

AAV9 BINDING POLYPEPTIDES

TECHNICAL FIELD

The present invention relates to an antigen-binding polypeptide capable of binding an adeno-associated virus (AAV) and a separation matrix comprising said antigen-binding polypeptide, and to use thereof in the context of affinity capture or affinity separation.

BACKGROUND

Advanced therapy medicinal products (ATMPs) have emerged as an area of great promise for the treatment or prophylaxis of severe diseases. It includes for instance gene and cell therapies aimed at restoring or replacing defective genes, as well as other therapies based on recombinant nucleic acids.

To deliver a recombinant nucleic acid to a recipient cell, a vector is typically needed. Depending on the kind of therapy, the target cell type and the nucleic acid to be delivered, different vectors may be used.

Adeno-associated viruses (AAV) are small, non-enveloped viruses of the *Parvoviridae* family, and are by nature non-pathogenic and of low immunogenicity. AAV can be engineered to deliver DNA to target cells, and recombinant adeno-associated virus (rAAV) vectors have emerged as one of the most versatile and successful gene therapy delivery vehicles.

Adeno-associated viruses have a linear single-stranded DNA (ssDNA) genome contained in a capsid formed of capsid protein subunits. To date, AAV capsid serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 (AAVrh10), 11 and 12 have been identified. AAV of different serotypes have different tropism, that is, specificity for infecting a cell or tissue type. Most of the earlier AAV gene transfer studies used AAV serotype 2 (AAV2), however, later research and development has revealed that other serotypes may be more efficient as therapeutic vectors. In recent years, AAV9 has attracted much interest. AAV9 is considered very promising for many gene therapies targeting the lung, liver, heart, skeletal muscle, or the CNS.

AAV vectors, engineered to contain a gene of interest, can be produced in mammalian cells, such as human embryonic kidney cells. The AAV vectors are harvested from the cell culture, clarified and purified to remove cell debris, host cell DNA and protein, and any other impurities. Purity, efficacy and safety of clinical grade vectors is crucial, as impurities remaining from the production stage may cause adverse effects, reduce transduction efficiency, and even elicit a systemic immune

response or inflammatory response. The vectors also need to retain viral activity throughout the purification process.

Normally, several filtration steps and several chromatography steps are used to separate AAV particles from cell cultures. Ultracentrifugation can be efficient, but is not scalable. Chromatographic techniques are used for adenovirus purification, but the results have often been insufficient in
5 respect of purity, yield and capacity.

Affinity chromatography is a specific mode of chromatography in which an affinity ligand interacts with a target entity via biological affinity in a "lock-key" fashion. General examples of useful interactions in affinity chromatography are e.g., enzyme-substrate interaction, biotin-avidin
10 interaction, antibody-antigen interaction, etc. The affinity ligand is capable of specifically and reversibly binding the desired target entity and is immobilized on a chromatographic support material. When contacted with a sample containing the target entity under binding conditions, the ligand selectively binds the target entity, while other species of the sample can be washed away. The captured target entity can then be eluted, typically by changing buffer conditions, such as
15 conductivity or salt concentration, and/or pH. After subsequent cleaning and regeneration of the chromatography material, the same chromatography material can be used for a new cycle of affinity purification. Affinity chromatography can potentially yield a target of very high purity.

For an efficient affinity capture process, a high target binding capacity of the ligand is desired. The binding capacity is generally expected to deteriorate over the course of an affinity
20 purification process involving many purification cycles, due to exposure to alkaline conditions during the aforementioned cleaning of the chromatography material.

There is a need in the art for improved means of purification of viral vectors based on AAV, in particular AAV9.

SUMMARY OF THE INVENTION

25 It is an object of the invention to overcome or at least partly alleviate drawbacks of the prior art.

Accordingly, it is an object of the invention to provide an AAV affinity capture material that allows more efficient purification of AAV vectors.

In a first aspect, these and other objects are achieved by an antigen-binding polypeptide
30 capable of binding adeno-associated virus serotype 9 (AAV9), the polypeptide comprising a single-domain antibody (sdAb) variant having complementarity determining regions CDR1, CDR2 and CDR3 as defined herein.

CDR1 comprises an amino acid sequence selected from the group of sequences defined by SEQ ID NO: 1:

$X_1X_2X_3SX_5X_6TMX_9$ (SEQ ID NO: 1)

wherein, independently, X_1 is R, L or S, preferably R or L; X_2 is T or R, preferably T; X_3 is L or F, preferably L; X_5 is D, N or E, preferably D; X_6 is Y, N or F, preferably Y or F; and X_9 is G or A, preferably G. For example, X_6 may be F and X_9 may be G or A.

CDR2 comprises a sequence selected from the group of sequences defined by defined by SEQ ID NO: 2:

$X_1X_2SWSGX_7X_8TX_{10}$ (SEQ ID NO: 2)

wherein, independently, X_1 is A, S, L, V or I, preferably A; X_2 is I or V, preferably I; X_7 is A or S, preferably A; X_8 is Y or F, preferably Y; and X_{10} is K, F or Y. In embodiments, X_2 is I, X_7 is A, X_8 is Y and/or X_{10} is K.

CDR3 comprises a sequence selected from the group of sequences defined by defined by SEQ ID NO: 3:

$X_1X_2TX_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}$ (SEQ ID NO: 3)

wherein, independently, X_1 is G or A, preferably G; X_2 is P or S, preferably P; X_4 is G or P, preferably G; X_5 is L, T or P, preferably L or P; X_6 is L or I, preferably L; X_7 is S, T or A, preferably S or A; X_8 is K, R, N or Q; X_9 is K, H or R, preferably K or R; X_{10} is A, S or T, preferably A or T; X_{11} is T, S, P or A, preferably T; X_{12} is P, A, T or S, preferably P; X_{13} is A, P, R or G, preferably A or P; X_{14} is D, N, E or Q, preferably D or E; X_{15} is Y or F, preferably Y; X_{16} is D, N, E or Q, preferably D; and X_{17} is Y, F or R, preferably Y or F.

In another aspect, the present disclosure provides a multimeric polypeptide comprising at least two moieties, each moiety comprising a single-domain antibody variant as defined herein.

In another aspect, there is provided a fusion protein comprising at least one antigen-binding polypeptide or a multimeric polypeptide as described herein and a further polypeptide moiety.

In other aspects, the present disclosure provides an isolated nucleic acid encoding the antigen-binding polypeptide, the multimeric polypeptide or the fusion protein, as well as an expression vector comprising said nucleic acid, and a recombinant host cell comprising the expression vector.

In another aspect a method of producing the antigen-binding polypeptide, the multimeric polypeptide the fusion protein is provided, the method comprising i) providing recombinant host cells; ii) culturing the host cell under conditions enabling expression of the antigen-binding polypeptide, the multimeric polypeptide or the fusion protein; and iii) isolating the antigen-binding polypeptide, the multimeric polypeptide or the fusion protein. Step iii) may comprise purifying the

antigen-binding polypeptide, the multimeric polypeptide or the fusion protein by chromatography, such as immobilized metal affinity chromatography (IMAC) or affinity chromatography.

In a further aspect the disclosure provides the use of the antigen-binding polypeptide, the multimer or the fusion protein as an affinity ligand for binding of adeno-associated virus serotype 9 (AAV9). Said use may be e.g. for in vitro detection of AAV9 in a sample, or for purification of AAV9 from a sample, optionally in the context of a method of providing a therapeutic composition comprising AAV9 as a viral vector.

In other aspects the disclosure provides a separation matrix and a method of separation.

The separation matrix comprises the antigen-binding polypeptide, the multimeric polypeptide or the fusion protein coupled to a support material, thus providing an affinity separation matrix employing the antigen-binding polypeptide as an affinity ligand. The support material may be selected from the group consisting of a particle, a bead, a fiber, a fibrous membrane, a filter, a sheet, a porous monolith, a chip, a plate, and a well. The support material may be a chromatography matrix. For example, the chromatography matrix may comprise a polysaccharide material, such as beads comprising agarose or a derivative thereof. Alternatively, the chromatography matrix may comprise a polymeric fibrous matrix or membrane, such as a nonwoven fibrous matrix.

The method of separation comprises the steps of

- (a) providing a separation matrix of the present disclosure,
 - (b) contacting the separation matrix with a liquid sample comprising said AAV9 viral particles vector under conditions allowing the antigen-binding polypeptide to bind to the AAV9 viral particles,
 - (c) optionally washing the chromatography material,
 - (d) eluting the bound AAV9 viral particles from the separation matrix, and optionally
 - (e) cleaning the separation matrix material with a cleaning liquid.
- Step (e) typically comprises cleaning the separation matrix with an alkaline cleaning liquid, wherein the cleaning liquid preferably comprises from 0.05 to 0.5 M of NaOH.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other aspects of the present invention will now be described in more detail, with reference to the appended drawings showing embodiment(s) of the invention, in which:

Fig. 1 is a schematic illustration of the arrangement of framework regions and complementarity determining regions of a single-domain antibody.

Fig. 2a-c are schematic illustrations of various structures of antigen-binding polypeptides according to the present disclosure.

Fig. 3a-c are schematic illustrations of a fusion protein comprising an antigen-binding polypeptide as disclosed herein fused to another polypeptide moiety.

Fig. 4a-b are sensorgrams showing the AAV9 binding capacity of two exemplary polypeptides.

Fig. 5 is a graph showing the alkaline stability of exemplary antigen-binding polypeptides.

5 Fig. 6 is a graph showing the alkaline stability of exemplary antigen-binding polypeptides and a commercially available affinity ligand.

Figs. 7a-h are sensorgrams showing binding to AAV9 and other AAV serotypes for exemplary antigen-binding polypeptides and a commercially available affinity ligand.

10 Fig. 8a is a graph plotting the AAV9 binding response of an antigen binding polypeptide having a fusion partner (solid line) or no fusion partner (dashed line), respectively, against the number of binding cycles on Biacore, each cycle involving 0.5 M NaOH exposure. The binding response was normalized against the response of the second cycle (i.e., first cycle excluded). Fig. 8b shows the binding response from the same experiment, normalized to the response of the first cycles.

15 Fig. 9a is a picture of a PAGE gel demonstrating stronger protein expression of multimeric fusion proteins according to embodiments of the invention and an antigen binding polypeptide alone (lacking fusion partner). Fig. 9b is a graph plotting the AAV9 binding response of the multimeric fusion proteins and the multimer lacking a fusion partner polypeptide, respectively, against the number of binding cycles on Biacore, each cycle involving 0.3 M NaOH exposure. The binding
20 response was normalized against the response of the second cycle (i.e., first cycle excluded).

Fig. 10 shows AAV9 binding responses obtained for increasing number of cycles involving NaOH exposure for two fusion proteins according to embodiments of the invention.

Fig. 11a is a chromatogram showing the elution peak from a chromatography column using an AAV9 binding polypeptide according to embodiments of the present invention as affinity ligand
25 immobilized on the chromatography matrix. Fig. 11b is a closer view of the elution peak.

Fig. 12 is a photograph of an SDS-PAGE gel showing the protein content of the eluate fractions from the chromatography run of Example 8.

Fig. 13 is a graph plotting the dynamic binding capacity as evaluated in Example 8.

30 As illustrated in the figures, some features may be exaggerated for illustrative purposes and, thus, are provided to illustrate the general structures of embodiments of the present invention. Like reference numerals refer to like elements throughout.

DEFINITIONS

As used herein, the terms "peptide" and "polypeptide" are used synonymously herein to refer to compounds formed of sequences of amino acids, without restriction as to size. "Protein" may be used to refer to the larger compounds of this class. Amino acid sequences are written left to right in the direction from the amino (N) to the carboxy (C) terminus. In accordance with standard nomenclature, amino acid residue sequences are denominated by either a three letter or a single letter code as indicated as follows: Alanine (Ala, A), Arginine (Arg, R), Asparagine (Asn, N), Aspartic Acid (Asp, D), Cysteine (Cys, C), Glutamine (Gln, Q), Glutamic Acid (Glu, E), Glycine (Gly, G), Histidine (His, H), Isoleucine (Ile, I), Leucine (Leu, L), Lysine (Lys, K), Methionine (Met, M), Phenylalanine (Phe, F), Proline (Pro, P), Serine (Ser, S), Threonine (Thr, T), Tryptophan (Trp, W), Tyrosine (Tyr, Y), and Valine (Val, V). "Peptides" include any oligopeptide, polypeptide, gene product, expression product, or protein. A peptide is comprised of consecutive amino acids and encompasses naturally occurring or synthetic molecules. In addition, as used herein, the term "peptide" refers to amino acids joined to each other by peptide bonds or modified peptide bonds, e.g., peptide isosteres, etc. and may contain modified amino acids other than the 20 gene-encoded amino acids. The peptides can be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art.

A "single-chain polypeptide" herein refers to a polypeptide that is formed of a single amino acid sequence, in which the amino acid residues are connected via peptide bonds. A single-chain polypeptide may, upon folding, additionally form other internal bonds such as disulfide bonds.

The expression "antigen-binding polypeptide" generally refers to a polypeptide that possesses at least one binding region, and typically at least two, such as three binding regions, such that the polypeptide has a binding affinity for a molecule (referred to as antigen or "target"). An antigen-binding polypeptide may have any protein structure, as long as there is a binding affinity for the antigen. However, some classes of antigen-binding polypeptides that have frequently been exploited and used as a basis for engineered antigen-binding polypeptides often have a scaffold or framework structure that is generally conserved between antigen-binding polypeptides of the same class, but which bind to different antigens. The binding regions of an antigen-binding molecule can typically be engineered or evolved to bind to a particular antigen, while the scaffold or framework structure remains essentially the same. Non-limiting examples of antigen-binding polypeptides include natural or engineered (i) antibodies, such as monoclonal antibodies, (ii) antibody fragments that contain the light chain and/or heavy chain variable region(s) with complementarity determining regions (CDRs), such as Fab (fragment antigen-binding), Fv (variable fragment), scFab (single chain antigen-binding fragment) and scFv (single chain variable fragment), (iii) single-domain antibodies,

and iv) polypeptides having a scaffold derived from bacteria, such as immunoglobulin-binding bacterial proteins (such as *Finagoldia magna* protein L, and staphylococcal protein A and protein G) or domains thereof, including the wild types as well as variants in which one or more of the antigen binding regions have been engineered.

5 The expression "single-domain polypeptide" or "single-domain amino acid sequence" refers to a polypeptide which forms an individual protein domain, and which does not comprise any other individual protein domain. Examples of single-domain polypeptides include antibody fragments such as heavy chain variable domain and light chain variable domain, single-domain antibodies (sdAb), albumin binding domain (ABD) and polypeptides derived from a domain of SpA, such as the B, C or Z
10 domain of SpA. Single-domain antibodies are particularly contemplated. A single-domain polypeptide typically consists of a single-chain polypeptide. A single-domain polypeptide is in itself monomeric, but it may form part of a multimer, as described elsewhere herein. A single-domain polypeptide may be joined to another polypeptide, for example via a peptide bond or via a disulfide bond. Preferably, the antigen-binding polypeptide of the present disclosure may consist of a single polypeptide chain.

15 The term "single-domain antibody" refers to a variable domain which is or is derived from a heavy chain variable domain (VH) of an antibody devoid of light chains. Hence, a single domain antibody lacks antibody light chains entirely, including the light chain variable domain (VL). A single-domain antibody may also lack such structural features that are needed for a functional VH/VL interaction, and which are present in, e.g., the conventional VH of IgG1. Hence, a single domain
20 antibody does not form part of a dimeric structure with an antibody light chain variable domain. Single-domain antibodies include antibodies naturally devoid of antibody light chains, such as sdAb derived from IgG2 or IgG3 of camelids (*Camelidae*), e.g. dromedary, camel, llama and alpaca, which are also referred to as VHH (heavy chain variable domain of a heavy chain antibody. Single domain antibodies also include antibodies derived from cartilaginous fishes, such as sharks, usually
25 termed VNARs (variable new antigen receptors). Alternatively, single domain antibodies may be synthetic variants based on an amino acid sequence of an antibody heavy chain variable domain from a species that naturally does not produce sdAb (e.g. cow, rat, mouse or rabbit) and which are modified e.g. with respect to amino acids in the natural VH/VL interaction of such antibodies, so as to mimic an sdAb. Such modification may involve substitution of amino acids of the VH/VL interface
30 region to increase the hydrophilicity or solubility.

Generally, terms such as "polypeptide", "protein", "antibody", "single-domain polypeptide", "single-domain antibody", etc. also include synthetic variants that are recombinantly produced and whose amino acid sequence may have been modified in relation to the amino acid sequence of a naturally occurring counterpart. Where such modified variants are particularly intended, the term

“variant” may also be used. The term “wild type” or “wt” refers to the typical naturally occurring form. Single-domain antibody variants of the present invention are synthetic, non-naturally occurring amino acid sequences.

Complementary determining regions (CDRs) are hypervariable regions of an antibody or part thereof which participate in binding to a target epitope. The CDRs are typically defined by their separate amino acid sequences, although in an antibody, the CDRs together form a three-dimensional binding site for the target antigen. A variable domain of an antibody heavy chain (VH) has three CDRs, referred to as CDR1, CDR2 and CDR3, as positioned from the N terminus of the polypeptide chain. The sequence length of CDRs may vary, and CDR1, CDR2 and especially CDR3 of a VH may be of different lengths.

The portions of an antibody VH not forming the CDRs are referred to as framework regions. The framework regions are typically four and referred to as framework region (FW) 1 to 4, as counted from the N terminus. The framework regions are responsible for the general secondary and tertiary structure of the domain and thus for positioning and orientation of the CDR regions. The framework regions may be referred to as a scaffold structure. Although part of the variable domain, the framework regions are less variable than the CDRs. Generally, amino acid sequence variations that do not significantly alter the secondary or tertiary structure of the framework may however be tolerated. Some parts or amino acid positions of the framework regions may be conserved. The general stability of the framework regions allows for a high degree of variation in the CDRs.

As used herein, the term “ligand” is a molecule that has a known or unknown affinity for a given entity. The term “ligand” may herein be used interchangeably with the terms “affinity ligand”, “selective binding molecule”, “selective binding partner”, “capturing molecule” and “capturing agent”. “Affinity ligand” refers to a moiety or molecule that binds reversibly and selectively or preferentially with high affinity to a target entity through a specific interaction with a binding site of the component. In the context of the present invention, an affinity ligand is a polypeptide, and may also be referred to as an “affinity protein”. An affinity ligand may be immobilized to a solid support such as a resin.

The term “target entity” herein refers to an entity that forms a specific binding partner to the ligand, and may also be referred to as an “analyte”. The analytes or target entities of interest according to the present disclosure are adeno-associated virus vectors, in particular AAV9 vectors.

As used herein, “affinity” in the context of polypeptides denotes the strength of interaction between two molecules, wherein at least one molecule is a polypeptide. The interaction is selective, i.e. discriminates between the affinity binding partner and other molecules present. Binding affinity is also expressed as the dissociation equilibrium constant (KD).

As used herein, the term “binding capacity” refers to the capability of a ligand to bind the target molecules when the ligand is immobilized on a surface. The binding capacity of a ligand can differ depending on the type of surface on which it is immobilized. For the purpose of the present invention, the binding capacity can be measured by surface plasmon resonance (SPR) technology using e.g. a Biacore instrument as described in the present Examples. The binding capacity for a certain concentration of analyte is influenced by the density of immobilized ligands on the surface. In the context of the present invention the binding capacity is preferably determined at a ligand density of at least about 3000 response units (RU), such as in the range of from 3000-4000 RU.

The term “dynamic binding capacity” (abbreviated “DBC”) of a chromatography column in the context of protein purification is the binding capacity under operating conditions, i.e., in a packed affinity chromatography column during sample application. The DBC of a chromatography resin is expressed as the amount of analyte that binds to the resin under given flow conditions before a significant breakthrough of unbound analyte occurs. DBC is determined by loading a sample containing a known concentration of the analyte and monitoring the flow-through. The analyte will bind to the resin to a certain break point before unbound analyte will flow through the column. The DBC can be determined on the breakthrough curve at a loss of, for example, 10% analyte. This is referred to as the QB10% value. A sample is applied to a chromatography resin column during a specific residence time and the dynamic binding capacity for each resin is calculated at 10% of the breakthrough capacity i.e., the amount of analyte sample that is loaded onto the column until the concentration of analyte in the column effluent is 10% of the analyte sample concentration in the feed. If the dynamic binding capacity for each resin is calculated at 80% of the breakthrough capacity, this is referred to as the QB80% value.

The term “alkaline stability” as used herein, refers to a property of the antigen-binding polypeptides which relates to its ability to withstand alkali exposure without deleterious effect on structure and/or function of the polypeptide. For polypeptides capable of binding a target entity, the alkaline stability is determined based on the affinity for the target entity after exposure to NaOH. The alkaline stability of an antigen-binding polypeptide can be evaluated immobilizing the antigen-binding polypeptide on a support and measuring the target binding capacity before and after one or more cycles of exposure to NaOH, using methods described in the Examples below. In particular the evaluation is made on an SPR chip onto which the antigen-binding polypeptide is immobilized by covalent coupling with thiol, N-hydroxysuccinimide (NHS), streptavidin-biotin binding, or another coupling that is alkali resistant in itself, at a ligand density corresponding to at least 1000 RU, and preferably at least 3000 RU, and for a predetermined analyte concentration. Often, the first alkaline cleaning cycle may have a unique, large impact on binding capacity, e.g. due to removal of non-

covalently bound antigen-binding polypeptide from the support surface, and therefore the binding capacity is sometimes represented as normalized to the binding capacity measured after the first cycle of alkali exposure.

In the present context, an antigen-binding polypeptide is considered to be alkali stable if, after 10 cleaning cycles, preferably after 15 cleaning cycles, the immobilized antigen-binding polypeptide retains at least 50 % of the target binding capacity compared to its binding capacity after the first cleaning cycle (i.e. disregarding the binding capacity of the first cycle, prior to the first alkali exposure event), where a cleaning cycle involves 600 s of exposure to at least 0.3 M NaOH, such as at least 0.5 M NaOH.

The term "solid support" herein refers to a non-aqueous matrix of a solid phase material. Suitable solid phase materials include, but are not limited to, glass, silica (e.g., silica gel), polysaccharides (e.g., a polysaccharide matrix) such as agarose and cellulose, organic polymers such as polyacrylamide, methacrylate, and polystyrene-divinylbenzene copolymers. The solid phase can be of porous or nonporous character and can be compressible or incompressible. For example, the solid phase can be a polymeric matrix or an agarose particle or bead. Preferred solid support materials will be physically and chemically resilient to the conditions employed in a purification process including pumping and cross-flow filtration, and temperatures, pH, and other aspects of the liquids employed.

The term "surface" herein means all external surfaces of a solid structure. In the case of a porous support, such as a bead used e.g. in the field of chromatography, the term "surface" includes outer surfaces as well as pore surfaces. In the case of a fibrous membrane, the term "surface" encompasses the external surface of individual fibers.

The term "separation matrix" is used herein to denote a material comprising a solid support to which one or more ligand(s) have been coupled. The ligand(s) are capable of binding target entities herein also called analytes, which are to be separated from their surroundings, e.g. a liquid sample, and/or which are to be separated from other components present in the liquid sample. A separation matrix may be employed in various contexts, including analytical assays and purification of target for analytical or preparative purposes. The type of support may be selected depending on the intended use.

A separation matrix may further comprise a compound which couples the ligand(s) to the support. The terms "spacer", "extender", and "surface extender" may be used to describe such a compound, as further described herein. The term "resin" is sometimes used for a separation matrix in this field. The terms "chromatography material" and "chromatography matrix" are used herein to

denote a type of separation matrix. An affinity separation matrix is a separation matrix where the ligand is an affinity ligand.

The term "spacer" refers to a peptide or other chemical linkage or element that extends the structure of an entity. A spacer attached to a polypeptide, in particular an amino acid spacer, may be provided at the N terminus or the C terminus of the polypeptide. A spacer may connect a polypeptide to a solid support. Suitable spacers for coupling a polypeptide to a support may generally be any spacer used in the art to connect peptides, proteins or other organic molecules to a support. A spacer may also serve to connect two polypeptides or polypeptide domains. A spacer connecting two polypeptide moieties may also be referred to as a linker.

The terms "multimer" and "multimeric protein" as used herein refer to a protein comprising at least two repeating units of the single-domain antibody disclosed herein. Hence, the single domain antibody may be regarded as a monomeric polypeptide unit, which may be combined into a multimeric protein, such as a dimer, a trimer, a tetramer, a pentamer etc. The single-domain antibody units of a multimer may be identical, or may differ from one another with regard to their amino acid sequence. In addition to said single-domain antibody units, a multimer may contain one or more further polypeptide unit(s), such as a fusion partner polypeptide or stabilizing polypeptide unit as described herein, which is not a single-domain antibody. Such a further polypeptide unit may be present in a single copy or at multiple copies in the multimer.

Within a multimer, individual polypeptide units may be linked via peptide bonds, typically via a linker peptide. Thus, a multimeric protein may be formed of a single polypeptide chain.

The term "fusion protein" refers to a protein formed of two or more than two separate proteins (fusion partners) produced by recombinant protein expression as a single polypeptide containing both fusion partners. The fusion partners may be joined sequentially one after the other. The genes encoding the respective proteins are joined at the genetic level. The fusion partners typically do not naturally occur as fused to each other. A fusion protein may contain additional amino acids sequences, such as linker in between the fusion partners.

