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(54) Title: ALBUMIN VARIANTS AND CONJUGATES

(57) Abstract: The present invention relates to conjugation-competent albumins and albumin-related polypeptides, and their conjugates with at least one moiety, and to polynucleotides encoding them.

# TITLE: ALBUMIN VARIANTS AND CONJUGATES

## Reference to sequence listing

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

## FIELD OF THE INVENTION

The present invention relates to conjugation-competent albumins and albumin-related polypeptides, and their conjugates with at least one (*e.g.* several) moiety, and to polynucleotides encoding them.

## BACKGROUND OF THE INVENTION

Serum albumins provide valuable scaffolds to which bioactive molecules may be fused, either through genetic fusions or chemical fusions to improve the properties of the fused molecule(s) (Leger, R. *et al*. (2004), Bioorg Med Chem Lett 14(17): 4395-8; Thibaudeau, K., *et al*. (2005). Bioconjug Chem 16(4): 1000-8; Balan, V. *et al*. (2006), Antivir Ther 11(1): 35-45; EP 0413622; WO 90/13653; EP 1681304; WO 1997/024445). Albumin has a long plasma half-life of about 19 days and because of this property it has been suggested for use in drug delivery.

The human serum albumin (HSA) polypeptide chain has 35 cysteine residues, which form 17 disulphide bonds and one unpaired (free) cysteine at position 34 of the mature protein (SEQ ID NO. 2). Cysteine-34 has been used for conjugation of molecules to albumin (Leger *et al*. (2004) Bioorg Med Chem Lett 14(17): 4395-8; Thibaudeau *et al*. (2005), Bioconjug Chem 16(4): 1000-8), and provides a precise, well defined site for conjugation. However, conjugation at cysteine-34 provides only one site for attachment of a single moiety and thus there is no choice of conjugation site. Also, the provision of a single conjugation site means that only one moiety can be conjugated to each albumin molecule. WO 2009/126920 and WO 2010/059315 propose the substitution for cysteine of one or more (*e.g.* several) selected surface-exposed threonine or serine residues in albumin. However, the actual production of such variants is not disclosed. WO 2010/092135 discloses albumin variants comprising three or more (several) conjugation-competent cysteine residues: cysteine-34 and at least two further cysteine residues; or variants in which another amino acid is substituted for the cysteine-34, and there are at least three further free cysteines.

Pharmaceutical agents, or their precursors, are generally prepared as homogeneous species, to allow for quality control. In HSA, the free cysteine at position 34 is located in a hydrophobic crevice with a depth of 9.5Å (Cornell CN, Chang R, Kaplan LJ. 1981. Arch. Biochem. Biophys. 209(1):1–6.), and is not thought to be involved in homodimerization of HSA. However, surface-exposed cysteine residues in polypeptides may form stable inter-molecular disulphide bridges, as occur naturally for example between the heavy and light chains of

immunoglobulin. It is desirable to provide albumin variants having introduced cysteine residues which have a low propensity to form dimers or oligomers.

WO 2000/69902 discloses conjugation of pharmaceutically beneficial compounds to HSA at cysteine-34, and it was found that the conjugates maintained the long plasma half-life of albumin. The resulting plasma half-life of the conjugate was generally considerably longer than the plasma half-life of the beneficial therapeutic compound alone. Further, albumin has been genetically fused to therapeutically beneficial peptides (WO 2001/79271A and WO 2003/59934) with the typical result that the fusion has the activity of the therapeutically beneficial peptide and a considerably longer plasma half-life than the plasma half-life of the therapeutically beneficial peptide alone.

Albumin binds *in vivo* to its receptor, the neonatal Fc receptor (FcRn) "Brambell" and this interaction is known to be important for the plasma half-life of albumin. FcRn is a membrane bound protein, expressed in many cell and tissue types. FcRn has been found to salvage albumin from intracellular degradation (Roopenian D. C. and Akilesh, S. (2007), *Nat. Rev. Immunol* **7**, 715-725.). FcRn is a bifunctional molecule that contributes to maintaining a high level of IgGs and albumin in plasma in mammals such as humans. Data indicate that IgG and albumin bind non-cooperatively to distinct sites on FcRn (Andersen *et al.* (2006), *Eur. J. Immunol* **36**, 3044-3051; Chaudhury *et al.* (2006), *Biochemistry* **45**, 4983-4990). Andersen *et al.* (2010), Journal of Biological Chemistry 285(7): 4826-36, describes the affinity of human and mouse FcRn for each of mouse and human albumin (all possible combinations). No binding of albumin from either species was observed at physiological pH to either receptor. At acidic pH, a 100-fold difference in binding affinity was observed.

The major FcRn receptor binding site in albumin is localized within Domain III (DIII, 381-585), (Andersen *et al.* (2010), Clinical Biochemistry **43**, 367–372). A number of key amino acid residues have been shown to be important in binding, notably histidines H464, H510 and H536 and lysine K500 of human albumin (Andersen *et al.* (2010), Nat. Commun. **3**:610. DOI:10.1038/ncomms1607). Generally, the higher the affinity of an albumin for FcRn, the longer is its plasma half-life. WO 2011/124718 discloses a class of variant albumins having modulated binding affinity to FcRn; the variants comprise domain III of an albumin with one or more (*e.g.* several) other domains of albumin and optionally include one or more (*e.g.* several) point mutations. WO 2012/059486 discloses variants of albumin in which a C-terminal portion of Domain III is swapped with a corresponding portion of an albumin of a different animal species. WO 2013/075066, WO 2011/103076, WO 2012/112188, WO 2011/051489 and WO 2014/072481 disclose point mutations within Domain III, or combinations of such point mutations, which alter the binding affinity of albumin to FcRn.

Various amino acid residues of albumin located in Domain I or Domain II have also recently been found to affect its interaction with FcRn. WO 2013/135896 discloses albumin

variants having one or more (*e.g.* several) alterations in Domain I and one or more (*e.g.* several) alterations in Domain III. WO 2015/036579 discloses albumin variants having one or more (*e.g.* several) alterations in Domain II.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

It is desirable to provide albumin variants having one or more (*e.g.* several) introduced cysteine residues in which an introduced free cysteine residue does not itself have a major impact on FcRn binding of albumin, or be positioned such that conjugation of a partner molecule to the free cysteine will sterically hinder FcRn binding. Such considerations could reduce the risk of unpredictable effects when introducing combinations of more than one free cysteine in a single albumin variant. Such variant polypeptides may be further modified to include alterations known to affect the binding affinity of albumin for FcRn, so as to allow the plasma half-life of the polypeptide, or conjugates thereof, to be tailored for specific applications.

## SUMMARY OF THE INVENTION

Based on an analysis of the three-dimensional structure of a human serum albumin (HSA) bound to FcRn, the inventors have designed variant polypeptides (muteins) of albumin which have one or more (*e.g.* several) conjugation-competent cysteine residues. The term 'thio-albumin' is used herein to describe an albumin variant which comprises one or more (*e.g.* several) unpaired cysteine residues, particularly an albumin variant in which one or more (*e.g.* several) of the unpaired cysteine residues does not occur in a naturally occurring variant of an albumin. Thus a thio-albumin is a 'conjugation-competent albumin'. A thio-albumin may be referred to as a 'cysteine variant of an albumin'. More particularly, the invention relates to a conjugation-competent polypeptide comprising an amino acid sequence which is at least 60% identical to human albumin, particularly residues 1 to 585 of the mature human albumin polypeptide sequence of SEQ ID NO. 2, or a fragment thereof; wherein at least one position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue; and wherein the conjugation-competent polypeptide preferably has a tendency to exist as a monomer in solution which is at least 70% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution.

More preferably, the polypeptide has a tendency to exist as a monomer in solution which is at least 75% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution and at least one position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262,

N267, Q268, L275, L284, K317, A322, E333, D340, E354, K359, A362, E382, and L398 comprises a conjugation-competent cysteine residue.

The invention also relates to a conjugation-competent polypeptide comprising an amino acid sequence as defined above, and at least one (*e.g.* several) further modification compared to SEQ ID NO. 2, such as a further modification which causes the polypeptide to have at least one (*e.g.* several) further conjugation-competent cysteine, or alters the binding affinity of the polypeptide for FcRn, or alters the plasma half-life of the polypeptide. The present invention also relates to isolated polynucleotides encoding the variants; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of producing the variants.

The invention also relates to conjugates or associates comprising the variant albumin or fragment thereof according to the invention and a beneficial therapeutic moiety or to a fusion polypeptide comprising a variant albumin or fragment thereof of the invention and a fusion partner polypeptide.

The invention further relates to compositions comprising the variant albumin, fragment thereof, fusion polypeptide comprising variant albumin or fragment thereof or conjugates comprising the variant albumin or fragment thereof, according to the invention or associates comprising the variant albumin or fragment thereof, according to the invention. The compositions are preferably pharmaceutical compositions.

The invention further relates to a pharmaceutical composition comprising a variant albumin, fragment thereof, fusion polypeptide comprising variant albumin or fragment thereof or conjugates comprising the variant albumin or fragment thereof, or associates comprising the variant albumin or fragment thereof.

The invention also relates to the use of the variants, fragments, fusion polypeptides, conjugates, associates, nanoparticles and microparticles.

The invention also relates to a method for preparing a variant albumin, fragment thereof, fusion polypeptide comprising variant albumin or fragment thereof or conjugates comprising the variant albumin or fragment thereof, or associates comprising the variant albumin or fragment thereof.

## BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Multiple alignment of amino acid sequences of (i) full length mature HSA (Hu_1_2_3), (ii) an albumin variant comprising domain I and domain III of HSA (Hu_1_3), (iii) an albumin variant comprising domain II and domain III of HSA (Hu_2_3), (iv) full-length *Macaca mulatta* albumin (Mac_mul), (v) full-length *Rattus norvegicus* albumin (Rat) and (vi) full-length *Mus musculus* albumin (Mouse). Positions 500, 550 and 573 (relative to full length HSA) are indicated by arrows.

Figure 2. Multiple alignment of amino acid sequence of mature albumin from human, sheep, mouse, rabbit and goat and immature albumins from chimpanzee ("Chimp"), macaque, hamster, guinea pig, rat, cow, horse, donkey, dog, chicken, and pig. The Start and End amino acids of domains 1, 2 and 3 (as defined by Dockal *et al* (The Journal of Biological Chemistry, 1999, Vol. 274(41): 29303–29310)) are indicated with respect to mature human albumin.

Figure 3. Venn diagram showing the classes of and relationship between twenty amino acids.

Figure 4. A: Reaction scheme for biotinylation of a protein comprising a free thiol group with maleimide-PEG2-biotin. B: Schematic illustrating potential retro-Michael and succinimide hydrolysis reactions of conjugates formed in scheme A.

In A, the maleimide forms an adduct with the thiol group, thus forming a succinimide moiety with a thio-ether bond.

B illustrates adduct formation. The adduct may revert back to maleimide and free thiol *via* a retro-Michael pathway. Alternatively, the succinimide moiety may undergo stabilizing ring opening to succinic acid, by hydrolysis at pH 9. The thio-ether bond of the conjugate is retained and the succinic acid moiety is unreactive to other thiol compounds which may be present. Free maleimide, when subjected to hydrolysis, also becomes thiol unreactive.

Figure 5. MS spectra of purified variants (A: C34A+I271C variant; B: C34A+K93C variant) conjugated with maleimide-PEG2-biotin. A: The conjugate peak is 66924.1. The shorter peak is unconjugated protein. The relative peak heights indicate a conjugated proportion of 72%. +MS, 7.7-9.2min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). B: The conjugate peak is 66908.3, and there is no free proportion, indicating 100% conjugation. +MS, 7.6-9.4min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

Figure 6. MS spectra of purified albumins (A: wild type; B: C34A+E294C variant) conjugated with maleimide-PEG2-biotin and subjected to controlled hydrolysis. In A, 53% of the albumin is present as a thiol-stable conjugate with a peak of 66978.4; and 47% is present as a free albumin following retro-Michael deconjugation. +MS, 7.0-9.6min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). In B, 100% of the C34A+E294C variant is present as a thiol-stable conjugate with a peak of 66925.7. +MS, 7.6-9.5min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

Figure 7. MS spectra of purified albumin variants (A: K93C+E294C; B: K93C+E294C; C: C34A+K93C+E294C) conjugated with maleimide-PEG2-biotin and subjected to controlled hydrolysis (B and C). In A, a single peak of 67967.7 for K93C+E294C indicates 100% conjugation to each of the three free thiols. +MS, 1.6-2.6min, Baseline subtracted (0.40), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). In B, 20% of the triple conjugate of K93C+E294C is thiol stable after hydrolysis. The main peak, at 67476.2, is indicative of two

thiol stable conjugate bonds, and the loss of one maleimide-PEG2-biotin through retro-Michael deconjugation. +MS, 1.8-2.9min, Baseline subtracted (0.40), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). In C, the double conjugate of C34A+K93C+E294C is the major species, at a peak of 67443.1, and the other species is the single conjugate at a peak of 66894.6. +MS, 1.7-2.8min, Baseline subtracted (0.40), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

Figure 8. MS spectra of purified albumin variant K93C+E294C+K573P (which includes native Cys34). A: indicates 100% conjugation to each of the three free thiols. +MS, 7.3-9.7min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). In B, 23% of the triple conjugate of K93C+E294C+K573P (which includes native Cys34) is thiol stable after hydrolysis. The main peak, at 67447.3, is indicative of two thiol stable conjugate bonds, and the loss of one maleimide-PEG2-biotin through retro-Michael deconjugation. +MS, 7.4-9.5min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

Figure 9. A: Schematic illustrating Alexa Fluor® 488-PEG4-Lys(monobromomaleimide)-NH2 dye. The MS spectra of purified albumin variants (B: K573P; C: K93C+E294C+K573P) conjugated with Alexa Fluor® 488-PEG4-Lys(monobromomaleimide)-NH2 dye are shown. In B, a single peak of 67468.5 for K573P indicates 100% conjugation to the single free thiol at Cys34. +MS, 7.6-9.7min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). In C, the triple conjugate of K93C+E294C+K573P (which includes native Cys34) is the major species, at a peak of 69535.8. The shorter peak is double conjugate. The relative peak heights indicate 58% triple conjugate and 42% double conjugate respectively. +MS, 7.6-9.3min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

Figure 10. A: Schematic illustrating 5-carboxyfluorescein-PEG4-Lys(monobromomaleimide)-NH2 dye. The MS spectra of purified albumin variants (B: K573P; C: C34A+K93C+E294C+K573P) conjugated with 5-carboxyfluorescein-PEG4-Lys(monobromomaleimide)-NH2 dye are shown. In B, a single peak of 67310.6 for K573P indicates 100% conjugation to the single free thiol at Cys34. +MS, 7.2-9.3min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). In C, the double conjugate of C34A+K93C+E294C+K573P is the major species, at a peak of 68129.7. The shorter peak is single conjugated protein. The relative peak heights indicate 91% double conjugate and 9% single conjugated protein respectively. +MS, 7.3-9.3min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

Figure 11. A: Schematic illustrating monobromomaleimide-paclitaxel. The MS spectra of purified albumin variants (B: K573P; C: K93C+E294C+K573P) conjugated with monobromomaleimide-paclitaxel are shown. In B, a peak of 67412.2 for K573P indicates conjugation to the single free thiol at Cys34. The shorter peak is unconjugated protein. The relative peak heights indicate 77% single conjugate and 23% unconjugated protein respectively +MS, 7.1-8.9min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

In C, the double conjugate of K93C+E294C+K573P is the major species which is at a peak of 68364.2. The shorter peak is triple conjugated protein. The relative peak heights indicate 60% double conjugated and 30% triple conjugate protein respectively. +MS, 7.2-9.0min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

Figure 12. A: Schematic illustrating monobromomaleimide-PEG2-exenatide peptide. The MS spectra of purified albumin variants (B: K573P; C: C34A+K93C+E294C+K573P) conjugated with monobromomaleimide-PEG2-exenatide peptide are shown. In B, a peak of 71018.7 for K573P indicates conjugation to the single free thiol at Cys34. The main peak, at 66409.2 is unconjugated protein. The relative peak heights indicate single 33% conjugate and 67% unconjugated protein respectively. +MS, 7.2-8.8min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). In C, the double conjugate of C34A+K93C+E294C+K573P is 75557.3. The main peak, at 70941.7 is single conjugate. The shortest peak at 66322.4 is unconjugated protein. The relative peak heights indicate 33% double conjugate, 45% single conjugate and 22% unconjugated protein respectively. +MS, 7.2-9.2min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

Figure 13. A: Schematic illustrating maleimide-propyl-FLAG peptide. The MS spectra of purified albumin variants (B: K573P; C: K93C+E294C+K573P) conjugated with maleimide-propyl-FLAG peptide are shown. In B, a peak of 67573.4 for K573P indicates conjugation to the single free thiol at Cys34. The main peak is unconjugated protein. The relative peak heights indicate 29% single conjugate and 71% unconjugated protein respectively. +MS, 7.3-8.7min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA). In C, the triple conjugate of K93C+E294C+K573P (which includes native Cys34) is 69850.5. The main peak, at 68685.5 is double conjugate. The peak at 67520.3 is single conjugate. The shortest peak, at 66350.2 is unconjugated protein. The relative peak heights indicate 29% triple conjugate, 50% double conjugate, 20% single conjugate and 2% unconjugated protein respectively. +MS, 7.2-8.8min, Baseline subtracted (0.50), Deconvoluted (MaxEnt), Smoothed (0.00,1,GA).

**Definitions**

**Variant:** The term “variant” means a polypeptide derived from a parent albumin by one or more (*e.g.* several) alteration(s), *i.e.* a substitution, insertion, and/or deletion, at one or more (*e.g.* several) positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an amino acid occupying a position; and an insertion means adding 1 or more (*e.g.* several), such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, preferably 1-3 amino acids immediately adjacent an amino acid occupying a position. In relation to insertion, ‘immediately adjacent’ may be to the N-side (‘upstream’) or C-side (‘downstream’) of the amino acid occupying a position (‘the named amino acid’). Therefore, for an amino acid named/numbered ‘X’, the insertion may be at position ‘X+1’ (‘downstream’) or at

position 'X-1' ('upstream').

**Mutant:** The term "mutant" means a polynucleotide encoding a variant.

**Wild-Type Albumin:** The term "wild-type" (WT) albumin means albumin having the same amino acid sequence as naturally found in an animal or in a human being.

**Parent Albumin:** The term "parent" or "parent albumin" means an albumin to which an alteration is made by the hand of man to produce the albumin variants of the invention. The parent may be a naturally occurring (wild-type) polypeptide or an allele thereof, or even a variant thereof.

**Albumin:** Albumins are proteins and constitute the most abundant protein in plasma in mammals and albumins from a long number of mammals have been characterized by biochemical methods and/or by sequence information. Several albumins, *e.g.* HSA, have also been characterized crystallographically and the structure determined (HSA: He XM, Carter DC (July 1992), "Atomic structure and chemistry of human serum albumin", Nature 358 (6383): 209–15; horse albumin: Ho, J.X. *et al.* (2001). X-ray and primary structure of horse serum albumin (*Equus caballus*) at 0.27-nm resolution. Eur J Biochem. 215(1):205-12). The invention relates to all albumins and their structures.

The term "albumin" means a protein having the same and/or very similar three dimensional (tertiary) structure as HSA or HSA domains and having similar properties to HSA or to the relevant domains. Similar three dimensional structures are for example the structures of the albumins from the species mentioned herein. Some of the major properties of albumin are i) its ability to regulate plasma volume (oncotic activity), ii) a long plasma half-life of around 19 days ± 5 days, iii) binding to FcRn, iv) ligand-binding, *e.g.* binding of endogenous molecules such as acidic, lipophilic compounds including bilirubin, fatty acids, hemin and thyroxine (see also Table 1 of Kragh-Hansen *et al.*, 2002, Biol. Pharm. Bull. 25, 695, hereby incorporated by reference), v) binding of small organic compounds with acidic or electronegative features *e.g.* drugs such as warfarin, diazepam, ibuprofen and paclitaxel (see also Table 1 of Kragh-Hansen *et al.*, 2002, Biol. Pharm. Bull. 25, 695, hereby incorporated by reference), vi) binding to gp60, also known as albondin. Not all of these properties need to be fulfilled in order to characterize a protein or fragment as an albumin. If a fragment, for example, does not comprise a domain responsible for binding of certain ligands or organic compounds the variant of such a fragment will not be expected to have these properties either.

Albumins have generally a long plasma half-life of approximately 20 days or longer, *e.g.* HSA has a plasma half-life of 19 days. It is known that the long plasma half-life of HSA is mediated *via* interaction with its receptor FcRn, however, an understanding or knowledge of the exact mechanism behind the long half-life of HSA is not essential for the invention.

As examples of albumin proteins as starting parent "backbones" for making albumin variants according to the invention can be mentioned HSA (*e.g.* AAA98797 or P02768-1, SEQ

ID NO. 2 (mature), SEQ ID NO. 3 (immature)), primate serum albumin, (such as chimpanzee serum albumin (*e.g.* predicted sequence XP_517233.2 SEQ ID NO. 4), gorilla serum albumin or macaque serum albumin (*e.g.* NP_001182578, SEQ ID NO. 5), rodent serum albumin (such as hamster serum albumin (*e.g.* A6YF56, SEQ ID NO. 6), guinea pig serum albumin (*e.g.* Q6WDN9-1, SEQ ID NO. 7), mouse serum albumin (*e.g.* AAH49971 or P07724-1 Version 3, SEQ ID NO. 8) and rat serum albumin (*e.g.* AAH85359 or P02770-1 Version 2, SEQ ID NO. 9), bovine serum albumin (*e.g.* cow serum albumin P02769-1, SEQ ID NO. 10), equine serum albumin such as horse serum albumin (*e.g.* P35747-1, SEQ ID NO. 11) or donkey serum albumin (*e.g.* Q5XLE4-1, SEQ ID NO. 12), rabbit serum albumin (*e.g.* P49065-1 Version 2, SEQ ID NO. 13), goat serum albumin (*e.g.* ACF10391, SEQ ID NO. 14), sheep serum albumin (*e.g.* P14639-1, SEQ ID NO. 15), dog serum albumin (*e.g.* P49822-1, SEQ ID NO. 16), chicken serum albumin (*e.g.* P19121-1 Version 2, SEQ ID NO. 17) and pig serum albumin (*e.g.* P08835-1 Version 2, SEQ ID NO. 18) or a polypeptide having at least 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.2, 99.4, 99.6, or at least 99.8% amino acid identity to such an albumin. Other examples of albumin, which are also included in the scope of this application, include ovalbumin (*e.g.* P01012.pro: chicken ovalbumin; O73860.pro: turkey ovalbumin). A mature albumin sequence can be identified from an immature albumin sequence using techniques known to the skilled person, for example alignment with HSA (for which the mature and immature regions are known). For example, immature HSA is 609 amino acids long in which amino acids 1 to 19 are a signal sequence (also known as a leader sequence or pre sequence), amino acids 20 to 24 are a pro sequence and amino acids 25 to 609 are the mature protein. The alignment in Figure 2 allows the skilled person to predict mature sequences for several animal albumins (see “D1 Start”).

HSA as disclosed in SEQ ID NO. 2, or any naturally occurring allele thereof, is the preferred parent albumin according to the invention. HSA is a protein consisting of 585 amino acid residues and has a molecular weight of 67 kDa. In its natural form it is not glycosylated. The skilled person will appreciate that natural alleles may exist having essentially the same properties as HSA but having one or more (*e.g.* several) amino acid changes compared to SEQ ID NO. 2, and the inventors also contemplate the use of such natural alleles as parent albumins according to the invention.

The parent albumin, a fragment thereof, or conjugation-competent albumin variant, or albumin part of a fusion polypeptide or conjugate comprising albumin or a fragment thereof according to the invention preferably has a sequence identity to the sequence of HSA shown in SEQ ID NO. 2 of at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 85%, preferably at least 86%, preferably at least 87%, preferably at least 88%, preferably at least 89%, preferably at least 90%, preferably at least 91%, preferably at least 92%, preferably at least 93%, preferably at least 94%, preferably at least 95%, more preferred at least

96%, more preferred at least 97%, more preferred at least 98% and most preferred at least 99%, at least 99.2%, at least 99.4%, at least 99.6% or at least 99.8% or 100%. It is preferred that the parent albumin maintains at least one of the major properties of albumin or a similar tertiary structure as an albumin, such as HSA. The sequence identity may be over the full-length of SEQ ID NO. 2 or over a molecule consisting or comprising of a fragment such as one or more (*e.g.* several) domains of SEQ ID NO. 2, such as a molecule consisting of or comprising Domain III (*e.g.* SEQ ID NO. 19), a molecule consisting of or comprising Domain II and Domain III (*e.g.* SEQ ID NO. 20), a molecule consisting of or comprising Domain I and Domain III (*e.g.* SEQ ID NO. 21), a molecule consisting of or comprising two copies of Domain III (*e.g.* SEQ ID NO. 22), a molecule consisting of or comprising three copies of Domain III (*e.g.* SEQ ID NO. 23) or a molecule consisting of or comprising Domain I and two copies of Domain III (*e.g.* SEQ ID NO. 24).

The parent albumin, a fragment thereof, or conjugation-competent albumin variant, or albumin part of a fusion polypeptide or conjugate comprising albumin or a fragment thereof according to the invention, when folded, may have several, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and suitably all 17, of the native disulphide bonds of the polypeptide of SEQ ID NO. 2.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO. 3 (immature sequence of HSA) or SEQ ID NO. 2 (mature sequence of HSA).

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO. 2.

The parent albumin may be encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO. 2, or (ii) the full-length complementary strand of (i) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotide of SEQ ID NO. 1 or a subsequence thereof, as well as the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 3 or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding a parent from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, *e.g.* at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, *e.g.* at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least

700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labelled for detecting the corresponding gene (for example, with $^{32}P$, $^{3}H$, $^{35}S$, biotin, or avidin). Such probes are encompassed by the invention.

