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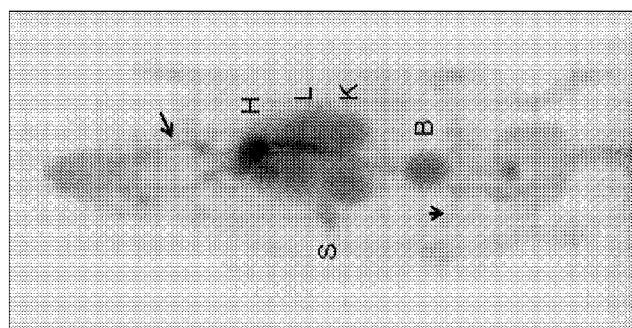


Figure 12

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(57) Abstract: The present disclosure relates to a class of engineered polypeptides having a binding affinity for albumin. It also relates to new methods and uses that exploit binding by these and other compounds to albumin in different contexts, some of which have significance for treatment or diagnosis of disease in mammals including humans.

POLYPEPTIDESTechnical field

The present disclosure relates to a class of engineered polypeptides having a binding affinity for albumin. It also relates to new methods and uses 5 that exploit binding by these and other compounds to albumin in different contexts, some of which have significance for the treatment of disease in mammals including humans.

Background10 *Serum albumin*

Serum albumin is the most abundant protein in mammalian sera (40 g/l; approximately 0.7 mM in humans), and one of its functions is to bind molecules such as lipids and bilirubin (Peters, *Advances in Protein Chemistry* 37:1 61, 1985). Serum albumin is devoid of any enzymatic or immunological 15 function. Furthermore, human serum albumin (HSA) is a natural carrier involved in the endogenous transport and delivery of numerous natural as well as therapeutic molecules (Sellers and Koch-Weser, *Albumin Structure, Function and Uses*, eds Rosener *et al*, Pergamon, Oxford, p 159, 1977). The half life of serum albumin is directly proportional to the size of the animal, 20 where for example human serum albumin has a half life of 19 days and rabbit serum albumin has a half life of about 5 days (McCurdy *et al*, *J Lab Clin Med* 143:1 15, 2004). HSA is widely distributed throughout the body, in particular in the interstitial and blood compartments, where it is mainly involved in the maintenance of osmolarity. Structurally, albumins are single-chain proteins 25 comprising three homologous domains and in total 584 or 585 amino acids (Dugaiczyk *et al*, *Proc Natl Acad Sci USA* 79:71, 1982). Albumins contain 17 disulfide bridges and a single reactive thiol, cysteine in position 34, but lack N-linked and O-linked carbohydrate moieties (Peters, 1985, *supra*; Nicholson *et al*, *Br J Anaesth* 85:599, 2000).

30

*Fusion or association with HSA results in increased in vivo half life of proteins*

Several strategies have been reported to either covalently couple 35 proteins directly to serum albumins or to a peptide or protein that will allow *in vivo* association to serum albumins. Examples of the latter approach have been described e.g. in WO91/01 743, in WO01/45746 and in Dennis *et al* (J

Biol Chem 277:35035-43, 2002). The first document describes *inter alia* the use of albumin binding peptides or proteins derived from streptococcal protein G (SpG) for increasing the half life of other proteins. The idea is to fuse the bacterially derived, albumin binding peptide/protein to a therapeutically interesting peptide/protein, which has been shown to have a rapid elimination from blood. The thus generated fusion protein binds to serum albumin *in vivo*, and benefits from its longer half life, which increases the net half life of the fused therapeutically interesting peptide/protein. WO01/45746 and Dennis *et al* relate to the same concept, but here, the authors utilize relatively short peptides to bind serum albumin. The peptides were selected from a phage displayed peptide library. In Dennis *et al*, earlier work is mentioned in which the enhancement of an immunological response to a recombinant fusion of the albumin binding domain of streptococcal protein G to human complement receptor Type 1 was found. US patent application published as US2004/0001 827 (Dennis) also discloses the use of constructs comprising peptide ligands, again identified by phage display technology, which bind to serum albumin and which are conjugated to bioactive compounds for tumor targeting.

20 *Albumin binding domains of bacterial receptor proteins*

Streptococcal protein G (SpG) is a bi-functional receptor present on the surface of certain strains of streptococci and is capable of binding to both IgG and serum albumin (Bjorck *et al*, Mol Immunol 24:1 113, 1987). The structure is highly repetitive with several structurally and functionally different domains (Guss *et al*, EMBO J 5:1 567, 1986), more precisely three Ig-binding domains and three serum albumin binding domains (Olsson *et al*, Eur J Biochem 168:31 9, 1987). The structure of one of the three serum albumin binding domains in SpG has been determined, showing a three-helix bundle fold (Kraulis *et al*, FEBS Lett 378:1 90, 1996, Johansson *et al*, J. Biol. Chem. 277:81 14-20, 2002). A 46 amino acid motif was defined as ABD (albumin binding c/omain) and has subsequently also been designated G148-GA3 (GA for protein G-related albumin binding). In for example WO09/01 6043, albumin binding variants of the 46 amino acid motif ABD are disclosed.

Other bacterial albumin binding domains than the ones in protein G have also been identified, some of which are structurally similar to the ones of protein G. Examples of proteins containing such albumin binding domains are the PAB, PPL, MAG and ZAG proteins (Rozak *et al*, Biochemistry 45:3263-

3271, 2006). Studies of structure and function of such albumin binding domains have been carried out and reported e.g. by Johansson and co-workers (Johansson *et al*, *J Mol Biol* 266:859-865, 1997). Furthermore, Rozak *et al* have reported on the creation of artificial variants of G148-GA3, which 5 were selected and studied with regard to different species specificity and stability (Rozak *et al*, *Biochemistry* 45:3263-3271, 2006), whereas Jonsson *et al* developed artificial variants of G148-GA3 having very much improved affinity for human serum albumin (Jonsson *et al*, *Prot Eng Des Sel* 21:515-27, 2008). For some of the variants a higher affinity was achieved at the cost of 10 reduced thermal stability.

In addition to the three-helix containing proteins described above, there are also other unrelated bacterial proteins that bind albumin.

#### *ABD and immunization*

15 Recently, a few T- and B-cell epitopes were experimentally identified within the albumin binding region of Streptococcal protein G strain 148 (G148) (Goetsch *et al*, *Clin Diagn Lab Immunol* 10:125-32, 2003). The authors behind the study were interested in utilizing the T-cell epitopes of G148 in vaccines, i.e. to utilize the inherent immune-stimulatory property of the 20 albumin binding region. Goetsch *et al* additionally found a B-cell epitope, i.e. a region bound by antibodies after immunization, in the sequence of G148.

In pharmaceutical compositions for human administration no immune-response is desired. Therefore, the albumin binding domain G148 is as such 25 unsuitable for use in such compositions due to its abovementioned immune-stimulatory properties.

#### Description

The above drawbacks and deficiencies of the prior art are overcome or 30 alleviated by, in a first aspect, an albumin binding polypeptide, comprising an amino acid sequence selected from

i) LAX<sub>3</sub>AKX<sub>6</sub>X<sub>7</sub>ANX<sub>10</sub> ELDXi<sub>4</sub>YGVSDF YKRLIX<sub>26</sub>KAKT VEGVEALKX<sub>39</sub>X<sub>40</sub> ILX<sub>43</sub>X<sub>44</sub>LP

35 wherein independently of each other

X<sub>3</sub> is selected from E, S, Q and C;

X<sub>6</sub> is selected from E, S and C;

X<sub>7</sub> is selected from A and S;  
X<sub>10</sub> is selected from A, S and R;  
X<sub>i<sub>4</sub></sub> is selected from A, S, C and K;  
x<sub>26</sub> is selected from D and E;  
5 x<sub>39</sub> is selected from D and E;  
x<sub>40</sub> is selected from A and E;  
x<sub>43</sub> is selected from A and K;  
x<sub>44</sub> is selected from A, S and E;  
L in position 45 is present or absent; and  
10 P in position 46 is present or absent;

and

ii) an amino acid sequence which has at least 95 % identity to the sequence defined in i).

15 The above defined class of sequence related polypeptides having a binding affinity for albumin is derived from a common parent polypeptide sequence, which folds into a three alpha helix bundle domain. More specifically, the polypeptides as described above are derived from a model building based on a structure of a complex between serum albumin and the 20 albumin binding domain G148-GA3 (Lejon *et al*, J Biol Chem 279:42924-8, 2004), as well as analyses of binding and structural properties of a number of mutational variants of the common parent polypeptide sequence. The above defined amino acid sequence i) comprises amino acid substitutions as compared to the parent polypeptide sequence that result in a class of 25 polypeptides which are expected to fold into an almost identical three helix bundle domain. While the parent polypeptide sequence already comprises a binding surface for interaction with albumin, that binding surface is modified by some of the substitutions according to the above definition. The substitutions according to the above definition provide an improved albumin 30 binding ability as compared to the parent polypeptide sequence.

The albumin binding polypeptides according to the first aspect of the disclosure exhibit a set of characteristics, which, for example, make them suitable for use as fusion or conjugate partners for therapeutic molecules for human administration. The albumin binding polypeptides according to the 35 present disclosure demonstrate, for example in comparison with related albumin binding polypeptides such as the albumin binding domain G148-GA3

and the albumin binding polypeptides disclosed in WO09/016043, at least five of the following six characteristics:

- The polypeptides display a different surface compared to, for example, G148-GA3 and other bacterially derived albumin binding domains. The difference may decrease or eliminate any risk for antibody reactions in a subject, such as a human, which has been previously exposed to such bacterial proteins.
- The polypeptides comprise fewer potential T-cell epitopes than, for example, G148-GA3 and other related, but different, mutational variants of the common parent polypeptide sequence, and hence exhibit low immunogenicity when administered to a subject, such as a human.
- The polypeptides display a lower reactivity with circulating antibodies when administered to a subject, such as a human. Thus, by amino acid substitutions in the surface of the polypeptides exposed to circulating antibodies, i.e. in the polypeptide surface not involved in the binding interaction with albumin, antibody cross-reactivity is reduced as compared to, for example, antibody cross-reactivity caused by G148-GA3 as measured in a test set of human sera.
- The polypeptides have a high albumin binding ability, both in terms of a higher binding affinity, as defined by a  $K_D$  value, and in terms of a slower off-rate, as defined by a  $k_{off}$  value, than, for example, known naturally occurring albumin binding polypeptides, such as the albumin binding domains derived from bacterial proteins.
- The polypeptides comprise fewer amino acid residues that are associated with stability problems of polypeptides than, for example, known naturally occurring albumin binding polypeptides, such as the albumin binding domains derived from bacterial proteins. Thus, the polypeptides comprise, for example, no oxidation-prone methionines or tryptophanes and only one asparagine.
- The polypeptides have a higher structural stability, as defined by a melting point of above 55 °C, than previous albumin binding polypeptides, such as those disclosed in WO09/01 6043.

In one embodiment, the albumin binding polypeptide according to the first aspect display all six of the above listed characteristics. In another embodiment, the albumin binding polypeptide according to the first aspect displays, when bound to albumin, a more hydrophilic profile than, for

example, previous albumin binding polypeptides, such as those disclosed in WO09/01 6043. The surface of the albumin binding polypeptide which is exposed to the surroundings when the polypeptide interacts with albumin comprises fewer amino acid residues that confer surface hydrophobicity.

5 As the skilled person will realize, the function of any polypeptide, such as the albumin binding capacity of the polypeptides according to the first aspect, is dependent on the tertiary structure of the polypeptide. It is however possible to make changes to the sequence of amino acids in an  $\alpha$ -helical polypeptide without affecting the structure thereof (Taverna and Goldstein, J  
10 Mol Biol 315(3):479-84, 2002; He *et al*, Proc Natl Acad Sci USA 105(38):1441 2-17, 2008). Thus, modified variants of i), which are such that the resulting sequence is at least 95 % identical to a sequence belonging to the class defined by i), are also encompassed by the first aspect. For example, it is possible that an amino acid residue belonging to a certain  
15 functional grouping of amino acid residues (e.g. hydrophobic, hydrophilic, polar etc) could be exchanged for another amino acid residue from the same functional group.

The term "% identical" or "% identity", as used in the specification and claims, is calculated as follows. The query sequence is aligned to the target  
20 sequence using the CLUSTAL W algorithm (Thompson, J.D., Higgins, D.G. and Gibson, T.J., 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 aligned sequences may in some instances be the target sequence, such as the albumin binding polypeptide  
25 disclosed herein. In other instances, the query sequence may constitute the shortest of the aligned sequences. The query sequence may for example consist of at least 10 amino acid residues, such as at least 20 amino acid residues, such as at least 30 amino acid residues, such as at least 40 amino acid residues, for example 45 amino acid residues. The amino acid residues  
30 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.

In one embodiment of the albumin binding polypeptide according to the first aspect,  $X_6$  is E.  
35 In another embodiment of the albumin binding polypeptide according to this aspect,  $X_3$  is S.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_3$  is E.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_7$  is A.

5 In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{14}$  is S.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{14}$  is C.

10 In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{10}$  is A.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{10}$  is S.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{26}$  is D.

15 In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{26}$  is E.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{3g}$  is D.

20 In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{3g}$  is E.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{40}$  is A.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{43}$  is A.

25 In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{44}$  is A.

In another embodiment of the albumin binding polypeptide according to this aspect,  $X_{44}$  is S.

30 In another embodiment of the albumin binding polypeptide according to this aspect, the L residue in position 45 is present.

In another embodiment of the albumin binding polypeptide according to this aspect, the P residue in position 46 is present.

In another embodiment of the albumin binding polypeptide according to this aspect, the P residue in position 46 is absent.

35 In another embodiment, the albumin binding polypeptide according to this aspect is subject to the proviso that  $X_7$  is neither L, E nor D.

The albumin binding polypeptide according to the first aspect may be prepared for conjugation with a suitable conjugation partner by the replacement of surface exposed amino acid residues with, for example, either a cysteine or a lysine. These replacements may be introduced into the N-terminal helix, i.e. helix one, of the polypeptide, which is the helix situated furthest away from the serum albumin when the albumin binding polypeptide is bound to serum albumin. Thus, a lysine residue in position  $X_{14}$  of the sequence defined in i) may be used to enable site-directed conjugation. This may furthermore be advantageous when the molecule is made by chemical peptide synthesis, since orthogonal protection of the epsilon-amino group of said lysine may be utilized. Furthermore, a cysteine residue may be introduced into the amino acid sequence to enable site-directed conjugation. For example, a cysteine residue may be introduced into any one of the positions  $x_3$ ,  $X_6$  and/or  $X_{14}$  in accordance with the above definition.

15        Coupling of a conjugation partner to the epsilon-amine of a lysine or the thiol group of a cysteine represents two chemically different alternatives to obtain site-directed conjugation using an amino acid residue within the amino acid sequence i). As the skilled person understands, other chemical alternatives for preparing an amino acid sequence for conjugation exist, and 20 are as such also within the scope of the present disclosure. One example of such a chemistry is the click-like chemistry enabled by the introduction of a tyrosine as presented by Ban *et al* (J Am Chem Soc 132:1 523-5, 2009).

25        The terms "albumin binding" and "binding affinity for albumin" as used in this specification refer to a property of a polypeptide which may be tested for example by the use of surface plasmon resonance technology, such as in a Biacore instrument. For example as described in the examples below, albumin binding affinity may be tested in an experiment in which albumin, or a fragment thereof, is immobilized on a sensor chip of the instrument, and the sample containing the polypeptide to be tested is passed over the chip.

30        Alternatively, the polypeptide to be tested is immobilized on a sensor chip of the instrument, and a sample containing albumin, or a fragment thereof, is passed over the chip. Albumin may, in this regard, be a serum albumin from a mammal, such as human serum albumin. The skilled person may then interpret the results obtained by such experiments to establish at least a 35 qualitative measure of the binding affinity of the polypeptide for albumin. If a quantitative measure is desired, for example to determine a  $K_D$  value for the interaction, surface plasmon resonance methods may also be used. Binding

values may for example be defined in a Biacore2000 instrument (GE Healthcare). Albumin is suitably immobilized on a sensor chip of the measurement, and samples of the polypeptide whose affinity is to be determined are prepared by serial dilution and injected.  $K_D$  values may then 5 be calculated from the results using for example the 1:1 Langmuir binding model of the BIAevaluation 4.1 software provided by the instrument manufacturer (GE Healthcare).

In one embodiment, the albumin binding polypeptide according to this aspect binds to albumin such that the  $k_{off}$  value of the interaction is at most 10  $5 \times 10^{-5} \text{ s}^{-1}$ , such as at most  $5 \times 10^{-6} \text{ s}^{-1}$ .

As described above, the albumin binding polypeptides as defined by the amino acid sequence i) are derived from a common parent polypeptide sequence which folds into a three alpha helix bundle domain. In one embodiment, the three helix domain of this parent polypeptide sequence 15 forms part of protein G from *Streptococcus* strain G148, in particular domain GA3.

In another embodiment, the amino acid sequence of the albumin binding polypeptide is selected from any one of SEQ ID NO:1-144 and SEQ ID NO:1 64-203, such as selected from any one of SEQ ID NO:1-144. More 20 specifically, the amino acid sequence is selected from SEQ ID NO:4-5, SEQ ID NO:7-8, SEQ ID NO:1 0-1 1, SEQ ID NO:13-14, SEQ ID NO:1 6-1 7, SEQ ID NO:1 9-20, SEQ ID NO:22-23, SEQ ID NO:25-26, SEQ ID NO:28-29, SEQ ID NO:31-32, SEQ ID NO:34-35, SEQ ID NO:37-38, SEQ ID NO:41-42, SEQ ID NO:49-50, SEQ ID NO:164-170 and SEQ ID NO:1 92-203. Thus, the amino 25 acid sequence may be selected from SEQ ID NO:4-5, SEQ ID NO:7-8, SEQ ID NO:1 0-1 1, SEQ ID NO:1 3-14, SEQ ID NO:1 6-1 7, SEQ ID NO:19-20, SEQ ID NO:22-23, SEQ ID NO:25-26, SEQ ID NO:28-29, SEQ ID NO:31-32, SEQ ID NO:34-35, SEQ ID NO:37-38, SEQ ID NO:41-42 and SEQ ID NO:49-50.

In one embodiment, the albumin binding polypeptide according to this 30 aspect further comprises one or more additional amino acid residues positioned at the N- and/or the C-terminal of the sequence defined in i). These additional amino acid residues may play a role in enhancing the binding of albumin by the polypeptide, and improving the conformational stability of the folded albumin binding domain, but may equally well serve 35 other purposes, related for example to one or more of production, purification, stabilization *in vivo* or *in vitro*, coupling, labeling or detection of the polypeptide, as well as any combination thereof. Such additional amino acid

residues may comprise one or more amino acid residue(s) added for purposes of chemical coupling, e.g. to a chromatographic resin to obtain an affinity matrix or to a chelating moiety for complexing with a radiometal.

The amino acids directly preceding or following the alpha helix at the

5 N-or C-terminus of the amino acid sequence i) may thus in one embodiment affect the conformational stability. One example of an amino acid residue which may contribute to improved conformational stability is a serine residue positioned at the N-terminal of the amino acid sequence i) as defined above. The N-terminal serine residue may in some cases form a canonical S-X-X-E

10 capping box, by involving hydrogen bonding between the gamma oxygen of the serine side chain and the polypeptide backbone NH of the glutamic acid residue. This N-terminal capping may contribute to stabilization of the first alpha helix of the three helix domain constituting the albumin binding polypeptide according to the first aspect of the disclosure.

15 Thus, in one embodiment, the additional amino acids comprise at least one serine residue at the N-terminal of the polypeptide. The amino acid sequence is in other words preceded by one or more serine residue(s). In another embodiment of the albumin binding polypeptide, the additional amino acids comprise a glycine residue at the N-terminal of the polypeptide. It is

20 understood that the amino acid sequence i) may be preceded by one, two, three, four or any suitable number of amino acid residues. Thus, the amino acid sequence may be preceded by a single serine residue, a single glycine residue or a combination of the two, such as a glycine-serine (GS) combination or a glycine-serine-serine (GSS) combination. Examples of

25 albumin binding polypeptides comprising additional amino residues at the N-terminal are set out in SEQ ID NO:145-163, such as in SEQ ID NO:145-148 and SEQ ID NO:1 62-1 63. In yet another embodiment, the additional amino acid residues comprise a glutamic acid at the N-terminal of the polypeptide as defined by the sequence i).

30 Similarly, C-terminal capping may be exploited to improve stability of the third alpha helix of the three helix domain constituting the albumin binding polypeptide. A proline residue, when present at the C-terminal of the amino acid sequence defined in i), may at least partly function as a capping residue. In such a case, a lysine residue following the proline residue at the C-terminal

35 may contribute to further stabilization of the third helix of the albumin binding polypeptide, by hydrogen bonding between the epsilon amino group of the lysine residue and the carbonyl groups of the amino acids located two and

three residues before the lysine in the polypeptide backbone, e.g., when both L45 and P46 are present, the carbonyl groups of the leucine and alanine residues of the amino acid sequence defined in i). Thus, in one embodiment, the additional amino acids comprise a lysine residue at the C-terminal of the 5 polypeptide.

As discussed above, the additional amino acids may be related to the production of the albumin binding polypeptide. In particular, when an albumin binding polypeptide according to an embodiment in which P46 is present is produced by chemical peptide synthesis, one or more optional amino acid 10 residues following the C-terminal proline may provide advantages. Such additional amino acid residues may for example prevent formation of undesired substances, such as diketopiperazine at the dipeptide stage of the synthesis. One example of such an amino acid residue is glycine. Thus, in one embodiment, the additional amino acids comprise a glycine residue at the 15 C-terminal of the polypeptide, directly following the proline residue or following an additional lysine and/or glycine residue as accounted for above. Alternatively, polypeptide production may benefit from amidation of the C-terminal proline residue of the amino acid sequence i), when present. In this case, the C-terminal proline comprises an additional amine group at the 20 carboxyl carbon. In one embodiment of the polypeptides described herein, particularly those ending at its C-terminus with proline or other amino acid known to racemize during peptide synthesis, the above-mentioned addition of a glycine to the C-terminus or amidation of the proline, when present, can also counter potential problems with racemization of the C-terminal amino 25 acid residue. If the polypeptide, amidated in this way, is intended to be produced by recombinant means, rather than by chemical synthesis, amidation of the C-terminal amino acid can be performed by several methods known in the art, e.g. through the use of amidating PAM enzyme.

Examples of albumin binding polypeptides comprising additional amino 30 acid residues at the C-terminal are set out in SEQ ID NO:145-1 52, such as in SEQ ID NO:1 48-1 50. The skilled person is aware of methods for accomplishing C-terminal modification, such as by different types of pre-made matrices for peptide synthesis.

In another embodiment, the additional amino acid residues comprise a 35 cysteine residue at the N- and/or C-terminal of the polypeptide. Such a cysteine residue may directly precede and/or follow the amino acid sequence as defined in i) or may precede and/or follow any other additional amino acid

residues as described above. Examples of albumin binding polypeptides comprising a cysteine residue at the N- and/or C-terminal of the polypeptide chain are set out in SEQ ID NO:149-1 50 (C-terminal) and SEQ ID NO:1 51-152 (N-terminal). By the addition of a cysteine residue to the polypeptide 5 chain, a thiol group for site directed conjugation of the albumin binding polypeptide may be obtained. Alternatively, a selenocysteine residue may be introduced at the C-terminal of the polypeptide chain, in a similar fashion as for the introduction of a cysteine residue, to facilitate site-specific conjugation (Cheng *et al*, Nat Prot 1:2, 2006).

