(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 November 2017 (16.11.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/194597 A1

(51) International Patent Classification:
C07K 14/31 (2006.01)

(21) International Application Number:
PCT/EP2017/061164

(22) International Filing Date:
10 May 2017 (10.05.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

1608229.9	11 May 2016 (11.05.2016)	GB
1608232.3	11 May 2016 (11.05.2016)	GB
15/282,367	30 September 2016 (30.09.2016)	US

(71) Applicant: **GE HEALTHCARE BIOPROCESS R&D AB** [SE/SE]; Björkgatan 30, 751 84 Uppsala (SE).

(72) Inventors: **RODRIGO, Gustav, José**; GE Healthcare, Bjorkgatan 30, 751 84 Uppsala (SE). **BJORKMAN, Tomas**; GE Healthcare, Bjorkgatan 30, 751 84 Uppsala (SE). **ANDER, Mats**; GE Healthcare, Bjorkgatan 30, 751 84 Uppsala (SE). **HANSSON, Jesper, Ulf**; GE Healthcare, Bjorkgatan 30, 751 84 Uppsala (SE).

(74) Agent: **LARSSON, Anders** et al.; GE HEALTHCARE BIOPROCESS R&D AB, Patent Department, Björkgatan 30, 751 84 Uppsala (SE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: SEPARATION MATRIX

(57) Abstract: The invention relates to a separation matrix comprising at least 11 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein: a) the ligands comprise multimers of alkali-stabilized Protein A domains, and b) the porous support comprises cross-linked polymer particles having a volume-weighted median diameter (d50,v) of 56-70 micrometers and a dry solids weight of 55-80 mg/ml.

WO 2017/194597 A1

SEPARATION MATRIX

Technical field of the invention

[0001] The present invention relates to the field of affinity chromatography, and more specifically to mutated immunoglobulin-binding domains of Protein A, which are useful in affinity chromatography of immunoglobulins. The invention also relates to multimers of the mutated domains and to separation matrices containing the mutated domains or multimers.

Background of the invention

[0002] Immunoglobulins represent the most prevalent biopharmaceutical 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 mAb manufacturing processes whilst controlling the associated costs.

[0003] Affinity chromatography is used in most cases, as one of the key steps in the purification of these immunoglobulin molecules, such as monoclonal or polyclonal antibodies. A particularly interesting class of affinity reagents is proteins capable of specific binding to invariable parts of an immunoglobulin molecule, such interaction being independent on 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 cell culture derived feed stocks. An example of such a protein is staphylococcal protein A, 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.

[0004] Staphylococcal protein A (SpA) based reagents have due to their high affinity and selectivity found a widespread use in the field of biotechnology, e.g. in affinity chromatography for capture and purification of antibodies as well as for detection or quantification. At present, SpA-based affinity medium probably is 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-ligands are

commercially available, for example, in the form of native protein A (e.g. Protein A SEPHAROSE™, GE Healthcare, Uppsala, Sweden) and also comprised of recombinant protein A (e.g. rProtein A-SEPHAROSE™, GE Healthcare). More specifically, the genetic manipulation performed in the commercial recombinant protein A product is aimed at facilitating the attachment thereof to a support and at increasing the productivity of the ligand.

[0005] These applications, like other affinity chromatography applications, require comprehensive attention to definite removal of contaminants. Such contaminants can for example be non-eluted molecules adsorbed to the stationary phase or matrix in a chromatographic procedure, such as non-desired biomolecules or microorganisms, including for example proteins, carbohydrates, lipids, bacteria and viruses. The removal of such contaminants from the matrix is usually performed after a first elution of the desired product, in order to regenerate the matrix before subsequent use. Such removal usually involves a procedure known as cleaning-in-place (CIP), wherein agents capable of eluting contaminants from the stationary phase are used. One such class of agents often used is alkaline solutions that are passed over said stationary phase. At present the most extensively used cleaning and sanitizing agent is NaOH, and the concentration thereof can range from 0.1 up to e.g. 1 M, depending on the degree and nature of contamination. This strategy is associated with exposing the matrix to solutions with pH-values above 13. For many affinity chromatography matrices containing proteinaceous affinity ligands such alkaline environment is a very harsh condition and consequently results in decreased capacities owing to instability of the ligand to the high pH involved.

[0006] An extensive research has therefore been focused on the development of engineered protein ligands that exhibit an improved capacity to withstand alkaline pH-values. For example, Gülich et al. (Susanne Gülich, Martin Linhult, Per-Åke Nygren, Mathias Uhlén, Sophia Hober, Journal of Biotechnology 80 (2000), 169-178) suggested protein engineering to improve the stability properties of a Streptococcal albumin-binding domain (ABD) in alkaline environments. Gülich et al. created a mutant of ABD, wherein all the four asparagine residues have been replaced by leucine (one residue), aspartate (two residues) and lysine (one residue). Further, Gülich et al. report that their mutant exhibits a target protein binding behavior similar to that of the native protein, and that affinity columns containing the engineered ligand show higher binding capacities after repeated exposure to alkaline conditions than columns prepared

using the parental non-engineered ligand. Thus, it is concluded therein that all four asparagine residues can be replaced without any significant effect on structure and function.

[0007] Recent work shows that changes can also be made to protein A (SpA) to effect similar properties. US patent application publication US 2005/0143566, which is hereby incorporated by reference in its entirety, discloses that when at least one asparagine residue is mutated to an amino acid other than glutamine or aspartic acid, the mutation confers an increased chemical stability at pH-values of up to about 13-14 compared to the parental SpA, such as the B-domain of SpA, or Protein Z, a synthetic construct derived from the B-domain of SpA (US 5,143,844, incorporated by reference in its entirety). The authors show that when these mutated proteins are used as affinity ligands, the separation media as expected can better withstand cleaning procedures using alkaline agents. Further mutations of protein A domains with the purpose of increasing the alkali stability have also been published in US 8,329,860, JP 2006304633A, US 8,674,073, US 2010/0221844, US 2012/0208234, US 9,051,375, US 2014/0031522, US 2013/0274451 and WO 2014/146350, all of which are hereby incorporated by reference in their entireties. However, the currently available mutants are still sensitive to alkaline pH and the NaOH concentration during cleaning is usually limited to 0.1 M, which means that complete cleaning is difficult to achieve. Higher NaOH concentrations, which would improve the cleaning, lead to unacceptable capacity losses.

[0008] There is thus still a need in this field to obtain a separation matrix containing protein ligands having a further improved stability towards alkaline cleaning procedures. There is also a need for such separation matrices with an improved binding capacity to allow for economically efficient purification of therapeutic antibodies.

Summary of the invention

[0009] One aspect of the invention is to provide a polypeptide with improved alkaline stability. This is achieved with an Fc-binding polypeptide comprising a mutant of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 80% such as at least 90%, 95% or 98% identity to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine,

tryptophan, methionine, valine, alanine, histidine and arginine. Alternatively, the polypeptide comprises a sequence as defined by, or having at least 80% or at least 90%, 95% or 98% identity to SEQ ID NO 53.

X_1Q X_2AFYEILX_3LP NLTEEQRX_4X_5F IX_6X_7LKDX_8PSX_9 S$X_{10}X_{11}X_{12}$LAEAKX_{13} X_{14}NX_{15}AQ (SEQ ID NO 53)

wherein individually of each other:

X_1=A or Q or is deleted

X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R

X_3=H or K

X_4=A or N

X_5=A, G, S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K

X_6=Q or E

X_7=S or K

X_8=E or D

X_9=Q or V or is deleted

X_{10}=K,R or A or is deleted

X_{11}=A,E or N or is deleted

X_{12}=I or L

X_{13}=K or R

X_{14}=L or Y

X_{15}=D, F,Y,W,K or R

[00010] One advantage is that the alkaline stability is improved over the parental polypeptides, with a maintained highly selective binding towards immunoglobulins and other Fc-containing proteins.

[00011] A second aspect of the invention is to provide a multimer with improved alkaline stability, comprising a plurality of polypeptides. This is achieved with a multimer of the polypeptide disclosed above.

[00012] A third aspect of the invention is to provide a nucleic acid or a vector encoding a polypeptide or multimer with improved alkaline stability. This is achieved with a nucleic acid or vector encoding a polypeptide or multimer as disclosed above.

[00013] A fourth aspect of the invention is to provide an expression system capable of expressing a polypeptide or multimer with improved alkaline stability. This is achieved with an expression system comprising a nucleic acid or vector as disclosed above.

[00014] A fifth aspect of the invention is to provide a separation matrix capable of selectively binding immunoglobulins and other Fc-containing proteins and exhibiting an improved alkaline stability. This is achieved with a separation matrix comprising at least 11 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein:
a) the ligands comprise multimers of alkali-stabilized Protein A domains,
b) the porous support comprises cross-linked polymer particles having a volume-weighted median diameter (d50,v) of 56-70 micrometers and a dry solids weight of 55-80 mg/ml. Alternatively, it is achieved with a separation matrix comprising at least 15 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein said ligands comprise multimers of alkali-stabilized Protein A domains.

[00015] One advantage is that a high dynamic binding capacity is provided. A further advantage is that a high degree of alkali stability is achieved.

[00016] A sixth aspect of the invention is to provide an efficient and economical method of isolating an immunoglobulin or other Fc-containing protein. This is achieved with a method comprising the steps of:
a) contacting a liquid sample comprising an immunoglobulin with a separation matrix as disclosed above,
b) washing the separation matrix with a washing liquid,
c) eluting the immunoglobulin from the separation matrix with an elution liquid, and
d) cleaning the separation matrix with a cleaning liquid.

[00017] Further suitable embodiments of the invention are described in the dependent claims. Co-pending applications PCT EP2015/076639, PCT EP2015/076642, GB 1608229.9 and GB 1608232.3 are hereby incorporated by reference in their entireties.

Definitions

[00018] The terms “antibody” and “immunoglobulin” are used interchangeably herein, and are understood to include also fragments of antibodies, fusion proteins comprising antibodies or antibody fragments and conjugates comprising antibodies or antibody fragments.

[00019] The terms an “Fc-binding polypeptide” and “Fc-binding protein” mean a polypeptide or protein respectively, capable of binding to the crystallisable part (Fc) of an antibody and includes e.g. Protein A and Protein G, or any fragment or fusion protein thereof that has maintained said binding property.

[00020] The term “linker” herein means an element linking two polypeptide units, monomers or domains to each other in a multimer.

[00021] The term “spacer” herein means an element connecting a polypeptide or a polypeptide multimer to a support.

[00022] The term “% identity” with respect to comparisons of amino acid sequences is determined by standard alignment algorithms such as, for example, Basic Local Alignment Tool (BLAST™) described in Altshul et al. (1990) J. Mol. Biol., 215: 403-410. A web-based software for this is freely available from the US National Library of Medicine at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome . Here, the algorithm “blastp (protein-protein BLAST)” is used for alignment of a query sequence with a subject sequence and determining i.a. the % identity.

[00023] As used herein, the terms "comprises," "comprising," "containing," "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of' or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

Brief description of figures

[00024] Fig. 1 shows an alignment of the Fc-binding domains as defined by SEQ ID NO:1-7 and 51-52.

[00025] Fig. 2 shows results from Example 2 for the alkali stability of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to an SPR biosensor chip.

[00026] Fig. 3 shows results from Example 4 for the alkali stability (0.5 M NaOH) of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to agarose beads.

[00027] Fig. 4 shows results from Example 4 for the alkali stability (1.0 M NaOH) of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to agarose beads.

[00028] Fig. 5 shows results from Example 7 for the alkali stability (1.0 M NaOH) of agarose beads with different amounts of mutated multimer variants (SEQ ID NO. 20) coupled. The results are plotted as the relative remaining dynamic capacity (Qb10%, 6 min residence time) vs. incubation time in 1 M NaOH.

[00029] Fig. 6 shows results from Example 7 for the alkali stability (1.0 M NaOH) of agarose beads with different amounts of mutated multimer variants (SEQ ID NO. 20) coupled. The results are plotted as the relative remaining dynamic capacity (Qb10%, 6 min residence time) after 31 h incubation in 1 M NaOH vs. the ligand content of the prototypes.

Detailed description of embodiments

[00030] In one aspect the present invention discloses an Fc-binding polypeptide, which comprises, or consists essentially of, a mutant of an Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 90%, at least 95% or at least 98% identity to, SEQ ID NO: 1 (E-domain), SEQ ID NO: 2 (D-domain), SEQ ID NO:3 (A-domain), SEQ ID NO:22 (variant A-domain), SEQ ID NO: 4 (B-domain), SEQ ID NO: 5 (C-domain), SEQ ID NO:6 (Protein Z) , SEQ ID NO:7 (Zvar), SEQ ID NO 51 (Zvar without the linker region amino acids 1-8 and 56-58) or SEQ ID NO 52 (C-domain without the linker region amino acids 1-8

and 56-58) as illustrated in Fig. 1, wherein at least the asparagine (or serine, in the case of SEQ ID NO 2) residue at the position* corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine. Protein Z (SEQ ID NO:6) is a mutated B-domain as disclosed in US5143844, hereby incorporated by reference in its entirety, while SEQ ID NO 7 denotes a further mutated variant of Protein Z, here called Zvar, with the mutations N3A,N6D,N23T. SEQ ID NO:22 is a natural variant of the A-domain in Protein A from Staphylococcus aureus strain N315, having an A46S mutation, using the position terminology of Fig. 1. The mutation of N11 in these domains confers an improved alkali stability in comparison with the parental domain/polypeptide, without impairing the immunoglobulin-binding properties. Hence, the polypeptide can also be described as an Fc- or immunoglobulin-binding polypeptide, or alternatively as an Fc- or immunoglobulin-binding polypeptide unit.

[00031] *Throughout this description, the amino acid residue position numbering convention of Fig 1 is used, and the position numbers are designated as corresponding to those in SEQ ID NO 4-7. This applies also to multimers, where the position numbers designate the positions in the polypeptide units or monomers according to the convention of Fig. 1.

[00032] SEQ ID NO 51 (truncated Zvar)

[00033] QQ NAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQ

[00034] SEQ ID NO 52 (truncated C domain)

[00035] QQ NAFYEILHLP NLTEEQRNGF IQSLKDDPSV SKEILAEAKK LNDAQ

[00036] In alternative language, the invention discloses an Fc-binding polypeptide which comprises a sequence as defined by, or having at least 90%, at least 95% or at least 98% identity to SEQ ID NO 53.

[00037] SEQ ID NO 53

[00038] X_1Q X_2AFYEILX_3LP NLTEEQRX_4X_5F IX_6X_7LKDX_8PSX_9 S$X_{10}X_{11}X_{12}$LAEAKX_{13} X_{14}NX_{15}AQ

wherein individually of each other:

X_1=A, Q or is deleted

X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R

X_3=H or K

X_4=A or N

X_5=A, G, S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K, such as S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K

X_6=Q or E

X_7=S or K

X_8=E or D

X_9=Q, V or is deleted

X_{10}=K, R, A or is deleted

X_{11}=A, E, N or is deleted

X_{12}=I or L

X_{13}=K or R

X_{14}=L or Y

X_{15}=D, F,Y,W,K or R

[00039] Specifically, the amino acid residues in SEQ ID NO 53 may individually of each other be:

X_1 = A or is deleted

X_2 = E

X_3 = H

X_4 = N

X_6 = Q

X_7 = S

X_8 = D

X_9 = V or is deleted

X_{10} = K or is deleted

X_{11} = A or is deleted

X_{12} = I

X_{13} = K

X_{14} = L.

[00040] In certain embodiments, the amino acid residues in SEQ ID NO 53 may be:

X_1=A, X_2 = E, X_3 = H, X_4 = N, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L. In some embodiments X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D and one or more of X_1, X_9, X_{10} and X_{11} is deleted. In further embodiments, X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5= S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K , X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D, or alternatively X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}= F,Y,W,K or R.

[00041] The N11 (X_2) mutation (e.g. a N11E or N11K mutation) may be the only mutation or the polypeptide may also comprise further mutations, such as substitutions in at least one of the positions corresponding to positions 3, 6, 9, 10, 15, 18, 23, 28, 29, 32, 33, 36, 37, 40, 42, 43, 44, 47, 50, 51, 55 and 57 in SEQ ID NO:4-7. In one or more of these positions, the original amino acid residue may e.g. be substituted with an amino acid which is not asparagine, proline or cysteine. The original amino acid residue may e.g. be substituted with an alanine, a valine, a threonine, a serine, a lysine, a glutamic acid or an aspartic acid. Further, one or more amino acid residues may be deleted, e.g. from positions 1-6 and/or from positions 56-58.