A genomic DNA or cDNA library prepared from such other organisms may be screened for DNA that hybridizes with the probes described above and encodes a parent. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO. 1 or a subsequence thereof, the carrier material is used in a Southern blot.

For purposes of the invention, hybridization indicates that the polynucleotide hybridizes to a labelled nucleotide probe corresponding to the polynucleotide shown in SEQ ID NO. 1, its complementary strand, or a subsequence thereof, under low to very high stringency conditions. Molecules to which the probe hybridizes can be detected using, for example, X-ray film or any other detection means known in the art.

The nucleic acid probe may comprise or consist of the mature polypeptide coding sequence of SEQ ID NO. 1, *i.e.* nucleotides 1 to 1785 of SEQ ID NO. 1. The nucleic acid probe may comprise or consist of a polynucleotide of SEQ ID NO. 25 (nucleotide sequence encoding HSA, the nucleotide sequence has been engineered to introduce restriction enzyme sites) or a fragment thereof.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as pre-hybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/mL sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C (very low stringency), 50°C (low stringency), 55°C (medium stringency), 60°C (medium-high stringency), 65°C (high stringency), or 70°C (very high stringency).

For short probes that are about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as pre-hybridization and hybridization at about 5°C to about 10°C below the calculated $T_m$ using the calculation according to Bolton and McCarthy (1962, *Proc. Natl. Acad. Sci. USA* 48: 1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per mL following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed once in 6X SCC

plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated $T_m$.

The parent or conjugation-competent albumin may be encoded by a polynucleotide with a sequence identity to the mature polypeptide coding sequence of SEQ ID NO. 1 of at least 60%, *e.g.* at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which encodes a polypeptide which is able to function as an albumin. In an embodiment, the parent is encoded by a polynucleotide comprising or consisting of SEQ ID NO 1.

Three dimensional (3D) Models

The present disclosure makes reference to the crystal structure of HSA from the RCSB Protein Databank (PDB, which can be viewed at http://www.rcsb.org/pdb/) with the entry with PDB identity 1AO6 or 1ao6 (Sugio, S., A. Kashima, *et al.* (1999), Protein Eng **12**(6): 439-46). Compared to the mature HSA sequence (SEQ ID NO. 2), the 1AO6 structure starts at residue S5 (with the first 4 amino acids absent from the structure) and finishes at A582 of SEQ ID NO. 2 (with the last 3 amino acids absent from the structure). The amino acid positions used herein to describe positions to alter to generate conjugation-competent cysteines are referring to the positions in SEQ ID NO. 2, not 1ao6. Further structures of albumin are available to the skilled person, for example the atomic coordinates for the tertiary structure of human albumin are available at the GenBank DNA database which can be viewed at www.ncbi.nlm.nih.gov. Structures may be viewed using suitable software such as RasM.1 Chime (Sayle, TIBS 20, 374, 1995). Available albumin coordinates include:

**1AO6**, **1BM0** (Sugio *et al.* (1999), Protein Eng **12**(6): 439-46), which was among the top 17 requested proteins.

**1UOR**, He & Carter (1992), Nature **358**(6383): 209-15.

**1bj5** ***and*** **1bke**, Curry *et al.* (1998), Nat Struct Biol **5**(9): 827-35.

**1e7a**,**1e7b, 1e7c,** Bhattacharya *et al.* (2000), J Biol Chem **275**(49): 38731-8.

**1e7e**, **1e7f**, **1e7g**, **1e7h** and **1e7i**, Bhattacharya *et al.* (2000), J Mol Biol **303**(5): 721-32.

**1GNJ**, Petitpas *et al.* (2001), J Mol Biol **314**(5): 955-60.

**1HA2** and **1H9Z** Petitpas *et al.* (2001), J Biol Chem **276**(25): 22804-9.

**4K71**, Schmidt *et al.* (2013),. Structure **21**:1966-1978

**4N0F** and **4N0U**, Oganesyan *et al.* (2014), J Biol Chem **289**(11):7812-24.

**Albumin moiety:** The albumin part of a fusion polypeptide, conjugate, associate, nanoparticle or composition comprising the albumin variant or fragment thereof according to the invention, may be referred to as an 'albumin moiety' or 'albumin component'. A polypeptide according to the invention may comprise or consist of an albumin moiety.

**Isolated variant:** The term "isolated variant" means a variant in a form or environment which does not occur in nature. Non-limiting examples of isolated variants include (1) any non-naturally occurring variant; (2) any variant that is at least partially removed from one or more (*e.g.* several) or all of the naturally occurring constituents with which it is associated in nature; (3) any variant modified by the hand of man relative to the polypeptide from which it is derived (*e.g.* the polypeptide from which it is derived as found in nature); or (4) any variant modified by increasing the amount of the variant relative to other components with which it is naturally associated (*e.g.* multiple copies of a gene encoding the substance; use of a stronger promoter than the promoter naturally associated with the gene encoding the substance). An isolated variant may be present in a fermentation broth sample. Isolated variants may be recombinant or synthetic.

**Substantially pure variant:** The term "substantially pure variant" means a preparation that contains at most 10%, at most 8%, at most 6%, at most 5%, at most 4%, at most 3%, at most 2%, at most 1%, and at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. Preferably, the variant is at least 92% pure, *e.g.* at least 94% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99%, at least 99.5% pure, and 100% pure by weight of the total polypeptide material present in the preparation. Purity may be determined by SDS-PAGE or GP-HPLC. The variants of the invention are preferably in a substantially pure form. This can be accomplished, for example, by preparing the variant by well-known recombinant methods and by purification methods.

**Mature polypeptide:** The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, *etc*. The mature polypeptide may be amino acids 1 to 585 of SEQ ID NO. 2, *e.g.* with the inclusion of alterations according to the invention and/or any post-translational modifications.

**Mature polypeptide coding sequence:** The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature albumin polypeptide. The mature polypeptide coding sequence may be nucleotides 1 to 1758 of SEQ ID NO. 1 *e.g.* with the alterations required to encode a variant according to the invention.

**Sequence Identity:** The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 3.0.0 or later, more preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the

EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labelled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 3.0.0 or later, more preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labelled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

**Fragment:** The term "fragment" as used herein includes any fragment of full-length albumin or a variant thereof, so long as at least one (*e.g.* several) basic property, for example binding activity (type of and specific activity *e.g.* binding to bilirubin), osmolarity (oncotic pressure, colloid osmotic pressure), behaviour in a certain pH-range (pH-stability) has not significantly been changed. "Significantly" in this context means that one skilled in the art would say that the properties of the variant may still be different but would not be unobvious over the ones of the original protein. A fragment may consist of one uninterrupted sequence derived from HSA or it may comprise two or more (*e.g.* several) sequences derived from HSA. The fragments according to the invention have a size of more than approximately 20 amino acid residues, preferably more than 30 amino acid residues, more preferred more than 40 amino acid residues, more preferred more than 50 amino acid residues, more preferred more than 75 amino acid residues, more preferred more than 100 amino acid residues, more preferred more than 200 amino acid residues, more preferred more than 300 amino acid residues, even more preferred more than 400 amino acid residues and most preferred more than 500 amino acid residues. A fragment may comprise or consist of at least 50, 60, 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% of an albumin or of a domain of an albumin. Preferred albumin domains of the invention are domains having at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.5% or 100% identity to HSA domain I consisting of amino acid residues 1 to 194 ± 1 to 15 amino acids of SEQ ID NO. 2; at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.5% or 100% identity to HSA domain II consisting of amino acid residues 192 to 387 ± 1 to 15 amino acids of SEQ ID NO. 2 and at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.5% or 100% identity to HSA domain III consisting of amino acid residues 381 to 585 ± 1 to 15 amino acids of SEQ ID NO. 2.

Domains I, II and III may be defined with reference to HSA (SEQ ID NO. 2). For example, HSA Domain I may consist of or comprise amino acids 1 to 194 (± 1 to 15 amino acids) of SEQ ID NO. 2, HSA Domain II may consist of or comprise amino acids 192 (± 1 to 15 amino acids) to 387 (± 1 to 15 amino acids) of SEQ ID NO. 2 and Domain III may consist of or comprise amino acid residues 381 (± 1 to 15 amino acids) to 585 (± 1 to 15 amino acids) of SEQ ID NO. 2. "± 1 to 15 amino acids" means that the residue number may deviate by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids to the C-terminus and/or to the N-terminus of the stated amino acid position. Examples of domains I, II and III are described by Dockal *et al.* (The Journal of Biological Chemistry, 1999, Vol. 274(41): 29303–29310) and Kjeldsen *et al.* (Protein Expression and Purification, 1998, Vol 13: 163–169) and are tabulated below.

**Table 1**

| **Amino acid residues of HSA domains I, II and III with reference to SEQ ID NO. 2** | **Dockal *et al*** | **Kjeldsen *et al*** |
|---|---|---|
| Domain I | 1 to 197 | 1 to 192 |
| Domain II | 189 to 385 | 193 to 382 |
| Domain III | 381 to 585 | 383 to 585 |

A fragment may comprise or consist of one or more (*e.g.* several) domains of albumin described herein such as DI + DII, DI + DIII, DII + DIII, DIII + DIII, DI + DIII + DIII, DIII + DIII + DIII, or fragments of such domains or combinations of domains.

The skilled person can identify domains I, II and III in non-human albumins by amino acid sequence alignment with HSA, for example using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 3.0.0 or later, more preferably version 5.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. Other suitable software includes MUSCLE ((Multiple sequence comparison by log-expectation, Robert C. Edgar, Version 3.6, http://www.drive5.com/muscle; Edgar (2004) Nucleic Acids Research 32(5), 1792-97 and Edgar (2004) BMC Bioinformatics, 5(1):113) which may be used with the default settings as described in the User Guide (Version 3.6, September 2005). Versions of MUSCLE later than 3.6 may also be used for any aspect of the invention). Examples of suitable alignments are provided in Figures 1 and 2.

It is preferred that domains have at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.5% identity or 100% identity to Domain I, II or III of HSA (SEQ ID NO. 2).

Additionally, single or multiple heterologous fusions comprising any of the above; or single or multiple heterologous fusions to albumin, or a variant or fragment of any of these may be used. Such fusions include albumin N-terminal fusions, albumin C-terminal fusions and co-N-terminal and C-terminal albumin fusions as exemplified by WO 01/79271 (incorporated herein by reference).

**Equivalent amino acid positions:** Throughout this specification amino acid positions are defined in relation to full-length mature HSA (*i.e.* without leader sequence, SEQ ID NO. 2). However, the skilled person understands that the invention also relates to variants of non-human albumins (*e.g.* those disclosed herein) and/or fragments of a human or non-human albumin. For clarity, for albumins other than HSA (SEQ ID NO. 2), equivalent residues are favoured for mutation. Equivalent positions can be identified in fragments of HSA, in animal albumins and in fragments, fusions and other derivatives or variants thereof by comparing amino acid sequences using pairwise (*e.g.* ClustalW) or multiple (*e.g.* MUSCLE) alignments. For example, Figure 1 shows that positions equivalent to 500, 550 and 573 in full length HSA are easily identified in fragments of HSA and in albumins of other species. Positions 500, 550 and 573 are indicated by arrows. Further details are provided in Table 2 below.

**Table 2: Example of identification of equivalent positions in HSA, animal albumins and albumin fragments**

| **Organism (accession number of protein)** | **Albumin** | | | **Position equivalent to HSA (native amino acid):** | | |
|---|---|---|---|---|---|---|
| | **Full length or fragment** | **Fragment details** | **Total length of mature protein** | **500 (K)** | **550 (D)** | **573 (K)** |
| *Homo sapiens* (AAA98797) | Full length | - | 585 | 500 (K) | 550 (D) | 573 (K) |
| *Homo sapiens* | Fragment | DI, DIII | 399 | 314 (K) | 364 (D) | 387 (K) |
| *Homo sapiens* | Fragment | DI, DIII | 403 | 318 (K) | 368 (D) | 391 (K) |
| *Macaca mulatta* (NP_001182578) | Full length | - | 584 | 500 (K) | 550 (N) | 573 (P) |
| *Rattus* | Full length | - | 584 | 500 (K) | 550 (D) | 573 (P) |

| Organism (accession number of protein) | Albumin | | | Position equivalent to HSA (native amino acid): | | |
|---|---|---|---|---|---|---|
| | Full length or fragment | Fragment details | Total length of mature protein | 500 (K) | 550 (D) | 573 (K) |
| *norvegicus* (AAH85359) | | | | | | |
| *Mus musculus* (AAH49971) | Full length | - | 584 | 500 (K) | 550 (D) | 573 (P) |

Figure 1 was generated by MUSCLE using the default parameters including output in ClustalW 1.81 format. The raw output data was shaded using BoxShade 3.21 (which can be accessed at http://www.ch.embnet.org/software/BOX_form.html) using Output Format: RTF_new; Font Size: 10; Consensus Line: no consensus line; Fraction of sequences (that must agree for shading): 0.5; Input sequence format: ALN. Therefore, throughout this specification amino acid positions defined in HSA also apply to equivalent positions in fragments, derivatives or variants and fusions of HSA, albumins from other species and fragments and fusions thereof. Such equivalent positions may have (i) a different residue number in its native protein and/or (ii) a different native amino acid in its native protein. Likewise, Figure 2 shows that equivalent positions can be identified in fragments (*e.g.* domains) of an albumin with reference to SEQ ID NO. 2 (HSA).

**Conservative substitution:** As used herein, the term "conservative" amino acid substitutions refers to substitutions made within the same group, and which typically do not substantially affect protein function. By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such variants may be made by techniques well known in the art, such as by site-directed mutagenesis as disclosed in US Patent No 4,302,386 issued 24 November 1981 to Stevens, incorporated herein by reference.

In one embodiment, the Venn diagram of Figure 3 may be used to determine conservative amino acid substitutions: Using Figure 3, a conservation mutation score (ranging from 0 to 5) may be calculated. A score of 0 is the highest conservation, which, for cysteine, is only assigned for substitution of a cysteine residue with another cysteine residue. For changes from any other amino acid to a cysteine (or for a cysteine to any other amino acid), the score may be 1, 2, 3, 4, 5. A score of 1 is a more conservative substitution than a score of 2, 3, 4 or 5. A score of 5 is assigned to the lowest conservation between a substituted amino acid and

the cysteine. The score of 0 to 5 is calculated from Figure 3 as the number of boundaries (*i.e.* lines) crossed to go from cysteine to the appropriate amino acid. Thus the score for cysteine is 0 as no boundaries are crossed. Likewise, the score of aspartic acid (D) is 3, since 3 boundaries are crossed. The conservation mutation score (with respect to Figure 3) for the 20 different amino acids are defined as (using one-letter codes for the amino acids): A=1, C=0, D=3, E=4, F=4, G=2, H=5, I=4, K=4, L=4, M=3, N=2, P=3, Q=3, R=5, S=1, T=1, V=3, W=3, Y=3.

Alternatively, or in addition, "conservative" amino acid substitutions refers to substitutions made within the same group such as within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

For example, a conservative substitution of alanine-2 in SEQ ID NO. 2 can include glycine or serine. Non-conservative substitutions encompass substitutions of amino acids in one group by amino acids in another group. For example, a non-conservative substitution could include the substitution of a polar amino acid for a hydrophobic amino acid.

**Conventions for Designation of Variants**

For purposes of the present invention, the mature polypeptide disclosed in SEQ ID NO. 2 is used to determine the corresponding amino acid residue in another albumin. The amino acid sequence of another albumin is aligned with the mature polypeptide disclosed in SEQ ID NO. 2, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the mature polypeptide disclosed in SEQ ID NO. 2 is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* **48**: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* **16**: 276-277), preferably version 3.0.0 or later, more preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix.

Identification of the corresponding amino acid residue in another albumin can be determined or confirmed by an alignment of multiple polypeptide sequences using several computer programs including, but not limited to, MUSCLE (multiple sequence comparison by log-expectation; version 3.5 or later; Edgar, 2004, *Nucleic Acids Research* 32: 1792-1797), MAFFT (version 6.857 or later; Katoh and Kuma, 2002, *Nucleic Acids Research* 30: 3059-3066; Katoh *et al.*, 2005, *Nucleic Acids Research* 33: 511-518; Katoh and Toh, 2007, *Bioinformatics* 23: 372-374; Katoh *et al.*, 2009, *Methods in Molecular Biology* 537: 39-64; Katoh and Toh,

2010, *Bioinformatics* 26: 1899-1900), and EMBOSS EMMA employing ClustalW (1.83 or later; Thompson *et al.*, 1994, *Nucleic Acids Research* 22: 4673-4680), using their respective default parameters.

When the other polypeptide (or protein) has diverged from the mature polypeptide of SEQ ID NO. 2 such that traditional sequence-based comparison fails to detect their relationship (Lindahl and Elofsson, 2000, *J. Mol. Biol.* **295**: 613-615), other pairwise sequence comparison algorithms can be used. Greater sensitivity in sequence-based searching can be attained using search programs that utilize probabilistic representations of polypeptide families (profiles) to search databases. For example, the PSI-BLAST program generates profiles through an iterative database search process and is capable of detecting remote homologs (Altschul *et al.*, 1997, *Nucleic Acids Res.* **25**: 3389-3402). Even greater sensitivity can be achieved if the family or superfamily for the polypeptide has one or more (*e.g.* several) representatives in the protein structure databases. Programs such as GenTHREADER (Jones, 1999, *J. Mol. Biol.* **287**: 797-815; McGuffin and Jones, 2003, *Bioinformatics* **19**: 874-881) utilize information from a variety of sources (PSI-BLAST, secondary structure prediction, structural alignment profiles, and solvation potentials) as input to a neural network that predicts the structural fold for a query sequence. Similarly, the method of Gough *et al.*, 2000, *J. Mol. Biol.* **313**: 903-919, can be used to align a sequence of unknown structure with the superfamily models present in the SCOP database. These alignments can in turn be used to generate homology models for the polypeptide, and such models can be assessed for accuracy using a variety of tools developed for that purpose.

For proteins of known structure, several tools and resources are available for retrieving and generating structural alignments. For example the SCOP superfamilies of proteins have been structurally aligned, and those alignments are accessible and downloadable. Two or more (*e.g.* several) protein structures can be aligned using a variety of algorithms such as the distance alignment matrix (Holm and Sander, 1998, *Proteins* **33**: 88-96) or combinatorial extension (Shindyalov and Bourne, 1998, *Protein Engineering* **11**: 739-747), and implementation of these algorithms can additionally be utilized to query structure databases with a structure of interest in order to discover possible structural homologs (*e.g.* Holm and Park, 2000, *Bioinformatics* **16**: 566-567).

In describing the albumin variants of the present invention, the nomenclature described below is adapted for ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviation is employed. The term 'point mutation' and/or 'alteration' includes deletions, insertions and substitutions.

Substitutions. For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine at position 226 with alanine is designated as "Thr326Ala" or "T326A". Multiple mutations (or alterations) are separated by addition marks ("+"), *e.g.* "Gly205Arg + Ser411Phe" or "G205R +

S411F", representing substitutions at positions 205 and 411 of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively. The Figures also use ("/"), *e.g.* "E492T/N503D" this should be viewed as interchangeable with ("+").

Deletions. For an amino acid deletion, the following nomenclature is used: Original amino acid, position*. Accordingly, the deletion of glycine at position 195 is designated as "Gly195*" or "G195*". Multiple deletions are separated by addition marks ("+"), *e.g.* "Gly195* + Ser411*" or "G195* + S411*".

Insertions. As disclosed above, an insertion may be to the N-side ('upstream', 'X-1') or C-side ('downstream', 'X+1') of the amino acid occupying a position ('the named (or original) amino acid', 'X').

For an amino acid insertion to the C-side ('downstream', 'X+1') of the original amino acid ('X'), the following nomenclature is used: Original amino acid, position, original amino acid, inserted amino acid. Accordingly the insertion of lysine after glycine at position 195 is designated "Gly195GlyLys" or "G195GK". An insertion of multiple amino acids is designated [Original amino acid, position, original amino acid, inserted amino acid #1, inserted amino acid #2; *etc.*]. For example, the insertion of lysine and alanine after glycine at position 195 is indicated as "Gly195GlyLysAla" or "G195GKA".

In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example, the sequence would thus be:

| Parent: | Variant: |
|---|---|
| 195 | 195 195a 195b |
| G | G - K - A |

For an amino acid insertion to the N-side ('upstream', 'X-1') of the original amino acid (X), the following nomenclature is used: Original amino acid, position, inserted amino acid, original amino acid. Accordingly the insertion of lysine (K) before glycine (G) at position 195 is designated "Gly195LysGly" or "G195KG". An insertion of multiple amino acids is designated [Original amino acid, position, inserted amino acid #1, inserted amino acid #2; *etc.*, original amino acid]. For example, the insertion of lysine (K) and alanine (A) before glycine at position 195 is indicated as "Gly195LysAlaGly" or "G195KAG". In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters with 'prime' to the position number of the amino acid residue following the inserted amino acid residue(s). In the above example, the sequence would thus be:

| Parent: | Variant: |
|---|---|
| 195 | 195a' 195b' 195 |
| G | K - A - G |

Multiple alterations. Variants comprising multiple alterations are separated by addition marks ("+"), *e.g.* "Arg170Tyr+Gly195Glu" or "R170Y+G195E" representing a substitution of arginine and glycine at positions 170 and 195 tyrosine and glutamic acid, respectively.

Different alterations. Where different alterations can be introduced at a position, the different alterations are separated by a comma, *e.g.* "Arg170Tyr,Glu" represents a substitution of arginine at position 170 with tyrosine or glutamic acid. Thus, "Tyr167Gly,Ala + Arg170Gly,Ala" designates the following variants: "Tyr167Gly+Arg170Gly", "Tyr167Gly+Arg170Ala", "Tyr167Ala+Arg170Gly", and "Tyr167Ala+Arg170Ala".

**Conjugation competence:** A conjugation-competent cysteine is a cysteine residue which is capable of forming an intermolecular bond with a conjugation partner, particularly a conjugation partner that is not an albumin. A conjugation-competent polypeptide, *i.e.* thio-albumin, is capable of forming an intermolecular bond with a conjugation partner by virtue of the conjugation-competent cysteine residue. The thio-albumin may or may not have a high level of conjugation competence, for example at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99 or 100% relative to the conjugation competence of an albumin consisting of SEQ ID NO. 2 having only one conjugation competent cysteine at Cys-34. Conjugation competence may be determined relative to any conjugatable molecule (conjugation partner) of interest, for example a bioactive molecule or a fluorescent dye. Determination may be through mass spectrometry (MS) analysis or quantification of the activity of the bioactive compound such as its fluorescence. Conjugation competence of albumin and biotin or HRP may be determined by assaying the mass of the resultant conjugate and/or the enzyme activity of the conjugated compound. Determination by fluorescent labelling and cellular uptake is described by McGraw *et al.*, (1987), *The Journal of Cell Biology*, 105, 207-214; and Presley *et al.*, (1993), *The Journal of Cell Biology*, 122, 1231-1241. An advantage of a thio-albumin having a high conjugation competence is that it may allow efficient conjugation of molecules to the thio-albumin. Conjugation competence may be measured with respect to time. Favoured thio-albumins may be (a) those which achieve maximal conjugation quickly or (b) slowly. The conjugation competence of a specific cysteine may be determined by methods known to those skilled in the art – for example, the protein may be digested post-conjugation and peptide mapping performed to determine the degree of conjugation at the specific cysteine.

A **bioactive agent** or **bioactive compound** is one which has the ability to interact with a living organism, system or cell. It may, for example, be a biological or chemical agent or

compound.

**Ligand binding**: The ligand binding properties of albumin include binding to anionic and neutral ligands such as long-chain fatty acids, bilirubin and other miscellaneous ligands. The long-chain fatty acids, oleic (C18:1), palmitic (C16:0), linoleic (C18:2), stearic (C18:0), arachidonic (C20:4) and palmitoleic (C16:1) are known to bind HSA. Ligand binding studies can be performed on HSA and thio-albumins using an isothermal titration calorimetry method that had been suitably qualified for this purpose. Samples can be pre-treated by defatting (Sogami, M. and J. F. Foster (1968). Biochemistry 7(6): 2172-82, incorporated herein by reference) followed by thiol blocking (Sogami, M., H. A. Petersen, *et al.* (1969). Biochemistry 8(1): 49-58, incorporated herein by reference) and subsequent gel permeation chromatography. The binding curves generated for thio-albumins and HSA with octanoate, for example, may subsequently be compared, and functional similarity established. Conjugated- and/or non-conjugated thio-albumin may have at least 5%, 10%, 15%, 20%, 30%, 40% or 50%, 60%, 70%, at least 80%, 90%, 95%, 100%, 105% or more of HSA's receptor binding activity, mole for mole, to bilirubin and/or a fatty acid.

**FcRn and shFcRn:** The term “FcRn” means the neonatal Fc receptor (FcRn), particularly the human neonatal Fc receptor. shFcRn is a soluble recombinant form of FcRn. shFcRn is a heterodimer of SEQ ID NO. 26 (truncated heavy chain of the major histocompatibility complex class I-like Fc receptor (FCGRT)) and SEQ ID NO. 27 (beta-2-microglobulin). Together, SEQ ID NO. 26 and 27 form hFcRn.

The conjugated- and/or non-conjugated thio-albumin may or may not have an altered binding affinity to FcRn.

The thio-albumin or conjugate thereof may have a binding to FcRn that is stronger or weaker (and, preferably, is stronger) than that of the parent albumin or conjugate thereof.

The thio-albumin or conjugate thereof may have a KD to FcRn (*e.g.* shFcRn) that is lower than the corresponding KD for HSA or conjugate thereof to. Preferably, the KD for the thio-albumin or conjugate is less than 0.9X KD for HSA to FcRn, more preferred less than 0.5X KD for HSA to FcRn, more preferred less than 0.1X KD for HSA to FcRn, even more preferred less than 0.05X KD for HSA to FcRn, even more preferred less than 0.02X KD for HSA to FcRn, even more preferred less than 0.01X KD for HSA to FcRn and most preferred less than 0.001X KD for HSA to FcRn (where X means ‘multiplied by’).