10 In one embodiment, the albumin binding polypeptide comprises no more than two cysteine residues. In another embodiment, the albumin binding polypeptide comprises no more than one cysteine residue.

15 In another embodiment, the additional amino acid residues of the albumin binding polypeptide comprise a "tag" for purification or detection of the polypeptide, such as a hexahistidyl (His6) tag, or a "myc" ("c-Myc") tag or a "FLAG" tag for interaction with antibodies specific to the tag and/or to be used in purification. The skilled person is aware of other alternatives.

20 In yet another embodiment, the albumin binding polypeptide according to this aspect binds to human serum albumin. In other embodiments, the albumin binding polypeptide binds to albumin from other species than the human species, such as albumin from mouse, rat, dog and cynomolgus macaques.

25 The "additional amino acid residues" discussed above may also constitute one or more polypeptide domain(s) with any desired function, such as the same binding function as the first, albumin binding domain, or another binding function, or a therapeutic function, or an enzymatic function, or a fluorescent function, or mixtures thereof.

30 There is consequently in another, related aspect, provided a fusion protein or conjugate comprising

- 30 i) a first moiety consisting of an albumin binding polypeptide according to the first aspect as described herein; and
- ii) a second moiety consisting of a polypeptide having a desired biological activity.

35 A fusion protein or conjugate comprising an albumin binding polypeptide according to the first aspect of the disclosure and a second moiety may increase the *in vitro* and/or the *in vivo* half life of the second moiety, as compared to the *in vivo* half life of the second moiety *per se*. As a

consequence, when a fusion protein or conjugate according to this aspect is administered to a subject, such as a human subject, the *in vivo* exposure to the second moiety may increase, which may lead to improved potency of the biological activity of the second moiety, as compared to the potency of *in vivo* exposure of the second moiety when administered by itself.

The desired biological activity may, for example, be a therapeutic activity, a binding activity or an enzymatic activity. When the desired biological activity is a therapeutic activity, the second moiety showing this activity may be a therapeutically active polypeptide. Non-limiting examples of 10 therapeutically active polypeptides are biomolecules, such as molecules selected from the group consisting of human endogenous enzymes, hormones, growth factors, chemokines, cytokines and lymphokines, and non-human biologically active proteins, such as proteins selected from the group consisting of bacterial toxins (e.g. pseudomonas exotoxin and staphylococcal 15 and streptococcal superantigens), enzymes (e.g. RNase and beta-lactamase) and activating proteins (e.g. streptokinase). Non-limiting examples of therapeutically active biomolecules which may prove useful in a fusion or conjugate with the albumin binding polypeptide are selected from the group consisting of IL-2, GLP-1, BNP (Alb-beta-natriuretic peptide), IL-1-RA 20 (interleukin-1 receptor antagonist), KGF (keratinocyte growth factor), Stemgen®, growth hormone (GH), G-CSF, CTLA-4, myostatin, Factor VII, Factor VIII and Factor IX.

The leaky defective blood vessels of tumor tissue make its vasculature (endothelial barrier) permeable for macromolecules, whereas in blood vessels 25 of healthy tissue only small molecules can pass the endothelial barrier. Likewise, the permeability of the blood-joint barrier for albumin in inflamed joints of rheumatoid arthritis patients is markedly increased. Thus, fusion proteins or conjugates according to this aspect are likely to permeate blood vessels in tumor tissue and the blood-joint barrier in inflamed joints.

30 When said desired biological activity of the second moiety is a binding activity, said second moiety may be a binding polypeptide capable of selective interaction with a target molecule. Such a binding polypeptide may for example be selected from the group consisting of antibodies and fragments and domains thereof substantially retaining antibody binding 35 activity; microbodies, maxybodies, avimers and other small disulfide-bonded proteins; and binding proteins derived from a scaffold selected from the group consisting of staphylococcal protein A and domains thereof, other three helix

domains, lipocalins, ankyrin repeat domains, cellulose binding domains,  $\gamma$  crystallines, green fluorescent protein, human cytotoxic T lymphocyte-associated antigen 4, protease inhibitors such as Kunitz domains, PDZ domains, SH3 domains, peptide aptamers, staphylococcal nuclease, 5 tendamistats, fibronectin type III domain, transferrin, zinc fingers and conotoxins.

In some embodiments, the target molecule for binding of said target binding polypeptide may be selected from the group consisting of amyloid  $\beta$  (A $\beta$ ) peptide of Alzheimer's disease; other disease-associated amyloid 10 peptides; toxins, such as bacterial toxins and snake venoms; blood clotting factors, such as von Willebrand factor; interleukins, such as IL-13; myostatin; pro-inflammatory factors, such as TNF-a, TNF-a receptor, IL-1, IL-8 and IL-23; complement factors, such as C3 and C5; hypersensitivity mediators, such as histamine and IgE; tumor-related antigens, such as CD19, CD20, 15 CD22, CD30, CD33, CD40, CD52, CD70, cMet, HER1, HER2, HER3, HER4, CAIX (carbonic anhydrase IX), CEA, IL-2 receptor, MUC1, PSMA, TAG-72; and other biological molecules such as G-CSF, GM-CSF, growth hormone (GH), insulin and somatostatin.

As the skilled person understands, the albumin binding polypeptide 20 according to the first aspect may be useful in a fusion protein or as a conjugate partner to any other moiety. Therefore, the above lists of therapeutically active polypeptides, binding polypeptides and target molecules should not be construed as limiting in any way.

In one embodiment of a fusion protein or conjugate according to the 25 present disclosure, the second moiety is conjugated to the albumin binding polypeptide via a lysine or cysteine residue added to the N- or C-terminal of the albumin binding polypeptide or via a lysine or cysteine residue at a position within the albumin binding polypeptide, such as at a position selected from X<sub>3</sub>, X<sub>6</sub> and **Xi<sub>4</sub>**. If the conjugation site is one within the amino acid 30 sequence i) of the albumin binding polypeptide, such as a cysteine in position **Xi<sub>4</sub>**, no additional amino acids need to be added to the albumin binding polypeptide for the purpose of enabling conjugation to the second moiety. A conjugation site within the polypeptide chain as defined by i) may moreover shield the polypeptide against cross-reacting antibodies, in particular the 35 portion of the polypeptide close to the conjugation site. Without wishing to be bound by theory, when the conjugate via the albumin binding polypeptide is bound to serum albumin *in vivo*, i.e. situated in the binding pocket of serum

albumin, the second moiety, conjugated at a position within for example helix one of the three helix domain constituting the albumin binding polypeptide, is likely to point away from the serum albumin to which the albumin binding polypeptide is bound. In addition, a conjugation site within the albumin binding 5 polypeptide may impair the presentation of the portion of the peptides otherwise derived from this portion of the polypeptide to T-cells due to, for example, effects on processing in the antigen presenting cell, impaired fit of potential peptides in the peptide binding groove of the MCH class II molecule, and remodeled peptide surface available to the T-cell receptor (due to the 10 conjugated portion sticking out). Thus, the immunogenicity of the portion of the conjugate near the conjugation site is expected to become further reduced after conjugation.

Due to the high affinity between the albumin binding polypeptide of the present disclosure and serum albumin, a conjugate or fusion protein 15 comprising such an albumin binding polypeptide might be regarded as an indirect complex with serum albumin. A conjugate or a fusion protein according to the present disclosure thus provides an alternative to the frequently used method of exploiting direct conjugates or fusions with serum albumin. Such direct conjugates with serum albumin are frequently 20 inhomogeneous, irrespective of what method is used for coupling. When a specific molecule is coupled to serum albumin via an amino group of a lysine residue, any one of a large number of lysines on the surface of the serum albumin molecule may be targeted, which gives a random conjugation site and a random product. Although thiol coupling via the single unpaired 25 cysteine in human serum albumin (in position 34, Peters, 1985, *supra*) seems to offer an alternative method for obtaining a direct conjugate, such a methodology frequently does not lead to a homogeneous product. Only 20- 60 % of the molecules in commercially available (human) serum albumin 30 display a free thiol group, whereas the rest are blocked by thiol compounds such as cysteine, homocysteine or glutathione. In contrast, conjugation to the three helix domain of the albumin binding polypeptide according to the present disclosure may be performed site-specifically. This may be accomplished, as discussed above, either by coupling to one or more cysteines, to a selenocysteine, or to a designated lysine (orthogonally 35 protected during synthesis).

According to this aspect of the present disclosure, the second moiety having the desired biological activity may either be conjugated to the three

helix domain of the albumin binding polypeptide or produced as a fusion protein with the same. A non-limiting example of a conjugate according to the present disclosure is given below. Glucagon-like peptide 1 (GLP-1), or a derivative thereof, is a small polypeptide which may suitably be present as a 5 second moiety in a conjugate with the albumin binding polypeptide.

Conjugation of GLP-1 to the albumin binding polypeptide may be performed in any one of the positions of the polypeptide sequence as described above. The conjugate may as such be produced in a non-biological process and is expected to display a significantly enhanced potency as compared to the 10 potency of GLP-1 *per se*. Conjugation may be employed with both small polypeptides or proteins, such as GLP-1, or with larger polypeptides or proteins. A conjugate according to the present disclosure may typically comprise a non-amino acid spacer moiety, such as polyethylene glycol (PEG).

15 Other polypeptides or proteins may be combined with the amino acid sequence of the albumin binding polypeptide in the form of a fusion protein. Such a fusion protein may furthermore comprise one or more spacer amino acid residues between the first and second moieties.

As described above, the albumin binding polypeptide according to the 20 first aspect binds serum albumin from several species, including mouse, rat, dog and cynomolgus macaques. Thus, a fusion protein or conjugate according to the present disclosure may contribute to enhancing the biological effect of a second moiety, not only in a human subject, but also in animal models. A number of endogenous proteins have been produced as direct 25 fusions with human serum albumin, examples of such proteins include G-CSF, GH, interferons, CD4, IL-2, insulin, glucagon, GLP-1, antibody Fab fragments and protease inhibitors like Kunitz-domain derived proteins. Such direct fusions may however not be fully evaluated in animal models. This is due to the fact that human serum albumin does not interact properly with the 30 endogenous Fc neonatal receptor (FcRn), e.g. in the commonly used animal models mouse and rat, and that this interaction is an important factor contributing to the long circulation time of serum albumin. As described above, a conjugate or a fusion protein according to the present disclosure may, in the presence of serum albumin, combine with albumin and function as 35 an indirect fusion with albumin. This makes a conjugate or a fusion protein comprising an albumin binding polypeptide according to the first aspect useful

in preclinical model species, provided that the second moiety is biologically active in the selected species.

In one embodiment, there is provided a fusion protein or conjugate comprising a further moiety consisting of a polypeptide having a further, 5 desired biological activity, which may be the same as or different from that of the second moiety. One specific example of such a fusion protein or conjugate comprises a therapeutically active polypeptide as defined above as a second moiety and a binding polypeptide as defined above as a further moiety.

10 With regard to the description above of fusion proteins or conjugates incorporating an albumin binding polypeptide according to the first aspect, it is to be noted that the designation of first, second and further moieties is made for clarity reasons to distinguish between albumin binding polypeptide or polypeptides according to the present disclosure on the one hand, and 15 moieties exhibiting other functions on the other hand. These designations are not intended to refer to the actual order of the different domains in the polypeptide chain of the fusion protein or conjugate. Thus, for example, said first moiety may without restriction appear at the N-terminal end, in the middle, or at the C-terminal end of the fusion protein or conjugate.

20 In a related aspect, there is provided an albumin binding polypeptide, fusion protein or conjugate as defined in the present disclosure, further comprising an organic molecule, such as a cytotoxic agent. Non-limiting examples of cytotoxic agents which may be fused or conjugated to an albumin binding polypeptide according to the first aspect, or combined with a 25 fusion protein or conjugate according to the second aspect, are selected from calicheamycin, auristatin, doxorubicin, maytansinoid, taxol, ecteinascidin, geldanamycin, methotrexate and their derivatives, and combinations thereof. Previously, attempts have been made to treat various disorders with direct albumin conjugates. Such direct albumin conjugates have been exploited e.g. 30 with doxorubicin in cancer (Kratz *et al*, J Med Chem 45: 5523-33, 2002) and methotrexate in rheumatoid arthritis (Wunder *et al*, J Immunol 170:4793-4801, 2003). It is to be understood that the albumin binding polypeptide, either by itself or as a moiety in a fusion protein or conjugate, by its high albumin binding ability provides indirect means of construing albumin complexes, and 35 thus may provide an alternative treatment method compared to the attempts mentioned above.

The above aspects furthermore encompass polypeptides in which the albumin binding polypeptide according to the first aspect, or the albumin binding polypeptide as comprised in a fusion protein or conjugate according to the second aspect, has been provided with a label group, such as a label

5 selected from the group consisting of fluorescent dyes and metals, chromophoric dyes, chemiluminescent compounds and bioluminescent proteins, enzymes, radionuclides and particles, for example for purposes of detection of the polypeptide. In particular, the disclosure encompasses a radiolabeled polypeptide consisting of a radiochelate of an albumin binding

10 polypeptide, fusion protein or conjugate as described herein and a radionuclide, such as a radioactive metal.

In embodiments where the labeled albumin binding polypeptide comprises an albumin binding polypeptide according to the first aspect of the disclosure and a label, the labeled polypeptide may for example be used for labeling serum albumin indirectly. Due to the strong association between the labeled polypeptide and serum albumin, the labeled polypeptide may be used for example to study vascular permeability and blood pool.

In other embodiments, the labeled albumin binding polypeptide is present as a moiety in a fusion protein or conjugate also comprising a second moiety having a desired biological activity. The label may in some instances be coupled only to the albumin binding polypeptide, and in some instances both to the albumin binding polypeptide and to the second moiety of the conjugate or fusion protein. When reference is made to a labeled polypeptide, this should be understood as a reference to all aspects of polypeptides as described herein, including fusion proteins and conjugates comprising an albumin binding polypeptide and a second and optionally further moieties. Thus, a labeled polypeptide may contain only the albumin binding polypeptide and e.g. a therapeutic radionuclide, which may be chelated or covalently coupled to the albumin binding polypeptide, or contain the albumin binding polypeptide, a therapeutic radionuclide and a second moiety such as a small molecule having a desired biological activity such as therapeutic efficacy.

In embodiments where the albumin binding polypeptide, fusion protein or conjugate is radiolabeled, such a radiolabeled polypeptide may comprise a radionuclide. A majority of radionuclides have a metallic nature and metals are typically incapable of forming stable covalent bonds with elements presented in proteins and peptides. For this reason, labeling of proteins and peptides with radioactive metals is performed with the use of chelators, i.e.

multidentate ligands, which form non-covalent compounds, called chelates, with the metal ions. In an embodiment of the albumin binding polypeptide, fusion protein or conjugate, the incorporation of a radionuclide is enabled through the provision of a chelating environment, through which the 5 radionuclide may be coordinated, chelated or complexed to the polypeptide.

One example of a chelator is the polyaminopolycarboxylate type of chelator. Two classes of such polyaminopolycarboxylate chelators can be distinguished: macrocyclic and acyclic chelators. In one embodiment, the albumin binding polypeptide, fusion protein or conjugate comprises a 10 chelating environment provided by a polyaminopolycarboxylate chelator conjugated to the albumin binding polypeptide via a thiol group of a cysteine residue or an epsilon amine group of a lysine residue.

The most commonly used macrocyclic chelators for radioisotopes of indium, gallium, yttrium, bismuth, radioactinides and radiolanthanides are 15 different derivatives of DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid). In one embodiment, the chelating environment of the albumin binding polypeptide, fusion protein or conjugate is provided by DOTA or a derivative thereof. More specifically, one group of chelating polypeptides encompassed by the present disclosure is made by reacting the DOTA 20 derivative 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide (maleimidomonoamide-DOTA) with, for example, a thiol group of the albumin binding polypeptide, for example as described in Example 5.

The high kinetic inertness, i.e. the slow rate of dissociation of metal 25 from DOTA, favors stable attachment of a radionuclide. However, elevated temperatures are required for labeling due to a slow association rate. For this reason, DOTA derivatives are widely used for labeling of short peptides, such as the albumin binding polypeptides of the present disclosure, which display binding functionality following incubation at temperatures required for the 30 labeling reaction.

The most commonly used acyclic polyaminopolycarboxylate chelators are different derivatives of DTPA (diethylenetriamine-pentaacetic acid). Hence, polypeptides having a chelating environment provided by diethylenetriaminepentaacetic acid or derivatives thereof are also 35 encompassed by the present disclosure.

It has been found that backbone-modified semi-rigid variants of DTPA provide adequate stability for labeling with <sup>90</sup>Y of e.g. Zevalin®. Though

acyclic chelators are less inert, and consequently, less stable than macrocyclic ones, their labeling is rapid enough even at ambient temperature. For this reason, they might be used for labeling of fusion proteins or conjugates according to the present disclosure. Detailed protocols for

5 coupling of polyaminopolycarboxylate chelators to targeting proteins and peptides have been published by Cooper *et al* (Nat Prot 1: 314-7, 2006) and by Sosabowski and Mather (Nat Prot 1:972-6, 2006).

An albumin binding polypeptide, a fusion protein or conjugate according to the aspects described herein coupled to a

10 polyaminopolycarboxylate chelator may be used to provide a radiolabeled polypeptide consisting of a radiochelate of the albumin binding polypeptide, fusion protein or conjugate coupled to the chelator and a radionuclide suitable for medical imaging, the radionuclide being selected from the group consisting of  $^{61}\text{Cu}$ ,  $^{64}\text{Cu}$ ,  $^{66}\text{Ga}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{110\text{m}}\text{In}$ ,  $^{111}\text{In}$ ,  $^{44}\text{Sc}$  and  $^{86}\text{Y}$ , or with a

15 radionuclide suitable for therapy, the radionuclide being selected from the group consisting of  $^{225}\text{Ac}$ ,  $^{212}\text{Bi}$ ,  $^{213}\text{Bi}$ ,  $^{67}\text{Cu}$ ,  $^{166}\text{Ho}$ ,  $^{177}\text{Lu}$ ,  $^{212}\text{Pb}$ ,  $^{149}\text{Pm}$ ,  $^{153}\text{Sm}$ ,  $^{227}\text{Th}$  and  $^{90}\text{Y}$ , wherein the radionuclide is complexed with the albumin binding polypeptide via a chelator.

In embodiments thereof, the polypeptide may also be radiolabeled with

20 non-metal radioisotopes using so called indirect labeling. Thus, for labeling with for example  $^{18}\text{F}$ ,  $^{76}\text{Br}$ , different iodine isotopes and  $^{211}\text{At}$ , intermediate "linker molecules" are used for labeling. Such a linker molecule should contain two functional moieties, one providing rapid and efficient radiolabeling, and another enabling rapid and efficient coupling to the

25 proteins, e.g. to amine groups, or preferably to the thiol group of a unique cysteine, such as in position  $\text{X}_{14}$  of the albumin binding polypeptide. For example a maleimide group reacts with thiol groups to form a stable thioether bond. The "linker molecule" may first be reacted with the radiolabel and subsequently with the thiol or the selenothiol group of the protein.

30 In another aspect, there is provided a polynucleotide encoding an albumin binding polypeptide or a fusion protein as described herein. Also encompassed is a method of producing an albumin binding polypeptide or a fusion protein as described above, comprising expressing the polynucleotide, an expression vector comprising the polynucleotide and a host cell

35 comprising the expression vector.

The albumin binding polypeptide of the present disclosure may alternatively be produced by non-biological peptide synthesis using amino

acids and/or amino acid derivatives having protected reactive side-chains, the non-biological peptide synthesis comprising

step-wise coupling of the amino acids and/or the amino acid derivatives to form a polypeptide according to the first aspect having

5 protected reactive side-chains,

removal of the protecting groups from the reactive side-chains of the polypeptide, and

folding of the polypeptide in aqueous solution.

Thus, normal amino acids (e.g. glycine, alanine, phenylalanine, 10 isoleucine, leucine and valine) and pre-protected amino acid derivatives are used to sequentially build a polypeptide sequence, in solution or on a solid support in an organic solvent. One specific example of peptide synthesis on solid support is described in Example 5. When a complete polypeptide sequence is built, the protecting groups are removed and the polypeptide is 15 allowed to fold in an aqueous solution. Each polypeptide according to the present disclosure reversibly folds into a three helix bundle domain without added factors, and hence folds spontaneously.

The conjugate according to the second aspect may be produced by a method comprising producing an albumin binding polypeptide according to 20 any of the methods described above, such as by non-biological peptide synthesis, and conjugating the produced albumin binding polypeptide with a second and/or further moiety as defined in connection with the second aspect.

In one embodiment of a fusion protein or conjugate, there is moreover 25 provided a fusion protein or conjugate as defined herein for use in therapy, e.g. for use as a medicament. Such a fusion protein or conjugate may exhibit a half-life *in vivo* which is longer than the half-life *in vivo* of the polypeptide having a desired biological activity *per se*. The fusion protein or conjugate may moreover elicit no or a reduced immune response upon administration to 30 the mammal, such as a human, as compared to the immune response elicited upon administration to the mammal of the polypeptide having a desired biological activity *per se*. Alternatively speaking, this provides a method for decreasing the immunogenicity of a polypeptide having a desired biological activity, through the fusion or conjugation of such a polypeptide to an albumin 35 binding polypeptide, fusion protein or conjugate according to aspects disclosed herein. In addition, this may enable enhancement of the biological activity of a second moiety.

In another embodiment, there is provided a fusion protein or conjugate according to the present disclosure, for use in diagnosis, e.g. for use as a diagnostic agent.

The present disclosure also concerns different aspects of using the

5 above-described albumin binding polypeptide, as well as various methods for treatment, diagnosis and detection in which the polypeptide is useful due to its binding and other characteristics. When referring to the "albumin binding polypeptide" in the following description of these uses and methods, this term is intended to encompass the albumin binding polypeptide alone, but also all

10 those molecules based on this polypeptide described above that e.g. incorporate the albumin binding polypeptide as a moiety in a fusion protein or conjugate, and/or are conjugated to a label, a chelator, a therapeutic and/or diagnostic agent and/or are provided with additional amino acid residues as a tag or for other purposes. As explained above, such fusion proteins,

15 derivatives etc form a part of the present disclosure.

Another set of aspects concern the provision of new means to increase the solubility in aqueous solution of a poorly soluble compound, through conjugation thereof to an albumin binding polypeptide, fusion protein or conjugate. The ensuing complex of poorly soluble compound and an albumin

20 binding polypeptide, alone or incorporated as a moiety in a fusion protein or conjugate, is able to associate with albumin *in vivo* or *in vitro*, which association increases the solubility in aqueous solution. Thus, in an embodiment of this further aspect, there is provided a composition, comprising

25 a compound which *per se* has a solubility in water of no more than 100 µg/ml; coupled to

an albumin binding polypeptide, a fusion protein or conjugate as described herein, wherein the compound and the albumin binding polypeptide, fusion protein or conjugate are covalently coupled.