[00042] In some embodiments, the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 (X_1) is an amino acid other than glutamine, asparagine, proline or cysteine, such as an alanine or it can be deleted. The combination of the mutations at positions 9 and 11 provides particularly good alkali stability, as shown by the examples. In specific embodiments, in SEQ ID NO: 7 the amino acid residue at position 9 is an alanine and the amino acid residue at position 11 is a lysine or glutamic acid, such as a lysine. Mutations at position 9 are also discussed in copending application PCT/SE2014/050872, which is hereby incorporated by reference in its entirety.

[00043] In some embodiments, the amino acid residue at the position corresponding to position 50 in SEQ ID NO:4-7 (X_{13}) is an arginine or a glutamic acid.

[00044] In certain embodiments, the amino acid residue at the position corresponding to position 3 in SEQ ID NO:4-7 is an alanine and/or the amino acid residue at the position corresponding to position 6 in SEQ ID NO:4-7 is an aspartic acid. One of the amino acid residues at positions 3 and 6 may be an asparagine and in an alternative embodiment both amino acid residues at positions 3 and 6 may be asparagines.

[00045] In some embodiments the amino acid residue at the position corresponding to position 43 in SEQ ID NO:4-7 (X_{11}) is an alanine or a glutamic acid, such as an alanine or it can be deleted. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and lysine/glutamic acid respectively, while the amino acid residue at position 43 is alanine or glutamic acid.

[00046] In certain embodiments the amino acid residue at the position corresponding to position 28 in SEQ ID NO:4-7 (X_5) is an alanine or an asparagine, such as an alanine.

[00047] In some embodiments the amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 (X_9) is selected from the group consisting of asparagine, alanine, glutamic acid and valine, or from the group consisting of glutamic acid and valine or it can be deleted. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and glutamic acid respectively, while the amino acid residue at position 40 is valine. Optionally, the amino acid residue at position 43 may then be alanine or glutamic acid.

[00048] In certain embodiments, the amino acid residue at the position corresponding to position 42 in SEQ ID NO:4-7 (X_{10}) is an alanine, lysine or arginine or it can be deleted.

[00049] In some embodiments the amino acid residue at the position corresponding to position 18 in SEQ ID NO:4-7 (X_3) is a lysine or a histidine, such as a lysine.

[00050] In certain embodiments the amino acid residue at the position corresponding to position 33 in SEQ ID NO:4-7 (X_7) is a lysine or a serine, such as a lysine.

In some embodiments the amino acid residue at the position corresponding to position 37 in SEQ ID NO:4-7 (X_8) is a glutamic acid or an aspartic acid, such as a glutamic acid.

[00051] In certain embodiments the amino acid residue at the position corresponding to position 51 in SEQ ID NO:4-7 (X_{14}) is a tyrosine or a leucine, such as a tyrosine.

[00052] In some embodiments, the amino acid residue at the position corresponding to position 44 in SEQ ID NO:4-7 (X_{12}) is a leucine or an isoleucine. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and lysine/glutamic acid

respectively, while the amino acid residue at position 44 is isoleucine. Optionally, the amino acid residue at position 43 may then be alanine or glutamic acid.

[00053] In some embodiments, the amino acid residues at the positions corresponding to positions 1, 2, 3 and 4 or to positions 3, 4, 5 and 6 in SEQ ID NO: 4-7 have been deleted. In specific variants of these embodiments, the parental polypeptide is the C domain of Protein A (SEQ ID NO: 5). The effects of these deletions on the native C domain are described in US9018305 and US8329860, which are hereby incorporated by reference in their entireties.

[00054] In certain embodiments, the mutation in SEQ ID NO 4-7, such as in SEQ ID NO 7, is selected from the group consisting of:N11K; N11E; N11Y; N11T; N11F; N11L; N11W; N11I; N11M; N11V; N11A; N11H; N11R; N11E,Q32A; N11E,Q32E,Q40E; N11E,Q32E,K50R; Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I; Q9A,N11E,Q40V,A42K,N43A,L44I; N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I; Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R; Q9A,N11K,H18K,D37E,A42R; Q9A,N11E,D37E,Q40V,A42K,N43A,L44I and Q9A,N11E,D37E,Q40V,A42R,N43A,L44I. These mutations provide particularly high alkaline stabilities. The mutation in SEQ ID NO 4-7, such as in SEQ ID NO 7, can also be selected from the group consisting of N11K; N11Y; N11F; N11L; N11W; N11I; N11M; N11V; N11A; N11H; N11R; Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I; Q9A,N11E,Q40V,A42K,N43A,L44I; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I; N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I and Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R.

[00055] In some embodiments, the polypeptide comprises or consists essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ

ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 29, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 38, SEQ ID NO 39, SEQ ID NO 40, SEQ ID NO 41, SEQ ID NO 42, SEQ ID NO 43, SEQ ID NO 44, SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO 47, SEQ ID NO 48, SEQ ID NO 49 and SEQ ID NO 50. It may e.g. comprise or consist essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28 and SEQ ID NO 29. It can also comprise or consist essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 38, SEQ ID NO 40; SEQ ID NO 41; SEQ ID NO 42; SEQ NO 43, SEQ ID NO 44, SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO 47 and SEQ ID NO 48.

[00056] In certain embodiments, the polypeptide comprises or consists essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of SEQ ID NO 54-70. comprises or consists essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of SEQ ID NO 71-75, or it may comprise or consist essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of SEQ ID NO 76-79. It may further comprise or consist essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of SEQ ID NO 89-95.

[00057] The polypeptide may e.g. be defined by a sequence selected from the groups above or from subsets of these groups, but it may also comprise additional amino acid residues at the N- and/or C-terminal end, e.g. a leader sequence at the N-terminal end and/or a tail sequence at the C-terminal end.

SEQ ID NO 8 Zvar(Q9A,N11E,N43A)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SA**A**LLAEAKK LNDAQAPK

SEQ ID NO 9 Zvar(Q9A,N11E,N28A,N43A)

VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQR**A**AF IQSLKDDPSQ SA**A**LLAEAKK LNDAQAPK

SEQ ID NO 10 Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KEI**LAEAKK LNDAQAPK

SEQ ID NO 11 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 12 Zvar(N11E,Q32A)
VDAKFDKEQQ **E**AFYEILHLP NLTEEQRNAF I**A**SLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 13 Zvar(N11E)
VDAKFDKEQQ **E**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 14 Zvar(N11E,Q32E,Q40E)
VDAKFDKEQQ **E**AFYEILHLP NLTEEQRNAF I**E**SLKDDPS**E** SANLLAEAKK LNDAQAPK

SEQ ID NO 15 Zvar(N11E,Q32E,K50R)
VDAKFDKEQQ **E**AFYEILHLP NLTEEQRNAF I**E**SLKDDPSQ SANLLAEAK**R** LNDAQAPK

SEQ ID NO 16 Zvar(N11K)
VDAKFDKEQQ **K**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 23 Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)
VDAKFDKEQQ **K**AFYEIL**K**LP NLTEEQRNAF IQ**K**LKD**E**PSQ S**RAI**LAEAK**R** **Y**NDAQAPK

SEQ ID NO 24 Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQR**A**AF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 25 Zvar(Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)
VDAKFDKE**A**Q **K**AFYEIL**K**LP NLTEEQR**A**AF IQ**K**LKD**E**PSQ S**RAI**LAEAK**R** **Y**NDAQAPK

SEQ ID NO 26 Zvar(N11K, H18K, D37E, A42R, N43A, L44I)
VDAKFDKEQQ **K**AFYEIL**K**LP NLTEEQRNAF IQSLKD**E**PSQ S**RAI**LAEAKK LNDAQAPK

SEQ ID NO 27 Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)
VDAKFDKE**A**Q **K**AFYEIL**K**LP NLTEEQRNAF IQSLKD**E**PSQ S**RAI**LAEAKK LNDAQAPK

SEQ ID NO 28 Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)
VDAKFDKE**A**Q **K**AFYEIL**K**LP NLTEEQRNAF IQSLKD**E**PSQ S**RAI**LAEAK**R**
LNDAQAPK

SEQ ID NO 29 Zvar(Q9A,N11K,H18K,D37E,A42R)
VDAKFDKE**A**Q **K**AFYEIL**K**LP NLTEEQRNAF IQSLKD**E**PSQ S**R**NLLAEAKK
LNDAQAPK

SEQ ID NO 36 B(Q9A,N11E,Q40V,A42K,N43A,L44I)
ADNKFNKE**A**Q **E**AFYEILHLP NLNEEQRNGF IQSLKDDPS**V** S**KAI**LAEAKK
LNDAQAPK

SEQ ID NO 37 C(Q9A,N11E,E43A)
ADNKFNKE**A**Q **E**AFYEILHLP NLTEEQRNGF IQSLKDDPSV SK**A**ILAEAKK
LNDAQAPK

SEQ ID NO 38 Zvar(N11Y)
VDAKFDKEQQ **Y**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 39 Zvar(N11T)
VDAKFDKEQQ **T**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 40 Zvar(N11F)
VDAKFDKEQQ **F**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 41 Zvar(N11L)
VDAKFDKEQQ **L**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 42 Zvar(N11W)
VDAKFDKEQQ **W**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 43 Zvar(N11I)
VDAKFDKEQQ **I**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 44 Zvar(N11M)
VDAKFDKEQQ **M**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 45 Zvar(N11V)
VDAKFDKEQQ **V**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 46 Zvar(N11A)
VDAKFDKEQQ **A**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK
LNDAQAPK

SEQ ID NO 47 Zvar(N11H)
VDAKFDKEQQ **H**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 48 Zvar(N11R)
VDAKFDKEQQ **R**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 49 Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 50 Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPK

SEQ ID NO 54 Zvar(Q9A,N11E, A29G,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**G**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 55 Zvar(Q9A,N11E, A29S,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**S**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 56 Zvar(Q9A,N11E, A29Y,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**Y**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 57 Zvar(Q9A,N11E, A29Q,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**Q**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 58 Zvar(Q9A,N11E, A29T,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**T**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 59 Zvar(Q9A,N11E, A29N,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**N**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 60 Zvar(Q9A,N11E, A29F,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**F**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 61 Zvar(Q9A,N11E, A29L,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**L**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 62 Zvar(Q9A,N11E, A29W,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**W**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 63 Zvar(Q9A,N11E, A29I,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**I**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 64 Zvar(Q9A,N11E, A29M,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**M**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 65 Zvar(Q9A,N11E, A29V,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**V**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 66 Zvar(Q9A,N11E, A29D,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**D**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 67 Zvar(Q9A,N11E, A29E,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**E**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 68 Zvar(Q9A,N11E, A29H,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**H**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 69 Zvar(Q9A,N11E, A29R,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**R**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 70 Zvar(Q9A,N11E, A29K,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRN**K**F IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 71 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53F)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LN**F**AQAPK

SEQ ID NO 72 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53Y)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LN**Y**AQAPK

SEQ ID NO 73 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53W)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LN**W**AQAPK

SEQ ID NO 74 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53K)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LN**K**AQAPK

SEQ ID NO 75 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53R)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LN**R**AQAPK

SEQ ID NO 76 Zvar(Q9del,N11E, Q40V,A42K,N43A,L44I)
VDAKFDKE_Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 77 Zvar(Q9A,N11E, Q40del,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS_ S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 78 Zvar(Q9A,N11E, Q40V,A42del,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S_**AI**LAEAKK LNDAQAPK

SEQ ID NO 79 Zvar(Q9A,N11E, Q40V,A42K,N43del,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**K**_**I**LAEAKK LNDAQAPK

SEQ ID NO 89 Zvar(D2del,A3del,K4del,Q9A,N11E,Q40V,A42K,N43A,L44I)
V___FDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 90 Zvar(V1del,D2del,Q9A,N11E,Q40V,A42K,N43A,L44I,K58del)
__AKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAP_

SEQ ID NO 91 Zvar(K4del,F5del,D6del,K7del,E8del,Q9A,N11E,Q40V,A42K,N43A,L44I)
VDA_____**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 92 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,A56del,P57del,K58del)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQ___

SEQ ID NO 93 Zvar(V1del,,D2del,A3del,Q9A,N11E,Q40V,A42K,N43A,L44I)
___KFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 94
Zvar(V1del,D2del,A3del,K4del,F5del,D6del,K7del,E8del,Q9A,N11E,Q40V,A42K,N43A,L44I)
_____**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 95 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,K58_insYEDG)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKYE DG

[00058] In a second aspect the present invention discloses a multimer comprising, or consisting essentially of, a plurality of polypeptide units as defined by any embodiment disclosed above. The use of multimers may increase the immunoglobulin binding capacity and multimers may also have a higher alkali stability than monomers. The multimer can e.g. be a dimer, a trimer, a tetramer, a pentamer, a hexamer, a heptamer, an octamer or a nonamer. It can be a homomultimer, where all the units in the multimer are identical or it can be a heteromultimer, where at least one unit differs from the others. Advantageously, all the units in the multimer are alkali stable, such as by comprising the mutations disclosed above. The polypeptides can be linked to each other directly by peptide bonds between the C-terminal and N-terminal ends of the polypeptides. Alternatively, two or more units in the multimer can be linked by linkers comprising oligomeric or polymeric species, such as linkers comprising peptides with up to 25 or 30 amino acids, such as 3-25 or 3-20 amino acids. The linkers may e.g. comprise or consist essentially of a peptide sequence defined by, or having at least 90% identity or at least 95% identity, with an amino acid sequence selected from the group consisting of APKVDAKFDKE, APKVDNKFNKE, APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APK, APKYEDGVDAKFDKE and YEDG or alternatively selected from the group consisting of APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APKYEDGVDAKFDKE and YEDG. They can also consist essentially of a peptide sequence defined by or having at least 90% identity or at least 95% identity with an amino acid sequence selected from the group consisting of APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APK and APKYEDGVDAKFDKE. In some embodiments the linkers do not consist of the peptides APKVDAKFDKE or APKVDNKFNKE, or alternatively do not consist of the peptides APKVDAKFDKE, APKVDNKFNKE , APKFNKE, APKFDKE, APKVDKE or APKADKE.

[00059] The nature of such a linker should preferably not destabilize the spatial conformation of the protein units. This can e.g. be achieved by avoiding the presence of proline in the linkers. Furthermore, said linker should preferably also be sufficiently stable in alkaline environments not to impair the properties of the mutated protein units. For this purpose, it is advantageous if the linkers do not contain asparagine. It can additionally be advantageous if the linkers do not contain glutamine. The multimer may further at the N-terminal end comprise a plurality of amino acid residues e.g. originating from the cloning process or constituting a

residue from a cleaved off signaling sequence. The number of additional amino acid residues may e.g. be 20 or less, such as 15 or less, such as 10 or less or 5 or less. As a specific example, the multimer may comprise an AQ, AQGT, VDAKFDKE, AQVDAKFDKE or AQGTVDAKFDKE sequence at the N-terminal end.

[00060] In certain embodiments, the multimer may comprise, or consist essentially, of a sequence selected from the group consisting of: SEQ ID NO 80-87. These and additional sequences are listed below and named as Parent(Mutations)n, where n is the number of monomer units in a multimer.

SEQ ID NO 17 Zvar(Q9A,N11E,N43A)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPKC

SEQ ID NO 18 Zvar(Q9A,N11E,N28A,N43A)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPKC

SEQ ID NO 19 Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPKC

SEQ ID NO 20 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 30 Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)4
AQGT VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPKC

SEQ ID NO 31 Zvar(Q9A,N11K,H18K,D37E,A42R)4

AQGT VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAQAPK VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAQAPK VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAQAPK VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAQAPKC

SEQ ID NO 32 Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 33 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)6
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 34 Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)4
AQGT VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPKC

SEQ ID NO 35 Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)4
AQGT VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPKC

SEQ ID NO 80 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with D2, A3 and K4 in linker deleted
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDA**QAPK VFDKE**AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 81 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with K58, V1 and D2 in linker deleted
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDA**QAP AKFDKE**AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 82 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with P57, K58, V1, D2 and A3 in linker deleted

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDA**QAP AKFDKE**AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 83 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with K4, F5, D6, K7 and E8 in linker deleted
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDA**QAPK VDA**AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 84 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with A56, P57 and K58 in linker deleted
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDA**Q VDAKFDKE**AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 85 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with V1, D2 and A3 in linker deleted
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDA**QAPK KFDKE**AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 86 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with V1, D2, A3, K4, F5, D6, K7 and E8 in linker deleted
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDA**QAPK** AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 87 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with YEDG inserted in linker between K58 and V1
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDA**QAPK YEDG VDAKFDKE**AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 88 Zvar2
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

[00061] In some embodiments, the polypeptide and/or multimer, as disclosed above, further comprises at the C-terminal or N-terminal end one or more coupling elements, selected from the group consisting of one or more cysteine residues, a plurality of lysine residues and a plurality of histidine residues. The coupling element(s) may also be located within 1-5 amino acid residues, such as within 1-3 or 1-2 amino acid residues from the C-terminal or N-terminal end. The coupling element may e.g. be a single cysteine at the C-terminal end. The coupling element(s) may be directly linked to the C- or N-terminal end, or it/they may be linked via a stretch 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 not to impair the properties of the mutated protein. 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-terminal cysteine is that endpoint coupling of the protein can be achieved through reaction of the cysteine thiol with an electrophilic group on a support. This provides excellent mobility of the coupled protein which is important for the binding capacity.