For a conjugate comprising a thio-albumin, preferably the KD for the conjugate is less than 0.9X KD for the corresponding conjugate comprising HSA to FcRn, more preferred less than 0.5X KD for the corresponding conjugate to FcRn, more preferred less than 0.1X KD for the corresponding conjugate to FcRn, even more preferred less than 0.05X KD for the corresponding conjugate to FcRn, even more preferred less than 0.02X KD for the corresponding conjugate to FcRn, even more preferred less than 0.01X KD for the

corresponding conjugate to FcRn and most preferred less than 0.001X KD for the corresponding conjugate to FcRn (where X means 'multiplied by'). 'Corresponding conjugate' means a conjugate comprising HSA (*e.g.* SEQ ID NO. 2) instead of the thio-albumin (*i.e.* albumin variant).

The thio-albumin or conjugate thereof may have a KD to FcRn that is higher than the corresponding KD for HSA or conjugate thereof to FcRn. Preferably, the KD for the thio-albumin or conjugate is more than 2X KD for HSA to FcRn, more preferred more than 5X KD for HSA to FcRn, more preferred more than 10X KD for HSA to FcRn, even more preferred more than 25X KD for HSA to FcRn, most preferred more than 50X KD for HSA to FcRn. The thio-albumin or conjugate may be a null binder to FcRn.

For a conjugate comprising a thio-albumin, prefererably the KD for the conjugate, Preferably, the KD for the corresponding conjugate comprising HSA is more than 2X KD for the corresponding conjugate to FcRn, more preferred more than 5X KD for the corresponding conjugate to FcRn, more preferred more than 10X KD for the corresponding conjugate to FcRn, even more preferred more than 25X KD for the corresponding conjugate to FcRn, most preferred more than 50X KD for the corresponding conjugate to FcRn. Corresponding conjugate' means a conjugate comprising HSA (*e.g.* SEQ ID NO. 2) instead of the thio-albumin (*i.e.* albumin variant).

When determining and/or comparing KD, one or more (*e.g.* several) (and preferably all) of the following parameters may be used:

Instrument: Biacore 3000 instrument (GE Healthcare)

Flow cell: CM5 sensor chip

FcRn: human FcRn, preferably soluble human FcRn, optionally coupled to a tag such as Glutathione S Transferase (GST) or Histidine (His), most preferably His such as 6 histidine residues at the C-terminus of the beta-2-microglobulin.

Quantity of FcRn: 1200-2500 RU

Coupling chemistry: amine coupling chemistry (*e.g.* as described in the protocol provided by the manufacturer of the instrument).

Coupling method: The coupling may be performed by injecting 20 µg/mL of the protein in 10 mM sodium acetate pH 5.0 (GE Healthcare). Phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005% Tween 20) at pH 5.5 may be used as running buffer and dilution buffer. Regeneration of the surfaces may be done using injections of HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) at pH 7.4 (Biacore AB).

Quantity of injection of test molecule (*e.g.* HSA or variant) 20-0.032µM

Flow rate of injection: constant, *e.g.* 30 µL/mL

Temperature of injection: 25 °C

Data evaluation software: BIAevaluation 4.1 software (BIAcore AB).

**Plasma half-life:** Plasma half-life is ideally determined *in vivo* in suitable individuals. However, since it is time consuming and expensive and inevitably there are ethical concerns connected with doing experiments in animals or man, it is desirable to use an *in vitro* assay for determining whether plasma half-life is extended or reduced. It is known that the binding of albumin to its receptor (FcRn) is important for plasma half-life and the correlation between receptor binding and plasma half-life is that a higher affinity of albumin to its receptor leads to longer plasma half-life. Thus for the invention a higher affinity of albumin to FcRn is considered indicative of an increased plasma half-life and a lower affinity of albumin to its receptor is considered indicative of a reduced plasma half-life.

The binding of albumin to its receptor FcRn may be described using the term affinity and the expressions “stronger” or “weaker”. Thus, it should be understood that a molecule having a higher affinity to FcRn than HSA is considered to bind more strongly to FcRn than HSA and a molecule having a lower affinity to FcRn than HSA is considered to bind more weakly to FcRn than HSA. The term ‘binding coefficient’ can be used instead of the term ‘binding affinity’.

The terms “longer plasma half-life” or “shorter plasma half-life” and similar expressions are understood to be in relationship to the corresponding parent or reference or corresponding albumin molecule. Thus, a longer plasma half-life with respect to a variant albumin of the invention means that the variant has longer plasma half-life than that of the corresponding albumin having the same sequences except for the alteration(s) described herein.

**Reference:** a reference is an albumin, fusion, conjugate, composition, associate, nanoparticle or microparticle to which an albumin variant, fusion, conjugate, composition, associate, nanoparticle or microparticle is compared. The reference may comprise or consist of full length albumin (such as HSA or a natural allele thereof) or a fragment thereof. A reference may also be referred to as a ‘corresponding’ albumin, fusion, conjugate, composition, associate or nanoparticle to which an albumin variant, fusion, conjugate, composition, associate or nanoparticle is compared. A reference may comprise or consist of HSA (SEQ ID NO. 2) or a fragment, fusion, conjugate, associate, nanoparticle or microparticle thereof. Preferably, the reference is identical to the polypeptide, fusion polypeptide, conjugate, composition, associate, nanoparticle or microparticle according to the invention (“being studied”) with the exception of the albumin moiety. Preferably the albumin moiety of the reference comprises or consists of an albumin (*e.g.* HSA, SEQ ID NO. 2) or a fragment thereof. The amino acid sequence of the albumin moiety of the reference may be longer than, shorter than or, preferably, the same (± 1 to 15 amino acids) length as the amino sequence of the albumin moiety of the polypeptide, fusion polypeptide, conjugate, composition, associate, nanoparticle or microparticle according to the invention (“being studied”).

**Allelic variant:** The term "allelic variant" means any of two or more (several) alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene. Polymorphisms known for HSA (SEQ ID NO. 2) are discussed in Minchiotti *et al.* (2008). Hum Mutat 29(8): 1007-16 and at http://www.uniprot.org/uniprot/P02768.

**Coding sequence:** The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of its translated polypeptide product. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG and ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a DNA, cDNA, synthetic, or recombinant polynucleotide.

**cDNA:** The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

**Nucleic acid construct:** The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic. The term nucleic acid construct is synonymous with the term "expression cassette" when the nucleic acid construct contains the control sequences required for expression of a coding sequence of the invention.

**Control sequences:** The term "control sequences" means all nucleic acid sequences necessary for the expression of a polynucleotide encoding a variant of the invention. Each control sequence may be native (*i.e.* from the same gene) or foreign (*i.e.* from a different gene) to the polynucleotide encoding the variant or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a variant.

**Operably linked:** The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs the expression of the coding sequence.

**Expression:** The term “expression” includes any step involved in the production of the variant including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion. The thio-albumin may or may not be capable of being expressed at a level of at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% relative to the expression of an unmodified albumin (such as SEQ ID NO. 2) from a suitable expression system, such as yeast (*e.g. Saccharomyces*, *e.g. S. cerevisiae*) or an Aspergillus. Relative expression levels can be determined, for example, by expression of the protein followed by quantification by SDS-PAGE, GP-HPLC or Western Blotting.

**Expression vector:** The term “expression vector” means a linear or circular DNA molecule that comprises a polynucleotide encoding a variant and is operably linked to control sequences that provide for its expression.

**Host cell:** The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term “host cell” encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

## DETAILED DESCRIPTION OF THE INVENTION

### Conjugation-competent polypeptides I

A first aspect of the invention provides a conjugation-competent polypeptide comprising an amino acid sequence which is at least 60% identical to human albumin, particularly residues 1 to 585 of the mature human albumin polypeptide sequence of SEQ ID NO. 2, or a fragment thereof;

wherein at least one (*e.g.* several) position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue;

preferably wherein the conjugation-competent polypeptide has a tendency to exist as a monomer in solution which is at least 70% of the tendency of the parent polypeptide (such as the polypeptide of SEQ ID NO. 2) to exist as a monomer in solution, more preferably at least 75, 80, 85, 90, 95, 96, 97, 98, at least 99 or 100% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution. Preferably the parent polypeptide does not contain the conjugation-competent Cys residue or residues described herein. Preferably the parent polypeptide does not contain the additional mutation or mutations described herein. That is, preferably the parent polypeptide is identical to the conjugation-competent polypeptide with the

exception of the introduced cysteine residue or residues and, if present, the introduced other mutation or mutations.

Suitably, the at least one (*e.g.* several) position is selected from K93, E294, A226, E230, I271, and E358, particularly from K93, E294, A226, E230, and I271.

Preferably the conjugation-competent polypeptide has at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.2, 99.4, 99.6, 99.8% sequence identity to SEQ ID NO. 2. For example, in addition to the introduced Cys residue or Cys residues, the conjugation-competent polypeptide may have at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 (*e.g.* several) other mutations relative to SEQ ID NO. 2. Alternatively, in addition to the introduced Cys residue or Cys residues, the conjugation-competent polypeptide may have zero other mutations relative to SEQ ID NO. 2.

Preferably, the conjugation-competent polypeptide has a tendency to exist as a monomer in solution which is at least 75% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution and at least one position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, L284, K317, A322, E333, D340, E354, K359, A362, E382, and L398 comprises a conjugation-competent cysteine residue.

Preferably the polypeptide is a recombinant polypeptide. Preferably the polypeptide is an isolated and/or purified polypeptide. Preferably the polypeptide is synthetic and/or does not naturally occur in nature.

A conjugation-competent cysteine at the position defined above may or may not be created in an albumin by *insertion*, for example by adding a cysteine with or without one or more (*e.g.* several) additional residues and without removal of an amino acid residue from the albumin sequence; or by substituting one or more (*e.g.* several) adjacent amino acids with a larger number of residues containing at least one (*e.g.* several) cysteine, thus extending the overall length of the polypeptide. For example, a cysteine residue may be introduced immediately adjacent an albumin residue identified herein. The cysteine residue may be introduced as a single cysteine residue or within a polypeptide. The polypeptide may be from 2 to 50 amino acids long, preferably from 2, 10, 20, 30, or 40 to 10, 20, 30, 40 or 50 amino acids long.

Suitably, the polypeptide comprises one or more (*e.g.* several) of:

a) substitution of an amino acid, other than cysteine, with a cysteine at a position corresponding to a position equivalent to any of residues K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2; and/or

b) insertion of a cysteine at a position adjacent the N- or C- side of an amino acid corresponding to a position equivalent to any of residues K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2.

Substitutions are preferred, and the following disclosure of selected positions should be understood to specifically encompass substitutions, without limitation.

Suitably 2, 3, 4, 5 or more (*e.g.* several) positions equivalent to positions selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2 comprise a conjugation-competent cysteine residue. Suitably the 2, 3, 4, 5 or more (*e.g.* several) positions are selected from K93, E294, A226, E230, I271, and E358, particularly from K93, E294, A226, E230 and I271.

For a polypeptide comprising a Cys at a position equivalent to position E294 of SEQ ID NO. 1, preferably the polypeptide also comprises a Cys at a position equivalent to one or more of K93, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, or E382.

The inventors have found that variants of HSA in which cysteine has been substituted at a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, and E382 have the beneficial property of a tendency to exist as a monomer in solution which is at least 70% of the tendency of the HSA polypeptide of SEQ ID NO. 2 to exist as a monomer in solution. A cysteine introduced at one of the selected positions therefore has a low tendency to cause the variant to form dimers or higher order oligomers in solution. This beneficial effect is also noted in variants in which there are cysteines at more than one selected position. Without wishing to be bound by theory, the inventors ascribe the monomer tendencies of the polypeptides of the invention to the flexibility of the polypeptide chain in the region of, and surface exposure at, the site of cysteine substitution. This reflects an exercise of inventive skill, based on years of experience in protein structural biology, in the choices applied by the inventors in selecting positions within HSA for substitution with cysteine.

The tendency of albumin or variants thereof to exist as a monomer, rather than a dimer or higher order oligomer, can be determined based on measurement of monomer, dimer and higher order oligomer quantities in solutions of the albumin or variant under similar conditions.

Suitable techniques for performing such measurements include Gel Permeation High

Pressure Liquid Chromatography, as described in the Examples. Results are typically expressed as "percentage monomer", which is calculated as:

amount of monomeric albumin by mass X 100 / (amount of monomeric albumin by mass + amount of dimeric albumin by mass + amount of higher order oligomer by mass).

Alternatively, the tendency to form non-monomers in solution, that is dimers and/or higher order oligomers, may be expressed. The "percentage non-monomer" is 100% minus percentage monomer.

Samples may be tested shortly after purification (for example, within 24 hours after purification) following production in shake flasks or 10L bioreactors, or following storage at 2-8 °C, *e.g.* 5 °C, for time periods of up to or including 1 week, 1 month, 2 months, 3 months or 6 months. Samples are typically tested, and optionally stored, in a solution of one or more (*e.g.* several) salts and at a pH of about 7.0±0.5. The solution may comprise a buffer comprising 50 mM ammonium acetate, 10 mM sodium octanoate, pH 7.0, preferably at a polypeptide concentration of from about 0.2 to about 2.5 mg/mL. The solution may comprise a buffer comprising 25 mM sodium phosphate, 215 mM sodium chloride, pH 6.5, preferably at a polypeptide concentration of from about 5 to about 50 mg/mL.

The percentage monomer for a given albumin may differ depending on the albumin purity and concentration. Albumin produced in shake flask culture is typically purified using a single AlbuPure® (Prometic Life Sciences Inc. or Albumedix Ltd (formerly Novozymes Biopharma UK Ltd)) chromatography step, and typically is obtained at a concentration of about 0.2 to 2.0 mg/mL, more preferably 1±0.5 mg/mL and a protein purity of >95% by SDS reducing PAGE. AlbuPure® is a high-performance affinity capture adsorbent designed for albumin fusion protein purification, which comprises a synthetic triazine ligand coupled to a base matrix. Under these conditions, percentage monomer of HSA was found to be about 87%, rising to about 89% upon storage at 6 months at 2-8 °C *e.g.* 5 °C. Albumin produced in 10L bioreactor culture is typically purified by a AlbuPure® chromatography step followed by an ion exchange chromatography, is ultrafiltered, and then formulated at 50 mg/mL, and has a protein purity of >99% by SDS reducing PAGE. Under these conditions, percentage monomer of HSA was found to be about 94%, and was stable at two months of storage at 2-8 °C and at 6 months storage at 2-8 °C. A variant having at least 70% of the tendency of HSA to exist as a monomer in solution may therefore be found to be at least 60% monomer, preferably at least 69% monomer (less than 40% non-monomer, preferably less than 31% non-monomer) when tested after typical shake flask production and purification as described above, for samples tested shortly after purification or stored for up to two or up to six months. For a variant having at least 80% of the tendency of HSA to exist as a monomer in solution, the percentage monomer should be at least 70% preferably at least 79% monomer, and the percentage non-monomer less than

30%, preferably less than 21%. A variant having at least 70% of the tendency of HSA to exist as a monomer in solution may be found to be at least 65% monomer, preferably at least 69% monomer, when tested after typical 10L bioreactor production and purification as described above, for samples tested shortly after purification or stored for up to two months. For a variant having at least 80% of the tendency of HSA to exist as a monomer in solution, the percentage monomer should be at least 75% preferably at least 79%. The tendency is preferably measured at day 0, *e.g.* the day that the variant is produced, however it may also be measured later *e.g.* at day 1, 2, 3, 4, 5, 6, 7 or after 2, 3, 4, 5, 6, 7 weeks or after 1 or 2 months storage *e.g.* at 2-8 °C *e.g.* 5 °C. Suitably, the percentage monomer should be stable upon storage for up to seven weeks or two months, meaning that it does not reduce by more than 10, more than 9, 8, 7, 6, 5, 4, 3, 2 or 1 percentage points between testing shortly after purification and testing after two months of storage *e.g.* at 2-8 °C *e.g.* 5 °C. Preferably the percentage monomer should not reduce by more than 5 percentage points between testing shortly after purification and testing after 7 weeks of storage at 2-8 °C *e.g.* 5 °C.

The variant may or may not have a tendency to exist as a monomer in solution which is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution. This tendency may be tested shortly after purification or after storage for up to six months *e.g.* at 2-8 °C *e.g.* 5 °C.

The tendency of the polypeptide to exist as monomer in solution may be measured following storage for at least 7 weeks at a temperature from 2 to 8°C such as 5°C, at least 8 weeks at a temperature from 2 to 8°C such as 5°C, at least 3 months at a temperature from 2 to 8°C such as 5°C, at least 4 months at a temperature from 2 to 8°C such as 5°C, at least 6 months storage at a tempature from 2 to 8°C such as 5°C, or at least 3 months storage at a tempature of about 40 °C. Most preferably the tendency of the polypeptide to exist as monomer in solution is measured following storage for at least 3 months at a temperature from 2 to 8°C such as 5°C.

The tendency of the polypeptide to exist as a monomer in solution may be measured at a polypeptide concentration of from 0.2 to 50 mg/mL, for example at about 5 mg/mL.

The tendency of the polypeptide to exist as a monomer in solution may be measured at a pH from about 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, or 7.4 to about 6.1, 6.2, 6.3, 6.4, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4 or 7.5, preferably about pH 7.

The tendency of the polypeptide to exist as a monomer in solution may be measured in a buffer comprising 50 mM ammonium acetate, 10 mM sodium octanoate, pH 7.0, preferably at a polypeptide concentration of from about 0.5 to about 5 mg/mL.

The tendency of the polypeptide to exist as a monomer in solution may be measured in a buffer comprising 25 mM sodium phosphate, 215 mM sodium chloride, pH 6.5, preferably at a

polypeptide concentration of from about 5 to about 50 mg/mL.

The conjugation-competent polypeptide may, prior to storage, be purified for example using a triazine (such as AlbuPure®) chromatography matrix or DE-FF chromatography matrix, more preferably by triazine (such as AlbuPure®) chromatography matrix followed by DE-FF chromatography matrix. Suitable methods are disclosed in Example 10

The polypeptide sample storage may be static. The polypeptide sample storage may be vertical.

Where a variant comprises more than one conjugation-competent cysteine as provided above, the tendency to exist as a monomer may be reduced compared to the variant which differs only by virtue of having one fewer such cysteines. For example, a variant albumin having the substitutions E294C + K93C has a lower tendency to exist as a monomer than a variant albumin having either substitution alone. Suitably, the variant comprises a conjugation-competent cysteine residue at two positions selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2, wherein the variant has a tendency to exist as a monomer in solution which is at least 75% of the tendency of a variant which differs only by virtue of comprising a conjugation-competent cysteine residue at only one of the two positions.

Suitably, the variant comprises a conjugation-competent cysteine residue at two positions selected from K93, E294, A226, E230, I271, E358, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2, wherein the variant has a tendency to exist as a monomer in solution which is at least 75% of the tendency of a variant which differs only by virtue of comprising a conjugation-competent cysteine residue at only one of the two positions.

Higher monomer tendencies are preferred, such as at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100%. For example, HSA comprising the substitution E294C + K93C has a tendency to exist as a monomer in solution which is at least 90% of the tendency of HSA comprising the substitution K93C, or at least 85% of the tendency of HSA comprising the substitution E294C, to exist as a monomer in solution. These results are illustrated in the Examples, with material purified from 10L bioreactor preparations, and tested shortly after purification, or after storage for seven weeks or two months at 2-8 °C *e.g.* 5 °C. The same samples were also stable following storage for 6 months. Albumin variants having more than one conjugation-competent cysteine can be prepared by introducing a further conjugation-competent cysteine residue into a variant which already has at least one (*e.g.* several) conjugation-competent cysteine residue. Variants comprising a further conjugation-competent cysteine residue which have at least 75% of the tendency of the reference albumin lacking the further conjugation-competent cysteine residue to

exist as a monomer in solution may be preferred.

Suitable variants may comprise a conjugation-competent cysteine residue at one or two or more (*e.g.* several) positions selected from K93, E294, A226, E230, I271 and E358 of SEQ ID NO. 2. Suitable combinations of positions are (i) K93 + E294, A226, E230, I271, or E358; (ii) E294 + K93, A226, E230, I271, or E358; (iii) A226 + K93, E294, E230, I271, or E358; (iv) E230 + K93, E294, A226, I271, or E358; (v) I271 + K93, E294, A226, E230, or E358; (vi) K93 + E294 + A226, E230, I271, or E358 of SEQ ID NO. 2. Suitable variants may comprise a conjugation-competent cysteine residue at one or two or more (*e.g.* several) positions selected from L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, D237, E230, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, L398 of SEQ ID NO. 2. Suitable combinations of positions are: (1) L24 + F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (2) F49 + L24, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (3) V54 + L24, F49, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (4) D56 + L24, F49, V54, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (5) L66 + L24, F49, V54, D56, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (6) A92 + L24, F49, V54, D56, L66, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (7) Q94 + L24, F49, V54, D56, L66, A92, K93, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (8) E97 + L24, F49, V54, D56, L66, A92, K93, Q94, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (9) H128 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (10) F156 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (11) E227 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E230, D237, K240, D259, K262, N267,

Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (12) D237 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E230, E227, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (13) K240 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E230, E227, D237, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (14) D259 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E230, E227, D237, K240, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (15) K262 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E230, E227, D237, K240, D259, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (16) N267 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E230, E227, D237, K240, D259, K262, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (17) Q268 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (18) L275 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (19) E277 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (20) L284 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (21) E311 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, K317, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (22) K317 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, A322, E333, D340, E354, E358, K359, A362, E382, or L398; (23) A322 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, E333, D340, E354, E358, K359, A362, E382, or L398; (24) E333 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, D340, E354, E358, K359, A362, E382, or L398; (25) D340 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, E227, D237, E230, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, E354, E358, K359, A362, E382, or L398; (26) E354 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226,

E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E358, K359, A362, E382, or L398; (27) K359 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, A362, E382, or L398; (28) A362 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, E382, or L398; (29) E382 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, or L398; (30) L398 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, or E382; (31) K93 + L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382 or L398; (32) E294 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382 or L398; (33) A226 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382 or L398; (34) E230 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382 or L398; (35) I271 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, E358, K359, A362, E382 or L398; and (36) E358 + L24, F49, V54, D56, L66, A92, K93, Q94, E97, H128, F156, A226, E227, E230, D237, K240, D259, K262, N267, Q268, I271, L275, E277, L284, E294, E311, K317, A322, E333, D340, E354, K359, A362, E382 or L398.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine provided at a position equivalent to K93 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E294 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to A226 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E230 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to I271 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E358 in SEQ ID NO. 2.

A particularly preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K93 in SEQ ID NO. 2 and a cysteine at a position equivalent to E294 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to L24 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to F49 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to V54 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to D56 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to L66 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to A92 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to Q94 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E97 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to H128 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to F156 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E227 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to D237 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to K240 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to D259 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to K262 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to N267 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to Q268 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to L275 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E277 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to L284 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E311 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to K317 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to A322 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E333 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to D340 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E354 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to K359 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to A362 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to E382 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 and a cysteine at a position equivalent to L398 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine provided at a position equivalent to K93 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A particularly preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 a cysteine at a position equivalent to E294 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to A226 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E230 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to I271 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E358 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K93 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2, a cysteine at a position equivalent to E294 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L24 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to F49 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to V54 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to D56 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L66 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to A92 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to Q94 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E97 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a

position equivalent to H128 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to F156 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E227 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to D237 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K240 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to D259 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K262 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to N267 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to Q268 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L275 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E277 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L284 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E311 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in

SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K317 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to A322 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E333 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to D340 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E354 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K359 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to A362 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E382 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L398 in SEQ ID NO. 2 and a cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine provided at a position equivalent to K93 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A particularly preferred polypeptide may have at least 90% identity to SEQ ID NO. 2 a cysteine at a position equivalent to E294 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to A226 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E230 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to I271 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E358 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K93 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2, a cysteine at a position equivalent to E294 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L24 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to F49 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to V54 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to D56 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L66 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to A92 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to Q94 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E97 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a

position equivalent to H128 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to F156 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E227 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to D237 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K240 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to D259 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K262 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to N267 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to Q268 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L275 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E277 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L284 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E311 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in

SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K317 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to A322 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E333 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to D340 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E354 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to K359 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to A362 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to E382 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

A preferred polypeptide may have at least 90% identity to SEQ ID NO. 2, a cysteine at a position equivalent to L398 in SEQ ID NO. 2 and no cysteine at a position equivalent to C34 in SEQ ID NO. 2.

The 'no cysteine' at a position equivalent to C34 in SEQ ID NO. 2 may be provided, for example, by a substitution of C34 to an amino acid, such as a natural amino acid, for example, A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y. Such a substitution may be described as C34X. The substitution C34A is preferred. The 'no cysteine' at a position equivalent to C34 in SEQ ID NO. 2 may be provided, for example, by deletion of the cysteine at this position.

A thio-albumin may or may not include a polypeptide where one or more (*e.g.* several) naturally occurring free-thiol group(s), such as cysteine-34 in HSA (SEQ ID NO. 2), is modified to an amino acid which is not cysteine. For example, cysteine may or may not be replaced by an amino acid which has a relatively high conservation score (*e.g.* 1, 2 or 3 as calculated

according to Fig. 3) such as alanine or serine. A thio-albumin may or may not include a polypeptide where one or more (*e.g.* several) naturally occurring free-thiol group(s), such as cysteine-34 in HSA (SEQ ID NO. 2) are present. Thus, the conjugation-competent polypeptide of any of the above embodiments may comprise, at a position equivalent to position 34 of SEQ ID NO. 2, a conjugation-competent cysteine. Alternatively, there may not be a conjugation-competent cysteine at a position equivalent to position 34 of SEQ ID NO. 2.