As used herein a "linker" refers to a peptide or other chemical linkage that functions to link otherwise independent functional domains. A linker may be located between two polypeptide units, monomers or domains, such as between a ligand (e.g., a sdAb) and another polypeptide component containing an otherwise independent functional or structural domain, or between two ligands. Suitable linkers for coupling two or more linked units may generally be any linker used in the art to link peptides, proteins or other organic molecules. A linker peptide may be a stretch of amino acids preferably ranging from 1 to 20 amino acids, such as 2-15, such as 2-8, 2-4 or 4-12 amino acids.

The term "vector" is herein used to denote a virus particle, normally a recombinant virus particle, which is intended for use to achieve gene transfer to modify specific cell type or tissue. A virus particle can for example be engineered to provide a vector expressing therapeutic genes. Several virus types are currently being investigated for use to deliver genetic material (e.g., genes) to
5 cells to provide either transient or permanent transgene expression. These include adenoviruses, retroviruses (γ -retroviruses and lentiviruses), poxviruses, adeno-associated viruses (AAV), baculoviruses, and herpes simplex viruses. The term "capsid" means the shell of a virus particle.

Sequence identity may be assessed by any convenient method. However, for determining the degree of sequence identity between sequences, computer programmes that make pairwise or
10 multiple alignments of sequences are useful, for instance EMBOSS Needle or EMBOSS stretcher may be used for pairwise sequence alignments while Clustal or MUSCLE may be used for multiple sequence alignments, though any other appropriate programme may be used. Whether the alignment is pairwise or multiple, it must be performed globally (i.e. across the entirety of the reference sequence) rather than locally. Sequence alignments and % identity calculations may be
15 determined using for instance standard Clustal Omega parameters: matrix Gonnet, gap opening penalty 6, gap extension penalty 1. The term "% identity", as used throughout the disclosure, may for example be calculated as follows. The query sequence is aligned to the target sequence using the CLUSTAL W algorithm (Thompson et al, Nucleic Acids Research, 22: 4673-4680 (1994)). A comparison is made over the window corresponding to the shortest of the aligned sequences. The shortest of the
20 aligned sequences may in some instances be the target sequence. In other instances, the query sequence may constitute the shortest of the aligned sequences. The amino acid residues at each position are compared and the percentage of positions in the query sequence that have identical correspondences in the target sequence is reported as % identity.

DETAILED DESCRIPTION

25 The present inventors have found that a novel antigen-binding polypeptide, capable of binding adeno-associated virus (AAV) serotype 9 (AAV9), is useful for affinity capture of AAV9-based vectors. The antigen-binding polypeptide is typically a single-chain polypeptide. The antigen-binding polypeptide is based on a single-domain antibody (sdAb), but may optionally comprise additional amino acids, e.g. positioned N-terminally or C-terminally thereof. The antigen-binding polypeptide of
30 the present invention may be derived from a camelid single-domain antibody, also referred to as a VHH (variable domain of a heavy chain of a heavy chain antibody).

The single-domain antibody variant lacks the light chains of conventional antibodies such as IgG1, and also lacks the heavy chain constant domains. Thus, a single-domain antibody of the

antigen-binding polypeptide disclosed herein comprises only a heavy chain variable domain, including three complementarity determining regions (CDRs). Thus, the antigen-binding polypeptide contains no antibody light chain variable region or light chain CDRs.

In general, single domain antibodies are easier to express in prokaryotic and eukaryotic cells
5 as compared to full-size antibodies such as IgG, as well as Fab fragments or single-chain fragments including both light and heavy chain portions. Despite having only three CDRs, as opposed to the six CDRs of larger antibodies and antibody fragments, the target binding properties of can be satisfactory.

The portions of the sdAb not forming the CDRs are referred to as framework regions. The
10 framework regions of an sdAb, which are typically four and referred herein to as framework region (FW) 1 to 4, are responsible for the general secondary and tertiary structure of the sdAb. The framework regions form two beta sheets forming a sandwich and connected via a cysteine bridge. The CDRs are typically presented as three surface loops, one CDR per loop.

Another advantage of the sdAb of the present disclosure is that the CDR sequences can be
15 longer than the CDRs of conventional antibodies. The surface loops may thus spatially extend further from the body of the sdAb as have a better ability to bind into pockets of the target epitope. Interestingly, these the relatively long CDR sequences do not render the sdAb unstable, as may be the case with long CDRs in antigen-binding scaffolds derived from the tenth type III domain of human fibronectin (Adnektin™).

20 Generally, the sdAb variant may comprise framework regions and complementary determining regions in the following order from N-terminus to C-terminus: [FWR1]-[CDR1]-[FWR2]-[CDR2]-[FWR3]-[CDR3]-[FWR4]. Fig. 1 schematically illustrates the sequence of framework regions and complementarity determining regions (CDRs) of an sdAb, from the direction of the N terminus to the C terminus of the amino acid sequence. Further, Table 1 outlines the FWRs and CDRs as defined
25 herein in relation to the Kabat amino acid numbering system (Kabat et al., 1991, *J. Immunol.* 147(5), 1709-1719). For an sdAb variant of the present disclosure, Framework region 1 (FWR1) is formed of the amino acids in positions 1 to 26. Next, amino acids in Kabat positions 27 to 35d form CDR1, Kabat positions 35a-d being optional. Framework region 2 (FWR2) is formed of the amino acids in Kabat positions 36 to 49. CDR2 is formed of the amino acids in Kabat positions 50 to 58 (52a being
30 optional). Framework region 3 (FWR3) is formed of the amino acids in Kabat positions 59 to 94. CDR3 is formed of the amino acids in Kabat positions 95 to 102 (100a-j being optional). Lastly, framework region 4 (FWR4) is formed of amino acids in Kabat positions 103 to 113. It may be noted that the definition of CDRs and framework regions within the present disclosure does not fully correspond to the definition of CDRs and framework regions of Kabat et al; however, the same amino acid

numbering system is used. The framework regions may collectively be referred to as the “framework”.

Table 1

Amino acid positions (Kabat)	1-26	27-35d	36-49	50-58	59-94	95-102	103-113
Region	FWR1	CDR1	FWR2	CDR2	FWR3	CDR3	FWR4
Example sequence	SEQ ID NO:5		SEQ ID NO:6		SEQ ID NO:7		SEQ ID NO:8

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The sdAb of the present invention may have a framework that has a certain degree of sequence identity to a native camelid VHH framework. For example, each of the framework regions of the present sdAb may have a sequence identity in the range of 70-100 % to the respective framework regions of SEQ ID NO: 4, which are represented by SEQ ID NO:5 (FWR1), SEQ ID NO:6 (FWR2), SEQ ID NO:7 (FWR3) and SEQ ID NO:8 (FWR4), respectively. Advantageously, at least one of the framework regions of the present sdAb may comprise an amino acid substitution relative to SEQ ID NO: 4 (i.e. one or more of SEQ ID NO: 5-8, such as preferably one or more of SEQ ID NO: 5-7, such as at least SEQ ID NO: 7), wherein the sdAb exhibits improved alkaline stability (e.g. assessed by exposure to at least 0.1 M NaOH) compared to SEQ ID NO: 4. Stabilized sdAb frameworks are described in more detail elsewhere herein.

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Optionally, the framework regions of the sdAbs of the present invention may have a certain degree of sequence identity with the synthetic framework regions of SEQ ID NO: 91 or SEQ ID NO: 92. The individual framework regions of SEQ ID NO: 92 are represented by SEQ ID NO:151 (FWR1), SEQ ID NO:154 (FWR2), SEQ ID NO:157 (FWR3) and SEQ ID NO:8 (FWR4), and the individual framework regions of SEQ ID NO: 91 are represented by SEQ ID NO:149 (FWR1), SEQ ID NO:152 (FWR2), SEQ ID NO:155 (FWR3) and SEQ ID NO:8 (FWR4). Alternatively, the framework regions of the sdAbs of the present invention may have a certain degree of sequence identity with the individual framework regions of SEQ ID NO: 150 (FWR1), SEQ ID NO:153 (FWR2), SEQ ID NO:156 (FWR3) and SEQ ID NO:8 (FWR4). Alternatively, the framework regions of the sdAbs of the present invention may have a certain degree of sequence identity with the synthetic framework regions of any one of SEQ ID NO: 177-181. The individual framework regions for FW1 are defined by SEQ ID NO: 183-186, for FW2 by SEQ ID NO: 187-189, and FW3 by SEQ ID NO: 190-192.

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For example, each of the framework regions 1, 2 and 3 of the present sdAb may have at least 70% sequence identity with the reference framework region. FWR4 may have less than 70 % sequence identity with the reference framework region. Optionally also FWR4 may have at least 70% sequence identity with the reference framework region.

30

Parts of the framework regions of an sdAb are relatively conserved and it may be preferable to make only few or no modifications in these parts in relation to e.g. SEQ ID NO: 4, 91 or 92. These parts are herein defined by Kabat positions 1-5, 7-14, 25-26, 41-42, 46, 48-49, 59, 61-75, 77-78, 80-82a, 82c-94 and 103-113. Advantageously, each of FWR1-3, and optionally also FWR4, may have at least 80 %, such as at least 85 %, such as at least 90 %, sequence identity with the corresponding framework region of SEQ ID NO: 4, 91 or 92 over the conserved amino acid positions (i.e., omitting the other positions from the comparison). As an example, FWR1 contains 26 aa, whereof 21 amino acids represent the conserved positions as defined above. Substituting 2 of these amino acids may thus result in 90.5 % identity, whereas substituting 3 amino acids may result in 85.7 % identity. The sdAb variant may have an amino acid sequence wherein Kabat positions 1-5, 7-13, 25-26, 36-39, 41-42, 46, 48-49, 59, 61-75, 77-78, 80-82a and 82c-94 may include up to 15, such as up to 14, such as up to 13, such as 12, 11, or 10 amino acid residues that are substituted in relation to at least one of SEQ ID NOs: 4, 91, 92, 131, 171 and 172. For example, in FWR1, positions 1-5, 7-13 and 25-26 (21 residues) may together have up to 4, such as up to 3, amino acid residues that differ from the corresponding position of SEQ ID NO: 4, 91, 92 or 171. In FWR2, Kabat positions 36-39, 41-42, 46 and 48-49 (14 residues) may together have 1 or 2 amino acid residues that differ from the corresponding position of SEQ ID NO: 4, 91 or 92. In FWR3, Kabat positions 59, 61-75, 77-78, 80-82a, 82c-94 (34 residues) may have up to 6 amino acid residues that differ from the corresponding position of SEQ ID NO: 4, 91 or 92.

The amino acid in Kabat position 1 may advantageously be selected from Q, V, D and E, and may in particular be Q or E. For example, any sdAb amino acid sequence disclosed herein in which Kabat position 1 is not E (e.g., is Q), may instead have E in Kabat position 1.

The framework regions of sdAbs typically have a secondary structure comprising β -strands and/or β -sheets. The framework regions typically form a β -sandwich structure often comprising a cysteine bridge. The CDRs are typically presented as three surface loops at one end of the structure, one CDR per loop.

An advantage of using sdAbs as affinity binders is that the CDR sequences can be more diverse with regard to their length than the CDRs of conventional antibodies. The surface loops formed of the CDRs can thus spatially extend further from the main body of the sdAb and thus have a better ability to bind into pockets of a target epitope, or the surface loops may be shorter, forming flatter surfaces which may facilitate binding to other epitopes.

The present inventors have identified novel amino acid sequences which have excellent AAV9 binding properties when used as CDRs in an sdAb framework. When the polypeptide comprising the sdAb is immobilized on a support, these binding properties provide surprisingly good

capacity for binding AAV9. Also, compared to other sdAbs binding AAVs, the present sdAbs can differentiate different AAV serotypes such that they bind selectively to AAV9, while other AAV sdAbs bind several serotypes. The binding of the present sdAbs is thus more specific compared to the prior art.

5 Furthermore, CDR sequences disclosed herein have been found to contribute to the overall alkaline stability of the sdAb. It is a well-known fact in the art that sdAbs (also known as “nanobodies”) have poor alkaline stability. It is also a common feature of previous AAV sdAb ligands, as expressed e.g. in patent application WO2020242988A2, which relates to affinity agents comprising ligands that bind AAV, where it in [0005] is stated “Currently, there are only 3 affinity resins available
10 for the purification of AAV, POROS™, CaptureSelect™ AAV9, POROS™ CaptureSelect™ AAVX and AVB Sepharose. These resins have 2 major shortcomings, they cannot be cleaned with sodium hydroxide and can only be reused for a few cycles. This increases resin consumption and hence leads to high resin costs for the purification.” Thus, it was surprisingly found that the present sdAb not only bind AAV selectively and with high capacity (good affinity), but that they also are alkaline stable. It was
15 identified that certain CDR sequences greatly increased alkali stability especially when combined with a modified sdAb framework, where the resulting sdAbs became even more stable. In addition, when fusing the sdAbs with a further polypeptide moiety derived from a protein domain of staphylococcal protein A (SpA), the resulting fusion protein showed even better properties.

Previous sdAbs capable of binding AAV9 exist in the art, such as disclosed in CN116751284,
20 but they are not specific for AAV9, but most importantly, they are not alkali stable as the present sdAbs. CN116396381 discloses sdAbs alleged as capable of binding a plurality of serotypes, including AAV9, and having acid base tolerance. However, when tested, the sdAbs has very low affinity towards AAV9. Five variants (sequences denoted SEQ ID NO: 7, 14, 21, 28 and 35 as described in claim 2 of CN116396381) have been expressed and analysed in same way as the AAV9 selective
25 single domain antibodies of the present invention, and out of these five one (SEQ ID NO: 14) did not express and the other four showed very low interaction (~100 Ru instead of above 1000Ru as for the polypeptides of the present disclosure), which binding affinity is similar “sticky/unspecific” interaction, i.e. no affinity. Thus, the affinity is so low binding to AAV9 can be disputed. In addition, regarding the alleged “acid base tolerance”, such as in example 7 of CN116396381, CBS buffer is used
30 (the buffer is not further specified, only pH is noted), presumably a sodium carbonate bicarbonate buffer at different pH. It is well known that the type of buffer, ionic strength and molarity greatly affect the stability of a polypeptide. The buffer used in CN116396381 is not corresponding or similar to the NaOH buffer used for cleaning in place (CIP), and to establish alkaline stability of a protein. It may be noted that for achieving the alkaline stability as defined in the present disclosure, the

polypeptide should be able to undergo CIP with NaOH or KOH of at least 0.05 M, such as 0.05-1 M. Thus, no alkaline wash is performed, and no alkaline stability was shown.

The CDRs of an sdAb can have a variable number of amino acids. CDR1 may include a sequence of between 9 and 13 amino acids. In the exemplary polypeptides shown herein, CDR1 is formed of 9 amino acids. However, it is contemplated that the amino acid sequences for CDR1 described herein may be supplemented with up to 4 additional amino acids, especially at the C-terminal. In the Kabat numbering system, the CDR1 may be formed of amino acid positions 27, 28, 29, 30, 31, 32, 33, 34 and 35 (9 amino acids) or the positions from 27-35 and up to 35d (thus up to 13 amino acids).

CDR2 may include between 10 and 13 amino acids. In the exemplary polypeptides shown herein, CDR2 is formed of 10 amino acids. In the Kabat numbering system, the CDR2 may constitute positions 50, 51, 52, 52a, 53, 54, 55, 56, 57 and 58.

CDR3 may include up to 18 amino acids. In the exemplary polypeptides shown herein, CDR3 is formed of 17 amino acids. In the Kabat numbering system, the CDR3 may be formed of amino acid positions 95, 96, 97, 98, 99, 100, 100a, 100b, 100c, 100d, 100e, 100f, 100g, 100h, 100i, 101 and 102.

In more detail, in the present sdAb variant CDR1 may be constituted by amino acid positions according to the Kabat numbering system as outlined in the Table 2a below.

Table 2a. CDR1 positions

Position (Kabat)	27	28	29	30	31	32	33	34	35	35a	35b	35c	35d
Position in SEQ ID NO: 1	1	2	3	4	5	6	7	8	9	-	-	-	-
Useful amino acids according to the present disclosure	R, L, S	T, R	L, F	S	D, N, E	Y, N, F	T	M	A, G				
Preferred amino acid(s)	R	T	L	S	D	Y, F	T	M	G	-	-	-	-

Thus, in CDR1/SEQ ID NO:1, amino acids may be selected, for each position independently, from the useful and preferred amino acids indicated in Table 2a. The present inventors found that the amino acid sequence of CDR1 had a substantial influence on the binding properties and stability of the antigen-binding polypeptide, with the potential to improve one or both of these characteristics. Amino acid substitutions in CDR1 were in general found to impact binding capacity and stability to a greater extent than amino acid substitutions in CDR3, which appears to tolerate an even higher degree of variation.

In particular, it was found that the amino acids in Kabat positions 30, 33 and 34 (corresponding to positions 4, 7 and 8 of SEQ ID NO: 1) were advantageously fixed as serine (S), threonine (T) and methionine (M), respectively. Meanwhile, other positions of CDR1 (e.g. Kabat position 32) were found to allow some variability, with acceptable or further improved binding

capacity. In Kabat positions 27-29, the amino acid sequence RTL is considered particularly advantageous, however RTF is also contemplated.

Table 2b shows exemplary CDR1 sequences of the sdAb disclosed herein.

Table 2b. Exemplary CDR1 sequences.

CDR1 variant	SEQ ID NO:
RTFSDYTMG	9
LTLSDYTMG	10
RTLSNYTMG	11
RTLSEYTMG	12
RTLSDFTMG	13
RTLSDNTMG	14
RTLSDYTMA	15
RTLSDYTMG	16
RTLSDFTMA	17

5

In some embodiments, CDR1 may be selected from the group consisting of SEQ ID NOs:9-17, such as SEQ ID NOs:13, 14, 16 and 17.

In the present sdAb variant, CDR2 may be formed of amino acid positions according to the Kabat numbering system as outlined in the Table 3a below.

10

Table 3a. CDR2 positions

Position (Kabat)	50	51	52	52a	52b	52c	52d	53	54	55	56	57	58
Position in SEQ ID NO: 2	1	2	3	4	-	-	-	5	6	7	8	9	10
Useful amino acid(s) according to the present disclosure	A, S, L, I, V	I, V	S, T	W				S	G	A, S	Y, F	T	H, K, Y, F
Preferred amino acid(s)	A	I	S	W	-	-	-	S	G	A	Y	T	K, Y, F

It was found that certain amino acid substitutions in CDR2 had an influence on the binding properties and stability of the antigen-binding polypeptide, with the potential to improve one or both of these characteristics. On the other hand, amino acid substitutions in some positions were found to not to improve binding capacity and/or stability, or were even found to impair binding capacity and/or stability, and therefore positions 4, 5, 6, and 9 of CDR2 were selected as indicated in Table 3a.

Thus, in CDR2/SEQ ID NO:2, amino acids may be selected, for each position independently, from the useful and preferred amino acids indicated in Table 2a. For example, optionally, in SEQ ID NO: 2 X₂ may be I; X₇ may be A; X₈ may be Y; and/or X₁₀ may be K. In some embodiments, in

20

CDR2/SEQ ID NO: 2 X₂ is I and X₈ is Y. In some embodiments, in CDR2/SEQ ID NO:2, either i) X₁ is A, or ii) X₁ is S, L or I, and X₇ is A.

Table 3b shows exemplary CDR2 sequences of the sdAb variants disclosed herein.

5 Table 3b. Exemplary CDR2 amino acid sequences

CDR2 variant	SEQ ID NO
AVSWGGSFTY	21
AISWGGSFTY	22
SISWGSAYTY	23
LISWGSAYTY	24
IISWGSAYTY	25
AISWGSAYTY	26
AVSWGGSAYTY	27
AVSWGGSAYTK	28
AISWGGSAYTK	29
AISWGGSAYTH	30
AISWGGSAYTF	31

Thus, in some embodiments, CDR2 may be selected from the group consisting of SEQ ID NOs: 21-31, such as selected from the group consisting of SEQ ID NOs: 26, 28, 29, and 31.

10 In the sdAb variants of the present disclosure, CDR3 may be constituted by amino acid positions according to the Kabat numbering system as outlined in the Table 4a below. The present sdAbs tolerate a relatively high degree of variability in the CDR3 amino acid sequence without deterioration of binding capacity or alkaline stability. Nevertheless, it was found that for some positions, certain amino acids are particularly advantageous for the purpose of enhancing binding capacity or alkaline stability. Hence, for CDR3, amino acids are selected (useful and preferred, 15 respectively) as indicated in Table 4a.

Table 4a. CDR3 positions

Position (Kabat)	95	96	97	98	99	100	100a	100b	100c	100d	100e	100f	100g	100h	100i	100j	101	102
Position in SEQ ID NO: 3	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	-	16	17
Useful amino acids according to the present disclosure	G, A	S, P	T	G, P	P, T, L	I, L	A, T, S	K, R; N, Q	K, H, R	T, A, S	T, S, A, P	P, A, T, S	A, P, R, G	D, N, E, Q	Y, F		D, N, E, Q	Y, F, R
Preferred amino acid(s)	G	P	T	G	L, P	L	S, A	K, N, Q	K, R	A, T	T	P	A, P	D, E	Y	-	D	Y, F

Thus, in CDR3/SEQ ID NO:3, amino acids may be selected, for each position independently, from the useful and preferred amino acids indicated in Table 4a. For example, optionally, in SEQ ID NO: 3 X₂ is P; X₇ is S; X₈ is K, N or R, such as K or R; X₉ is K; X₁₀ is A or T; X₁₁ is T; X₁₃ is A; and/or X₁₇ is Y.

Optionally, in SEQ ID NO: 3 at least one of X₈ and X₉ may be K.

5 Optionally, in SEQ ID NO: 3, at least one of X₅ and X₆ may be L, and preferably both X₅ and X₆ are L.

Optionally, in SEQ ID NO: 3 X₁₃ is A and, preferably, X₁₀ is A.

Optionally, in SEQ ID NO: 3 X₁₄ is D and X₁₆ is D.

Table 4b shows exemplary CDR3 sequences of the sdAb disclosed herein.

10

Table 4b. Exemplary CDR3 sequences

CDR3 Sequence	SEQ ID NO	CDR3 Sequence	SEQ ID NO
GPTGPLSRSSPPDYDY	34	GPTGLLSRKSTPADYDY	54
GSTGPLTRRSTPPDYDY	35	GPTGLLSKKATPADYDY	55
APTGLLSKKATPADYDY	36	GPTGLLSKKTTPADYDY	56
GSTGLLSKKATPADYDY	37	GPTGLLSKKAAPADYDY	57
GPTGLLSKKSTPADYDY	38	GPTGLLSKKATSADYDY	58
GPTGPLSKKATPADYDY	39	GPTGLLSKKATTADYDY	59
GPTGTLSKKATPADYDY	40	GPTGLLSKKATPRDYDY	60
GPTGLISKKATPADYDY	41	GPTGLLSKKATPANYDY	61
GPTGLLAKKATPADYDY	42	GPTGLLSKKATPAEYDY	62
GPTGLLSNKATPADYDY	43	GPTGLLSKKATPAQYDY	63
GPTGLLSQKATPADYDY	44	GPTGLLSKKATPADFDY	64
GPTGLLSRKATPADYDY	45	GPTGLLSKKATPADYNY	65
GPTGLLSKRATPADYDY	46	GPTGLLSKKATPADYEY	66
GPTGLLSKHATPADYDY	47	GPTGLLSKKATPADYQY	67
GPTGLLSNRATPADYDY	48	GPTGLLSKKATPADYDF	68
GPTGLLSQRATPADYDY	49	GPTGLLSKKATPADYDR	69
GPTGLLSRRATPADYDY	50	GPTGLLSRKTTPADYQY	70
GPTGLLSNHATPADYDY	51	GPTGLLSKKATPPDYDY	71
GPTGLLSQHATPADYDY	52	GPTGPLSKKTTPADYDY	72
GPTGLLSRHATPADYDY	53	GPTGLLSQKTTPADYDY	173

Thus, in some embodiments, CDR3 may be selected from the group consisting of SEQ ID NOs: 34-72 and 173, such as selected from SEQ ID NOs: 36, 37, 39, 40, 42-46, 48-50, 55, 56, 62, 64, 68, 70-72, and 173.

15

Exemplary combinations of CDR sequences are listed in Table 5. The table also refers to exemplary sdAb variant sequences having said combination of CDRs. All of the listed polypeptides exhibited good or excellent binding to AAV9, as demonstrated in the Examples.