[00062] The alkali stability of the polypeptide or multimer can be assessed by coupling it to an SPR chip, e.g. to Biacore CM5 sensor chips as described in the examples, using e.g. NHS- or maleimide coupling chemistries, and measuring the immunoglobulin-binding capacity of the chip, typically using polyclonal human IgG, before and after incubation in alkaline solutions at a specified temperature, e.g. 22 +/- 2 °C. The incubation can e.g. be performed in 0.5 M NaOH for a number of 10 min cycles, such as 100, 200 or 300 cycles. The IgG capacity of the matrix after 100 10 min incubation cycles in 0.5 M NaOH at 22 +/- 2 °C can be at least 55, such as at least 60, at least 80 or at least 90% of the IgG capacity before the incubation. Alternatively, the remaining IgG capacity after 100 cycles for a particular mutant measured as above can be compared with the remaining IgG capacity for the parental polypeptide/multimer. In this case, the remaining IgG capacity for the mutant may be at least 105%, such as at least 110%, at least 125%, at least 150% or at least 200% of the parental polypeptide/multimer.

[00063] In a third aspect the present invention discloses a nucleic acid encoding a polypeptide or multimer according to any embodiment disclosed above. Thus, the invention encompasses all forms of the present nucleic acid sequence such as the RNA and the DNA encoding the polypeptide or multimer. The invention embraces a vector, such as a plasmid, which in addition to the coding sequence comprises the required signal sequences for expression of the polypeptide or multimer according the invention. In one embodiment, the vector comprises nucleic acid encoding a multimer according to the invention, wherein the separate nucleic acids encoding each unit may have homologous or heterologous DNA sequences.

[00064] In a fourth aspect the present invention discloses an expression system, which comprises, a nucleic acid or a vector as disclosed above. The expression system may e.g. be a gram-positive or gram-negative prokaryotic host cell system, e.g. *E.coli* or *Bacillus sp.* which has been modified to express the present polypeptide or multimer. In an alternative embodiment,

the expression system is a eukaryotic host cell system, such as a yeast, e.g. *Pichia pastoris* or *Saccharomyces cerevisiae,* or mammalian cells, e.g. CHO cells.

[00065] In a fifth aspect, the present invention discloses a separation matrix, wherein a plurality of polypeptides or multimers according to any embodiment disclosed above have been coupled to a solid support. The separation matrix may comprise at least 11, such as 11-21, 15-21 or 15-18 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein:

a) the ligands comprise multimers of alkali-stabilized Protein A domains,

b) the porous support comprises cross-linked polymer particles having a volume-weighted median diameter (d50,v) of 56-70, such as 56-66, micrometers and a dry solids weight of 55-80, such as 60-78 or 65-78, mg/ml. The cross-linked polymer particles may further have a pore size corresponding to an inverse gel filtration chromatography Kd value of 0.69-0.85, such as 0.70-0.85 or 0.69-0.80, for dextran of Mw 110 kDa. Suitably, the cross-linked polymer particles can have a high rigidity, to be able to withstand high flow rates. The rigidity can be measured with a pressure-flow test as further described in Example 8, where a column packed with the matrix is subjected to increasing flow rates of distilled water. The pressure is increased stepwise and the flow rate and back pressure measured, until the flow rate starts to decrease with increasing pressures. The maximum flow rate achieved and the maximum pressure (the back pressure corresponding to the maximum flow rate) are measured and used as measures of the rigidity. When measured in a FineLine^{TM} 35 column (GE Healthcare Life Sciences) at a bed height of 300 +/- 10 mm, the max pressure can suitably be at least 0.58 MPa, such as at least 0.60 MPa. This allows for the use of smaller particle diameters, which is beneficial for the dynamic capacity. The multimers may e.g. comprise tetramers, pentamers, hexamers or heptamers of alkali-stabilized Protein A domains, such as hexamers of alkali-stabilized Protein A domains. The combination of the high ligand contents with the particle size range, the dry solids weight range and the optional Kd range provides for a high binding capacity, e.g. such that the 10% breakthrough dynamic binding capacity for IgG is at least 45 mg/ml, such as at least 50 or at least 55 mg/ml at 2.4 min residence time. Alternatively, or additionally, the 10% breakthrough dynamic binding capacity for IgG may be at least 60 mg/ml, such as at least 65, at least 70 or at least 75 mg/ml at 6 min residence time.

The alkali-stabilized Protein A multimers are highly selective for IgG and the separation matrix can suitably have a dissociation constant for human IgG2 of below 0.2 mg/ml, such as below 0.1 mg/ml, in 20 mM phosphate buffer, 180 mM NaCl, pH 7.5. This can be determined according to the adsorption isotherm method described in N Pakiman et al: J Appl Sci 12, 1136-1141 (2012).

[00066] In certain embodiments the alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 80% such as at least 90%, 95% or 98% identity to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine, such as an amino acid selected from the group consisting of glutamic acid and lysine. The amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 may further be, or be mutated to, a valine. The alkali-stabilized Protein A domains may also comprise any mutations as described in the polypeptide and/or multimer embodiments above.

[00067] In some embodiments the alkali-stabilized Protein A domains comprise an Fc-binding polypeptide having an amino acid sequence as defined by, or having at least 80% or at least 90, 95% or 98% identity to SEQ ID NO 53.

X_1Q X_2AFYEILX_3LP NLTEEQRX_4X_5F IX_6X_7LKDX_8PSX_9 S$X_{10}X_{11}X_{12}$LAEAKX_{13} X_{14}NX_{15}AQ (SEQ ID NO 53)

wherein individually of each other:

X_1=A or Q or is deleted

X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R

X_3=H or K

X_4=A or N

X_5=A, G, S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K

X_6=Q or E

X_7=S or K

X_8=E or D

X_9=Q or V or is deleted

X_{10}=K,R or A or is deleted

X_{11}=A,E or N or is deleted

X_{12}=I or L

X_{13}=K or R

X_{14}=L or Y

X_{15}=D, F,Y,W,K or R

[00068] In some embodiments, the amino acid residues may individually of each other be:

a) X_1 = A or is deleted, X_2 = E, X_3 = H, X_4 = N, X_6 = Q, X_7 = S, X_8 = D, X_9 = V or is deleted, X_{10} = K or is deleted, X_{11} = A or is deleted, X_{12} = I, X_{13} = K, X_{14} = L.

b) X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

c) X_1 is A, X_2 = E, X_3 = H, X_4 = N, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D or

d) X_1 is A, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

[00069] In certain embodiments the invention discloses a separation matrix comprising at least 15, such as 15-21 or 15-18 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein the ligands comprise multimers of alkali-stabilized Protein A domains. These multimers can suitably be as disclosed in any of the embodiments described above or as specified below.

[00070] Such a matrix is useful for separation of immunoglobulins or other Fc-containing proteins and, due to the improved alkali stability of the polypeptides/multimers, the matrix will withstand highly alkaline conditions during cleaning, which is essential for long-term repeated use in a bioprocess separation setting. The alkali stability of the matrix can be assessed by measuring the immunoglobulin-binding capacity, typically using polyclonal human IgG, before and after incubation in alkaline solutions at a specified temperature, e.g. 22 +/- 2 °C. The incubation can e.g. be performed in 0.5 M or 1.0 M NaOH for a number of 15 min cycles, such as 100, 200 or 300 cycles, corresponding to a total incubation time of 25, 50 or 75 h. The IgG capacity of the matrix after 96-100 15 min incubation cycles or a total incubation time of 24 or 25 h in 0.5 M NaOH at 22 +/- 2 °C can be at least 80, such as at least 85, at least 90 or at least 95% of the IgG capacity before the incubation. The capacity of the matrix after a total incubation time of 24 h in 1.0 M NaOH at 22 +/- 2 °C can be at least 70, such as at least 80 or at least 90% of the IgG capacity before the incubation. The the 10% breakthrough dynamic binding capacity (Qb10%) for IgG at 2.4 min or 6 min residence time may e.g. be reduced by less than 20 % after incubation 31 h in 1.0 M aqueous NaOH at 22 +/- 2 C.

[00071] As the skilled person will understand, the expressed polypeptide or multimer should be purified to an appropriate extent before being immobilized to a support. Such purification methods are well known in the field, and the immobilization of protein-based ligands to supports is easily carried out using standard methods. Suitable methods and supports will be discussed below in more detail.

[00072] 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 (-$CONH_2$, possibly in N- substituted forms), amino (-NH_2, possibly in substituted form), oligo- or polyethylenoxy groups on their external and, if present, also on internal surfaces. The solid support can suitably be porous. The porosity can be expressed as a Kav or Kd 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 6-13. Kav is determined as the ratio $(V_e-V_0)/(V_t-V_0)$, where Ve is the elution volume of a probe molecule (e.g. Dextran 110 kD), V_0 is the void volume of the column (e.g. the elution volume of a high Mw void marker, such as raw dextran) and V_t is the total volume of the column. Kd can be determined as $(V_e-V_0)/V_i$, where V_i is the elution volume of a salt (e.g. NaCl) able to access all the volume except the matrix volume (the volume occupied by the matrix polymer molecules). By definition, both Kd and Kav values always lie within the range 0 – 1. The Kav value can advantageously be 0.6 – 0.95, e.g. 0.7 – 0.90 or 0.6 – 0.8, as measured with dextran of Mw 110 kDa as a probe molecule. The Kd value as measured with dextran of Mw 110 kDa can suitably be 0.68-0.90, such as 0.68-0.85 or 0.70-0.85. An advantage of this is that the support has a large fraction of pores able to accommodate both the polypeptides/multimers of the invention and immunoglobulins binding to the polypeptides/multimers and to provide mass transport of the immunoglobulins to and from the binding sites.

[00073] The polypeptides or multimers may be attached to the support via conventional coupling techniques utilising e.g. thiol, amino and/or carboxy groups present in the ligand. Bisepoxides, epichlorohydrin, CNBr, N-hydroxysuccinimide (NHS) etc are well-known coupling reagents. Between the support and the polypeptide/multimer, a molecule known as a

spacer can be introduced, which improves the availability of the polypeptide/multimer and facilitates the chemical coupling of the polypeptide/multimer to the support. Depending on the nature of the polypeptide/multimer and the coupling conditions, the coupling may be a multipoint coupling (e.g. via a plurality of lysines) or a single point coupling (e.g. via a single cysteine). Alternatively, the polypeptide/multimer may be attached to the support by non-covalent bonding, such as physical adsorption or biospecific adsorption.

[00074] In some embodiments the matrix comprises 5 – 25, such as 5-20 mg/ml, 5 – 15 mg/ml, 5 – 11 mg/ml or 6 – 11 mg/ml of the polypeptide or multimer coupled to the support. The amount of coupled polypeptide/multimer can be controlled by the concentration of polypeptide/multimer used in the coupling process, by the activation and 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 polypeptide/multimer, at least up to a point where the pores become significantly constricted by the coupled polypeptide/multimer. Without being bound by theory, it appears though that for the Kd values recited for the support, the constriction of the pores by coupled ligand is of lower significance. The relative binding capacity per mg coupled polypeptide/multimer will decrease at high coupling levels, resulting in a cost-benefit optimum within the ranges specified above.

[00075] In certain embodiments the polypeptides or multimers are 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 polypeptide or multimer, the mobility of the coupled polypeptide/multimer is enhanced which provides improved binding capacity and binding kinetics. In some embodiments the polypeptide/multimer is coupled via a C-terminal cysteine provided on the protein as described above. 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.

[00076] In certain embodiments the support comprises 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.

[00077] In some embodiments the support comprises agar or agarose. The supports used in the present invention can easily be prepared according to standard methods, such as inverse suspension gelation (S Hjertén: 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 (GE Healthcare). 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 entireties, and hence renders the matrix more suitable for high flow rates.

[00078] In certain embodiments the support, such as a polymer, polysaccharide or agarose support, is 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.

[00079] Alternatively, the solid support is 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™ (GE Healthcare) is used. In another alternative, the solid support according to the invention comprises a support of inorganic nature, e.g. silica, zirconium oxide etc.

[00080] In yet another embodiment, the solid support is in another form such as a surface, a chip, capillaries, or a filter (e.g. a membrane or a depth filter matrix).

[00081] As regards the shape of the matrix according to the invention, in one embodiment the matrix is in the form of a porous monolith. In an alternative embodiment, the matrix is in beaded or particle form that can be porous or non-porous. 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 monoliths, 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.

[00082] In a sixth aspect, the present invention discloses a method of isolating an immunoglobulin, wherein a separation matrix as disclosed above is used. The method may comprise the steps of:

a) contacting a liquid sample comprising an immunoglobulin with a separation matrix as disclosed above,

b) washing the separation matrix with a washing liquid,

c) eluting the immunoglobulin from the separation matrix with an elution liquid, and

d) cleaning the separation matrix with a cleaning liquid, which may comprise 0.1 – 1.0 M NaOH or KOH, such as 0.4 – 1.0 M NaOH or KOH.

Steps a) – d) may be repeated at least 10 times, such as at least 50 times or 50 – 200 times.

[00083] In certain embodiments, the method comprises the steps of:

a) contacting a liquid sample comprising an immunoglobulin with a separation matrix as disclosed above,

b) washing said separation matrix with a washing liquid,

c) eluting the immunoglobulin from the separation matrix with an elution liquid, and

d) cleaning the separation matrix with a cleaning liquid, which can alternatively be called a cleaning-in-place (CIP) liquid, e.g. with a contact (incubation) time of at least 10 min.

The method may also comprise steps of, before step a), providing an affinity separation matrix according to any of the embodiments described above and providing a solution comprising an immunoglobulin and at least one other substance as a liquid sample and of, after step c), recovering the eluate and optionally subjecting the eluate to further separation steps, e.g. by anion or cation exchange chromatography, multimodal chromatography and/or hydrophobic interaction chromatography. Suitable compositions of the liquid sample, the washing liquid and the elution liquid, as well as the general conditions for performing the separation are well known

in the art of affinity chromatography and in particular in the art of Protein A chromatography. The liquid sample comprising an Fc-containing protein and at least one other substance may comprise host cell proteins (HCP), such as CHO cell, E Coli or yeast proteins. Contents of CHO cell and E Coli proteins can conveniently be determined by immunoassays directed towards these proteins, e.g. the CHO HCP or E Coli HCP ELISA kits from Cygnus Technologies. The host cell proteins or CHO cell/E Coli proteins may be desorbed during step b).

[00084] The elution may be performed by using any suitable solution used for elution from Protein A media. This can e.g. be a solution or buffer with pH 5 or lower, such as pH 2.5 – 5 or 3 – 5. It can also in some cases be a solution or buffer with pH 11 or higher, such as pH 11 – 14 or pH 11 - 13. In some embodiments the elution buffer or the elution buffer gradient comprises at least one mono- di- or trifunctional carboxylic acid or salt of such a carboxylic acid. In certain embodiments the elution buffer or the elution buffer gradient comprises at least one anion species selected from the group consisting of acetate, citrate, glycine, succinate, phosphate, and formiate.

[00085] In some embodiments, the cleaning liquid is alkaline, such as with a pH of 13 – 14. Such solutions provide efficient cleaning of the matrix, in particular at the upper end of the interval

[00086] In certain embodiments, the cleaning liquid comprises 0.1 – 2.0 M NaOH or KOH, such as 0.5 – 2.0 or 0.5 – 1.0 M NaOH or KOH. These are efficient cleaning solutions, and in particular so when the NaOH or KOH concentration is above 0.1 M or at least 0.5 M. The high stability of the polypeptides of the invention enables the use of such strongly alkaline solutions.

