Dynamic binding capacity at 10% breakthrough (Qb10) at retention time (RT) of 6 minutes was evaluated by employing DBC extension tool in UNICORN™ 7.7 (Cytiva™).

Table 8. UNICORN™ method used in DBC test.

Step	Buffer	Length (CV)	Flow rate (mL/min)	RT (Min.)
Equilibration	PBS pH 7.4	3	0.25	4
Sample application	2 mg/mL test molecule	-	0.166	6
Column wash	PBS pH 7.4	7	0.166	6
Elution	50 mM Na-acetate, pH 3.5	5	0.166	6
CIP	0.1 NaOH	3	0.2	5
Re-Equilibration	PBS pH 7.4	8	0.2	5

The molecules tested on the prototype VH3 and the above-mentioned reference resins were the mAb Trastuzumab, Fab-fragment of Trastuzumab, and VHH, listed in Table 7 above. The results are shown in Fig.5A-5C.

Fig. 5A shows DBC for Trastuzumab at 6 min RT. Here it is demonstrated that the prototype VH3 shows similar dynamic binding capacity to Trastuzumab as the references MabSelect Prisma™ (Cytiva™) and Praesto® Jetted A50 (Purolite) and has a better dynamic binding capacity than JSR Amsphere™ A3 (JSR Life Sciences).

- 5 Fig. 5B shows DBC for Fab-fragment of Trastuzumab at 6 min RT. Here it is demonstrated that the prototype VH3 has a higher dynamic binding capacity to the Fab fragment than any of the reference resins. The prototype VH3 has a 37% higher dynamic binding capacity than Amsphere™ A3, and a 32% higher dynamic binding capacity than Praesto® Jetted A50 for the Fab fragment. This is indicative of a better binding to the VH3 chain, which is present on the Fab fragment of Trastuzumab,
10 for the prototype. Furthermore, it is indicative that the prototype is not dependent on an Fc binding to separate a target molecule.

- Fig 5C shows DBC for a VHH. Again, it is demonstrated that the prototype VH3 has a higher dynamic binding capacity than the references. The prototype VH3 has a 34% higher binding capacity than Amsphere™ A3, and a 38% higher binding capacity than Praesto® Jetted A50 for the VHH. Again, this
15 is indicative of a better binding to VH3 for the prototype.

Example 5 - Separation of Bispecific Antibodies

- For this example, the antibody Emicizumab (Hemlibra®) was used. Emicizumab is an asymmetric bispecific antibody with two different heavy chains, of which one has a variable region of class VH1
20 and the other one has variable region of class VH3 (**Figure 6A**). The heavy chains are paired with identical light chains. Mutations in heavy chains (CH3) facilitate the heavy chains hetero-dimerization through electric repulsion and attractions. There are two possible mispairings present in the feed when producing Emicizumab in the form of two homodimers: VH1:VH1 and VH3:VH3 (**Figure 6B and 6C**).

- 25 On the VH3 binding prototype, which is lacking Fc interaction, the homodimer VH1:VH1 should not bind and therefore go in flow-through, while the other homodimer VH3:VH3 binds with two interactions, thus creating avidity effect and therefore binds more stringent compared to the correctly paired heterodimer VH1:VH3. The VH1:VH3 heterodimer should elute at a milder pH. By eluting in a gradient or two step elution with different pH's, a separation between VH1:VH3 and
30 VH3:VH3 should be feasible.

The prototype was packed in Tricorn™ 50/100 column, 2ml resin.

5A. Stepwise elution

30 mg Emicizumab/ml resin was added, and elution was performed stepwise with a first elution at pH 4.0 and a second elution at pH 3.5. ÄKTA pure™ 25 chromatography systems and the UNICORN™ method is disclosed in Table 9.

5 **Table 9.** UNICORN™ method used for bispecific separation of high load.