Table 5

CDR1 sequence	CDR2 sequence	CDR3 sequence	Exemplary sdAb
RTLSDYTMG (SEQ ID NO:16)	LISWSGAYTY (SEQ ID NO:24)	GPTGLLSKKSTPADYDY (SEQ ID NO:38)	vh29 (SEQ ID NO:78)
	IISWSGAYTY (SEQ ID NO:25)	GPTGLLSKKSTPADYDY (SEQ ID NO:38)	vh30 (SEQ ID NO:79)
	IISWSGAYTY (SEQ ID NO:25)	GPTGLLSRKSTPADYDY (SEQ ID NO:54)	vh33 (SEQ ID NO:82)
	LISWSGAYTY (SEQ ID NO:24)	GPTGLLSKKATPADYDY (SEQ ID NO:55)	vh35 (SEQ ID NO:84)
	IISWSGAYTY (SEQ ID NO:25)	GPTGLLSKKATPADYDY (SEQ ID NO:55)	vh36 (SEQ ID NO:85)
	SISWSGAYTY (SEQ ID NO:23)	GPTGLLSRKATPADYDY (SEQ ID NO:45)	vh37 (SEQ ID NO:86)
	LISWSGAYTY (SEQ ID NO:24)	GPTGLLSRKATPADYDY (SEQ ID NO:45)	vh38 (SEQ ID NO:87)
	IISWSGAYTY (SEQ ID NO:25)	GPTGLLSRKATPADYDY (SEQ ID NO:45)	vh39 (SEQ ID NO:88)
	AISWSGAYTY (SEQ ID NO:26)	GPTGLLSKKSTPADYDY (SEQ ID NO:38)	vh40 (SEQ ID NO:89)
		GPTGLLSKKATPADYDY (SEQ ID NO:55)	vh41 (SEQ ID NO:90)
	AISWSGAYTK (SEQ ID NO:29)	GPTGLLSKKATPADYDY (SEQ ID NO:55)	vh118 (SEQ ID NO:92)
		GPTGPLSKKATPADYDY (SEQ ID NO:39)	vh214 (SEQ ID NO:109)
		GPTGLLSKKTTPADYDY (SEQ ID NO:56)	vh221 (SEQ ID NO:116)
		GPTGLLSKRATPADYDY (SEQ ID NO:46)	vh334 (SEQ ID NO:131)
		GPTGLLSQRATPADYDY (SEQ ID NO: 49)	vh336 (SEQ ID NO:135)
RTFSDYTMG (SEQ ID NO:9)	AVSWGSFTY (SEQ ID NO:21)	GPTGPLSRSSPPDYDY (SEQ ID NO:34)	vh50 (SEQ ID NO:75)
	AVSWSGAYTY (SEQ ID NO:27)	GSTGPLTRRSTPPDYDY (SEQ ID NO:35)	vh58 (SEQ ID NO:76)
RTLSDFTMG (SEQ ID NO:13)	AISWSGAYTK (SEQ ID NO:29)	GPTGLLSKKATPADYDY (SEQ ID NO:55)	vh199 (SEQ ID NO:97)
		GPTGLLSKKTTPADYDY (SEQ ID NO:56)	vh324 (SEQ ID NO:131) vh511-515 (SEQ ID NO: 177-181)
		GPTGLLSQKTTTPADYDY (SEQ ID NO:173)	vh499 (SEQ ID NO:174) vh516 (SEQ ID NO: 182)
RTLSDFTMA (SEQ ID NO:17)	AISWSGAYTY (SEQ ID NO: 26)	GPTGLLSRKTTTPADYQY (SEQ ID NO:70)	vh374 (SEQ ID NO:141) vh359 (SEQ ID NO:140) vh428 (SEQ ID NO:146)

For example, in sdAb variants of the present disclosure may have any combination of CDRs in which CDR1 is selected from SEQ ID NOs: 9-17, such as selected from SEQ ID NO: 13, 14 16 and 17;

CDR2 is selected from SEQ ID NOs: 21-31, such as selected from SEQ ID NO: 26, 28, 29 and 31; and CDR3 is selected from SEQ ID NOs: 34-72, such as selected from SEQ ID NO: 36, 37, 39, 40, 42-46, 48-50, 55, 56, 62, 64, 68, 70-72, and 173.

Further variants that share CDR1 and CDR2 of SEQ ID NO:92 and differ only slightly in CDR3 are represented by SEQ ID NOs: 106, 107, 109-124, 126-130 and 132-139.

Thus, for example, the sdAb variant may comprise a) an amino acid sequence selected from SEQ ID NOs: 75-93, 95-98, 101, 102, 105-108, 109-124, 126-146, 174, such as selected from SEQ ID NOs: SEQ ID NO: 92, 97, 109, 116, 131, 140-146 and 174; or b) an amino acid sequences having at least 90 %, such as at least 95 %, identity to a sequence as defined in a), provided that the CDRs are as defined for SEQ ID NO:1-3. Optionally, an sdAb variant according to option b) which has less than 100 % identity to any one of said sequences may differ with regard to the amino acid residues of the CDRs (still being within the definition of SEQ ID NOs: 1-3), and/or the framework amino acid residues of Kabat positions 6, 14, 19, 23, 24, 40, 43, 44, 45, 47, 60, 76, 79, 82b and/or 89.

The terms "capable of binding AAV9", "AAV9 binding", "capable of interacting with AAV9" and "interaction with AAV9" as used in this disclosure refer to an event or a property of a polypeptide which may be tested for example by ELISA or by surface plasmon resonance (SPR) technology.

AAV9 interaction may be tested in an experiment in which the polypeptide to be tested is immobilized on a sensor chip of the surface plasmon resonance (SPR) instrument, and a liquid sample containing AAV9 (the target or analyte) is passed over the chip. Alternatively, the target (here AAV9) may be immobilized on the surface, and the polypeptide to be tested is contained in a liquid sample that is passed over the surface. The skilled person may then interpret the results obtained by such experiments to establish at least a qualitative measure of the binding affinity of the polypeptide for AAV9.

The AAV9 binding polypeptide may bind AAV9 "selectively", meaning that it may bind to AAV9, while it does not bind to other AAV serotypes, or binds to other AAV serotypes to a much lower degree. The term "selectivity", sometimes referred to as "specificity", of the binding polypeptide for a target refers to a binding polypeptide which will bind to the target with high affinity, but typically not to other antigens. A selective or specific binding polypeptide/single domain antibody will not, or to a low extent, cross-react with other targets than the intended antigen. Thus, by binding "specifically" it is meant that the binding protein binds to its target (i.e. AAV9) in a manner that can be distinguished from binding to non-target molecules, more particularly that the binding polypeptide binds its target (AAV9) with greater binding affinity than with which it binds other molecules. That is, the binding polypeptide does not bind to other, non-target, molecules, or does

not do so to an appreciable or significant degree, or binds with lower affinity to such other molecules than with which it binds AAV9. In the present case, the binding polypeptides bind AAV9, but not other serotypes (or to a very low degree). For example, the interaction with other AAV serotypes may be less than 20 %, such as less than 10 % or less than 5 %, of the AAV9 interaction response as measured on a Biacore instrument as detailed in the examples below. With other serotypes is referred to other distinct serotypes, such as for example AAV2, AAV5, and AAV8. As shown in example 5B below, the present polypeptides selectively bind AAV9, with no or very low affinity for other serotypes. It may be noted that closely related sequence variants, such as AAV-PHP.eB, are not seen as a distinct serotype in this sense, and hence not included in the "other serotypes" not bound.

10 The AAV9 binding polypeptide disclosed herein exhibit an improved AAV9 binding property in relation to known sdAb based affinity ligands to AAV9. In particular, a binding property that may be improved is binding capacity. Thus, the present AAV9 binding polypeptides may have improved AAV9 binding capacity.

15 Further, the inventors found that in addition to having an initial high binding capacity for AAV9, the polypeptides can retain a high AAV9 binding capacity also after alkali treatment. Improved alkaline stability is a great benefit in the context of affinity chromatography, as the chromatography material is conventionally subjected to cleaning using an alkaline agent (such as NaOH or KOH) between the purification cycles. Increased alkaline stability of the affinity ligand means that the chromatography material can be used for a higher number of purification cycles before binding capacity becomes unacceptably low.

20 The alkaline stability is usually determined based on the binding capacity after a certain number of alkali treatment cycles. In this context, the terms "treatment", "exposure" and "cleaning" are used interchangeably. For example, "improved alkaline stability" can mean that the polypeptide can withstand a higher number of alkali cleaning cycles, or the same number of cycles but with harsher alkali conditions, without a deterioration in binding capacity. Alternatively, "improved alkaline stability" can mean that the polypeptide will retain a higher percentage of the initial binding capacity after an equal number of cleaning cycles using the same conditions. The alkaline stability may be particularly relevant for a polypeptide intended to be immobilized on a solid support.

25 The alkali treatment may involve contact or incubation with 0.05-1 M NaOH or 0.1-0.5 M NaOH, such as 0.3-0.5 M NaOH for a time period of 5-15 minutes, such as 10 minutes (600 seconds) or about 10 minutes.

30 Preferably the AAV9 polypeptides of the invention retain a high AAV9 binding capacity after repeated cleaning cycles involving 600 s exposure to 0.3 or 0.5 M NaOH. For example, compared to the AAV9 binding capacity after the first cycle of NaOH exposure (i.e., excluding the binding capacity

recorded for the first cycle from the comparison), the AAV9 binding polypeptide as disclosed herein may retain at least 50 % of the AAV9 binding capability after at least 8 repeated binding cycles followed by cleaning with 0.5 M NaOH.

In embodiments, the AAV9 binding polypeptide may retain at least 50 % binding capability after at least 10 cycles, such as after at least 12 cycles of alkali exposure. More preferably, the AAV9 binding polypeptide retains at least 50 % binding capability after at least 20, 22 or 24 cycles of exposure to 0.5 M NaOH. A higher number of cycles with at least 50 % binding capability retained indicates an improved alkaline stability. Furthermore, a higher retained binding capacity after the same number of cycles of exposure to cleaning liquid (e.g. at least 0.1 M NaOH such as 0.3 M NaOH or 0.5 M NaOH) indicates an improved alkaline stability. In embodiments, after 10 cycles of contact with cleaning liquid the polypeptide may retain at least 50 %, such as at least 60 %, at least 70 %, at least 80 % or at least 90 %, of its initial target entity binding capacity, and/or after 20 cycles of contact with cleaning liquid the polypeptide retains at least 50 %, at least 60 %, such as at least 70 % or at least 80 % of its initial target entity binding capacity. In embodiments, after 10 cycles of contact with alkaline cleaning liquid, the polypeptide may retain at least 60 %, at least 70 %, at least 80 %, at least 90 %, such as at least 95 %, of the target entity binding capacity of the 2nd cycle, and/or after 20 cycles of contact with alkaline cleaning liquid, the polypeptide may retain at least 60 %, at least 70 %, at least 80 %, at least 90 %, such as at least 95 %, of the target entity binding capacity of the 2nd cycle. A cycle of exposure to alkaline cleaning liquid may be a chromatography cycle as described herein, or a cycle of an alkaline stability assessment as described elsewhere herein.

It will be understood that each binding cycle is followed by a cleaning step. A cleaning step may be for example approximately 5-15 minutes incubation in contact with 0.1-0.5 M NaOH, such as for example approximately 5 minutes or 10 minutes or 15 minutes. For example, the time of incubation in contact with 0.5 M NaOH may be 5 ± 0.5 minutes, 6 ± 0.5 minutes, 7 ± 0.5 minutes, or 8 ± 0.5 minutes, 9 ± 0.5 minutes, 10 ± 0.5 minutes, 11 ± 0.5 minutes, 13 ± 0.5 minutes, 14 ± 0.5 minutes, or 15 ± 0.5 minutes. Said incubation may be for example at 22 ± 2 °C.

The polypeptides of the present invention may have CDRs which contribute to improved alkaline stability.

Furthermore, the polypeptides disclosed herein may have a stabilized framework, meaning that at least one of the framework regions is modified relative to the corresponding framework region of SEQ ID NO: 4 in such a way that it contributes to an increased alkaline stability of the polypeptide. Thus, as a result, the polypeptide as a whole may have an improved alkaline stability.

For example, it has been found that in some amino acid positions of the framework regions of an sdAb, certain mutations of the amino acid residue in that position are not only tolerated

without compromising functionality, but may even lead to improved performance, especially an improved alkaline stability, of the resulting sdAb variant. In particular the following amino acid positions according to the Kabat numbering system have been identified as variable and potentially useful to improve alkaline stability of an sdAb variant: position 6, 14, 19, 23, 24, 40, 43, 44, 45, 47, 60, 76, 79, 82b and 89. Thus, the antigen-binding polypeptide may comprise a single-domain antibody variant comprising an amino acid sequence in which at least one of the amino acid positions 6, 14, 19, 23, 24, 40, 43, 44, 45, 47, 60, 76, 79, 82b and 89 is modified in relation to the corresponding amino acid of SEQ ID NO: 4 and where the sdAb framework has increased alkaline stability compared to SEQ ID NO:4.

Particularly advantageous modifications are outlined in Table 6. Thus, the antigen-binding polypeptide may optionally comprise a single-domain antibody variant comprising an amino acid sequence in which at least one, such as two, such as three, four five, six, seven, eight, nine or ten, of the residues in Kabat positions 19, 23, 24, 40, 43, 44, 45, 76, 79 and/or 89 are selected from the stabilizing amino acids indicated in Table 6. For example, at least eight, such as at least nine, of these positions may have amino acids selected accordingly.

Table 6. Exemplary stabilizing framework modifications

Kabat position	Stabilizing amino acids	Preferred stabilizing amino acid(s)
19	R, S, K, T	R, T
23	T, S, V	T
24	I, V, S	
40	R, I, K, T	R, I
43	R, K, Q, E, G	R, K
44	E, Q, A, D, G	
45	R, I, L	R
76	N, Q	
79	Y, W, F	Y, W, F
89	V, I	V, I

Fig. 2a-c schematically illustrate exemplary structures of antigen-binding polypeptides of the present disclosure. In Fig 2a, an antigen-binding polypeptide 200 comprises an N-terminal amino acid sequence 201, an sdAb variant 202 and a C-terminal amino acid sequence 203. Notably, both the N-terminal amino acid sequence 201 and the C-terminal sequence 203 are optional.

The antigen-binding polypeptide may optionally be provided in the form of a multimeric antigen-binding polypeptide (“multimer”) schematically illustrated in Fig. 2b-c. In Fig. 2b, a multimeric polypeptide 220 comprises multiple single-domain antibody variants 202 as disclosed herein, which may optionally be joined by a linker sequence 204. N-terminal amino acid sequence 201’ is optional and can be a leader peptide of e.g. 2-6, such as 4, amino acids. C-terminal sequence

203 is optional and may comprise a purification tag, optionally including a linker. The sdAb variant units 202 may be identical, or may differ from one another with regard to their amino acid sequence. For example, the polypeptide may comprise two sdAb variants ("dimer"), or three sdAb variants ("trimer"). Fig. 2c shows an exemplary trimer 230, comprising three sdAb variants 202.

5 Optionally a multimeric antigen-binding polypeptide may contain at least one additional amino acid sequence which does not represent an sdAb variant, for example a fusion partner polypeptide as described in connection with the fusion protein elsewhere herein.

 Multimeric variants of the present polypeptide may be advantageous in that they can provide an increased binding capacity, and/or an improved stability, in relation to a polypeptide
10 comprising a single copy of an sdAb variant.

 As indicated above, the antigen-binding polypeptide or multimer may, in addition to the sdAb variant(s), optionally comprise additional amino acids located N- and/or C-terminally thereof, such as illustrated in Fig. 2a-d. Such a polypeptide or multimer should be understood as a polypeptide or multimer having one or more additional amino acid residues at the very first and/or
15 the very last position in the polypeptide chain, i.e. at the N- and/or C-terminus of the polypeptide or multimer. Thus, the antigen-binding polypeptides as defined herein may comprise any suitable number of additional amino acid residues, for example one, two, three, four, five, six, seven, eight, nine, ten or more, such as up to 20, additional amino acid residues optionally in a consecutive sequence. For example, a sequence of additional amino acids may consist of from 1 to 12 amino
20 acids, and antigen binding polypeptide may comprise several such sequences. The additional amino acid may be a legacy from recombinant protein expression, a leader peptide, a signal peptide (e.g. PelB or OmpA), a purification tag, a peptide intended for coupling to support, or the like. The additional amino acid may be a spacer or linker as defined elsewhere herein, or have the ability to serve as spacer or linker. The additional amino acid residues may individually or collectively improve
25 production, purification, stabilization in vitro or coupling of the polypeptide to substrates of interest, for example to a solid support, such as a solid support described in connection to the aspect of a separation matrix. Such additional N-terminal or C-terminal amino acid residues do typically not affect the functional properties, such as binding affinity, of the antigen-binding polypeptide or the single domain antibody as such.

30 Furthermore, when the antigen-binding polypeptide is provided in the form of a multimer, comprising a plurality of single-domain antibody units, said additional amino acid residues may for example not be repeated for each occurrence of the single-domain antibody. For example, where the antigen-binding polypeptide is provided in the form of a multimer, said additional amino acids may occur only at the N-terminus or the C-terminus of the multimer.

Said additional amino residues may be coupled to the sdAb or multimer by means of chemical conjugation (using known organic chemistry methods) or by any other means, such as expression as a fusion protein or joined in any other fashion, either directly or via a linker, for example a peptide linker as described above.

5 In some embodiments, the polypeptide and/or multimer, as disclosed above, further comprises at the C-terminal or N-terminal end one or more tags or coupling elements, selected from the group consisting of a cysteine residue, a plurality of lysine residues and a plurality of histidine residues, and combinations thereof. Exemplary tags or coupling peptides include (His)₆, (His)₆-Cys and AEAAAKHHHHHC (SEQ ID NO: 159).

10 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 linker comprising up to 15 amino acids, such as 1-5, 1-10 or 5-10 amino acids. This stretch should preferably also be sufficiently stable in alkaline environments not to impair the properties of the protein. For this purpose, it is advantageous if the stretch does not contain asparagine. It can additionally be
15 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.

As the skilled person understands, the construction of a multimer, for example as a fusion
20 protein, often involves use of linkers between the monomer moieties to be fused. A monomer moiety may refer to a single-domain polypeptide as described herein. The skilled person is aware of different kinds of linkers with different properties, such as flexible peptide linkers, rigid peptide linkers and cleavable peptide linkers. Linkers may be used in order to for example increase stability or improve folding of fusion proteins. A linker may also ensure a certain spatial distance between two
25 sdAb variants, or between the sdAb variant and a fusion partner polypeptide, which in either case may be beneficial for the access of the target to the binding surface of the sdAb variant. The presence of a linker within a fusion protein generally does not substantially affect the target binding capacity of a correctly folded antigen-binding polypeptide monomer, such as a single-domain antibody.

30 Thus, a multimer as defined herein may further comprise at least one linker. For example, a linker is present between each monomer within the multimer. The linker may for example be selected from the group consisting of flexible amino acid linkers, rigid amino acid linkers and cleavable amino acid linkers. Peptide linkers may be structured or unstructured. Typically, unstructured linkers are more flexible and less rigid than structured linkers. Alternatively,

the linker may be a non-peptidic linker. Thus, polypeptides disclosed herein, or sub-units thereof, may be linked to each directly by peptide bonds between the C-terminal and N-terminal ends of the polypeptides. Alternatively, two or more monomers, in other words monomer units or moieties, within the multimer can be linked by elements comprising oligomeric or polymeric species, such as elements comprising up to 15 or 30 amino acids, such as 1-5, 1-10 or 5-10 amino acids. In 5 embodiments, said linker comprises up to 15 amino acid residues. The nature of such a link should preferably not destabilize the spatial conformation of the protein units, which is of the monomers within the multimer. This can e.g. be achieved by avoiding the presence of proline in the linkers. Furthermore, said linkers should preferably also be sufficiently stable in alkaline environments not to 10 impair the properties of the protein units. For this purpose, it is advantageous if the linkers do not contain asparagine. It can additionally be advantageous if the linker does not contain glutamine. The multimer may further at the N-terminal end comprise additional amino acid residues as described above, 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 15 or less, such as 10 or less or 15 5 or less.

The antigen-binding polypeptide may be provided in the form of a fusion protein. As indicated above, it may contain a plurality of sdAb variants produced as a single fusion protein, optionally with additional amino acids, including linkers, as described above.

In some embodiments the antigen-binding polypeptide may be a fusion protein containing at 20 least one sdAb variant as disclosed herein and a further polypeptide moiety, also referred to as a fusion partner polypeptide. Fig. 3a schematically illustrates a fusion protein 300 comprising, in the N- to C-terminal direction, a first polypeptide moiety 200 and a second polypeptide moiety 301, joined by an optional peptide linker 204. In this figure, the second polypeptide moiety 301 is located C-terminally of the first polypeptide moiety 200. It is also contemplated that the second polypeptide moiety may be located N-terminally of the first polypeptide moiety. The first polypeptide moiety 200 25 may comprise or consist of only a single-chain polypeptide 202 as illustrated in Fig. 2a, or may comprise or consist of a single-chain polypeptide 202 and additional amino acids, such as the additional amino acid sequence 201 and/or linker 204. The second polypeptide moiety 301 may be referred to as a fusion partner polypeptide.

30 The fusion partner polypeptide 301 may be a polypeptide that improves or adds at least one functionality or desirable property. For example, the fusion partner polypeptide may be a stabilizing polypeptide, which is a polypeptide that further improves stability, such as alkaline stability, of the antigen-binding polypeptide. As another example, the fusion partner polypeptide may be a polypeptide that improves expression of the antigen-polypeptide in a recombinant cell, such as *E.*

coli. As yet another example, a fusion partner polypeptide may provide an opportunity for purification of the antigen-binding polypeptide by affinity chromatography.

A fusion protein of the present disclosure may in particular comprise an sdAb variant, or a plurality of sdAb variants, fused to an α -helix-containing polypeptide, such as a protein domain. The α -helix containing polypeptide may contain at least one α -helix, such as at least 2 α -helices, such as 3, 4, 5 or 6 α -helices, such as up to 10 α -helices. In embodiments, it may be an α -helix bundle domain, such as a three-helix bundle domain. In an α -helix bundle domain, the α -helices are spatially grouped together and form the major part of the protein domain, which may lack other prominent unstructured or beta-structured regions. Examples of α -helix bundle domains include protein domains of *Staphylococcus aureus* protein A (SpA) and albumin binding domain (ABD). For example, the fusion partner polypeptide of the fusion protein of the present disclosure may comprise an amino acid sequence derived from SpA domain A, domain B, domain C, domain D or domain E. For example, the fusion partner polypeptide may comprise an amino acid sequence having at least 80 % identity, such as at least 85 % or at least 90 % identity, to SEQ ID NO: 161 or 162.

Also, the fusion protein of the present disclosure may be a multimeric protein, meaning that it may comprise more than one sdAb, or more than one fusion partner polypeptide. Multimeric variants containing multiple copies of an sdAb may be advantageous in that they can provide an increased binding capacity in relation to a fusion protein containing only one copy. A multimeric fusion protein containing multiple fusion partner polypeptides may provide an improved stability.

Thus, the fusion protein may optionally be provided in the form of a multimeric fusion protein ("multimer") containing at least two antigen-binding polypeptides, and/or at least two fusion partner polypeptides, as schematically illustrated in Fig. 3b-c. In Fig. 3b, a multimeric fusion protein 310 comprises multiple units of the antigen-binding polypeptide 200 arranged sequentially in the N- to C-terminal direction, and which may optionally be joined to one another by linker sequences 204, which may be the same or different for each occurrence. The antigen-binding polypeptides 200 may be identical, or may be different from one another with regard to their amino acid sequence. Fig. 3b shows three antigen-binding polypeptides 200 ("trimer"), but it is envisaged that the fusion protein may comprise any suitable number of said antigen-binding polypeptide 200. For example, the fusion protein may contain at least two, or at least 3, 4, 5 or 6 units of the antigen-binding polypeptide. The fusion protein may contain e.g. up to 10 antigen-binding polypeptides 200.

Furthermore, the fusion protein may contain at least 2, such as 3, 4, 5 or 6 units, e.g. up to 10 units, of the fusion partner polypeptide. Fig. 3c illustrates a fusion protein 320 containing one first antigen-binding polypeptide 200 and three polypeptide moieties 301. Linkers 204 are optional, and may be the same or different for each occurrence. The polypeptide moieties 301 may be identical, or

may be different with regard to their amino acid sequences. In other embodiments, the fusion protein may contain two fusion partner polypeptides and any suitable number of the antigen-binding polypeptide 200 as described above. For example, the fusion protein may contain one antigen-binding polypeptide flanked by two fusion partner polypeptides. Where a fusion protein comprises multiple antigen-binding polypeptides and multiple fusion partner polypeptides, for example 2 or 3 of each, it is envisaged that the different polypeptide moieties 200, 301 may be arranged in an alternating fashion instead of polypeptides of the same kind being arranged sequentially as depicted in Fig. 3b (for the antigen-binding polypeptide 200) and Fig. 3c (for the fusion partner polypeptide 301). For example, a multimeric fusion protein containing two units of each polypeptide may have the following general structure: [antigen-binding polypeptide]-[fusion partner polypeptide]-[antigen-binding polypeptide]-[fusion partner polypeptide].

More generally, a fusion protein according to the present disclosure may have a structure as follows:

$$([A-L1]_m-[Z-L2]_n)_p$$

wherein A represents a polypeptide as described herein; Z represents a further polypeptide moiety (fusion partner polypeptide) as described herein; L1 for each occurrence may be present or absent, and where present, represents a linker or spacer; L2 for each occurrence may be present or absent, and where present, represents a linker or spacer; m represents an integer of from 1 to 4; n represents an integer of from 1 to 4, or when $m \geq 2$, n represents 0 or an integer of from 1 to 4; and p represents an integer of from 1 to 4. Preferably $(m+n)*p$, that is, the total number of polypeptide moieties A and Z in the fusion protein, may be up to 10, such as up to 8.