[00087] The method may also include a step of sanitizing the matrix with a sanitization liquid, which may e.g. comprise a peroxide, such as hydrogen peroxide and/or a peracid, such as peracetic acid or performic acid.

[00088] In some embodiments, steps a) – d) are repeated at least 10 times, such as at least 50 times, 50 – 200, 50-300 or 50-500 times. This is important for the process economy in that the matrix can be re-used many times.

[00089] Steps a) – c) can also be repeated at least 10 times, such as at least 50 times, 50 – 200, 50-300 or 50-500 times, with step d) being performed after a plurality of instances of step c), such that step d) is performed at least 10 times, such as at least 50 times. Step d) can e.g. be performed every second to twentieth instance of step c).

Examples

Mutagenesis of protein

[00090] Site-directed mutagenesis was performed by a two-step PCR using oligonucleotides coding for the mutations. As template a plasmid containing a single domain of either Z, B or C was used. The PCR fragments were ligated into an E. coli expression vector. DNA sequencing was used to verify the correct sequence of inserted fragments.
To form multimers of mutants an Acc I site located in the starting codons (GTA GAC) of the B, C or Z domain was used, corresponding to amino acids VD. The vector for the monomeric domain was digested with Acc I and phosphatase treated. Acc I sticky-ends primers were designed, specific for each variant, and two overlapping PCR products were generated from each template. The PCR products were purified and the concentration was estimated by comparing the PCR products on a 2% agarose gel. Equal amounts of the pair wise PCR products were hybridized (90°C -> 25°C in 45min) in ligation buffer. The resulting product consists approximately to ¼ of fragments likely to be ligated into an Acc I site (correct PCR fragments and/or the digested vector). After ligation and transformation colonies were PCR screened to identify constructs containing the desired mutant. Positive clones were verified by DNA sequencing.

Construct expression and purification

[00091] The constructs were expressed in the bacterial periplasm by fermentation of *E. coli* K12 in standard media. After fermentation the cells were heat-treated to release the periplasm content into the media. The constructs released into the medium were recovered by microfiltration with a membrane having a 0.2 μm pore size.

[00092] Each construct, now in the permeate from the filtration step, was purified by affinity. The permeate was loaded onto a chromatography medium containing immobilized IgG (IgG Sepharose 6FF, GE Healthcare). The loaded product was washed with phosphate buffered saline and eluted by lowering the pH.

[00093] The elution pool was adjusted to a neutral pH (pH 8) and reduced by addition of dithiothreitol. The sample was then loaded onto an anion exchanger. After a wash step the construct was eluted in a NaCl gradient to separate it from any contaminants. The elution pool was concentrated by ultrafiltration to 40-50 mg/ml. It should be noted that the successful affinity purification of a construct on an immobilized IgG medium indicates that the construct in question has a high affinity to IgG.

[00094] The purified ligands were analyzed with RPC LC-MS to determine the purity and to ascertain that the molecular weight corresponded to the expected (based on the amino acid sequence).

Example 1

[00095] The purified monomeric ligands listed in Table 1, further comprising for SEQ ID NO 8-16, 23-28 and 36-48 an AQGT leader sequence at the N-terminus and a cysteine at the C terminus, were immobilized on Biacore CM5 sensor chips (GE Healthcare, Sweden), using the amine coupling kit of GE Healthcare (for carbodiimide coupling of amines on the carboxymethyl groups on the chip) in an amount sufficient to give a signal strength of about 200-1500 RU in a Biacore surface plasmon resonance (SPR) instrument (GE Healthcare, Sweden) . To follow the IgG binding capacity of the immobilized surface 1mg/ml human polyclonal IgG (Gammanorm) was flowed over the chip and the signal strength (proportional to the amount of binding) was noted. The surface was then cleaned-in-place (CIP), i.e. flushed with 500mM NaOH for 10 minutes at room temperature (22 +/- 2°C). This was repeated for 96-100 cycles and the immobilized ligand alkaline stability was followed as the remaining IgG binding capacity (signal strength) after each cycle. The results are shown in Table 1 and indicate that at least the ligands Zvar(N11K)1, Zvar(N11E)1, Zvar(N11Y)1, Zvar(N11T)1, Zvar(N11F)1, Zvar(N11L)1, Zvar(N11W)1, ZN11I)1, Zvar(N11M)1, Zvar(N11V)1, Zvar(N11A)1, Zvar(N11H1), Zvar(N11R)1, Zvar(N11E,Q32A)1, Zvar(N11E,Q32E,Q40E)1 and Zvar(N11E,Q32E,K50R)1, Zvar(Q9A,N11E,N43A)1, Zvar(Q9A,N11E,N28A,N43A)1, Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)1, Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1, Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)1, Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1, Zvar(Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1, Zvar(N11K, H18K, D37E, A42R, N43A, L44I)1, Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)1 and Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)1, as well as the varieties of

Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1 having G,S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K in position 29, the varieties of Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1 having F,Y,W,K or R in position 53 and the varieties of Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1 where Q9, Q40, A42 or N43 has been deleted, have an improved alkali stability compared to the parental structure Zvar1, used as the reference. Further, the ligands B(Q9A,N11E,Q40V,A42K,N43A,L44I)1 and C(Q9A,N11E,E43A)1 have an improved stability compared to the parental B and C domains, used as references.

Table 1. Monomeric ligands, evaluated by Biacore (0.5 M NaOH).

Ligand	**Sequence**	**Capacity after 96-100 cycles**	**Reference capacity after 96-100 cycles**	**Capacity relative to reference**
Zvar(N11E,Q32A)1	SEQ ID NO 12	57%	55%	1.036
Zvar(N11E)1	SEQ ID NO 13	59%	55%	1.073
Zvar(N11E,Q32E,Q40E)1	SEQ ID NO 14	52%	51%	1.020
Zvar(N11E,Q32E,K50R)1	SEQ ID NO 15	53%	51%	1.039
Zvar(N11K)1	SEQ ID NO 16	62%	49%	1.270
Zvar(N11Y)1	SEQ ID NO 38	55%	46%	1.20
Zvar(N11T)1	SEQ ID NO 39	50%	46%	1.09
Zvar(N11F)1	SEQ ID NO 40	55%	46%	1.20
Zvar(N11L)1	SEQ ID NO 41	57%	47%	1.21
Zvar(N11W)1	SEQ ID NO 42	57%	47%	1.21
Zvar(N11I)1	SEQ ID NO 43	57%	47%	1.21
Zvar(N11M)1	SEQ ID NO 44	58%	46%	1.26
Zvar(N11V)1	SEQ ID NO 45	56%	46%	1.22
Zvar(N11A)1	SEQ ID NO 46	58%	46%	1.26
Zvar(N11H)1	SEQ ID NO 47	57%	46%	1.24
Zvar(N11R)1	SEQ ID NO 48	59%	46%	1.28
Zvar(Q9A,N11E,N43A)1	SEQ ID NO 8	70%	47%	1.49
Zvar(Q9A,N11E,N28A,N43A)1	SEQ ID NO 9	68%	47%	1.45
Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)1	SEQ ID NO 10	67%	47%	1.43
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1	SEQ ID NO 11	66%	47%	1.40

Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)1	SEQ ID NO 24	65%	48%	1.35
Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1	SEQ ID NO 23	67%	46%	1.46
Zvar(Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1	SEQ ID NO 25	59%	46%	1.28
Zvar(N11K, H18K, D37E, A42R, N43A, L44I)1	SEQ ID NO 26	59%	45%	1.31
Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)1	SEQ ID NO 27	63%	45%	1.40
Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)1	SEQ ID NO 28	67%	45%	1.49
B(Q9A,N11E,Q40V,A42K,N43A,L44I)1	SEQ ID NO 36	39%	35%	1.11
C(Q9A,N11E,E43A)1	SEQ ID NO 37	60%	49%	1.22
Zvar(Q9A,N11E,**A29G**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 54	69%	48%	1.44
Zvar(Q9A,N11E,**A29S**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 55	66%	48%	1.38
Zvar(Q9A,N11E,**A29Y**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 56	61%	48%	1.27
Zvar(Q9A,N11E,**A29Q,**Q40V,A42K,N43A,L44I)1	SEQ ID NO 57	60%	47%	1.28
Zvar(Q9A,N11E,**A29T**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 58	60%	47%	1.28
Zvar(Q9A,N11E,**A29N**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 59	61%	47%	1.30
Zvar(Q9A,N11E,**A29F**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 60	62%	46%	1.35
Zvar(Q9A,N11E,**A29L**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 61	61%	46%	1.33
Zvar(Q9A,N11E,**A29W**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 62	60%	46%	1.30
Zvar(Q9A,N11E,**A29I**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 63	58%	47%	1.23
Zvar(Q9A,N11E,**A29M**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 64	62%	47%	1.32
Zvar(Q9A,N11E,**A29V**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 65	62%	47%	1.32
Zvar(Q9A,N11E,**A29D**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 66	56%	47%	1.19
Zvar(Q9A,N11E,**A29E**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 67	57%	47%	1.21
Zvar(Q9A,N11E,**A29H**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 68	57%	47%	1.21
Zvar(Q9A,N11E,**A29R**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 69	58%	46%	1.26
Zvar(Q9A,N11E,**A29K**,Q40V,A42K,N43A,L44I)1	SEQ ID NO 70	59%	46%	1.28
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,**D53F**)1	SEQ ID NO 71	58%	46%	1.26
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,**D53Y**)1	SEQ ID NO 72	59%	46%	1.28
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,**D53W**)1	SEQ ID NO 73	62%	46%	1.35
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,**D53K**)1	SEQ ID NO 74	65%	46%	1.41
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,**D53R**)1	SEQ ID NO 75	60%	46%	1.30

Zvar(**Q9del**,N11E,Q40V,A42K,N43A,L44I)1	SEQ ID NO 76	60%	46%	1.30
Zvar(Q9A,N11E,**Q40del**,A42K,N43A,L44I)1	SEQ ID NO 77	59%	46%	1.28
Zvar(Q9A,N11E,Q40V,**A42del**,N43A,L44I)1	SEQ ID NO 78	57%	46%	1.24
Zvar(Q9A,N11E,Q40V,A42K,**N43del**,L44I)1	SEQ ID NO 79	55%	46%	1.20

[00096] The Biacore experiment can also be used to determine the binding and dissociation rates between the ligand and IgG. This was used with the set-up as described above and with an IgG1 monoclonal antibody as probe molecule. For the reference Zvar1, the on-rate (10^5 $M^{-1}s^{-1}$) was 3.1 and the off-rate (10^5 s^{-1}) was 22.1, giving an affinity (off-rate/on-rate) of 713 pM. For Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1 (SEQ ID NO. 11), the on-rate was 4.1 and the off-rate 43.7, with affinity 1070 pM. The IgG affinity was thus somewhat higher for the mutated variant.

Example 2

[00097] The purified dimeric, tetrameric and hexameric ligands listed in Table 2 were immobilized on Biacore CM5 sensor chips (GE Healthcare, Sweden), using the amine coupling kit of GE Healthcare (for carbodiimide coupling of amines on the carboxymethyl groups on the chip) in an amount sufficient to give a signal strength of about 200-1500 RU in a Biacore instrument (GE Healthcare, Sweden) . To follow the IgG binding capacity of the immobilized surface 1mg/ml human polyclonal IgG (Gammanorm) was flowed over the chip and the signal strength (proportional to the amount of binding) was noted. The surface was then cleaned-in-place (CIP), i.e. flushed with 500mM NaOH for 10 minutes at room temperature (22 +/- 2°C). This was repeated for 300 cycles and the immobilized ligand alkaline stability was followed as the remaining IgG binding capacity (signal strength) after each cycle. The results are shown in Table 2 and in Fig. 2 and indicate that at least the ligands Zvar(Q9A,N11E,N43A)4, Zvar(Q9A,N11E,N28A,N43A)4, Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)4 and Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4, Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)4 and Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)4 have an improved alkali stability compared to the parental structure Zvar4, which was used as a reference. The hexameric ligand Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)6 also has improved alkali stability compared to the parental structure Zvar6, used as a reference. Further, Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I) dimers with deletions of a) D2,A3,K4; b) K58,V1,D2; c) P57,K58,V1,D2,A3; d) K4,F5,D6,K7,E8; e) A56,P57,K58; V1,D2,A3 or f)

V1,D2,A3,K4,F5,D6,K7,E8 from the linker region between the two monomer units have improved alkali stability compared to the parental structure Zvar2, used as a reference. Also Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I) dimers with an insertion of YEDG between K58 and V1 in the linker region have improved alkali stability compared to Zvar2.

Table 2. Dimeric, tetrameric and hexameric ligands, evaluated by Biacore (0.5M NaOH).

Ligand	**SEQ ID NO:**	**Remaining capacity 100 cycles (%)**	**Capacity relative to ref. 100 cycles**	**Remaining capacity 200 cycles (%)**	**Capacity relative to ref. 200 cycles**	**Remaining capacity 300 cycles (%)**	**Capacity relative to ref. 300 cycles**
Zvar4	21	67	1	36	1	16	1
Zvar(Q9A,N11E,N43A)4	17	81	1.21	62	1.72	41	2.56
Zvar(Q9A,N11E,N28A,N43A)4	18	80	1.19	62	1.72	42	2.62
Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)4	19	84	1.25	65	1.81	48	3.00
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4	20	90	1.34	74	2.06	57	3.56
Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)4	32	84	1.24	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)6	33	87	1.30	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)4	34	81	1.13	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)4	35	84	1.17	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with D2, A3 and K4 in linker deleted	80	70	1.27	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with K58, V1 and D2 in linker deleted	81	76	1.38	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with P57, K58, V1, D2 and A3 in linker deleted	82	74	1.35	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with K4, F5, D6, K7 and E8 in linker deleted	83	70	1.30	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with A56, P57 and K58 in linker deleted	84	68	1.26	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with V1, D2 and A3 in linker deleted	85	75	1.39	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with V1, D2, A3, K4, F5, D6,	86	62	1.13	Not tested	Not tested	Not tested	Not tested

K7 and E8 in linker deleted							
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with YEDG inserted in linker between K58 and V1	87	72	1.31	Not tested	Not tested	Not tested	Not tested
Zvar2	88	55	1	Not tested	Not tested	Not tested	Not tested

Example 3

[00098] Example 2 was repeated with 100 CIP cycles of three ligands using 1 M NaOH instead of 500 mM as in Example 2. The results are shown in Table 3 and show that all three ligands have an improved alkali stability also in 1M NaOH, compared to the parental structure Zvar4 which was used as a reference.

Table 3. Tetrameric ligands, evaluated by Biacore (1M NaOH).

Ligand	**Sequence**	**Remaining capacity 100 cycles (%)**	**Capacity relative to ref. 100 cycles**
Zvar4	SEQ ID NO 21	27	1
Zvar(Q9A,N11E,N28A,N43A)4	SEQ ID NO 18	55	2.04
Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)4	SEQ ID NO 19	54	2.00
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4	SEQ ID NO 20	56	2.07

Example 4

[00099] The purified tetrameric ligands of Table 2 (all with an additional N-terminal cysteine) were immobilized on agarose beads using the methods described below and assessed for capacity and stability. The results are shown in Table 4 and Fig. 3.

Table 4. Matrices with tetrametric ligands, evaluated in columns (0.5 M NaOH).

Ligand	**SEQ ID NO.**	**Ligand content (mg/ml)**	**Initial IgG capacity Qb10 (mg/ml)**	**Remaining IgG capacity Qb10 after six 4 h cycles (mg/ml)**	**Remaining IgG capacity after six 4 h cycles (%)**	**Capacity retention relative to ref. after six 4 h cycles**
Zvar4	21	7	52.5	36.5	60	1
Zvar4	21	12	61.1	43.4	71	1
Zvar(Q9A,N11E,N28A,N43A)4	18	7.0	49.1	44.1	90	1.50
Zvar(Q9A,N11E,N28A,N43A)4	18	12.1	50.0	46.2	93	1.31

Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4	20	7.2	49.0	44.2	90	1.50
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4	20	12.8	56.3	53.6	95	1.34
Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)4	30	9.7	56.3	52.0	92	1.53
Zvar(Q9A,N11K,H18K,D37E,A42R)4	31	10.8	56.9	52.5	92	1.30

Activation

[000100] The base matrix used was rigid cross-linked agarose beads of 85 micrometers (volume-weighted, d50V) median diameter, prepared according to the methods of US6602990, hereby incorporated by reference in its entirety, and with a pore size corresponding to an inverse gel filtration chromatography Kav value of 0.70 for dextran of Mw 110 kDa, according to the methods described in Gel Filtration Principles and Methods, Pharmacia LKB Biotechnology 1991, pp 6-13.