Step	Inlet	Buffer	Length (CV)	Flow rate (mL/min)	comments
Equilibration	A1	20 mM Na-phosphate, 150 mM NaCl, pH 7.2	5	1	
Sample application	N/A	Superloop, 50 ml. Sample volume is 49.5 mL (CCCF)	varied	0.5; RT 4 min	Collect FT, peak fractionation
Column wash 1	A1	20 mM Na-phosphate, 150 mM NaCl, pH 7.2	5	0.5	
Column wash 2	A2	20 mM Na-citrate, pH 6.0	5	0.5	
Elution block 1	B1	20 mM Na-citrate, pH 4.0	10	0.5	Collect 1 mL fraction
Elution block 2		20 mM Na-citrate, pH 3.5	7	0.5	Collect 1 mL fraction
Column strip	A4	100 mM acetic acid	3	0.5	
CIP	A3	0.5 M NaOH	3	0.40	Up flow
Re-equilibration 1	A1	20 mM phosphate, 150 mM NaCl, pH 7.2	2	0.40	Up flow
Re-equilibration 2	A1	20 mM phosphate, 150 mM NaCl, pH 7.2	5	1	Up flow

The result is shown in Fig. 7. Two distinct peaks are shown between 70-80 ml and between 90-100 ml, respectively. The first peak between 70-80 ml is eluted at pH 4.0, and the second peak between 90-100 ml is eluted at pH 3.5. The first peak corresponds to the heterodimer and the second peak to the homodimer (LC-MS results not shown). Thus, a separation between VH1:VH3 and VH3:VH3 as discussed above was achieved.

Example 6 - Purification performance

To test purification performance e.g., yield, elution pool, aggregates content, and host cell protein (HCP) clearance, the prototype was compared with MabSelect PrismA™ (Cytiva™). 1 mL column of each resin types (VH3 prototype and MabSelect™ PrismA) were packed and a Trastuzumab clarified cell culture feed was loaded on the columns at 70% of DBC at Qb (10).

Table 10. UNICORN™ method used in purification performance study.

Step	Buffer	Length (CV)	Flow rate (ml/min)	RT	Comment
Equilibration	20 mM Na-phosphate, 150 mM NaCl, pH 7.0	5	0.166	6 min	
Sample application	Superloop, 50 ml. Sample volume is 70% of determined DBC at 10% breakthrough	varied	0.166	6 min	Collect FT
Column wash 1	20 mM Na-phosphate, 150 mM NaCl, pH 7.0	1.5	0.166	6 min	
Column wash 2	20 mM Na-phosphate, 500 mM NaCl, pH 7.0	5	0.166	6 min	
Column wash 3	50 mM Na-acetate, pH 6.0	2	0.166	6 min	
Elution	50 mM Na-acetate, pH 3.5	5	0.166	6 min	Peak fractions, start 100 mAU- stop 95 mAU
Column strip	100 mM acetic acid	2	0.166	6 min	
Column CIP	0.5 M NaOH	3	0.20	5 min	Up flow
Re-equilibration at low flow	20 mM Na-phosphate, 150 mM NaCl, pH 7.0	1.5	0.20	5 min	Up flow
Re-equilibration	20 mM Na-phosphate, 150 mM NaCl, pH 7.0	3	0.5	2 min	Up flow

Yield was calculated in percentage from total amount of antibody found in elution pool where the initial load was considered as 100%. [Eluate]*pool volume / [Max load].

Aggregation content in elution was determined by loading 600 µg fresh eluate onto a Superdex 200 10/300 GL column and from chromatogram by peak integration. HCP levels were measured on a

5 Gyrolab xP GW10224 (ID: 37640) using Gyrolab CHO-HCP kit as a standard.

Table 11. UNICORN™ method used SEC analyses of eluate for aggregates content from purification performance run.

Phase and inlet	Buffer	Volume (mL or CV)	Flow rate (mL/min)	Comments
Equilibration A11	200 mM Potassium phosphate, pH 6.8	1 mL	0.5	Auto zero UV
Sample application A1	Eluate from purification run	50 µL /600 µg	0.5	Auto zero UV
Elution B1	200 mM Potassium phosphate, pH 6.8	run length: 25 mL	0.5	

The results are shown in Table 12.

10 **Table 12.** Comparison of purification performance data from Prototype and MabSelect Prisma™ (MS Prisma).

Resin	Yield (%)	Elution pool Vol (CV)	Aggregates (%)	HCP (ppm)*
MS Prisma	99,0	1,81	1,3	596
Prototype VH3	97,8	1,85	1,1	275

*HCP in start material, 152085 ppm

As can be seen in Table 12, the prototype is comparable with MabSelect Prisma™ in all aspects analyzed. Elution pool volume for both columns is a bit larger (1.3–1.5 CV is expected) because of 1 mL column used. System dead volume is also a contributor to large elution pool. System dead volume is added to pool volume as dead volume divided by pool volume. Thus, use of a smaller column and a system with larger dead volume results in larger pool volume. When a larger column is used the system dead volume is diluted and keeps the pool volume smaller.