For a polypeptide comprising two or more (several) conjugation competent cysteine residues, when the polypeptide is folded, the conjugation competent cysteine residues may or may not be relatively evenly distributed over the surface of the folded protein. The term 'folded' includes folding of a polypeptide/protein into its natural configuration, for example the most thermodynamically stable folded configuration. An advantage of relatively even distribution is that it allows conjugation of two or more (several) moieties to the thio-albumin with minimal steric hindrance or without steric hindrance between two or more (several) of the conjugated moieties. This has the advantage of minimising, and optionally eliminating, potential loss of activity due to issues such as steric hindrance between adjacent moieties (conjugation partners) which may be conjugated to the thio-albumin. Such moieties, for example bioactive molecules, may be relatively bulky.

Preferably the two or more (several) conjugation-competent cysteines are distributed over the surface of the thio-albumin molecule such that they are spaced as far from each other as possible, for example geometrically possible. Preferably the distance between two or more (several) conjugation-competent cysteines is at least 5, 10, 20, 30, 40, 50, 60, 70, or 80 Angstroms. Preferably each conjugation competent cysteine is at least 5, 10, 20, 30, 40, 50, 60, 70, or 80 Angstroms distant from one or several or all other conjugation-competent cysteines in the molecule. The distance between two conjugation-competent cysteines is preferably a distance which is at least 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 95% and most preferably 100% of the length of the longest axis of the folded albumin molecule, for example as shown in a model of an albumin. For example, the longest axis of SEQ ID NO. 2 as shown in protein structure 1AO6 is approximately 85 Angstroms. Therefore, it is preferred that the two or more (several) of the cysteine residues are at least 65, 70, 75 or most preferably 80 Angstroms apart. Most preferably each conjugation-competent cysteine residue is at a distance of at least 80, 90, or 95% and most preferably 100% of the length of the longest axis of the folded albumin molecule.

Preferably the side chains of conjugation-competent cysteines are directed away from each other and/or directed so that a moiety conjugated to the cysteine will be directed away from the centre of the albumin structure. This provides the advantage of preventing interactions between the conjugated moieties and/or the albumin moiety itself.

With reference to an amino acid sequence, candidate amino acid residues may be visually inspected using software such as Yasara (Krieger and Vriend, 2014, Bioinformatics 30(20) 2981-2982; and described at http://www.yasara.org/).

Suitably, the polypeptide comprises substitution of an amino acid, other than cysteine, with a cysteine at one or both positions corresponding to a position equivalent to residues K93 or E294 of SEQ ID NO. 2. The Cα-Cα distance between C34 and K93 is 20.3 Å, between C34 and E294 is 39.9 Å and K93 and E294 45.9 Å in WT HSA (SEQ ID NO. 2).

Maleimide conjugation is a convenient means of conjugating a conjugation partner to an albumin. Capability to form a conjugate with maleimide-polyethylenglycol2-biotin is believed to be indicative of capability to form a conjugate with other conjugation partners containing a maleimide group. Conversely, if a conjugation-competent polypeptide has a low efficiency of conjugation with maleimide-polyethylenglycol2-biotin, or fails to conjugate, this is not indicative that it is poorly capable or not capable of conjugating with a different chemical group. Maleimide conjugates form a thio-ether bond, which may or may not be capable of stabilisation upon controlled hydrolysis. Stable conjugate formation may be preferred, such that the conjugate does not release a reactive maleimide conjugation partner during storage or use. The latter could potentially form unwanted conjugates with thiol-reactive species encountered *in vivo*.

As shown in the Examples, native HSA having a single free thiol at cysteine 34 forms approximately 50% stable conjugate upon maleimide conjugation and controlled hydrolysis. In contrast, polypeptides of the invention may form stable conjugates at higher efficiencies. In particular, albumins comprising a free thiol group at a position selected from those equivalent to K93, E294, and E358 of SEQ ID NO. 2 form stable maleimide conjugates at high efficiency, as shown in the Examples. Albumins comprising two or more (several) such thiols also may also form stable maleimide conjugates.

A conjugation-competent polypeptide of the invention may or may not be capable of forming a conjugate with maleimide-polyethylenglycol2-biotin (maleimide-PEG2-biotin) at a conjugation efficiency of at least 90%, preferably at least 95%, which conjugate may or may not be at least 90%, preferably at least 95% stable upon controlled hydrolysis. Figure 4 illustrates the conjugation of maleimide-PEG2-biotin to a free thiol of a protein, and reactions which may occur to the formed conjugate.

A conjugation efficiency of a particular percentage indicates that the specified percentage of free thiol groups in the albumin form an adduct with the maleimide moiety, under suitable reaction conditions. The maleimide group reacts with thiols in the pH range 6.5-7.5 to form a thio-ether linkage with very little cross-reactivity with amines at this pH. The use of 20mM sodium phosphate, 150mM sodium chloride, pH 7.2 works well for this reaction. The

concentration of protein should ideally be in the range of 1-10mg/mL. Lower concentrations of protein may result in the need to increase the molar excess of reagent to obtain an acceptable level of modification (Hermanson, Greg T. (2008), Bioconjugate Techniques. Second Edition, Academic Press, San Diego, CA). The formation of the adduct results in an increase in mass which can be measured, for example by mass spectrometry, as in the Examples. Conveniently, the percentage conjugation efficiency is in relation to all free thiols of the albumin. Where the albumin has more than one such free thiol, a different percentage conjugation efficiency may pertain to each free thiol, and may be expressed in relation either to each individual free thiol, or collectively to all free thiols. Thus, if an albumin has two free thiols, one having 50% conjugation efficiency and the other having 100% conjugation efficiency, the overall conjugation efficiency for the albumin is the average of the two conjugation efficiencies, in this case 75%.

A stability of a particular percentage upon controlled hydrolysis indicates that the specified percentage of thiol-maleimide adduct undergoes ring-opening stabilisation, that is, the succinimide ring moiety is hydrolysed to a succinic acid moiety, and the thio-ether bond of the conjugate is maintained, as illustrated in Figure 4. The percentage stability may be expressed in relation either to each individual free thiol or the albumin, or collectively to all free thiols. Controlled hydrolysis may be performed at alkaline pH and above ambient temperature. Suitably, adducts are incubated at pH 9.0 and 37 °C for at least 18 hours, preferably 24 hours in a buffered salts solution, such as phosphate buffered saline. The hydrolysis of the succinimide moiety to a succinic acid moiety by the addition of $H_2O$ has the effect of increasing the mass of the conjugate, which can be measured, for example by mass spectrometry, as in the Examples. Where conjugation efficiency is incomplete, this must be taken into account in determining the percentage stability. For example, if 50% of an albumin having one free thiol forms a conjugate, and 40% of the albumin is conjugated following controlled hydrolysis, this represents a stability of 80%. In these circumstances, 50% of the albumin is initially unconjugated, and therefore has a mass indicative of free albumin. The mass does not change upon controlled hydrolysis. Of the 50% of the albumin that is initially conjugated, a portion, 40% of the total albumin, has an increased mass of 18 Da due to the addition of $H_2O$. The other portion, 10% of the total albumin, does not undergo hydrolysis and therefore its mass does not change. Although this albumin is still conjugated, it may be unstable during storage or use, because it can undergo de-conjugation *via* the retro-Michael pathway, as illustrated in Figure 4. In contrast, the stably hydrolysed conjugate can be expected to remain stable during storage or use (Fontaine, S. *et al*, *Bioconjugate Chem.* 2015, 26, 145-152).

Suitably conjugation efficiencies for a polypeptide of the invention may be at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or substantially 100%. Suitably conjugation efficiencies for an individual free thiol of a polypeptide of the invention may be at least 50%, at least 60%, at least

70%, at least 80%, at least 90%, at least 95% at least 96%, at least 97%, at least 98%, at least 99%, or substantially 100%. Suitable stabilities of a polypeptide conjugate upon controlled hydrolysis may be at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or substantially 100%.

As shown in the Examples, native HSA having a single free thiol at cysteine 34 forms greater than about 90% conjugate. Albumins comprising a free thiol group at a position selected from those equivalent to K93, E294, E358, L24, V54, H128, E227, K240, K262, Q268, E277, K317, A322, K359, and A362 of SEQ ID NO. 2 form maleimide conjugates greater than about 90% efficiency, those with a free thiol group at a position selected from those equivalent to L24, V54, H128, E227, K240, K262, K359, and A362 form maleimide conjugates greater than about 95% efficiency.

Suitable stabilities of a particular thiol-ether conjugate bond of a polypeptide conjugate upon controlled hydrolysis may be at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or substantially 100%.

The polypeptide may or may not further comprise a further linker to which a conjugation partner, such as a bioactive compound, radiopharmaceutical or imaging agent, may be linked. For example a linker may comprise a primary amine such as a lysine.

It is preferred that the conjugation-competent polypeptide has an acceptable immunogenicity, particularly in humans. More preferably the conjugation-competent polypeptide has an immunogenicity that is comparable to or lower than that of a parent albumin such as WT HSA (SEQ ID NO. 2). Therefore, preferably the alteration(s) to provide a conjugation competent cysteine residue(s) do not adversely affect the immunogenicity of the polypeptide relative to the parent albumin such as WT HSA.

Preferably, the alteration(s) made to provide the conjugation competent cysteine residue(s) do not adversely affect the immunogenicity of the polypeptide in human, *e.g.* relative to the immunogenicity of wild-type HSA (SEQ ID NO. 2).

The immunogenicity of the polypeptide may be determined or predicted by screening for T-cell epitopes and/or for B-cell epitopes. Screening may be *in silico*, *in vitro* or *ex vivo.* For example, the immunogenicity of the polypeptide may be determined or predicted by an *ex vivo* T cell activation assay. The T cell activation assay may comprise measuring T cell responses using a proliferation assay, *e.g.* [3H]-thymidine uptake. Preferably, the polypeptide has less than 10% reactivity in the T cell proliferation assay, preferably less than 8, 6, 4, or 2 % reactivity, most preferably 0%. 'Reactivity' means that a positive response was observed. Therefore 10% reactivity means that a positive response was observed in 10% of the donor samples.

The T cell activation assay may comprise measuring T cell responses using a cytokine secretion assay, *e.g.* IL-2 ELISpot. Preferably the polypeptide has less than 10% reactivity in the cytokine secretion assay, preferably less than 8, 6, 4, or 2 % reactivity, most preferably 0%. 'Reactivity' means that a positive response was observed. Therefore 10% reactivity means that a positive response was observed in 10% of the donor samples.

More preferred, the conjugation-competent polypeptide has less than 10% reactivity in a T cell proliferation assay and in a cytokine secretion assay, *e.g.* an EpiScreen™ assay (Abzena, Cambridge, UK).

The T cell assays may comprise CD4+ T cells.

The T cell assays may use peripheral blood mononuclear cells from a cohort of 50 healthy donors representing the European and North American population (based on HLA allotypes).

Preferably, the polypeptide does not stimulate an adverse antibody response in human, such as a specific antibody response.

For a conjugate comprising the conjugation-competent polypeptide, preferably the conjugate has an immunogenicity that is comparable to or lower than that of a corresponding conjugate comprising a parent albumin such as WT HSA (SEQ ID NO. 2) instead of the conjugation-competent polypeptide. Consequently, the properties mentioned for the conjugation-competent polypeptide also apply to a conjugate comprising the conjugation-competent polypeptide, however the 'control' may be a parent albumin such as WT HSA or a corresponding conjugate comprising a parent albumin such as WT HSA.

**Conjugation-competent polypeptides II**

A second aspect of the invention provides a conjugation-competent polypeptide comprising an amino acid sequence according to the first aspect of the invention, and at least one (*e.g.* several) further modification compared to SEQ ID NO. 2, such as a further modification which causes the polypeptide to have at least one (*e.g.* several) further conjugation-competent cysteine, or alters the binding affinity of the polypeptide for FcRn, or alters the plasma half-life of the polypeptide.

The second aspect of the invention allows for the favoured conjugation-competent cysteines as defined in relation to the first aspect of the invention to be combined with other modifications in an albumin background, and provides the option to further tailor the albumin for specific applications.

<u>Further conjugation-competent cysteines</u>

The at least one (*e.g.* several) further modification may or may not cause the polypeptide to have at least one (*e.g.* several) further conjugation-competent cysteine. The polypeptide may

or may not comprise a total of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 conjugation competent cysteine residues. The polypeptide may or may not comprise at least one (*e.g.* several) further conjugation-competent cysteine as defined in relation to the first aspect of the invention.

The polypeptide may or may not comprise at least one (*e.g.* several) further conjugation-competent cysteine, other than at a position corresponding to least one position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2. Suitable conjugation-competent cysteines are disclosed in WO 2010/092135 (incorporated by reference, particularly Figures 5 and 6). Suitably, at least one (*e.g.* several) position equivalent to a position selected from D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371, N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2 may comprise a conjugation-competent cysteine. Suitably, the polypeptide may comprise one or more (*e.g.* several) of: (a) substitution of an amino acid, other than cysteine, with a cysteine at a position corresponding to a position equivalent to any of residues D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371, N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2; (b) insertion of a cysteine at a position adjacent the N- or C- side of an amino acid corresponding to a position equivalent to any of residues D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371, N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2 so as to generate a conjugation competent cysteine at any of C369, C361, C91, C177, C567, C316, C75, C169, C124 and C558; and (c) addition of a cysteine to the N- side of the N-terminal residue of an albumin sequence or to the C- side of the C-terminal residue of an albumin sequence. Exemplary combinations include conjugation-competent cysteines located at: (a) A2 + L585, (b) A2 + A364 + D562 + L585C, (c) A2 and adjacent the C-side of the C-terminus of the albumin (d) T79 + A364; (e) A364 + D1; (f) T79 + D562 + A364; (g) D562 + A364 + D1; (h) T79 + D562 + A364 + A504; (i) T79 + D562 + A364 + L585; (j) T79 + D562 + A364 + D1; (k) T79 + D562 + A364 +L585 + D1; (l) E86 + D562 + A364 +A504 + A2; (m) S270 + A581; (n) S270 + D129; (o) S270 + A581 + E82; (p) S270 + A581 + D129; (q) S270 + A581 + E82 + D129; (r) S270 + A581 + E82 + D129 + Q397; (s) C369 +

C177; (t) A364 + A581; (u) T79 + A364 + A581; (v) A364 + A581 + D129; (w) A364 + C177; (x) D562 + C369; (y) D129 + C369; (z) A581 + C369; or (aa) D562 + D129 + C369.

Further suitable cysteine residues may be introduced as disclosed in WO 2009/126920 or WO 2010/059315 (incorporated herein by reference). Specifically, one or more (*e.g.* several) surface-exposed amino acid residues may be substituted for a cysteine residue, corresponding to one or more (*e.g.* several) positions corresponding S58, T76, T79, T83, T125, T236, S270, S273, S304, S435, T478, T496, T506 and T508 of SEQ ID NO. 2.

As noted in relation to the first aspect of the invention, increasing the number of conjugation-competent cysteine residues in an albumin variant may reduce its tendency to exist as a monomer in solution. It is preferred that the conjugation-competent polypeptide of the second aspect of the invention has a tendency to exist as a monomer in solution which is at least 70% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution, and optionally at least 75%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100%. This preference applies whether or not the polypeptide comprises a further conjugation-competent cysteine as defined in relation to the second aspect. Nevertheless, useful conjugation-competent polypeptides may still be provided which have a lower tendency to exist as a monomer in solution. Because the conjugation-competent cysteine residues defined in relation to the first aspect of the invention themselves contribute relatively minimally to non-monomer formation, combining one or more (*e.g.* several) of them with one or more (*e.g.* several) other conjugation-competent cysteine residues can be expected to result in a variant having increased monomer percentage compared to a variant having the same number of conjugation-competent cysteine residues selected from the prior art.

Albumin variants with altered binding to FcRn and/or altered plasma half-life

The at least one (*e.g.* several) further modification may or may not alter the binding affinity of the albumin variant to FcRn and/or alter the plasma half-life. Preferably the albumin variant may have at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.2, 99.4, 99.6, 99.8% sequence identity to SEQ ID NO. 2. For example, in addition to the introduced Cys residue or Cys residues, the albumin variant may have at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 (*e.g.* several) other mutations relative to SEQ ID NO. 2. Alternatively, in addition to the introduced Cys residue or Cys residues, the albumin variant may have zero other mutations relative to SEQ ID NO. 2.

The thio-albumin or conjugate may have a plasma half-life that is either longer or shorter, preferably longer, than that of the parent albumin or conjugate thereof, or a binding to FcRn that is stronger or weaker, preferably stronger. Preferably the thio-albumin or conjugate has a plasma half-life that is longer than that of HSA or the corresponding conjugate thereof.

Alternatively, this may be expressed as the thio-albumin or conjugate having a KD to FcRn (*e.g.* shFcRn) that is lower than the corresponding KD for HSA or conjugate thereof to. Preferably, the KD for the thio-albumin or conjugate is less than 0.9X KD for HSA to FcRn, more preferred less than 0.5X KD for HSA to FcRn, more preferred less than 0.1X KD for HSA to FcRn, even more preferred less than 0.05X KD for HSA to FcRn, even more preferred less than 0.02X KD for HSA to FcRn, even more preferred less than 0.01X KD for HSA to FcRn and most preferred less than 0.001X KD for HSA to FcRn (where X means 'multiplied by').

For a conjugate comprising a thio-albumin, prefererably the KD for the conjugate is less than 0.9X KD for the corresponding conjugate comprising HSA to FcRn, more preferred less than 0.5X KD for the corresponding conjugate to FcRn, more preferred less than 0.1X KD for the corresponding conjugate to FcRn, even more preferred less than 0.05X KD for the corresponding conjugate to FcRn, even more preferred less than 0.02X KD for the corresponding conjugate to FcRn, even more preferred less than 0.01X KD for the corresponding conjugate to FcRn and most preferred less than 0.001X KD for the corresponding conjugate to FcRn (where X means 'multiplied by'). 'Corresponding conjugate' means a conjugate comprising HSA (*e.g.* SEQ ID NO. 2) instead of the thio-albumin (*i.e.* albumin variant).

Alternatively, the thio-albumin or conjugate may have a plasma half-life that is shorter than that of HSA or the conjugate thereof.

This may be expressed as the thio-albumin or conjugate having a KD to FcRn that is higher than the corresponding KD for HSA or conjugate thereof to FcRn. Preferably, the KD for the thio-albumin or conjugate is more than 2X KD for HSA to FcRn, more preferred more than 5X KD for HSA to FcRn, more preferred more than 10X KD for HSA to FcRn, even more preferred more than 25X KD for HSA to FcRn, most preferred more than 50X KD for HSA to FcRn. The thio-albumin or conjugate may be a null binder to FcRn.

For a conjugate comprising a thio-albumin, prefererably the KD for the conjugate, Preferably, the KD for the corresponding conjugate comprising HSA is more than 2X KD for the corresponding conjugate to FcRn, more preferred more than 5X KD for the corresponding conjugate to FcRn, more preferred more than 10X KD for the corresponding conjugate to FcRn, even more preferred more than 25X KD for the corresponding conjugate to FcRn, most preferred more than 50X KD for the corresponding conjugate to FcRn. Corresponding conjugate' means a conjugate comprising HSA (*e.g.* SEQ ID NO. 2) instead of the thio-albumin (*i.e.* albumin variant).

The half-life of the thio-albumin or conjugate or product made from associate, nanoparticle, microparticle or liposome may be tailored in order to achieve a binding affinity or half-life which meets the needs of the user.

When determining and/or comparing KD, one or more (*e.g.* several) (and preferably all) of the following parameters may be used:

Instrument: Biacore 3000 instrument (GE Healthcare)

Flow cell: CM5 sensor chip

FcRn: human FcRn, preferably soluble human FcRn, optionally coupled to a tag such as Glutathione S Transferase (GST) or Histidine (His), most preferably His such as 6 histidine residues at the C-terminus of the beta-2-microglobulin.

Quantity of FcRn: 1200-2500 RU

Coupling chemistry: amine coupling chemistry (*e.g.* as described in the protocol provided by the manufacturer of the instrument).

Coupling method: The coupling may be performed by injecting 20 µg/mL of the protein in 10 mM sodium acetate pH 5.0 (GE Healthcare). Phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005% Tween 20) at pH 5.5 may be used as running buffer and dilution buffer. Regeneration of the surfaces may be done using injections of HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) at pH 7.4 (Biacore AB).

Quantity of injection of test molecule (*e.g.* HSA or variant) 20-0.032µM

Flow rate of injection: constant, *e.g.* 30 µL/mL

Temperature of injection: 25 °C

Data evaluation software: BIAevaluation 4.1 software (BIAcore AB).

Domain III of albumin is primarily responsible for binding FcRn. The conjugation-competent polypeptide may or may not comprise or consist of albumin domain III or a variant thereof and at least one (*e.g.* several) additional albumin domain or fragment thereof, such as a second albumin domain III or a variant thereof, as disclosed in WO 2011/124718 (incorporated herein by reference). Suitably, the polypeptide comprises or consists of at least one (*e.g.* several) albumin domain III or variant or fragment thereof, wherein at least one (*e.g.* several) albumin domain III comprises one or more (*e.g.* several) substitutions in positions corresponding to the positions in SEQ ID NO. 2 selected among: 573, 500, 550, 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 574, 575, 577, 578, 579, 580, 581, 582 and 584, as disclosed in WO 2011/051489 (incorporated herein by reference). Suitable substitutions include one or more (*e.g.* several) substitutions in positions corresponding to the positions in SEQ ID NO. 2 selected among: K573Y, W, P, H, F, V, I, T, N, S, G, M, C, A, E, Q, R, L, D, K500E, G, D, A, S, C, P, H, F, N, W, T, M, Y, V, Q, L, I, R, Q417A, H440A, H464Q, E492G, D494N,Q,A, E495Q,A, T496A, D494E+Q417H, D494N+T496A, E492G+V493P, P499A, E501A,Q, N503H,K, H510Q, H535Q, K536A, P537A, K538A, K541G,D, D550E,N, E492G+K573P,A, or E492G/N503H/K573P.

In an alternative embodiment, the polypeptide may comprise alterations at two or more (several) positions selected from positions corresponding to positions (a) 492 and 580; (b) 492 and 574; (c) 492 and 550; (d) 550 and 573; (e) 550 and 574; (f) 550 and 580 in SEQ ID NO. 2, as disclosed in WO 2014/072481 (incorporated herein by reference).

In an alternative embodiment, the conjugation-competent polypeptide may comprise: (i) an N-terminal region comprising a first albumin which is a human albumin variant, in which the N-terminal of the first albumin comprises all amino acids of the human albumin variant except the C-terminal 2 to 30 amino acids; and (ii) a C-terminal region of a second albumin, which is selected from macaque albumin, mouse albumin, rabbit albumin, sheep albumin, human albumin, goat albumin, chimpanzee albumin, hamster albumin, guinea pig albumin, rat albumin, cow albumin, horse albumin, donkey albumin, dog albumin, chicken albumin, or pig albumin, or a variant thereof, in which the C-terminal of the second albumin or albumin variant comprises the C-terminal 2 to 30 amino acids of the second albumin or albumin variant; wherein the polypeptide has (i) an altered plasma half-life compared with the human albumin variant and/or (ii) an altered binding affinity to FcRn compared with the human albumin variant, as disclosed in WO 2012/059486 (incorporated herein by reference).

In an alternative embodiment, the polypeptide may comprise one or more (*e.g.* several) alterations in Domain I of the mature human albumin polypeptide sequence of SEQ ID NO. 2; and one or more (*e.g.* several) alterations in Domain III of the mature human albumin polypeptide sequence of SEQ ID NO. 2, wherein the one or more (*e.g.* several) alterations cause the polypeptide to have an altered binding affinity to FcRn, as disclosed in WO 2013/135896 (incorporated herein by reference). Suitably, the alteration(s) in Domain I are selected from positions corresponding to any of positions 78 to 120 of SEQ ID NO. 2, such as any of positions 78 to 88 and/or from any of 105 to 120; and the alteration(s) in Domain III are selected from positions corresponding to any of positions 425, 505, 510, 512, 524, 527, 531, 534, 569, 573, 575 of SEQ ID NO. 2. Suitably, the alteration at the position corresponding to positions 78 to 120 or 425, 505, 510, 512, 524, 527, 531, 534, 569, 573, and/or 575 of SEQ ID NO. 2 is a substitution; and the alteration is optionally a substitution selected from (i) 83N, K or S; (ii) 111D, G, H, R, Q or E; or (iii) 573P, Y, W, H, F, T, I or V.

In an alternative embodiment, the polypeptide may comprise one or more (*e.g.* several) alterations in Domain II of the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions 349, 342, 381, 345, 384, 198, 206, 340, 341, 343, 344, 352, 382, 348, and/or 383 in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn, as disclosed in WO 2015/036579 (incorporated herein by reference). Suitably, the alteration at the position corresponding to position 349, 342, 381, 345, 384, 198, 206, 340, 341, 343, 344, 352, 382, 348,

and/or 383 is a substitution; and the alteration is optionally a substitution selected from (i) 349F, W, Y, H, P, K or Q, preferably F; (ii) 342Y, W, F, H, T, N, Q, A, C, I, L, P, V, preferably Y; (iii) 381G or A, preferably G; or (iv) 345E, H, I or Q.

In an alternative embodiment, the polypeptide may comprise a variant Domain III of an albumin, or fragment thereof, comprising a mutation, such as a substitution, corresponding to one or more (*e.g.* several) positions corresponding to V418, T420, V424, E505 and V547 of SEQ ID NO. 2. These mutations are disclosed in WO 2013/075066 (incorporated herein by reference). Substitutions may be at one, two or more (several, *e.g.* at two, three, four, or five) of the positions corresponding to V418, T420, V424, E505 and V547; for example, there may be one or more (*e.g.* several) substitutions selected from V418M, T420A, V424I, E505(R/K/G) and V547A. In a particular embodiment, the albumin comprises the substitutions V418M, T420A and E505R; or V418M, T420A, E505G and V547A. The albumin may comprise one or more (*e.g.* several) additional substitutions at positions selected from N429, M446, A449, T467, and A552; such as selected from N429D, M446V, A449V, T467M, and A552T.