In some embodiments, $n=1$, $m=1$ and $p=1$. In other embodiments, m is 2 or 3, n is 1 and p is 1. In yet other embodiments, m is 1, n is 1 and p is 2 or 3. For reference, in the exemplary fusion protein of Fig. 3a, $m=1$, $n=1$ and $p=1$. In the exemplary fusion protein of Fig. 3b, $m=3$, $n=1$ and $p=1$. In the exemplary fusion protein of Fig. 3c, $m=1$, $n=3$ and $p=1$. In candidate construct "6B-3" of Example 6B (see Table 17), $m=1$, $n=1$ and $p=3$. In examples where the fusion protein comprises at least two fusion partner polypeptides, it may be preferred that at least one is located at the C terminus of the fusion protein. Optionally, at least two fusion partner polypeptides may be arranged sequentially at the C terminus of the fusion protein.

At the N-terminus of a fusion protein, such as a multimeric fusion protein, there may be a short amino acid sequence, such as a leader or signal peptide. At the C-terminus a fusion protein, such as a multimeric fusion protein, a tag or spacer, or another short amino acid sequence may optionally be provided as desired.

In embodiments, the fusion partner polypeptide is not located at or near the N-terminus of the fusion protein.

For clarity, any reference herein to the antigen-binding polypeptide of the present disclosure encompasses also multimeric variants and fusion proteins thereof, unless stated otherwise.

5 The antigen-binding polypeptide of the present invention can be produced by conventional biotechnological methods, by recombinant protein production using genetically engineered host cells.

10 Thus, in further aspects, the present disclosure provides an isolated nucleic acid encoding an antigen-binding polypeptide as described herein; an expression vector comprising said nucleic acid; and a host cell comprising said expression vector.

Also encompassed by this disclosure is a method of producing the antigen-binding polypeptide as described herein, comprising culturing said host cell under conditions allowing expression of said antigen-binding polypeptide from its expression vector, and isolating the antigen-binding polypeptide.

15 The host cells may be prokaryotic cells, such as bacteria, or eukaryotic cells. Exemplary prokaryotic host cells are *Escherichia coli*. Suitable eukaryotic cells may be yeast cells, such as *Saccharomyces cerevisiae* and *Pichia pastoris*, or animal cells, such as insect cells or mammalian cells commonly used in the field of biotechnology for the production of proteins, e.g. Chinese Hamster Ovary (CHO) cells or human embryonic kidney (HEK) cells.

20 Following isolation of the antigen-binding polypeptide from the host cells, which can be done by conventional means of harvesting, clarification and/or filtration etc, the antigen-binding polypeptide can be purified, e.g. by conventional means known in the art. For example, where the antigen-binding polypeptide comprises a histidine tag, a step of purifying the antigen-binding polypeptide may comprise immobilized metal affinity chromatography (IMAC). Other methods of purification that may be used include affinity chromatography. For example, an affinity ligand that
25 binds to a framework region of the sdAb variant may be used, or the affinity chromatography may use an affinity ligand based on a domain of SpA, wherein the affinity ligand binds to an VH region, such as VH3, of the antigen-binding polypeptide. Thus, the sdAb variant of the present disclosure may be capable of VH3 interaction with SpA domain or variant thereof.

30 Alternatively, where the antigen-binding polypeptide forms part of a fusion protein, the affinity ligand may bind to a fusion partner, such as to an affinity tag included in the amino acid sequence of a fusion partner polypeptide.

The antigen-binding polypeptide as disclosed herein may alternatively be produced by *in vitro* translation, or by non-biological peptide synthesis using amino acids and/or amino acid derivatives having protected reactive side-chains. Non-biological peptide synthesis may comprise:

- step-wise coupling of the amino acids and/or the amino acid derivatives to form a
- 5 polypeptide having protected reactive side-chains,
- removal of the protecting groups from the reactive side-chains of the, and
- folding of the polypeptide in aqueous solution.

In general, the present antigen-binding polypeptide may be used for capture of the target entity for which it has an affinity. However, preferably the antigen-binding polypeptide as such is not

10 intended for use as a therapeutic compound, and is preferably not intended for use *in vivo*. Hence, the present antigen-binding polypeptide may be used for *in vitro* capture of the target entity.

The capture may be for the detection of the target entity within a sample, or for separation of the target entity from other components in a sample. The antigen-binding polypeptide may be used for analytical separation of the target entity, or it may be used for preparative purification of

15 the target entity. The term "preparative" refers to the process of preparing a purified target entity, in particular during the manufacture of a product, such as a pharmaceutical product or a therapeutic composition, which comprises the target entity. Separation, detection and/or quantification using the antigen-binding polypeptide of the present disclosure may alternatively be performed in the context of a sensor application.

20 An *in vitro* method of detecting AAV9 viral particles present in a sample may comprise: providing a sample containing AAV9 viral particles; contacting the sample with an antigen-binding polypeptide, multimer or fusion protein as described herein, under conditions allowing the sdAb variant to bind to the AAV9 viral particles, wherein the binding event generates a detectable signal; and detecting the detectable signal. Suitable means for generation of a detectable signal in response

25 to a binding event are known in the art.

In particular, the antigen-binding polypeptide of the present invention may be used in applications involving interaction between antigen-binding polypeptide and its target entity, where either the antigen-binding polypeptide or the target entity is coupled to a support. When the antigen-binding polypeptide is coupled or immobilized to a support, it is still capable of binding its

30 target entity.

Where the antigen-binding polypeptide is coupled to a support, this may be referred to as an adsorbent material or an affinity capture material. Adsorbent materials may be used in various industrial or laboratory applications, including separation, purification, detection and/or quantification of the target entity. A separation matrix as described herein is an adsorbent material

intended for use in separation of a target entity from other components. The coupling of the antigen-binding polypeptide to the support may optionally be provided via a C-terminal amino acid sequence suitable for coupling to a support. For example, a tag or spacer ending with a cysteine may be provided for coupling via a thioether bond, as described in more detail below. Where the antigen-binding polypeptide forms part of a fusion protein, a C-terminal fusion partner polypeptide may have such a C-terminal amino acid sequence suitable for coupling to a support as described below.

Where the antigen-binding polypeptide is not coupled to a support, it may be used e.g. in a detection assay where the target or analyte is present on a surface and is contacted with the antigen-binding polypeptide which is present in a solution. In such a case, it is envisaged that a reporter entity may be used for generating a detectable signal, wherein the reporter entity is capable of binding to the antigen binding polypeptide, or capable of binding to the fusion partner in the case of a fusion protein. The reporter entity may be e.g. a fluorescent labelled or radiolabelled entity as known in the art. If the antigen binding polypeptide or fusion protein is capable of binding to IgG, the reporter entity may be based on IgG.

The antigen-binding polypeptide may be used in applications where it may be exposed to alkaline conditions, such as treatment with NaOH. Such treatments are frequently used in the field of chromatography, to strip a chromatography matrix of bound impurities before the next purification cycle (cleaning-in-place, as described above). However, alkali exposure or cleaning of a binding surface may be used also in other contexts.

The support may be a solid support and may optionally be a porous material.

The support may be or comprise a surface onto which the antigen-binding polypeptide is coupled. Examples of such support materials include conventional protein-binding support and surfaces such as a chip, a plate, a well, and a sheet. The support may optionally be provided in other forms such as a fiber, a membrane, a fibrous matrix, a filter, a porous monolith, a particle or a bead, such as a gel bead as used in chromatography resins. Particles or beads can be porous or non-porous. Particles or beads may include magnetic beads. Supports in the form of beads or particles 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, a separation procedure commonly follows conventional chromatography with a concentration gradient. In case of pure suspension, batch-wise mode will be used.

The support may be made of any suitable material, as outlined in more detail below. As a non-limiting example, a conventional affinity separation matrix is often of organic nature and based on polymers that expose a hydrophilic surface to the aqueous media used, i.e. expose hydroxy (-OH),

carboxy (-COOH), carboxamido (-CONH₂, possibly in N-substituted forms), amino (-NH₂, possibly in substituted form), oligo- or polyethylenoxy groups on their external and, if present, also on internal surfaces.

The antigen-binding polypeptide may be attached to the support via known coupling techniques utilizing e.g. thiol, amino and/or carboxy groups present in the antigen-binding polypeptide. Bisepoxides, epichlorohydrin, CNBr, N-hydroxysuccinimide (NHS) etc. are well-known coupling reagents. Between the support and the antigen-binding polypeptide, a spacer molecule can be introduced, which improves the availability of the first polypeptide moiety and/or facilitates the chemical coupling of the antigen-binding polypeptide to the support. Depending on the nature of the antigen-binding polypeptide and the coupling conditions, the coupling may be a random or multipoint coupling (e.g. via a plurality of lysines or histidines) or a single point coupling (e.g. via a single cysteine). In embodiments, to increase coupling control, it may be preferably that the sdAb variant as such does not comprise any histidine residues. However also in such cases, the antigen-binding polypeptide as a whole may comprise a histidine tag, such as a (His)₆ tag. Alternatively, the antigen-binding polypeptide may be attached to the support by non-covalent bonding, such as physical adsorption or biospecific adsorption.

The antigen-binding polypeptide may be coupled to the support via thioether bonds. Methods for performing such coupling are well-known in this field and easily performed by the skilled person in this field using standard techniques and equipment. Thioether bonds are flexible and stable and generally suited for use in affinity chromatography. In particular when the thioether bond is via a terminal or near-terminal cysteine residue on the antigen-binding polypeptide, the mobility of the coupled antigen-binding polypeptide is enhanced which provides improved binding capacity and binding kinetics. In some embodiments the antigen-binding polypeptide 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.

With regard to specific materials, the support may comprise a polymeric material. Polymeric materials include materials of natural or synthetic polymers, and combinations thereof. For example, the support may comprise a polyhydroxy polymer, such as a polysaccharide. Examples of polysaccharides include e.g. dextran, starch, cellulose, pullulan, agar, agarose etc, including derivatives thereof. The beneficial features of these polysaccharides could further be improved by means of covalent cross-linking, rendering them particularly suitable for enzyme immobilization with a wide range of derivatization methods taking advantage of chemical modification of a fraction of the polymer hydroxyls, where the hydroxyl functions could be partially or totally derivatized. These

derivatives and methods for derivatizing the polysaccharides are well known to the skilled person. For example, agarose derivatives include e.g. Agarose-Protein A/G/L, Agarose-Biotin, Agarose-Carboxyl, Agarose-Amines, Agarose-Sulfonate and Agarose-Maltose. Polysaccharides are inherently hydrophilic with low degrees of nonspecific interactions, they provide a high content of reactive (activatable) hydroxyl groups and they are generally stable towards alkaline cleaning solutions used in bioprocessing. The support may comprise agar or agarose, such as crosslinked agarose. Such supports used in the present invention can easily be prepared according to standard methods, such as inverse suspension gelation (S Hjerten: Biochim Biophys Acta 79(2), 393-398 (1964)). Alternatively, the base matrices are commercially available products, such as crosslinked agarose beads sold under the name of SEPHAROSE™ FF (Cytiva™). In an embodiment, which is especially advantageous for large-scale separations, the support has been adapted to increase its rigidity using the methods described in US6602990 or US7396467, which are hereby incorporated by reference in their entirety, and hence renders the matrix more suitable for high flow rates.

Synthetic polymers useful as material for the support include 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 can be hydrophilized to expose hydrophilic groups as defined above to a surrounding aqueous liquid. Such polymers are easily produced according to standard methods. As an alternative, a commercially available product, such as SOURCE™ (Cytiva™) may be used.

Alternatively, the solid support according to the invention comprises a support of inorganic nature, e.g. silica, zirconium oxide etc.

In embodiments, the antigen-binding polypeptide may be coupled to a support which is a convection-based chromatography matrix. Such convection-based chromatography matrix may comprise a porous polymer membrane, a filter, a fibrous matrix, or a porous monolith. Examples of a porous polymer membrane include Mustang™ membranes (Cytiva) and Sartobind™ membranes (Sartorius). A fibrous support may be based on electrospun polymeric fibers or cellulose fibers, optionally non-woven fibers. A fibrous matrix may thus be a non-woven fibrous matrix. The fibers may have a cross-sectional diameter of 10-1000 nm, such as 200-800 nm, 200-400 nm or 300-400 nm. Such a fibrous support can be found in a HiTrap Fibro™ unit (Cytiva™). Alternative fibrous supports are disclosed in e.g. WO2019/137869 and WO2018/011600.

An adsorbent material or separation matrix as described herein may be used for the same purposes as mentioned above for the antigen-binding polypeptide.

Thus in one aspect, the invention provides a chromatography material comprising a separation matrix as disclosed herein, as well as chromatography columns or devices incorporating such separation matrix.

In another aspect, the adsorbent material or separation matrix may be a sensor surface
5 intended for use in a sensor or other detection or quantification devices.

The present invention provides a method of separating or isolating a target entity, wherein a separation matrix as disclosed herein is used. In some embodiments, the method comprises contacting a liquid sample comprising the target entity with a separation matrix as disclosed herein. The contacting is performed under conditions under which the target entity can bind to the first
10 polypeptide moiety. The method may furthermore comprise washing said separation matrix with a washing liquid, eluting the target entity from the separation matrix with an elution liquid, and optionally cleaning the separation matrix with a cleaning liquid. The cleaning liquid can alternatively be called a cleaning-in-place (CIP) liquid. The cleaning liquid typically is an alkaline solution, such as comprising NaOH or KOH of at least 0.05 M, such as 0.05-1 M, such as 0.05-0.5 M, such as at least
15 0.1 M, such as 0.1-0.5 M, e.g. 0.3 M or 0.5 M. The contact (incubation) time may be at least 10 min. The binding, eluting and cleaning steps may advantageously be repeated at least 10 times, such as at least 20 times.

A skilled person will understand that the liquid sample to be purified may be any sample comprising the target entity in an environment from which it is to be purified or separated. The
20 sample may be obtained from a cell culture, such as a clarified cell culture harvest, and may have been subjected to one or more conventional steps of filtration, concentration, dilution and/or buffer exchange, and optionally one or more initial chromatography steps, in particular a step which is not based on affinity chromatography, such as ion exchange chromatography, hydrophobic interaction chromatography, multimodal chromatography, or size exclusion chromatography. For example, the
25 sample may be a clarified and filtered cell culture harvest. In some cases the sample may have been subjected to one or more steps of filtration by tangential flow filtration (TFF).

Prior to contacting with the present separation matrix, liquid sample contains the target entity and at least one impurity, such as host cell protein (HCP) or host cell nucleic acids. The present separation matrix is useful for separating the target entity from such impurities, and after performing
30 the above process, the eluate containing the target entity has a reduced level of at least one such impurity.

Optionally, after performing a separation based on affinity capture as described herein, the eluate containing purified target entity may be subjected to one or more steps of filtration, concentration, dilution or buffer exchange, or chromatography step(s) not based on affinity

chromatography, such as ion exchange chromatography, hydrophobic interaction chromatography, multimodal chromatography, or size exclusion chromatography. A further chromatography step performed after the affinity separation of the present disclosure may be referred to as a polishing step.

5 While the invention is described herein with respect to exemplary embodiments, the skilled person will appreciate that the invention is not limited to these. As the skilled person will realize, the function of any polypeptide is dependent on the tertiary structure of the polypeptide. It is therefore possible to make minor changes to the sequence of amino acids in a polypeptide without affecting the function thereof. Thus, the disclosure encompasses modified variants of the antigen-binding
10 polypeptide. Such modified variants are such that their affinity for the target AAV vector, in particular AAV9, is at least retained.

 Use of the verb “to comprise” and its conjugations does not exclude the presence of elements or steps other than those stated. For example, a polypeptide “comprising” a complementarity determining region 1 (CDR1) can also have further CDRs, such as a CDR2 and a
15 CDR3, and may have other regions, such as FWRs, and optionally other structures or sequences. The article “a” or “an” preceding an element does not exclude the presence of a plurality of such elements. The mere fact that certain measures are recited in mutually different dependent claims does not indicate that a combination of these measures cannot be used to advantage.

EXAMPLES

20 **Example 1: Production of polypeptides**

 This example describes the generation of glycerol stock solution for the production of candidate polypeptides used in Examples 3-6 and 8.

 Materials and equipment used were as follows: plasmid DNA for the candidate polypeptides to be produced; chemically competent BL21(DE3) *E. coli* (prepared in-house), KCM buffer (5X) 0.5M
25 KCl, 0.15M CaCl₂, 0.25M MgCl₂, LB culture medium (Invitrogen™), carbenicillin (100 mg/ml), agar plates (BD BACTO™ Agar) supplemented with carbenicillin (100 µg/ml) (PanReac AppliChem™), 14 ml Falcon™ Round-Bottom (Corning™), Infors HT Shaking incubator.

 Desired amino acid sequences of candidate polypeptides were back-translated to DNA and optimized to remove rare codons. Constructs were ordered as plasmid DNA based on an expression
30 vector with T5 promotor, OmpA signal peptide, ampicillin resistance and pUC origin of replication. Plasmid DNA was used for transformation into chemically competent BL21(DE3) *E. coli*, plated on agar plate supplemented with 100 µg/ml carbenicillin and grown overnight at 37°C. For each candidate, a single colony was used to inoculate 4 mL LB media supplemented with 100 µg/ml

carbenicillin in a 14 ml round-bottom tube and grown over night at 37°C, 200 rpm agitation. A glycerol stock of each variant was prepared with 900 µl overnight culture and 500 µl glycerol 50% and stored at -80°C until further use.

5 **Example 2: AAV9 binding of sdAb variants having CDR2/CDR3 variations**

The ability of the candidate polypeptides to bind AAV9 was investigated by surface plasmon resonance analysis using a Biacore™ instrument (Cytiva, Sweden). Each candidate polypeptide contained an sdAb with variations in the CDR2 and CDR3 amino acid sequences as outlined in Table 7. All candidates had the same CDR1 amino acid sequence RTLSDYTMG (SEQ ID NO: 16).

10

Table 7

Candidate	sdAb SEQ ID NO:	CDR2 amino acid sequence	CDR3 amino acid sequence
vh28	77	SISWSGAYTY (SEQ ID NO:23)	GPTGLLSKKSTPADYDY (SEQ ID NO:38)
vh29	78	LISWSGAYTY (SEQ ID NO:24)	
vh30	79	IISWSGAYTY (SEQ ID NO:25)	
vh31	80	SISWSGAYTY (SEQ ID NO:23)	GPTGLLSRKSTPADYDY (SEQ ID NO:54)
vh32	81	LISWSGAYTY (SEQ ID NO:24)	
vh33	82	IISWSGAYTY (SEQ ID NO:25)	
vh34	83	SISWSGAYTY (SEQ ID NO:23)	GPTGLLSKKATPADYDY (SEQ ID NO:55)
vh35	84	LISWSGAYTY (SEQ ID NO:24)	
vh36	85	IISWSGAYTY (SEQ ID NO:25)	
vh37	86	SISWSGAYTY (SEQ ID NO:23)	GPTGLLSRKATPADYDY (SEQ ID NO:45)
vh38	87	LISWSGAYTY (SEQ ID NO:24)	
vh39	88	IISWSGAYTY (SEQ ID NO:25)	
vh40	89	AISWSGAYTY (SEQ ID NO:26)	GPTGLLSKKSTPADYDY (SEQ ID NO:38)
vh41	90		GPTGLLSKKATPADYDY (SEQ ID NO:55)

Materials and methods

Generation of purified candidate polypeptides

15 Candidate polypeptides were ordered from GenScript Biotech for protein expression and purification. Constructs were expressed with pelB signal peptide and a C-terminal tag (AEAAAKHHHHHC, SEQ ID NO: 161) in *E. coli*. Protein purification was performed using immobilized metal affinity chromatography (IMAC). Purified candidate polypeptides were shipped to Cytiva, Sweden.

20

Biotinylation of purified candidate polypeptides

Purified candidate polypeptides were biotinylated at the C-terminal cysteine using EZ-Link™ Maleimide-PEG2-Biotin, No-Weigh™ Format (ThermoFisher Scientific) with a 5X molar excess of biotin. After biotinylation, buffer exchange to PBS was performed using PD MidiTrap™ G-25 columns (Cytiva).

Biacore analysis of binding affinity for AAV9

To assess binding to AAV9, the candidate polypeptides were immobilized on a Biacore™ Series S SA chip. AAV9 (2E12 viral particles (vp)/ml) was used as analyte.

Materials and equipment used were as follows: Biacore™ Series S SA sensor chips, Biacore™ 8K+ instrument (all from Cytiva, Sweden); AAV9 (prepared in-house); candidate polypeptides generated as described above.

Immobilization was performed using a standard method in Biacore™ software with coupling of biotinylated polypeptides in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1).

Biotinylated AAV9 binding polypeptide variants were diluted in PBS at a concentration of 100 µg/ml. The immobilization levels obtained were 5503 +/- 324 RU.

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

Biacore™ method for binding analysis:

Running buffer: PBS-P+; Flow rate: 5 µl/min; Sample injection: 2400 s/40 min over both Flow Cells (FC1 and FC2); Dissociation time: 2400 s/40 min; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. Injections of analyte (AAV9) for each channel (cycles) were as follows: running buffer, AAV9 2.5E11 vp/ml, AAV9 1E12 vp/ml, AAV9 2E12 vp/ml.

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

Results

Table 8 below reports the immobilization level and maximum binding responses recorded for the respective analyte concentrations. All candidates showed binding to AAV9 and in particular vh29, vh30, vh33, and vh35-41 demonstrated excellent binding capacity, and the binding capacity was higher for the two highest AAV9 concentrations tested.

Table 8

Candidate	Immobilization level (RU)	Response (RU) 2.5E11 vp/ml	Response (RU) 1E12 vp/ml	Response (RU) 2E12 vp/ml
vh28	5096	4214	7347	7680
vh29	5373	5421	9279	9505
vh30	4879	5542	9102	9306
vh31	5310	4829	7887	8191
vh32	5751	4651	7153	7394
vh33	5274	5327	9096	9295
vh34	4734	4602	7461	7715
vh35	6078	6892	11028	11353
vh36	5787	6829	10743	11052
vh37	5384	6633	10067	10385
vh38	5804	6195	9963	10322
vh39	5452	6702	10551	10954
vh40	5846	7189	10876	11170
vh41	5268	6832	10954	11281

Figures 4a-b are representative sensorgrams, showing the binding capacity of vh36 (Fig. 4a) and vh41 (Fig. 4b) for the respective AAV9 concentrations 2E12 vp/ml (top curve), 1E12 v/(ml (middle curve) and 2.5E11 vp/ml (lower curve). vh28-vh35 and vh37-40 generated similarly shaped curves.

Example 3A: Binding and stability of further candidate polypeptides with CDR point modifications

This example further investigated the effect of single-point amino acid modifications in the CDR regions of the candidate polypeptides on alkaline stability and target binding. The candidate polypeptides tested were expressed as fusion protein in which the respective sdAb had SEQ ID NO:161 fused to its C-terminal via a linker. All candidates had an N-terminal OmpA signal peptide and a C-terminal (His)₆ tag. The sdAb sequences are presented in Tables 9a-c. The sdAb of SEQ ID NO:92 was used as the reference, into which selected single point mutations were introduced. As indicated in Tables 9a-c below, each candidate differed from SEQ ID NO:92 in one amino acid position, either in CDR1, CDR2 or CDR3.

The following aspects were evaluated: (1) Affinity assessment of AAV9 Interaction (tested through high concentration injection of AAV9). (2) Alkaline stability (tested by reduction of AAV9 binding after increasing number of treatments with 0.5 M NaOH).

*Materials and methods*Generation of MabSelect PrismA™ purified candidate polypeptides

For each candidate, 5 µl of the glycerol stock produced as described in Example 1 above was inoculated in 4 ml LB culture medium supplemented with 100 µg/ml carbenicillin in 14 ml round-bottom tube and grown over night at 37°C, 200 rpm agitation. Protein expression culture medium (TB medium supplemented with 100 µg/ml carbenicillin and 2 mM MgCl₂) was prepared and added to filled 500 ml baffled glass shake flasks (50 ml per flask, 43 flasks in total). Each flask was inoculated with approximately 500 µl from the previous overnight culture to obtain a starting OD₆₀₀ of 0.05. The flasks were incubated in Infors HT shaking incubator for approximately 3.5 hours at 37°C and 140 rpm shaking until OD₆₀₀ reached 1.0. Thereafter 50 µl IPTG (1 M) was added to a final concentration of 1 mM and the flasks were incubated in Infors HT shaking incubator at 27°C and 140 rpm shaking for 18 hours, whereafter the cultures were transferred to Falcon tubes (50 ml). To generate crude variant lysates the Falcon tubes were incubated in 48°C water bath for 2 hours followed by pelleting of cell debris by centrifugation at 8000 g for 10 min and filtration of supernatant with a 0.45 µm filter.

The samples were purified using HiTrap MabSelect PrismA™ chromatography columns (Cytiva). Clarified lysate for each sample was loaded onto a 1 mL column, equilibrated in phosphate buffered saline (PBS) pH 7.4, allowing a residence time of about 2.4 min. The column was then washed with 5 column volumes (CV) of PBS pH 7.4, followed by 5 CV of 50 mM sodium acetate pH 6. Protein was eluted using 50 mM sodium acetate pH 3.5 in a step gradient over 5 CV.

Concentration of the purified samples was measured using NanoDrop (Thermo Scientific) according to manufacturer's instructions. Following purification, samples were kept in refrigerator in 41ntarctic tubes until all measurements had been performed and were then kept in freezer until Biacore analysis. Protein mass was determined by liquid chromatography–mass spectrometry on a ACQUITY Rda Detector (Waters™) using a BioResolve column (Waters™).