[000101] 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.

Coupling

[000102] To 20 mL of ligand solution (50 mg/mL) in a 50 ml Falcon tube, 169 mg $NaHCO_3$, 21 mg Na_2CO_3, 175 mg NaCl and 7 mg EDTA, was added. The Falcon tube was placed on a roller table for 5-10 min, and then 77 mg of DTE was added. Reduction proceeded for >45 min. The ligand solution was then desalted on a PD10 column packed with Sephadex G-25. The ligand content in the desalted solution was determined by measuring the 276 nm UV absorption.

[000103] The activated gel was washed with 3-5 GV {0.1 M phosphate/1 mM EDTA pH 8.6} and the ligand was then coupled according to the method described in US6399750, hereby incorporated by reference in its entirety. All buffers used in the experiments had been degassed by nitrogen gas for at least 5-10 min. The ligand content of the gels could be controlled by varying the amount and concentration of the ligand solution.

[000104] After immobilization the gels were washed 3xGV with distilled water. The gels + 1 GV {0.1 M phosphate/1 mM EDTA/10% thioglycerol pH 8.6} was mixed and the tubes were left in a shaking table at room temperature overnight. The gels were then washed alternately

with 3xGV {0.1 M TRIS/0.15 M NaCl pH 8.6} and 0.5 M HAc and then 8-10xGV with distilled water. Gel samples were sent to an external laboratory for amino acid analysis and the ligand content (mg/ml gel) was calculated from the total amino acid content.

Protein

[000105] Gammanorm 165 mg/ml (Octapharma), diluted to 2mg/ml in Equilibration buffer.

Equilibration buffer

[000106] PBS Phosphate buffer 10 mM + 0.14 M NaCl + 0.0027 M KCl, pH 7,4 (Medicago)

Adsorption buffer

PBS Phosphate buffer 10 mM + 0.14 M NaCl + 0.0027 M KCl, pH 7,4 (Medicago)

Elution buffers

[000107] 100 mM acetate pH 2.9

Dynamic binding capacity

[000108] 2 ml of resin was packed in TRICORN™ 5 100 columns. The breakthrough capacity was determined with an ÄKTAExplorer 10 system at a residence time of 6 minutes (0.33 ml/min flow rate). Equilibration buffer was run through the bypass column until a stable baseline was obtained. This was done prior to auto zeroing. Sample was applied to the column until a 100% UV signal was obtained. Then, equilibration buffer was applied again until a stable baseline was obtained.

[000109] Sample was loaded onto the column until a UV signal of 85% of maximum absorbance was reached. The column was then washed with 5 column volumes (CV) equilibration buffer at flow rate 0.5ml/min. The protein was eluted with 5 CV elution buffer at a flow rate of 0.5 ml/min. Then the column was cleaned with 0.5M NaOH at flow rate 0.2 ml/min and re-equilibrated with equilibration buffer.

[000110] For calculation of breakthrough capacity at 10%, the equation below was used. That is the amount of IgG that is loaded onto the column until the concentration of IgG in the column effluent is 10% of the IgG 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 IgG subclass;

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.

[000111] The dynamic binding capacity (DBC) at 10% breakthrough was calculated. The dynamic binding capacity (DBC) was calculated for 10 and 80% breakthrough.

CIP - 0.5 M NaOH

[000112] The 10% breakthrough DBC (Qb10) was determined both before and after repeated exposures to alkaline cleaning solutions. Each cycle included a CIP step with 0.5 M NaOH pumped through the column at a rate of 0.5/min for 20 min, after which the column was left standing for 4 h. The exposure took place at room temperature (22 +/- 2°C). After this incubation, the column was washed with equilibration buffer for 20 min at a flow rate of 0.5 ml/min. Table 4 shows the remaining capacity after six 4 h cycles (i.e. 24 h cumulative exposure time to 0.5 M NaOH), both in absolute numbers and relative to the initial capacity.

Example 5

[000113] Example 4 was repeated with the tetrameric ligands shown in Table 5, but with 1.0 M NaOH used in the CIP steps instead of 0.5 M. The results are shown in Table 5 and in Fig. 4.

Table 5. Matrices with tetrametric ligands, evaluated in columns – 1.0 M NaOH.

Ligand	**SEQ ID NO.**	**Ligand content (mg/ml)**	**Initial IgG capacity Qb10 (mg/ml)**	**Remaining IgG capacity Qb10 after six 4 h cycles (mg/ml)**	**Remaining IgG capacity after six 4 h cycles (%)**	**Capacity retention relative to ref. after six 4 h cycles**
Zvar4	21	12	60.1	33.5	56	1
Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4	20	12.8	60.3	56.0	93	1.67
Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)4	30	9.7	62.1	48.1	77	1.44

Example 6

Base matrices

[000114] The base matrices used were a set of rigid cross-linked agarose bead samples of 59-93 micrometers (volume-weighted, d50V) median diameter (determined on a Malvern Mastersizer 2000 laser diffraction instrument), prepared according to the methods of US6602990 and with a pore size corresponding to an inverse gel filtration chromatography Kd value of 0.62-0.82 for dextran of Mw 110 kDa, according to the methods described above, using HR10/30 columns (GE Healthcare) packed with the prototypes in 0.2 M NaCl and with a range of dextran fractions as probe molecules (flow rate 0.2 ml/min). The dry weight of the bead samples ranged from 53 to 86 mg/ml, as determined by drying 1.0 ml drained filter cake samples at 105 °C over night and weighing.

Table 6. Base matrix samples

Base matrix	**Kd**	**d50v (µm)**	**Dry weight (mg/ml)**
A18	0.704	59.0	56.0
A20	0.70	69.2	55.8
A27	0.633	87.2	74.2
A28	0.638	67.4	70.2
A29	0.655	92.6	57.5
A32	0.654	73.0	70.5
A33	0.760	73.1	55.5
A38	0.657	70.9	56.2
A39	0.654	66.0	79.1
A40	0.687	64.9	74.9
A41	0.708	81.7	67.0
A42	0.638	88.0	59.4
A43	0.689	87.5	77.0
A45	0.670	56.6	66.0
A52	0.620	53.10	63.70
A53	0.630	52.6	86.0
A54	0.670	61.3	75.3
A55	0.640	62.0	69.6

A56	0.740	61.0	56.0
A56-2	0.740	51.0	56.0
A62a	0.788	48.8	70.1
A62b	0.823	50.0	46.9
A63a	0.790	66.8	59.6
A63b	0.765	54.0	79.0
A65a	0.796	58.0	60.0
A65b	0.805	57.3	46.0
B5	0.793	69.0	84.4
C1	0.699	71.0	73.4
C2	0.642	66.5	81.1
C3	0.711	62.0	82.0
C4	0.760	62.0	82.0
H31	0.717	82.0	59.0
H35	0.710	81.1	61.0
H40	0.650	52.8	65.0
I1	0.640	50.0	67.0
41	0.702	81.6	60.6
517	0.685	87.9	64.4
106	0.692	86.7	64.6
531C	0.661	51.7	63.8
P10	0.741	59.3	70.0
S9	0.736	64.1	72.2

Coupling

[000115] 100 ml base matrix was washed with 10 gel volumes distilled water on a glass filter. The gel was weighed (1 g = 1 ml) and mixed with 30 ml distilled water and 8.08 g NaOH (0.202 mol) in a 250 ml flask with an agitator. The temperature was adjusted to 27 +/- 2 °C in a water bath. 16 ml epichlorohydrin (0.202 mol) was added under vigorous agitation (about 250 rpm) during 90 +/- 10 minutes. The reaction was allowed to continue for another 80 +/- 10 minutes and the gel was then washed with >10 gel volumes distilled water on a glass filter until neutral pH was reached. This activated gel was used directly for coupling as below.

[000116] To 16.4 mL of ligand solution (50 mg/mL) in a 50 ml Falcon tube, 139 mg $NaHCO_3$, 17.4 mg Na_2CO_3, 143.8 mg NaCl and 141 mg EDTA, was added. The Falcon tube was placed on a roller table for 5-10 min, and then 63 mg of DTE was added. Reduction proceeded for >45 min. The ligand solution was then desalted on a PD10 column packed with Sephadex G-25. The ligand content in the desalted solution was determined by measuring the 276 nm UV absorption.

[000117] The activated gel was washed with 3-5 GV {0.1 M phosphate/1 mM EDTA pH 8.6} and the ligand was then coupled according to the method described in US6399750 5.2.2, although with considerably higher ligand amounts (see below). All buffers used in the experiments had been degassed by nitrogen gas for at least 5-10 min. The ligand content of the gels was controlled by varying the amount and concentration of the ligand solution, adding 5-20 mg ligand per ml gel. The ligand was either a tetramer (SEQ ID NO. 20) or a hexamer (SEQ ID NO. 33) of an alkali-stabilized mutant.

[000118] After immobilization the gels were washed 3xGV with distilled water. The gels + 1 GV {0.1 M phosphate/1 mM EDTA/10% thioglycerol pH 8.6} was mixed and the tubes were left in a shaking table at room temperature overnight. The gels were then washed alternately with 3xGV {0.1 M TRIS/0.15 M NaCl pH 8.6} and 0.5 M HAc and then 8-10xGV with distilled water. Gel samples were sent to an external laboratory for amino acid analysis and the ligand content (mg/ml gel) was calculated from the total amino acid content.

Evaluation

[000119] The Qb10 % dynamic capacity for polyclonal human IgG at 2.4 and 6 min residence time was determined as outlined in Example 4.

Table 7. Prototype results

Prototype	**Base matrix**	**Ligand content (mg/ml)**	**Multimer**	**Qb10% 2.4 min (mg/ml)**	**Qb10% 6 min (mg/ml)**
N1	A38	7.45	tetramer	44.4	58.25
N2	A20	7.3	tetramer	45.12	57.21
N3	A42	6.72	tetramer	33.56	50.02
N4	A29	7.3	tetramer	36.34	51.8

N5	A28	7.9	tetramer	42.38	58.25
N6	A39	6.96	tetramer	41.88	54.67
N7	A27	7.5	tetramer	29.19	48.73
N8	A43	6.99	tetramer	33.43	49.79
N9	A38	11.34	tetramer	48.1	72.78
N10	A20	10.6	tetramer	50.66	70.07
N11	A42	11.1	tetramer	32.25	57.78
N12	A29	11	tetramer	34.85	64.68
N13	A28	11.9	tetramer	39.92	63.75
N14	A39	10.48	tetramer	44.37	64.79
N15	A27	12.1	tetramer	24.8	55.56
N16	A43	10.51	tetramer	31.82	58.04
N17	A41	8.83	tetramer	38.5	56.8
N18	A41	8.83	tetramer	37.84	58.6
N19	A41	8.83	tetramer	35.06	57.23
N20	A41	5.0	tetramer	35.64	46.04
N21	A41	13.0	tetramer	34.95	62.23
N22	A40	13.15	tetramer	56.85	71.09
N23	A33	7.33	tetramer	48.69	55.76
N24	A40	11.03	tetramer	54.96	73.8
033A	A38	7.5	tetramer	44	58
033B	A38	11.3	tetramer	48	73
097A	A20	7.3	tetramer	45	57
097B	A20	10.6	tetramer	51	70
003A	A28	7.9	tetramer	42	58
003B	A28	11.9	tetramer	40	64
003C	A28	15.8	tetramer	37	67
038A	A39	7.0	tetramer	42	55
038B	A39	10.5	tetramer	44	65
074	A40	13.2	tetramer	57	71
093	A33	7.3	tetramer	49	56
058A	A40	11.0	tetramer	55	74
077	A18	8.2	tetramer	52	59

010	A32	10.7	tetramer	40	57
099	A32	13.3	tetramer	37	66
030A	B5	6.3	tetramer	32	38
030B	B5	9.6	tetramer	45	47
293A	C1	5.4	tetramer	38	47
293B	C1	10.8	tetramer	43	60
294A	C2	5.1	tetramer	39	46
294B	C2	10.5	tetramer	42	57
336A	H40	5.6	tetramer	47	52
336B	H40	9.1	tetramer	52	67
091	A18	13.4	tetramer	N/A	63
092	A20	12.8	tetramer	49	67
080	A33	9.4	tetramer	51	58
089	A40	6.1	tetramer	49	59
688A	A62a	6.6	tetramer	41	46
688B	A62a	14.8	tetramer	55	62
871	A62a	9.7	tetramer	48	60
934A	A63a	6.6	tetramer	40	44
934B	A63a	14.0	tetramer	48	56
017B	A65a	13.1	tetramer	56	64
041A	A62b	5.2	tetramer	40	N/A
041B	A62b	11.1	tetramer	52	N/A
116A	A65b	5.8	tetramer	42	46
116B	A65b	8.8	tetramer	49	56
017A	A65a	6.1	tetramer	40	44
387A	A62a	6.4	tetramer	43	45
387B	A62a	7.5	tetramer	47	56
432	A63a	6.1	tetramer	39	44
433A	A65a	6.6	tetramer	42	47
433B	A65a	13.6	tetramer	52	61
579A	I1	6.1	tetramer	45	51
579B	I1	11.2	tetramer	57	68
064A	C3	5.9	tetramer	44	52

064B	C3	9.0	tetramer	49	62
064C	C3	14.3	tetramer	51	70
352A	C4	10.1	tetramer	55	63
352B	C4	14.4	tetramer	59	67
066A	C3	6.8	hexamer	48	59
066B	C3	11.9	hexamer	51	73
066C	C3	15.1	hexamer	43	61
353A	C4	11.2	hexamer	62	74
353B	C4	15.2	hexamer	57	82
872A	A62a	9.6	hexamer	56	72
872B	A62a	14.5	hexamer	62	84
869A	H40	6.9	hexamer	50	56
869B	H40	14.3	hexamer	56	75
869C	H40	23.0	hexamer	41	65
962A	H35	6.8	hexamer	36	49
962B	H35	12.3	hexamer	31	54
962C	H35	20.3	hexamer	20	43
112A	A56	7.9	hexamer	47	55
112B	A56	12.4	hexamer	57	73
112C	A56	19.2	hexamer	55	80
113A	A56	7.1	hexamer	48	57
113B	A56	12.4	hexamer	53	73
113C	A56	15.2	hexamer	48	76
212A	H31	6.5	hexamer	37	38
212B	H31	10.4	hexamer	50	61
212C	H31	20.0	hexamer	31	52
213A	A33	6.5	hexamer	44	53
213B	A33	10.9	hexamer	50	65
213C	A33	11.1	hexamer	50	68
432A	A20	6.4	hexamer	41	56
432B	A20	12.4	hexamer	38	64
432C	A20	21.1	hexamer	44	43
433A	A38	5.9	hexamer	47	57

433B	A38	11.6	hexamer	48	72
433C	A38	15.8	hexamer	36	62
742A	A54	6.7	hexamer	38	46
742B	A54	12.6	hexamer	45	52
742C	A54	21.1	hexamer	38	65
726A	A63b	6.4	hexamer	42	46
726B	A63b	10.6	hexamer	49	60
726C	A63b	16.7	hexamer	53	69
793A	A56-2	6.8	hexamer	50	58
793B	A56-2	12.5	hexamer	59	72
793C	A56-2	19.2	hexamer	61	82
517	517	12.0	tetramer*	35	56
106	106	5.8	tetramer*	33	45
531C	531C	11.2	tetramer*	54	65
P10	P10	19.0	hexamer		76
S9	S9	18.4	hexamer	56	75

*SEQ ID NO 21

Example 7

[000120] A series of prototypes, prepared as above, with different ligand content (tetramer, SEQ ID NO:20) were incubated in 1 M NaOH for 4, 8 and 31 hours at 22 +/- 2 °C and the dynamic IgG capacity (Qb10%, 6 min residence time) was measured before and after incubation. The prototypes are shown in Table 8 and the results in Figs. 5 and 6. It can be seen that the stability towards this harsh alkali treatment increases with increasing ligand content.

Table 8 Samples for incubation in 1 M NaOH

Prototype	**Ligand content (mg/ml)**	**Qb10%, 6 min, before incubation (mg/ml)**
N1	12	78
LE28	13	79
N17	16	73
N16	20	73

Example 8

Pressure-flow testing of matrices

300 ml sedimented matrix was packed in a FineLine™ 35 column (GE Healthcare Life Sciences, Uppsala, Sweden), with 35 mm inner diameter and 330 mm tube height. The gel was suspended in distilled water to produce a slurry volume of 620 ml and the height of the packed bed was 300 +/- 10 mm. The packing pressure was 0.10 +/- 0.02 bar (10 +/- 2 kPa).