In an alternative embodiment, the variant may comprise a variant Domain III of an albumin, or fragment thereof, comprising one to eighteen amino acid substitutions to increase one or both of affinity for FcRn and serum half-life of the polypeptide, as disclosed in WO 2011/103076 (incorporated herein by reference). Substitutions may be at any one or more (*e.g.* several) of positions corresponding to positions 381, 383, 391, 401, 402, 407, 411, 413, 414, 415, 416, 424, 426, 434, 442, 445, 447, 450, 454, 455, 456, 457, 459, 463, 495, 506, 508, 509, 511, 512, 515, 516, 517, 519, 521, 523, 524, 525, 526, 527, 531, 535, 538, 539, 541, 557, 561, 566 or 569 of SEQ ID NO. 2. Suitable substitutions may be selected from V381N, V381Q, E383A, E383G, E383I, E383L, E383V, N391A, N391G, N391I, N391L, N391V, Y401D, Y401E, K402A, K402G, K402I, K402L, K402V, L407F, L407N, L407Q, L407W, L407Y, Y411Q, Y411N, K413C, K413S, K413T, K414S, K414T, V415C, V415S, V415T, Q416H, Q416P, V424A, V424G, V424I, V424L, V424N, V424Q, V426D, V426E, V426H, V426P, G434C, G434S, G434T, E442K, E442R, R445F, R445W, R445Y, P447S, P447T, E450D, E450E, S454C, S454M, S454T, V455N, V455Q, V456N, V456Q, L457F, L457W, L457Y, Q459K, Q459R, L463N, L463Q, E495D, T506F, T506W, T506Y, T508K, T508R, T508S, F509C, F509I, F509L, F509M, F509V, F509W, F509Y, A511F, A511W, A511Y, D512F, D512W, D512Y, T515C, T515H, T515N, T515P, T515Q, T515S, L516F, L516S, L516T, L516W, L516Y, S517C, S517F, S517M, S517T, S517W, S517Y, K519A, K519G, K519I, K519L, K519V, R521F, R521W, R521Y, I523A, I523D, I523E, I523F, I523G, I523K, I523L, I523N, I523Q, I523R, I523V, I523W, I523Y, K524A, K524G, K524I, K524L, K524V, K525A, K525G, K525I, K525L, K525V, Q526C, Q526M, Q526S, Q526T, Q526Y, T527F, T527W, T527Y, E531A, E531G, E531I, E531L, E531V, H535D, H535E, H535P, K538F, K538W, K538Y, A539I, A539L, A539V, K541F, K541W, K541Y, K557A, K557G, K557I, K557L, K557V, A561F, A561W, A561Y, T566F,

T566W, T566Y, A569H, and A569P; such as selected from L407N, L407Y, V415T, V424I, V424Q, V426E, V426H, P447S, V455N, V456N, L463N, E495D, T506Y, T508R, F509M, F509W, A511F, D512Y, T515Q, L516T, L516W, S517W, R521W, I523D, I523E, I523G, I523K, I523R, K524L, Q526M, T527Y, H535P and K557G.

The variant may comprise a variant Domain III of an albumin, or fragment thereof, comprising amino acid substitutions at positions corresponding to the following positions of SEQ ID NO. 2: (a) residues 383 and 413; (b) residues 401 and 523; (c) residues 407 and 447; (d) residues 407 and 447 and 539; (e) residues 407 and 509; (f) residues 407 and 526; (g) residues 411 and 535; (h) residues 414 and 456; (i) residues 415 and 569; (j) residues 426 and 526; (k) residues 442 and 450 and 459; (l) residues 463 and 508; (m) residues 508 and 519 and 525; (n) residues 509 and 527; (o) residues 523 and 538; (p) residues 526 and 557; (q) residues 541 and 561; (r) residues 463 and 523; (s) residues 508 and 523; (t) residues 508 and 524; (u) residues 463, 508 and 523; (v) residues 463, 508 and 524; (w) residue 508, 523 and 524; (x) residue 463, 508, 523 and 524; (y) residues 463 and 524; (z) residues 523 and 524; and (aa) residues 463, 523, and 524, wherein the substitutions increase one or both of affinity for FcRn and serum half-life of the polypeptide, as disclosed in WO 2012/112188 (incorporated herein by reference). Suitable substitutions may be selected from (a) L463C, F, G, H, I, N, S or Q; (b) T508C, E, I, K, R or S; (c) I523A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W or Y; (d) K524A, F, G, H, I, L, M, Q, T or V; (e) L463F or N; (f) T508R or S; (g) I523D, E, F, G, K or R; and (h) K524L.

The variant albumin may comprise one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V418, T420, V424, E505, V547, K573 in SEQ ID NO. 2; wherein the one or more (several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

The variant albumin may comprise one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V381, preferably V381N or Q; E383, preferably E383A, G, I, L, or V; N391, preferably N391A, G, I, L or V; Y401 preferably Y401D or E; K402, preferably K402A, G, I, L, or V; L407, preferably L407F, N, Q, W, or Y; Y411, preferably Y411Q, or N; K413, preferably K413C, S, or T; K414, preferably K414S or T; V415C, preferably V415C, S, or T; Q416, preferably Q416H or P; V424, preferably V424A, G, I, L, N, or Q; V426D, preferably V426D, E, H, or P; G434, preferably G434C, S, or T; E442, preferably E442K or R; R445, preferably R445F, W or Y; P447, preferably P447S or T; E450, preferably E450D or E; S454, preferably S454C, M or T; V455, preferably V455N or Q; V456, preferably V456N or Q; L457, preferably L457F, W or Y; Q459, preferably Q459K or R; L463, preferably L463N or Q; E495, preferably E495D; T506, preferably T506F, W or Y; T508, preferably T508K, R, or S; F509,

preferably F509C, I, L, M, V, W or Y; A511, preferably A511F, W, or Y; D512, preferably D512F, W or Y; T515, preferably T515C, H, N, P, Q or S; L516, preferably L516F, S, T, W or Y; S517, preferably S517C, F, M, T, W or Y; K519, preferably K519A, G, I, L, or V; R521, preferably R521F, W or Y; I523, preferably I523A, D, E, F, G, K, L, N, Q, R, V, W or Y; K524, preferably K524A, G, I, L or V; K525, preferably K525A, G, I, L or V; Q526, preferably Q526C, M, S, T or Y; T527, preferably T527F, W or Y; E531, preferably E531A, G, I, L or V; H535, preferably H535D, E or P; K538, preferably K538F, W or Y; A539, preferably A539I, L or V; K541, preferably, K541F, W or Y; K557, preferably K557A, G, I, L or V; A561, preferably A561F, W or Y; T566, preferably T566F, W or Y; A569, preferably A569H or P in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

The variant albumin may comprise one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V547, preferably V457A; K573, preferably K573P or Y; I523, preferably I523A or G, T527, preferably T527M, K500, preferably K500A; or E505, preferably E505Q in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

The variant albumin may comprise one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions 573, 523, 527 or 505 of SEQ ID NO. 2, preferably K573Y; I523G; I523A; T527M; E505Q; or K573P, for example K573Y and I523G; K573Y, I523G and T527M; K573Y, E505Q and T527M; K573Y and T527M; K573P and I523G; K573P, I523G and T527M; K573P, E505Q and T527M; K573P and T527M; V547A; V547A and K573P; V547A, E505Q, K573P and T527M; or K500A and H510Q of SEQ ID NO. 2.

Other modifications

The second aspect of the invention encompasses other modifications. For example, the polypeptide may or may not comprise at least one (*e.g.* several) mutation that reduces glycosylation.

**Fusion polypeptide**

A third aspect of the invention provides a fusion polypeptide comprising a conjugation-competent polypeptide of either the first or the second aspect of the invention.

Polypeptides of the invention may be fused with a non-albumin polypeptide fusion partner. The fusion partner may in principle be any polypeptide but generally it is preferred that the fusion partner is a polypeptide having bioactive, therapeutic, prophylactic (including

vaccine), diagnostic, imaging or other beneficial properties. Such properties may be referred to as 'pharmaceutically beneficial properties'. Fusion polypeptides comprising albumin or fragments thereof are known in the art. It has been found that such fusion polypeptides comprising albumin or a fragment thereof and a fusion partner polypeptide have a longer plasma half-life compared to the unfused fusion partner polypeptide alone.

One or more (*e.g.* several) bioactive, therapeutic, prophylactic (including vaccine), diagnostic, imaging or other beneficial polypeptides may be fused to the N-terminus, the C-terminus of albumin, inserted into a loop in the albumin structure or any combination thereof. It may or it may not comprise linker sequences separating the various components of the fusion polypeptide. By way of non-limiting examples, a fusion may comprise N'-partner-albumin-C', N'-albumin-partner-C', N'-albumin-partner-albumin-C', N'- partner-albumin- partner-C' where 'partner' is the fusion partner.

Teachings relating to fusions of albumin or a fragment thereof are known in the art and the skilled person will appreciate that such teachings can also be applied to the invention. WO 2001/79271A (particularly page 9 and/or Table 1), WO 2003/59934 (particularly Table 1), WO 03/060071 (particularly Table 1) and WO 01/079480 (particularly Table 1) (each incorporated herein by reference in their entirety) also contain examples of bioactive, therapeutic, prophylactic (including vaccine), diagnostic, imaging or other beneficial polypeptides that may be fused to albumin or fragments thereof, and these examples apply also to the invention.

An advantage of using a genetically or chemically fused albumin is that either or all of the molecules which contribute to the fusion may have improved properties relative to the unfused molecule(s) (Balan *et al*. (2006), Antivir Ther 11(1): 35-45). Albumins and albumin particles are also important for carrying and delivering drugs and prodrugs to their sites of action (Kratz, F. (2008), Journal of Controlled Release, 132 (3), p.171-183). Fusion and particle technologies offer improved dosing regimens due to improved pharmacokinetic properties, such as half-life extension, and may improve bioavailability and protect the fused conjugation partner, for example bioactive molecule, radiopharmaceutical or imaging agent, from inactivation.

The polypeptide may also be fused to one or more (*e.g.* several) purification tags such as $(Ala\text{-}Trp\text{-}Trp\text{-}Pro)_n$, avidin/streptavidin/Strep-tag, FLAG™ peptide (DYKDDDDK), His-tag.

Further preferences for the third aspect of the invention include those of the first and second aspects of the invention. The skilled person understands that any aspect of the invention may be combined with another aspect or aspects of the invention and/or with one or more (*e.g.* several) of the preferences for the aspects of the invention and/or other disclosures made herein.

**Polynucleotides**

A fourth aspect of the invention provides a polynucleotide which encodes the polypeptide according to the first, second or third aspects of the invention.

The polynucleotide may be an isolated polynucleotide. The polynucleotide may be comprised in a vector (such as a plasmid) and/or in a host cell.

The polynucleotide may or may not be codon-optimised relative to the host from which it is to be expressed. SEQ ID NO. 1 provides the usual coding sequence of HSA (SEQ ID NO. 2). SEQ ID NO. 28 provides a coding sequence of HSA (SEQ ID NO. 1) which is codon-optimised for expression from *S. cerevisiae*. SEQ ID NO. 1 or SEQ ID NO. 28 may be mutated in order to provide a polynucleotide which encodes a polypeptide according to the invention. Preferably the polynucleotide is synthetic and/or recombinant. Preferably the polynucleotide is an isolated polynucleotide. The polynucleotide may encode an HSA with or without a leader sequence. For example, the polynucleotide may encode an HSA with the natural leader sequence of HSA (amino acids 1 to 24 of SEQ ID NO. 3) or an HSA with a fusion leader sequence (amino acids 1 to 24 of SEQ ID NO. 29).

The polypeptide may be provided as a nucleic acid construct comprising a polynucleotide encoding a polypeptide of the invention operably linked to one or more (*e.g.* several) control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

A polynucleotide may be manipulated in a variety of ways to provide for expression of a variant. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

The control sequence may be a promoter sequence, which is recognized by a host cell for expression of the polynucleotide. The promoter sequence contains transcriptional control sequences that mediate the expression of the variant. The promoter may be any nucleic acid sequence that shows transcriptional activity in the host cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* protease A (PRA1), *Saccharomyces cerevisiae* protease B (PRB1), *Saccharomyces cerevisiae* translation elongation factor (TEF1), *Saccharomyces cerevisiae* translation elongation factor (TEF2), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces*

*cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-488.

The skilled person knows useful promoters for use in rice and mammalian cells, such as CHO or HEK. In a rice host, useful promoters are obtained from cauliflower mosaic virus 35S RNA gene (CaMV35S), maize alcohol dehydrogenase (Adh1) and alpha Amy3.

In a mammalian host cell, such as CHO or HEK, useful promoters are obtained from Cytomegalovirus (CMV) and CAG hybrid promoter (hybrid of CMV early enhancer element and chicken beta-actin promoter), Simian vacuolating virus 40 (SV40).

The control sequence may also be a suitable transcription terminator sequence, which is recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3'-terminus of the polynucleotide encoding the variant. Any terminator that is functional in the host cell may be used.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), *Saccharomyces cerevisiae* alcohol dehydrogenase (ADH1) and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*. The skilled person knows useful terminators for use in rice and mammalian cells, such as CHO or HEK. For example, in a rice host, preferred terminators are obtained from *Agrobacterium tumefaciens* nopaline synthase (Nos) and cauliflower mosaic virus 35S RNA gene (CaMV35S).

The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5'-terminus of the polynucleotide encoding the variant. Any leader sequence that is functional in the host cell may be used.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the variant-encoding sequence and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a variant and directs the variant into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a

signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the variant. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding region that is foreign to the coding sequence. The foreign signal peptide coding region may be required where the coding sequence does not naturally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the variant. However, any signal peptide coding region that directs the expressed variant into the secretory pathway of a host cell may be used.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*. The skilled person knows useful signal peptides for use in rice and mammalian cells, such as CHO or HEK.

Where both signal peptide and propeptide regions are present at the N-terminus of a variant, the propeptide region is positioned next to the N-terminus of the variant and the signal peptide region is positioned next to the N-terminus of the propeptide region.

**Plasmids**

A fifth aspect of the invention provides a plasmid comprising the polynucleotide of the fourth aspect of the invention. The plasmid may be a 2 micron based plasmid such as those described in WO 2005/061719, WO 2005/061718 and WO 2006/067511 (all incorporated herein by reference). The plasmid may exhibit enhanced chaperone activity, for example through over expression of a chaperone, particularly PDI. Preferred helper proteins include *PDI1*, *AHA1*, *ATP11*, *CCT2*, *CCT3*, *CCT4*, *CCT5*, *CCT6*, *CCT7*, *CCT8*, *CNS1*, *CPR3*, *CPR6*, *DER1, DER3, DOA4*, *ERO1*, *EUG1*, *ERV2, EPS1, FKB2*, *FMO1*, *HCH1*, *HRD3*, *HSP10*, *HSP12*, *HSP104*, *HSP26*, *HSP30*, *HSP42*, *HSP60*, *HSP78*, *HSP82*, *KAR2*, *JEM1*, *MDJ1*, *MDJ2*, *MPD1*, *MPD2*, *PDI1*, *PFD1*, *ABC1*, *APJ1*, *ATP11*, *ATP12*, *BTT1*, *CDC37*, *CPR7*, *HSC82*, *KAR2*, *LHS1*, *MGE1*, *MRS11*, *NOB1*, *ECM10*, *SCJ1*, *SSA1*, *SSA2*, *SSA3*, *SSA4*, SSB1, SSB2, *SSC1*, *SSE2*, *SIL1*, *SLS1*, *ORM1*, *ORM2*, *PER1*, *PTC2, PSE1, UBC7, UBI4* and *HAC1* or a truncated intronless *HAC1* (Valkonen *et al*. 2003, *Applied Environ. Micro*., 69, 2065). Such helper proteins are disclosed in WO 2005/061718, WO 2006/067511 and WO 2006/136831 (all incorporated herein by reference).

**Host cells**

A sixth aspect of the invention provides an expression system such as a host cell comprising a polynucleotide according to the fourth aspect of the invention and/or a plasmid of the fifth aspect of the invention. Preferably the host cell is a mammalian cell such as a human or bovine cell, or a fungal cell such as a yeast cell. Alternatively, the host cell may be a

bacterial cell such as a *Bacillus* or *Escherichia coli* or a viral cell such as Baculovirus or a plant cell such as a rice *e.g. Oryza sativa*. Most preferably, the cell is a yeast cell such as a *Saccharomyces* (*e.g. S. cerevisiae*), a *Pichia* or an Aspergillus cell.

**Conjugates**

A seventh aspect of the invention provides a conjugate which comprises a conjugation partner, such as a bioactive compound, radiopharmaceutical or imaging agent, and a polypeptide according to the first, second or third aspect of the invention, wherein the conjugation partner is linked to the polypeptide through a conjugation-competent cysteine residue of the polypeptide. The conjugation partner may be a bioactive, therapeutic, diagnostic or imaging compound such as those mentioned herein. The conjugate may comprise 2 or more, (several, for example 2, 3, 4, 5, 6, 7,8, 9 or 10), conjugation partners which may each be different and/or may be multiple copies of the same compound. Preferably, each conjugation partner is linked to the polypeptide through a conjugation-competent cysteine residue of the polypeptide, however conjugation partners may be linked by other means for example by a genetic fusion or covalent bonds to non-cysteine amino acids such as lysine.

A related aspect provides a use of a polypeptide according to the invention for the production of a thio-albumin-conjugate.

*Conjugation partner*

The term 'conjugation partner' includes bioactive agents, imaging agents, diagnostic agents, contrast agents, radiopharmaceuticals and therapeutic compounds such as chemotherapeutic drugs and radiopharmaceuticals. A thio-albumin of the invention may be conjugated to one or more (*e.g.* several) conjugation partners.

*Imaging agents, diagnostic compounds, contrast agents and therapeutic compounds*

The use of diagnostic agents, imaging agents and biological "contrast" agents are well known to the art. A diagnostic agent is any pharmaceutical product used as part of a diagnostic test (*i.e.* together with the equipment and procedures that are needed to assess the test result). The diagnostic agent may be used *in vivo, ex vivo* or *in vitro*.

The ability of albumin to accumulate in damaged muscle fibres of dystrophic muscle has been well described. For example, a Gadolinium-DTPA-albumin conjugate may be used as a combined diagnostic and therapeutic tool to visualize and monitor, for example, dystrophic muscle by magnetic resonance imaging (MRI) and for the delivery of putative therapeutics bound to albumin for effective targeting to dystrophic muscle (Amthor *et al.* (2004), Neuromuscular Disorders 14912: 791-796). Malignant tumours often show an increased uptake and metabolism of albumin. The use of gadolinium-albumin conjugate has also been described

for improved imaging of malignant tumours and to determine by MRI tumours sensitive to a therapy with drug-conjugated albumin (Kiessling *et al.* (2002), Investigative Radiology 37(4): 93-198).

Current imaging agents often degrade quickly whilst longer-lasting agents are often toxic. The use of albumin conjugates may be especially useful to increase the half-life of imaging agents and would therefore permit imaging over an extended period of time. WO 2005/082423 (incorporated herein by reference) describes the use of serum albumin conjugated to fluorescent substances for imaging.

A thio-albumin of this invention may be conjugated to two or more (several) molecules selected from bioactive, imaging agents, diagnostic agents, therapeutic compounds and contrast agents.

Tumours (and muscle degeneration) show enhanced uptake of albumin (EPR: Enhanced Permeation and Retention). Albumin conjugates may be used for enhanced imaging, and also to assess whether tumours (or other tissues and organs) would be suitable for albumin conjugated drugs.

*Bioactive compounds*

The bioactive compound may be a therapeutic or diagnostic compound. The therapeutic compound may be a chemotherapy drug for use in cancer chemotherapy. It may be cytostatic or cytotoxic; it may be a tumor-inhibiting agent.

The bioactive compound may already contain a free thiol group, *e.g.* a polypeptide containing a Cysteine residue with a free thiol group. Alternatively, the bioactive compound may be modified so as to contain a free thiol group. Thus, the amino acid sequence of a polypeptide may be altered so as to include a Cysteine residue with a free thiol group, or the bioactive compound may be chemically derivatized to include a free thiol group.

The bioactive compound may be a polypeptide (protein), particularly a recombinant protein pharmaceutical. It may be a chemotherapy or radiotherapy drug used to treat cancers and other related diseases.

The free thiol containing albumin mutein of the invention (thio-albumin) can be conjugated *via* the free thiol group, or groups if the albumin mutein of the invention contains more than one free thiol, to at least one (*e.g.* several) bioactive compound by methods know to the art. The bioactive compound includes but is not limited to, peptides, polypeptides or proteins (either natural, recombinant, or synthetic) (Debinski, (2002) Cancer Investigation 20, 801-809, O'Keefe and Draper *et al.*, (1985) JBC 260, 932-937, Xia *et al.*, (2000) J. Pharmacology Experimental Therapeutics 295, 594-600, Kavimandan *et al.*, (2006), Bioconjugate Chem. 17, 1376-1384, Humphries, *et al.*, (1994) J. Tissue Culture Methods 16, 239-242, Wenning *et al.*, (1998) Biotech. Bioeng. 57, 484-496, Yazdi and Murphy, (1994),

Cancer Research 54, 6387-6394, Weaver and Laske (2003) J. Neuro-Oncology 65, 3-13, Widera *et al.*, (2003) Pharmaceutical Research 20, 1231-1238, Daniels, T.R. *et al.* (2006) Clinical Immunology 121, 159-176 and the references included therein); therapeutic and diagnostic drugs or compounds (Mishra *et al.*, (2006) J. Drug Targeting 14, 45-53, Lim and Shen, (2004) Pharmaceutical Research 21, 1985-1992, Fritzer *et al.*, (1996) Biochemical Pharmacology 51, 489-493, Lubgan and Jozwiak (2002) Cell. Mol. Biol. Lett. 7, 98, Daniels, T.R. *et al.* (2006) Clinical Immunology 121, 159-176 and the references included therein); high molecular weight complexes including but not limited to liposomes, viruses and nanoparticles (Mishra *et al.*, (2006) J. Drug Targeting 14, 45-53, Daniels, T.R. *et al.* (2006) Clinical Immunology 121, 159-176 and the references included therein); nucleic acids and radionuclides, including DNA, RNA (including siRNA) and their analogs (Lee *et al.*, (2005), Arch. Pharm. Res. 28, 722-729, Huang *et al.*, (2007) FASEB J. 21, 1117-1125, Daniels, T.R. *et al.* (2006) Clinical Immunology 121, 159-176 and the references included therein) and devices (Humphries, *et al.*, (1994) J. Tissue Culture Methods 16, 239-242 and the references included therein). Additionally the entity can itself be modified by methods known to the art.