Biacore analysis of binding affinity for AAV9

To assess binding to AAV9, the candidate polypeptides were immobilized on a Biacore™ Series S CM5 chip. AAV9 (2E12 viral particles (vp)/ml) was used as analyte.

Materials and equipment used were as follows: Biacore™ Series S CM5 sensor chips, Biacore™ Amine coupling kit, Biacore™ Acetate buffer pH 5.0, Biacore™ 8K+ instrument (all from Cytiva, Sweden); AAV9 (prepared in-house); candidate polypeptides generated as described above.

Immobilization was performed using a standard method in Biacore™ software with coupling of AAV9 binding polypeptide variants in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1

(FC1). AAV9 binding polypeptide variants were diluted in Biacore™ Acetate buffer pH 5.0 at a concentration of 25 µg/ml. The immobilization levels obtained were 3484 +/- 197 RU.

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

5 Running buffer: PBS-P+; Flow rate: 5 µl/min; Sample injection: 2400 s/40 min over both Flow Cells (FC1 and FC2); Dissociation time: 2400 s/40 min; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. Injections of analyte (AAV9) for each channel (cycles) were as follows: running buffer, 2E11 vp/ml, 5E11 vp/ml, 2E12 vp/ml.

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

Biacore analysis of alkaline stability

15 Assessment of alkaline stability was done by immobilizing the candidate polypeptides on the Biacore™ chips. After evaluation of AAV9 binding (see above) the same chips were subjected to repeated cycles of binding to AAV9 (5E11 vp/ml) followed by injection of NaOH (0.5 M).

 Biacore method for alkaline stability (per cycle):

20 Running buffer: PBS-P+; Flow rate: 10 µl/min; Sample injection 1 (AAV9 5E11 vp/ml): 300 s over both Flow Cells; Dissociation time 1: 30 s; Sample injection 2 (0.5 M NaOH): 600 s over both flow cells; Dissociation time 2: 0 s; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. This cycle was repeated 40 times to follow stability of AAV9 response values.

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

Results

25 The response levels of AAV9 interactions of the candidate polypeptides using the maximum AAV9 concentration (2E12 vp/ml) was normalized to the response of the candidate vh118 (having the sdAb sequence of SEQ ID NO: 92) and summarized in Tables 9a-c.

30 Alkaline stability was recorded as the number of cycles with at least 50 % binding capacity retained. The first cycle was excluded, and binding capacity values were normalized against the binding capacity of the second cycle, i.e. binding after the first exposure to NaOH. In each cycle, 0.5 M NaOH was injected with a contact time of 10 min at 10 µl/min. The result for each candidate is presented in Tables 9A-C.

 It was seen that a satisfactory AAV9 binding capacity was achieved for many of the investigated point mutations. A binding capacity of at least 80 % of that of vh118 is considered

satisfactory. Looking at the alkaline stability, it was found that some point mutations in the CDR regions (cf. SEQ ID NOs:13, 14, 37, 39, 42-44, 56), were particularly beneficial. In this regard, maintaining a binding capacity of at least 50 % after 10 cycles is considered acceptable; a retained binding capacity of at least 50 % after 15 cycles being preferred.

5 Particularly preferred are those variants that exhibited a normalized binding capacity of at least 100 % and maintained at least 50 % binding capacity for 20 cycles or more such as at least 24 cycles.

 On the other hand, non-claimed variants such as vh194, having Y in position 3 of CDR1, and vh206 having R in position 4 of CDR2, showed poor or very poor binding and alkaline stability.

10

Table 9a. CDR1 variants tested

sdAb	CDR1 amino acid sequence	CDR1 SEQ ID NO:	sdAb SEQ ID NO:	Normalized AAV9 binding capacity (%)	NaOH stability (no. of exposure cycles with at least 50% binding retained)
vh118	RTLSDYTMG	16	92	100.0	24
vh193	LTLSDYTMG	10	93	89.1	17
vh194	RTYSDYTMG	18	94	37.9	5
vh197	RTLSNYTMG	11	95	104.0	15
vh198	RTLSEYTMG	12	96	102.5	15
vh199	RTLSDFTMG	13	97	115.1	≥40**
vh200	RTLSDNTMG	14	98	85.5	24
vh202	RTLSDYAMG	19	99	16.4	1
vh203	RTLSDYTIG	20	100	72.3	8
vh204	RTLSDYTMA	15	101	110.0	18

** 62 % binding capacity was retained after 40 cycles

Table 9b. CDR2 variants tested

sdAb	CDR2 sequence	CDR2 SEQ ID NO:	sdAb SEQ ID NO:	Normalized AAV9 binding capacity (%)	NaOH stability (no. of exposure cycles with at least 50% binding maintained)
vh118	AISWSGAYTK	29	92	100.0	24
vh210	AISWSGAYTF	31	105	111.1	22
vh205	AITWSGAYTK	169	102	78.6	8
vh206	AISRSGAYTK	32	103	0.4	N/A
vh208	AISWSAAYTK	33	104	51.8	6

15

Table 9c. CDR3 variants tested

sdAb	CDR3 amino acid sequence	CDR3 SEQ ID NO	sdAb SEQ ID NO	Normalized AAV9 binding capacity (%)	NaOH stability (no. of exposure cycles with at
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					least 50% binding maintained
vh118	GPTGLLSKKATPADYDY	55	92	100.0	24
vh211	APTGLLSKKATPADYDY	36	106	103.1	22
vh212	GSTGLLSKKATPADYDY	37	107	112.8	28
vh213	GPSGLLSKKATPADYDY	73	108	40.5	3
vh214	GPTGPLSKKATPADYDY	39	109	110.8	38
vh215	GPTGTLSKKATPADYDY	40	110	109.0	23
vh216	GPTGLISKKATPADYDY	41	111	114.3	16
vh217	GPTGLLAKKATPADYDY	42	112	116.4	25
vh218	GPTGLLSNKATPADYDY	43	113	107.1	27
vh219	GPTGLLSQKATPADYDY	44	114	107.3	29
vh220	GPTGLLSKHATPADYDY	47	115	103.1	16
vh221	GPTGLLSKKTTPADYDY	56	116	101.6	≥40*
vh222	GPTGLLSKKAAPADYDY	57	117	112.0	18
vh223	GPTGLLSKKATSADYDY	58	118	110.0	15
vh224	GPTGLLSKKATTADYDY	59	119	108.0	17
vh225	GPTGLLSKKATPRDYDY	60	120	107.9	18
vh226	GPTGLLSKKATPANYDY	61	121	109.5	15
vh227	GPTGLLSKKATPAEYDY	62	122	105.0	24
vh228	GPTGLLSKKATPAQYDY	63	123	106.5	18
vh229	GPTGLLSKKATPADFDY	64	124	102.7	22
vh230	GPTGLLSKKATPADRDY	74	125	31.1	3
vh231	GPTGLLSKKATPADYNY	65	126	103.9	18
vh232	GPTGLLSKKATPADYEY	66	127	108.6	18
vh233	GPTGLLSKKATPADYQY	67	128	111.7	17
vh234	GPTGLLSKKATPADYDF	68	129	106.9	23
vh235	GPTGLLSKKATPADYDR	69	130	106.8	15

* 51 % binding capacity was retained after 40 cycles

Example 3B: Binding and stability of further candidate polypeptides with CDR3 point modifications

This example further investigated the effect of amino acid modifications in the CDR3 region of the candidate polypeptides on alkaline stability and target binding. The candidate polypeptides tested were expressed as fusion protein in which the respective sdAb had SEQ ID NO: 161 fused to its C-terminal via a linker. All candidates had an N-terminal OmpA signal peptide and a C-terminal (His)₆ tag. The sdAb sequences tested are presented in Table 9d. The sdAb of SEQ ID NO:92 (vh118) was used as the reference, into which point mutations were introduced in amino acid positions 100b and/or 100c.

The following aspects were evaluated: (1) Affinity assessment of AAV9 interaction (tested through high concentration injection of AAV9). (2) Alkaline stability (tested by reduction of AAV9 binding after increasing number of treatments with 0.5 M NaOH).

Materials and methods

Candidate polypeptides were designed, generated and evaluated for binding affinity and alkaline stability on a Biacore instrument, as described in Example 3A.

5 *Results*

The immobilization levels obtained were 3394 +/- 183 RU.

The response levels of AAV9 interactions of the candidate polypeptides using the maximum AAV9 concentration (2E12 vp/ml) was normalized to the response of the candidate vh118 and summarized in Table 9d. It was seen that a satisfactory AAV9 binding capacity was achieved for many
10 of the investigated point mutations.

Alkaline stability was recorded as the number of cycles with at least 50 % binding capacity retained. The first cycle was excluded, and binding capacity values were normalized against the binding capacity recorded for the second cycle. In each cycle, 0.5 M NaOH was injected with a contact time of 10 min at 10 µl/min. The result for each candidate is presented in Table 9d.

15 It was noted that the candidate polypeptides having R instead of K in position 9 of CDR3 performed even better than vh118.

Table 9d. Further CDR3 variants

sdAb	CDR3 amino acid sequence	CDR3 SEQ ID NO	sdAb SEQ ID NO	Normalized AAV9 binding capacity (%)	NaOH stability (no. of exposure cycles with at least 50% binding maintained)
vh118	GPTGLLSKKATPADYDY	55	92	100.0	22
vh333	GPTGLLSRKATPADYDY	45	132	116.7	20
vh334	GPTGLLSKRATPADYDY	46	133	117.3	32
vh335	GPTGLLSNRATPADYDY	48	134	114.9	28
vh336	GPTGLLSQRATPADYDY	49	135	106.5	33
vh337	GPTGLLSRRATPADYDY	50	136	120.6	27
vh338	GPTGLLSNHATPADYDY	51	137	104.4	14
vh339	GPTGLLSQHATPADYDY	52	138	104.2	18
vh340	GPTGLLSRHATPADYDY	53	139	98.6	13

20 **Example 4: AAV9 binding polypeptides having variations in CDR1, CDR2, CDR3 and different frameworks**

This example further investigated a different set of CDR sequences in different sdAb frameworks. The candidate polypeptides tested were expressed as fusion protein in which the respective sdAb had SEQ ID NO: 161 fused to its C-terminal via a linker, and had a C-terminal (His)₆-
25 tag. The sdAb sequences variants are presented in Tables 10a-b.

The following aspects were evaluated: (1) Affinity assessment of AAV9 interaction (tested through high concentration injection of AAV9). (2) Alkaline stability (tested by reduction of AAV9 binding after increasing number of treatments with 0.5 M NaOH).

5 Table 10a

sdAb	FW variant	CDR1	CDR2	CDR3	sdAb SEQ ID NO:
vh118	1	RTLSDYTMG (SEQ ID NO: 16)	AISWSGAYTK (SEQ ID NO: 29)	GPTGLLSKKATPADYDY (SEQ ID NO: 55)	92
vh374	2	RTLSDFDMA (SEQ ID NO: 17)	AISWSGAYTY (SEQ ID NO: 26)	GPTGLLSRKTPADYQY (SEQ ID NO: 70)	141
vh359	3				140
vh428	1				146

Table 10b

FW variant	FWR1	FWR2	FWR3	FWR4
1	QVQLQQSGGGSVQ AGGSLRLSCTISG (SEQ ID NO:149)	WFRQRPKEREFA (SEQ ID NO:152)	YADSVKGRFTISRDNAKNTVY LQMNSLKPEDTAVYYCAA (SEQ ID NO: 155)	WGQGTQVTVSS (SEQ ID NO: 8)
2	QVQLQQSGGGSVQ AGGSLRLSCTSSG (SEQ ID NO:150)	WFRQRPGRAREFA (SEQ ID NO:153)	YTDSVKGRFTISRDNAKNTVF LQMQLKPEDTAIYYCAA (SEQ ID NO:156)	WGQGTQVTVSS (SEQ ID NO: 8)
3	QVQLVESGGGSVQA GGSLSLSCTISG (SEQ ID NO:151)	WFRQRPKEREFA (SEQ ID NO:154)	YTDSVKGRFTISRDNAKNTVY LQMQLKPEDTAVYYCAA (SEQ ID NO:157)	WGQGTQVTVSS (SEQ ID NO: 8)

Materials and methods

10 Generation of Immobilized Metal Affinity Chromatography (IMAC) purified candidate polypeptides

For each variant, 5 µl glycerol stock produced as described in Example 1 was inoculated in 4 ml LB culture medium supplemented with 100 µg/ml carbenicillin in 14 ml round-bottom tubes and grown over night at 37°C, 200 rpm agitation. Protein expression culture medium (TB medium supplemented with 100 µg/ml carbenicillin and 2 mM MgCl₂) was prepared and added to filled 500 ml baffled glass shake flasks (50 ml per flask, 7 flasks in total). Each flask was inoculated with approximately 500 µl from the previous overnight culture to obtain a starting OD600 of 0.05. The flasks were incubated in Infors HT shaking incubator for approximately 3.5 hours at 37°C and 140 rpm shaking until OD600 reached 1.0. Thereafter 50 µl IPTG (1 M) was added to a final concentration of 1 mM and the flasks were incubated in Infors HT shaking incubator at 27°C and 140 rpm shaking for 18 hours, whereafter the cultures were transferred to Falcon tubes (50 mL). To

generate crude variant lysates the Falcon tubes were incubated in 48°C water bath for 2 hours followed by pelleting of cell debris by centrifugation at 8000 g for 10 min and filtration of supernatant with a 0.45 µm filter.

Clarified lysate was loaded on HisTrap™ FF 1 mL chromatography columns (Cytiva™), equilibrated in 50 mM sodium phosphate pH 7.5, 500 mM NaCl, allowing a residence time of about 2 min. The column was then washed with 15 column volumes (CV) of 50 mM sodium phosphate pH 7.5, 500 mM NaCl. Protein was eluted using 0.5 M imidazole pH 7.5-8, linear gradient (0-100 %) over 10 CV. Eluted protein was buffer-exchanged into phosphate buffered saline (Medicago) pH 7.4 using gravity flow columns prepacked with Sephadex™ G-25 resin (Cytiva) prior to further analysis.

Concentration of HisTrap™ FF purified samples was measured using NanoDrop (Thermo Scientific) according to manufacturer's instructions. Following purification, samples were kept in refrigerator in Eppendorf tubes until all measurements were performed and were then kept in freezer until Biacore analysis. Protein mass was determined by liquid chromatography–mass spectrometry on a ACQUITY Rda Detector (Waters™) using a BioResolve column (Waters™).

Biacore analysis of binding affinity for AAV9

To assess binding to AAV9, the AAV9 binding polypeptides were immobilized on a Biacore™ Series S CM5 chip. AAV9 (2E12 viral particles (vp)/ml) was used as analyte.

Materials and equipment used were as follows: Biacore™ Series S CM5 sensor chips, Biacore™ Amine coupling kit, Biacore™ Acetate buffer pH 5.0, Biacore™ 8K+ instrument (all from Cytiva, Sweden); AAV9 (prepared in-house); candidate polypeptides generated as described above.

Immobilization was performed using a standard method in Biacore™ software with coupling of AAV9 binding polypeptide variants in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1). AAV9 binding polypeptide variants were diluted in acetate buffer pH 5.0 at a concentration of 25 µg/ml. The immobilization levels obtained were 3001 +/- 159 RU.

In each run the polypeptide was immobilized in FC2.

Running buffer: PBS-P+; Flow rate: 5 µl/min; Sample injection: 2400 s/40 min over both Flow Cells (FC1 and FC2); Dissociation time: 2400 s/40 min; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. Injections of analyte (AAV9) for each channel (cycles) were as follows: running buffer, AAV9 2E11 vp/ml, 5E11 vp/ml, 2E12 vp/ml.

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

Biacore analysis of alkaline stability

Assessment of alkaline stability was done on the Biacore™ chips prepared above. After evaluation of AAV9 binding (see above), the samples were subjected to followed by repeated cycles of binding to AAV9 (5E11 vp/ml) followed by injection of NaOH (0.5 M).

5 Biacore method for alkaline stability (per cycle): Running buffer: PBS-P+; Flow rate: 10 µl/min; Sample injection 1 (AAV9 5E11 vp/ml): 300 s over both Flow Cells; Dissociation time 1: 30 s; Sample injection 2 (0.5 M NaOH): 600 s over both flow cells; Dissociation time 2: 0 s; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. This cycle was repeated 40 times to follow stability of AAV9 response values.

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

Results

15 The response levels of AAV9 interactions of the candidate polypeptides using the maximum AAV9 concentration (2E12 vp/ml) was normalized to the response of vh118 and summarized in Table 11, recording alkaline stability as the number of cycles with at least 50 % binding capacity retained. Fig. 5 presents the result of the alkaline stability assessment, plotting the binding capacity against the no. of cycles. The binding response values were normalized against the binding capacity recorded for the second cycle (i.e., excluding the first cycle). In each cycle, 0.5 M NaOH was injected with a
20 contact time of 10 min at 10 µl/min.

 It was seen that candidates vh374, vh359 and vh428, which had the same set of CDRs but differed in the framework regions, had even higher alkaline stability than vh118. Comparing vh118 with vh428, which had identical framework regions, it was concluded that the CDRs of vh428/vh374/vh359 had a positive contribution to alkaline stability.

25

Table 11

	Normalized AAV9 binding capacity (%)	NaOH stability (no. of exposure cycles with at least 50% binding maintained)
vh118	100.0	22
vh374	102.2	30
vh359	86.4	34
vh428	107.6	40

Example 5A: Comparative study of AAV9 binding and stability

In this example, 7 candidate polypeptides were tested and compared to a commercially available affinity ligand (CaptureSelect™ Biotin Anti-AAV9 Conjugate, ThermoFischer Scientific). The candidate polypeptides were expressed as fusion proteins in which the respective sdAb had SEQ ID NO: 161 fused to its C-terminal via a peptide linker (AA), and all constructs had a C-terminal tag (HHHHHHC). The sdAb sequences variants are presented in Table 12.

Table 12

	CDR1	CDR2	CDR3	sdAb SEQ ID NO:
vh118	RTLSDYTMG	AISWSGAYTK	GPTGLLSKKATPADYDY	92
vh97	(SEQ ID NO:16)	(SEQ ID NO:29)	(SEQ ID NO:55)	91
vh376	RTLSDFTMA	AVSWSGAYTK	GPTGLLSKKATPPDYDY	142
	(SEQ ID NO:17)	(SEQ ID NO:28)	(SEQ ID NO:71)	
vh324	RTLSDFTMG (SEQ ID NO:13)	AISWSGAYTK (SEQ ID NO:29)	GPTGLLSKKTPADYDY (SEQ ID NO:56)	131
vh377			GPTGPLSKKTPADYDY (SEQ ID NO:72)	143
vh378			GPTGLLSKKTPADYDY	144
vh379			(SEQ ID NO:56)	145

Materials and methods

IMAC purified candidate polypeptides were generated as described in Example 4.

Biotinylation of purified candidate polypeptides

Purified candidate polypeptides were biotinylated at the C-terminal cysteine using EZ-Link™ Maleimide-PEG2-Biotin, No-Weigh™ Format (ThermoFisher Scientific) with a 2X molar excess of biotin.

SEC purification of biotinylated candidate polypeptides

Proteins were additionally purified by size exclusion chromatography (SEC) for separation from excess biotin, non-biotinylated protein dimers and other impurities. SEC was performed in phosphate buffered saline (Medicago) pH 7.4 at a flow rate of 0.75 mL/min using Superdex™ 75 Increase 10/300 GL chromatography column (Cytiva). Proteins of interest eluted as sharp symmetric peak. Purest fractions (as analyzed by SDS-PAGE) were pooled and used for further analyses.

Biacore analysis of binding affinity for AAV9

To assess binding to AAV9, the AAV9 binding polypeptide candidates and the reference CaptureSelect™ Biotin Anti-AAV9 Conjugate (Thermo Scientific™) were immobilized on a Biacore™ Series S SA chip. AAV9 (2E12 viral particles (vp)/ml) was used as analyte.

5 Materials and equipment used were as follows: Biacore™ Series S SA sensor chips, Biacore™ 8K+ instrument (all from Cytiva, Sweden); AAV9 (prepared in-house); candidate polypeptides generated as described above; CaptureSelect™ Biotin Anti-AAV9 Conjugate (Thermo Scientific™).

10 Immobilization was performed using a standard method in Biacore™ software with coupling of biotinylated AAV9 binding polypeptide variants and CaptureSelect™ Biotin Anti-AAV9 Conjugate (Thermo Scientific™) in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1). Biotinylated AAV9 binding polypeptide variants were diluted in PBS at a concentration of 100 µg/ml and CaptureSelect™ Biotin Anti-AAV9 Conjugate (Thermo Scientific™) was diluted in PBS at a concentration of 10 µg/ml.

15 Biacore™ method for binding analysis: Running buffer: PBS-P+; Flow rate: 5 µl/min; Sample injection: 2400 s/40 min over both Flow Cells (FC1 and FC2); Dissociation time: 2400 s/40 min; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. Injections of analyte (AAV9) for each channel (cycles) were as follows: running buffer, 2E11 vp/ml, 5E11 vp/ml, 2E12 vp/ml.

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

Biacore analysis of alkaline stability

25 Assessment of alkaline stability was done on the same Biacore™ chips as used for binding analysis as described above. After evaluation of AAV9 binding (see above), the samples were subjected to 88 repeating cycles of exposure to NaOH (0.3 M). Every second cycle included with binding to AAV9 (5E11 vp/ml) otherwise running buffer PBS-P+ was used as sample injection to reduce analyte consumption.

30 Biacore method for alkaline stability (per cycle): Running buffer: PBS-P+; Flow rate: 10 µl/min; Sample injection 1 (AAV9 5E11 vp/ml or running buffer PBS-P+ every second cycle to reduce analyte consumption): 300 s over both flow cells; Dissociation time 1: 30 s; Sample injection 2 (0.3 M NaOH): 600 s over both flow cells; Dissociation time 2: 0 s; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. This cycle was repeated 88 times to follow stability of AAV9 response values.

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

Results

Table 13 shows the immobilization levels obtained for the tested polypeptides, as well as the AAV9 binding capacity using the maximum AAV9 concentration, and the NaOH stability. The candidate polypeptides were expressed as fusion with SEQ ID NO: 161, and hence had a molecular weight that was higher than for an sdAb as such. The relative immobilization level is the immobilization level divided by the molecular weight.

Table 13

	MW (Da)	Immobilization level (RU)	Relative immobilization level (RU/Da)	AAV9 capacity, 2E12 vp/ml (RU)	NaOH stability (no. of exposure cycles with at least 50% binding retained)
vh118	21385	3479	0.16	12487	34
vh97	21215	6056	0.29	13109	26
vh376	21284	2032	0.10	10236	28
vh324	21386	2127	0.10	10722	36
vh377	21373	4598	0.22	12694	>88
vh378	21478	3568	0.17	12669	66
vh379	21161	5237	0.25	13808	72
CaptureSelect™ Biotin Anti-AAV9 Conjugate*	13000	4128	0.32	8855	4

*prior art

It was seen that despite being immobilized at lower molar levels than the prior art reference, CaptureSelect™ Biotin Anti-AAV9 Conjugate, the tested AAV9 binding polypeptides of the present invention had a markedly higher AAV9 binding capacity. Furthermore, the alkaline stability of the inventive candidates was exceedingly high. In fact, for one candidate, vh377, the AAV9 response never dropped below 50 % during the 88 cycles.

The result of the alkaline stability assessment is also shown in Fig. 6. During the alkaline stability analysis, the Biacore run stopped between cycle 28 and 30, hence the data point at cycle 30 was not measured. The run was continued, and next binding data was measured at cycle 32.

Example 5B: Comparative study of target selectivity

In this example, the binding selectivity for AAV9 was tested for the 7 candidate polypeptides of Example 5A and a commercially available AAV9 binding affinity ligand. The candidate polypeptides were expressed as fusion proteins in which the respective sdAb had SEQ ID NO: 161 fused to its C-terminal via a peptide linker (AA), and all constructs had a C-terminal tag (HHHHHC).

Materials and methods

IMAC purified candidate polypeptides generated in Example 5A were used.

Biotinylation of purified candidate polypeptides

Purified candidate polypeptides were biotinylated at the C-terminal cysteine using EZ-Link™

- 5 Maleimide-PEG2-Biotin, No-Weigh™ Format (ThermoFisher Scientific) with a 2X molar excess of biotin.

Dialysis of biotinylated candidate polypeptides

Proteins were separated from excess biotin by dialysis. Dialysis of biotinylated protein samples was performed according to Slide-A-Lyzer™ G2 Dialysis Cassette 3 mL 10 kDa MWCO
10 (Thermo Scientific™) protocol using PBS as dialysis buffer. Concentration of dialyzed samples were measured using NanoDrop (Thermo Scientific) according to manufacturer's instructions and were then kept in freezer until further analysis.

Biacore analysis of binding selectivity for different AAV serotypes

- To assess binding to different AAV serotypes, the candidate polypeptides as well as
15 CaptureSelect™ Biotin Anti-AAV9 Conjugate (Thermo Scientific™) were immobilized on a Biacore™ Series S SA chip. Tangential flow filtrated (TFF) AAV material for serotype AAV2, AAV5, AAV8 and AAV9 were used as analytes.