30 In one embodiment, the compound *per se* has a solubility of no more than 10 µg/ml. In yet another embodiment, said solubility is no more than 1 µg/ml.

35 In some embodiments, the compound may be a pharmaceutically active compound, for example a cytotoxic agent. Non-limiting examples of cytotoxic agents are those selected from calicheamycin, auristatin, doxorubicin, maytansinoid, taxol, ecteinascidin, geldanamycin and their derivatives, and combinations thereof. Alternatively, the cytotoxic agent may

be a synthetic chemotoxin made by organic synthesis and not derived from a naturally occurring compound.

In addition to the poorly soluble compound and albumin binding polypeptide, fusion protein or conjugate, the composition according to this 5 aspect of the disclosure may, in some embodiments, also comprise a binding polypeptide with an affinity for a clinically relevant target. This binding polypeptide is suitably different from the albumin binding polypeptide, and may be non-covalently or covalently coupled to the other components of the inventive composition. As non-limiting examples, the binding polypeptide with 10 an affinity for a clinically relevant target may be selected from the group consisting of antibodies and fragments and domains thereof substantially retaining antibody binding activity; microbodies, maxybodies, avimers and other small disulfide-bonded proteins; and binding proteins derived from a scaffold selected from the group consisting of staphylococcal protein A and 15 domains thereof, other three helix domains, lipocalins, ankyrin repeat domains, cellulose binding domains,  $\gamma$  crystallines, green fluorescent protein, human cytotoxic T lymphocyte-associated antigen 4, protease inhibitors such as Kunitz domains, PDZ domains, SH3 domains, peptide aptamers, staphylococcal nuclease, tendamistats, fibronectin type III domain, transferrin, 20 zinc fingers and conotoxins.

The composition according to the above aspect of the present disclosure has an ability to associate with albumin *in vivo* or *in vitro*, through the provision in the composition of an albumin binding polypeptide, by itself or as present in a fusion protein or conjugate. In certain cases, it may be of 25 benefit to form a complex of the composition with albumin outside of a living organism, i.e. to add exogenous albumin to the composition. Such a composition may be lyophilized, providing a formulation that is suitable for storage at ambient temperature. Thus, the present disclosure also provides a composition as defined above which further comprises albumin, such as 30 human serum albumin.

The present disclosure also provides the composition according to the above aspect for use as a medicament, i.e. for use in therapy, in cases where the compound is a therapeutically active compound. Suitably, the provision of an albumin binding polypeptide, fusion protein or conjugate and optionally 35 albumin does not deleteriously affect the therapeutic efficacy of the active compound, so the inventive composition will be useful in those therapeutic or prophylactic settings where the compound *per se* is indicated.

In another embodiment, there is provided the composition according to the above aspect for use as a diagnostic agent, i.e. for use in diagnosis.

A related aspect of the present disclosure provides a method of preparation of a composition as described immediately above. The method

5 comprises

providing a compound which *per se* has a solubility in water of no more than 100 pg/ml; and

covalently coupling the compound to an albumin binding polypeptide, fusion protein or conjugate according aspects as described herein, thus

10 forming a composition comprising a covalent complex of compound and albumin binding polypeptide, fusion protein or conjugate.

In embodiments of the present disclosure where albumin is included into the composition, the method may comprise the additional step of mixing said complex of compound and albumin binding polypeptide, fusion protein or conjugate with albumin, thus forming a composition comprising a non-covalent complex of i) the covalent complex of compound and albumin binding polypeptide, fusion protein or conjugate, and ii) albumin. The relative proportions of the two components of this non-covalent complex may for example be 1:1, so that one unit of the complex of poorly soluble compound

15 and albumin binding polypeptide, fusion protein or conjugate is associated with one molecule of albumin. In one embodiment, the method additionally comprises lyophilizing the non-covalent complex to obtain a lyophilized composition.

In another closely related aspect, the present disclosure provides a

25 method of increasing the aqueous solubility of a compound, comprising

providing a compound which *per se* has a solubility in water of no more than 100 pg/ml;

covalently coupling the compound to an albumin binding polypeptide, fusion protein or conjugate according aspects as described herein, thus

30 forming a covalent complex of compound and albumin binding polypeptide, fusion protein or conjugate; and

mixing said complex of compound and albumin binding polypeptide, fusion protein or conjugate with albumin under conditions that promote the non-covalent association of the albumin binding polypeptide with albumin;

35 whereby the solubility in water of the compound in said complex is greater than the solubility in water of the compound *per se*.

In these method aspects concerning the solubility of a poorly soluble compound, the optional features of the various components are as described in connection with the immediately preceding composition aspect.

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

15 Figures

Figure 1 is a listing of the amino acid sequences of examples of albumin binding polypeptides of the present disclosure (SEQ ID NO:1-1 52, SEQ ID NO:1 55-203), the GA3 domain from protein G of *Streptococcus* strain G148 extended by a N-terminal glycine residue (SEQ ID NO:1 53) and an albumin binding polypeptide derived from G148-GA3 as previously described by Jonsson *et al* (*supra*, SEQ ID NO:154).

Figure 2 shows the result of binding analysis performed in a Biacore instrument for investigating the binding of the albumin binding polypeptide PEP0791 2 (SEQ ID NO:1 57) to human serum albumin. Three different concentrations of purified protein (40 nM, fat gray line; 10 nM, black line; and 2.5 nM, gray line) were injected over a surface with 955 RU of immobilized human serum albumin.

Figures 3A-C show the result of binding analysis performed by ELISA for investigating the binding of the albumin binding polypeptides PEP0791 3 (SEQ ID NO:1 53), PEP06923 (SEQ ID NO:1 54), PEP07271 (SEQ ID NO:1 55), PEP0791 2 (SEQ ID NO:1 57), PEP07554 (SEQ ID NO:1 56), PEP07914 (SEQ ID NO:1 58), PEP07968 (DOTA-conjugated PEP0791 1, SEQ ID NO:1 59) and PEP07844 (SEQ ID NO:1 61), to IgG molecules present in 126 individual normal human sera, where A) shows the average OD-value, B) shows the percentage of negative sera (defined as OD < 0.15), and C) shows the percentage of positive sera (defined as OD > 1.0).

Figures 4A-B are chromatograms showing analysis of purified, chemically produced albumin binding polypeptide PEP07834 (SEQ ID NO:1 60), where A) shows the absorbance signal at 220 nm, blank subtracted, and B) shows the absorbance signal at 280 nm, blank subtracted. Two peaks 5 appeared at both wavelengths.

Figures 5A-B are spectrograms showing massspectrometric analysis of the two peaks identified in Figure 4A) and B). A) is the spectrogram of the first peak, i.e. the monomer of PEP07834 (SEQ ID NO:1 60), and B) is the spectrogram of the dimer of PEP07834.

10 Figures 6A-C are diagrams showing an immunogenicity assessment of albumin binding polypeptides PEP0791 3 (SEQ ID NO:1 53), PEP0791 2 (SEQ ID NO:1 57), PEP07914 (SEQ ID NO:1 58) and PEP07968 (DOTA-conjugated PEP0791 1, SEQ ID NO:1 59) in a CD3<sup>+</sup> CD4<sup>+</sup> T cell proliferation assay. A) shows the number of individuals responding to the albumin binding 15 polypeptides compared to recombinant human albumin in a cohort of 52 Caucasian donors. B) shows the average stimulation indices (SI) for PEP0791 3, PEP0791 2, PEP07914 and PEP07968 compared to the negative control containing recombinant human albumin. C) shows the number of responding individuals against all proteins in the study as compared to the 20 buffer control .

Figures 7A-C shows the result of binding analysis performed in a Biacore instrument for investigating the binding of the albumin binding polypeptides A) PEP07986 (SEQ ID NO:1 63), B) PEP08296 (DOTA-conjugated PEP081 85, SEQ ID NO:148) and C) PEP06923 (SEQ ID NO:1 54) 25 to albumin from different species. The sensorgrams shown correspond to protein injected at a concentration of 40 nM over surfaces immobilized with albumin from human (1130 RU), thin gray line; cynomolgus monkey (1046 RU), thick gray line; rat (831 RU), thick light gray line; dog (1053 RU), thin black line; and mouse (858 RU), thick black line.

30 Figure 8 shows the inhibitory effect of  $Z_x$ -PP01 3 (open circles),  $Z_y$ -PP013 (open squares) and  $Z_{neg}$ -PP013 (closed triangles) on cytokine induced TF-1 cell proliferation in the presence of five times molar excess of HSA.

Figure 9 shows the maximum binding responses obtained by Biacore analysis of PEP07986 (SEQ ID NO:1 63) stored at 4, 25 or 40 °C for one 35 week, two weeks, one month and three months as indicated, at a concentration of 2 mg/ml, injected over immobilized HSA (704 RU) at a

concentration of 10 nM. Non-treated samples from time = 0 are shown as references.

Figure 10 shows the result of binding analysis performed in a Biacore instrument for investigating the binding of the albumin binding polypeptide

5 PEP08296 (DOTA-conjugated PEP08185, SEQ ID NO:148) to human serum albumin before and after heat treatment. Two concentrations of PEP08296 (0.8 nM, grey lines; 4 nM, black lines) were injected over a surface with 724 RU of immobilized human serum albumin. Solid lines are before heat treatment and hatched lines after heat treatment for 10 minutes at 90 °C.

10 Figures 11A-B show the overlay of two CD spectra of PEP08296 (DOTA-conjugated PEP08185, SEQ ID NO:148) before and after heat treatment for 12 min at 90 °C. A) Sample incubated in PBS pH 7.2. B) Sample incubated in PBS pH 4.0.

15 Figure 12 shows the maximum intensity projection (MIP) image of the whole body distribution of <sup>68</sup>Ga-PEP08296 in a healthy rat, summed during 1.5 h of data collection immediately following intravenous injection (tail vein). Circulating radioactivity in the major vessels (e.g. the jugular (long arrow) and femoral (short arrow)), the heart (H), liver (L), spleen (S), kidney (K) and bladder (B) are readily delineated.

20 Figure 13 shows a gel filtration chromatogram of PEP07986 (SEQ ID NO:163) injected at a concentration of 42 mg/ml, black solid line. A chromatogram of ovalbumin (Mw 43 kDa) injected at a concentration of 5 mg/ml, gray broken line, is included for comparison, confirming that the peak for PEP07986 is not an aggregate, which would have been expected in the

25 void volume eluted at an earlier time point than ovalbumin.

30 The invention will now be illustrated further through the non-limiting description of experiments conducted in accordance therewith. Unless otherwise specified, conventional chemistry and molecular biology methods were used throughout.

ExamplesExample 1:Cloning, expression, purification and characterization of albumin binding polypeptides

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In this example, ten different albumin binding polypeptides, PEP0791 3 (SEQ ID NO:1 53), PEP0791 2 (SEQ ID NO:1 56), PEP07914 (SEQ ID NO:1 58), PEP07968 (DOTA-conjugated PEP0791 1, SEQ ID NO:1 59), PEP06923 (SEQ ID NO:1 54), PEP07271 (SEQ ID NO:1 55), PEP07554 (SEQ ID NO:1 56), PEP07844 (SEQ ID NO:1 61), PEP07986 (SEQ ID NO:163) and PEP08296 (DOTA-conjugated PEP081 85, SEQ ID NO:148), the amino acid sequences of which are set out in Figure 1 and in the appended sequence listing, were cloned, purified and characterized.

15 *Material and methods**Cloning of albumin binding polypeptide variants*

Mutations in G148-GA3 were generated using site directed mutagenesis with the appropriate oligonucleotides to obtain the desired albumin binding polypeptide variants. The gene fragments were amplified by PCR with primers adding specific endonuclease sites as well as an N-terminal MGSS sequence preceding the albumin binding polypeptide variants. The fragments were cleaved with *Nde*I and *Not*I, purified and ligated to a cloning vector, the plasmid pAY02556 (containing an origin of replication from pBR322, a kanamycin resistance gene and a T7 promoter for expression of the gene of interest), restricted with the same enzymes. Ligations were transformed to electrocompetent *E. coli* TOP10 cells. The transformed cells were spread on TBAB plates (30 g/l tryptose blood agar base) supplemented with 50 µg/ml of kanamycin, followed by incubation at 37 °C overnight. The colonies were screened using PCR and sequencing of amplified fragments was performed using the biotinylated oligonucleotide and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), used in accordance with the manufacturer's protocol. The sequencing reactions were purified by binding to magnetic streptavidin coated beads using a Magnatrix 8000 (NorDiag AB), and analyzed on ABI PRISM® 3100 Genetic Analyzer (PE Applied Biosystems). All albumin binding polypeptide variants were subcloned as monomers and the constructs encoded by the expression

vectors were MGSS-[PP###], where PP### corresponds to the amino acid residues constituting the sequence of the albumin binding polypeptide.

In addition, the gene fragments of G148-GA3, PP007 (SEQ ID NO:7), PP01 3 (SEQ ID NO:1 3) and PP037 (SEQ ID NO:37) were amplified by PCR 5 with primers adding specific endonuclease sites as well as a hexahistidin sequence, a TEV protease site and a glycine residue before the amino acid residues constituting the sequence of the albumin binding polypeptide. The polypeptides PEP0791 3 (SEQ ID NO:1 53), PEP0791 2 (SEQ ID NO:1 57), PEP07914 (SEQ ID NO:1 58) and PEP07968 (SEQ ID NO:1 59) correspond to 10 the albumin binding polypeptides G148-GA3, PP007 (SEQ ID NO:7), PP01 3 (SEQ ID NO:1 3) and PP037 (SEQ ID NO:37) with glycine residues added. The fragments were cleaved with *Xba*1 and *Not*1, purified and ligated to a cloning vector, the plasmid pAY0251 2 (containing an origin of replication from pBR322, a kanamycin resistance gene and a T7 promoter for expression of 15 the gene of interest. The cloning site is preceded by a sequence encoding a peptide containing a hexahistidine tag followed by a cleavage site for the TEV protease), restricted with the same enzymes. Ligation, transformation and sequence verification were performed as described above. The four albumin binding polypeptide variants G148-GA3, PP007, PP01 3 and PP037 were 20 subcloned as monomers and the constructs encoded by the expression vectors were MGSSHHHHHHLQSSGVDLGTENLYFQG-[PP###].

The expression vector encoding MGSSHHHHHHLQSSGVDLGTENLY-FQG-[PP01 3] was further modified by site directed mutagenesis using oligonucleotides, resulting in the insertion of a serine residue before the 25 amino acid residues constituting the sequence of the albumin binding polypeptide, to obtain the construct MGSSHHHHHHLQSSGVDLGTENLYFQ-GS-[PP01 3]. This construct was further modified by 1) site directed mutagenesis to replace the serine residue at position 14 (within PP013) with a cysteine residue, generating MGSSHHHHHHLQSSGVDLGTENLYFQGS-[PP049], and 2) addition of a glycine residue C-terminally, generating 30 MGSSHHHHHHLQSSGVDLGTENLYFQGS-[PP049]-G. The addition of glycine C-terminally was accomplished by PCR amplification with primers including nucleotides encoding the glycine residue and specific endonuclease sites. The fragment was cleaved with *Xba*1 and *Not*1, purified and ligated to a cloning vector, the plasmid pAY02641 (containing an origin of replication from pBR322, a kanamycin resistance gene and a T7 promoter for expression of 35 the gene of interest), restricted with the same enzymes. Ligation,

transformation and sequence verification were performed as described above.

*Protein expression*

5 The albumin binding polypeptide variants were expressed in *E. coli* BL21 (DE3) either with an N-terminal MGSS-extension or with an N-terminal His6-tag followed by a TEV-protease recognition site and a glycine residue. A colony of each albumin binding polypeptide variant was used to inoculate 4 ml TSB+YE medium supplemented with kanamycin to a concentration of 50 µg/ml. The cultures were grown at 37 °C for approximately 5 hours. 3 ml from each of the cultures was used to inoculate 800 ml TSB+YE supplemented with kanamycin to a concentration of 50 µg/ml in parallel bio reactors (Greta system, Belach Bioteknik AB). The cultivations were performed at 37 °C, with aeration at 800 ml/minute and an agitation profile to keep dissolved oxygen

10 levels above 30 %, to an OD600 of 2, which was followed by addition of IPTG to a final concentration of 0.5 mM. Cultivation was continued for five hours after which the cultivation was cooled to 10 °C, aeration was stopped and agitation lowered to 300 rpm. Cell pellets were harvested by centrifugation (15600 × g, 4 °C, 20 minutes) and stored at -20 °C until purification.

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*Purification of albumin binding polypeptide variants with a His<sub>6</sub>-tag and a TEV-protease site*

25 Frozen cell pellets harboring soluble hexahistidine-tagged polypeptides PEP0791 3 (SEQ ID NO:1 53), PEP0791 2 (SEQ ID NO:1 56), PEP07914 (SEQ ID NO:1 58), PEP07968 (SEQ ID NO:1 59), PEP07986 (SEQ ID NO:1 63) and PEP081 85 (SEQ ID NO:148) were suspended in 35 ml binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) with an addition of 1000 U Benzonase® (1.01 654.001, Merck) and disrupted by ultrasonication. For each of the polypeptides, the ultrasonicated suspension was clarified by 30 centrifugation (1 h, 37000 × g, 4 °C) and the supernatant was loaded onto a His GraviTrap™ column (11-0033-99, GE Healthcare). The column was washed with 10 ml washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 60 mM imidazole, pH 7.4), before eluting the polypeptide with 3 ml elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). The buffer 35 was exchanged to a cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8) using PD-10 desalting column (17-0851 -01, GE Healthcare). The amount of polypeptide product was determined by measuring the absorbance at 280 nm

before adding DTT to a final concentration of 5 mM. His6-tagged TEV protease was added to the cleavage buffer at a 1:10 mass ratio relative to the polypeptide product. The cleavage was performed over night under slow mixing at 4 °C. Imidazole was added to the cleavage mix, to a concentration of 20 mM, before loading the mix onto a His GraviTrap™ column (11-0033-99, GE Healthcare) for removing cleaved His6-tags, His6-tagged TEV protease and His6-tagged uncleaved product.

For each variant, the flow-through, containing the albumin binding polypeptide variant, was further purified by reversed phase chromatography (RPC), as follows. The flow-through fraction was loaded on 1 ml Resource 15 RPC column (GE Healthcare), previously equilibrated with RPC A Buffer (0.1 % TFA in water). After column wash with 10 column volumes (CV) RPC A Buffer, bound polypeptides were eluted with a linear gradient of 0-50 % RPC B Buffer (0.1 % TFA in acetonitrile) during 10 CV. The flow rate was 2 ml/min and the absorbance at 280 nm was monitored. Fractions containing albumin binding polypeptide variant were identified by SDS-PAGE analysis and pooled.

The RPC-purified albumin binding polypeptide variants were further purified by gel filtration on 120 ml Superdex 75 (GE Healthcare) packed in an XK16 column (GE Healthcare). The running buffer was 1xPBS, and the flow rate 2 ml/min. Fractions containing pure albumin binding polypeptide variant were pooled and concentrated to approximately 1.3 mg/ml. Finally, the concentrate was purified from trace amounts of remaining endotoxins by using 1 ml columns of AffinityPak Detoxi-Gel Endotoxin removing gel (Pierce, prod#20344), according to the manufacture's recommendations.

The albumin binding polypeptide variants PEP0791 1 and PEP081 85 were conjugated with Mal-DOTA before the RPC-purification step, as follows. The buffer of the flow-through fraction from the IMAC-FT purification step was exchanged to 0.2 M NaAc, pH 5.5, using a disposable PD-10 desalting column (GE Healthcare). Maleimido-mono-amide-DOTA (Macrocyclics, cat. no. B-272) was added at 5-fold molar excess and incubated for 60 minutes at 30 °C under continuous shaking. The resulting polypeptide were denoted PEP07968 and PEP08296, respectively.

35 *Purification of albumin binding polypeptide-variants without His<sub>6</sub>-tag*

Frozen cell pellets harboring soluble albumin binding polypeptide variants PEP06923 (SEQ ID NO:154), PEP07271 (SEQ ID NO:155),

PEP07554 (SEQ ID NO:1 56) and PEP07844 (SEQ ID NO:1 61) were suspended in 20 mM Tris-HCl, pH 8 and disrupted by ultrasonication. For each of the polypeptide variants, the ultrasonicated suspension was clarified by centrifugation (30 min, 32000 x g, 4 °C) and the supernatant was loaded 5 onto a HSA-Sepharose column (GE Healthcare). After washing with TST-buffer (25 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, 0.05% Tween 20, pH 8.0), followed by 5 mM NH<sub>4</sub>Ac, pH 5.5, bound albumin binding polypeptide variant was eluted with 0.5 M HAc, pH 3.2.

The albumin binding polypeptide variants were further purified by 10 reversed phase chromatography (RPC), as follows. For each of the variants, the eluate from the HSA-affinity purification step was loaded on 1 ml Resource 15 RPC column (GE Healthcare), previously equilibrated with RPC A Buffer (0.1 % TFA in water). After column wash with 10 CV RPC A Buffer, bound polypeptides were eluted with a linear gradient of 0-50 % RPC B Buffer 15 (0.1 % TFA in acetonitrile) during 10 CV. The flow rate was 2 ml/min and the absorbance at 280 nm was monitored. Fractions containing pure albumin binding polypeptide variants were identified by SDS-PAGE analysis and pooled. Finally, the buffer was exchanged to 1xPBS (2.68 mM KCl, 137 mM 20 NaCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) using a disposable PD-10 desalting column (GE Healthcare).

*Characterization of purified albumin binding polypeptide-variants*

The concentration was assessed by measuring the absorbance at 280 nm using a NanoDrop® ND-1 000 Spectrophotometer. The proteins were 25 further analyzed with SDS-PAGE and LC-MS.

For the SDS-PAGE analysis, approximately 10 µg of each albumin binding polypeptide variant was mixed with NuPAGE LDS Sample Buffer (Invitrogen), incubated at 70 °C for 15 min and loaded onto NuPAGE 4-12 % Bis-Tris Gels (Invitrogen). The gels were run with NuPAGE MES SDS 30 Running Buffer (Invitrogen) in an XCell II SureLock Electrophoresis Cell (Novex) employing the Sharp Prestained Standard (Invitrogen) as molecular weight marker and using PhastGel BlueR (GE Healthcare) for staining.

To verify the identity of the albumin binding polypeptide variants, LC/MS analyses were performed using an Agilent 1100 LC/MSD system, 35 equipped with API-ESI and a single quadruple mass analyzer. Approximately 10 µg of each of the purified albumin binding polypeptide variants was loaded on a Zorbax 300SB-C8 Narrow-Bore column (2.1 x 150 mm, 3.5 µm, Agilent

Technologies) at a flow-rate of 0.5 ml/min. Polypeptides were eluted using a linear gradient of 10-70 % solution B for 15 min at 0.5 ml/min. The separation was performed at 30 °C. The ion signal and the absorbance at 280 and 220 nm were monitored. The molecular weights of the purified albumin binding 5 polypeptide variants were confirmed by MS.

### *Results*

The expression levels of the albumin binding polypeptide variants were 10-30 mg product/g cell pellet, as estimated from SDS-PAGE analysis.

10 For all variants, the purity, as determined by SDS-PAGE analysis, exceeded 95 % and the LC/MS analysis verified the correct molecular weights. After purification, between 1 and 8 mg of pure polypeptide was obtained for each of the ten albumin binding polypeptide variants.