*Therapeutic compounds*

Examples of therapeutic compounds include: 4-1BB ligand, 5-helix, A human C-C chemokine, A human L105 chemokine, A human L105 chemokine designated huL105_3, A monokine induced by gamma-interferon (MIG), A partial CXCR4B protein, A platelet basic protein (PBP), α1-antitrypsin, ACRP-30 Homologue, Complement Component C1q C, Adenoid-expressed chemokine (ADEC), aFGF, FGF-1, AGF, AGF Protein, albumin, an etoposide, angiostatin, Anthrax vaccine, Antibodies specific for collapsin, antistasin, Anti-TGF beta family antibodies, antithrombin III, APM-1, ACRP-30, Famoxin, apo-lipoprotein species, Arylsulfatase B, b57 Protein, BCMA, Beta-thromboglobulin protein (beta-TG), bFGF, FGF2, Blood coagulation factors, BMP Processing Enzyme Furin, BMP-10, BMP-12, BMP-15, BMP-17, BMP-18, BMP-2B, BMP-4, BMP-5, BMP-6, BMP-9, Bone Morphogenic Protein-2, calcitonin, Calpain-10a, Calpain-10b, Calpain-10c, Cancer Vaccine, Carboxypeptidase, C-C chemokine, MCP2, CCR5 variant, CCR7, CCR7, CD11a Mab, CD137, 4-1BB Receptor Protein, CD20 Mab, CD27, CD27L, CD30, CD30 ligand, CD33 immunotoxin, CD40, CD40L, CD52 Mab, Cerebus Protein, Chemokine Eotaxin, Chemokine hIL-8, Chemokine hMCP1, Chemokine hMCP1a, Chemokine hMCP1b, Chemokine hMCP2, Chemokine hMCP3, Chemokine hSDF1b, Chemokine MCP-4, chemokine TECK and TECK variant, Chemokine-like protein IL-8M1 Full-Length and Mature, Chemokine-like protein IL-8M10 Full-Length and Mature, Chemokine-like protein IL-8M3, Chemokine-like protein IL-8M8 Full-Length and Mature, Chemokine-like protein IL-8M9 Full-Length and Mature, Chemokine-like protein PF4-414 Full-Length and Mature, Chemokine-like protein PF4-426 Full-Length and Mature, Chemokine-like protein PF4-M2 Full-Length and

Mature, Cholera vaccine, Chondromodulin-like protein, c-kit ligand, SCF, Mast cell growth factor, MGF, Fibrosarcoma-derived stem cell factor, CNTF and fragment thereof (such as CNTFAx15`(Axokine™)), coagulation factors in both pre and active forms, collagens, Complement C5 Mab, Connective tissue activating protein-III, CTAA16.88 Mab, CTAP-III, CTLA4-Ig, CTLA-8, CXCR3, CXC chemokine receptor 3, cyanovirin-N, Darbepoetin, designated exodus, designated huL105_7, DIL-40, Dnase, EDAR, EGF Receptor Mab, ENA-78, Endostatin, Eotaxin, Epithelial neutrophil activating protein-78, EPO receptor, EPOR, erythropoietin (EPO) and EPO mimics, Eutropin, Exodus protein, Factor IX, Factor VII, Factor VIII, Factor X and Factor XIII, FAS Ligand Inhibitory Protein (DcR3), FasL, FGF, FGF-12, Fibroblast growth factor homologous factor-1, FGF-15, FGF-16, FGF-18, FGF-3, INT-2, FGF-4, gelonin, HST-1, HBGF-4, FGF-5, FGF-6, Heparin binding secreted transforming factor-2, FGF-8, FGF-9, Glia activating factor, fibrinogen, flt-1, flt-3 ligand, Follicle stimulating hormone Alpha subunit, Follicle stimulating hormone Beta subunit, Follitropin, Fractalkine, fragment. myofibrillar protein Troponin I, FSH, Galactosidase, Galectin-4, G-CSF, GDF-1, Gene therapy, Glioma-derived growth factor, glucagon, glucagon-like peptides, Glucocerebrosidase, glucose oxidase, Glucosidase, Glycodelin-A, Progesterone-associated endometrial protein, GM-CSF, gonadotropin, Granulocyte chemotactic protein-2 (GCP-2), Granulocyte-macrophage colony stimulating factor, growth hormone, Growth related oncogene-alpha (GRO-alpha), Growth related oncogene-beta (GRO-beta), Growth related oncogene-gamma (GRO-gamma), hAPO-4, TROY, hCG, Hepatitus B surface Antigen, Hepatitus B Vaccine, HER2 Receptor Mab, hirudin, HIV gp120, HIV gp41, HIV Inhibitor Peptide, HIV Inhibitor Peptide, HIV Inhibitor Peptide, HIV protease inhibiting peptides, HIV-1 protease inhibitors, HPV vaccine, Human 6CKine protein, Human Act-2 protein, Human adipogenesis inhibitory factor, human B cell stimulating factor-2 receptor, Human beta-chemokine H1305 (MCP-2), Human C-C chemokine DGWCC, Human CC chemokine ELC protein, Human CC type chemokine interleukin C, Human CCC3 protein, Human CCF18 chemokine, Human CC-type chemokine protein designated SLC (secondary lymphoid chemokine), Human chemokine beta-8 short forms, Human chemokine C10, Human chemokine CC-2, Human chemokine CC-3, Human chemokine CCR-2, Human chemokine Ckbeta-7, Human chemokine ENA-78, Human chemokine eotaxin, Human chemokine GRO alpha, Human chemokine GROalpha, Human chemokine GRObeta, Human chemokine HCC-1, Human chemokine HCC-1, Human chemokine I-309, Human chemokine IP-10, Human chemokine L105_3, Human chemokine L105_7, Human chemokine MIG, Human chemokine MIG-beta protein, Human chemokine MIP-1alpha, Human chemokine MIP1beta, Human chemokine MIP-3alpha, Human chemokine MIP-3beta, Human chemokine PF4, Human chemokine protein 331D5, Human chemokine protein 61164, Human chemokine receptor CXCR3, Human chemokine SDF1alpha, Human chemokine SDF1beta, Human chemokine ZSIG-35, Human Chr19Kine protein, Human CKbeta-9, Human CX3C 111 amino acid

chemokine, Human DNAX interleukin-40, Human DVic-1 C-C chemokine, Human EDIRF I protein sequence, Human EDIRF II protein sequence, Human eosinocyte CC type chemokine eotaxin, Human eosinophil-expressed chemokine (EEC), Human fast twitch skeletal muscle troponin C, Human fast twitch skeletal muscle troponin I, Human fast twitch skeletal muscle Troponin subunit C, Human fast twitch skeletal muscle Troponin subunit I Protein, Human fast twitch skeletal muscle Troponin subunit T, Human fast twitch skeletal muscle troponin T, Human foetal spleen expressed chemokine, FSEC, Human GM-CSF receptor, Human gro-alpha chemokine, Human gro-beta chemokine, Human gro-gamma chemokine, Human IL-16 protein, Human IL-1RD10 protein sequence, Human IL-1RD9, Human IL-5 receptor alpha chain, Human IL-6 receptor, Human IL-8 receptor protein hIL8RA, Human IL-8 receptor protein hIL8RB, Human IL-9 receptor protein, Human IL-9 receptor protein variant #3, Human IL-9 receptor protein variant fragment, Human IL-9 receptor protein variant fragment #3, Human interleukin 1 delta, Human interleukin 10, Human interleukin 18, Human interleukin 18 derivatives, Human interleukin-1 beta precursor, Human interleukin-1 beta precursor, Human interleukin-1 receptor accessory protein, Human interleukin-1 receptor antagonist beta, Human interleukin-1 type-3 receptor, Human interleukin-10 (precursor), Human interleukin-11 receptor, Human interleukin-12 40 kD subunit, Human interleukin-12 beta-1 receptor, Human interleukin-12 beta-2 receptor, Human interleukin-12 p35 protein, Human interleukin-12 p40 protein, Human interleukin-12 receptor, Human interleukin-13 alpha receptor, Human interleukin-13 beta receptor, Human interleukin-15, Human interleukin-15 receptor from clone P1, Human interleukin-17 receptor, Human interleukin-18 protein (IL-18), Human interleukin-3, human interleukin-3 receptor, Human interleukin-3 variant, Human interleukin-4 receptor, Human interleukin-5, Human interleukin-6, Human interleukin-7, Human interleukin-7, Human interleukin-8 (IL-8), Human intracellular IL-1 receptor antagonist, Human IP-10 and HIV-1 gp120 hypervariable region fusion protein, Human IP-10 and human Muc-1 core epitope (VNT) fusion protein, human liver and activation regulated chemokine (LARC), Human Lkn-1 Full-Length and Mature protein, Human mammary associated chemokine (MACK) protein Full-Length and Mature, Human mature chemokine Ckbeta-7, Human mature gro-alpha, Human mature gro-gamma polypeptide used to treat sepsis, Human MCP-3 and human Muc-1 core epitope (VNT) fusion protein, Human MI10 protein, Human MI1A protein, Human monocyte chemoattractant factor hMCP-1, Human monocyte chemoattractant factor hMCP-3, Human monocyte chemotactic proprotein (MCPP) sequence, Human neurotactin chemokine like domain, Human non-ELR CXC chemokine H174, Human non-ELR CXC chemokine IP10, Human non-ELR CXC chemokine Mig, Human PAI-1 mutants, Human protein with IL-16 activity, Human protein with IL-16 activity, Human secondary lymphoid chemokine (SLC), Human SISD protein, Human STCP-1, Human stromal cell-derived chemokine, SDF-1, Human T cell mixed lymphocyte reaction expressed chemokine (TMEC), Human thymus and activation regulated cytokine (TARC), Human thymus

expressed, Human TNF-alpha, Human TNF-beta (LT-alpha), Human type CC chemokine eotaxin 3 protein sequence, Human type II interleukin-1 receptor, Human wild-type interleukin-4 (hIL-4) protein, Human ZCHEMO-8 protein, Humanized Anti-VEGF Antibodies, and fragments thereof, Humanized Anti-VEGF Antibodies, and fragments thereof, Hyaluronidase, ICE 10 kD subunit, ICE 20 kD subunit, ICE 22 kD subunit, Iduronate-2-sulfatase, Iduronidase, IL-1 alpha, IL-1 beta, IL-1 inhibitor (IL-1i), IL-1 mature, IL-10 receptor, IL-11, IL-11, IL-12 p40 subunit, IL-13, IL-14, IL-15, IL-15 receptor, IL-17, IL-17 receptor, IL-19, IL-1i fragments, IL1-receptor antagonist, IL-21 (TIF), IL-3 containing fusion protein, IL-3 mutant proteins, IL-3 variants, IL-4, IL-4 muteins, IL-4 mutein Y124G, IL-4 mutein Y124X, IL-5, IL-5 muteins, Il-5 receptor, IL-6, Il-6 receptor, IL-7 receptor clone, IL-8 receptor, IL-9 mature protein variant (Met117 version), immunoglobulins or immunoglobulin-based molecules or fragment of either (*e.g.* a Small Modular ImmunoPharmaceutical™ ("SMIP") or dAb, Fab' fragments, F(ab')2, scAb, scFv or scFv fragment), including but not limited to plasminogen, Influenza Vaccine, Inhibin alpha, Inhibin beta, insulin, insulin-like growth factor, Integrin Mab, inter-alpha trypsin inhibitor, inter-alpha trypsin inhibitor, Interferon gamma-inducible protein (IP-10), interferons (such as interferon alpha species and sub-species, interferon beta species and sub-species, interferon gamma species and sub-species), interleukin 6, interleukin 8 (IL-8) receptor, interleukin 8 receptor B, interleukin-1alpha, interleukin-2 receptor associated protein p43, interleukin-3, interleukin-4 muteins, interleukin-8 (IL-8) protein, interleukin-9, interleukin-9 (IL-9) mature protein (Thr117 version), interleukins (such as IL10, IL11 and IL2), Japanese encephalitis vaccine, Kalikrein Inhibitor, Keratinocyte growth factor, Kunitz domain protein (such as aprotinin, amyloid precursor protein and those described in WO 03/066824, with or without albumin fusions), LACI, lactoferrin, Latent TGF-beta binding protein II, leptin, Liver expressed chemokine-1 (LVEC-1), Liver expressed chemokine-2 (LVEC-2), LT-alpha, LT-beta, Luteinization Hormone, Lyme Vaccine, Lymphotactin, Macrophage derived chemokine analogue MDC (n+1), Macrophage derived chemokine analogue MDC-eyfy, Macrophage derived chemokine analogue MDC-yl, Macrophage-derived chemokine (MDC), Maspin, Protease Inhibitor 5, MCP-1 receptor, MCP-1a, MCP-1b, MCP-3, MCP-4 receptor, M-CSF, Melanoma inhibiting protein, Membrane-bound proteins, Met117 human interleukin 9, MIP-3 alpha, MIP-3 beta, MIP-Gamma, MIRAP, Modified Rantes, monoclonal antibody, MP52, Mutant interleukin 6 S176R, myofibrillar contractile protein Troponin I, Natriuretic Peptide, Nerve Growth Factor-beta, Nerve Growth Factor-beta2, Neuropilin-1, Neuropilin-2, Neurotactin, Neurotrophin-3, Neurotrophin-4, Neurotrophin-4a, Neurotrophin-4b, Neurotrophin-4c, Neurotrophin-4d, Neutrophil activating peptide-2 (NAP-2), NOGO-66 Receptor, NOGO-A, NOGO-B, NOGO-C, Novel beta-chemokine designated PTEC, N-terminal modified chemokine GroHEK/hSDF-1alpha, N-terminal modified chemokine GroHEK/hSDF-1beta, N-terminal modified chemokine met-hSDF-1 alpha, N-terminal modified chemokine met-hSDF-1 beta,

OPGL, Osteogenic Protein-1 (OP-1), BMP-7, Osteogenic Protein-2, OX40, ACT-4, OX40L, Oxytocin (Neurophysin I), parathyroid hormone, Patched, Patched-2, PDGF-D, Pertussis toxoid, Pituitary expressed chemokine (PGEC), Placental Growth Factor, Placental Growth Factor-2, Plasminogen Activator Inhibitor-1 (PAI-1), Plasminogen Activator Inhibitor-2 (PAI-2), Platelet derived growth factor, Platelet derived growth factor Bv-sis, Platelet derived growth factor precursor A, Platelet derived growth factor precursor B, Platelet Mab, platelet-derived endothelial cell growth factor (PD-ECGF), Platelet-Derived Growth Factor A chain, Platelet-Derived Growth Factor B chain, polypeptide used to treat sepsis, Preproapolipoprotein "milano" variant, Preproapolipoprotein "paris" variant, pre-thrombin, Primate CC chemokine "ILINCK", Primate CXC chemokine "IBICK", proinsulin, Prolactin, Prolactin2, prosaptide, Protease inhibitor peptides, Protein C, Protein S, pro-thrombin, prourokinase, RANTES, RANTES 8-68, RANTES 9-68, RANTES peptide, RANTES receptor, Recombinant interleukin-16, Resistin, restrictocin, Retroviral protease inhibitors, ricin, Rotavirus Vaccine, RSV Mab, saporin, sarcin, Secreted and Transmembrane polypeptides, serum cholinesterase, serum protein (such as a blood clotting factor), Soluble BMP Receptor Kinase Protein-3, Soluble VEGF Receptor, Stem Cell Inhibitory Factor, Straphylococcus Vaccine, Stromal Derived Factor-1 alpha, Stromal Derived Factor-1 beta, Substance P (tachykinin), T1249 peptide, T20 peptide, T4 Endonuclease, TACI, Tarc, TGF-beta 1, TGF-beta 2, Thr117 human interleukin 9, thrombin, thrombopoietin, thrombopoietin derivative 1, thrombopoietin derivative 2, thrombopoietin derivative 3, thrombopoietin derivative 4, thrombopoietin derivative 5, thrombopoietin derivative 6, thrombopoietin derivative 7, Thymus expressed chemokine (TECK), Thyroid stimulating Hormone, tick anticoagulant peptide, Tim-1 protein, TNF-alpha precursor, TNF-R, TNF-RII, TNF p75 Receptor, Death Receptor, tissue plasminogen activator (tPA), transferrin, transforming growth factor beta, Troponin peptides, Truncated monocyte chemotactic protein 2 (6-76), Truncated RANTES protein (3-68), tumour necrosis factor, Urate Oxidase, urokinase, Vasopressin (Neurophysin II), VEGF R-3, flt-4, VEGF Receptor, KDR, flk-1, VEGF-110, VEGF-121, VEGF-138, VEGF-145, VEGF-162, VEGF-165, VEGF-182, VEGF-189, VEGF-206, VEGF-D, VEGF-E, VEGF-X, von Willebrand's factor, Wild type monocyte chemotactic protein 2, ZTGF-beta 9.

*Chemotherapy drugs*

Examples of chemotherapy drugs include: 13-cis-Retinoic Acid, 2-CdA, 2-Chlorodeoxyadenosine, 5-Azacitidine, 5-Fluorouracil, 5-FU, 6-Mercaptopurine, 6-MP, 6-TG, 6-Thioguanine, Abraxane, Accutane®, Actinomycin-D, Adriamycin®, Adrucil®, Agrylin®, Ala-Cort®, Aldesleukin, Alemtuzumab, ALIMTA, Alitretinoin, Alkaban-AQ®, Alkeran®, All-transretinoic Acid, Alpha Interferon, Altretamine, Amethopterin, Amifostine, Aminoglutethimide, Anagrelide, Anandron®, Anastrozole, Arabinosylcytosine, Ara-C, Aranesp®, Aredia®, Arimidex®, Aromasin®, Arranon®, Arsenic Trioxide, Asparaginase, ATRA, Avastin®, Azacitidine, BCG, BCNU,

Bevacizumab, Bexarotene, BEXXAR®, Bicalutamide, BiCNU, Blenoxane®, Bleomycin, Bortezomib, Busulfan, Busulfex®, C225, Calcium Leucovorin, Campath®, Camptosar®, Camptothecin-11, Capecitabine, Carac™, Carboplatin, Carmustine, Carmustine Wafer, Casodex®, CC-5013, CCNU, CDDP, CeeNU, Cerubidine®, Cetuximab, Chlorambucil, Cisplatin, Citrovorum Factor, Cladribine, Cortisone, Cosmegen®, CPT-11, Cyclophosphamide, Cytadren®, Cytarabine, Cytarabine Liposomal, Cytosar-U®, Cytoxan®, Dacarbazine, Dacogen, Dactinomycin, Darbepoetin Alfa, Dasatinib, Daunomycin, Daunorubicin, Daunorubicin Hydrochloride, Daunorubicin Liposomal, DaunoXome®, Decadron, Decitabine, Delta-Cortef®, Deltasone®, Denileukin diftitox, DepoCyt™, Dexamethasone, Dexamethasone acetate, Dexamethasone Sodium Phosphate, Dexasone, Dexrazoxane, DHAD, DIC, Diodex, Docetaxel, Doxil®, Doxorubicin, Doxorubicin liposomal, Droxia, DTIC, DTIC-Dome®, Duralone®, Efudex®, Eligard™, Ellence™, Eloxatin™, Elspar®, Emcyt®, Epirubicin, Epoetin alfa, Erbitux™, Erlotinib, Erwinia L-asparaginase, Estramustine, Ethyol, Etopophos®, Etoposide, Etoposide Phosphate, Eulexin®, Evista®, Exemestane, Fareston®, Faslodex®, Femara®, Filgrastim, Floxuridine, Fludara®, Fludarabine, Fluoroplex®, Fluorouracil, Fluoxymesterone, Flutamide, Folinic Acid, FUDR®, Fulvestrant, G-CSF, Gefitinib, Gemcitabine, Gemtuzumab ozogamicin, Gemzar®, Gleevec™, Gliadel® Wafer, GM-CSF, Goserelin, Granulocyte-Colony Stimulating Factor, Granulocyte Macrophage Colony Stimulating Factor, Halotestin®, Herceptin®, Hexadrol, Hexalen®, Hexamethylmelamine, HMM, Hycamtin®, Hydrea®, Hydrocort Acetate®, Hydrocortisone, Hydrocortisone Sodium Phosphate, Hydrocortisone Sodium Succinate, Hydrocortone Phosphate, Hydroxyurea, Ibritumomab, Ibritumomab Tiuxetan, Idamycin®, Idarubicin, Ifex®, IFN-alpha, Ifosfamide, IL-11, IL-2, Imatinib mesylate, Imidazole Carboxamide, Interferon alfa, Interferon Alfa-2b (PEG Conjugate), interleukin-2, interleukin-11, Intron A® (interferon alfa-2b), Iressa®, Irinotecan, Isotretinoin, Kidrolase®, Lanacort®, Lapatinib, L-asparaginase, LCR, Lenalidomide, Letrozole, Leucovorin, Leukeran, Leukine™, Leuprolide, Leurocristine, Leustatin™, Liposomal Ara-C, Liquid Pred®, Lomustine, L-PAM, L-Sarcolysin, Lupron®, Lupron Depot®, Matulane®, Maxidex, Mechlorethamine, Mechlorethamine Hydrochloride, Medralone®, Medrol®, Megace®, Megestrol, Megestrol Acetate, Melphalan, Mercaptopurine, Mesna, Mesnex™, Methotrexate, Methotrexate Sodium, Methylprednisolone, Meticorten®, Mitomycin, Mitomycin-C, Mitoxantrone, M-Prednisol®, MTC, MTX, Mustargen®, Mustine, Mutamycin®, Myleran®, Mylocel™, Mylotarg®, Navelbine®, Nelarabine, Neosar®, Neulasta™, Neumega®, Neupogen®, Nexavar®, Nilandron®, Nilutamide, Nipent®, Nitrogen Mustard, Novaldex®, Novantrone®, Octreotide, Octreotide acetate, Oncospar®, Oncovin®, Ontak®, Onxal™, Oprevelkin, Orapred®, Orasone®, Oxaliplatin, Paclitaxel, Paclitaxel Protein-bound, Pamidronate, Panitumumab, Panretin®, Paraplatin®, Pediapred®, PEG Interferon, Pegaspargase, Pegfilgrastim, PEG-INTRON™, PEG-L-asparaginase, PEMETREXED, Pentostatin, Phenylalanine Mustard, Platinol®, Platinol-AQ®, Prednisolone, Prednisone,

Prelone®, Procarbazine, PROCRIT®, Proleukin®, Prolifeprospan 20 with Carmustine Implant, Purinethol®, Raloxifene, Revlimid®, Rheumatrex®, Rituxan®, Rituximab, Roferon-A® (Interferon Alfa-2a), Rubex®, Rubidomycin hydrochloride, Sandostatin®, Sandostatin LAR®, Sargramostim, Solu-Cortef®, Solu-Medrol®, Sorafenib, SPRYCEL™, STI-571, Streptozocin, SU11248, Sunitinib, Sutent®, Tamoxifen, Tarceva®, Targretin®, Taxol®, Taxotere®, Temodar®, Temozolomide, Teniposide, TESPA, Thalidomide, Thalomid®, TheraCys®, Thioguanine, Thioguanine Tabloid®, Thiophosphoamide, Thioplex®, Thiotepa, TICE®, Toposar®, Topotecan, Toremifene, Tositumomab, Trastuzumab, Tretinoin, Trexall™, Trisenox®, TSPA, TYKERB®, VCR, Vectibix™, Velban®, Velcade®, VePesid®, Vesanoid®, Viadur™, Vidaza®, Vinblastine, Vinblastine Sulfate, Vincasar Pfs®, Vincristine, Vinorelbine, Vinorelbine tartrate, VLB, VM-26, Vorinostat, VP-16, Vumon®, Xeloda®, Zanosar®, Zevalin™, Zinecard®, Zoladex®, Zoledronic acid, Zolinza, Zometa®.

*Radiopharmaceuticals*

Examples of radiopharmaceuticals include: Carbon-11, Carbon-14, Chromium-51, Cobalt-57, Cobalt-58, Erbium-169, Fluorine-18, Gallium-67, Gold-198, Indium-111, Indium-113m, Iodine-123, Iodine-125, Iodine-131, Iron-59, Krypton-81m, Nitrogen-13, Oxygen-15, Phosphorous-32, Rhenium-186, Rubidium-82, Samarium-153, Selenium-75, Strontium-89, Technetium-99m, Thallium-201, Tritium, Xenon-127, Xenon-133, Yttrium-90.

*Imaging agents*

Examples of imaging agents include: Gadolinium, magnetite, manganese, technetium, I125, I131, P32, Tl201, Iopamidol, PET-FDG.

**Preparation of a polynucleotide**

An eighth aspect of the invention provides a method of producing a polynucleotide comprising:

(a) providing a nucleic acid molecule encoding a parent albumin or fragment thereof; and

(b) modifying the nucleic acid sequence of the nucleic acid molecule to encode a conjugation-competent polypeptide which is at least 60% identical to human albumin, particularly residues 1 to 585 of the mature human albumin polypeptide sequence of SEQ ID NO. 2, or a fragment thereof, wherein at least one (*e.g.* several) position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue.

Suitably, modifying the nucleic acid sequence comprises introducing an alteration such that at least one (*e.g.* several) conjugation-competent cysteine as provided for in step (b) is introduced into the encoded polypeptide. Preferred alterations are as described in relation to the first and second aspects of the invention.

It is preferred that the parent albumin comprises or consists of:

(a) a polypeptide having at least 70% sequence identity to the mature polypeptide of SEQ ID NO. 2;

(b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO. 2, or (ii) the full-length complement of (i);

(c) a polypeptide encoded by a polynucleotide having at least 60% identity to the mature polypeptide coding sequence of SEQ ID NO. 2; and/or

(d) a fragment of the mature polypeptide of SEQ ID NO. 2.

Suitably, the parent albumin comprises or consists of the HSA polypeptide sequence of SEQ ID NO. 2 or a variant or fragment thereof.

The variant polynucleotides can be prepared by those skilled persons using any mutagenesis procedure known in the art, such as site-directed mutagenesis, synthetic gene construction, semi-synthetic gene construction, random mutagenesis, shuffling, *etc.*

Site-directed mutagenesis is a technique in which one or more (*e.g.* several) mutations (alterations) are created at one or more (*e.g.* several) defined sites in a polynucleotide encoding the parent.

Site-directed mutagenesis can be accomplished *in vitro* by PCR involving the use of oligonucleotide primers containing the desired mutation. Site-directed mutagenesis can also be performed *in vitro* by cassette mutagenesis involving the cleavage by a restriction enzyme at a site in the plasmid comprising a polynucleotide encoding the parent and subsequent ligation of an oligonucleotide containing the mutation in the polynucleotide. Usually the restriction enzyme that digests at the plasmid and the oligonucleotide is the same, permitting ligation of the plasmid and insert to one another. See, *e.g.* Scherer and Davis, 1979, *Proc. Natl. Acad. Sci. USA* 76: 4949-4955; and Barton *et al.*, 1990, *Nucleic Acids Res.* 18: 7349-4966.

Site-directed mutagenesis can also be accomplished *in vivo* by methods known in the art, see, *e.g.* U.S. Patent Application Publication: 2004/0171154; Storici *et al.*, 2001, *Nature Biotechnol.* 19: 773-776; Kren *et al.*, 1998, *Nat. Med.* 4: 285-290; and Calissano and Macino, 1996, *Fungal Genet. Newslett.* 43: 15-16.

Any site-directed mutagenesis procedure can be used in the invention. There are many commercial kits available that can be used to prepare variants.

Synthetic gene construction entails *in vitro* synthesis of a designed polynucleotide molecule to encode a polypeptide of interest. Gene synthesis can be performed utilizing a

number of techniques, such as the multiplex microchip-based technology described by Tian *et al.* (2004, *Nature* 432: 1050-1054) and similar technologies wherein oligonucleotides are synthesized and assembled upon photo-programmable microfluidic chips.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (*e.g.* Lowman *et al.*, 1991, Biochemistry 30: 10832-10837; U.S. Patent: 5,223,409; WO 92/06204) and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

Semi-synthetic gene construction is accomplished by combining aspects of synthetic gene construction, and/or site-directed mutagenesis, and/or random mutagenesis, and/or shuffling. Semi-synthetic construction is typified by a process utilizing polynucleotide fragments that are synthesized, in combination with PCR techniques. Defined regions of genes may thus be synthesized *de novo*, while other regions may be amplified using site-specific mutagenic primers, while yet other regions may be subjected to error-prone PCR or non-error prone PCR amplification. Polynucleotide sub sequences may then be shuffled.

**Method of producing a polypeptide**

A ninth aspect of the invention provides a method of producing a polypeptide of the invention comprising:

(a) culturing a host cell according to the invention under conditions that allow expression of the polypeptide; and

(b) recovering the polypeptide from the host cell and/or from host cell growth medium.

The method may or may not further comprise determining the receptor binding capacity and/or the conjugation competence of the polypeptide and/or the tendency to exist as a monomer in solution, and optionally selecting a polypeptide which does or does not have a receptor binding capacity and/or conjugation competence and/or selected range of percentage monomer tendency.

The variants of the invention can be prepared using techniques well known to the skilled person. One convenient way is by cloning a nucleic acid molecule encoding a parent albumin or a fragment thereof and modifying the sequence of the nucleic acid molecule according to the method of the eighth aspect of the invention, preparing a suitable genetic construct where the modified nucleic acid molecule is placed in operative connection with suitable regulatory genetic elements, such as promoter, terminator, activation sites, ribosome binding sites *etc.*, introducing the genetic construct into a suitable host organism, culturing the transformed host organism under conditions leading to expression of the variant and recovering the variant. All these techniques are known in the art and it is within the skills of the average practitioner to design a suitable method for preparing a particular variant according to the invention.