Distilled water was then pumped through the column at increasing pump rates and the flow rate (expressed as the linear flow velocity, cm/h) and back pressure (MPa) was measured after 5 min for each pump setting. The measurements were continued until a max flow rate and a max pressure was reached, i.e. the flow rate and back pressure achieved when the flow rate starts to diminish at increasing back pressures.

Table 9 Pressure flow performance of matrices

Matrix	**Max flow velocity, cm/h**	**Max pressure (MPa)**
517	1343	0.56
106	1306	0.56
531C	513	0.51
P10	862	0.60
S9	1172	0.64

The P10 and S9 matrices have a higher rigidity, as indicated by the max pressure, and can thus sustain comparatively high flow velocities despite their low (59-64 micrometers) median particle diameters.

[000121] This written description uses examples to disclose the invention, including the best mode, and also to enable any person skilled in the art to practice the invention, including making and using any devices or systems and performing any incorporated methods. The patentable scope of the invention is defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal languages of the claims. All patents and patent applications mentioned in the text are hereby incorporated by reference in their entireties, as if they were individually incorporated.

ITEMIZED LIST OF EMBODIMENTS

i. An Fc-binding polypeptide which comprises a sequence as defined by, or having at least 90% or at least 95% or 98% identity to SEQ ID NO 53.

$X_1Q\ X_2AFYEILX_3LP\ NLTEEQRX_4X_5F\ IX_6X_7LKDX_8PSX_9\ SX_{10}X_{11}X_{12}LAEAKX_{13}\ X_{14}NX_{15}AQ$ (SEQ ID NO 53)

wherein individually of each other:

X_1=A or Q or is deleted

X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R

X_3=H or K

X_4=A or N

X_5=A, G, S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K

X_6=Q or E

X_7=S or K

X_8=E or D

X_9=Q or V or is deleted

X_{10}=K,R or A or is deleted

X_{11}=A,E or N or is deleted

X_{12}=I or L

X_{13}=K or R

X_{14}=L or Y

X_{15}=D, F,Y,W,K or R

ii. The polypeptide of embodiment i, wherein:

X_1 = A or is deleted, X_2 = E, X_3 = H, X_4 = N, X_6 = Q, X_7 = S, X_8 = D, X_9 = V or is deleted, X_{10} = K or is deleted, X_{11} = A or is deleted, X_{12} = I, X_{13} = K, X_{14} = L.

iii. The polypeptide of embodiment i or ii, wherein X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

iv. The polypeptide of embodiment i or ii, wherein X_1 is deleted, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

v. The polypeptide of embodiment i or ii, wherein X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5= S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K , X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

vi. The polypeptide of embodiment i or ii, wherein X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 is deleted, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

vii. The polypeptide of embodiment i or ii, wherein X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} is deleted, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

viii. The polypeptide of embodiment i or ii, wherein X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} is deleted, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

ix. The polypeptide of embodiment i or ii, wherein X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}= F,Y,W,K or R.

x. An Fc-binding polypeptide comprising a mutant of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 90% such as at least 95% or 98% identity to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.

xi. The polypeptide of embodiment x, comprising a mutant of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 90% such as at least 95% or 98% identity to, SEQ ID NO 51 or SEQ ID NO 52.

xii. The polypeptide of embodiment x or xi, wherein the amino acid residue at the position corresponding to position 11 in SEQ ID NO:4-7 is a glutamic acid.

xiii. The polypeptide of any one of embodiments x-xii, wherein the amino acid residue at the position corresponding to position 11 in SEQ ID NO:4-7 is a lysine.

xiv. The polypeptide of any one of embodiments x-xiii, wherein the amino acid residue at the position corresponding to position 29 in SEQ ID NO:4-7 is a glycine, serine, tyrosine, glutamine, threonine, asparagine, phenylalanine, leucine, tryptophan, isoleucine, methionine, valine, aspartic acid, glutamic acid, histidine, arginine or lysine.

xv. The polypeptide of any one of embodiments x-xiv, wherein the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 is an alanine.

xvi. The polypeptide of any one of embodiments x-xv, wherein the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 has been deleted.

xvii. The polypeptide of any one of embodiments x-xvi, wherein the amino acid residue at the position corresponding to position 50 in SEQ ID NO:4-7 is an arginine or a glutamic acid, such as an arginine.

xviii. The polypeptide of any one of embodiments x-xvii, wherein the amino acid residue at the position corresponding to position 43 in SEQ ID NO:4-7 has been deleted.

xix. The polypeptide of any one of embodiments x-xviii, wherein the amino acid residue at the position corresponding to position 28 in SEQ ID NO:4-7 is an alanine or an asparagine.

xx. The polypeptide of any one of embodiments x-xix, wherein the amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 is selected from the group consisting of asparagine, alanine, glutamic acid and valine.

xxi. The polypeptide of any one of embodiments x-xx, wherein the amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 has been deleted.

xxii. The polypeptide according to any one of embodiments x-xxi, wherein the amino acid residue at the position corresponding to position 42 in SEQ ID NO:4-7 is an alanine, lysine or arginine, such as an arginine.

xxiii. The polypeptide according to any one of embodiments x-xxii, wherein the amino acid residue at the position corresponding to position 42 in SEQ ID NO:4-7 has been deleted.

xxiv. The polypeptide according to any one of embodiments x-xxiii, wherein the amino acid residue at the position corresponding to position 44 in SEQ ID NO:4-7 is a leucine or an isoleucine, such as an isoleucine.

xxv. The polypeptide according to any one of embodiments x-xxiv, wherein the amino acid residue at the position corresponding to position 44 in SEQ ID NO:4-7 has been deleted.

xxvi. The polypeptide according to any one of embodiments x-xxv, wherein the amino acid residue at the position corresponding to position 53 in SEQ ID NO:4-7 is a phenylalanine, a tyrosine, a tryptophan, an arginine or a lysine.

xxvii. The polypeptide according to any one of embodiments x-xxvi, wherein the amino acid residue at the position corresponding to position 18 in SEQ ID NO:4-7 is a lysine or a histidine, such as a lysine.

xxviii. The polypeptide according to any one of embodiments x-xxvii, wherein the amino acid residue at the position corresponding to position 33 in SEQ ID NO:4-7 is a lysine or a serine, such as a lysine.

xxix. The polypeptide according to any one of embodiments x-xxviii, wherein the amino acid residue at the position corresponding to position 37 in SEQ ID NO:4-7 is a glutamic acid or an aspartic acid, such as a glutamic acid.

xxx. The polypeptide according to any one of embodiments x-xxix, wherein the amino acid residue at the position corresponding to position 51 in SEQ ID NO:4-7 is a tyrosine or a leucine, such as a tyrosine.

xxxi. The polypeptide according to any one of embodiments x-xxx, wherein one or more of the amino acid residues at the positions corresponding to positions 1, 2, 3, 4, 5, 6, 7, 8, 56, 57 or 58 in SEQ ID NO: 4-7 have been deleted.

xxxii. The polypeptide according to any one of embodiments x-xxxi, wherein the mutation is selected from the group consisting of:

Q9A,N11E, A29G,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29S,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29Y,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29Q,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29T,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29N,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29F,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29L,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29W,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29I,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29M,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29V,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29D,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29E,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29H,Q40V,A42K,N43A,L44I;
Q9A,N11E, A29R,Q40V,A42K,N43A,L44I; and
Q9A,N11E, A29K,Q40V,A42K,N43A,L44I.

xxxiii. The polypeptide according to any one of embodiments x-xxxii, wherein the mutation is selected from the group consisting of:
Q9A,N11E, Q40V,A42K,N43A,L44I,D53F;
Q9A,N11E, Q40V,A42K,N43A,L44I,D53Y;
Q9A,N11E, Q40V,A42K,N43A,L44I,D53W;
Q9A,N11E, Q40V,A42K,N43A,L44I,D53K; and
Q9A,N11E, Q40V,A42K,N43A,L44I,D53R.

xxxiv. The polypeptide according to any one of embodiments x-xxxiii, wherein the mutation is selected from the group consisting of:
Q9del,N11E, Q40V,A42K,N43A,L44I;
Q9A,N11E, Q40del,A42K,N43A,L44I;
Q9A,N11E, Q40V,A42del,N43A,L44I; and
Q9A,N11E, Q40V,A42K,N43del,L44I.

xxxv. The polypeptide according to any one of embodiments x-xxxiv, wherein the mutation is selected from the group consisting of:
D2del,A3del,K4del,Q9A,N11E,Q40V,A42K,N43A,L44I;
V1del,D2del,Q9A,N11E,Q40V,A42K,N43A,L44I,K58del;
V1del,D2del,A3del,Q9A,N11E,Q40V,A42K,N43A,L44I,P57del,K58del;
K4del,F5del,D6del,K7del,E8del,Q9A,N11E,Q40V,A42K,N43A,L44I;
Q9A,N11E,Q40V,A42K,N43A,L44I,A56del,P57del,K58del;
V1del,,D2del,A3del,Q9A,N11E,Q40V,A42K,N43A,L44I;
V1del,D2del,A3del,K4del,F5del,D6del,K7del,E8del,Q9A,N11E,Q40V,A42K,N43A,L44I;and
Q9A,N11E,Q40V,A42K,N43A,L44I,K58_insYEDG.

xxxvi. The polypeptide according to any one of embodiments i-xxxi, comprising or consisting essentially of a sequence having at least 90% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 54, SEQ ID NO 55, SEQ ID NO 56, SEQ ID NO 57, SEQ ID NO 58, SEQ ID NO 59, SEQ ID NO 60, SEQ ID NO 61, SEQ ID NO 62, SEQ ID NO 63, SEQ ID NO 64, SEQ ID NO 65, SEQ ID NO 66, SEQ ID NO 67, SEQ ID NO 68, SEQ ID NO 69 and SEQ ID NO 70.

xxxvii. The polypeptide according to any one of embodiments i-xxxi, comprising or consisting essentially of a sequence having at least 90% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 71, SEQ ID NO 72, SEQ ID NO 73, SEQ ID NO 74 and SEQ ID NO 75.

xxxviii. The polypeptide according to any one of embodiments i-xxxi, comprising or consisting essentially of a sequence having at least 90% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 76, SEQ ID NO 77, SEQ ID NO 78 and SEQ ID NO 79.

xxxix. The polypeptide according to any one of embodiments i-xxxi, comprising or consisting essentially of a sequence having at least 90% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 89, SEQ ID NO 90, SEQ ID NO 91, SEQ ID NO 92, SEQ ID NO 93, SEQ ID NO 94 and SEQ ID NO 95.

xl. The polypeptide according to any preceding embodiment, which polypeptide has an improved alkaline stability compared to a polypeptide as defined by SEQ ID NO 1, SEQ ID NO

2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 or SEQ ID NO 7, such as by SEQ ID NO 7.

xli. The polypeptide according to any preceding embodiment, which polypeptide has an improved alkaline stability compared to a parental polypeptide as defined by SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 or SEQ ID NO 7, such as by SEQ ID NO 7.

xlii. The polypeptide according to embodiment xl or xli, wherein the alkaline stability is improved as measured by the remaining IgG-binding capacity, after 24, 25 h incubation in 0.5 M or 1.0 M aqueous NaOH at 22 +/- 2 °C.

xliii. A multimer comprising or consisting essentially of a plurality of polypeptides as defined by any preceding embodiment.

xliv. The multimer according to embodiment xliii, wherein the polypeptides are linked by linkers comprising up to 25 amino acids, such as 3-25 or 3-20 amino acids.

xlv. The multimer of embodiment xliii or xliv, wherein at least two polypeptides are linked by linkers comprising or consisting essentially of a sequence having at least 90% identity with an amino acid sequence selected from the group consisting of APKVDAKFDKE, APKVDNKFNKE, APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APK, APKYEDGVDAKFDKE and YEDG.

xlvi. The multimer according to embodiment xliv or xlv, which is a dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer or nonamer.

xlvii. The multimer according to any one of embodiments xliv-xlvi, which comprises or consists essentially of a sequence selected from the group of sequences defined by SEQ ID NO 80, SEQ ID NO 81, SEQ ID NO 82, SEQ ID NO 83, SEQ ID NO 84, SEQ ID NO 85, SEQ ID NO 86 and SEQ ID NO 87.

xlviii. The polypeptide or multimer according to any preceding embodiment, further comprising at, or within 1-5 amino acid residues from, the C-terminal or N-terminal one or more coupling

element, selected from the group consisting of one or more cysteine residues, a plurality of lysine residues and a plurality of histidine residues.

xlix. A nucleic acid or a vector encoding a polypeptide or multimer according to any preceding embodiment.

l. An expression system, which comprises a nucleic acid or vector according to embodiment xlix.

li. A separation matrix, wherein a plurality of polypeptides or multimers according to any one of embodiment i – xlviii have been coupled to a solid support.

lii. A separation matrix comprising at least 11 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein:
a) said ligands comprise multimers of alkali-stabilized Protein A domains,
b) said porous support comprises cross-linked polymer particles having a volume-weighted median diameter (d50,v) of 55-70 micrometers and a dry solids weight of 55-80 mg/ml.

liii. A separation matrix comprising at least 15, such as 15-21 or 15-18 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein said ligands comprise multimers of alkali-stabilized Protein A domains.

liv. The separation matrix of embodiment li or liii, wherein said cross-linked polymer particles comprise cross-linked polysaccharide particles.

lv. The separation matrix of any one of embodiments li-liv, wherein said cross-linked polymer particles comprise cross-linked agarose particles.

lvi. The separation matrix of any one of embodiments li-lv, wherein said cross-linked polymer particles have a pore size corresponding to an inverse gel filtration chromatography Kd value of 0.70-0.85 for dextran of Mw 110 kDa.

lvii. The separation matrix of any one of embodiments li-lvi, which has a max pressure of at least 0.58, such as at least 0.60, MPa when packed at 300 +/-10 mm bed height in a FineLine™ 35 column.

lviii. The separation matrix of any one of embodiments li-lvii, wherein said multimers comprise tetramers, pentamers, hexamers or heptamers of alkali-stabilized Protein A domains.

lix. The separation matrix of any one of embodiments li-lviii, wherein said multimers comprise hexamers of alkali-stabilized Protein A domains.

lx. The separation matrix of any one of embodiments li-lix, wherein the polypeptides are linked by linkers comprising up to 25 amino acids, such as 3-25 or 3-20 amino acids.

lxi. The separation matrix of any one of embodiments li-lx, wherein at least two polypeptides are linked by linkers comprising or consisting essentially of a sequence having at least 90% identity with an amino acid sequence selected from the group consisting of APKVDAKFDKE, APKVDNKFNKE, APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APK, APKYEDGVDAKFDKE and YEDG.

lxii. The separation matrix of any one of embodiments li-lxi, having a 10% breakthrough dynamic binding capacity for IgG of at least 45 mg/ml, such as at least 50 or at least 55 mg/ml mg/ml at 2.4 min residence time.

lxiii. The separation matrix of any one of embodiments li-lxii, having a 10% breakthrough dynamic binding capacity for IgG of at least 60 mg/ml, such as at least 65, at least 70 or at least 75 mg/ml at 6 min residence time.

lxiv. The separation matrix of any one of embodiments li-lxiii, wherein the 10% breakthrough dynamic binding capacity for IgG at 2.4 or 6 min residence time is reduced by less than 20 % after incubation 31 h in 1.0 M aqueous NaOH at 22 +/- 2 C.

lxv. The separation matrix of any one of embodiments li-lxiv, having a dissociation constant for IgG2 of below 0.2 mg/ml, such as below 0.1 mg/ml, in 20 mM phosphate buffer, 180 mM NaCl, pH 7.5.

lxvi. The separation matrix according to any one of embodiments li-lxv, wherein the polypeptides or multimers have been coupled to the solid support or porous support via thioether bonds.

lxvii. The separation matrix according to any one of embodiments li - lxvi, wherein the solid support or porous support is a polysaccharide.

lxviii. The separation matrix according to any one of embodiments li-lxvii, wherein the IgG capacity of the matrix after 24 incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 80, such as at least 85, at least 90 or at least 95% of the IgG capacity before the incubation.

lxix. The separation matrix according to any one of embodiments li-lxviii, wherein the IgG capacity of the matrix after 24 incubation in 1.0 M NaOH at 22 +/- 2 °C is at least 70, such as at least 80 or at least 90% of the IgG capacity before the incubation.

lxx. The separation matrix of any one of embodiments li-lxix, wherein said alkali-stabilized Protein A domains or plurality of polypeptides/multimers comprise(s) mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 80% such as at least 90%, 95% or 98% identity to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.

lxxi. The separation matrix of embodiment lxx, wherein the amino acid residue at the position corresponding to position 11 in SEQ ID NO:4-7 is, or has been mutated to, a glutamic acid or a lysine.

lxxii. The separation matrix of embodiment lxx or lxxi, wherein the amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 is, or has been mutated to, a valine.

lxxiii. The separation matrix of any one of embodiments li-lxix, wherein said alkali-stabilized Protein A domains or plurality of polypeptides/multimers comprise(s) an Fc-binding

polypeptide having an amino acid sequence as defined by, or having at least 80%, such as at least 90, 95 or 98%, identity to SEQ ID NO 53.