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### Example 2:

#### Affinity determination for albumin binding polypeptides

In this example, PEP06923 (SEQ ID NO:154), PEP07271 (SEQ ID 20 NO:1 55), PEP07844 (SEQ ID NO:1 61), PEP0791 2 (SEQ ID NO:1 57), PEP0791 3 (SEQ ID NO:1 53), PEP07914 (SEQ ID NO:1 58) and PEP07968, (DOTA-conjugated PEP0791 1, SEQ ID NO:1 59), synthesized or expressed and purified in Example 1 were characterized for affinity to human serum albumin (HSA) using a Biacore instrument. PEP0791 3 corresponds to the 25 amino acid sequence of G148-GA3 with addition of a N-terminal glycine residue, whereas PEP07271 , PEP07844, PEP0791 2, PEP07914 and PEP07968 correspond to the albumin binding polypeptides of PP001 (SEQ ID NO:1 ), PP043 (SEQ ID NO:43), PP007 (SEQ ID NO:7), PP01 3 (SEQ ID NO:1 3) and PP037 (SEQ ID NO:37) with different N-terminal amino acid 30 additions.

### *Material and methods*

Biosensor analysis on a Biacore2000 instrument (GE Healthcare) was performed with HSA (Albucult®, Novozymes), immobilized by amine coupling 35 onto the carboxylated dextran layer of the surfaces of CM-5 chips (research grade; GE Healthcare) according to the manufacturer's recommendations. Surface 1 of the chip was activated and deactivated and used as a reference

cell (blank surface) during injections, whereas surface 2 comprised HSA immobilized to 731 resonance units (RU) and surface 4 comprised HSA immobilized to 955 RU. The purified albumin binding polypeptide variants were diluted in running buffer HBS-EP (GE Healthcare) to 2.5 nM, 10 nM and 5 40 nM, and injected at a constant flow-rate of 50  $\mu$ l/min for 5 minutes, followed by injection of HBS-EP for 60 minutes. The surfaces were regenerated with one injection of 25  $\mu$ l HCl, 10 mM. The affinity measurements were performed in two sets; in the first set HBS-EP, PEP06923, PEP07271, PEP0791 2, PEP0791 3, PEP07914 and PEP07968 10 were injected (chip surface 2), and in the second set HBS-EP, PEP06923, PEP07844, PEP0791 2 and PEP07914 were injected (chip surface 4). PEP06923 was injected twice in each run as a control. The results were analyzed with a BiaEvaluation software (GE Healthcare). Curves of the blank surface were subtracted from the curves of the ligand surfaces.

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### Results

The Biacore 2000 instrument has a technical limitation, hindering measurements of very high affinity. Hence, the purpose of the Biacore study was not to determine the exact kinetic parameters of the albumin binding 20 polypeptide variants' affinity for HSA. However, the results provide a quantitative estimation of the relative affinities of these polypeptides for albumin. After subtraction of reference surface and buffer injection, curves were fitted to a 1:1 (Langmuir) binding model using BIAevaluation software with correction for mass transfer and with RUmax set as a local parameter. 25 Curves are shown in Figure 2. The relative  $K_D$ ,  $k_a$  ( $k_{on}$ ) and  $k_d$  ( $k_{off}$ ) values were estimated and are presented in the Tables below.

Table 1:  
Kinetic parameters ( $k_a$ ,  $k_d$  and  $K_D$ ) of albumin bineling polypeptides to HSA, 1st set

	$k_a$ ( $M s^{-1}$ )	$k_d$ ( $s^{-1}$ )	$K_D$ ( $M$ )
PEP07913	$5.7 \times 10^5$	$9.3 \times 10^{-4}$	$1.6 \times 10^{-9}$
PEP06923 (1)	$2.9 \times 10^7$	$2.9 \times 10^{-5}$	$9.9 \times 10^{-13}$
PEP06923 (2)	$2.6 \times 10^7$	$2.8 \times 10^{-5}$	$1.1 \times 10^{-12}$
PEP07271	$3.9 \times 10^6$	$2.9 \times 10^{-5}$	$7.5 \times 10^{-12}$
PEP07912	$4.6 \times 10^6$	$2.8 \times 10^{-5}$	$6.2 \times 10^{-12}$
PEP07914	$3.5 \times 10^6$	$2.5 \times 10^{-5}$	$7.2 \times 10^{-12}$
PEP07968	$3.0 \times 10^6$	$2.7 \times 10^{-5}$	$9.0 \times 10^{-12}$

Table 2:  
Kinetic parameters ( $k_a$ ,  $k_d$  and  $K_D$ ) of albumin binding polypeptides to HSA, 2nd set

	$k_a$ (Ms <sup>-1</sup> )	$k_d$ (s <sup>-1</sup> )	$K_D$ (M)
PEP06923 (1)	$2.0 \times 10^7$	$2.6 \times 10^{-5}$	$1.3 \times 10^{-12}$
PEP06923 (2)	$2.1 \times 10^7$	$2.5 \times 10^{-5}$	$1.2 \times 10^{-12}$
PEP07912	$5.4 \times 10^6$	$2.8 \times 10^{-5}$	$5.2 \times 10^{-12}$
PEP07914	$3.8 \times 10^6$	$2.6 \times 10^{-5}$	$6.9 \times 10^{-12}$
PEP07844	$5.4 \times 10^6$	$2.3 \times 10^{-5}$	$4.4 \times 10^{-12}$

As shown in Tables 1 and 2, PEP07271 (SEQ ID NO:1 55), PEP07844 (SEQ ID NO:1 61), PEP07912 (SEQ ID NO:1 57), PEP07914 (SEQ ID NO:1 58) and PEP07968 (DOTA-conjugated PEP07911, SEQ ID NO:1 59) all seem to have approximately the same affinity for HSA, widely exceeding the affinity of the parent G148-GA3 (PEP07913; SEQ ID NO:1 53). The HSA affinity of these polypeptides is slightly lower compared to PEP06923 (SEQ ID NO:1 54), despite similar off-rate.

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Example 3:

Determination of melting temperature (Tm) for albumin binding polypeptides

15 In this example, the albumin binding polypeptide variants PEP07913 (SEQ ID NO:1 53), PEP06923 (SEQ ID NO:1 54), PEP07271 (SEQ ID NO:1 55), PEP07554 (SEQ ID NO:1 56), PEP07912 (SEQ ID NO:1 57), PEP07914 (SEQ ID NO:1 58), PEP07968 (DOTA-conjugated PEP07911, SEQ ID NO:1 59), PEP07844 (SEQ ID NO:161) and PEP07986 (SEQ ID NO:1 63), expressed and purified as described in Example 1, and the albumin polypeptide variant PEP07975 (DOTA-conjugated PEP07834, SEQ ID NO:1 60), produced as described in Example 5, were analyzed by CD analysis. PEP07913 corresponds to the sequence of G148-GA3 having an N-terminal glycine residue, PEP06923 is an engineered high affinity derivative previously described by Jonsson *et al, supra*, whereas PEP07271, PEP07554, PEP07912, PEP07914, PEP07968, PEP07844 and PEP07975 are examples of the albumin binding polypeptides of PP001 (SEQ ID NO:1), PP007 (SEQ ID NO:7), PP013 (SEQ ID NO:13), PP037 (SEQ ID NO:37) and PP043 (SEQ ID NO:43) having different N-terminal amino acid additions according to the present disclosure.

### Material and methods

Purified albumin binding polypeptide variants were diluted in 1xPBS, to final concentrations between 0.4 and 0.5 mg/ml. Circular dichroism (CD) analysis was performed on a Jasco J-810 spectropolarimeter in a cell with an 5 optical path-length of 1 mm. In the variable temperature measurements, the absorbance was measured at 221 nm from 20 °C to 90 °C, with a temperature slope of 5 °C/min.

### Results

10 The melting temperatures (Tm) of the different albumin binding polypeptide variants were calculated by determining the midpoint of the transition in the CD vs. temperature plot. The results are summarized in Table 3 below.

15 Table 3. Determined Tm values of tested albumin binding polypeptide variants

Variant	SEQ ID NO:#	N-terminal sequence <sup>3</sup>	Tm (°C)
PEP07913	SEQ ID NO:153	<u>GL</u>	61
PEP06923	SEQ ID NO:154	<u>GSSL</u>	57
PEP07271	SEQ ID NO:155	<u>GSSL</u>	65
PEP07554	SEQ ID NO:156	<u>GSSL</u>	58
PEP07912	SEQ ID NO:157	<u>GL</u>	53
PEP07914	SEQ ID NO:158	<u>GL</u>	59
PEP07968	SEQ ID NO:159 <sup>1</sup>	<u>GL</u>	53
PEP07975	SEQ ID NO:160 <sup>1,2</sup>	<u>AL</u>	50
PEP07844	SEQ ID NO:161	<u>GSSL</u>	65
PEP07986	SEQ ID NO:163	<u>GSL</u>	61

<sup>1)</sup>The peptide is conjugated with maleimide-DOTA at the cysteine

<sup>2)</sup>The peptide is amidated at the C-terminus

<sup>3)</sup> Leucine (underlined) is the residue in position 1 of the amino acid sequence of the albumin binding polypeptide as defined in the first aspect of the present disclosure

20 The polypeptide PEP07968 is identical to PEP07912, except for the former having a cysteine residue in position 14 conjugated with maleimide DOTA, and the latter a serine residue. Thus, the DOTA modification should not affect the melting temperature. Also PEP07975 is conjugated with DOTA 25 using C<sub>14</sub>, and is identical to PEP07968 except for the C-terminal amide (resulting from the peptide synthesis in Example 5) and for having an N-

terminal alanine instead of a glycine. Furthermore, comparing PEP0791 2 and PEP07554 reveals that an N-terminal serine gives a higher melting temperature than a glycine in the same position (5 °C difference in Tm). Thus, all albumin binding polypeptide variants according to the present disclosure 5 show Tm above 55 °C, except PEP0791 2 and DOTA-conjugated variants. Taking into consideration the importance of the N-terminal portion, all the tested albumin binding polypeptides are superior to the prior art derivative of Jonsson *et al*, i.e. PEP06923.

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Example 4:  
Serum response analysis

15 The percentage of human serum containing IgG, capable of binding to a set of albumin binding polypeptides as disclosed herein was analyzed by ELISA. In total, 149 serum samples corresponding to 127 individuals were screened.

*Material and methods*

20 ELISA plates (96-well, half area plates (Costar, cat. No. 3690)) were coated with 50 µl/well of Albucult® (Novozymes) diluted to 8 µg/ml in coating buffer (Sigma, cat. No. 3041). The plates were coated over night for three days at 4 °C. On the day of analysis, the plates were washed twice with tap water and blocked for 2 hours with 100 µl of phosphate buffered saline (PBS) 25 containing 0.05 % casein (PBSC). The plates were emptied and 50 µl/well of the albumin binding polypeptides PEP0791 3 (SEQ ID NO:1 53), PEP06923 (SEQ ID NO:1 54), PEP07271 (SEQ ID NO:1 55), PEP0791 2 (SEQ ID NO:1 57), PEP07554 (SEQ ID NO:1 56), PEP07914 (SEQ ID NO:1 58), PEP07968 (DOTA-conjugated PEP0791 1, SEQ ID NO:159) and PEP07844 (SEQ ID NO:1 61), diluted to 2 µg/ml in PBSC were added according to a pre-made plate layout. After incubation for two hours at room temperature (RT), the plates were washed in PBSC four times using an automated ELISA washer. The 149 serum samples from 129 individuals were diluted 50 times in PBSC by adding 24 µl serum to 1174 µl PBSC. 50 µl of the diluted sera was 30 added per well according to the pre-made plate layout. Each serum sample was tested as a singlet. Positive and negative controls were included on each plate and for each albumin binding polypeptide. Albumin binding antibodies 35

(50  $\mu$ l, 0.5  $\mu$ l/ml immunoglobulin solution prepared in house from sera from primates immunized with PEP06923) was added as a positive control and 50  $\mu$ l PBSC was used as a negative control. The plates were incubated for one hour at RT and subsequently washed four times in PBSC using an automated 5 ELISA washer. The bound IgG was detected with 50  $\mu$ l/well of anti-human IgG (Southern Biotech, cat no 2040-05) diluted 10 000 times in PBSC. After washing four times in PBSC using an automated ELISA washer, 50  $\mu$ l/well of substrate was added (Pierce cat. No. 34021). The reaction was stopped after 10-15 minutes by the addition of 50  $\mu$ l H<sub>2</sub>SO<sub>4</sub> to each well, prior to measuring 10 the absorbance using a multi-well plate reader (Victor3, Perkin Elmer).

### Results

Of the 149 sera screened for IgG binding to the albumin binding polypeptides, 23 were negative for all eight polypeptides (OD-value < 0.1), i.e. 15 showed no IgG bound to the polypeptides. The analysis was performed with the 126 sera that were positive for one or more albumin binding polypeptides. The average absorbance was calculated (Figure 3A) and the percentage of sera with OD-values values either < 0.15 (Figure 3B) or > 1.0 (Figure 3C). The highest average OD-value and the highest percentage of serum with IgG 20 binding were obtained with PEP0791 3 (SEQ ID NO:153), PEP06923 (SEQ ID NO:154) and PEP07844 (SEQ ID NO:161), whereas least reactivity was found against PEP07968 (DOTA-conjugated PEP0791 1, SEQ ID NO:159), PEP07914 (SEQ ID NO:158) and PEP07954 (SEQ ID NO:156).

Thus, the most reactive albumin binding polypeptides were the 25 parental G148-GA3 (PEP0791 3, SEQ ID NO:153) and the previously affinity improved derivative (PEP06923, SEQ ID NO:154), having helix 1 retained from G148-GA3. The third of the more reactive polypeptides (PEP07844, SEQ ID NO:161) contains the original lysine in position 14 in helix 1. This residue is intended for conjugation, and will therefore not be exposed in the 30 final context. The identical albumin binding polypeptide variant, except for having an alanine in position 14 (PEP07554, SEQ ID NO:156), is one of the least reactive.

Example 5:Chemical synthesis of a DOTA-conjugated albumin binding polypeptide*Material and methods*

5 The albumin binding polypeptide PEP07834 (SEQ ID NO:1 60) was synthesized by solid phase peptide synthesis (SPPS, as described by Quibell, M. & Johnson, T., in Fmoc Solid Phase Peptide Synthesis-A Practical Approach, W.C. Chan, P.D. White Eds, Oxford University Press 2000, 115-135) in a 433 A Peptide Synthesizer reactor (Applied Biosystems, Foster City, 10 CA) on a 0.1 mmol scale, i.e. with a theoretical possible yield of 0.1 mmol peptide, using standard Fmoc chemistry. An acid-labile Fmoc amide resin was used as solid support throughout the synthesis (Rink Amide MBHA Resin LL (100-200 mesh), loading 0.39 mmol amide/g resin (Novabiochem)).

15 47 amino acid residues according to the sequence below were coupled to the amide resin by acylation reactions in the reactor for 10 minutes at room temperature (RT) and mixing. The acylation reactions were performed with a ten-fold molecular excess of Fmoc protected amino acids in NMP (N-methylpyrrolidone, Merck), activated with 1 eq of 2-(1 H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU, IRIS Biotech), 1 20 eq of 1-hydroxybenzotriazole (HOEt, IRIS Biotech) and 2 eq of diisopropylethylamine (DIEA, Applied Biosystems). In addition, all reactive amino acid side chains were protected with standard side chain protection groups (tert-butyl (fBu) for Asp, Glu, Ser, Thr and Tyr, tert-butyloxycarbonyl (Boc) for Lys, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for 25 Arg, and trityl (Trt) for Asn and Cys) prior to activation and coupling. In order to diminish the amount of incomplete couplings leading to truncated peptides, a minor amount of selected amino acid residues were subjected to coupling by acylation twice, without Fmoc deprotection as described below between the first and second coupling. The amino acid sequence of the synthesized 30 albumin binding polypeptide PEP07834 was

ALASAKEAAN AELDCYGVSD FYKRLIDKAK TVEGVEALKD AILAALP-NH<sub>2</sub> (SEQ ID NO:1 60-NH<sub>2</sub>).

35 The underlined amino acid residues were double coupled. Any remaining unreacted amino groups on the resin bound peptides were capped with acetic anhydride (0.5 M acetic anhydride (AlfaAesar), 0.1 25 M DIEA, 0.01 5 M HOEt

in NMP) for 5 min. Following every coupling, deprotection of the N-terminal Fmoc group on the resin bound peptides were performed by treatment with 20% piperidine (Sigma-Aldrich) in NMP for 10 min.

After completed synthesis, the peptides were cleaved from the solid support and simultaneously the side chain protection groups were cleaved off by treatment with TFA/EDT/H<sub>2</sub>O/TIS (94:2.5:2.5:1) (TFA: trifluoroacetic acid (Apollo), EDT: 1,2-ethanedithiol (Aldrich), TIS: triisopropylsilane (Aldrich)) at RT for 2 h with occasional mixing. After TFA treatment, the peptides were extracted three times using 20 % acetonitrile (Merck) in water and *tert*-butyl methyl ether (Merck). The aqueous phases were combined, filtered and lyophilized.

The crude peptides were analyzed and purified by semi-preparative RP-HPLC (Reprosil GOLD C 18 300, 250\*10 mm, 5  $\mu$ m particle size) and a gradient of 32-55% B (A: 0.1 % TFA-H<sub>2</sub>O; B: 0.1 % TFA-CH<sub>3</sub>CN) during 25 min at a flow rate of 2.5 ml min<sup>-1</sup>, followed by lyophilization.

The synthetic yield was determined by calculation of the integrated areas under the peaks from the 220 nm signal from the crude analysis on RP-HPLC. The correct molecular weight was verified using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) on a 6520 Accurate Mass Q-TOF LC/MS (Agilent Technologies). The purity of the product was verified using RP-HPLC (Reprosil GOLD C 18 300, 250\*4.6 mm, 3  $\mu$ m particle size) using a gradient of 35-55 % B over 25 min at a flow rate of 1.0 ml min<sup>-1</sup>.

#### *DOTA conjugation*

3 mg of PEP07834-amide (SEQ ID NO:1 60-amide) was reduced with 20 mM DTT at 40 °C for 30 minutes. Excess DTT was removed by buffer exchange on a PD-10 column (GE Healthcare) to 0.2 M ammonium acetate, pH 5.5. The coupling was performed with a 5-fold molar excess of chelator, maleimido-mono-amide-DOTA (Macrocyclics, Cat. No. B-272) solution in water (1 mg/ml). The mixture was incubated for 1 hour at 30 °C under continuous shaking. Purification from non-conjugated chelators was made on a semi-preparative RPC column (Zorbax 300SB C18, 9.4x250 mm, 5 pm). The coupling degree of the purified material was analyzed by HPLC-MS on a Zorbax 300SB C8 150 x 2.1 mm, 3.5  $\mu$ m analytical column. Only maleimide-DOTA-conjugated PEP07834, denoted PEP07975, was detected by the method.

### Results

Based on the elution profile of the crude material, the synthetic yield of the albumin binding polypeptide PEP07834-amide (SEQ ID NO:1 60-amide) was determined to be 8 %. The found molecular weight was 4952.9 Da, which 5 is in good agreement with the theoretical molecular weight calculated to 4952.6 Da. When analyzing the purified product, approximately 10-15 % of the protein was found to be a disulfide linked homodimer (Figure 4 and 5). The binding activity of the DOTA-conjugated peptide (PEP07975) was confirmed as described in Example 2 (data not shown), and the melting 10 temperature determined as described in Example 3.

### Example 6:

#### Immunoactivity testing of albumin binding polypeptides

15 PEP0791 3 (SEQ ID NO:1 53), PEP0791 2 (SEQ ID NO:1 57), PEP07914 (SEQ ID NO:1 58), and PEP07968 (DOTA-conjugated PEP0791 1, SEQ ID NO:1 59) were screened for their ability to induce T cell proliferation in peripheral blood mononuclear cells (PBMC) from 52 human Caucasian 20 individuals (obtained from CRI-Labo Medische Analyse, Gent, Belgium). PEP0791 3 corresponds to the sequence of G148-GA3 having an N-terminal glycine residue, whereas PEP0791 2, PEP07914 and PEP07968 are examples of the albumin binding polypeptides of PP007 (SEQ ID NO:7), PP01 3 (SEQ ID NO:1 3) and PP037 (SEQ ID NO:37) having different N- 25 terminal amino acid additions according to the present disclosure.

### *Materials and methods*

PBMCs, prepared according to standard cell biological methods, were added to a tissue culture (TC) treated 96-well round bottom plate (Falcon) in 30 an amount of 300 000 PBMCs/ well. The cells were stimulated by addition of 100  $\mu$ l/well of albumin binding polypeptides PEP0791 3, PEP0791 2, PEP07914 and PEP07968 in AIMV medium (Invitrogen) additionally containing 900  $\mu$ g/ml (3-fold molar excess) of recombinant human albumin (Albucult®, Novozymes). This corresponded to a final concentration of 35 albumin binding polypeptide of 30  $\mu$ g/ml. The stimulation was done in eight-plicates, i.e. the same albumin binding polypeptide were added to eight wells in identical amounts and under the same conditions. In positive control wells,

the cells were stimulated with either 30 µg/ml Keyhole Limpet Hemocyanin (KLH, Calbiochem) or 30 µg/ml tetanus toxoid (TT, Statens Serum Institut). In negative control wells, only AIMV medium with or without 900 µg/ml of albumin were added.

5 Cell proliferation was assessed after seven days of culturing using Alexa Fluor 488 Click-iT EdU flow cytometry assay kit (Invitrogen). 1 µM/well of EdU incorporation marker was added on day six. On day seven, cells were washed, dissociated from the plate, washed again and stained for 30 minutes with anti-CD3-PerCP reagent (Becton Dickinson) and anti-CD4-Alexa647  
10 reagent (Becton Dickinson). Following staining, the cells were washed, fixed (BD cellfix, BD biosciences), permeabilized (using saponin) and stained for EdU by addition of Click-iT reagent according to the manufacturer's protocol (Invitrogen). After completed staining, cells were washed again and analyzed using flow cytometry (FACSCantoll, BD Biosciences). To assess the number  
15 of proliferating cells, a fixed number of fluospheres (Invitrogen) was added to each well before analysis. All staining procedures and washes were performed directly in the 96-well plate.

20 The raw FACSCantoll data were gated hierarchically on CD3<sup>+</sup> CD4<sup>+</sup> T cells and the number of gated cells as well as their fluorescence intensity of EdU-Alexa Flour 488 incorporation marker were recorded. The mean values  
25 of the number of proliferating cells/eight-plicate of protein treated wells were compared to the positive and negative controls and the resulting ratios, described as stimulation indices (SI), were calculated. Based on the SI and the variation between replicates, threshold SI-values were set to 2.0 and 0.5 for stimulation and inhibition, respectively.

### Results

30 The albumin binding polypeptides PEP0791 3, PEP0791 2, PEP07914 and PEP07968 were assessed for their immunogenic potential in the presence of 3-fold excess of recombinant human albumin in a target human population using an *in vitro* PBMC proliferation assay. Compared to the albumin control, PEP0791 3 induced CD3<sup>+</sup>CD4<sup>+</sup> T cells proliferation in 6 of 52 donors, PEP0791 2 in 5 of 52 donors and PEP07914 and PEP07968 in 1 of 52 donors (Figure 6A).