The variant polypeptide of the invention may also be connected to a signal sequence in order to have the variant polypeptide secreted into the growth medium during culturing of the transformed host organism. It is generally advantageous to have the variant polypeptide secreted into the growth medium in order to ease recovery and purification. The polypeptide may be prepared as a fusion polypeptide as described in relation to the third aspect of the invention. Techniques for preparing variant polypeptides have been disclosed in WO 2009/019314 (included by reference) and these techniques may also be applied to the invention.

Albumins have been successfully expressed as recombinant proteins in a range of hosts including fungi (including but not limited to *Aspergillus* (WO 06066595), *Kluyveromyces* (Fleer 1991, *Bio/technology* 9, 968-975), *Pichi*a (Kobayashi 1998 *Therapeutic Apheresis* 2, 257-262) and *Saccharomyces* (Sleep 1990, *Bio/technology* 8, 42-46)), bacteria (Pandjaitab 2000, *J. Allergy Clin. Immunol.* 105, 279-285)), animals (Barash 1993, *Transgenic Research* 2, 266-276) and plants (including but not limited to potato and tobacco (Sijmons 1990, *Bio/technology* 8, 217 and Farran 2002, *Transgenic Research* 11, 337-346) and rice *e.g. Oryza sativa*) and mammalian cells such as CHO and HEK. The variant polypeptide of the invention is preferably produced recombinantly in a suitable host cell. In principle any host cell capable of producing a polypeptide in suitable amounts may be used and it is within the skills of the average practitioner to select a suitable host cell according to the invention. A preferred host organism is yeast, preferably selected among *Saccharomycacae*, more preferred *Saccharomyces cerevisiae*.

The variant polypeptides of the invention may be recovered and purified from the growth medium using a combination of known separation techniques such as filtration, centrifugation, chromatography, and affinity separation techniques *etc.* It is within the skills of the average practitioner to purify the variants of the invention using a particular combination of such known separation steps. As an example of purification techniques that may be applied to the variants of the invention can be mentioned the teaching of WO 00/44772.

In the method of the invention, the host cell may or may not exhibit enhanced chaperone activity. Accordingly, the present invention also provides a method for producing a polypeptide (or protein) of the invention, the method comprising: (a) providing a host cell of the invention comprising a polynucleotide encoding protein product of choice as defined above; and (b) growing the host cell (for example, culturing the host cell in a culture medium); thereby to produce a cell culture or recombinant organism comprising an increased level of the protein product of choice compared to the level of production of the protein product of choice achieved by growing (for example, culturing), under the same conditions, the same host cell that has not been genetically modified to cause over-expression of one or more (*e.g.* several) helper proteins.

The step of growing the host cell may or may not involve allowing a host cell derived from a multicellular organism to be regrown into a multicellular recombinant organism (such as a plant or animal) and, optionally, producing one or more (*e.g.* several) generations of progeny therefrom.

The thio-albumin may or may not be capable of being expressed at a level of at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% relative to the expression of an unmodified albumin (such as SEQ ID NO. 2) from a suitable expression system, such as yeast (*e.g. Saccharomyces*, *e.g. S. cerevisiae*) or an Aspergillus. Relative expression levels can be determined, for example, by expression of the protein followed by quantification by SDS-PAGE, HPLC or Western Blotting. Relative expression levels may be determined in at least 10 liter scale.

The method may or may not further comprise the step of purifying the thus expressed protein product of choice from the cultured host cell, recombinant organism or culture medium.

The production method may comprise linking a conjugation partner to the polypeptide of the invention through a conjugation competent cysteine residue of the polypeptide. Suitable conjugation methods and conjugation partners are described herein.

The thio-albumin or fusions of thio-albumin and another protein or proteins can be expressed as variants with reduced N-linked glycosylation. Accordingly, in case of HSA, it may be particularly advantageous to use a yeast deficient in one or more (*e.g.* several) protein mannosyl transferases involved in O-glycosylation of proteins, for instance by disruption of the gene coding sequence. Recombinantly expressed proteins can be subject to undesirable post-translational modifications by the producing host cell. The mannosylated albumin would be able to bind to the lectin Concanavalin A. The amount of mannosylated albumin produced by the yeast can be reduced by using a yeast strain deficient in one or more (*e.g.* several) of the *PMT* genes (WO 94/04687). The most convenient way of achieving this is to create a yeast which has a defect in its genome such that a reduced level of one of the Pmt proteins is produced. For example, there may or may not be a deletion, insertion or transposition in the coding

sequence or the regulatory regions (or in another gene regulating the expression of one of the *PMT* genes) such that little or no Pmt protein is produced. Alternatively, the yeast could be transformed to produce an anti-Pmt agent, such as an anti-Pmt antibody. Alternatively, the yeast could be cultured in the presence of a compound that inhibits the activity of one of the PMT genes (Duffy *et al*, "*Inhibition of protein mannosyltransferase 1 (PMT1) activity in the pathogenic yeast Candida albicans*", International Conference on Molecular Mechanisms of Fungal Cell Wall Biogenesis, 26-31 August 2001, Monte Verita, Switzerland, Poster Abstract P38). If a yeast other than *S. cerevisiae* is used, disruption of one or more (*e.g.* several) of the genes equivalent to the *PMT* genes of *S. cerevisiae* is also beneficial, *e.g.* in *Pichia pastoris* or *Kluyveromyces lactis*. The sequence of *PMT1* (or any other *PMT* gene) isolated from *S. cerevisiae* may be used for the identification or disruption of genes encoding similar enzymatic activities in other fungal species. The cloning of the *PMT1* homologue of *Kluyveromyces lactis* is described in WO 94/04687.

The variant polypeptides of the invention may be used for delivering a therapeutically beneficial compound (including prophylactically beneficial compound such as a vaccine) to an animal or a human individual in need thereof. Such therapeutically beneficial compounds include, but are not limited to, labels and readily detectable compounds for use in diagnostics, such as various imaging techniques; pharmaceutical active compounds such as drugs, or specifically binding moieties such as antibodies. The variants of the invention may even be connected to two or more (several) different therapeutically beneficial compounds, *e.g.* an antibody and a drug, which gives the combined molecule the ability to bind specifically to a desired target and thereby provide a high concentration of the connected drug at that particular target.

The method may further comprise the step of purifying the polypeptide recovered from the host cell and/or from the host cell growth medium. The purification step optionally comprises cell immobilisation, cell separation and/or cell breakage, but always comprises at least one (*e.g.* several) other purification step different from the step or steps of cell immobilisation, separation and/or breakage.

Thio-albumin of the invention may be purified from the culture medium by any technique that has been found to be useful for purifying such proteins. Similarly, cell separation techniques, such as centrifugation, filtration (*e.g.* cross-flow filtration, expanded bed chromatography and the like) are well known in the art. Likewise, methods of cell breakage, including beadmilling, sonication, enzymatic exposure and the like are well known in the art.

The "at least one (*e.g.* several) other purification step" may be any other step suitable for protein purification known in the art. For example purification techniques for the recovery of recombinantly expressed albumin have been disclosed in: WO 92/04367, removal of matrix-derived dye; EP 464590, removal of yeast-derived colorants; EP 319067, alkaline precipitation

and subsequent application of the albumin to a lipophilic phase; and WO 96/37515, US 5 728 553 and WO 00/44772, which describe complete purification processes; all of which are incorporated herein by reference. Suitable methods include ammonium sulphate or ethanol precipitation, acid or solvent extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography, lectin chromatography, concentration, dilution, pH adjustment, diafiltration, ultrafiltration, high performance liquid chromatography ("HPLC"), reverse phase HPLC, conductivity adjustment and the like.

The polypeptide may be purified to a commercially or industrially acceptable level of purity. By commercially or industrially acceptable level of purity, we include the provision of the thio-albumin and/or thio-albumin-conjugate in which other material (for example, one or more (*e.g.* several) contaminants) are present at a level of less than 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001%, or 0.000001% and, most preferably at a level of 0%.

A commercially or industrially acceptable level of purity may be obtained by a relatively crude purification method by which the protein product of choice is put into a form suitable for its intended purpose. A protein preparation that has been purified to a commercially or industrially acceptable level of purity may, in addition to the protein product of choice, also comprise, for example, cell culture components such as host cells or debris derived therefrom. Alternatively, high molecular weight components (such as host cells or debris derived therefrom) may or may not be removed (such as by filtration or centrifugation) to obtain a composition comprising the protein product of choice and, optionally, a functionally acceptable level of low molecular weight contaminants derived from the cell culture process.

The protein may or may not be purified to achieve a pharmaceutically acceptable level of purity. A protein has a pharmaceutically acceptable level of purity if it is essentially pyrogen free and can be used for its intended purpose and hence be administered in a pharmaceutically efficacious amount without causing medical effects not associated with the activity of the protein.

The thio-albumin and/or thio-albumin-conjugate may be provided at a concentration of at least $10^{-4}$ g.L$^{-1}$, $10^{-3}$ g.L$^{-1}$, 0.01 g.L$^{-1}$, 0.02 g.L$^{-1}$, 0.03 g.L$^{-1}$, 0.04 g.L$^{-1}$, 0.05 g.L$^{-1}$, 0.06 g.L$^{-1}$,0.07 g.L$^{-1}$, 0.08 g.L$^{-1}$, 0.09 g.L$^{-1}$, 0.1 g.L$^{-1}$, 0.2 g.L$^{-1}$, 0.3 g.L$^{-1}$, 0.4 g.L$^{-1}$, 0.5 g.L$^{-1}$, 0.6 g.L$^{-1}$, 0.7 g.L$^{-1}$, 0.8 g.L$^{-1}$, 0.9 g.L$^{-1}$, 1 g.L$^{-1}$, 2 g.L$^{-1}$, 3 g.L$^{-1}$, 4 g.L$^{-1}$, 5 g.L$^{-1}$, 6 g.L$^{-1}$, 7 g.L$^{-1}$, 8 g.L$^{-1}$, 9 g.L$^{-1}$, 10 g.L$^{-1}$, 15 g.L$^{-1}$, 20 g.L$^{-1}$, 25 g.L$^{-1}$, 30 g.L$^{-1}$, 40 g.L$^{-1}$, 50 g.L$^{-1}$, 60 g.L$^{-1}$, 70 g.L$^{-1}$, 80 g.L$^{-1}$, 90 g.L$^{-1}$, 100 g.L$^{-1}$, 150 g.L$^{-1}$, 200 g.L$^{-1}$, 250 g.L$^{-1}$, 300 g.L$^{-1}$, 350 g.L$^{-1}$, 400 g.L$^{-1}$, 500 g.L$^{-1}$, 600 g.L$^{-1}$, 700 g.L$^{-1}$, 800 g.L$^{-1}$, 900 g.L$^{-1}$, 1000 g.L$^{-1}$.

A method of the present invention may or may not further comprise the step of formulating the purified protein product of choice with a carrier or diluent and optionally presenting the thus formulated protein in a unit dosage form.

Although it is possible for a therapeutically useful protein obtained by a process of the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more (*e.g.* several) acceptable carriers or diluents. The carrier(s) or diluent(s) must be "acceptable" in the sense of being compatible with the desired protein. Typically, the carriers or diluents will be water or saline which will be sterile and pyrogen free. Alternatively, a method of the present invention may or may not further comprise the step of lyophilising the thus purified protein product of choice.

The thio-albumin may be formulated by strategies given in "Protein Formulation and Delivery", E. J. McNally (Ed.), published by Marcel Dekker Inc. New York 2000 and "Rational Design of Stable Protein Formulations – Theory and Practice"; J. F. Carpenter and M. C. Manning (Ed.) Pharmaceutical Biotechnology Vol 13. Kluwer Academic/Plenum Publishers, New York 2002, Yazdi and Murphy, (1994), Cancer Research 54, 6387-6394, Widera *et al.*, (2003) Pharmaceutical Research 20, 1231-1238; Lee *et al.*, (2005), Arch. Pharm. Res. 28, 722-729. Examples of formulation methods are as follows:

Method #1: Following purification the free thiol containing albumin mutein of the invention or the conjugate can be stored at 4°C, -20°C or -80°C in 0.01 M – 0.1 M phosphate buffered saline (pH 7.0 - 8.0) containing 0.01 M – 0.25 M NaCl.

Method #2: Following purification the free thiol containing albumin mutein of the invention or the conjugate can be stored at 4°C, -20°C or -80°C in 0.01 M – 0.1 M phosphate buffered saline (pH 7.0 - 8.0) containing 0.01 M – 0.25 M NaCl and containing 10-20mg/L Polysorbate 80.

Method #3: Following purification the free thiol containing albumin mutein of the invention or the conjugate can be stored at 4°C, -20°C or -80°C in 0.01 M – 0.25 M NaCl (pH 7.0 - 8.0).

Method #4: Following purification the free thiol containing albumin mutein of the invention or the conjugate can be stored at 4°C, -20°C or -80°C in 0.01 M – 0.25 M NaCl (pH 7.0 - 8.0) containing 10-20mg/L Polysorbate 80.

*Freeze-dried formulations*

Method #5: Following purification the free thiol containing albumin mutein of the invention or the conjugate can be dialysed against water, freeze dried and stored at 4°C, -20°C or -80°C.

Method #6: Following purification the free thiol containing albumin mutein of the invention or the conjugate can be dialysed against 0.01 M – 0.25 M NaCl (pH 7.0 - 8.0), freeze dried and stored at 4°C, -20°C or -80°C.

**Conjugation methods**

A tenth aspect of the invention provides a method of producing the conjugate of the seventh aspect of the invention, the method comprising linking a polypeptide of the first, second or third aspect of the invention, or produced by the method of the ninth aspect of the invention, to a bioactive compound through a conjugation-competent cysteine residue of the polypeptide. The linking may be carried out using a linker.

The albumin mutein (thio-albumin) of the invention can be covalently linked to one or more (*e.g.* several) conjugation partners such as bioactive compounds by methods known in the art (for example those provided by Pierce, Thermo Fisher Scientific, Rockford, IL, USA; https://tools.lifetechnologies.com/content/sfs/brochures/1602163-Crosslinking-Reagents-Handbook.pdf). These include, but are not limited to incorporating or engineering a thiol reactive group into or onto the conjugation partner, for example by incorporating or engineering another free thiol present on the conjugation partner; or by incorporating or engineering a pyridyl disulphide group on the conjugation partner; or by incorporating or engineering an haloacetyl group on the bioactive compound or by incorporating or engineering a maleimide group on the conjugation partner, or by incorporating or engineering a thiosulfonate group on the conjugation partner, or by incorporating or engineering vinylsulfone group on the conjugation partner. For example, but not limited to, N-ethylmaleimide (NEM, Pierce), 2-amino-2'-aminoethanethiolsulfonate (Pierce), N-beta-maleimidoprpionic acid (BMPA Pierce), methyl methane thiosulfonate (MMTS, Pierce), fluorescein-5-maleimide (Pierce), 5-iodoacetamido-fluorescein (5-IAF, Pierce) or N-[6-7-amino-4-methylcoumarin-3-acetamido) hexyl]-3'-[2'-pyridyldithio] propionamide (AMCA-HPDP, Pierce).

If the conjugation partner contains at least one (*e.g.* several) thiol group, then the conjugation partner may be cross-linked to the albumin mutein of the invention by methods known to the art such as, but not limited to, oxidation or by the use of cross-linking reagents such as, but not limited to, 1,4-Bis-maleimidibutane (BMB, Pierce); 1,4-Bis-maleimidyl-2,3-dihydroxybutane (BMDB, Pierce); Bis-maleimidohexane (BMH, Pierce), Bis-maleimidoethane (BMOE, Pierce); 1,8-Bis-Maleimidotriethyleneglycol (BM[PEO]3 Pierce); 1,11-Bis-Maleimidotetraethyleneglycol (BM[PEO]4 Pierce); 1,4-Di-[3'-(2'-pyridyldithio)-propionamido]butane (DPDPB, Pierce); dithio-bis-maleimidoethane (DTME Pierce); 1,6-Hexane-bis-vinylsulfone (HBVS, Pierce) and Tris-[2-maleimimidoethyl]amine (TMEA, Pierce).

If the conjugation partner does not contain a thiol reactive group then it may be modified to incorporate one or more (*e.g.* several) such groups by either chemical modification or genetic engineering by methods know to the art (Chapman, A.P. (2002) Adv. Drug Deliv. Rev., 54 531–545: Humphreys, D.P. *et al.* Protein Engineering, Design & Selection vol. 20 no. 5 pp. 227–234, 2007). While these two references describe methodologies to cross-link PEG to an engineered

free thiol within an antibody or antibody fragment, the techniques may be used to cross-link a conjugation partner to an engineered free thiol within the albumin mutein of the invention. Alternatively the Drug Affinity Complex (DAC™) technology developed by ConjuChem Inc. (Montreal, Quebec, Canada, H2X 3Y8) may be used, *e.g.* as described in WO 200069902. There are three parts of each DAC™ construct: 1) the drug component (the portion responsible for biologic activity); 2) a linker attached to the drug component, and 3) a reactive chemistry group at the opposite end of the linker, usually a soft electrophile selective for thiols; a maleimide is the most useful embodiment. Other applicable conjugation methods are described in WO 2007/071068 incorporated herein by reference.

If the conjugation partner does not contain a thiol reactive group but does contain one or more (*e.g.* several) amino groups then it may be modified to incorporate one or more (*e.g.* several) thiol reactive groups by chemical modification by methods known to the art such as the use of cross-linking reagents such as, but not limited to, N-5-azido-2-nitrobenzoyloxysuccinimide (AMAS, Pierce), N-[beta-maleimidopropyloxy] succinimide ester (BMPS, Pierce), N-eta-maleimidocaproic acid (EMCA, Pierce), N-[eta-maleimidocaproyloxy]succinimide ester (EMCS, Pierce), N-[eta-maleimidocaproyloxy]sulfosuccinimide ester (sulfo-EMCS, Pierce), N-[gamma-maleimidobutyryloxy]succinimide ester (GMBS, Pierce), N-[gamma-maleimidobutyryloxy]sulfosuccinimide ester (sulfo-GMBS, Pierce), N-kappa-maleimidoundecanoic acid (KMUA, Pierce), N-[kappa -maleimidoundecanoyloxy]sulfosuccinimide ester (sulfo-KMUS, Pierce), m-maleimidobenzoyl-N-hydroxysuccinimide (MBS, Pierce), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS, Pierce), N-succinimidyl S-acetylthio-acetate (SATA, Pierce), N-succinimidyl S-acetylthiopropionate (SATP, Pierce), succinimidyl 3-[bromoacetamido]propionate (SBAP, Pierce), N-succinimidyl iodoacetate (SIA, Pierce), N-succinimidyl[4-iodoacetyl]aminobenzoate (SIAB, Pierce), sulfosuccinimidyl[4-iodoacetyl]aminobenzoate (sulfo-SIAB, Pierce), succinimidyl [4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC, Pierce), sulfosuccinimidyl [4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC, Pierce), succinimidyl-[4-[N-maleimidomethyl]cyclohexane-1-carboxy-[6-amidocaproate (LC-SMCC, Pierce), 4-succinimidyloxycarbonyl-methyl-alpha[2-pyridyldithio]toluene (SMPT, Pierce), sulfosuccinimidyl6-[alpha-methyl-alpha-(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT, Pierce), succinimidyl 4-[p-maleimidophenyl]-butyrate (SMPB, Pierce), sulfosuccinimidyl 4-[p-maleimidophenyl]-butyrate (sulfo-SMPB, Pierce), succinimidyl-6-[(beta-maleimidopropionamido)hexanoate] (SMPH, Pierce), N-succinimidyl 3-[2-pyridyldithio]propionate (SPDP, Pierce), succinimidyl [3-(2-pyridyldithio)propionamido]hexanoate (LC-SPDP, Pierce), sulfosuccinimidyl [3'-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP, Pierce) and N-succinimidyl-[4-

vinylsulfonyl]benzoate (SVSB Pierce). It may be advantageous to block certain amine residue as described by Kavimandan *et al.*, (2006), Bioconjugate Chem. 17, 1376-1384.

Suitable linkers include bromomaleimide linkers such as monobromomaleimide linkers. Monobromomaleimides are next generation maleimides for the construction of stable conjugates, as described in Smith *et al* Organic & Biomolecular Chemistry, (2015), 13, pages 7946-7949. Preferred monobromomaleimide linkers include those described in WO 2011/018611 (incorporated herein by reference).

If the conjugation partner does not contain a thiol reactive group but does contain one or more (*e.g.* several) carbonyl (oxidised carbohydrate) groups then it can be modified to incorporate one or more (*e.g.* several) thiol reactive groups by chemical modification by methods known to the art such as the use of cross-linking reagents such as, but not limited to, (N-β-maleimidopropionic acid hydrazide (BMPH, Pierce) N-[eta-maleimidocaproic acid]hydrazide (EMCH, Pierce), 4-[N-maleimidomethyl]cyclohexane-1carboxylhydrazide•HCl•1/2 dioxane (MMCCH, Pierce), 3-maleimidophenyl boronic acid (MPBH, Pierce), N-[kappa -maleimidoundecanoic acid]hydrazide (KMUH, Pierce) and 3-[2-pyridyldithio]propionyl hydrazide (PDPH, Pierce).

If the conjugation partner does not contain a thiol reactive group but does contain one or more (*e.g.* several) hydroxyl groups then it may be modified to incorporate one or more (*e.g.* several) thiol reactive groups by chemical modification by methods known to the art such as the use of cross-linking reagents such as, but not limited to, N-[p-maleimidophenyl]isocyanate (PMPI, Pierce).

**Associates**

An eleventh aspect of the invention provides an associate comprising the conjugate of the seventh aspect of the invention and a bioactive, therapeutic, prophylactic, diagnostic, imaging or other beneficial moiety.

The conjugates may further be used in the form of “associates”. In this connection the term “associate” is intended to mean a compound comprising a conjugate of a variant of albumin or a fragment thereof and another compound bound or associated to the conjugate by non-covalent binding. As an example of such an associate can be mentioned an associate consisting of a variant albumin conjugate and a lipid associated to albumin by a hydrophobic interaction. Such associates are known in the art and they may be prepared using well known techniques. As an example of a preferred associate according to the invention can be mentioned, an associate comprising a variant albumin conjugate and a taxane, a taxol or taxol derivative (*e.g.* paclitaxel). Further examples of associates comprise a bioactive, therapeutic, prophylactic (including vaccine), diagnostic, imaging or other beneficial moiety.

Methods for the preparation of associates are well-known to the skilled person, for example, formulation (by association) of HSA with lipo-compounds is described in Hussain, R. and Siligardi, G. (2006), International Journal of Peptide Research and Therapeutics, Vol. 12, NO: 3, pp. 311–315.

**Nanoparticle, microparticle or liposome**

A twelfth aspect of the invention provides a nanoparticle, a microparticle or a liposome comprising the polypeptide or the first, second or third aspect of the invention, the conjugate of the seventh aspect of the invention or the associate of the eleventh aspect of the invention.

Albumins and albumin particles are important for carrying and delivering drugs and prodrugs to their sites of action (Kratz (2008), Journal of Controlled Release, 132 (3), p.171-183). Fusion and particle technologies offer improved dosing regimens due to improved pharmacokinetic properties, such as plasma half-life extension, and may improve bioavailability and protect the fused bioactive molecule from inactivation.

Techniques for incorporation of a molecule into nano- or microparticles are known in the art. Preferred methods for preparing nano- or microparticles that may be applied to the variant albumin conjugate or associate thereof according to the invention are disclosed in WO 2004/071536 or WO 2008/007146 or Oner & Groves (Pharmaceutical Research, Vol 10(9), 1993, pages 1387 to 1388) which are incorporated herein by reference. Preferably the average diameter of a nano-particle is from 5 to 1000 nm, more preferably from 5, 10, 20, 30, 40, 50, 80, 100, 130, 150, 200, 300, 400, 500, 600, 700, 800, 900, or 999 to 5, 10, 20, 30, 40, 50, 80, 100, 130, 150, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nm. An advantage of a microparticle less than 200 nm diameter, and more particularly less than 130 nm, is that is amenable to sterilization by filtration through a 0.2 μm (micron) filter. Preferably, the average diameter of a microparticle is from 1000 nm (1 μm (micron)) to 100 μm (micron), more preferably from 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 to 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μm (micron).

The thio-albumin of the invention (and/or its conjugated form) may be used to produce nanoparticles and/or be entrapped within a nanoparticle or liposome.

The thio-albumin of the invention may be used with and/or in and/or as a nanoparticle and/or liposome. A problem of current conjugation strategies is maintaining both the pharmacological and immunological activity of the conjugation partner, such as a bioactive-targeting ligand conjugate. There is likely to be a maximum number of protein targeting ligand or bioactive moieties (conjugation partners) possible for conjugation to a protein and if this number is exceeded the targeting ligand does not retain its biological activity. Preferably the biological activity of the conjugation partner is not reduced by conjugation to an albumin of the invention.

Liposomes and nanoparticles may be used to entrap bioactive compounds. They provide a mechanism for enhanced delivery of drugs such as bioactive compounds, or uptake by target cells and/or a reduction in the toxicity of the free bioactive to non-target organs which may result in an increased therapeutic index and/or reduced side effects. In addition, many solvent-based formulations required for the delivery of some bioactive compounds (*e.g.* taxanes) are associated with toxicity which limits the maximum dose which can be given to a patient. Liposome and nanoparticle delivery may also be advantageous for such bioactive compounds, since they would allow larger amounts of the bioactive compound to be delivered whilst avoiding some of the toxicities of solvent-based formulations (Hawkins *et al* (2008), Advanced Drug Delivery Reviews, 60, 8, p876-885).

Methods for attaching targeting ligands to liposomes and nanoparticles are known in the art (reviewed in Nobs *et al* (2004), Journal of Pharmaceutical Sciences Vol 93 p1980-1992) and may be used in accordance with the invention. Attachment methods may be non-covalent or covalent. Covalent reactions appear to be favourable, because covalent linkage is more stable than noncovalent methods. Lipids for the covalent or non-covalent attachment of proteins, peptides, or drugs to the liposome surface are available commercially (for example Avanti Polar Lipids Inc Alabaster, Alabama, USA). There are 3 major classes of functionality: conjugation through disulphide or thioether formation, amide bond formation, or biotin/streptavidin binding, any of these may be used in the invention.