X_1Q X_2AFYEILX_3LP NLTEEQRX_4X_5F IX_6X_7LKDX_8PSX_9 S$X_{10}X_{11}X_{12}$LAEAKX_{13} X_{14}NX_{15}AQ (SEQ ID NO 53)

wherein individually of each other:

X_1=A or Q or is deleted

X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R

X_3=H or K

X_4=A or N

X_5=A, G, S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K

X_6=Q or E

X_7=S or K

X_8=E or D

X_9=Q or V or is deleted

X_{10}=K,R or A or is deleted

X_{11}=A,E or N or is deleted

X_{12}=I or L

X_{13}=K or R

X_{14}=L or Y

X_{15}=D, F,Y,W,K or R

lxxiv. The separation matrix of embodiment lxxiiii, wherein individually of each other:

X_1 = A or is deleted, X_2 = E, X_3 = H, X_4 = N, X_6 = Q, X_7 = S, X_8 = D, X_9 = V or is deleted, X_{10} = K or is deleted, X_{11} = A or is deleted, X_{12} = I, X_{13} = K, X_{14} = L.

lxxv. The separation matrix of embodiment lxxiii, wherein individually of each other:

X_1=A, X_2 = E, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

lxxvi. The separation matrix of embodiment lxxiii, wherein individually of each other:

wherein X_1 is A, X_2 = E, X_3 = H, X_4 = N, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

lxxvii. The separation matrix of embodiment lxxiii, wherein individually of each other:

wherein X_1 is A, X_3 = H, X_4 = N, X_5=A, X_6 = Q, X_7 = S, X_8 = D, X_9 = V, X_{10} = K, X_{11} = A, X_{12} = I, X_{13} = K, X_{14} = L and X_{15}=D.

lxxviii. The separation matrix according to any one of embodiments li-lxxvii, wherein said multimers or polypeptides further comprise at, or within 1-5 amino acid residues from, the C-terminal or N-terminal one or more coupling element, selected from the group consisting of one or more cysteine residues, a plurality of lysine residues and a plurality of histidine residues.

lxxix. The separation matrix according to any one of embodiments li-lxxviii wherein said multimers or polypeptides further comprise at the N-terminal a leader sequence, comprising 1-20 amino acid residues.

lxxx. A method of isolating an immunoglobulin, wherein a separation matrix according to any one of embodiments li-lxxix is used.

lxxxi. The method of embodiment lxxx, comprising the steps of:
a) contacting a liquid sample comprising an immunoglobulin with a separation matrix according to any one of embodiments li-lxxix,
b) washing said separation matrix with a washing liquid,
c) eluting the immunoglobulin from the separation matrix with an elution liquid, and
d) cleaning the separation matrix with a cleaning liquid.

lxxxii. The method of embodiment lxxxi, wherein the cleaning liquid is alkaline, such as with a pH of 13 – 14.

lxxxiii. The method of embodiment lxxxi or lxxxii, wherein the cleaning liquid comprises 0.1 – 1.0 M NaOH or KOH, such as 0.5 – 1.0 M or 0.4-1.0 M NaOH or KOH.

lxxxiv. The method of any one of embodiments lxxxi – lxxxiii, wherein steps a) – d) are repeated at least 10 times, such as at least 50 times or 50 – 200 times.

lxxxv. The method of any one of embodiments lxxxi –lxxxiv, wherein steps a) – c) are repeated at least 10 times, such as at least 50 times or 50 – 200 times and wherein step d) is performed after a plurality of instances of step c), such as at least 10 or at least 50 times.

CLAIMS

1. A separation matrix comprising at least 11, such as at least 15, 15-21, 17-21 or 18-20 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein:
a) said ligands comprise multimers of alkali-stabilized Protein A domains,
b) said porous support comprises cross-linked polymer particles having a volume-weighted median diameter (d50,v) of 56-70 micrometers and a dry solids weight of 55-80 mg/ml.

2. The separation matrix of claim 1, wherein said cross-linked polymer particles comprise cross-linked polysaccharide particles.

3. The separation matrix of claim 1 or 2, wherein said cross-linked polymer particles comprise cross-linked agarose particles.

4. The separation matrix of any preceding claim, wherein said cross-linked polymer particles have a pore size corresponding to an inverse gel filtration chromatography Kd value of 0.69-0.85 for dextran of Mw 110 kDa.

5. The separation matrix of any preceding claim, wherein said multimers comprise tetramers, pentamers, hexamers or heptamers of alkali-stabilized Protein A domains.

6. The separation matrix of any preceding claim, wherein said multimers comprise hexamers of alkali-stabilized Protein A domains.

7. The separation matrix of any preceding claim, having a 10% breakthrough dynamic binding capacity for IgG of at least 45 mg/ml, such as at least 50 or at least 55 mg/ml at 2.4 min residence time.

8. The separation matrix of any preceding claim, having a 10% breakthrough dynamic binding capacity for IgG of at least 60 mg/ml, such as at least 65, at least 70 or at least 75 mg/ml at 6 min residence time.

9. The separation matrix of any preceding claim, wherein the 10% breakthrough dynamic binding capacity for IgG at 2.4 min or 6 min residence time is reduced by less than 20 % after incubation 31 h in 1.0 M aqueous NaOH at 22 +/- 2 C.

10. The separation matrix of any preceding claim, having a dissociation constant for IgG2 of below 0.2 mg/ml, such as below 0.1 mg/ml, in 20 mM phosphate buffer, 180 mM NaCl, pH 7.5.

11. The separation matrix of any preceding claim, which has a max pressure of at least 0.58, such as at least 0.60, MPa when packed at 300 +/-10 mm bed height in a FineLine™ 35 column.

12. The separation matrix of any preceding claim, wherein said alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.

13. The separation matrix of claim 12, wherein the amino acid residue at the position corresponding to position 11 in SEQ ID NO:4-7 is, or has been mutated to, a glutamic acid or a lysine.

14. The separation matrix of claim 12 or 13, wherein the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 is an alanine

15. The separation matrix of claim 12 or 13, wherein the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 has been deleted.

16. The separation matrix of any one of claims 12-15, wherein the amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 is, or has been mutated to, a valine.

17. The separation matrix of any one of claims 12-16, wherein one or more of the amino acid residues at the positions corresponding to positions 1, 2, 3, 4, 5, 6, 7, 8, 56, 57 or 58 in SEQ ID NO: 4-7 have been deleted.

18. The separation matrix of any one of claims 1-11, wherein said alkali-stabilized Protein A domains comprise an Fc-binding polypeptide having an amino acid sequence as defined by, or having at least 80% or at least 90, 95% or 98% identity to SEQ ID NO 53.

X_1Q X_2AFYEILX_3LP NLTEEQRX_4X_5F IX_6X_7LKDX_8PSX_9 S$X_{10}X_{11}X_{12}$LAEAKX_{13} X_{14}NX_{15}AQ (SEQ ID NO 53)

wherein individually of each other:

X_1=A or Q or is deleted

X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R

X_3=H or K

X_4=A or N

X_5=A, G, S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K

X_6=Q or E

X_7=S or K

X_8=E or D

X_9=Q or V or is deleted

X_{10}=K,R or A or is deleted

X_{11}=A,E or N or is deleted

X_{12}=I or L

X_{13}=K or R

X_{14}=L or Y

X_{15}=D, F,Y,W,K or R

19. The separation matrix of claim 18, wherein individually of each other:

X_1 = A or is deleted, X_2 = E, X_3 = H, X_4 = N, X_6 = Q, X_7 = S, X_8 = D, X_9 = V or is deleted, X_{10} = K or is deleted, X_{11} = A or is deleted, X_{12} = I, X_{13} = K, X_{14} = L.

20. The separation matrix of any preceding claim, wherein said multimers comprise tetramers, pentamers, hexamers or heptamers of alkali-stabilized Protein A domains.

21. The separation matrix of any preceding claim, wherein said multimers comprise hexamers of alkali-stabilized Protein A domains.

22. The separation matrix of any preceding claim, wherein the polypeptides are linked by linkers comprising up to 25 amino acids, such as 3-25 or 3-20 amino acids.

23. The separation matrix of any preceding claim, wherein at least two polypeptides are linked by linkers comprising or consisting essentially of a sequence having at least 90% identity with an amino acid sequence selected from the group consisting of APKVDAKFDKE, APKVDNKFNKE, APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APK, APKYEDGVDAKFDKE and YEDG.

24. A method of isolating an immunoglobulin, comprising the steps of:
a) contacting a liquid sample comprising an immunoglobulin with a separation matrix according to any preceding claim,
b) washing said separation matrix with a washing liquid,
c) eluting the immunoglobulin from the separation matrix with an elution liquid, and
d) cleaning the separation matrix with a cleaning liquid.

25. The method of claim 24, wherein the cleaning liquid comprises 0.1 – 1.0 M NaOH or KOH, such as 0.4 – 1.0 M NaOH or KOH.

26. The method of claim 24 or 25, wherein steps a) – d) are repeated at least 10 times, such as at least 50 times or 50 – 200 times.

27. A separation matrix comprising at least 15, such as 15-21 or 15-18, mg/ml Fc-binding ligands covalently coupled to a porous support, wherein said ligands comprise multimers of alkali-stabilized Protein A domains.

28. The separation matrix of claim 27, wherein said alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 80% such as at least 90%, 95% or 98% identity to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.

29. The separation matrix of claim 27, wherein said alkali-stabilized Protein A domains comprise an Fc-binding polypeptide having an amino acid sequence as defined by, or having at least 80% or at least 90, 95% or 98% identity to SEQ ID NO 53.

$X_1Q\ X_2AFYEILX_3LP\ NLTEEQRX_4X_5F\ IX_6X_7LKDX_8PSX_9\ SX_{10}X_{11}X_{12}LAEAKX_{13}$
$X_{14}NX_{15}AQ$ (SEQ ID NO 53)

wherein individually of each other:

X_1=A or Q or is deleted

X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R

X_3=H or K

X_4=A or N

X_5=A, G, S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K

X_6=Q or E

X_7=S or K

X_8=E or D

X_9=Q or V or is deleted

X_{10}=K,R or A or is deleted

X_{11}=A,E or N or is deleted

X_{12}=I or L

X_{13}=K or R

X_{14}=L or Y

X_{15}=D, F,Y,W,K or R

Alignment of Fc-binding domains

```
E          --- -------AQQ  NAFYQVLNMP  NLNADQRNGF  IQSLKDDPSQ  SANVLGEAQK  LNDSQAPK  51  (SEQ ID NO: 1)
D          ADA QQNKFNKDQQ  SAFYEILNMP  NLNEEQRNGF  IQSLKDDPSQ  STNVLGEAKK  LNESQAPK  61  (SEQ ID NO: 2)
A          --A DNN-FNKEQQ  NAFYEILNMP  NLNEEQRNGF  IQSLKDDPSQ  SANLLAEAKK  LNESQAPK  58  (SEQ ID NO: 3)
B          --- ADNKFNKEQQ  NAFYEILHLP  NLNEEQRNGF  IQSLKDDPSQ  SANLLAEAKK  LNDAQAPK  58  (SEQ ID NO: 4)
C          --- ADNKFNKEQQ  NAFYEILHLP  NLTEEQRNGF  IQSLKDDPSV  SKEILAEAKK  LNDAQAPK  58  (SEQ ID NO: 5)
Z          --- VDNKFNKEQQ  NAFYEILHLP  NLNEEQRNAF  IQSLKDDPSQ  SANLLAEAKK  LNDAQAPK  58  (SEQ ID NO: 6)
Zvar       --- VDAKFDKEQQ  NAFYEILHLP  NLTEEQRNAF  IQSLKDDPSQ  SANLLAEAKK  LNDAQAPK  58  (SEQ ID NO: 7)

           --- --------QQ  NAFYEILHLP  NLTEEQRNAF  IQSLKDDPSQ  SANLLAEAKK  LNDAQ---  47  (SEQ ID NO: 51)
           --- --------QQ  NAFYEILHLP  NLTEEQRNGF  IQSLKDDPSV  SKEILAEAKK  LNDAQ---  47  (SEQ ID NO: 52)

Pos             1        10          20          30          40          50        58
```


Fig. 1

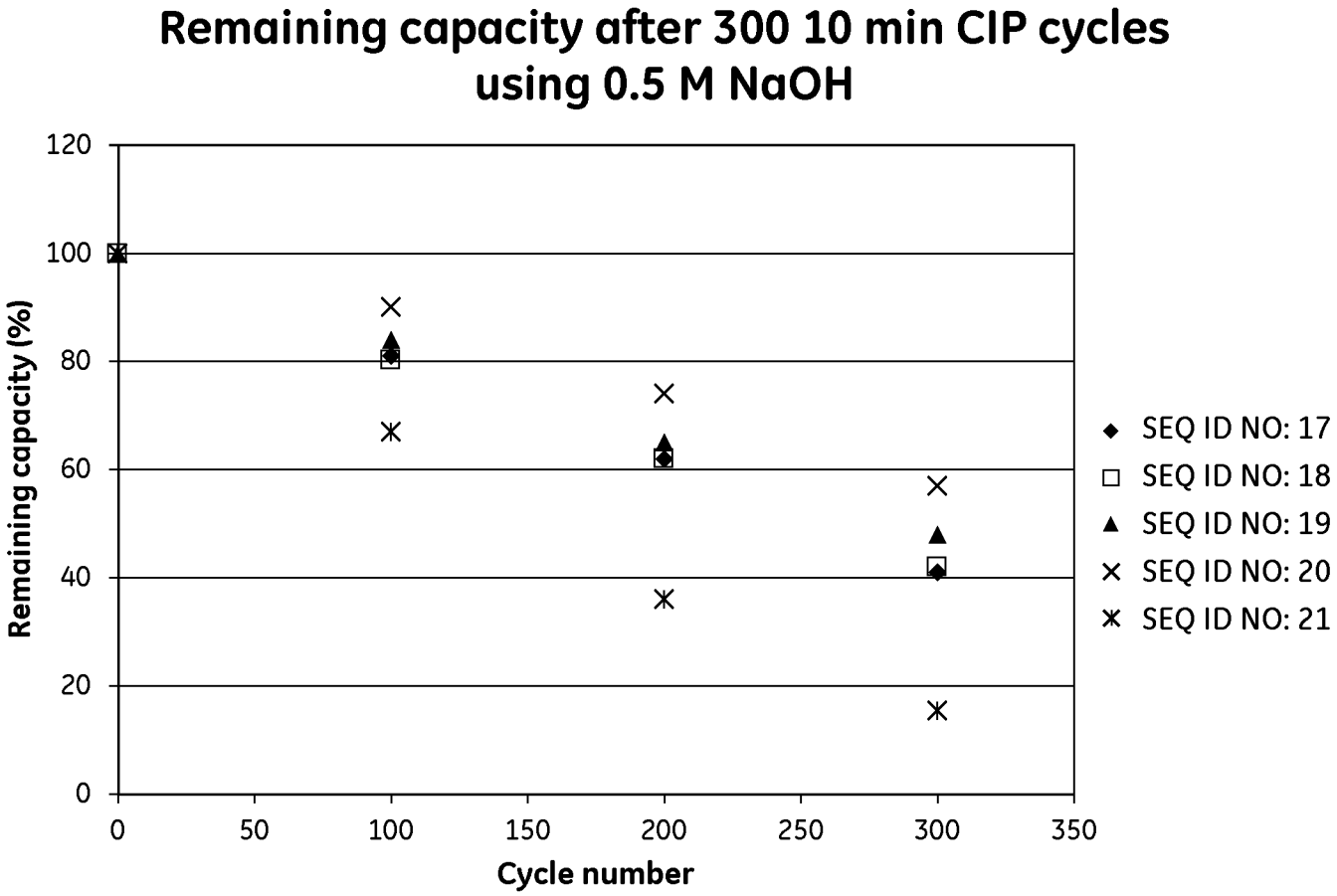
Remaining capacity after 300 10 min CIP cycles using 0.5 M NaOH
Remaining capacity (%)
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
Cycle number
SEQ ID NO: 17
SEQ ID NO: 18
SEQ ID NO: 19
SEQ ID NO: 20
SEQ ID NO: 21

Fig. 2.