- Materials and equipment used were as follows: Biacore™ Series S SA sensor chips, Biacore™
8K+ instrument (all from Cytiva, Sweden); TFF material of AAV2, AAV5, AAV8, AAV9 (around 1E12
20 vp/ml for all AAV serotypes) (prepared in-house); candidate polypeptides generated as described above; CaptureSelect™ Biotin Anti-AAV9 Conjugate (Thermo Scientific™).

- Immobilization was performed using a standard method in Biacore™ software with coupling of biotinylated AAV9 binding polypeptide variants and CaptureSelect™ Biotin Anti-AAV9 Conjugate in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1). Biotinylated AAV9 binding
25 polypeptide variants were diluted in PBS at a concentration of 100 µg/ml and CaptureSelect™ Biotin Anti-AAV9 Conjugate was diluted in PBS at a concentration of 10 µg/ml. Immobilization was performed to get similar molar amounts of AAV9-binding polypeptides, hence different immobilization levels depending on molecular weight.

- Biacore™ method for binding analysis: Running buffer: PBS-P+; Flow rate: 10 µl/min; Sample
30 injection: 500 s over both Flow Cells (FC1 and FC2); Dissociation time: 500 s; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. Injections of analyte for each channel (cycles) were as follows: running buffer, AAV2 TFF, AAV5 TFF, AAV8 TFF, AAV9 TFF.

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

Results

Immobilization levels obtained are shown in Table 14. The candidate polypeptides were expressed as fusion with SEQ ID NO: 161, and hence had a molecular weight that was higher than for an sdAb as such.

Table 14

	Molecular weight (Da)	Immobilization level (RU)	Relative immobilization level (RU/Da)
vh118	21385	1207	0.09
vh97	21215	1841	0.06
vh376	21284	1258	0.06
vh324	21386	1139	0.05
vh377	21373	1497	0.07
vh378	21478	1312	0.06
vh379	21161	1915	0.09
CaptureSelect™ Biotin Anti-AAV9 Conjugate	13000	983	0.08

Fig. 7a- h are sensorgrams showing the binding response to TFF samples of different AAV serotypes: AAV9 (dashed line), AAV8 (dotted line), AAV2 (solid line), AAV5 (solid line). Also the running buffer response is shown in solid line. As clearly seen in these graphs, the candidates vh97 (Fig. 7a), vh118 (Fig. 7b), vh324 (Fig. 7c), vh376 (Fig. 7d), vh377 (Fig. 7e), vh378 (Fig. 7f) and vh379 (Fig. 7g) were selective for AAV9, with no binding to AAV2, AAV5 or AAV8 samples. In contrast, the reference affinity ligand also showed high binding to both AAV9 and AAV8 samples (Fig. 7h).

Example 6A: AAV9 binding polypeptide with or without C-terminal fusion partner

A single-domain antibody capable of binding AAV9 was fused to a stabilizing polypeptide and the impact on protein expression, antigen binding capacity and alkaline stability was investigated.

The polypeptides tested are identified in Table 15. A single-domain antibody (sdAb) was compared to a fusion of the same single-domain antibody with a stabilizing polypeptide. The single domain antibody (denoted "sdAb") was an AAV9-binding VHH variant (SEQ ID NO:92). The stabilizing polypeptide (denoted "Z") was an SpA domain Z variant (SEQ ID NO: 161) in which binding to the Fc and VH3 portions of IgG had been abolished. Each construct had a C-terminal linker followed by a (His)₆ tag.

Table 15

Candidate no.	Construct description	Construct sequence
6A-1	[sdAb]	SEQ ID NO:147
6A-2	[sdAb]-[Z]	SEQ ID NO:165

In particular, the following aspects were evaluated: (1) Affinity assessment of AAV9 interaction (tested through high concentration injection of AAV9). (2) Alkaline stability (tested by reduction of AAV9 binding after increasing number of treatments with 0.5 M NaOH).

Materials and methods

Generation of MabSelect Prisma™ purified polypeptides

5 µl glycerol stock produced for the candidates 6A-1 and 6A-2 as described in Example 1 was inoculated in 4 ml LB culture medium supplemented with 100 µg/ml carbenicillin in 14 ml round-bottom tube and incubated at 37°C overnight at 160 rpm.

Protein expression culture medium (TB culture medium supplemented with 100 µg/ml carbenicillin and 2 mM MgCl₂) was prepared and added to filled 500 ml baffled glass shake flasks (50 ml per flask). Each flask was inoculated with approximately 500 µl from the previous overnight culture to obtain a starting OD₆₀₀ of 0.05. The flasks were incubated in an Infors HT shaking incubator for approximately 3.5 hours at 37°C and 140 rpm shaking until OD₆₀₀ reached 1.0. Thereafter 50 µl of IPTG (1 M) was added to a final concentration of 1 mM and the flasks were incubated in Infors HT shaking incubator at 27°C and 140 rpm shaking for 18 hours, whereafter the cultures were transferred to Falcon tubes (50 ml). To generate crude variant lysates the Falcon tubes were incubated in 48°C water bath for 2 hours followed by pelleting of cell debris by centrifugation at 8000 g for 10 min and filtration of supernatant with a 0.22 µm filter.

Clarified lysate for each sample was loaded onto a 1 ml HiTrap MabSelect™ Prisma chromatography column (Cytiva™), equilibrated in phosphate buffered saline (PBS) pH 7.4, allowing a residence time of about 2.4 min. The column was then washed with 5 column volumes (CV) of PBS pH 7.4, followed by 5 CV of 50 mM sodium acetate pH 6. Protein was eluted using 50 mM sodium acetate pH 3.5 in a step gradient over 5 CV.

Concentration of the purified samples was measured using NanoDrop (ThermoFisher Scientific) according to manufacturer's instructions. Following purification, samples were kept in refrigerator in Eppendorf tubes until all measurements had been performed and were then kept in freezer until Biacore analysis. Protein mass was determined by liquid chromatography–mass spectrometry on a ACQUITY Rda Detector (Waters™) using a BioResolve column (Waters™).

Biacore analysis of binding affinity for AAV9

To assess binding to AAV9, the candidates were immobilized on a Biacore™ Series S CM5 chip using Amine coupling kit (Cytiva™) and analyzed using Biacore™ 8K+ instrument (Cytiva™). A sample containing AAV9 viral particles (vp) at a concentration of 2E12 vp/ml was used as analyte.

5 Immobilization was performed using a standard method in Biacore software with coupling of the polypeptides in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1). Polypeptides were diluted in Biacore™ Acetate buffer pH 5.0 (Cytiva™) at a concentration of 17-30 µg/ml, depending on their molecular weight. The immobilization levels varied between different polypeptide variants corresponding to their molecular weight. In each run the polypeptide was
10 immobilized in FC2.

Biacore™ method for binding analysis: Running buffer: PBS-P+; Flow rate: 5 µl/min; Sample injection: 2400 s/40 min over both FC1 and FC2; Dissociation time: 2400 s/40 min; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. Injections of analyte (AAV9) for each channel (cycles) were as follows: running buffer, AAV9 2E11 vp/ml, 5E11 vp/ml, 2E12 vp/ml.

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

Biacore analysis of alkaline stability

After evaluation of AAV9 binding (see above) the same chips were subjected to repeated
20 cycles of binding to AAV9 (5E11 vp/ml) followed by injection of NaOH (0.5 M) for the assessment of alkaline stability.

Biacore method for alkaline stability (per cycle): Running buffer: PBS-P+; Flow rate: 10 µl/min; Sample injection 1 (AAV9 5E11 vp/ml): 300 s over both flow cells; Dissociation time 1: 30 s; Sample injection 2 (0.5 M NaOH): 600 s over both flow cells; Dissociation time 2: 0 s ;
25 Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. This cycle was repeated 40 times to follow stability of AAV9 response values.

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

30 *Results*

Analysis of expression level by Bis-Tris PAGE indicated that expression levels of polypeptide 6A-1 (sdAb, no fusion) and 6A-2 (sdAb with C-terminal stabilizing polypeptide) were comparable (data not shown).

Immobilization levels on CM5 chip were 2134 or 3560 RU, respectively. The AAV9 binding capacity at the maximum viral concentration (2E12 vp/ml) used was about 10 % higher for the tested fusion protein 6A-2 compared to the sdAb 6A-1.

Fig. 8A plots the AAV9 binding capacity for increasing number of cycles involving 0.5 M NaOH exposure (sample injection 2 above) normalized to the response after completing the first cycle (i.e., excluding the first cycle). As can be seen in this figure, the fusion protein 6A-2, comprising a single-domain antibody variant fused at its C-terminal to a stabilizing polypeptide, shows improved alkaline stability over the single-domain antibody 6A-1 alone. The improvement is even more pronounced if the response after each cycle is normalized to the response of the first cycle, before the first NaOH exposure, as shown in Fig. 6B. As can be seen in Fig. 6B, the drop in AAV9 binding capacity experienced between the first and second cycles is merely 20 % for fusion protein, compared to the 60 % reduction in response for the sdAb which is not part of a fusion protein.

Table 16 summarizes the results of the AAV9 binding capacity and alkaline stability measurements.

Table 16

Sample	AAV9 capacity (2E12 vp/ml) (RU)	Alkaline stability (0.5 M NaOH)	
		No. of cycles with at least 50% binding maintained (100%= response of 1 st cycle)	No. of cycles with at least 50% binding maintained (100%= response of 2 nd cycle)
6A-1	11292	1	10
6A-2	12421	14	19

Example 6B: AAV9 binding multimeric fusion proteins

Multimeric constructs of a single-domain antibody capable of binding AAV9 fused to a stabilizing polypeptide were investigated with respect to protein expression, antigen binding capacity and alkaline stability.

The polypeptides tested are outlined in Table 17 below. A single-domain antibody (sdAb) trimer was compared to trimeric fusion proteins containing one or three stabilizing polypeptides, respectively. The single domain antibody (denoted “sdAb”) was an AAV9-binding VHH variant (SEQ ID NO: 92). The stabilizing polypeptide (denoted “Z”) was a SpA domain Z variant (SEQ ID NO: 161) in which binding to the Fc and VH3 portions of IgG had been abolished. Each construct had a C-terminal linker followed by a (His)₆-Cys tag.

Table 17

Candidate no.	Description	Construct sequence
6B-1	[sdAb]-[sdAb]-[sdAb]	SEQ ID NO:166
6B-2	[sdAb]-[sdAb]-[sdAb]-[Z]	SEQ ID NO:167
6B-3	[sdAb]-[Z]-[sdAb]-[Z]-[sdAb]-[Z]	SEQ ID NO:168

The following aspects were evaluated: (1) Protein expression analysis by PAGE. (2) Affinity assessment of AAV9 interaction (tested through high concentration injection of AAV9). (3) Alkaline stability (tested by reduction of AAV9 binding after increasing number of treatments with 0.3 M NaOH).

Materials and methods

Generation of MabSelect Prisma™ purified and biotinylated polypeptides

10 Candidate polypeptides were generated and purified according to the method described in Example 6A.

Concentration of MabSelect Prisma™ purified samples was measured using NanoDrop (Thermo Scientific) according to manufacturer's instructions. Following purification, samples were reduced by adding 10 µl 0.5 M DTT dissolved in sterile water to 490 µl sample to reach a final DTT concentration of 10 mM. The samples were incubated in room temperature for 1 hour, followed by buffer exchange to PBS with Amersham NAP-5 columns (Cytiva™) according to protocol, resulting in 1 ml eluates. EZ-Link™ Maleimide-PEG2-Biotin, No weight™ format (Thermo Scientific) was dissolved in 190 µl PBS according to protocol and 5 µl dissolved biotin was added to each 1 ml eluate, to reach a final 2-5 molar excess of biotin. Samples were incubated on ice for 2 hours, followed by dialysis according to Slide-A-Lyzer™ G2 Dialysis Cassette (Thermo Scientific) protocol. The concentration of dialyzed samples was measured using NanoDrop (Thermo Scientific) according to manufacturer's instructions and kept in refrigerator in Eppendorf tubes until all measurements had been performed and were then kept in freezer until further analysis. Protein mass was determined by liquid chromatography-mass spectrometry on a ACQUITY Rda Detector (Waters™) using a BioResolve column (Waters™).

Bis-Tris PAGE analysis

To evaluate the protein expression levels, a Bis-Tris PAGE was performed on filtered supernatant post heat treatment. Materials and equipment used were as follows: NuPAGE LDS

sample buffer (x4), Page Ruler prestained, 4-12% Bis-Tris minigel, Invitrogen NP0321BOX, Mini-gel tank (Invitrogen), 20x MES buffer (Invitrogen), QuickBlue Dye.

15 µl of filtered supernatant from post heat treatment was mixed with 5 µl LDS sample buffer. The sample mix was heated to 70°C for 10 minutes and lightly centrifuged to collect condensate from the tube cap. 15 µl sample mix for each expressed construct was loaded on the gel. 5 µl PageRuler was loaded as marker. The gel was run at 200 V for 35 minutes and stained in QuickBlue dye for 2 hours. The gel was destained in deionized water overnight.

Analysis of binding affinity for AAV9

10 To assess binding to AAV9, the candidates were immobilized on a Biacore™ Series S SA chip (Cytiva™) and analyzed using Biacore™ 8K+ instrument (Cytiva™). A sample containing AAV9 viral particles (vp) at a concentration of 2E12 vp/ml was used as analyte.

Immobilization was performed using a standard SA coupling method in Biacore software with coupling of the polypeptides in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1). The polypeptides were diluted in PBS-P+ (Cytiva™) at a concentration of approximately 25 µg/ml. The immobilization levels varied somewhat between different polypeptide variants corresponding to their molecular weight. In each run the polypeptide was immobilized in FC2.

Biacore™ method for binding analysis: Running buffer: PBS-P+ ; Flow rate: 5 µl/min ; Sample injection: 2400 s/40 min over both FC1 and FC2; Dissociation time: 2400 s/40 min ; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. Injections of analyte (AAV9) for each channel (cycles) were as follows: running buffer, AAV9 2E11 vp/ml, 5E11 vp/ml, 2E12 vp/ml.

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

25 Analysis of alkaline stability

After evaluation of AAV9 binding (see above) the same chips were subjected to repeated cycles of binding to AAV9 (5E11 vp/ml) followed by injection of NaOH (0.3 M) for the assessment of alkaline stability.

Biacore™ method for alkaline stability (per cycle): Running buffer: PBS-P+ ; Flow rate: 10 µl/min ; Sample injection 1 (AAV9 5E11 vp/ml): 300 s over both flow cells ; Dissociation time 1: 30 s ; Sample injection 2 (0.3 M NaOH): 600 s over both flow cells ; Dissociation time 2: 0 s ; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. This cycle was repeated 40 times to follow stability of AAV9 response values.

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

Results

5 Analysis of expression level by Bis-Tris PAGE (Fig. 9A) indicated that the multimeric fusion proteins comprising a stabilizing polypeptide could improve protein expression.

Immobilization levels on SA chip were in the range of 3000-3500 RU. The AAV9 binding capacity at the maximum viral concentration used was higher for the tested fusion proteins 6B-2 and 6B-3 compared to the sdAb trimer without a stabilizing polypeptide.

10 Fig. 9B plots the AAV9 binding capacity for increasing number of cycles involving 0.3 M NaOH exposure (sample injection 2 above) normalized to the response after completing the first cycle (i.e., excluding the first cycle). The first cycle may remove loosely bound polypeptides from the surface. As can be seen in this figure, the fusion proteins 6B-2 and 6B-3 both show notable improvement in alkaline stability over the sdAb trimer 6B-1. Normalizing the response to the response of the first
15 cycle, it was found that 6B-3 experienced a larger drop in response between the first and second cycles than 6B-1 or 6B-2, but for the subsequent cycles remained more stable than 6B-1, which lost more of its binding capacity over the course of the test than the fusion proteins 6B-2 and 6B-3.

Table 18 summarizes the results of the AAV9 binding capacity and alkaline stability measurements.

20

Table 18

Sample	AAV9 response, report point analyte binding late (2E12 vp/ml, RU)	Alkaline stability (0.3 M NaOH)	
		No. of cycles with at least 50% binding maintained (100% = response of 1 st cycle)	No. of cycles with at least 50% binding maintained (100% = response of 2 nd cycle)
6B-1	13410	12	20
6B-2	14328	21	30
6B-3	14991	11	28

Example 7: AAV9 binding polypeptide with C-terminal fusion partner

This example compares single domain antibodies having different framework sequences,
25 wherein one candidate had a framework that was modified to enhance alkaline stability. The following aspects were evaluated: (1) Affinity assessment of AAV9 interaction (tested through high concentration injection of AAV9). (2) Alkaline stability (tested by reduction of target binding after increasing number of treatments with 0.5 M NaOH).

The single domain antibodies were VHH variants directed against AAV9. CDR regions were the same, but the candidates differed with regard to the amino acid sequence of the framework regions.

Each VHH was expressed as a fusion protein, fused C-terminally to an SpA domain Z variant (SEQ ID NO: 162) capable of binding to immunoglobulin Fc but not VH3.

The tested polypeptide candidates (fusion proteins) are outlined in Table 19.

Table 19

Candidate no.	VHH variant	Type of VHH framework	VHH sequence	Full protein sequence
7-1	vh97	Synthetic, partly stabilized	SEQ ID NO:91	SEQ ID NO:163
7-2	vh118	Synthetic, stabilized	SEQ ID NO:92	SEQ ID NO:164

10 *Materials and methods*

Preparation of glycerol stock solutions from IDT oligo

Materials and equipment used were as follows: vector plasmid with kanamycin resistance; G-block polypeptide candidates (IDT™); restriction enzymes KpnI-HF and HindIII-HF (NEB™); Antarctic phosphatase (NEB™); GFX™ PCR DNA and Gel Band Purification Kit (Cytiva™); T4 DNA ligase (New England Biolabs); Top10 *E. coli* cells (ThermoFisher™); chemically competent BL21(DE3) *E. coli* cells (prepared in-house); chemically competent K12-017 *E. coli* cells (prepared in-house); SOC-media (Invitrogen™); stock of kanamycin 50 mg/ml (PanReac AppliChem™); KCM buffer (5X) 0.5M KCl, 0.15M CaCl₂, 0.25M MgCl₂; Luria-Bertani (LB) culture medium (Invitrogen™); agar plate supplemented with kanamycin (50 µg/ml); 14 ml Falcon™ Round-Bottom tube (Corning™); PlasmidPrep Mini Spin Kit (Cytiva™); Infors HT Shaking incubator.

The vector plasmid and G-blocks containing the DNA sequences for all candidates were digested with restriction enzymes KpnI-HF and HindIII-HF. The plasmid was dephosphorylated using Antarctic phosphatase and gel purified according to manufacturer's protocols.

Restriction enzyme digested G-blocks were ligated into the gel purified plasmid using T4 DNA ligase for 2h at room temperature and transformed into Top10 *E. coli* cells. Briefly, cells and ligation mix were incubated for 20 min on ice and 10 min at room temperature in KCM-buffer. Cells were then transferred to SOC media and incubated at 37°C for 1h and then seeded on agar plates containing kanamycin 50 µg/ml and incubated at 37°C over night. A single colony was used to inoculate 4 mL LB media supplemented with 50 µg/ml kanamycin in a 14 ml round-bottom tube and grown over night at 37°C, 200 rpm. The plasmid was prepared according to manufacturer's protocols

and the purified plasmid was transformed into either KCM competent K12-017 E. coli or KCM competent BL21(DE3) E. coli. Cells and plasmid were incubated for 20 min on ice and 10 min at room temperature in KCM-buffer. Cells were then transferred to SOC media and incubated at 37°C for 1h and then seeded on agar plates containing kanamycin 50 µg/ml and incubated at 37°C over night. A single colony was used to inoculate 4 mL LB media supplemented with 50 µg/ml kanamycin in a 14 mL round-bottom tube and grown over night at 37°C, 200 rpm. A glycerol stock of each variant was prepared with 900 µl overnight culture and 500 µl 50% glycerol and stored at -80°C.

Protein expression and purification

For each candidate 5 µl BL21(DE3) glycerol stock produced as described above was inoculated in 4 ml LB culture medium supplemented with 100 µg/ml carbenicillin in 14 ml round-bottom tube and incubated at 37°C overnight at 160 rpm.

Protein expression culture medium (TB medium supplemented with 50 µg/ml kanamycin and 2 mM MgCl₂) was prepared and added to filled 100 ml baffled glass shake flasks (20 ml per flask). Each flask was inoculated with approximately 100 µl from the previous overnight culture to obtain a starting OD₆₀₀ of 0.05. The flasks were incubated in Infors HT shaking incubator for approximately 3 hours at 37°C and 140 rpm shaking until OD₆₀₀ reached 1.0. Thereafter 20 µl IPTG (1 M) was added to a final concentration of 1 mM and the flasks were incubated in Infors HT shaking incubator at 27°C and 140 rpm shaking for 18 hours, whereafter the cultures were transferred to Falcon tubes (50 mL). To generate crude variant lysates the Falcon tubes were incubated in 48°C water bath for 2 hours followed by pelleting of cell debris by centrifugation at 8000 g for 10 min and filtration of supernatant with a 0.22 µm filter.

The samples were purified using gravity flow in PD-10 columns (Cytiva™) packed in-house with IgG Sepharose 6FF chromatography resin (Cytiva™). Clarified lysate was loaded on the column, equilibrated in 50 mM Tris, 150 mM NaCl, 0.05% Tween20 (TST buffer). The column was washed with 10 column volumes (CV) of TST buffer followed by 2 CV of 2 CV 5 mM NH₄Ac pH 5. Protein was eluted using 2.5 ml 0.5 M Hac. Eluted protein was buffer-exchanged into phosphate buffered saline (Medicago) pH 7.4 using gravity flow columns PD-10 (Cytiva) prior to further analysis.

Concentration of IgG Sepharose purified samples was measured using NanoDrop (Thermo Scientific) according to manufacturer's instructions. Following purification, samples were kept in refrigerator in Eppendorf tubes until all measurements were performed and were then kept in freezer until Biacore analysis. Protein mass was determined by liquid chromatography–mass spectrometry on a ACQUITY Rda Detector (Waters™) using a BioResolve column (Waters™).

Biacore analysis of AAV9 binding affinity

To assess binding to AAV9, candidate polypeptides were immobilized on a Biacore™ Series S CM5 chip using Amine coupling kit and analyzed with a Biacore™ 8K+ instrument (all from Cytiva).

The analytes used was AAV9 (prepared in-house).

5 Immobilization was performed using a standard method in Biacore software with coupling of polypeptide variants in Flow Cell 2 (FC2) and activation/inactivation in Flow cell 1 (FC1). AAV9 binding candidates were diluted in Biacore™ Acetate buffer pH 5.0 (Cytiva™) at a concentration of 25 µg/ml, and the average immobilization levels was 4319 RU +/-640 RU. The polypeptides were immobilized in FC2.

10 Biacore™ method for binding analysis: Running buffer: PBS-P+ ; Flow rate: 5 µl/min ; Sample injection: 2400 s/40 min over both FC1 and FC2; Dissociation time: 2400 s/40 min ; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. Injections of analyte (AAV9) for each channel (cycles) were as follows: running buffer, 1.25E11 vp/ml, 5E11 vp/ml, 2E12 vp/ml.

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

Biacore analysis of alkaline stability

20 After the binding analysis (see above) the same CM5 chips having immobilized polypeptides were subjected to repeated cycles of binding to AAV9 followed by injection of NaOH for the assessment of alkaline stability (0.5 M).

 Biacore method (per cycle): Running buffer: PBS-P+ ; Flow rate: 10 µl/min ; Sample injection 1 (AAV9 3E11 vp/ml): 300 s over both flow cells ; Dissociation time 1: 30 s; Sample injection 2 (0.5 M NaOH): 600 s over both flow cells; Dissociation time 2: 0 s ; Regeneration: 10 mM Glycine-HCl pH 1.5, 30 µl/min, 2x30 s. This cycle was repeated 30 times to follow stability of target response values.

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

Results

 Both fusion proteins showed satisfactory binding to AAV9.

30 Table 20 summarizes the results of the binding capacity and alkaline stability measurements.

Table 20

Sample	AAV9 capacity (2E12 vp/ml) (RU)	Alkaline stability (0.5 M NaOH)	
		No. of cycles with at least 50% binding maintained (100%= response of 1 st cycle)	No. of cycles with at least 50% binding maintained (100%=response of 2 nd cycle)
7-1	14983	9	10
7-2	12193	22	24

Fig. 10 shows the binding capacities obtained for increasing number of cycles involving NaOH exposure (sample injection 2) as normalized to the response of the first cycle (i.e., including the first cycle). As can be seen in the figure, the AAV9-binding polypeptide which was based on stabilized VHH framework (7-2) showed improved alkaline stability in relation to the corresponding candidate (7-1) having a VHH framework that had not been subject to stabilizing modifications to the same extent.

Example 8: Functional evaluation – affinity chromatography

This example demonstrates with a basic experiment the functionality of an AAV9 binding polypeptide as an affinity ligand immobilized on a separation matrix.

Materials and methods

AAV9 binding candidate vh118 (SEQ ID NO:92) was produced as a fusion protein with SEQ ID NO: 161 and purified as described in Example 6A.

The fusion protein (SEQ ID NO:170) was immobilized on epoxy activated chromatography resin (highly crosslinked agarose beads) using cysteine coupling. 0.2 ml of the resin was packed in Tricorn 5/20 columns (Cytiva). Using an ÄKTA pure 25 system (Cytiva), 50 ml of tangential flow filtered (TFF) AAV9 material (prepared in-house) was loaded on the column with four minutes residence time and eluted with 100 mM citrate buffer pH 2.5.