15

Example 7 - Alkaline stability

1 mL of the prototype resin was packed in Tricorn™ 5/50 columns. The alkali stability was tested using Trastuzumab and VHH (see above in Table 4). A Trastuzumab solution of 2mg/mL was prepared
 5 in a PBS buffer, pH 7.4. VHH (VHH-EgA1) solution of 2 mg/ml was prepared in a 50 mM Acetate buffer, pH 6.0.

The breakthrough capacity was calculated using Extensions-DBC Calculations-Analyze in UNICORN™ 7.7.

For the calculation of breakthrough capacity at 10%, the equation below was used. That is the amount
 10 of target (mAb8/VHH) that is loaded onto the column until the concentration of target in the column effluent is 10% of the target concentration in the feed.

$$Q_{10\%} = \frac{C_0}{V_c} \left[V_{app} - V_{sys} - \int_{V_{sys}}^{V_{app}} \frac{A(V) - A_{sub}}{A_{100\%} - A_{sub}} * dV \right]$$

$A_{100\%}$ = 100% UV signal

A_{sub} = absorbance contribution from non-binding mAb

15 $A(V)$ = absorbance at a given applied volume

V_c = column volume

V_{app} = volume applied until 10% breakthrough

V_{sys} = system dead volume

C_0 = feed concentration

20

7A. Accelerated alkaline stability – 100 cycles

The test was made on an ÄKTA pure™ 25 chromatography system. Dynamic binding capacity at 10% breakthrough (Qb10) at retention time (RT) of 6 minutes was evaluated by employing DBC extension tool in UNICORN™ 7.7 (Cytiva™). The method, disclosed below in table13, was run 6 times, leading to
 25 a total NaOH exposure for the resin corresponding to 96 CIP of 15 minutes.

Table 13. Method for accelerated alkaline stability.

Step	Volume	Buffer	Flow direction	RT (min)
Equilibration*	5 CV	PBS pH 7.4/ 50 mM Na-acetate, pH 6.0		1
Sample*	20% of max	mAb8/ VHH-EgA1		6
Wash	3 CV	PBS pH 7.4/ 50 mM Na-acetate, pH 6.0		1
Elution	4 CV	50 mM Na-citrate, pH 2.5		1
CIP	5 CV	0.5 M NaOH	Up	1
CIP pause		0.5 M NaOH	Up	235
Equilibration*	10 CV	PBS pH 7.4/ 50 mM Na-acetate, pH 6.0	Up	1

* Equilibration buffer for Trastuzumab: PBS, pH 7.4. Equilibration buffer for VHH: 50 mM Acetate pH 6.0.

DBC was measured every 16th cycle. The results are shown in Fig. 8A. The remaining relative binding capacity after 100 cycles for both Trastuzumab and VHH was 93%.

7B. Alkaline stability – 200 cycles

This test was performed with Trastuzumab. ÄKTA pure™ 25 chromatography system and DBC extension tool in UNICORN™ 7.7 (Cytiva™) was used.

Table 14. UNICORN™ method for repeated CIP cycling.

Step	Buffer	Volume (CV)	Flow rate (ml/min)	Flow direction
Equilibration	PBS pH 7.4	2	0.25	Down
Elution	50 mM Na-acetate pH 3.5	5	0.166	Down
Equilibration	PBS pH 7.4	1.5	0.166	Down
CIP	0.5 M NaOH	3	0.2	Up
Re-equilibration	PBS pH 7.4	8.5	0.2	Up

Table 15. UNICORN™ method used for DBC run for Trastuzumab as a part of repeated CIP cycling study.