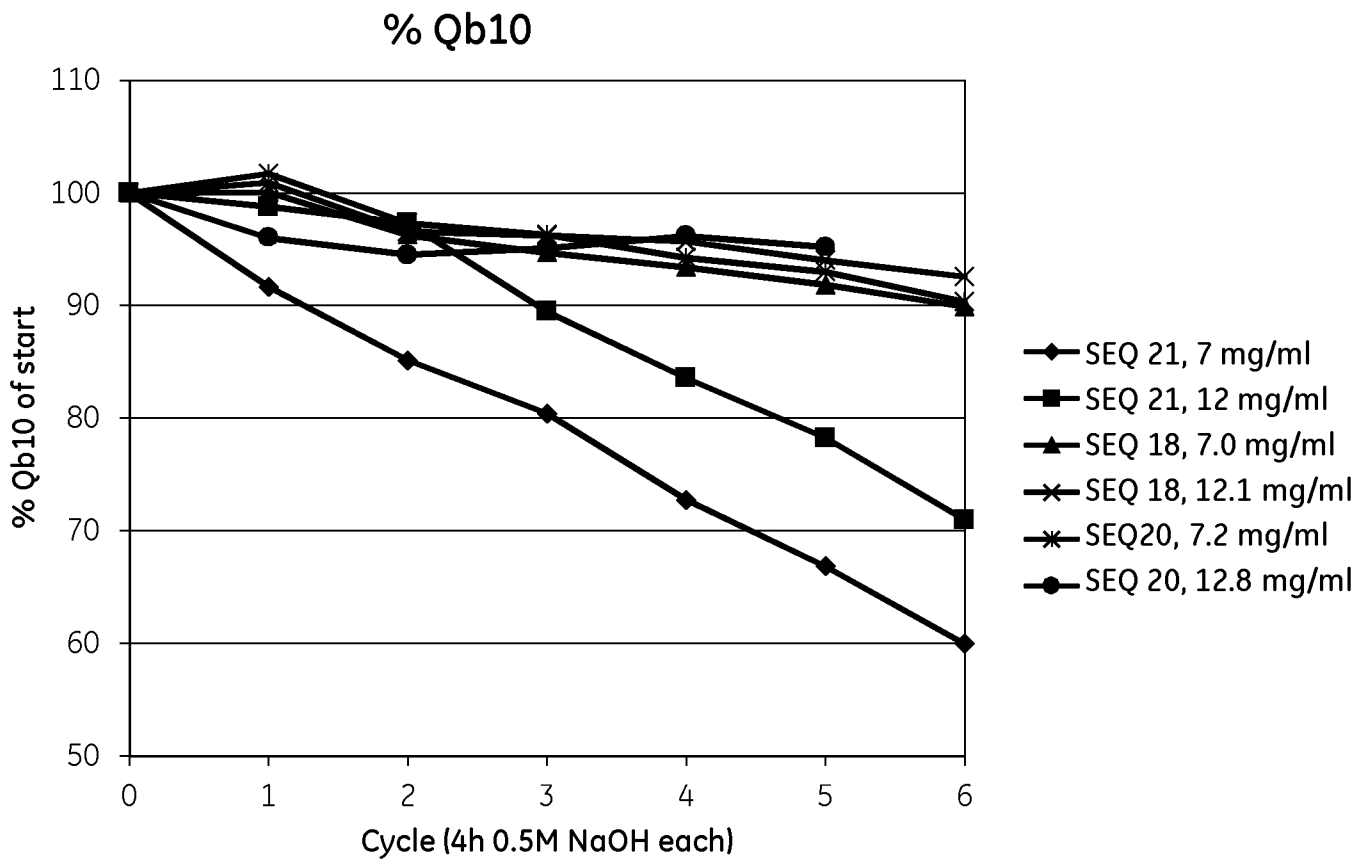
% Qb10
110
100
90
80
70
60
50
% Qb10 of start
0
1
2
3
4
5
6
Cycle (4h 0.5M NaOH each)
SEQ 21, 7 mg/ml
SEQ 21, 12 mg/ml
SEQ 18, 7.0 mg/ml
SEQ 18, 12.1 mg/ml
SEQ20, 7.2 mg/ml
SEQ 20, 12.8 mg/ml

Fig. 3.

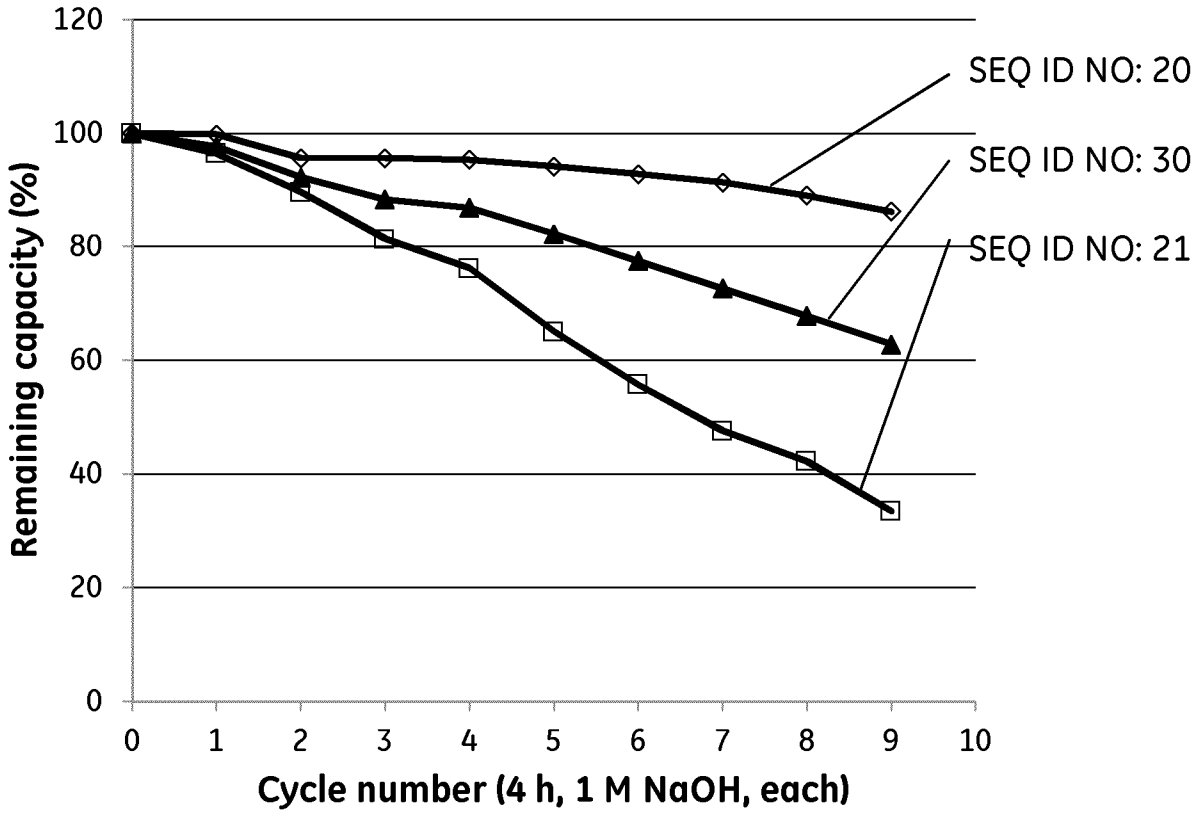
120
100
80
60
40
20
0
Remaining capacity (%)
0
1
2
3
4
5
6
7
8
9
10
Cycle number (4 h, 1 M NaOH, each)
SEQ ID NO: 20
SEQ ID NO: 30
SEQ ID NO: 21

Fig. 4.

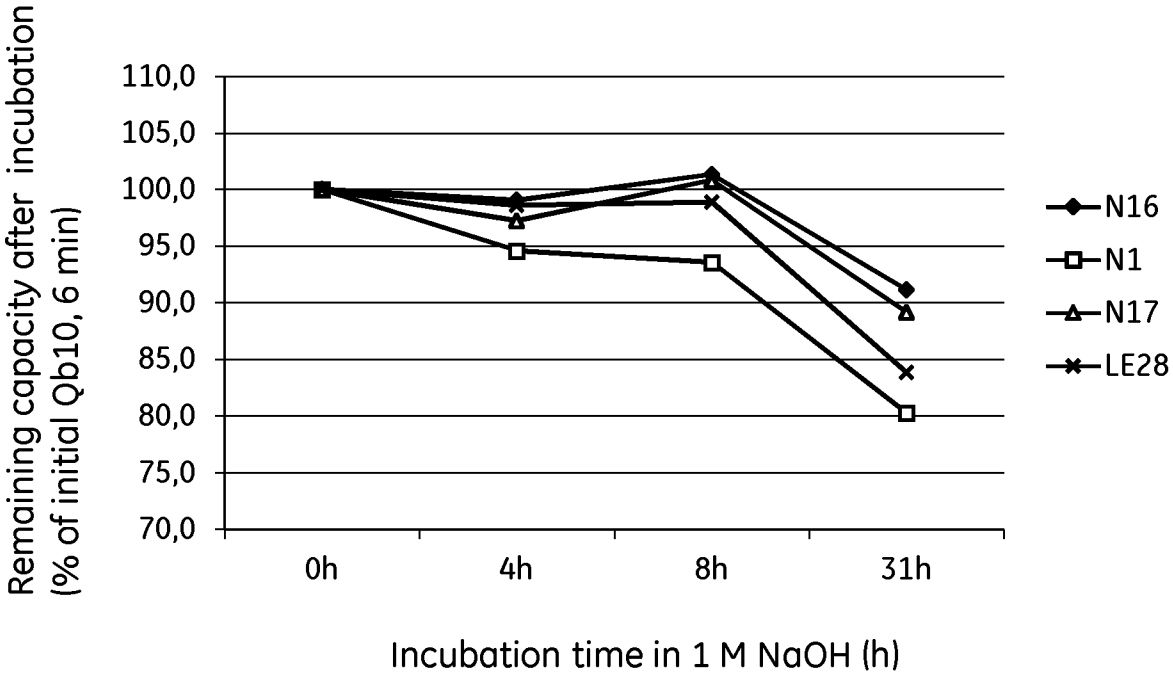
Remaining capacity after incubation
(% of initial Qb10, 6 min)
110,0
105,0
100,0
95,0
90,0
85,0
80,0
75,0
70,0
0h
4h
8h
31h
Incubation time in 1 M NaOH (h)
N16
N1
N17
LE28

Fig. 5.

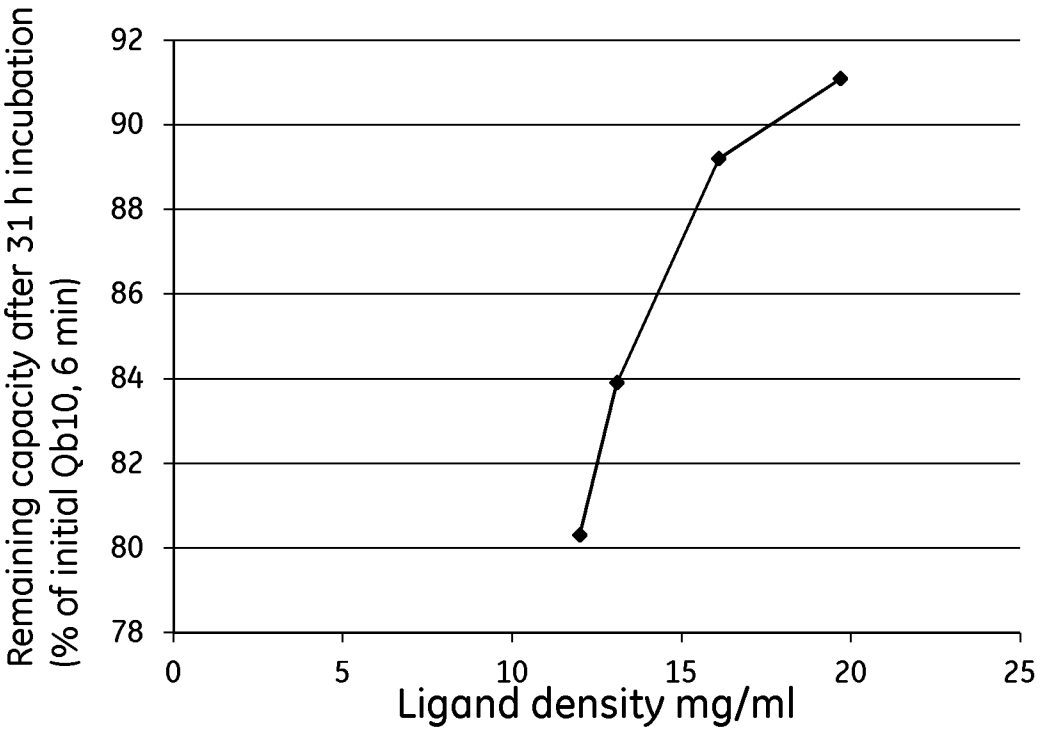
Remaining capacity after 31 h incubation
(% of initial Qb10, 6 min)
92
90
88
86
84
82
80
78
0
5
10
15
20
25
Ligand density mg/ml

Fig. 6.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/061164

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/31
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/005859 A1 (GE HEALTHCARE BIO SCIENCES AB [SE]) 15 January 2015 (2015-01-15) abstract page 3 page 5, line 6 page 5, line 32 - page 6, line 2 page 7, line 29 - page 9, line 18 page 10, line 26 - page 11, line 31 page 12, line 32 page 13, lines 20, 21 page 14, line 19 - line 20 examples 1, 2 claims 27-33 ----- -/--	1-29

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

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 August 2017	05/09/2017

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

2

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/083425 A1 (UNIV WESTERN ONTARIO [CA]; LI SHUN-CHENG [CA]; LI XING [CA]; VOSS COUR) 28 June 2012 (2012-06-28) abstract paragraphs [0001], [0006], [0007], [0011], [0030], [0031], [0034] - [0036], [0047] - [0049], [0055] examples 1, 2 -----	1-29
X,P	WO 2016/079033 A1 (GE HEALTHCARE BIOPROCESS R&D AB [SE]) 26 May 2016 (2016-05-26) abstract page 1, line 5 - line 8 page 3, line 26 - page 4, line 11 page 6, line 8 - page 15, line 16 page 16, lines 23, 28 page 18, line 10 - line 13 page 19, lines 6, 7 page 20, line 17 - line 20 examples 1-5 page 17, line 16 - line 17 -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/061164

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015005859 A1	15-01-2015	CN 105377880 A	02-03-2016
		CN 105377881 A	02-03-2016
		EP 3019519 A1	18-05-2016
		EP 3019520 A1	18-05-2016
		JP 2016523959 A	12-08-2016
		JP 2016525100 A	22-08-2016
		US 2016159855 A1	09-06-2016
		US 2016159857 A1	09-06-2016
		US 2016207966 A1	21-07-2016
		WO 2015005859 A1	15-01-2015
		WO 2015005862 A1	15-01-2015
WO 2012083425 A1	28-06-2012	CN 103403020 A	20-11-2013
		EP 2655404 A1	30-10-2013
		JP 5997176 B2	28-09-2016
		JP 2014508118 A	03-04-2014
		US 2014031522 A1	30-01-2014
		WO 2012083425 A1	28-06-2012
WO 2016079033 A1	26-05-2016	AU 2015348640 A1	27-04-2017
		AU 2015348641 A1	27-04-2017
		CN 107001432 A	01-08-2017
		CN 107001448 A	01-08-2017
		DE 112015005181 T5	24-08-2017
		EP 3221338 A1	27-09-2017
		EP 3221347 A1	27-09-2017
		KR 20170078693 A	07-07-2017
		KR 20170085502 A	24-07-2017
		SG 11201702796W A	30-05-2017
		SG 11201703053P A	30-05-2017
		WO 2016079033 A1	26-05-2016
		WO 2016079034 A1	26-05-2016