Collected eluate fractions were analyzed on a Coomassie SDS-PAGE gel. The low pH elution samples were neutralized with 1:1 volume of Tris buffer (400 mM). 15 µl of sample and 5 µl sample buffer + DTT was heated to 70 °C for 10 min. 5 µl sample was loaded and the gel was run for 35 minutes at 200V and then soaked in Coomassie solution overnight. The gel was then de-stained with water until satisfactory staining was achieved. The gel was photographed using ImageQuant 800.

To assess the alkaline stability of the immobilized fusion protein, the chromatography resin was subjected to cleaning-in-place (CIP) with 10 mM Glycine-HCl pH 1.5, followed by an alkaline stability study involving exposure to 0.5 M NaOH. The full stability study consisted of 40 cycles where each cycle involved contacting the chromatography material with 0.5 M NaOH for 30 min. A solution

containing pure AAV9 particles was added every 10th cycle with 15 seconds residence time. PBS buffer was used for all other cycles.

Results

5 Fig. 11a shows the chromatogram obtained for purification of AAV9 TFF material using the candidate antigen-binding polypeptide as an affinity ligand. The elution peak is marked with a rectangle. Fig. 11b shows only the region of the elution peak. Fig. 12 shows the photograph of the gel. VP1, VP2 and VP3 denotes the AAV9 virion proteins 1, 2 and 3, respectively.

The results show that the present candidate was able to successfully purify AAV9 viral
10 particles, with an estimated dynamic binding capacity (QB10%) of about 3E+14 viral particles/ml resin and estimated recovery of loaded virus particles of 70-80%.

The result of the alkaline stability assessment is shown in Fig. 13 which plots the binding capacity normalized to the binding capacity before the first NaOH exposure. Over the 40 cycles of exposure to 0.5 M NaOH, which corresponded to an accumulated exposure time of 20 hours, the
15 AAV9 binding capacity was reduced by less than 20 %.

Example 9: AAV9 binding polypeptides in different frameworks and two sets of CDRs

This example further investigated five additional distinct sdAb frameworks with the same sdAb CDR sequences. One framework was also tested with two different sets of CDR sequences. All
20 sdAbs have the same CDR1 and CDR2 sequences, while vh516 had a distinct CDR3 from vh511-vh515. Vh511-515 have the same CDRs as vh324 but have different frameworks. Vh324 has framework variant 1, while vh511-515 has five new framework permutations, as shown in the tables below.

The candidate polypeptides tested were expressed as fusion protein in which the respective sdAb had SEQ ID NO: 161 fused to its C-terminal via a linker, and had a C-terminal (His)₆-tag. The
25 sdAb sequences variants are presented in Tables 21 and 22.

The following aspects were evaluated: (1) Affinity assessment of AAV9 interaction (tested through high concentration injection of AAV9). (2) Alkaline stability (tested by reduction of AAV9 binding after increasing number of treatments with 0.3 M NaOH).

30 Table 21

sdAb	FW variant	CDR1	CDR2	CDR3	sdAb SEQ ID NO:
vh511	4	RTLSDFTMG (SEQ ID NO: 13)	AISWSGAYTK (SEQ ID NO: 29)	GPTGLLSKKTTPADYDY (SEQ ID NO: 56)	177
vh512	5				178
vh513	6				179

vh514	7				180
vh515	8				181
vh516	8	RTLSDFTMG (SEQ ID NO: 13)	AISWSGAYTK (SEQ ID NO: 29)	GPTGLLSQKTPADYDY (SEQ ID NO: 173)	182

Table 22

FW variant	FWR1	FWR2	FWR3	FWR4
1	QVQLQQSGGGSVQ AGGSLRLSCTISG (SEQ ID NO:149)	WFRQRP GKERE FVA (SEQ ID NO:152)	YADSVKGRFTISRDN AKNTV WLQMNSLKPEDTAVYYCAA (SEQ ID NO: 155)	WGQGTQVTVSS (SEQ ID NO: 8)
4	QVQLQQSGGGSVQ AGGSLSLSCTSSG (SEQ ID NO: 183)	WFRQRP GKERE FVA (SEQ ID NO: 187)	YADSVKGRFTISRDN AKNTV WLQMNSLKPEDTAVYYCAA (SEQ ID NO:190)	WGQGTQVTVSS (SEQ ID NO: 8)
5	QVQLQQSGGGSVQ AGGSLRLSCTSSG (SEQ ID NO:184)	WFRQIP GKERE FVA (SEQ ID NO:188)	YADSVKGRFTISRDN AKQTV WLQMNSLKPEDTAVYYCAA (SEQ ID NO:191)	WGQGTQVTVSS (SEQ ID NO: 8)
6	QVQLQQSGGGSVQ AGGSLRLSCTSSG (SEQ ID NO:184)	WFRQRP GKDL E FVA (SEQ ID NO:189)	YADSVKGRFTISRDN AKNTV WLQMNSLKPEDTAIYYCAA (SEQ ID NO: 192)	WGQGTQVTVSS (SEQ ID NO: 8)
7	QVQLQQSGGGSVQ AGGSLRLSCTVSG (SEQ ID NO: 185)	WFRQRP GKERE FVA (SEQ ID NO:187)	YADSVKGRFTISRDN AKNTV WLQMNSLKPEDTAVYYCAA (SEQ ID NO:190)	WGQGTQVTVSS (SEQ ID NO: 8)
8	QVQLQQSGGGSVQ AGGSLTL SCTVSG (SEQ ID NO:186)	WFRQRP GKERE FVA (SEQ ID NO:187)	YADSVKGRFTISRDN AKNTV WLQMNSLKPEDTAVYYCAA (SEQ ID NO:190)	WGQGTQVTVSS (SEQ ID NO: 8)
8	QVQLQQSGGGSVQ AGGSLTL SCTVSG (SEQ ID NO:186)	WFRQRP GKERE FVA (SEQ ID NO:187)	YADSVKGRFTISRDN AKNTV WLQMNSLKPEDTAVYYCAA (SEQ ID NO:190)	WGQGTQVTVSS (SEQ ID NO: 8)

Materials and methods

5 IMAC purified candidate polypeptides were generated as described in Example 5, and the Biacore analysis of binding affinity and stability were performed correspondingly.

Results

10 The response levels of AAV9 interactions of the candidate polypeptides using the maximum AAV9 concentration (2E12 vp/ml) was normalized to the response of vh377 and summarized in Table 23, recording alkaline stability as the number of cycles with at least 50 % binding capacity retained. The binding response values were normalized against the binding capacity recorded for the second cycle (i.e., excluding the first cycle). In each cycle, 0.3 M NaOH was injected with a contact time of 10 min at 10 µl/min.

It was seen that candidates vh511-515, which had the same set of CDRs but differed in the framework regions, had even higher alkaline stability than vh377. Comparing vh516 with vh515, which had identical framework regions, it was concluded that the different CDRs had an equal positive contribution to alkaline stability.

5

Table 23

	Normalized AAV9 binding capacity (%)	NaOH stability (no. of exposure cycles with at least 50% binding maintained)
vh377	100.0	76
vh511	119	46
vh512	111	56
vh513	118	60
vh514	113	69
vh515	122	46
vh516	119	46

Example 10: Measuring ligand alkaline stability

The alkaline stability was measured by assessing polypeptides with target binding to AAV9 (5e11 vp/ml) or binding to ZVH3 hexamer (MabSelect™ VH3 ligand) (50nM) that interacts with the ligand’s backbone. Alternating AAV9 or ZVH3 binding, the polypeptides were exposed to NaOH (0.5M) for 10 minutes for a total of 40 cycles. This was to show that both the target binding CDRs, and the framework, add to the alkaline stability of the polypeptide. The data attained from the present example was compared to the results for previously tested AAV9-binding sdAbs (results shown in patent application EP23204844.7).

15

Materials and methods

The ZVH3 hexamer was provided as described in WO2023174900A1, the hexamer having the sequence according to SEQ ID NO: 177 of WO2023174900A1, which is a hexamer of the monomer as defined by SEQ ID NO: 15 of WO2023174900A1, which is incorporated by reference herein.

Biacore analysis of alkali stability

20

Materials and equipment used were as follows: Biacore™ Series S CM5 sensor chips, Biacore™ Amine coupling kit, Biacore™ Acetate buffer pH 5.0, Biacore™ 8K+ instrument (all from Cytiva, Sweden); 0.5 M NaOH; ZVH3-ligand; candidate polypeptides generated as described above. Assessment of alkali stability was done on the Biacore™ chips prepared above. The polypeptides were subjected to 40 repeating cycles of binding to AAV9 (5e11vp/ml) or ZVH3 hexamer (50nM), followed by 10 minute

25

exposure to NaOH (0.5 M). The NaOH stability of the polypeptides was assessed by comparing the cycle number when the polypeptides had lost 50% of their initial binding to AAV9 or ZVH3 hexamer.

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

5 Results

The results of the alkaline stability assessment for the polypeptides are summarized in Table 24 below. The polypeptide CDR AAV9 binding is compared to the AAV9 ZVH3 backbone binding for the AAV9 binding polypeptides vh260-268. It may be seen that the alkaline stability is increased also for this target. The unstable polypeptides vh267 and vh268 are unstable for both bindings.

10 Table 24

	SEQ ID NO	NaOH stability cycle with 50% binding AAV9	NaOH stability cycle with 50% binding ZVH3
vh260	SEQ ID NO: 195	22	23
vh261	SEQ ID NO: 196	22	21
vh262	SEQ ID NO: 197	21	21
vh263	SEQ ID NO: 198	22	19
vh264	SEQ ID NO: 199	22	22
vh265	SEQ ID NO: 200	21	22
vh266	SEQ ID NO: 201	25	24
vh267	SEQ ID NO: 202	1	2
vh268	SEQ ID NO: 203	1	2

Example 11: Substituting first position of the framework from Q to E

In this experiment it was investigated whether using an E instead of a Q in the first position of the framework (FW1) would affect the stability of the polypeptide.

15 Materials and methods

Four polypeptides were tested, vh118 (SEQ ID NO:92) and vh498 (SEQ ID NO: 171), where vh498 differs from vh118 in that the first position is substituted from a Q to an E. Similarly, vh324 (SEQ ID NO: 131) and vh458 (SEQ ID NO: 172) were tested, where vh458 has the first position substituted from Q to E. The constructs were produced and tested as in Example 3A above, with purification on

20 PrismaA and coupling to CM5 chip.

Results

Even though the stability of the polypeptide was a little bit lower for the polypeptides having E as the first amino acid in the framework, it was demonstrated that also using E in the first position of FW1 give rise to highly stable polypeptides, as summarized in table 25 below. Vh118 substituted with E in position 1 is denoted vh118 (Q1E). vh324 is denoted correspondingly.

Table 25

	SEQ ID NO:	NaOH stability (no. of exposure cycles with at least80% binding maintained)
vh118	92	8
vh118 (Q1E)	171	7
Vh324	131	29
Vh324 (Q1E)	172	15

LIST OF ITEMIZED EMBODIMENTS

1. An antigen-binding polypeptide capable of binding adeno-associated virus serotype 9 (AAV9), the polypeptide comprising a single-domain antibody (sdAb) variant having complementarity determining regions CDR1, CDR2 and CDR3, wherein

- CDR1 comprises an amino acid sequence selected from the group of sequences defined by SEQ ID NO: 1:

$X_1X_2X_3SX_5X_6TMX_9$ (SEQ ID NO: 1)

wherein, independently,

- X_1 is R, L or S, preferably R or L;
- X_2 is T or R, preferably T;
- X_3 is L or F, preferably L;
- X_5 is D, N or E, preferably D;
- X_6 is Y, N or F, preferably Y or F;
- X_9 is G or A, preferably G;

- CDR2 comprises a sequence selected from the group of sequences defined by defined by SEQ ID NO: 2:

$X_1X_2SWSGX_7X_8TX_{10}$ (SEQ ID NO: 2)

wherein, independently,

- X_1 is A, S, L, V or I, preferably A;

X₂ is I or V, preferably I;
X₇ is A or S, preferably A;
X₈ is Y or F, preferably Y;
X₁₀ is K, F or Y;

5

and

- CDR3 comprises a sequence selected from the group of sequences defined by defined by SEQ ID NO: 3:

X₁X₂TX₄X₅X₆X₇X₈X₉X₁₀X₁₁X₁₂X₁₃X₁₄X₁₅X₁₆X₁₇ (SEQ ID NO: 3)

10

wherein, independently,

X₁ is G or A, preferably G;
X₂ is P or S, preferably P;
X₄ is G or P, preferably G;
X₅ is L, T or P, preferably L or P;
X₆ is L or I, preferably L;
X₇ is S, T or A, preferably S or A;
X₈ is K, R, N or Q;
X₉ is K, H or R, preferably K or R;
X₁₀ is A, S or T, preferably A or T;
X₁₁ is T, S, P or A, preferably T;
X₁₂ is P, A, T or S, preferably P;
X₁₃ is A, P, R or G, preferably A or P;
X₁₄ is D, N, E or Q, preferably D or E;
X₁₅ is Y or F, preferably Y;
X₁₆ is D, N, E or Q, preferably D; and
X₁₇ is Y, F or R, preferably Y or F.

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2. The antigen-binding polypeptide according to item 1, which is a single-chain antigen-binding polypeptide.

30

3. The antigen-binding polypeptide according to item 1 or 2, wherein the polypeptide lacks antibody heavy chain constant domains.

4. The antigen-binding polypeptide according to any one of the preceding items, wherein said sdAb variant is a variable domain of a heavy chain of a heavy chain antibody (VHH) variant.

5. The antigen-binding polypeptide according to any one of the preceding items, wherein the polypeptide does not contain an antibody light chain or domain thereof, such as a light chain variable fragment (VL).

6. The antigen-binding polypeptide according to any one of the preceding items, wherein the
5 sdAb variant comprises four framework regions FWR1, FWR2, FWR3 and FWR4.

7. The antigen-binding polypeptide according to any one of the preceding items wherein the sdAb variant comprises framework regions and complementary determining regions in the following order from N-terminus to C-terminus: [FWR1]-[CDR1]-[FWR2]-[CDR2]-[FWR3]-[CDR3]-[FWR4].

8. The antigen-binding polypeptide according to item 7, wherein at least one of said
10 framework regions comprises at least one an amino acid substitution relative to SEQ ID NO: 4 and wherein the antigen-binding polypeptide exhibits improved alkaline stability compared to SEQ ID NO: 4.

9. The antigen-binding polypeptide according to item 7 or 8, wherein each of FWR1, FWR2 and FWR3 has an amino acid sequence having at least 70% identity to a corresponding framework
15 region of an sdAb selected from the group consisting of SEQ ID NOs: 91 and 92.

10. The antigen-binding polypeptide according to any one of the preceding items, wherein said sdAb variant comprises an amino acid sequence in which at least one of the Kabat amino acid positions 6, 14, 19, 23, 24, 40, 43, 44, 45, 47, 60, 76, 79, 82b and 89 is modified in relation to the corresponding amino acid of SEQ ID NO: 4.

20 11. The antigen-binding polypeptide according to any one of the preceding items wherein the sdAb variant is capable of VH3 interaction with staphylococcal protein A domain or a variant thereof.

12. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 1 X₁ is R.

25 13. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 1 X₂ is T.

14. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 1 X₃ is L.

30 15. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 1 X₅ is D.

16. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 1 X₆ is Y or F.

17. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 1 X₉ is G.

18. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 1 X_1 is R, X_2 is T and X_3 is L or F.

19. The antigen-binding polypeptide according to any one of the preceding items wherein
CDR1 comprises a sequence selected from the group consisting of SEQ ID NOs: 9-17, such as selected
5 from the group consisting of SEQ ID NOs: 13, 14, 16 and 17.

20. The antigen-binding polypeptide according to any one of the preceding items wherein

i) in SEQ ID NO: 2 X_1 is A, or

ii) in SEQ ID NO: 2 X_1 is S, L or I, and in SEQ ID NO: 3 X_{10} is A.

21. The antigen-binding polypeptide according to any one of the preceding items wherein in
10 SEQ ID NO: 2 X_2 is I.

22. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 2 X_7 is A.

23. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 2 X_8 is Y.

15 24. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 2 X_{10} is K.

25. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 2 X_2 is I and X_8 is Y.

26. The antigen-binding polypeptide according to any one of the preceding items wherein
20 CDR2 comprises a sequence selected from the group consisting of SEQ ID NOs: 21-31, such as
selected from the group consisting of SEQ ID NOs: 26, 28, 29, and 31.

27. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 3 at least one of X_8 and X_9 is K.

28. The antigen-binding polypeptide according to any one of the preceding items wherein in
25 SEQ ID NO: 3 X_2 is P.

29. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 3, at least one of X_5 and X_6 is L, and preferably both X_5 and X_6 are L.

30. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 3 X_7 is S.

30 31. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 3 X_8 is K, N or R, such as K or R.

32. The antigen-binding polypeptide according to any one of the preceding items wherein in
SEQ ID NO: 3 X_9 is K.

33. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 3 X₁₀ is A or T.

34. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 3 X₁₁ is T.

5 35. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 3 X₁₃ is A and, preferably, X₁₀ is A.

36. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 3 X₁₄ is D and X₁₆ is D.

10 37. The antigen-binding polypeptide according to any one of the preceding items wherein in SEQ ID NO: 3 X₁₇ is Y.

38. The antigen-binding polypeptide according to any one of the preceding items wherein CDR3 comprises a sequence selected from the group consisting of SEQ ID NOs: 34-72, such as selected from SEQ ID NO: 36, 37, 39, 40, 42-46, 48-50, 55, 56, 62, 64, 68 and 70-72.

15 39. The antigen-binding polypeptide according to any one of the preceding items wherein CDR1 is selected from SEQ ID NOs: 9-17, such as selected from SEQ ID NO: 13, 14 16 and 17; CDR2 is selected from SEQ ID NOs: 21-31, such as selected from SEQ ID NO: 26, 28, 29 and 31; and CDR3 is selected from SEQ ID NOs: 34-72, such as selected from SEQ ID NO: 36, 37, 39, 40, 42-46, 48-50, 55, 56, 62, 64, 68 and 70-72.

20 40. The antigen-binding polypeptide according to any one of items 1-7, wherein the sdAb variant has

a) an amino acid sequence selected from SEQ ID NOs: 75-93, 95-98, 101, 102, 105-107, 109-124 and 126-146, such as selected from SEQ ID NOs: SEQ ID NO: 92, 97, 109, 116, 131 and 140-146; or

25 b) an amino acid sequences having at least 90 % identity, such as at least 95 % identity, to a sequence as defined in a) provided that the CDRs are as defined in item 1.

41. The antigen-binding polypeptide according to any one of the preceding items wherein the sdAb variant does not comprise any histidine (H) residues.

30 42. The antigen-binding polypeptide according to any one of the preceding items comprising at least one further amino acid sequence optionally selected from the group consisting of a leader peptide, a signal peptide, a purification tag, a coupling peptide, a spacer peptide and a linker peptide, each further amino acid sequence comprising up to 20 amino acids.

43. The antigen-binding polypeptide according to item 42 wherein said further amino acid sequence is positioned N-terminally or C-terminally of the sdAb variant.

44. The antigen-binding polypeptide according to item 43 wherein said further amino acid sequence is a PelB or OmpA signal peptide.

45. The antigen-binding polypeptide according to any one of items 42-44 wherein said further amino acid sequence comprises a plurality of histidines, such as a (His)₆ sequence, and optionally a C-terminal cysteine.

46. The antigen-binding polypeptide according to item 45 wherein said further amino acid sequence comprises a C-terminal tag, such as selected from the group consisting of HHHHHH, HHHHHHC and AEAAAKHHHHHC (SEQ ID NO: 159).

47. A multimeric polypeptide comprising at least two moieties, each moiety comprising a single-domain antibody variant as defined in any one of the preceding items, said moieties optionally being joined by a peptide linker.

48. A fusion protein comprising at least one antigen-binding polypeptide according to any one of items 1-46 or a multimeric polypeptide according to item 47 and a further polypeptide moiety.

49. The fusion protein according to item 48 wherein the further polypeptide moiety comprises an α -helix-containing polypeptide domain.

50. The fusion protein according to item 49 wherein the further polypeptide moiety comprises an α -helix bundle domain.

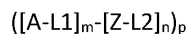
51. The fusion protein according to item 50 wherein the further polypeptide moiety is or is derived from a protein domain of staphylococcal protein A (SpA).

52. The fusion protein according to item 50 or 51, wherein the further polypeptide moiety comprises an amino acid sequence having least at least 80 % identity, such as at least 85 % or at least 90 % identity to SEQ ID NO: 161 or 162.

53. The fusion protein according to any one of items 48-52, wherein said further polypeptide moiety is positioned C-terminally of said antigen-binding polypeptide.

54. The fusion protein according to any one of items 48-52, comprising two or more copies of said further polypeptide moiety, optionally separated by a linker peptide.

55. The fusion protein according to item 48, comprising the following structure



wherein

A represents an antigen-binding polypeptide as defined in any one of items 1-46,

L1 for each occurrence may be present or absent, and where present, represents a linker or spacer,

Z represents a further polypeptide moiety according to any one of items 49-52,

L2 for each occurrence may be present or absent, and where present, represents a linker or spacer,

m represents an integer of from 1 to 4,

n represents an integer of from 1 to 4, or when $m \geq 2$, n represents 0 or an integer of from 1

5 to 4,

and

p represents an integer of from 1 to 4.

56. The fusion protein according to item 55, wherein m is 2 or 3, n is 1 and p is 1.

57. The fusion protein according to item 55, wherein m is 1, n is 1 and p is 2 or 3.

10 58. The fusion protein according to any one of items 55-57 comprising a C-terminal tag comprising a plurality of histidine residues, preferably at least 6, histidine residues, optionally wherein the fusion protein except for the tag does not contain any histidine residues.

59. An isolated nucleic acid encoding the antigen-binding polypeptide of any one of items 1-46, the multimeric polypeptide of item 47 or the fusion protein of any one of items 48-58.

15 60. An expression vector comprising the nucleic acid of item 59.

61. A recombinant host cell for the production of the antigen-binding polypeptide of any one of items 1-46 or the multimeric polypeptide of item 47 or the fusion protein of any one of items 48-58, comprising the expression vector of item 60.

20 62. A method of producing the antigen-binding polypeptide of any one of items 1-46 or the multimeric polypeptide of item 47 or the fusion protein of any one of items 48-58, comprising

- i. providing recombinant host cells according to item 61;
 - ii. culturing the host cell under conditions enabling expression of the antigen-binding polypeptide, the multimeric polypeptide or the fusion protein; and
 - iii. isolating the antigen-binding polypeptide, the multimeric polypeptide or the fusion
- 25 protein.

63. The method of item 62, wherein the cells are prokaryotic cells, such as *E. coli* cells.

64. The method of item 62, wherein the cells are eukaryotic cells.

65. The method of item 64, wherein the eukaryotic cells are yeast cells, such as *Saccharomyces cerevisiae* or *Pichia pastoris* cells.

30 66. The method of 64, wherein the eukaryotic cells are animal cells, such as mammalian cells, such as Chinese hamster ovary (CHO) cells or human embryonic kidney (HEK) cells.

67. The method of any one of items 62-66, wherein step iii comprises purifying the antigen-binding polypeptide, the multimeric polypeptide or the fusion protein by affinity chromatography.

68. The method of item 67, wherein the affinity chromatography uses an affinity ligand that binds to a framework region of the single-domain antibody variant.

69. Use of the antigen-binding polypeptide of any one of items 1-46 or the multimeric polypeptide of item 47 or the fusion protein of any one of items 48-58 as affinity ligand for binding of
5 adeno-associated virus serotype 9 (AAV9).

70. Use according to item 69 for *in vitro* detection and/or purification of AAV9.

71. Use according to item 69 in a method for preparing a therapeutic composition comprising AAV9 viral vector.

72. A separation matrix comprising the antigen-binding polypeptide of any one of items 1-46
10 or the multimeric polypeptide of item 47 or the fusion protein of any one of items 48-58 as an affinity ligand coupled to a support material.

73. The separation matrix according to item 72 wherein the support material is a surface.

74. The separation matrix according to item 72 wherein the support material is selected from a particle, a bead, a fiber, a fibrous membrane, a filter, a sheet, a porous monolith, a chip, a plate,
15 and a well.

75. The separation matrix according to item 74 wherein the support material is a chromatography matrix.

76. The separation matrix according to item 75 wherein the matrix comprises a polysaccharide-based material, such as agarose and derivatives thereof, or cellulose and derivatives
20 thereof.

77. The separation matrix according to item 76 wherein the matrix comprises crosslinked agarose.

78. The separation matrix according to item 75 wherein the support material is a chromatography matrix selected from the group consisting of a fibrous matrix, a membrane, a filter
25 and a porous monolith.

79. The separation matrix according to item 78 wherein the support material is a nonwoven fibrous matrix.

80. An *in vitro* method of detecting AAV9 viral particles present in a sample, comprising (a) providing a sample containing AAV9 viral particles; (b) contacting the sample with the antigen-
30 binding polypeptide of any one of items 1-46, the multimer of item 47 or the fusion protein of any one of items 48-58, under conditions allowing the sdAb variant to bind to the AAV9 viral particles, wherein the binding event generates a detectable signal; and (c) detecting the detectable signal.