Step	Buffer	Volume (CV)	Flow rate (ml/min)	Flow direction
Equilibration	PBS pH 7.4	3	0.25	Down
Sample Application	mAb8, ca. 2 mg/ml	varied	0.166	Down
Column wash	PBS pH 7.4	7	0.166	Down
Elution	50 mM Na-acetate pH 3.5	5	0.166	Down
CIP	0.5 M NaOH	3	0.2	Up
Re-equilibration	PBS pH 7.4	7.5	0.2	Up

9 CIP cycles (Table 14) were run before the first DBC measurement (Table 15). For each CIP, the contact time is 15 minutes. Thereafter, DBC was measured every 10th cycle with Trastuzumab at 6 min RT. The results are shown in Fig. 8B. The remaining relative binding capacity for Trastuzumab was 84% after 200 cycles.

Example 8 - Elution pH

For determination of elution pH of the VH3 binding prototype resin (Prototype VH3) and to compare with MabSelect Prisma™, 1 mL resin was packed in Tricorn™ 5/100 columns for both resins. A Trastuzumab solution of 2mg/mL was prepared in a PBS buffer, pH 7.2.

Five mg purified Trastuzumab was loaded on the columns and bound antibody was eluted with a 20 CV linear pH gradient from 100% Buffer A to 100% Buffer B where buffer A was 50 mM or 20 mM Na-citrate at pH 6.0 and buffer B was 50 mM or 20 mM Na-citrate at pH 2.5.

Table 16. UNICORN method used in elution pH study.

Phase and inlet	Buffer	Volume (CV)	Flow rate (mL/min)	Comments
Equilibration A1	20 mM Na-Phosphate, 150 mM NaCl pH 7.2	4 CV	0.25 mL/min	Reset UV monitor
Sample application S1	Trastuzumab 2mg/mL	5 mg	0.25 mL/min	
Wash 1	20 mM Na-Phosphate, 150 mM NaCl pH 7.2	3 CV	0.25 mL/min	

Wash 2	20 mM Na-Phosphate, 500 mM NaCl pH 7.2	3 CV	0.25 mL/min	
Wash 3 (Buffer A)	20 / 50 mM Na-Citrate pH 6	2 CV	0.25 mL/min	
Elution (Buffer B)	20 / 50 Na-Citrate pH 2.5	20 CV	0.25 mL/min	Gradient elution (100% B, A-B)
Strip	100 mM Na-Acetate pH 2.9	2 CV	0.25 mL/min	
CIP	0.5 M NaOH	3 CV	0.2 mL/min	Up flow
Re-equilibration	20 mM Na-phosphate, 150 mM NaCl pH 7.2	CV	0.2 mL/min	Up flow

Use of 50 mM buffer strength for elution is a common practice for protein A resin such as MabSelect PrismA™. However, more recently the inventors have found that on a Protein L resin, such as MabSelect™ VL (Cytiva™), use of 20 mM Na-citrate instead of 50 mM Na-citrate can result in milder elution (elution at higher pH). Hence, it was tested whether using 20 mM citrate for the present VH3 binding prototype could also result in milder elution.

Table 17. Elution pH for VH3 binding prototype (VH3) and MabSelect PrismA™ (MS PrismA).

Resin	Elution pH with 50 mM Na-Citrate	Elution pH with 20 mM Na-Citrate
VH3	3.61	3.85
MS PrismA	3.69	3.81

As Table 17 shows, the VH3 binding prototype has a similar elution pH as MabSelect PrismA™. As can be seen, lowering the concentration of the Na-citrate elution buffer, from 50mM to 20mM, leads to a slightly elevated, and thus milder, elution pH.

Thus, for a Protein A separation matrix, such as the present VH3 binding prototype, an elution pH can be achieved that is higher than that shown in for instance US 10,844,112 B2, by using a lower concentration of the citrate buffer. Thereby, a citrate buffer is shown to be advantageous for elution of an antibody or antibody fragment from a Protein A separation matrix, in contrast to the disclosure of US 10,844,112 B2. It is likely and probable that the use of an acetate buffer will behave in a similar manner.