35 The mean stimulation index (SI) for all 52 donors was not significantly different for PEP07914 and PEP07968 compared to the negative control

containing recombinant human albumin ( $p = 0.79$  and  $0.48$  respectively, Figure 6B). The SI for PEP0791 3 was significantly higher ( $p = 0.002$ ) whereas the SI for PEP0791 2 was higher but not significant ( $p=0.03$ , Figure 6B).

As compared to buffer only, the number of responding individuals was 5 10 for PEP0791 2, 7 for PEP0791 2, 2 for PEP0791 4, 1 for PEP07968, 2 for recombinant human albumin, and 49 and 51 for the two positive controls TT and KLH, respectively (Figure 6C). The albumin binding polypeptides were ranked according to their immunogenicity in the following order: PEP0791 3 > PEP0791 2 > PEP07914 > PEP07968. Both PEP07914 and PEP07968 were 10 defined as non-immunogenic. The above results thus demonstrate that the immunogenic potential of the albumin binding polypeptides of the present disclosure is low, as compared to the positive controls.

15

#### Example 7:

##### Albumin binding polypeptides' affinity to albumin from different species

In this example, PEP06923 (SEQ ID NO:154), PEP07986 (SEQ ID NO:163) and PEP08296, (DOTA-conjugated PEP081 85, SEQ ID NO:148), 20 expressed and purified as described in Example 1, were characterized for affinity to albumin from human (HSA), cynomolgus monkey (CSA), rat (RSA), mouse (MSA) and dog (DSA) using a Biacore instrument.

25

#### *Material and methods*

Biosensor analysis on a Biacore2000 instrument (GE Healthcare) was performed with HSA (Albucult®, Novozymes), CSA (purified *in-house* from cynomolgus serum), RSA (Sigma-Aldrich, Cat. No. A6272), MSA (Sigma-Aldrich, Cat. No. A3559) and DSA (MP Biomedicals, Cat. No. 55925), immobilized by amine coupling onto the carboxylated dextran layer of the 30 surfaces of CM-5 chips (research grade; GE Healthcare) according to the manufacturer's recommendations.

35

On chip 1, surface 1 was activated and deactivated and used as a reference cell (blank surface) during injections, whereas surface 2 comprised HSA immobilized to 1130 resonance units (RU), surface 3 comprised CSA immobilized to 1046 RU, surface 4 comprised RSA immobilized to 831 RU. On chip 2, surface 1 was used as blank surface, whereas surface 3

comprised MSA immobilized to 858 RU. On chip 3, surface 1 was used as blank surface, whereas surface 2 comprised DSA immobilized to 1053 RU.

For analysis of affinity for HSA, CSA, and RSA (chip 1), the purified albumin binding polypeptide variants were diluted in running buffer HBS-EP (GE Healthcare) to 40 nM, 10 nM and 2.5 nM; for analysis of affinity for MSA (chip 2) the albumin binding polypeptide variants were diluted to 1280 nM, 640 nM, 160 nM and 40 nM and for analysis of affinity for DSA (chip 3) albumin binding polypeptide variants were diluted to 1280 nM, 640 nM, 160 nM, 40 nM and 10 nM. The albumin binding polypeptides were injected at a constant flow-rate of 50  $\mu$ l/min for 5 minutes, followed by injection of HBS-EP for 60 minutes. The surfaces were regenerated with one injection of 25  $\mu$ l HCl, 10 mM. All samples were run in duplicates.

The results were analyzed with a BIAevaluation software (GE Healthcare). Curves of the blank surface were subtracted from the curves of the ligand surfaces.

### Results

The Biacore 2000 instrument has a technical limitation, hindering measurements of very high affinity. Hence, the purpose of the Biacore study was not to determine the exact kinetic parameters of the albumin binding polypeptide variants' affinity for HSA, CSA, RSA, MSA and DSA respectively. However, the results provide a quantitative estimation of the relative affinities of the enclosed polypeptides for albumin from these different species. After subtraction of reference surface and buffer injection, curves were fitted to a 1:1 (Langmuir) binding model using BIAevaluation software with correction for mass transfer and with RUmax set as a local parameter. Representative binding curves are shown in Figure 7.

PEP07986 and PEP08296 (DOTA-conjugated PEP081 85) bind with high affinity ( $K_D$  in the range from below picomolar to below nanomolar) to human serum albumin as well as to albumin from the frequent preclinical model species rat, cynomolgus monkey, mouse and dog. The relative affinities for the different species can be ranked as RSA  $\geq$  HSA/CSA  $>$  MSA/DSA, i.e. the  $K_D$  values ranked as  $K_{D\_RSA} \leq K_{D\_HSA}/K_{D\_CSA} < K_{D\_MSA}/K_{D\_DSA}$ . The affinities in terms of  $K_D$  values are the same or slightly lower (but in the same order of magnitude) as the affinity obtained for PEP06923 (non-inventive polypeptide).

Example 8:*In vitro* activity of protein Z variants fused to an albumin binding polypeptide

In this example, polypeptides comprising cytokine-specific protein Z (derivative of domain B of staphylococcal protein A) variants genetically fused to the albumin binding polypeptide variant PP01 3 (SEQ ID NO:1 3) were tested for their functionality, this being to block cytokine-induced proliferation of TF-1 cells in the presence of human serum albumin. Proliferation of TF-1 cells is dependent of the presence of any of several different types of cytokines and the proliferative response can be inhibited by blocking reagents such as the corresponding cytokine-specific protein Z variant. PP01 3 fused to a protein Z variant with specificity for an irrelevant protein was used as negative control.

15 *Materials and methods**Cloning of Z- PP013 fusion proteins*

Gene fragments of protein Z variants with specificity for cytokine X or Y respectively, or for an irrelevant protein (negative control), were amplified by PCR using primers adding *Pst*I and *Acc*I specific endonuclease sites. The fragments were cleaved with *Pst*I and *Acc*I, purified and ligated into an expression vector, the plasmid pAY02747, restricted with the same enzymes. pAY02747 contains an origin of replication from pBR322, a kanamycin resistance gene and a T7 promoter for expression of the gene of interest. The cloning site is preceded by a sequence encoding the amino acids MGSSLQ and succeeded by a sequence encoding VDSS-PP01 3, where PP01 3 is the disclosed albumin binding polypeptide with SEQ ID NO:1 3. Ligation, transformation and sequence verification were performed as described above. The encoded proteins were:

30           1) MGSSLQ-Z<sub>X</sub>-VDSS-PP01 3 (denoted Z<sub>X</sub>-PP01 3)  
          2) MGSSLQ-Z<sub>Y</sub>-VDSS-PP01 3 (denoted Z<sub>Y</sub>-PP01 3)  
          3) MGSSLQ-Z<sub>neg</sub>-VDSS-PP01 3 (denoted Z<sub>neg</sub>-PP01 3)

*Protein expression*

35           Z<sub>X</sub>-PP013, Z<sub>Y</sub>-PP01 3 and Z<sub>neg</sub>-PP01 3 were expressed in *E. coli* BL21 (DE3) cells. Colonies from the transformations of each fusion variant were used to inoculate starter cultures of 50 ml TSB+YE medium supplemented

with kanamycin to a concentration of 50 µg/ml. The cultures were grown at 37°C over night with agitation, 100 rpm. The starter cultures were then used to inoculate 900 ml TSB+YE medium supplemented with kanamycin to a concentration of 50 µg/ml. The cultures were grown for approximately 1.5 h to 5 an OD600 of >1.1, upon which IPTG was added to a final concentration of 0.2 mM. Cultivation was continued for five hours. Cell pellets were harvested by centrifugation (15600 g, 4 °C, 20 minutes) and stored at -20 °C until purification.

10 *Protein purification*

Frozen cell pellets harboring soluble fusion protein variants  $Z_X$ -PP01 3,  $Z_Y$ -PP01 3 and  $Z_{\text{neg}}$ -PP013 were resuspended in 50 mM Tris-HCl, 150 mM NaCl, pH 8 and 1000 U Benzonase® (Merck Cat. No. 1.01 654.0001) was added. The cells were disrupted by ultrasonication and for each of the fusion 15 protein variants, the ultrasonicated suspension was clarified by centrifugation (15 min, 37000 g, 4 °C). 20x TST-buffer (20x [25 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, 0.05% Tween 20, pH 8.0]) was added at a volume resulting in 1 × TST buffer in the clarified suspension. Each sample of fusion protein variant was loaded onto a HSA-Sepharose column (GE Healthcare). After 20 washing with TST-buffer, followed by 5 mM NH<sub>4</sub>Ac, pH 5.5, bound fusion protein variant was eluted with 0.5 M HAc, pH 2.5.

The fusion protein variants were further purified by reversed phase chromatography (RPC), as follows. For each of the variants, the eluate from the HSA-affinity purification step was loaded on a 1 ml Resource 15 RPC 25 column (GE Healthcare) previously equilibrated with RPC A Buffer (0.1 % TFA in water). After column wash with 10 CV RPC A Buffer and 5 CV of RPC B Buffer (0.1 % TFA in acetonitrile), bound fusion proteins were eluted with a linear gradient of 10-50 % RPC B Buffer over 20 CV. The flow rate was 2 ml/min and the absorbance at 280 nm was monitored. Fractions containing 30 pure fusion protein variants were identified by SDS-PAGE analysis and pooled. Finally, the buffer was exchanged to 1xPBS (2.68 mM KCl, 137 mM NaCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) using a disposable PD-10 desalting column (GE Healthcare). To verify the identity of the fusion protein variants, SDS-PAGE and LC/MS analyses were performed as described in 35 Example 1.

*In vitro cell assay of Z- PP013 fusion proteins*

The cell line TF-1 (CLS Cat. No. 300434) was propagated as recommended by the provider in RPMI 1640 medium + 10% fetal calf serum (Gibco) with the addition of 2 ng/ml of rhGM-CSF (Miltenyi). At the day of 5 experiment, the cells were washed in RPMI 1640 medium + 10% fetal calf serum to remove GM-CSF.

The ability of  $Z_X$ -PP013 and  $Z_Y$ -PP013 to block cytokine induced proliferation was analyzed by mixing the molecules  $Z_X$ -PP013,  $Z_Y$ -PP013 and  $Z_{\text{neg}}$ -PP013 with cytokines X and Y respectively, and with a five times molar 10 excess of HSA (Albucult®, Novozymes). The molecules were titrated in a 2-fold dilution series with a fixed concentration of cytokine (4.9 pM) and a five times molar excess of HSA. The titration was performed in 96-well plates in a volume of 100  $\mu\text{l}$ . 25 000 cells were added per well (100  $\mu\text{l}$ ) and plates were 15 incubated at 37 °C, 5% CO<sub>2</sub> for three days. To measure the proliferation, 19  $\mu\text{l}$  of CCK-8 cell proliferation reagent (Sigma) diluted two times in RPMI 1640 medium + 10% fetal calf serum, was added per well. The color reaction was monitored after 4 hours using 96-well plate reader (Victor3; PerkinElmer).

*Results*

20 As shown in Figure 8, both  $Z_X$ -PP013 and  $Z_Y$ -PP013 inhibited the respective cytokine induced proliferation in the presence of HSA whereas  $Z_{\text{neg}}$ -PP013, the negative control, did not affect proliferation of TF-1. Thus, the experiment shows that the function of the Z molecules was retained when incorporated into a fusion protein containing the albumin binding polypeptide, 25 and also when the fusion proteins were bound to albumin.

Example 9:Long-term stability of an albumin binding polypeptide

30 In this example, the stability of PEP07986 (SEQ ID NO:1 63), expressed and purified as described in Example 1, was investigated after storage at 4, 25, and 40 °C for up to three months. The status of the polypeptide after storage was investigated by measuring its binding to HSA 35 using a Biacore instrument.

*Material and methods*

Lyophilized PEP07986 was dissolved in sterile NaPi buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) at a concentration of 2 mg/ml. A reference sample (time = 0) was removed and stored at -80 °C.

5     Aliquots of 105  $\mu$ l were stored in sterile screw-cap eppendorf tubes sealed with parafilm at 4, 25, and 40 °C. After one week, two weeks, one month and three months, a sample stored at each temperature was cooled to 4 °C, centrifuged for 5 min at 13000 rpm and then stored at -80 °C awaiting Biosensor analysis.

10    Biosensor analysis was performed essentially as described in Example 2 but with HSA (Albucult®, Novozymes), immobilized to 704 resonance units (RU) and the albumin binding polypeptide variant was diluted to 10 nM and injected at a constant flow-rate of 20  $\mu$ l/min for 10 minutes, followed by injection of HBS-EP for 10 minutes.

15

*Results*

The binding to HSA of PEP07986 (SEQ ID NO:1 63) was retained after storage at 4, 25, and 40 °C for at least three months. The maximum binding responses to HSA obtained for PEP07986 stored at the various conditions

20    are shown in Figure 9.

Example 10:Stability of an albumin binding polypeptide under extreme conditions

25

In this example, biosensor and circular dichroism (CD) analysis of the albumin binding polypeptide PEP08296 (DOTA-conjugated PEP081 85, SEQ ID NO:148) after heat treatment (90 °C) in low pH (-4.0) buffer is described. Since such extreme reaction conditions have to be used for example for  $^{68}\text{Ga}$

30    labeling of DOTA-modified proteins, the influence of high heat and low pH treatment on the structural identity of the polypeptide and its capacity to bind HSA was investigated by measuring the melting temperature (Tm), refolding properties and binding to HSA.

*Material and methods**Biosensor analysis of heat stability*

Biosensor analysis on a Biacore 2000 instrument (GE Healthcare) was performed with HSA (Albucult®, Novozymes) immobilized by amine coupling onto the carboxylated dextran layer of the surfaces of CM-5 chip (research grade; GE Healthcare) according to the manufacturer's recommendations. Surface 1 of the chip was activated and deactivated and used as a reference cell (blank surface) during injections, whereas surface 2 comprised HSA immobilized to 724 resonance units (RU). PEP08296 (50  $\mu$ L, 100  $\mu$ g) in a 15 ml Falcon tube was diluted with 450  $\mu$ L 0.2 M sodium acetate (NaAc) pH 5.5 to a final peptide concentration of 0.2 mg/mL. After addition of 1.5 ml 0.05 M HCl (resembling the conditions and volume used for eluting a  $^{68}\text{Ge}/^{68}\text{Ga}$  generator) the sample was incubated for 10 minutes at 90 °C or RT (control) and then transferred to RT. 6 ml 0.1 M sodium citrate was added to neutralize the pH. The heat treated PEP08296 (0.8 and 4 nM) was injected at a constant flow-rate of 50  $\mu$ L/min for 5 minutes, followed by dissociation in HBS-EP for 15 minutes. The surfaces were regenerated with one injection of 25  $\mu$ L 10 mM HCl. The results were analyzed with BIAevaluation software (GE Healthcare). Curves of the blank surface were subtracted from the curves of the ligand surfaces.

*Determination of the melting temperature ( $T_m$ )*

PEP08296 was dissolved in PBS to a final concentration of 0.5 mg/ml. PBS with a pH of approximately 4.0 was prepared by adding 9.5  $\mu$ L 100 mM HCl to 100  $\mu$ L PBS. Circular dichroism (CD) analysis was performed as described in Example 3.

*CD analysis of heat stability*

To investigate structural reversibility of PEP08296 after heat treatment, two CD spectra between 195 and 250 nm were recorded per sample at 20 °C. After the first spectrum, a VTM cycle with heating to 90 °C was run as described above followed by collection of the second CD spectrum between 195 and 250 nm at 20 °C. In addition, PEP08296 was incubated in PBS pH 4.0 buffer or PBS pH 7.2 buffer for 12 minutes at 90 °C in a thermomixer (500 rpm, interval mixing 10 s on, 30 s off). After incubation, the samples were cooled on ice followed by centrifugation at 13000 rpm for 1 minute, and a CD spectrum between 195 and 250 nm was recorded at 20 °C.

*Results*

5 Biosensor analysis was used to investigate if heat treatment in combination with low pH, i.e. common conditions needed for  $^{68}\text{Ga}$ -labeling of polypeptide, would affect the capacity of PEP08296 to bind to HSA. Figure 10 shows the result of this binding analysis performed with a Biacore 2000 instrument. Two different concentrations of PEP08296, 0.8 nM and 4 nM, were injected over a surface with 724 RU of immobilized human serum albumin. Heat treatment for 10 min at 90 °C, pH 4.0, slightly reduced the 10 binding capacity of PEP08296 to HSA, indicating a potential structural change of the molecule.

15 CD was used to further investigate the potential structural change of the molecule. Similar CD spectra before and after heating would prove a sample to be structurally reversible. In the first experiment ,the samples were heated with a temperature gradient from 20 °C to 90 °C. The CD spectra before and after heat treatment were similar in the Tm determination experiment with the typical minima at 207 and 221 nm indicating a-helicity, i.e. short time heating to 90 °C in either pH 4 or pH 7.2 buffer had no effect on the structure of PEP08296.

20 However, pretreatment of PEP08296 for 12 minutes at 90 °C showed a slightly reduced alpha helix content of PEP08296 if incubated at pH 4.0, but no change in alpha helix content if incubated at pH 7.2. Typical overlays of two CD spectra before and after heating are shown in Figure 11.

25 The results from the melting temperature (Tm) determination are summarized in Table 4.

Table 4. Tm of PEP08296

Designation	Tm (°C)
PEP08296 at pH 7.2	59
PEP08296 at pH 4.0	62

Example 11:Blood pool imaging using a  $^{68}\text{Ga}$ -labeled albumin binding polypeptide

In the experiments making up this example, whole body distribution of  $^{68}\text{Ga}$ -labeled PEP08296 (DOTA-conjugated PEP08185, SEQ ID NO:148) in rats was followed by dynamic imaging over 1.5 hours. Due to the strong association between the labeled polypeptide and serum albumin, the labeled polypeptide can be used for example to study blood pool and tissue permeability.

10

*Material and methods* *$^{68}\text{Ga}$ -labeling of PEP08296*

$^{68}\text{Ga}$  was eluted as  $^{68}\text{GaCl}_3$  from the  $^{68}\text{Ge}/^{68}\text{Ga}$  generator (Obninsk, Russia) with 0.1 M HCl, converted to  $^{68}\text{GaCl}_4^-$  with concentrated HCl, trapped on an anionic exchange column (Chromafix-HCOs) and subsequently eluted with 18 MΩ water, as previously described (Velikyan *et al* (2008), Nucl Med Biol 35:529-536).

The labeling was performed essentially as described in Tolmachev *et al.* (EJNMMI 37:1 356-1 367, 2010). The concentrated  $^{68}\text{Ga}$ -eluate (150-200  $\mu\text{I}$ ) was added to PEP08296 (>1 00  $\mu\text{g}$  in 0.2 M sodium acetate buffer pH 5.5) and the pH was adjusted to 3.5-4 using sodium acetate (1.25 M) or HCl (0.1 M). The labeling mixture was incubated at 90 °C for 15 min before cooling, and the labeled protein was isolated by size exclusion purification on a NAP-5 column eluted with physiologically buffered saline.

25 The radiochemical purity and identity of the  $^{68}\text{Ga}$ -labeled protein was assessed by radio-HPLC using UV (210 nm) and radioactivity detectors in series and a Superdex Peptide 10/300 GL column (GE Healthcare) eluted with physiologically buffered saline.

30 *Small animal PET*

A rat (277 g) was anesthetized with isoflurane (initially 5 %, then 2 % blended with 7:3 air/02), controlled by an E-Z vaporizer using Microflex non-rebreather masks from Euthanex Corporation, and was kept on a heating pad (37 °C) while lying within a microPET Focus120 system (Siemens, CTI Concorde Microsystems).  $^{68}\text{Ga}$ -PEP08296, 33 MBq, was dispensed in a syringe, diluted with saline to 0.5 ml and injected via the tail vein. Data were acquired from the whole body by moving the bed in a constant bed motion

protocol for 1.5 h. Data were processed with MicroPET Manager and corrected for randoms, dead time and decay. Images were reconstructed by standard 2D filtered back projection using a ramp filter and evaluated using Inveon Research Workplace (Siemens Medical Solutions) software.

5

### Results

Basic distribution patterns (Figure 12) for PEP08296 were very similar to that of albumin labeled with radioisotopes such as <sup>68</sup>Ga-DOTA, <sup>64</sup>Cu-DOTA and <sup>11</sup>C (see e.g. Hoffend *et al* (2005), Nucl Med Biol 32:287-292 and Lu *et al* 10 (2008), "[1-<sup>11</sup>C]Butanol and [Methyl-<sup>11</sup>C]Albumin for Blood Flow and Blood Pool Imaging", poster at the XIth Turku PET Symposium, 24-27 May 2008). In brief, high radioactivity concentrations were observed in major blood vessels throughout the scan. Organs with large blood volumes (liver, spleen and kidney) were also clearly delineated, as was the cardiac blood pool 15 radioactivity. Radioactivity in the urinary bladder increased during the observation period, this observation of renal elimination being consistent with previous observations with labeled albumin-based tracers and with that of the metabolism of albumin itself.

The general distribution pattern of radioactivity and very slow plasma 20 clearance after intravenous injection of <sup>68</sup>Ga-PEP08296 is consistent with its expected very rapid and strong binding to albumin. These results therefore support further applications of the radiotracer as an *in vivo* blood pool imaging agent for use with positron emission tomography studies of tissue 25 permeability, both during the development of disease and during therapeutic intervention.

Example 12:  
Solubility of an albumin binding polypeptide

The solubility of PEP07986 (SEQ ID NO:1 63) in physiological buffer  
5 was investigated by consecutive concentrations of the sample using ultrafiltration, followed by concentration measurement and investigation of aggregation status. Concentrations determined by direct absorbance readings at 280 nm were consistent with concentrations determined by gel filtration, showing a solubility of more than 42 mg/ml with no aggregates detected.

10

*Material and methods*

Lyophilized PEP07986 was dissolved in NaPi buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) at a concentration of 3 mg/ml. Amicon Ultra centrifugal filter units, cut off of 3 kDa, (Millipore, Cat. No. UFC800324) were prerinse with 2 ml NaPi buffer by centrifugation at 4000 g for 20 min in a swinging bucket rotor centrifuge (Multifuge, Heraeus). 1620  $\mu$ l of 3 mg/ml PEP07986 was applied to a first centrifugal filter unit and centrifugation was performed at 4000 g, 20 °C, for 7 min. A 25  $\mu$ l sample was removed (UF sample 1) for further analysis and the rest of the sample was transferred to a second centrifugal filter unit. The centrifugation and sample removal were repeated three times with spinning times of 8, 9 and 20 min respectively (UF sample 2, 3 and 4 respectively). Absorbance readings were performed using a NanoDrop® ND-1 000 Spectrophotometer and by diluting UF samples 1-4 in NaPi buffer 2, 4, 6 and 12 times respectively. The concentrations were calculated using the extinction coefficient 1 Abs 280 = 1.955 mg/ml. Gel filtration was performed on a 1100 HPLC system (Agilent Technologies) using a Superdex75 10/300 GL column (GE Healthcare) which had been equilibrated in NaPi buffer. 10  $\mu$ l of each UF sample were applied to the column; NaPi buffer was used as running buffer and the flow rate was 0.5 ml/min. A chromatogram of the molecular weight standard ovalbumin (GE Healthcare), injected at a concentration of 5 mg/ml was collected as well. Concentrations were determined by integrating the area under the curve.