81. A method of chromatographic separation of AAV9 viral particles, comprising the steps of
(a) providing a separation matrix according to any one of items 72-79,

- (b) contacting the separation matrix with a liquid sample comprising said AAV9 viral particles under conditions allowing the antigen-binding polypeptide to bind to the AAV9 viral particles,
- (c) optionally washing the separation matrix,
- 5 (d) eluting the bound AAV9 viral particles from the separation matrix, and
- (e) cleaning the separation matrix material with a cleaning liquid.

82. The method of item 81 wherein step (e) comprises cleaning the separation matrix with an alkaline cleaning liquid, wherein the cleaning liquid preferably comprises from 0.05 to 0.5 M of NaOH.

- 10 83. The method of item 81 or 82 wherein steps (a)-(e) are repeated at least 10 times, such as at least 20 times.

84. The method of any one of items 81-83 wherein after 10 cycles of contact with cleaning liquid the polypeptide retains at least 50 %, such as at least 60 %, at least 70 %, at least 80 % or at least 90 %, of its initial target entity binding capacity.

- 15 85. The method of any one of items 81-84, wherein after 20 cycles of contact with cleaning liquid the polypeptide retains at least 50 %, at least 60 %, such as at least 70 % or at least 80 % of its initial target entity binding capacity.

86. The method of any one of items 81-83, wherein, after 10 cycles of contact with alkaline cleaning liquid, the polypeptide retains at least 60 %, at least 70 %, at least 80 %, at least 90 %, such as at least 95 %, of the target entity binding capacity of the 2nd cycle.
- 20

87. The method of item 86, wherein after 20 cycles of contact with alkaline cleaning liquid, the polypeptide retains at least 60 %, at least 70 %, at least 80 %, at least 90 %, such as at least 95 %, of the target entity binding capacity of the 2nd cycle.

Table 21. Exemplary amino acid sequences

SEQ ID NO	Amino acid sequence
4	EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFVVAINWSSGSTYYADSVKGRFTISRDNAKNTMYLQMNSLKPEDTAVYYCAAGYQINSGNYNFKDYEYDYWGQGTQVTVSS
5	EVQLVESGGGLVQAGGSLRLSCAASG
6	WFRQAPGKEREFV
7	YADSVKGRFTISRDNAKNTMYLQMNSLKPEDTAVYYCAA
8	WGQGTQVTVSS
9	RTFSDYTMG
10	LTLSDYTMG
11	RTLSNYTMG
12	RTLSEYTMG
13	RTLSDFTMG
14	RTLSDNTMG
15	RTLSDYTMA
16	RTLSDYTMG
17	RTLSDFTMA
21	AVSWGGSFTY
22	AISWGSFTY
23	SISWGSFTY
24	LISWGSFTY
25	IISWGSFTY
26	AISWGSFTY
27	AVSWGGSFTY
28	AVSWGGSFTK
29	AISWGSFTK
30	AISWGSFTH
31	AISWGSFTF
34	GPTGPLRRSSPPDYDY
35	GSTGPLRRSTPPDYDY
36	APTGLSKKATPADYDY
37	GSTGLSKKATPADYDY
38	GPTGLSKKSTPADYDY
39	GPTGPLSKKATPADYDY
40	GPTGTLSKKATPADYDY
41	GPTGLISKKATPADYDY
42	GPTGLLAKKATPADYDY
43	GPTGLLSNKATPADYDY
44	GPTGLLSQKATPADYDY
45	GPTGLLSRKATPADYDY
46	GPTGLLSKRATPADYDY
47	GPTGLLSKHATPADYDY
48	GPTGLLSNRATPADYDY
49	GPTGLLSQRATPADYDY
50	GPTGLLSRRATPADYDY
51	GPTGLLSNHATPADYDY
52	GPTGLLSQHATPADYDY
53	GPTGLLSRHATPADYDY

54	GPTGLLSRKSTPADYDY
55	GPTGLLSKKATPADYDY
56	GPTGLLSKKTPADYDY
57	GPTGLLSKKAAPADYDY
58	GPTGLLSKKATSADYDY
59	GPTGLLSKKATTADYDY
60	GPTGLLSKKATPRDYDY
61	GPTGLLSKKATPANYDY
62	GPTGLLSKKATPAEYDY
63	GPTGLLSKKATPAQYDY
64	GPTGLLSKKATPADFDY
65	GPTGLLSKKATPADYNY
66	GPTGLLSKKATPADYDY
67	GPTGLLSKKATPADYQY
68	GPTGLLSKKATPADYDF
69	GPTGLLSKKATPADYDR
70	GPTGLLSRKTPADYQY
71	GPTGLLSKKATPPDYDY
72	GPTGPLSKKTPADYDY
75	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDYTMGWFRQAPGKQREFVAAVSWSGSFTYYT DSVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGPLSRSSPPDYDYWGQGTQVTV SS
76	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDYTMGWFRQAPGKQREFVAAVSWSGAYTYYT DSVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGSTGPLTRRSTPPDYDYWGQGTQVTV SS
77	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVASISWSGAYTYYTD SVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKSTPADYDYWGQGTQVTVS S
78	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVALISWSGAYTYYTD SVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKSTPADYDYWGQGTQVTVS S
79	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVAIISWSGAYTYYTD SVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKSTPADYDYWGQGTQVTVS S
80	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVASISWSGAYTYYTD SVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSRKSTPADYDYWGQGTQVTVS S
81	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVALISWSGAYTYYTD SVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSRKSTPADYDYWGQGTQVTVS S
82	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVAIISWSGAYTYYTD SVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSRKSTPADYDYWGQGTQVTVS S
83	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVASISWSGAYTYYTD SVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
84	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVALISWSGAYTYYTD SVKGRFTISRDNAAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S

85	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVAIISWSGAYTTYTD SVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
86	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVASISWSGAYTTYTD SVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSRKATPADYDYWGQGTQVTVS S
87	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVALISWSGAYTTYTD SVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSRKATPADYDYWGQGTQVTVS S
88	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVAIISWSGAYTTYTD SVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSRKATPADYDYWGQGTQVTVS S
89	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVAAISWSGAYTTYTD SVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKSTPADYDYWGQGTQVTVS S
90	QVQLQESGGGLVQPGGSLRLSCAASGRTLSDYTMGWFRQAPGKQREFVAAISWSGAYTTYTD SVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
91	QVQLQESGGGSVQAGGSLRLSCVASGRTLSDYTMGWFRQPPGKAREFVAAISWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
92	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKEREFVAAISWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
93	QVQLQQSGGGSVQAGGSLRLSCTISGLTSDYTMGWFRQRP GKEREFVAAISWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
95	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSNYTMGWFRQRP GKEREFVAAISWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
96	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSEYTMGWFRQRP GKEREFVAAISWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
97	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDFTMGWFRQRP GKEREFVAAISWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
98	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDNMTGWFRQRP GKEREFVAAISWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
101	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMWFRQRP GKEREFVAAITWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
102	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKEREFVAAITWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
105	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKEREFVAAISWSGAYTFYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVS S
106	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKEREFVAAISWSGAYTKYAD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAAPTGLLSKKATPADYDYWGQGTQVTVS S

107	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGSTGLLSKKATPADYDYWGQGTQVTVS S
109	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGPLSKKATPADYDYWGQGTQVTV SS
110	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGTLSKKATPADYDYWGQGTQVTV SS
111	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLISKKATPADYDYWGQGTQVTVS S
112	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLAKKATPADYDYWGQGTQVTV SS
113	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSNKATPADYDYWGQGTQVTV SS
114	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSQKATPADYDYWGQGTQVTV SS
115	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKHATPADYDYWGQGTQVTV SS
116	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKTTPADYDYWGQGTQVTVS S
117	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKAAPADYDYWGQGTQVTV SS
118	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATSADYDYWGQGTQVTVS S
119	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATTADYDYWGQGTQVTVS S
120	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPRDYDYWGQGTQVTVS S
121	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPANYDYWGQGTQVTVS S
122	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPAEYDYWGQGTQVTVS S
123	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPAQYDYWGQGTQVTV SS
124	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADFDYWGQGTQVTVS S

126	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYNYWGQGTQVTVS S
127	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYEYWGQGTQVTVS S
128	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYQYWGQGTQVTV SS
129	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDFWGQGTQVTVS S
130	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDRWGQGTQVTV SS
131	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDFTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKTTPADYDYWGQGTQVTVS S
132	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSRKATPADYDYWGQGTQVTVS S
133	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKRATPADYDYWGQGTQVTVS S
134	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSNRATPADYDYWGQGTQVTV SS
135	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSQRATPADYDYWGQGTQVTV SS
136	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSRRATPADYDYWGQGTQVTV SS
137	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSNHATPADYDYWGQGTQVTV SS
138	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSQHATPADYDYWGQGTQVTV SS
139	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRP GKERE FVAAISWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSRHATPADYDYWGQGTQVTV SS
140	QVQLVESGGG SVQAGGSLSLCTISGRTLSDFTMAWFRQRP GKERE FVAAISWSGAYTYTDS VKGRFTISRDN AKNTVYLQMQLKPEDTAVYYCAAGPTGLLSRKTTTPADYQYWGQGTQVTVSS
141	QVQLQQSGGGSVQAGGSLRLSCTSSGRTLSDFTMAWFRQRP GRARE FVAAISWSGAYTYTDS SVKGRFTISRDN AKNTVFLQMQLKPEDTAIYYCAAGPTGLLSRKTTTPADYQYWGQGTQVTVS S
142	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDFTMAWFRQRP GKERE FVAAVSWSGAYTKYAD SVKGRFTISRDN AKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPPDYDYWGQGTQVTVS S

143	QVQLQQSGGGSVQAGGSLTSLCTVSGRTLSDFTMGWFRQRPKGKELEFVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVWLQMNSLKPEDTAVYYCAAGPTGPLSKKTPADYDYWGQGTQVTV SS
144	QVQLQQSGGGSVQAGGSLRLSCTSSGRTLSDFTMGWFRQRPKEREFEVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVWLQMNSLKPEDTAVYYCAAGPTGLLSKKTPADYDYWGQGTQVTV SS
145	QVQLQQSGGGSVQAGGSLRLSCTVSGRTLSDFTMGWFRQRPKEREFEVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVFLQMNSLKPEDTALYYCAAGPTGLLSKKTPADYDYWGQGTQVTVS S
146	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDFTMAWFRQRPKEREFEVAAISWSGAYTYYAD SVKGRFTISRDNNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSRKTPADYQYWGQGTQVTVS S
161	VDAKFDKEAYRALAEIARLPNLTEEQRAAFIQSLKDDPSGSKAILAEAKKVNDAAQAPK
162	VDAKFDKEAQEAFYEILHLPNLTEEQRNAFIQSLKDDPSGSKAILAEAKKVNDAAQAPK
171	EVQLQQSGGGSVQAGGSLRLSCTISGRTLSDYTMGWFRQRPKEREFEVAAISWSGAYTKYADS VKGRFTISRDNNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKATPADYDYWGQGTQVTVSS
172	EVQLQQSGGGSVQAGGSLRLSCTISGRTLSDFTMGWFRQRPKEREFEVAAISWSGAYTKYADS VKGRFTISRDNNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSKKTPADYDYWGQGTQVTVSS
173	GPTGLLSQKTPADYDY
174	QVQLQQSGGGSVQAGGSLRLSCTISGRTLSDFTMGWFRQRPKEREFEVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSQKTPADYDYWGQGTQVTV SS
175	EVQLQQSGGGSVQAGGSLRLSCTISGRTLSDFTMGWFRQRPKEREFEVAAISWSGAYTKYADS VKGRFTISRDNNAKNTVYLQMNSLKPEDTAVYYCAAGPTGLLSQKTPADYDYWGQGTQVTVS S
177	QVQLQQSGGGSVQAGGSLSLCTSSGRTLSDFTMGWFRQRPKGKELEFVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVWLQMNSLKPEDTAVYYCAAGPTGLLSKKTPADYDYWGQGTQVTV SS
178	QVQLQQSGGGSVQAGGSLRLSCTSSGRTLSDFTMGWFRQIPKEREFEVAAISWSGAYTKYAD SVKGRFTISRDNNAKQTVWLQMNSLKPEDTAVYYCAAGPTGLLSKKTPADYDYWGQGTQVTV SS
179	QVQLQQSGGGSVQAGGSLRLSCTSSGRTLSDFTMGWFRQRPKGDLEFVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVWLQMNSLKPEDTAIYYCAAGPTGLLSKKTPADYDYWGQGTQVTVS S
180	QVQLQQSGGGSVQAGGSLRLSCTVSGRTLSDFTMGWFRQRPKGKELEFVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVWLQMNSLKPEDTAVYYCAAGPTGLLSKKTPADYDYWGQGTQVTV SS
181	QVQLQQSGGGSVQAGGSLTSLCTVSGRTLSDFTMGWFRQRPKGKELEFVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVWLQMNSLKPEDTAVYYCAAGPTGLLSKKTPADYDYWGQGTQVTV SS
182	QVQLQQSGGGSVQAGGSLTSLCTVSGRTLSDFTMGWFRQRPKGKELEFVAAISWSGAYTKYAD SVKGRFTISRDNNAKNTVWLQMNSLKPEDTAVYYCAAGPTGLLSQKTPADYDYWGQGTQVTV SS

REFERENCES

Thompson et al, *Nucleic Acids Research*, 22: 4673-4680 (1994)

Kabat et al., *J. Immunol.* 147(5), 1709-1719 (1991)

S Hjerten, *Biochim Biophys Acta* 79(2), 393-398 (1964)

US6602990
US7396467
WO2019137869A1
WO2018011600A1
5 CN116396381A
CN116751284
WO2020242988A2
WO2023174900A1

CLAIMS

1. An antigen-binding polypeptide capable of binding adeno-associated virus serotype 9 (AAV9), the polypeptide comprising a single-domain antibody (sdAb) variant having complementarity
- 5 determining regions CDR1, CDR2 and CDR3, wherein
- CDR1 comprises an amino acid sequence selected from the group of sequences defined by SEQ ID NO: 1:
$$X_1X_2X_3SX_5X_6TMX_9 \quad \quad \quad (\text{SEQ ID NO: 1})$$

wherein, independently,

10 X_1 is R, L or S, preferably R or L;
 X_2 is T or R, preferably T;
 X_3 is L or F, preferably L;
 X_5 is D, N or E, preferably D;
 X_6 is Y, N or F, preferably Y or F;
15 X_9 is G or A, preferably G;
 - CDR2 comprises a sequence selected from the group of sequences defined by defined by SEQ ID NO: 2:
$$X_1X_2SWSGX_7X_8TX_{10} \quad \quad \quad (\text{SEQ ID NO: 2})$$

wherein, independently,

20 X_1 is A, S, L, V or I, preferably A;
 X_2 is I or V, preferably I;
 X_7 is A or S, preferably A;
 X_8 is Y or F, preferably Y;
 X_{10} is K, F or Y;
- 25 and
- CDR3 comprises a sequence selected from the group of sequences defined by defined by SEQ ID NO: 3:
$$X_1X_2TX_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17} \quad \quad \quad (\text{SEQ ID NO: 3})$$

wherein, independently,

30 X_1 is G or A, preferably G;
 X_2 is P or S, preferably P;
 X_4 is G or P, preferably G;

X₅ is L, T or P, preferably L or P;

X₆ is L or I, preferably L;

X₇ is S, T or A, preferably S or A;

X₈ is K, R, N or Q;

5 X₉ is K, H or R, preferably K or R;

X₁₀ is A, S or T, preferably A or T;

X₁₁ is T, S, P or A, preferably T;

X₁₂ is P, A, T or S, preferably P;

X₁₃ is A, P, R or G, preferably A or P;

10 X₁₄ is D, N, E or Q, preferably D or E;

X₁₅ is Y or F, preferably Y;

X₁₆ is D, N, E or Q, preferably D; and

X₁₇ is Y, F or R, preferably Y or F.

15 2. The antigen-binding polypeptide according to claim, wherein the antigen-binding polypeptide selectively binds AAV9.

3. The antigen-binding polypeptide according to claim 1 or 2, wherein said sdAb variant is a variable domain of a heavy chain of a heavy chain antibody (VHH) variant.

4. The antigen-binding polypeptide according to any one of claims 1-3, wherein said polypeptide is alkali stable.

20 5. The antigen-binding polypeptide according to any one of the preceding claims, wherein the sdAb variant comprises framework regions (FWRs) and complementary determining regions (CDRs) in the following order from N-terminus to C-terminus: [FWR1]-[CDR1]-[FWR2]-[CDR2]-[FWR3]-[CDR3]-[FWR4].

25 6. The antigen-binding polypeptide according to claim 5, wherein at least one of said framework regions comprises at least one an amino acid substitution relative to SEQ ID NO: 4 and wherein the antigen-binding polypeptide exhibits improved alkaline stability compared to SEQ ID NO: 4.

30 7. The antigen-binding polypeptide according to claims 5 or 6, wherein each of FWR1, FWR2 and FWR3 has an amino acid sequence having at least 70% identity to a corresponding framework region of an sdAb selected from the group consisting of SEQ ID NOs: 91 and 92.

8. The antigen-binding polypeptide according to any one of the preceding claims, wherein said sdAb variant comprises an amino acid sequence in which at least one of the Kabat amino acid

positions 6, 14, 19, 23, 24, 40, 43, 44, 45, 47, 60, 76, 79, 82b and 89 is modified in relation to the corresponding amino acid of SEQ ID NO: 4.

9. The antigen-binding polypeptide according to any one of the preceding claims, wherein in SEQ ID NO: 1, X₁ is R, X₂ is T, and X₃ is L.

5 10. The antigen-binding polypeptide according to any one of the preceding claims, wherein in SEQ ID NO: 1, X₆ is F and X₉ is G or A.

11. The antigen-binding polypeptide according to any one of the preceding claims, wherein in SEQ ID NO: 2 X₂ is I.

12. The antigen-binding polypeptide according to any one of the preceding claims, wherein in
10 SEQ ID NO: 2 X₇ is A, X₈ is Y, and/or X₁₀ is K.

13. The antigen-binding polypeptide according to any one of the preceding claims wherein SEQ ID NO: 3 X₅ is L or P and/or X₁₀ is T.

14. The antigen-binding polypeptide according to claim 1 or 2, wherein CDR1 is selected from SEQ ID NOs: 9-17, such as selected from SEQ ID NO: 13, 14 16 and 17; and/or CDR2 is selected from
15 SEQ ID NOs: 21-31, such as selected from SEQ ID NO: 26, 28, 29 and 31; and/or CDR3 is selected from SEQ ID NOs: 34-72 and 173, such as selected from SEQ ID NO: 36, 37, 39, 40, 42-46, 48-50, 55, 56, 62, 64, 68, 70-72 and 173.

15. The antigen-binding polypeptide according to claim 8, wherein the individual FWR are selected from SEQ ID NO: 149-151 (FWR1), SEQ ID NO: 152-154 (FWR2), SEQ ID NO: 155-157 (FWR3)
20 and SEQ ID NO: 8 (FWR4), and sequences having 95 % or more, such as 96 %, 97 %, 98 %, 99 %, identity thereto.

16. The antigen-binding polypeptide according to any preceding claim, wherein the sdAb variant has

a) an amino acid sequence selected from SEQ ID NOs: 75-93, 95-98, 101, 102, 105-107, 109-
25 124, 126-146 and 173, such as selected from SEQ ID NOs: SEQ ID NO: 92, 97, 109, 116, 131, 140-146 and 173; or

b) an amino acid sequences having at least 90 % identity, such as at least 95 % identity, to a sequence as defined in a) provided that the CDRs are as defined in claim 1.

17. The antigen-binding polypeptide according to any preceding claim, wherein the antigen-
30 binding polypeptide comprises a sequence selected from SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO:

97, SEQ ID NO: 131, SEQ ID NO: 173 and SEQ ID NO: 177-182, and sequences having 95 % or more, such as 96 %, 97 %, 98 %, 99 %, identity thereto.

18. The antigen-binding polypeptide according to any preceding claim, the antigen-binding polypeptide comprising at least one further amino acid sequence optionally selected from the group
5 consisting of a leader peptide, a signal peptide, a purification tag, a coupling peptide, a spacer peptide and a linker peptide, each further amino acid sequence comprising up to 20 amino acids.

19. The antigen-binding polypeptide according to claim 18, wherein said further amino acid sequence comprises a C-terminal tag, such as selected from the group consisting of HHHHHH, HHHHHHC and AEAAKHHHHHC (SEQ ID NO: 159).

10 20. A multimeric polypeptide comprising at least two moieties, each moiety comprising a single-domain antibody variant as defined in any one of the preceding claims, said moieties optionally being joined by a peptide linker.

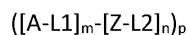
21. A fusion protein comprising at least one antigen-binding polypeptide according to any one of claims 1-19 or a multimeric polypeptide according to claim 20, and a further polypeptide
15 moiety.

22. The fusion protein according to claim 21, wherein the further polypeptide moiety comprises an α -helix-containing polypeptide domain, such as an α -helix bundle domain.

23. The fusion protein according to claim 22, wherein the further polypeptide moiety is or is derived from a protein domain of staphylococcal protein A (SpA).

20 24. The fusion protein according to claims 21-23, wherein the further polypeptide moiety comprises an amino acid sequence having least at least 80 % identity, such as at least 85 % or at least 90 % identity to SEQ ID NO: 161 or 162.

25. The fusion protein according to claim 21 or 22, comprising the following structure



25 wherein

A represents an antigen-binding polypeptide as defined in any one of claim 1-19,

L1 for each occurrence may be present or absent, and where present, represents a linker or spacer,

Z represents a further polypeptide moiety optionally as defined in any one of claim 22-24,

L2 for each occurrence may be present or absent, and where present, represents a linker or spacer,

m represents an integer of from 1 to 4,

n represents an integer of from 1 to 4, or when $m \geq 2$, n represents 0 or an integer of from 1

5 to 4,

and

p represents an integer of from 1 to 4.

26. An isolated nucleic acid encoding the antigen-binding polypeptide of any one of claims 1-19, the multimeric polypeptide of claim 20, or the fusion protein of any one of claims 21-25.

10 27. An expression vector comprising the nucleic acid of claim 26.

28. A recombinant host cell for the production of the antigen-binding polypeptide of any one of claims 1-19 or the multimeric polypeptide of claim 20 or the fusion protein of any one of claims 21-25, comprising the expression vector of claim 27.

15 29. A method of producing the antigen-binding polypeptide of any one of claims 1-19 or the multimeric polypeptide of claim 20 or the fusion protein of any one of claims 21-25, comprising

- i. providing recombinant host cells according to claim 28;
- ii. culturing the host cell under conditions enabling expression of the antigen-binding polypeptide, the multimeric polypeptide or the fusion protein; and
- iii. isolating the antigen-binding polypeptide, the multimeric polypeptide or the fusion

20 protein.

30. Use of the antigen-binding polypeptide of any one of claims 1-19, the multimeric polypeptide of claim 20 or the fusion protein of any one of claims 21-25 as affinity ligand for binding of adeno-associated virus serotype 9 (AAV9).

31. Use according to claim 03 for purification of AAV9 viral particles.

25 32. Use according to claim 30 for *in vitro* detection of AAV9.

33. A separation matrix comprising the antigen-binding polypeptide of any one of claims 1-19, the multimeric polypeptide of claim 20 or the fusion protein of any one of claims 21-25 coupled to a support material.

30 34. The separation matrix according to claim 33, wherein the support material is selected from a fibrous membrane, a particle or a bead, a fiber, a filter, a sheet, a porous monolith, a chip, a

plate, and a well.35. The separation matrix according to claim 133or 34, wherein the support material is a chromatography matrix.

36. The separation matrix according to claim 35, wherein the support material is a chromatography matrix selected from the group consisting of a fibrous matrix, a membrane, a filter
5 and a porous monolith.

37. The separation matrix according to claim 36, wherein the support material is a nonwoven fibrous matrix.

38. The separation matrix according to claim 35, wherein the support material comprises beads comprising agarose, or a derivative thereof.

10 39. A method of chromatographic separation of AAV9 viral particles, comprising the steps of
(a) providing a separation matrix according to any one of claims 33-38-19,
(b) contacting the separation matrix with a liquid sample comprising said AAV9 viral particles
under conditions allowing the antigen-binding polypeptide to bind to the AAV9 viral
particles,
15 (c) optionally washing the separation matrix, and
(d) eluting the bound AAV9 viral particles from the separation matrix.

40. The method of claim 39, further comprising:

(e) cleaning the separation matrix material with a cleaning liquid.

41. The method of claim 40, wherein step (e) comprises cleaning the separation matrix with
20 an alkaline cleaning liquid, wherein the cleaning liquid preferably comprises from 0.05 to 0.5 M of NaOH.

42. The method of any one of claims 40-41, wherein steps (a)-(e) are repeated at least 10 times, such as at least 20 times.

43. The method of any one of claims 40-42 wherein after 10 cycles of contact with cleaning
25 liquid the polypeptide retains at least 50 %, such as at least 60 %, at least 70 %, at least 80 % or at least 90 % of its initial target entity binding capacity.

44. The method of any one of claims 40-42, wherein, after 10 cycles of contact with alkaline cleaning liquid, the polypeptide retains at least 60 %, at least 70 %, at least 80 %, at least 90 %, such as at least 95 % of the target entity binding capacity of the 2nd cycle.