CLAIMS

1. A separation matrix for purification of antibodies or antibody fragments comprising at least one VH3 chain, said separation matrix comprising a VH3 binding ligand coupled to a porous support, wherein said VH3 binding ligand comprises at least one VH3 binding polypeptide having a
5 significantly reduced or abolished binding affinity for a Fc fragment, and wherein the porous support is in beaded or particle form.
2. The separation matrix according to claim 1, wherein the VH3 binding ligand comprises at least one VH3 binding polypeptide based on any of the native Protein A domains A_{wt} (SEQ ID NO 92),
10 B_{wt} (SEQ ID NO 91), C_{wt} (SEQ ID NO 90), D_{wt} (SEQ ID NO 93), E_{wt} (SEQ ID NO 94), or the engineered Protein A domains Z_{wt} (SEQ ID NO 89), Z_{var} (SEQ ID NO 88) or Z_{var2} (SEQ ID NO 95),
wherein the amino acid at the position corresponding to position 9 in SEQ ID NO 89 is selected from Q, Y and A, the amino acid at the position corresponding to position 10 in SEQ ID NO 89 is selected from Q and Y, the amino acid at the position corresponding to position 11 in SEQ ID NO 89 is
15 selected from T, E and R, the amino acid at the position corresponding to position 13 in SEQ ID NO 89 is selected from L, E, R, A and Q, , the amino acid at the position corresponding to position 14 in SEQ ID NO 89 is selected from L, E, R, A, Q and W, the amino acid at the position corresponding to position 17 in SEQ ID NO 89 is selected from A, H and L, the amino acid at the position corresponding to position 18 in SEQ ID NO 89 is selected from R, L and H, the amino acid at the position
20 corresponding to position 26 in SEQ ID NO 89 is selected from Q and S, the amino acid at the position corresponding to position 28 in SEQ ID NO 89 is selected from N and A, and the amino acid at the position corresponding to position 29 in SEQ ID NO 89 is selected from A and G.
3. The separation matrix according to any one of claims 1 or 2, wherein the porous support
25 comprises polymer particles having a Dry solids weight (D_w) of 50-200 mg/ml, a volume-weighted median diameter (D50v) of 30-100 µm.
4. The separation matrix according to any one of the preceding claims, wherein said matrix comprises at least 12 mg/ml VH3 binding ligand, such as at least 14 mg/ml, such as at least 14.5
30 mg/ml, at least 15 mg/ml, at least 15.5 mg/ml, at least 16 mg/ml, at least 16.5 mg/ml, at least 17 mg/ml, at least 17.5 mg/ml, at least 18 mg/ml, at least 18.5 mg/ml, at least 19 mg/ml, at least 19.5

mg/ml, at least 20 mg/ml, at least 20.5 mg/ml, at least 21 mg/ml, at least 21.5 mg/ml, or at least 22 mg/ml VH3 binding ligand.

5. The separation matrix according to any one of the preceding claims, wherein the porous support has a D_w of 50-150 mg/ml, 50-120 mg/ml, 50-100 mg/ml, 50-90 mg/ml, 60-80 mg/ml, or 60-75 mg/ml, such as at least 63 mg/ml, or at least 65 mg/ml, or at least 70 mg/ml.
6. The separation matrix according to any one of the preceding claims, wherein the porous support has a volume-weighted median diameter (D_{50v}) of 35-90 μm , 40-80 μm , 50-70 μm , 55-70 μm , 55-67 μm , 58-70 μm , or 58-67 μm , such as at least 60 μm , or at least 62 μm .
7. The separation matrix according to any one of the preceding claims, wherein the porous support has a K_d value, measured by inverse size exclusion chromatography with dextran of M_w 110 kDa as a probe molecule, of 0.6-0.95, such as a K_d value of 0.7-0.9, or a K_d value of 0.6-0.8, such as a K_d value of about 0,67, or a K_d value of about 0,72, or a K_d value of about 0,75.
8. The separation matrix according to any one of the claims 3-7, wherein the polymer particles are cross-linked.
9. The separation matrix according to any one of claims 2-8, wherein the amino acids at positions 9/10/11 are QQT, QYT, YQT, AQE, AQR or AYR; preferably QQT, QYT, AQE, AQR or AYR; more preferably AQE, AQR or AYR; most preferably AQR or AYR.
10. The separation matrix according to any one of claims 2-9, wherein the amino acids at positions 17/18 are AR, HL or LH; preferably HL or AR.
11. The separation matrix according to any one of claims 2-10, wherein the amino acids at positions 28/29 are NA, NG, AA or AG.

12. The separation matrix according to any one of claims 2-11, wherein independently of each other, the amino acids at positions 9/10/11 are AQE, AQR or AYR; and the amino acids at positions 13/14 are LA, AA, AE, AL, AQ, AR, EA, EE, EL, EQ, ER, LA, LE, LL, LQ, LR, QA, QE, QL, QQ, QR, RA, RE, RL, RQ, RR or LW; and the amino acids at positions 17/18 are LH or AR.