*Results*

Concentrations determined by direct absorbance readings at 280 nm and concentrations determined by gel filtration are shown in Table 5. The solubility of PEP07986 (SEQ ID NO:1 63) is at least 42 mg/ml in physiological buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.2). No aggregates were detected by gel filtration, as shown by Figure 13.

Table 5. Concentrations determined after consecutive concentration of PEP07986 (SEQ ID NO:1 63)

10

Sample	Concentrations (mg/ml) determined by	
	Spectrophotometer	Gel filtration
UF2	12.1	12.4
UF3	22.2	22.1
UF4	42.7	42.6

CLAIMS

1. Albumin binding polypeptide comprising an amino acid sequence selected from

5

i) LAX<sub>3</sub>AKX<sub>6</sub>X<sub>7</sub>ANX<sub>10</sub> ELDXi<sub>4</sub>YGVSDF YKRLIX<sub>26</sub>KAKT VEGVEALKX<sub>39</sub>X<sub>40</sub> ILX<sub>43</sub>X<sub>44</sub>LP

wherein independently of each other

10

X<sub>3</sub> is selected from E, S, Q and C;

X<sub>6</sub> is selected from E, S and C;

X<sub>7</sub> is selected from A and S;

X<sub>10</sub> is selected from A, S and R;

15 X<sub>i</sub><sub>4</sub> is selected from A, S, C and K;

X<sub>26</sub> is selected from D and E;

X<sub>39</sub> is selected from D and E;

X<sub>40</sub> is selected from A and E;

X<sub>43</sub> is selected from A and K;

20 X<sub>44</sub> is selected from A, S and E;

L in position 45 is present or absent; and

P in position 46 is present or absent;

and

25 ii) an amino acid sequence which has at least 95 % identity to the sequence defined in i).

30 2. Albumin binding polypeptide according to claim 1, wherein X<sub>6</sub> is E.

30 3. Albumin binding polypeptide according to any preceding claim, wherein X<sub>3</sub> is S.

35 4. Albumin binding polypeptide according to any one of claims 1-2, wherein X<sub>3</sub> is E.

35 5. Albumin binding polypeptide according to any preceding claim, wherein X<sub>7</sub> is A.

6. Albumin binding polypeptide according to any preceding claim,  
wherein  $X_{14}$  is S.

5 7. Albumin binding polypeptide according to any one of claims 1-5,  
wherein  $X_{14}$  is C.

8. Albumin binding polypeptide according to any preceding claim,  
wherein  $X_{10}$  is A.

10 9. Albumin binding polypeptide according to any one of claims 1-7,  
wherein  $X_{10}$  is S.

15 10. Albumin binding polypeptide according to any one of claims 1-9,  
wherein  $X_{26}$  is D.

11. Albumin binding polypeptide according to any one of claims 1-9,  
wherein  $X_{26}$  is E.

20 12. Albumin binding polypeptide according to any one of claims 1-11,  
wherein  $X_{39}$  is D.

13. Albumin binding polypeptide according to any one of claims 1-11,  
wherein  $X_{39}$  is E.

25 14. Albumin binding polypeptide according to any one of claims 1-13,  
wherein  $X_{40}$  is A.

15. Albumin binding polypeptide according to any one of claims 1-14,  
30 wherein  $X_{43}$  is A.

16. Albumin binding polypeptide according to any one of claims 1-15,  
wherein  $X_{44}$  is A.

35 17. Albumin binding polypeptide according to any one of claims 1-15,  
wherein  $X_{44}$  is S.

18. Albumin binding polypeptide according to any one of claims 1-17, wherein L in position 45 is present.

19. Albumin binding polypeptide according to any one of claims 1-18, 5 wherein P in position 46 is present.

20. Albumin binding polypeptide according to any preceding claim, which binds to albumin such that the  $k_{off}$  value of the interaction is at most  $5 \times 10^{-5} \text{ s}^{-1}$ .

10

21. Albumin binding polypeptide according to claim 20, which binds to albumin such that the  $k_{off}$  value of the interaction is at most  $5 \times 10^{-6} \text{ s}^{-1}$ .

15

22. Albumin binding polypeptide according to claim 1, whose amino acid sequence is selected from any one of SEQ ID NO:1 -144 and SEQ ID NO:1 64-203.

20

23. Albumin binding polypeptide according to claim 22, whose amino acid sequence is selected from any one of SEQ ID NO:4-5, SEQ ID NO:7-8, SEQ ID NO:1 0-1 1, SEQ ID NO:1 3-14, SEQ ID NO:1 6-1 7, SEQ ID NO:19-20, SEQ ID NO:22-23, SEQ ID NO:25-26, SEQ ID NO:28-29, SEQ ID NO:31 -32, SEQ ID NO:34-35, SEQ ID NO:37-38, SEQ ID NO:41 -42, SEQ ID NO:49-50, SEQ ID NO:1 64-1 70 and SEQ ID NO:1 92-203.

25

24. Albumin binding polypeptide according to claim 22, whose amino acid sequence is selected from any one of SEQ ID NO:1 -144.

30

25. Albumin binding polypeptide according to claim 24, whose amino acid sequence is selected from any one of SEQ ID NO:4-5, SEQ ID NO:7-8, SEQ ID NO:1 0-1 1, SEQ ID NO:1 3-14, SEQ ID NO:1 6-1 7, SEQ ID NO:19-20, SEQ ID NO:22-23, SEQ ID NO:25-26, SEQ ID NO:28-29, SEQ ID NO:31 -32, SEQ ID NO:34-35, SEQ ID NO:37-38, SEQ ID NO:41 -42 and SEQ ID NO:49-50.

35

26. Albumin binding polypeptide according to any preceding claim, further comprising one or more additional amino acid residues positioned at the N- and/or the C-terminal of the sequence defined in i).

27. Albumin binding polypeptide according to claim 26, in which the additional amino acids comprise at least one serine residue at the N-terminal of the polypeptide.

5

28. Albumin binding polypeptide according to any one of claims 26-27, in which the additional amino acids comprise a glycine residue at the N-terminal of the polypeptide.

10

29. Albumin binding polypeptide according to any one of claims 26-28, in which the additional amino acids comprise a cysteine residue at the N-terminal of the polypeptide.

15

30. Albumin binding polypeptide according to any one of claims 26-29, in which the additional amino acids comprise a lysine residue at the C-terminal of the polypeptide.

20

31. Albumin binding polypeptide according to any one of claims 26-30, in which the additional amino acids comprise a glycine residue at the C-terminal of the polypeptide.

25

32. Albumin binding polypeptide according to any one of claims 26-31, in which the additional amino acids comprise a cysteine residue at the C-terminal of the polypeptide.

30

33. Albumin binding polypeptide according to any one of claims 26-32, whose amino acid sequence is selected from any one of SEQ ID NO:145-1 50 and SEQ ID NO:1 62-1 63.

35

34. Albumin binding polypeptide according to any preceding claim, comprising no more than two cysteine residues.

35

35. Albumin binding polypeptide according to claim 34, comprising no more than one cysteine residue.

36. Albumin binding polypeptide according to any preceding claim, which binds to human serum albumin.

37. Fusion protein or conjugate comprising

- i) a first moiety consisting of an albumin binding polypeptide according to any preceding claim; and
- 5 ii) a second moiety consisting of a polypeptide having a desired biological activity.

10 38. Fusion protein or conjugate according to claim 37, in which the second moiety having a desired biological activity is a therapeutically active polypeptide.

15 39. Fusion protein or conjugate according to claim 38, in which the second moiety having a desired biological activity is selected from the group consisting of human endogenous enzymes, hormones, growth factors, chemokines, cytokines and lymphokines.

20 40. Fusion protein or conjugate according to claim 39, in which the second moiety is selected from the group consisting of IL-2, GLP-1, BNP, IL-1 -RA, KGF, Stemgen®, GH, G-CSF, CTLA-4, myostatin, Factor VII, Factor VIII and Factor IX.

25 41. Fusion protein or conjugate according to claim 38, in which the second moiety having a desired biological activity is a non-human biologically active protein, selected from the group consisting of bacterial toxins, enzymes and activating proteins.

30 42. Fusion protein or conjugate according to claim 37, in which the second moiety having a desired biological activity is a binding polypeptide capable of selective interaction with a target molecule.

35 43. Fusion protein or conjugate according to claim 42, in which the binding polypeptide is selected from the group consisting of antibodies and fragments and domains thereof substantially retaining antibody binding activity; microbodies, maxybodies, avimers and other small disulfide-bonded proteins; and binding proteins derived from a scaffold selected from the group consisting of staphylococcal protein A and domains thereof, other three helix domains, lipocalins, ankyrin repeat domains, cellulose binding domains,  $\gamma$

crystallines, green fluorescent protein, human cytotoxic T lymphocyte-associated antigen 4, protease inhibitors such as Kunitz domains, PDZ domains, SH3 domains, peptide aptamers, staphylococcal nuclease, tendamistats, fibronectin type III domain, transferrin, zinc fingers and 5 conotoxins.

44. Fusion protein or conjugate according to claim 43, in which said target molecule is selected from the group consisting of A $\beta$  peptide of Alzheimer's disease; other disease-associated amyloid peptides; toxins, such 10 as bacterial toxins and snake venoms; blood clotting factors, such as von Willebrand factor; interleukins, such as IL-1 3; myostatin; pro-inflammatory factors, such as TNF-a, TNF-a receptor, IL-1, IL-23 and IL-8; complement factors, such as C3 and C5; hypersensitivity mediators, such as histamine and IgE; tumor-related antigens, such as CD19, CD20, CD22, CD30, CD33, 15 CD40, CD52, CD70, cMet, HER1, HER2, HER3, HER4, CAIX, CEA, IL-2 receptor, MUC1, PSMA, TAG-72, and other biological molecules such as G-CSF, GM-CSF, GH, insulin and somatostatin.

45. Fusion protein or conjugate according to any one of claims 37-44, 20 comprising a further moiety consisting of a polypeptide having a further, desired biological activity, which may be the same as or different from that of the second moiety.

46. Fusion protein or conjugate according to claim 45, wherein the 25 second moiety is as defined in any one of claims 38-41, and the further moiety is as defined in any one of claim 42-44.

47. Conjugate according to any one of claims 37-46, in which the second moiety is conjugated to an albumin binding polypeptide according to 30 any one of claims 1-36 via the thiol group of any cysteine residue present at position X<sub>14</sub> of the polypeptide.

48. Albumin binding polypeptide, fusion protein or conjugate according 35 to any preceding claim, further comprising a cytotoxic agent.

49. Albumin binding polypeptide, fusion protein or conjugate according to claim 48, wherein the cytotoxic agent is selected from calicheamycin,

auristatin, doxorubicin, maytansinoid, taxol, ecteinascidin, geldanamycin, methotrexate and their derivatives, and combinations thereof.

50. Albumin binding polypeptide, fusion protein or conjugate according  
5 to any preceding claim further comprising a label.

51. Albumin binding polypeptide, fusion protein or conjugate according  
to claim 50, in which said label is selected from the group consisting of  
fluorescent dyes and metals, chromophoric dyes, chemiluminescent  
10 compounds and bioluminescent proteins, enzymes, radionuclides and  
particles.

52. Albumin binding polypeptide, fusion protein or conjugate according  
to claim 51, comprising a chelating environment provided by a  
15 polyaminopolycarboxylate chelator conjugated to the albumin binding  
polypeptide via a thiol group of a cysteine residue or an amine group of a  
lysine residue.

53. Albumin binding polypeptide, fusion protein or conjugate according  
20 to claim 52, wherein the polyaminopolycarboxylate chelator is 1,4,7,10-  
tetraazacyclododecane-1,4,7,10-tetraacetic acid or a derivative thereof.

54. Albumin binding polypeptide, fusion protein or conjugate according  
to claim 53, wherein the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic  
25 acid derivative is 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-  
maleimidoethylacetamide.

55. Albumin binding polypeptide, fusion protein or conjugate according  
to claim 52, wherein the polyaminopolycarboxylate chelator is  
30 diethylenetriaminepentaacetic acid or derivatives thereof.

56. Polynucleotide encoding an albumin binding polypeptide or a fusion  
protein according to any one of claims 1-46.

35 57. Method of producing a polypeptide according to any one of claims  
1-45, comprising expressing a polynucleotide according to claim 56.

56. Expression vector comprising a polynucleotide according to claim 56.

59. Host cell comprising an expression vector according to claim 58.

5           60. Method of producing a polypeptide according to any one of claims 1-36 by non-biological peptide synthesis using amino acids and/or amino acid derivatives having protected reactive side-chains, the non-biological peptide synthesis comprising  
10           step-wise coupling of the amino acids and/or the amino acid derivatives to form a polypeptide according to any one of claims 1-36 having protected reactive side-chains,  
              removal of the protecting groups from the reactive side-chains of the polypeptide, and  
15           folding of the polypeptide in aqueous solution.

61. Method of producing a conjugate according to any one of claims 37-48, comprising  
20           producing an albumin binding polypeptide by the method according to claim 60, and  
              conjugating the produced albumin binding polypeptide with a second and/or further moiety as defined in any one of claims 38-48.

62. Fusion protein or conjugate according to any one of claims 37-55  
25           for use as a medicament.

63. Fusion protein or conjugate according to claim 62, which elicits no or a reduced immune response upon administration to the mammal, as compared to the immune response elicited upon administration to the  
30           mammal of the polypeptide having a desired biological activity *per se*.

64. Fusion protein or conjugate according to any one of claims 37-55  
for use in diagnosis.

35           65. Composition, comprising  
              a compound which *per se* has a solubility in water of no more than 100 µg/ml; coupled to

an albumin binding polypeptide, a fusion protein or conjugate according to any one of claims 1-47, wherein the compound and the albumin binding polypeptide, fusion protein or conjugate are covalently coupled.

5 66. Composition according to claim 65, wherein said solubility is no more than 10 µg/ml.

67. Composition according to claim 66, wherein said solubility is no more than 1 µg/ml.

10 68. Composition according to any one of claims 65-67, wherein said compound is a pharmaceutically active compound.

15 69. Composition according to claim 68, wherein said pharmaceutically active compound is a cytotoxic agent.

70. Composition according to claim 69, wherein said cytotoxic agent is as defined in claim 49.

20 71. Composition according to claim 69, wherein said cytotoxic agent is a synthetic chemotoxin not derived from a naturally occurring compound.

25 72. Composition according to any one of claims 65-71, further comprising a binding polypeptide having a binding affinity for a clinically relevant target.

73. Composition according to claim 72, wherein said binding polypeptide is as defined in claim 43.

30 74. Composition according to any one of claims 65-73, further comprising human serum albumin.

75. Composition according to any one of claims 65-74 for use as a medicament.

35 76. Composition according to any one of claims 65-74 for use in diagnosis.

77. Method of preparation of a composition according to any one of claims 65-76, comprising

5        a) providing a compound which *per se* has a solubility in water of no more than 100 pg/ml; and

      b) covalently coupling the compound to an albumin binding polypeptide, fusion protein or conjugate according to any one of claims 1-47, thus forming a composition comprising a covalent complex of compound and an albumin binding polypeptide, fusion protein or conjugate.

10

78. Method according to claim 77, further comprising c) mixing said complex of compound and albumin binding polypeptide, fusion protein or conjugate with albumin, thus forming a composition comprising a non-covalent complex of i) the covalent complex of compound and albumin binding polypeptide, fusion protein or conjugate and ii) albumin.

79. Method according to claim 78, further comprising lyophilizing said composition comprising the non-covalent complex to obtain a lyophilized composition.

20

80. Method of increasing the aqueous solubility of a compound, comprising

      providing a compound which *per se* has a solubility in water of no more than 100 pg/ml;

25        covalently coupling the compound to an albumin binding polypeptide, fusion protein or conjugate according to any one of claims 1-47, thus forming a covalent complex of compound and albumin binding polypeptide; and

30        mixing said complex of compound and albumin binding polypeptide, fusion protein or conjugate with albumin under conditions that promote the non-covalent association of the albumin binding polypeptide with human serum albumin;

      whereby the solubility in water of the compound in said complex is greater than the solubility in water of the compound *per se*.

35        81. Method according to claim 80, wherein said solubility is as defined in any one of claims 66-67.

65

82. Method according to any one of claims 80-81, wherein said compound is as defined in any one of claims 68-71.

83. Method according to any one of claims 80-82, wherein said 5 complex further comprises a polypeptide having a binding affinity for a clinically relevant target.

84. Method according to claim 83, wherein said binding polypeptide is as defined in claim 43.

10

Polypeptide	Amino acid sequence	SEQ ID NO:
PP001	LASAKEAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:1
PP002	LASAKEAANS ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:2
PP003	LASAKESANS ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:3
PP004	LASAKESAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:4
PP005	LASAKSAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:5
PP006	LASAKSANS ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:6
PP007	LASAKEAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:7
PP008	LASAKEAANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:8
PP009	LASAKESANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:9
PP010	LASAKESAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:10
PP011	LASAKSAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:11
PP012	LASAKSANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:12
PP013	LAEAKEAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:13
PP014	LAEAKEAANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:14
PP015	LAEAKESANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:15
PP016	LAEAKESAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:16
PP017	LAEAKSAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:17
PP018	LAEAKSANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:18
PP019	LAEAKEAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:19
PP020	LAEAKEAANS ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:20
PP021	LAEAKESANS ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:21
PP022	LAQAKEAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:22
PP023	LAQAKEAANS ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:23
PP024	LAQAKESAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:24
PP025	LAQAKSAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:25
PP026	LAQAKSANS ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:26
PP027	LAQAKESAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:27
PP028	LAQAKESAANA ELDAYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:28
PP029	LAQAKSAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:29
PP030	LAQAKSANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:30
PP031	LAQAKESAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:31
PP032	LAQAKESAANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:32
PP033	LAQAKESANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:33
PP034	LAQAKESAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:34
PP035	LAQAKSAANA ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:35
PP036	LAQAKSANS ELDYGVSDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:36

Figure 1

PP037	LASAKEAANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:37
PP038	LASAKEAANS ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:38
PP039	LASAKESANS ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:39
PP040	LASAKESANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:40
PP041	LASAKSAANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:41
PP042	LASAKSAANS ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:42
PP043	LASAKEAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:43
PP044	LASAKEAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:44
PP045	LASAKESANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:45
PP046	LASAKESANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:46
PP047	LASAKSAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:47
PP048	LASAKSAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:48
PP049	LAEAKEAANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:49
PP050	LAEAKEAANS ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:50
PP051	LAEAKESANS ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:51
PP052	LAEAKESANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:52
PP053	LAEAKSAANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:53
PP054	LAEAKSAANS ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:54
PP055	LAEAKEAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:55
PP056	LAEAKEAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:56
PP057	LAEAKESANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:57
PP058	LAEAKESANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:58
PP059	LAEAKSAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:59
PP060	LAEAKSAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:60
PP061	LAQAKEAANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:61
PP062	LAQAKEAANS ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:62
PP063	LAQAKESANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:63
PP064	LAQAKESANA ELDGYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:64
PP065	LAQAKSAANA ELDGYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:65
PP066	LAQAKSAANS ELDGYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:66
PP067	LAQAKEAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:67
PP068	LAQAKEAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:68
PP069	LAQAKESANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:69
PP070	LAQAKESANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:70
PP071	LAQAKSAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:71
PP072	LAQAKSAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:72
PP073	LACAKEAANA ELDCYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:73

Figure 1

		SEQ ID NO:74
PP075	LACAKEAANS ELDICYGSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:75
PP076	LACAKESAANA ELDICYGSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:76
PP077	LACAKSAANA ELDICYGSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:77
PP078	LACAKSAANS ELDICYGSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:78
PP079	LACAKEAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:79
PP080	LACAKEAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:80
PP081	LACAKESAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:81
PP082	LACAKESAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:82
PP083	LACAKSAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:83
PP084	LACAKSAANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:84
PP085	LACAKEAANA ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:85
PP086	LACAKEAANS ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:86
PP087	LACAKESAANS ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:87
PP088	LACAKESAANA ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:88
PP089	LACAKSAANA ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:89
PP090	LACAKSAANS ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:90
PP091	LACAKEAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:91
PP092	LACAKEAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:92
PP093	LACAKESAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:93
PP094	LACAKESAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:94
PP095	LACAKSAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:95
PP096	LACAKSAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:96
PP097	LAQAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:97
PP098	LAQAKCANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:98
PP099	LAQAKCSANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:99
PP100	LAQAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:100
PP101	LAQAKCANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:101
PP102	LAQAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:102
PP103	LAQAKCAANA ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:103
PP104	LAQAKCAANS ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:104
PP105	LAQAKCSANS ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:105
PP106	LAQAKCSANA ELDSYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:106
PP107	LAQAKCAANA ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:107
PP108	LAQAKCANS ELDKYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:108
PP109	LASAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:109
PP110	LASAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:110

Figure 1

PP111	LASAKCSANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:111
PP112	LASAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:112
PP113	LASAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:113
PP114	LASAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:114
PP115	LASAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:115
PP116	LASAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:116
PP117	LASAKCSANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:117
PP118	LASAKCSANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:118
PP119	LASAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:119
PP120	LASAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:120
PP121	LAEAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:121
PP122	LAEAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:122
PP123	LAEAKCSANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:123
PP124	LAEAKCSANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:124
PP125	LAEAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:125
PP126	LAEAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:126
PP127	LAEAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:127
PP128	LAEAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:128
PP129	LAEAKCSANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:129
PP130	LAEAKCSANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:130
PP131	LAEAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:131
PP132	LAEAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:132
PP133	LACAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:133
PP134	LACAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:134
PP135	LACAKCSANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:135
PP136	LACAKCSANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:136
PP137	LACAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:137
PP138	LACAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:138
PP139	LACAKCSANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:139
PP140	LACAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:140
PP141	LACAKCSANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:141
PP142	LACAKCSANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:142
PP143	LACAKCAANA ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:143
PP144	LACAKCAANS ELDAYGVSDF YKRLLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:144
PP145	GSLASAKEAA NAELDSYGVs DFYKRLIDKA KTVEGVEALK DAIILALPG	SEQ ID NO:145
PP146	GSLAEAKEAA NAELDSYGVs DFYKRLIDKA KTVEGVEALK DAIILALPG	SEQ ID NO:146
PP147	GSLASAKEAA NAELDCYGVs DFYKRLIDKA KTVEGVEALK DAIILALPG	SEQ ID NO:147

Figure 1

SEQ ID NO:148	PEP08185	GSLAAKEAA NAEELDCYGV SDFYKRLLDKA KTVGEVEALK DATIILAALPG
SEQ ID NO:149	PP149	GSLAAKEAA NAEELDSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALPG
SEQ ID NO:150	PP150	GSLAAKEAA NAEELDSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALPG
SEQ ID NO:151	PP151	GCSLAAKEAA ANAEELDKYGV SDFYKRLLDKA KTVGEVEALK DATIILAALPG
SEQ ID NO:152	PP152	GCSLAAKEAA ANAEELDKYGV SDFYKRLLDKA KTVGEVEALK DATIILAALPG
SEQ ID NO:153	PEP07913	GAAEAKVLAN REIDKYGVSDF YYKNLINNAK TVEGVKALID EIIAALP
SEQ ID NO:154	PEP06923	GSSLAAKEA ANRELDKYGVSDF YKRLINK KTVGEVEALK KLHIIAALP
SEQ ID NO:155	PEP07271	GSSLAAKEA ANAEELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:156	PEP07554	GSSLAAKEA ANAEELDSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:157	PEP07912	GLASAAKEAN AEELDSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:158	PEP07914	GLAAEAKEAAN AEELDSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:159	PEP07911	GLASAAKEAN AEELDCYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:160	PEP07834	ALASAAKEAN AEELDCYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:161	PEP07844	GSSLAAKEA ANAEELDKYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:162	PEP07983	GSSLAAKEA NAEELDSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:163	PEP07986	GSSLAAKEA NAEELDSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:164	PP164	LAAEAKEANR ELDGSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:165	PP165	LAAEAKEAANA ELDGSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:166	PP166	LAAEAKEAANA ELDGSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:167	PP167	LAAEAKEAANA ELDGSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:168	PP168	LAAEAKEAANR ELDGSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:169	PP169	LAAEAKEAANA ELDGSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:170	PP170	LAAEAKEAANA ELDGSYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:171	PP171	LAAEAKEAANR ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:172	PP172	LAAEAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:173	PP173	LAAEAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:174	PP174	LAAEAKEAANR ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:175	PP175	LAAEAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:176	PP176	LAAEAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:177	PP177	LAAEAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:178	PP178	LAQAAKEAANR ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:179	PP179	LAQAAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:180	PP180	LAQAAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:181	PP181	LAQAAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:182	PP182	LAQAAKEAANR ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:183	PP183	LAQAAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP
SEQ ID NO:184	PP184	LAQAAKEAANA ELDAYGV SDFYKRLLDKA KTVGEVEALK DATIILAALP

Figure 1

PP185	LAQAKEAANR ELD SYGV SDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:185
PP186	LAQAKEAANA ELD SYGV SDF YKRLIEKAKT VEGVEALKDA IIAALP	SEQ ID NO:186
PP187	LAQAKEAANA ELD SYGV SDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:187
PP188	LAQAKEAANA ELD SYGV SDF YKRLIEKAKT VEGVEALKDA IIAALP	SEQ ID NO:188
PP189	LAQAKEAANR ELD SYGV SDF YKRLIEKAKT VEGVEALKDA IIAALP	SEQ ID NO:189
PP190	LAQAKEAANA ELD SYGV SDF YKRLIDKAKT VEGVEALKDA IILKALP	SEQ ID NO:190
PP191	LAQAKEAANA ELD SYGV SDF YKRLIDKAKT VEGVEALKDA ILASLP	SEQ ID NO:191
PP192	LAFAKEAANR ELD CYGV SDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:192
PP193	LAFAKEAANA ELD CYGV SDF YKRLIEKAKT VEGVEALKDA IIAALP	SEQ ID NO:193
PP194	LAFAKEAANA ELD CYGV SDF YKRLIDKAKT VEGVEALKDA IIAALP	SEQ ID NO:194
PP195	LAFAKEAANA ELD CYGV SDF YKRLIEKAKT VEGVEALKDA IIAALP	SEQ ID NO:195
PP196	LAFAKEAANR ELD CYGV SDF YKRLIEKAKT VEGVEALKDA IIAALP	SEQ ID NO:196
PP197	LAFAKEAANA ELD CYGV SDF YKRLIDKAKT VEGVEALKDA IILKALP	SEQ ID NO:197
PP198	LAFAKEAANA ELD CYGV SDF YKRLIDKAKT VEGVEALKDA ILASLP	SEQ ID NO:198
PP199	LAFAKEAANA ELD SYGV SDF YKRLIDKAKT VEGVEALKDA IIAAL	SEQ ID NO:199
PP200	LAFAKEAANA ELDAYGV SDF YKRLIDKAKT VEGVEALKDA IIAAL	SEQ ID NO:200
PP201	LAQAKEAANA ELDAYGV SDF YKRLIDKAKT VEGVEALKDA IIAAL	SEQ ID NO:201
PP202	LAQAKEAANA ELD SYGV SDF YKRLIDKAKT VEGVEALKDA IIAAL	SEQ ID NO:202
PP203	LAQAKEAANA ELD CYGV SDF YKRLIDKAKT VEGVEALKDA IIAAL	SEQ ID NO:203

Figure 1

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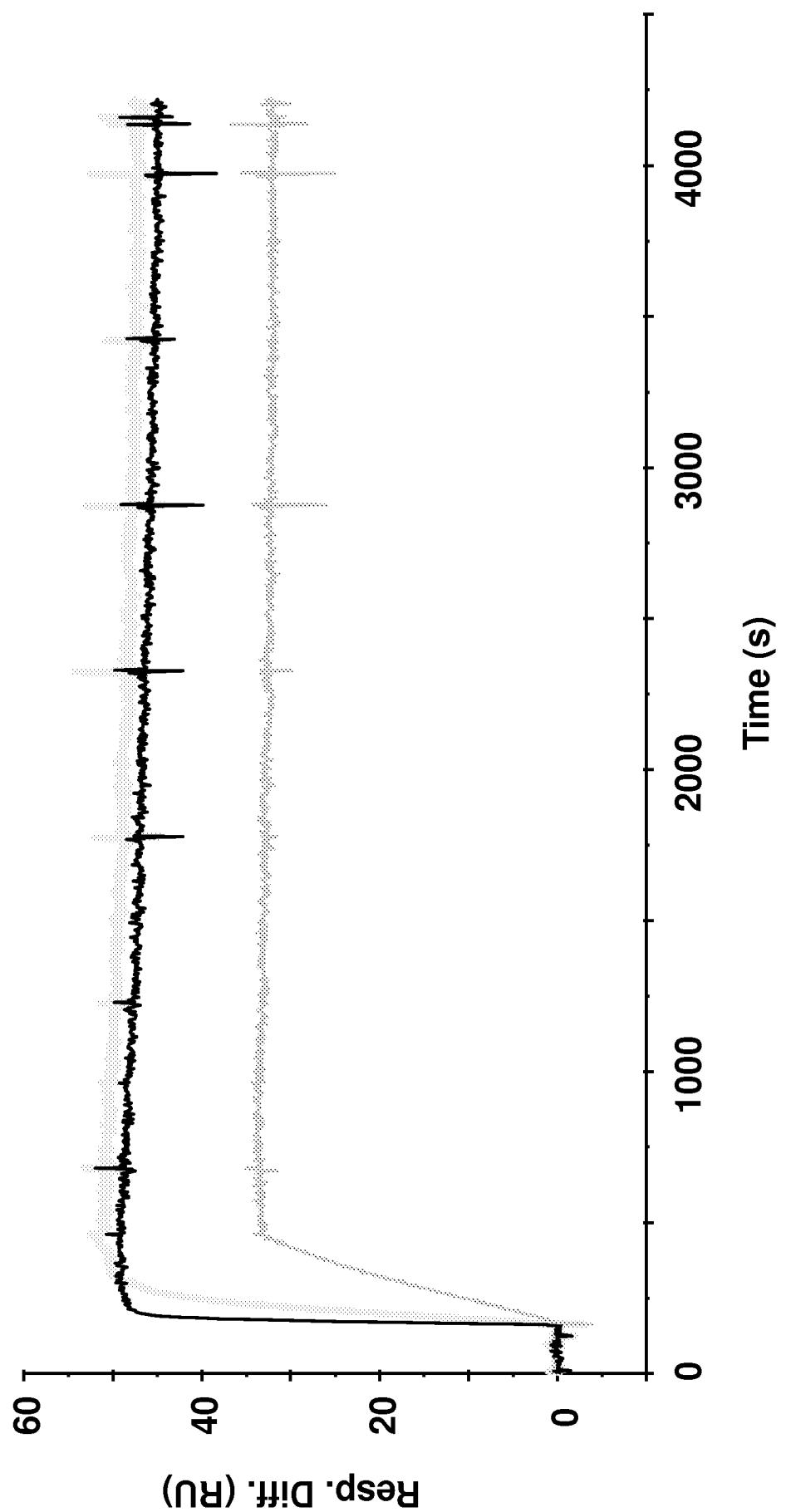
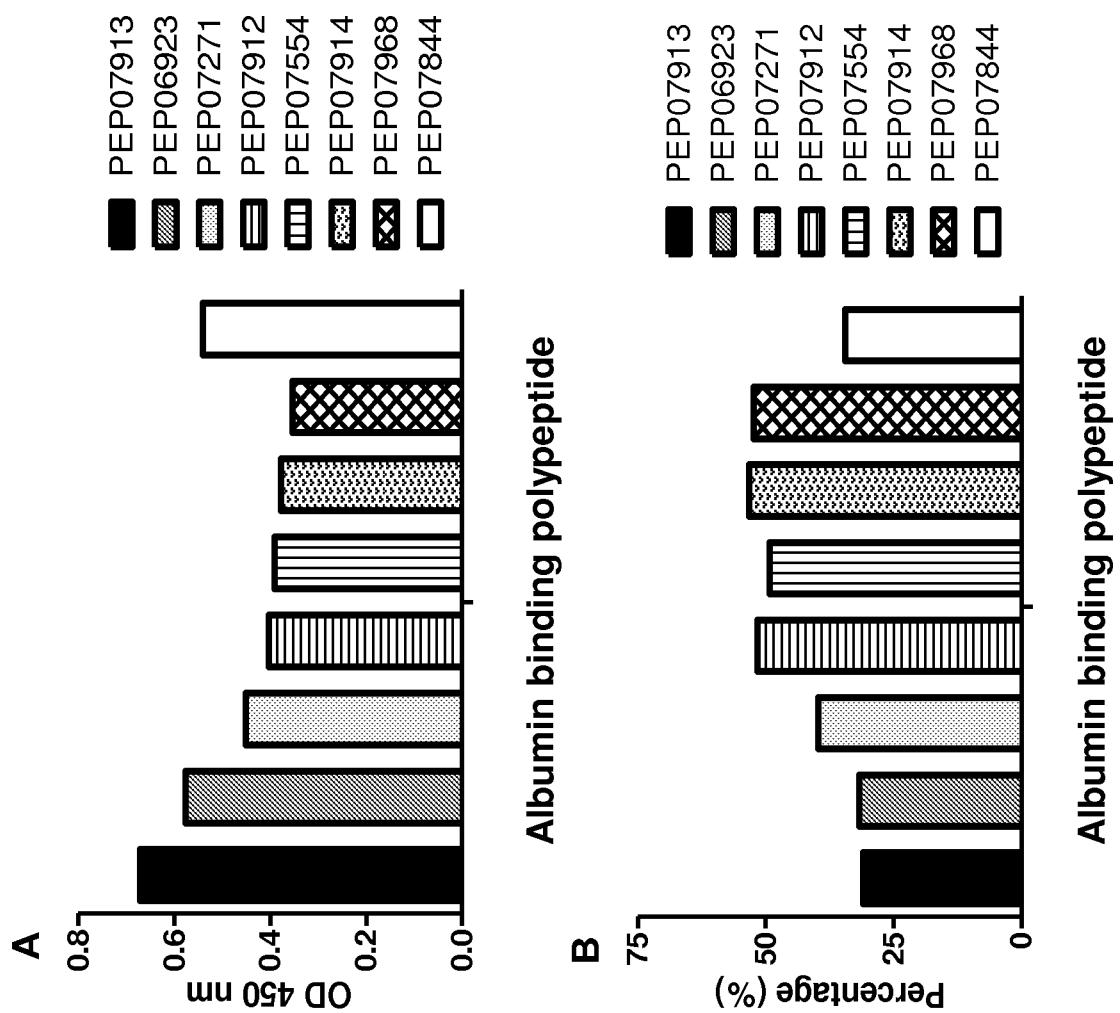


Figure 2

**Figure 3**

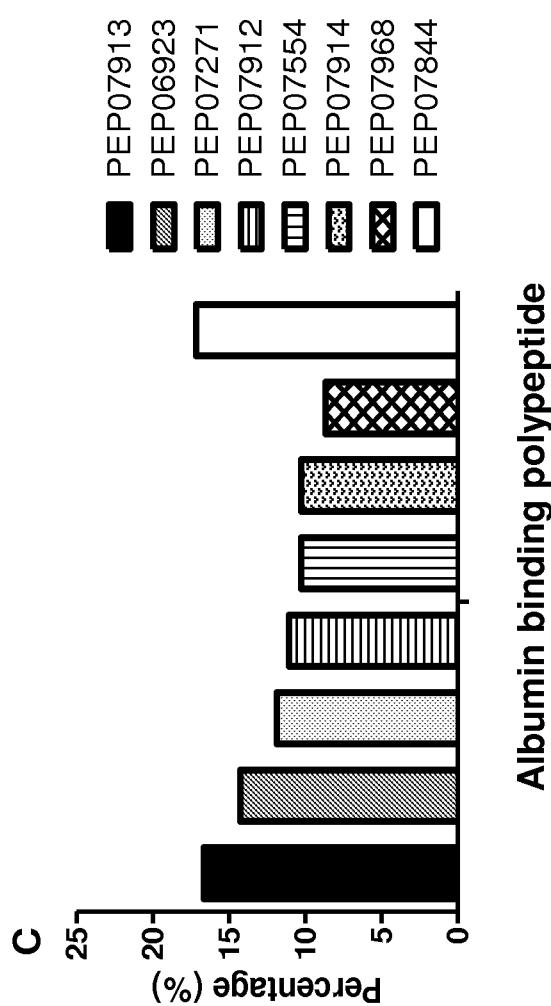
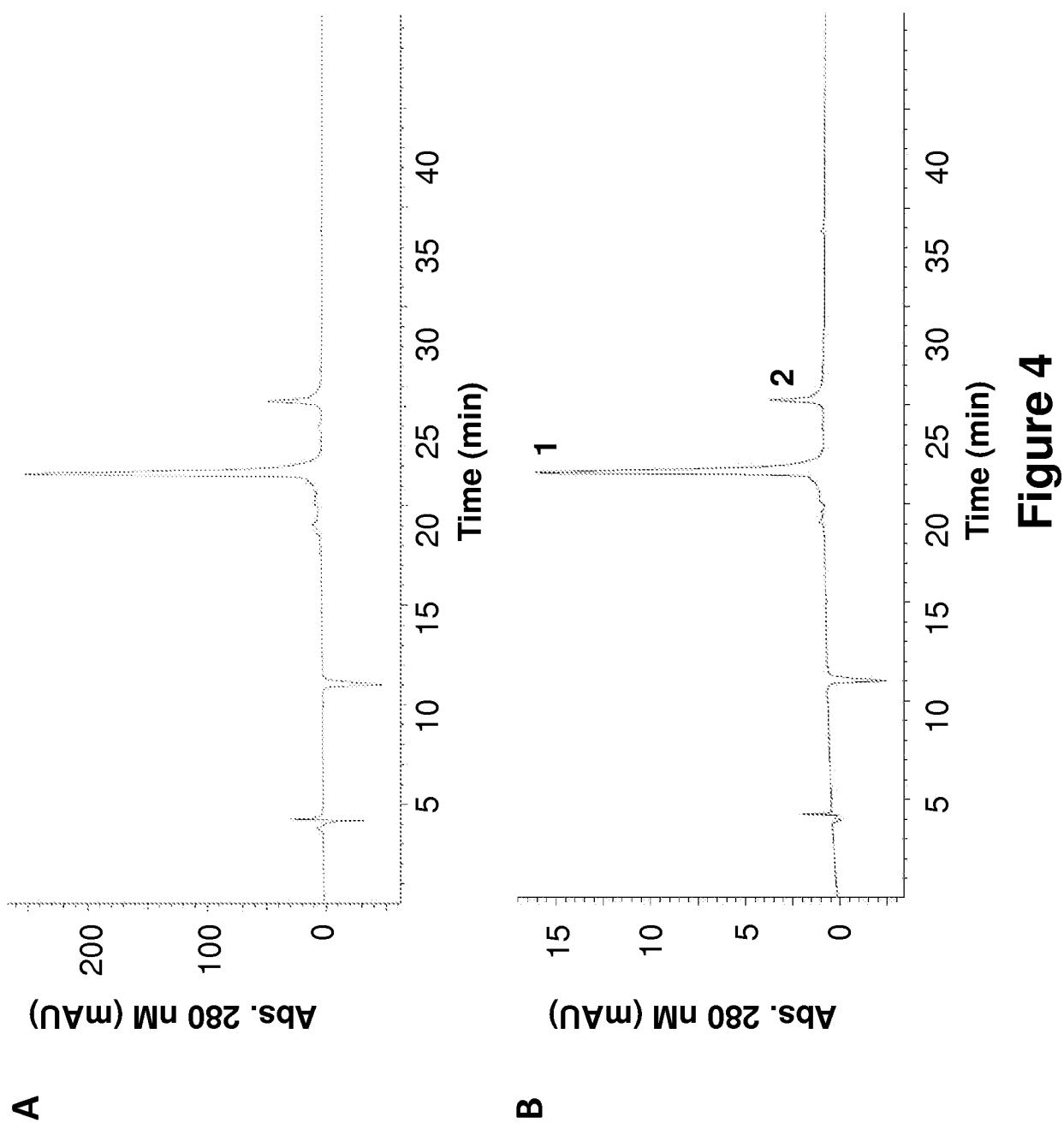
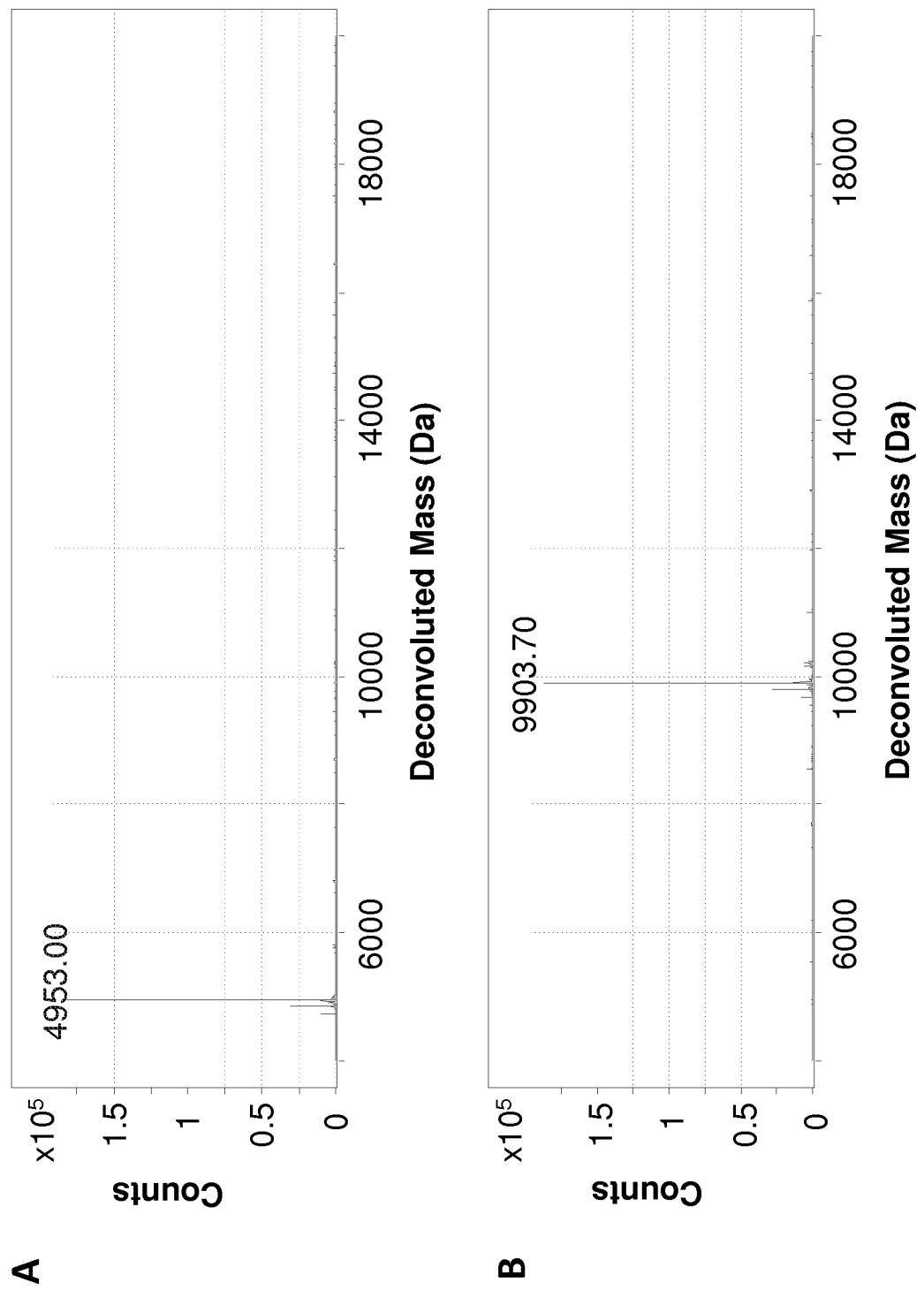