5

13. The separation matrix according to any one of claims 2-12, wherein the amino acids at positions 9/10/11/13/14/17/18 are QQTLALH, QYTLALH, YQTLALH, QQTLAAR, AQELALH, AYRLALH, AYRLWLH, AYRLWAR, AYRLAHL and AYRLWHL AQRLALH, AYRLAAR.

10 14. The separation matrix according to any one of claims 2-13, wherein the amino acids at positions 26/28/29 are QNG, QAA, QAG, QNA, or SAG; preferably QNG, QNA, SAG or QAG.

15 15. The separation matrix according to any one of claims 2-14, wherein the VH3 binding polypeptide is selected from SEQ ID NO:1 – SEQ ID NO:87 and SEQ ID NO: 99- SEQ ID NO:107, SEQ ID NO:111-SEQ ID NO:112, SEQ ID NO: 114-SEQ ID NO: 120, SEQ ID NO: 124-SEQ ID NO: 125 and SEQ ID NO:127-SEQ ID NO:144.

16. The separation matrix according to any of the preceding claims, wherein the VH3 binding ligand comprises multimers of the polypeptide, said multimers comprising at least two polypeptides.

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17. The separation matrix according to claim 16 wherein the multimers are homodimers or heterodimers.

25 18. The separation matrix according to any one of claims 16 or 17, wherein the multimer is a dimer, a trimer, a tetramer, a pentamer, a hexamer, a heptamer, an octamer or a nonamer.

19. The separation matrix according to any one of claims 16-18, wherein the VH3-binding ligand comprises any one of the polypeptides according to SEQ ID NO 96, SEQ ID NO 97 or SEQ ID NO 98.

20. The separation matrix according to any one of the preceding claims, wherein the ligand comprises a coupling element, said coupling element being one or more amino acid residues at the C-terminal or N-terminal end of the ligand, preferably one or more cysteine residues, one or more lysine residues, or one or more histidine residues, preferably at the C-terminal end of the ligand.

5

21. The separation matrix according to claim 20, wherein the ligand comprises one or more cysteine residues at the C-terminal end of the ligand.

22. A method for isolation of antibodies or antibody fragments comprising at least one VH3 chain, comprising the steps of:

10 a) contacting a liquid sample comprising a VH3 chain-containing antibody or antibody fragment with a separation matrix;

b) washing said separation matrix with one or a combination of several washing liquids;

c) eluting the VH3 chain-containing antibody or antibody fragment from the separation matrix with
15 an elution liquid; and

d) cleaning the separation matrix with a cleaning liquid;

wherein the separation matrix is according to any one of the claims 1-21.

23. The method according to claim 22, wherein an IgG capacity after 24 h incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 90% of the IgG capacity before the incubation, such as at least 91%, or at least 92%, or at least 93%.

20

24. The method according to claim 22, wherein an VHH capacity after 24 h incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 90% of the VHH capacity before the incubation, such as at least 91%, or at least 92%, or at least 93%.

25

25. The method according to claim 22, wherein an IgG capacity after 50 h incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 80% of the IgG capacity before the incubation, such as at least 81%, or at least 82%, or at least 83%, or at least 84%.

26. A method for separation of bispecific antibodies or antibody fragments comprising one VH3 chain from variants of the antibody or antibody fragment comprising two VH3 chains or no VH3 chain, comprising the steps of:

- 5 a) contacting a liquid sample comprising said bispecific antibodies with a separation matrix,
- b) washing said separation matrix with one or a combination of several washing liquids,
- c) eluting said bispecific antibody from the separation matrix with an elution liquid and at a decreasing pH and
- d) cleaning the separation matrix with a cleaning liquid,
- 10 wherein the separation matrix is according to any one of the claims 1-21.

27. The method according to claim 26, wherein an IgG capacity after 24 h incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 90% of the IgG capacity before the incubation, such as at least 91%, or at least 92%, or at least 93%.

15

28. The method according to claim 26, wherein an IgG capacity after 50 h incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 80% of the IgG capacity before the incubation, such as at least 81%, or at least 82%, or at least 83%, or at least 84%.

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