Figure 3

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**Figure 4**



**Figure 5**

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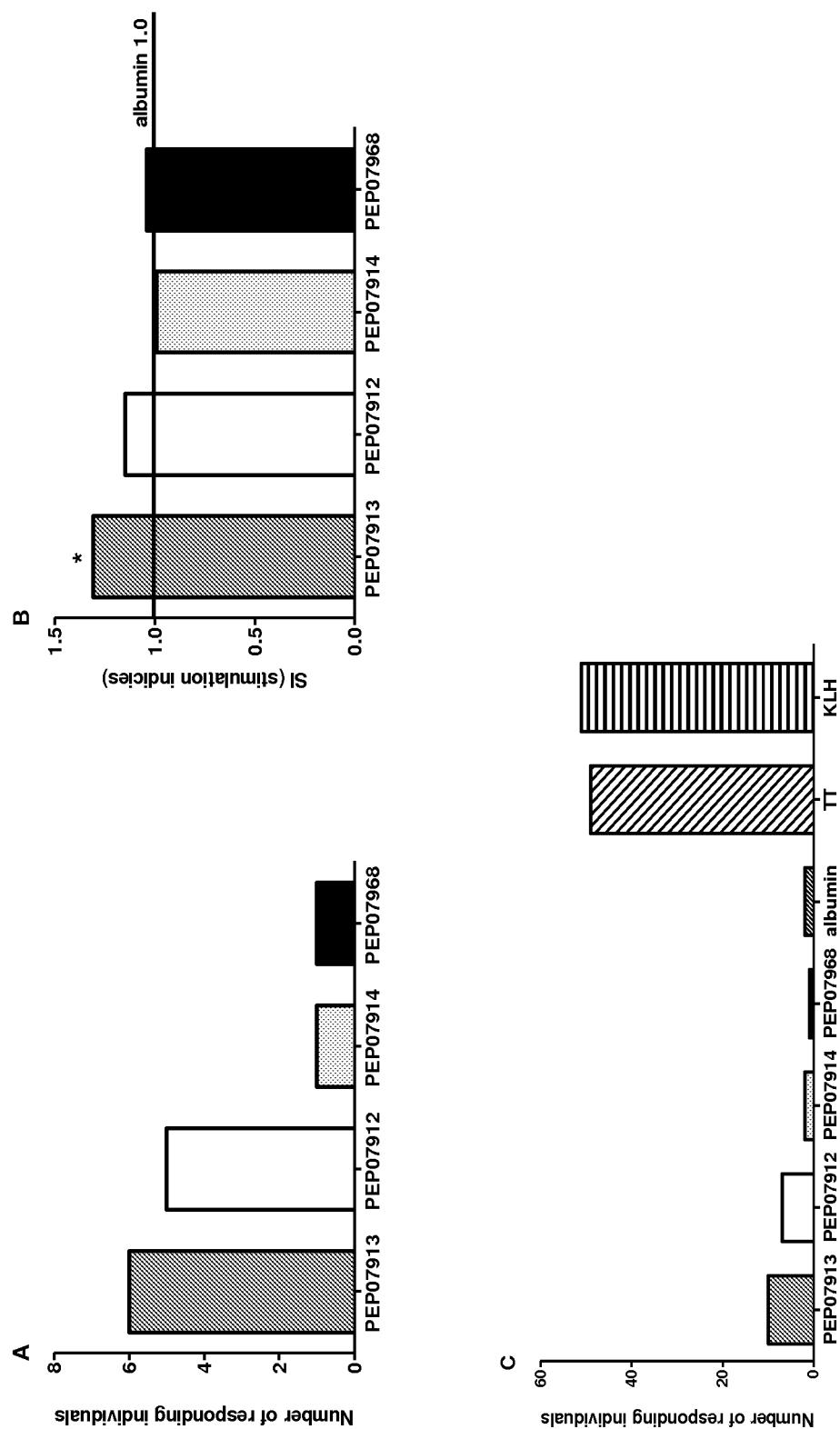
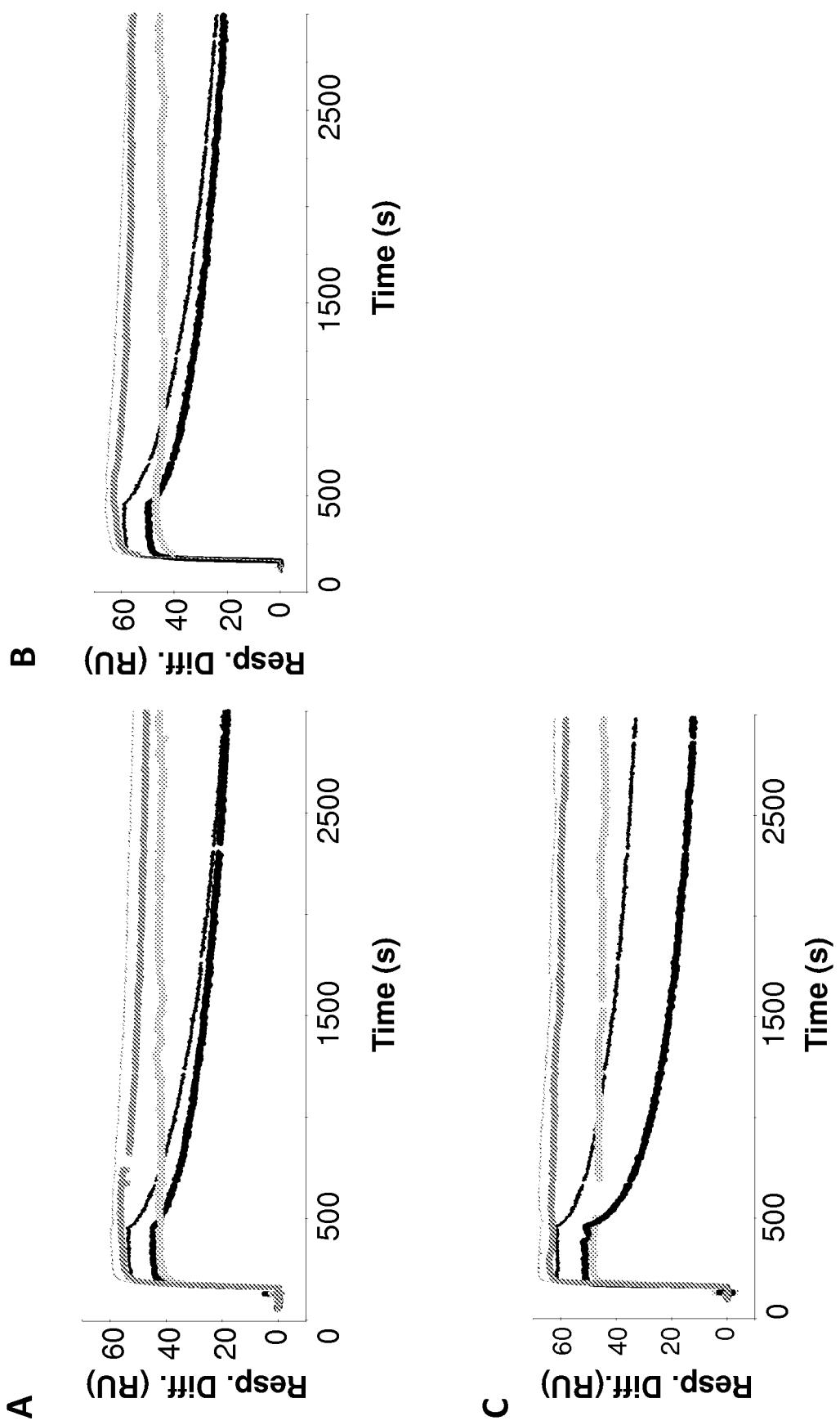


Figure 6

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**Figure 7**

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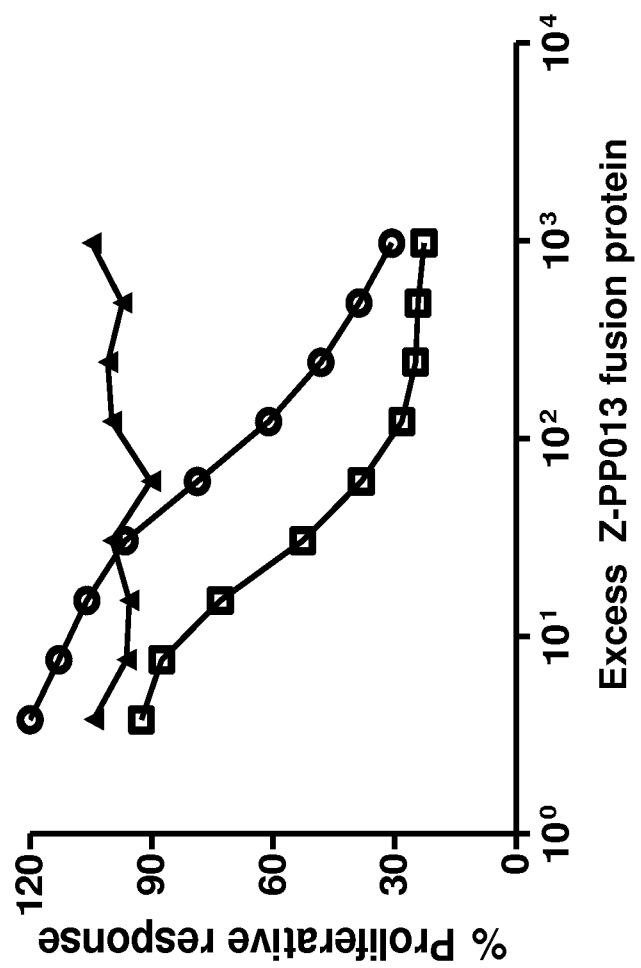
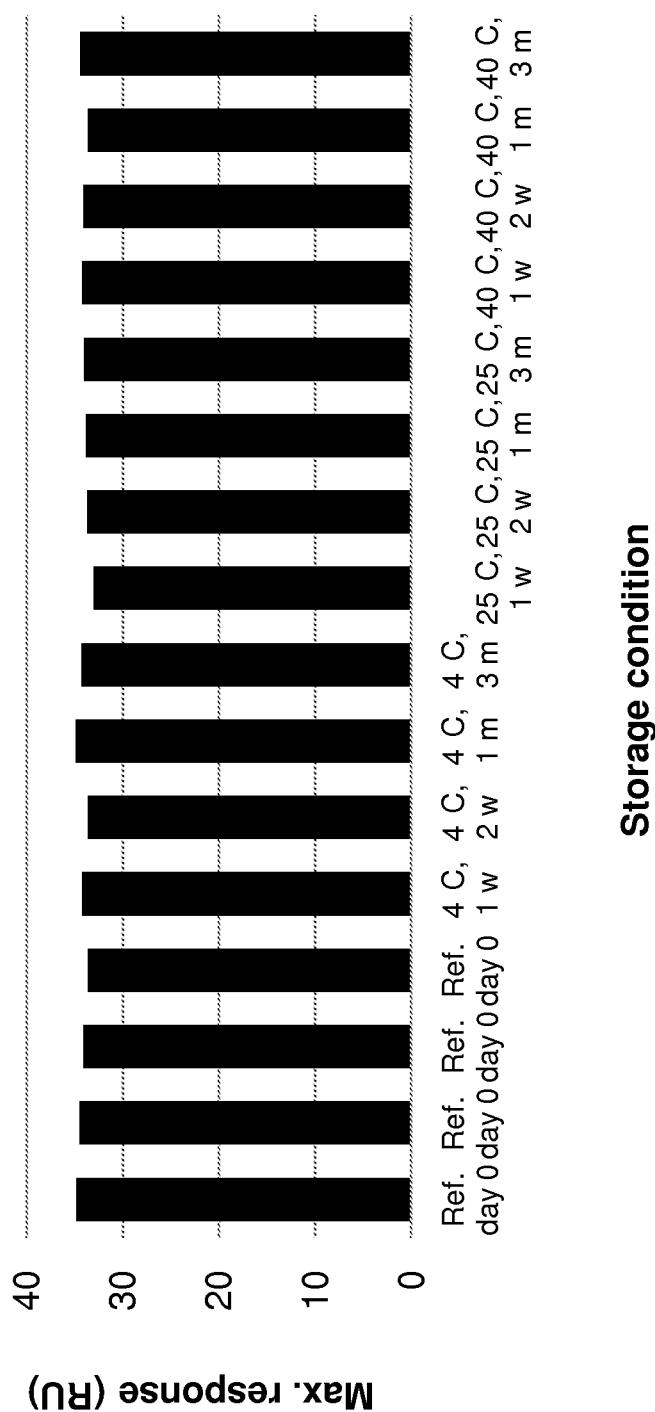


Figure 8

**Figure 9**

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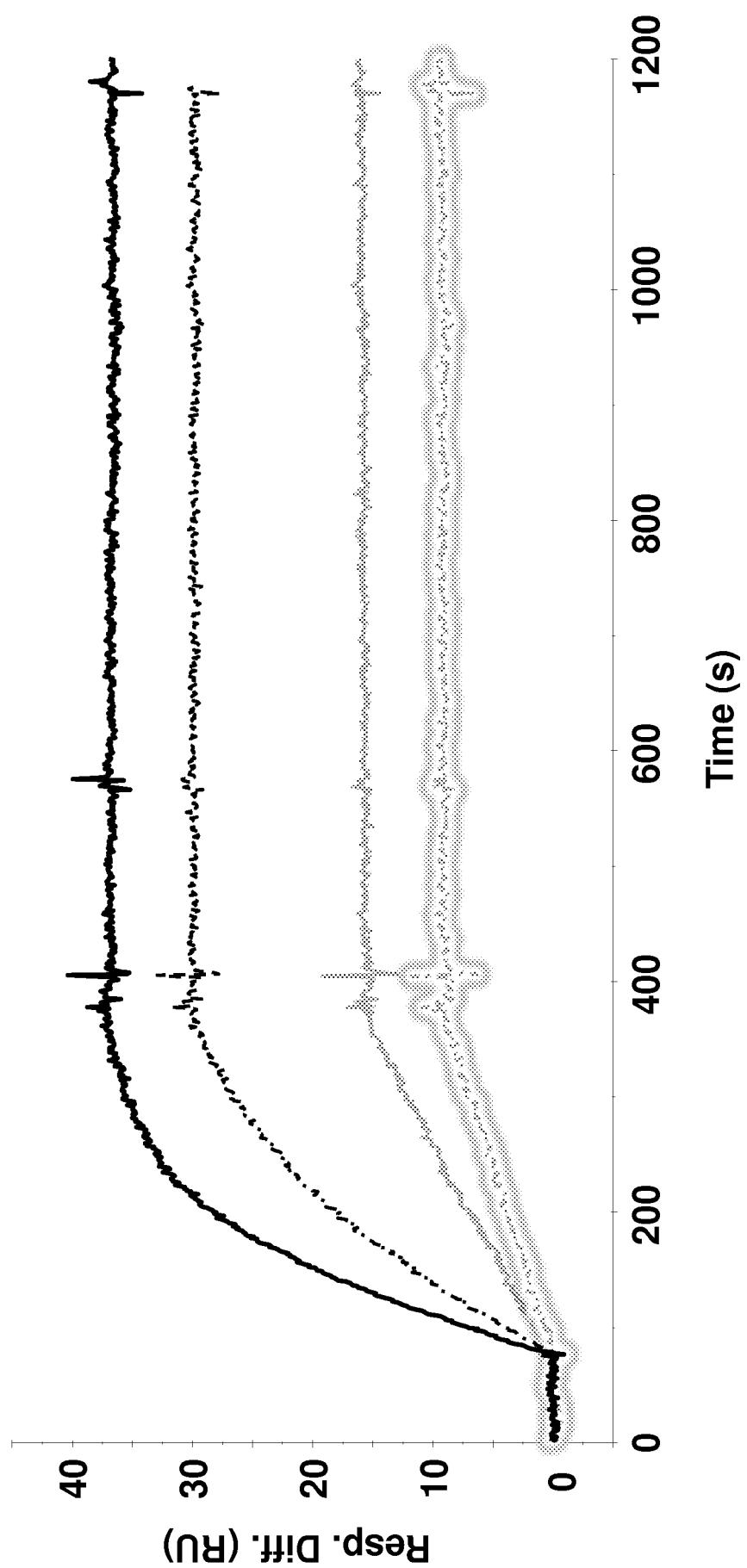
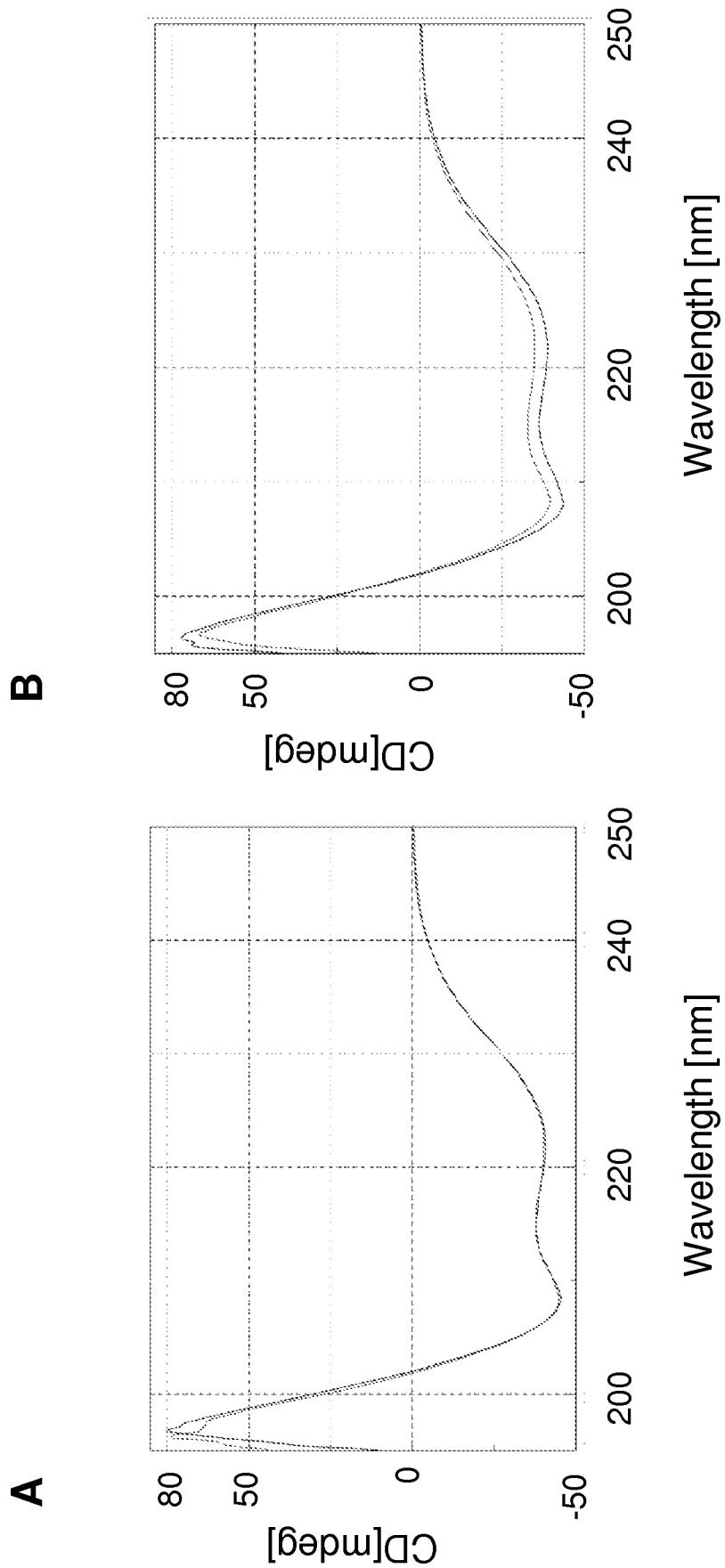
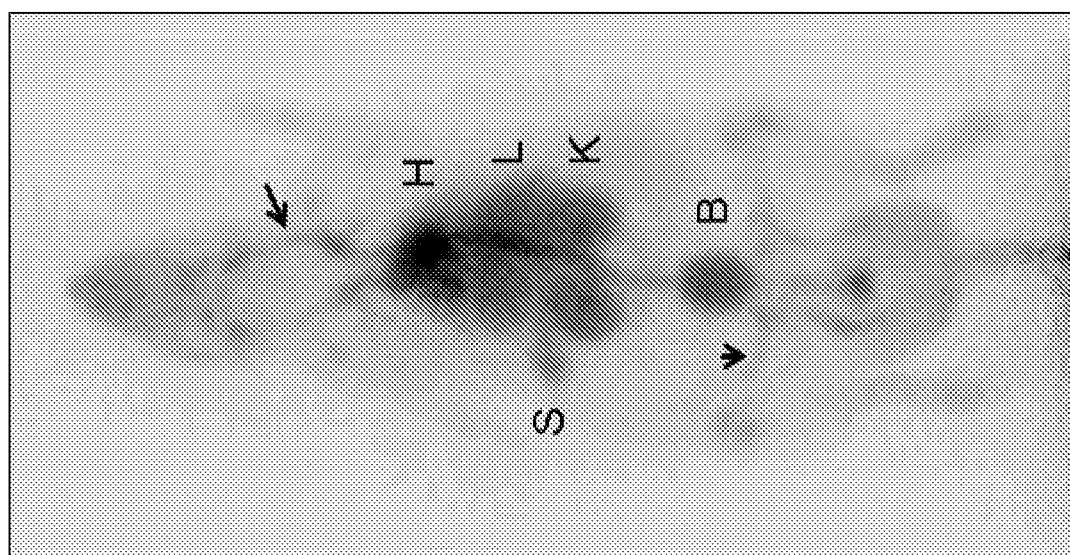


Figure 10

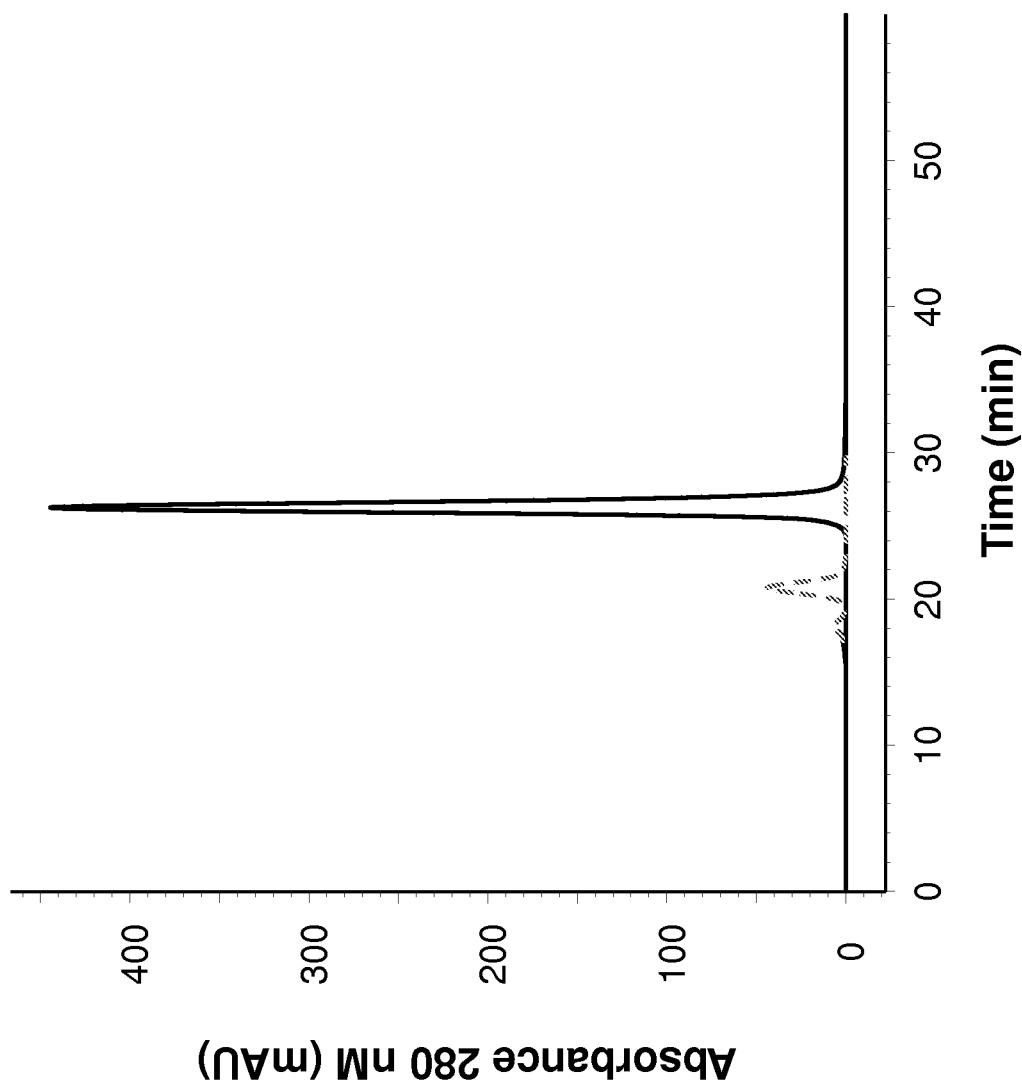
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**Figure 11**



**Figure 12**

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**Figure 13**