A number of methods relying on covalent coupling ligands to the surface of liposomes *via* thioether bonds have been described, most commonly utilizing the highly efficient reaction of maleimide with thiol groups. Functionalized lipid anchors commonly added to liposomes, and which may be used in or with the invention, include, but are not limited to those containing maleimide such as N-[4-(p-maleimidophenyl) butyramide]-PE (N-MPB]-PE) or N- [4-(p-maleimidomethyl) cyclohexane-carboxamide) (MCC-PE) which allow convenient covalent coupling of the targeting moiety *via* a stable thioether bond (Martin & Papahadjopoulos (1982), J. Biol. Chem. 257, 286- 288).

Method #7: Following purification the free thiol containing albumin mutein of the invention or the conjugate can be formulated into nanoparticles prepared according to known procedures for preparing nanoparticles, such as procedures disclosed in WO 2004/071536 A1 and WO 2008/007146 A1, both incorporated herein by reference.

Similarly materials for the formation of nanoparticles, including but are not limited to poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), and COOH-PLA are commercially available and may be functionalized with maleimide or other known chemistries according to known literature for nanoparticle formation. Any of these may be used in or with the invention.

Another convenient way for covalent coupling of ligands to liposomes involves conjugation of two thiols to form a disulphide; however under the reductive conditions in serum

more stable conjugation chemistries involving one free thiol group may be preferred. Chemistries such as (PDP-PE) allow covalent coupling *via* a disulphide bond. Modification of the ligand to introduce a free thiol group or a functionalized linker may be used. An advantage of the thio-albumin of the invention is that no ligand modification is required. However, ligand modification may optionally be used in addition to the invention.

Frequently thiol groups are not present in proteins, or are not present in sufficient amounts or at the desired location. Thus, most cases of covalent coupling of one of more ligands to a liposome *via* thioether or disulphide bonds requires the use of heterobifunctional cross linking agents (described herein with reference to conjugation). Some heterobifunctional cross linking agents (such as SPDP and SATA) require a de-protection step. The thio-albumin of the invention overcomes the requirement for this additional processing.

Alternatively thio-albumin could be conjugated to liposomes or nanoparticles by other chemistries, known to the art. For example, thio-albumin could be attached by an amide bond using a functionalised lipid anchor with either amine or carboxyl functional groups (examples include DSPE-PEG-COOH) which reacts with the primary amine of the ligand. Direct cross linking between primary amines and the surface of liposomes may also be used. The one or more (*e.g.* several) free thiol groups of thio-albumin would then be available for conjugation to another conjugation partner.

Following conjugation, a conjugation partner (*e.g.* bioactive molecule) may show a reduction in its activity (*e.g.* bioactivity). Thio-albumin described in this invention may overcome this problem by providing a conjugate, nanoparticle and/or liposome in which the conjugation partner is located and/or orientated with respect to a thio-albumin such that the conjugation partner retains at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% of its unconjugated activity.

Nanoparticles may be used, for example, in angiogenic applications, anti-angiogenic applications and to coat a medical device such as a stent. Nanoparticles are effective at targeting, for example to non tight–junctions, and therefore can be useful for targeting tumours such as cancerous tumours. Nanoparticles can also be useful to target antigen in order to provoke an immune response since nanoparticles are particularly susceptible to engulfment and presentation by phagocytes. The invention provides nanoparticles consisting only of thio-albumin according to the invention which may or may not be conjugated to a moiety (conjugation partner). The invention also provides nanoparticles comprising thio-albumin according to the invention, which may or may not be conjugated to a moiety, and one or more (*e.g.* several) other constituents of a nanoparticle which may or may not be albumin related. In a preferred embodiment, a thio-albumin according to the invention comprises at least two conjugation competent cysteine residues located on the surface of the polypeptide. Such a thio-albumin may be used for the preparation of nanoparticles in which one or more (*e.g.* several) conjugation competent cysteine residues may be used in the formation of a

nanoparticle and one or more (*e.g.* several) conjugation competent residues is used for conjugation to a conjugation partner, for example to a bioactive molecule.

**Compositions**

A thirteenth aspect of the invention provides a composition comprising a polypeptide, fusion polypeptide, conjugate, associate, nanoparticle, microparticle or liposome according to the invention and at least one (*e.g.* several) pharmaceutically acceptable carrier and/or diluent.

Various formulations are described herein in relation to the corresponding products.

A related aspect of the invention provides a method for making a pharmaceutical ingredient and/or a pharmaceutical product comprising making a thio-albumin according to the present invention, optionally conjugating a further molecule to the thio-albumin, optionally formulating the resultant conjugate with a pharmaceutically acceptable diluent and/or carrier and optionally preparing the product in unit dosage form.

**Medical uses**

A fourteenth aspect of the invention provides use of a polypeptide, fusion polypeptide, conjugate according to the invention and/or produced by a method according to the invention, or an associate, nanoparticle, microparticle or liposome for treatment of disease, treatment of illness and/or diagnosis.

Various medical uses are described herein in relation to the corresponding products.

In addition, in some embodiments, the thio-albumin or conjugate has a binding affinity to FcRn and/or plasma half-life that is altered compared to the parent or reference albumin or conjugate. This has the advantage that the binding affinity to FcRn and/or plasma half-life of conjugates, associates, nanoparticle, microparticle or liposome according to the invention can be selected in accordance with the particular therapeutic purpose. An increased half-life could have the benefit that the administration would be needed less frequently or at a reduced dose (and consequently with fewer side effects) compared to the situation where the reference molecule or composition was used. Alternatively, a shorter plasma half-life than the reference molecule or composition would have the benefit that the administration can be carried out at a higher dose compared to the situation where the reference molecule or composition was used with the benefit that the administered compound clears from the recipient more quickly than if the reference molecule or composition was used.

For example for a conjugate, associate or fusion polypeptide used for imaging purposes in animals or humans, where the imaging moiety has a very short half-life and a conjugate or a fusion polypeptide comprising HSA has a plasma half-life that is far longer than needed for the imaging purposes it would be advantageous to use a variant albumin or fragment thereof of the invention having a shorter plasma half-life than the parent or reference albumin or fragment

thereof, to provide conjugates or fusion polypeptides having a plasma half-life that is sufficiently long for the imaging purpose but sufficiently short to be cleared form the body of the particular patient on which it is applied.

In another example for a conjugate, an associate or fusion polypeptide comprising a therapeutic compound effective to treat or alleviate a particular condition in a patient in need for such a treatment it would be advantageous to use the variant albumin or fragment thereof having a longer plasma half-life than the parent or reference albumin or fragment thereof, to provide associates or conjugates or fusion polypeptides having longer plasma half-lives which would have the benefit that the administration of the associate or conjugate or fusion polypeptide of the invention would be needed less frequently or at reduced dose with less side effects compared to the situation where the parent or reference albumin or associates thereof or fragment thereof was used. For example, the invention provides a method of treating a proliferative disease in an individual, comprising administering the individual an effective amount of an associate according to the invention in which the associate comprises a taxane, a taxol or taxol derivative (*e.g.* paclitaxel).

**Use to increase half-life**

A fifteenth aspect of the invention provides for use of a polypeptide as defined in any previous aspect of the invention to increase the half-life of a molecule such as a bioactive agent, an imaging agent, a diagnostic agent, a contrast agent or a therapeutic compound such as a chemotherapeutic drug or radiopharmaceutical. Preferably, the half-life is increased by at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or at least 100% relative to the half-life of the molecule alone. Preferably, the half-life is increased by at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 hours or by at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or at least 14 days relative to the half-life of the molecule alone.

For example, the half-life of a molecule may be increased by conjugating it to the polypeptide as defined in any previous aspect of the invention for example *via* a conjugatable cysteine residue; by genetically fusing the molecule to the polypeptide, by associating the molecule with the polypeptide and/or by incorporating it into a particle according to any previous aspect of the invention.

**EMBODIMENTS OF THE INVENTION**

The invention is further described with reference to the following numbered paragraphs:

1. A conjugation-competent polypeptide comprising an amino acid sequence which is at least 70% identical to human albumin, particularly residues 1 to 585 of the mature human albumin polypeptide sequence of SEQ ID NO. 2, or a fragment thereof;

wherein at least one (*e.g.* several) position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue; and

preferably wherein the conjugation-competent polypeptide has a tendency to exist as a monomer in solution which is at least 70% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution.

2. The conjugation-competent polypeptide of Paragraph 1, wherein the polypeptide comprises one or more (*e.g.* several) of:

substitution of an amino acid, other than cysteine, with a cysteine at a position corresponding to a position equivalent to any of residues K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2; and/or

insertion of a cysteine at a position adjacent the N- or C- side of an amino acid corresponding to a position equivalent to any of residues K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2.

3. The conjugation-competent polypeptide of Paragraph 1 or 2 wherein two, three, four, five or more (*e.g.* several) positions equivalent to positions selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2 comprise a conjugation-competent cysteine residue.

4. The conjugation-competent polypeptide of any preceding Paragraph, wherein the polypeptide has a tendency to exist as a monomer in solution which is at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution.

5. The conjugation-competent polypeptide of any preceding Paragraph wherein the tendency of the polypeptide to exist as monomer in solution is measured following storage for at least 7 weeks at a temperature from 2 to 8°C such as 5°C, at least 8 weeks at a temperature

from 2 to 8°C such as 5°C, at least 3 months at a temperature from 2 to 8°C such as 5°C, at least 4 months at a temperature from 2 to 8°C such as 5°C, at least 6 months storage at a temperature from 2 to 8°C such as 5°C, or at least 3 months storage at a temperature of about 40 °C.

6. The conjugation-competent polypeptide of Paragraph 5 wherein the tendency of the polypeptide to exist as monomer in solution is measured following storage for at least 3 months at a temperature from 2 to 8°C, such as 5°C

7. The conjugation-competent polypeptide of paragraph 5 or 6, prior to storage, wherein the polypeptide is purified using triazine (such as AlbuPure®) chromatography matrix or DE-FF chromatography matrix prior to storage.

8. The conjugation-competent polypeptide of any of paragraphs 5, 6 or 7 wherein, prior to storage, the polypeptide is purified using triazine (such as AlbuPure®) chromatography matrix followed by DE-FF chromatography matrix.

9. The conjugation-competent polypeptide of any of paragraphs 5 to 8 wherein, prior to storage, the polypeptide is purified using triazine (such as AlbuPure®) chromatography matrix followed by DE-FF chromatography matrix followed by size exclusion (*e.g* size exclusion limit (Mr) of about 5 $x10^3$ to 2.5 $x10^5$ such as Sephacryl S-200 HR) chromatography.

10. The conjugation-competent polypeptide of any of Paragraphs 5 to 9 wherein the storage uses a polypeptide concentration of from 0.5 to 50 mg/mL.

11. The conjugation-competent polypeptide of any of Paragraphs 5 to 10 wherein the storage uses a polypeptide concentration of about 5 mg/mL.

12. The conjugation-competent polypeptide of any of Paragraphs 5 to 11 wherein the storage is at a pH between about 6.0 and about 7.5.

13. The conjugation-competent polypeptide of any of Paragraphs 5 to 12 wherein the storage is at a pH about 7.

14. The conjugation-competent polypeptide of any of Paragraphs 5 to 13 wherein the storage uses a buffer comprising 50 mM ammonium acetate, 10 mM sodium octanoate, pH 7.0, preferably at a polypeptide concentration of from about 0.2 to about 2.5 mg/mL.

15. The conjugation-competent polypeptide of any of Paragraphs 5 to 14 wherein the storage uses a buffer comprising 25 mM sodium phosphate, 215 mM sodium chloride, pH 6.5, preferably at a polypeptide concentration of from about 5 to about 50 mg/mL.

16. The conjugation-competent polypeptide of any preceding Paragraph, wherein at least one (*e.g.* several) position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, L284, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue; and wherein the tendency to exist as monomer in solution is at least 75% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution.

17. The conjugation-competent polypeptide of any preceding Paragraph wherein the amino acid sequence is at least 95% identical to human albumin, particularly residues 1 to 585 of the mature human albumin polypeptide sequence of SEQ ID NO. 2, or a fragment thereof and the conjugation-competent polypeptide has a tendency to exist as a monomer in solution which is at least 80% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution.

18. The conjugation-competent polypeptide of any preceding Paragraph, wherein at a position equivalent to position 34 of SEQ ID NO. 2 there is a conjugation-competent cysteine.

19. The conjugation-competent polypeptide of any of Paragraphs 1 to 18, wherein at a position equivalent to position 34 of SEQ ID NO. 2 there is not a conjugation-competent cysteine.

20. The conjugation-competent polypeptide of any preceding Paragraph in which the polypeptide comprises two or more (several) conjugation-competent cysteine residues wherein, when the polypeptide is folded, there is a distance of at least 5 Å between at least one pair of the conjugation-competent cysteine residues.

21. The conjugation-competent polypeptide of any preceding Paragraph, wherein the polypeptide comprises substitution of an amino acid, other than cysteine, with a cysteine at one or both positions corresponding to a position equivalent to residues K93 or E294 of SEQ ID NO. 2.

22. The conjugation-competent polypeptide of any preceding Paragraph which is capable of forming a conjugate with maleimide-polyethylenglycol2-biotin, at a conjugation efficiency of at least 90%, preferably at least 95%, suitably wherein the conjugate is at 90%, preferably at least 95% stable upon controlled hydrolysis.

23. The conjugation-competent polypeptide of Paragraph 22 wherein the capability of forming a conjugate with maleimide-polyethylenglycol2-biotin is determined by incubating at ambient temperature overnight in phosphate buffered saline buffer pH 7.4.

24. The conjugation-competent polypeptide of Paragraph 22 or 23 wherein stability is determined by incubating at pH 9.0 and 37 °C for at least 18 hours, preferably 24 hours, in a buffered salts solution, such as phosphate buffered saline.

25. A conjugation-competent polypeptide comprising an amino acid sequence which is at least 70% identical to human albumin (SEQ ID NO. 2), or a fragment thereof;

wherein at least one (*e.g.* several) position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue; and

comprising at least one (*e.g.* several) further conjugation-competent cysteine, or at least one (*e.g.* several) modification that alters the binding affinity of the polypeptide for FcRn, or alters the plasma half-life of the polypeptide.

26. The conjugation-competent polypeptide of Paragraph 25 wherein the at least one (*e.g.* several) further modification comprises at least one (*e.g.* several) further conjugation-competent cysteine as defined in any one of Paragraphs 1, 2, 3 or 21.

27. The conjugation-competent polypeptide of any preceding Paragraph wherein at least one (*e.g.* several) position equivalent to a position selected from D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371, N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2 comprises a conjugation-competent cysteine.

28. The conjugation-competent polypeptide of any preceding Paragraph in which the polypeptide comprises one or more (*e.g.* several) of:

substitution of an amino acid, other than cysteine, with a cysteine at a position corresponding to a position equivalent to any of residues D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371, N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2; and/or

insertion of a cysteine at a position adjacent the N- or C- side of an amino acid corresponding to a position equivalent to any of residues D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371, N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2; and/or

deletion or substitution of a cysteine at a position corresponding to any of C360, C316, C75, C168, C558, C361, C91, C124, C169 and C567 of SEQ ID NO. 2 so as to generate a conjugation competent cysteine at any of C369, C361, C91, C177, C567, C316, C75, C169, C124 and C558; and/or

addition of a cysteine to the N- side of the N-terminal residue of an albumin sequence or to the C- side of the C-terminal residue of an albumin sequence.

29. The conjugation-competent polypeptide of any preceding Paragraph in which the polypeptide comprises conjugation-competent cysteines located at: (a) A2 + L585, (b) A2 + A364 + D562 + L585C, (c) A2 and adjacent the C-side of the C-terminus of the albumin (d) T79 + A364; (e) A364 + D1; (f) T79 + D562 + A364; (g) D562 + A364 + D1; (h) T79 + D562 + A364 + A504; (i) T79 + D562 + A364 + L585; (j) T79 + D562 + A364 + D1; (k) T79 + D562 + A364 +L585 + D1; (l) E86 + D562 + A364 +A504 + A2; (m) S270 + A581; (n) S270 + D129; (o) S270 + A581 + E82; (p) S270 + A581 + D129; (q) S270 + A581 + E82 + D129; (r) S270 + A581 + E82 + D129 + Q397; (s) C369 + C177; (t) A364 + A581; (u) T79 + A364 + A581; (v) A364 + A581 + D129; (w) A364 + C177; (x) D562 + C369; (y) D129 + C369; (z) A581 + C369; or (aa) D562 + D129 + C369.

30. The conjugation-competent polypeptide of any preceding Paragraph which comprises or consists of albumin domain III or a variant thereof and at least one (*e.g.* several) additional albumin domain or fragment thereof, such as a second albumin domain III or a variant thereof.

31. The conjugation-competent polypeptide of any preceding Paragraph which comprises or consists of at least one (*e.g.* several) albumin domain III or variant or fragment thereof wherein at least one (*e.g.* several) albumin domain III comprises one or more (*e.g.* several) substitutions

in positions corresponding to the positions in SEQ ID NO. 2 selected among: 573, 500, 550, 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 574, 575, 577, 578, 579, 580, 581, 582 and 584.

32. The conjugation-competent polypeptide of Paragraph 31, wherein the one or more (*e.g.* several) substitutions in positions corresponding to the positions in SEQ ID NO. 2 is selected among: K573Y, W, P, H, F, V, I, T, N, S, G, M, C, A, E, Q, R, L, D, K500E, G, D, A, S, C, P, H, F, N, W, T, M, Y, V, Q, L, I, R, Q417A, H440A, H464Q, E492G, D494N,Q,A, E495Q,A, T496A, D494E+Q417H, D494N+T496A, E492G+V493P, P499A, E501A,Q, N503H,K, H510Q, H535Q, K536A, P537A, K538A, K541G,D, D550E,N, E492G+K573P,A, or E492G/N503H/K573P.

33. The conjugation-competent polypeptide of any preceding Paragraph wherein the polypeptide comprises alterations at two or more (*e.g.* several) positions selected from positions corresponding to positions (a) 492 and 580; (b) 492 and 574; (c) 492 and 550; (d) 550 and 573; (e) 550 and 574; (f) 550 and 580 in SEQ ID NO. 2.

34. The conjugation-competent polypeptide of any preceding Paragraph comprising: (i) an N-terminal region comprising a first albumin which is a human albumin variant, in which the N-terminal of the first albumin comprises all amino acids of the human albumin variant except the C-terminal 2 to 30 amino acids; and

(ii) a C-terminal region of a second albumin, which is selected from macaque albumin, mouse albumin, rabbit albumin, sheep albumin, human albumin, goat albumin, chimpanzee albumin, hamster albumin, guinea pig albumin, rat albumin, cow albumin, horse albumin, donkey albumin, dog albumin, chicken albumin, or pig albumin, or a variant thereof, in which the C-terminal of the second albumin or albumin variant comprises the C-terminal 2 to 30 amino acids of the second albumin or albumin variant;

wherein the polypeptide has (i) an altered plasma half-life compared with the human albumin variant and/or (ii) an altered binding affinity to FcRn compared with the human albumin variant.

35. The conjugation-competent polypeptide of any preceding Paragraph comprising one or more (*e.g.* several) alterations in Domain I of the mature human albumin polypeptide sequence of SEQ ID NO. 2; and one or more (*e.g.* several) alterations in Domain III of the mature human albumin polypeptide sequence of SEQ ID NO. 2, wherein the one or more (*e.g.* several) alterations cause the polypeptide to have an altered binding affinity to FcRn.

36. The conjugation-competent polypeptide of Paragraph 35 wherein the alteration(s) in Domain I are selected from positions corresponding to any of positions 78 to 120 of SEQ ID NO.

2, such as any of positions 78 to 88 and/or from any of 105 to 120; and the alteration(s) in Domain III are selected from positions corresponding to any of positions 425, 505, 510, 512, 524, 527, 531, 534, 569, 573, or 575 of SEQ ID NO. 2.

37. The conjugation-competent polypeptide of Paragraph 36 wherein the alteration at the position corresponding to positions is selected among 78 to 120 or 425, 505, 510, 512, 524, 527, 531, 534, 569, 573, and/or 575 of SEQ ID NO. 2 is a substitution; and the alteration is optionally a substitution selected from (i) 83N, K or S; (ii) 111D, G, H, R, Q or E; or (iii) 573P, Y, W, H, F, T, I or V.

38. The conjugation-competent polypeptide of any preceding Paragraph comprising one or more (*e.g.* several) alterations in Domain II of the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions 349, 342, 381, 345, 384, 198, 206, 340, 341, 343, 344, 352, 382, 348, and/or 383 in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

39. The conjugation-competent polypeptide of Paragraph 38 wherein the alteration at the position corresponding to position 349, 342, 381, 345, 384, 198, 206, 340, 341, 343, 344, 352, 382, 348, and/or 383 is a substitution; and the alteration is optionally a substitution selected from (i) 349F, W, Y, H, P, K or Q, preferably F; (ii) 342Y, W, F, H, T, N, Q, A, C, I, L, P, V, preferably Y; (iii) 381G or A, preferably G; or (iv) 345E, H, I or Q.

40. The conjugation-competent polypeptide of any preceding Paragraph comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V418, T420, V424, E505, V547, K573 in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

41. The conjugation-competent polypeptide of any preceding Paragraph comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V381, preferably V381N or Q; E383, preferably E383A, G, I, L, or V; N391, preferably N391A, G, I, L or V; Y401 preferably Y401D or E; K402, preferably K402A, G, I, L, or V; L407, preferably L407F, N, Q, W, or Y; Y411, preferably Y411Q, or N; K413, preferably K413C, S, or T; K414, preferably K414S or T; V415C, preferably V415C, S, or T; Q416, preferably Q416H or P; V424,

preferably V424A, G, I, L, N, or Q; V426D, preferably V426D, E, H, or P; G434, preferably G434C, S, or T; E442, preferably E442K or R; R445, preferably R445F, W or Y; P447, preferably P447S or T; E450, preferably E450D or E; S454, preferably S454C, M or T; V455, preferably V455N or Q; V456, preferably V456N or Q; L457, preferably L457F, W or Y; Q459, preferably Q459K or R; L463, preferably L463N or Q; E495, preferably E495D; T506, preferably T506F, W or Y; T508, preferably T508K, R, or S; F509, preferably F509C, I, L, M, V, W or Y; A511, preferably A511F, W, or Y; D512, preferably D512F, W or Y; T515, preferably T515C, H, N, P, Q or S; L516, preferably L516F, S, T, W or Y; S517, preferably S517C, F, M, T, W or Y; K519, preferably K519A, G, I, L, or V; R521, preferably R521F, W or Y; I523, preferably I523A, D, E, F, G, K, L, N, Q, R, V, W or Y; K524, preferably K524A, G, I, L or V; K525, preferably K525A, G, I, L or V; Q526, preferably Q526C, M, S, T or Y; T527, preferably T527F, W or Y; E531, preferably E531A, G, I, L or V; H535, preferably H535D, E or P; K538, preferably K538F, W or Y; A539, preferably A539I, L or V; K541, preferably, K541F, W or Y; K557, preferably K557A, G, I, L or V; A561, preferably A561F, W or Y; T566, preferably T566F, W or Y; A569, preferably A569H or P in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

42. The conjugation-competent polypeptide of any preceding Paragraph comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V547, preferably V457A; K573, preferably K573P or Y; I523, preferably I523A or G, T527, preferably T527M, K500, preferably K500A; or E505, preferably E505Q in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

43. The conjugation-competent polypeptide of any preceding Paragraph comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions 573, 523, 527 or 505 of SEQ ID NO. 2, preferably K573Y; I523G; I523A; T527M; E505Q; or K573P.

44. The conjugation-competent polypeptide of Paragraph 43 comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions K573Y and I523G; K573Y, I523G and T527M; K573Y, E505Q and T527M; K573Y and T527M; K573P and I523G; K573P, I523G and T527M; K573P, E505Q and T527M; K573P and T527M; V547A; V547A and K573P; V547A, E505Q, K573P and T527M; or K500A and H510Q.

45. The conjugation-competent polypeptide of any of Paragraphs 25 to 44 wherein the conjugation-competent polypeptide has a tendency to exist as a monomer in solution which is at least 70% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution, and optionally at least 75%, at least 80%, at least 90%, at least 95% or at least 100%.

46. The conjugation-competent polypeptide of any preceding Paragraph, in which the polypeptide has at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.2, 99.4, 99.6, 99.8% sequence identity to SEQ ID NO. 2.

47. The conjugation-competent polypeptide of any preceding Paragraph wherein, when the polypeptide is folded, there are at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and preferably all 17 of the native disulphide bonds of the polypeptide of SEQ ID NO. 2.

48. The conjugation-competent polypeptide of any preceding Paragraph in which the polypeptide further comprises a further linker to which a bioactive compound, radiopharmaceutical or imaging agent may be linked.

49. The conjugation-competent polypeptide of any preceding Paragraph wherein the alteration(s) to provide a conjugation competent cysteine residue(s) result in a polypeptide with acceptable immunogenicity in human, preferably an immunogenicity which is comparable to or lower than that of wild-type HSA (SEQ ID NO. 2).

50. The conjugation-competent polypeptide of any preceding Paragraph wherein the alteration(s) to provide a conjugation competent cysteine residue(s) does not adversely affect the immunogenicity of the polypeptide in human, *e.g.* relative to the immunogenicity of wild-type HSA (SEQ ID NO. 2).

51. The conjugation-competent polypeptide of Paragraph 49 or 50 wherein the immunogenicity of the polypeptide is determined or predicted by screening for T-cell epitopes and/or for B-cell epitopes.

52. The conjugation-competent polypeptide of any of Paragraphs 50 to 51 wherein the immunogenicity of the polypeptide is determined or predicted by an *ex vivo* T cell activation assay.

53. The conjugation-competent polypeptide of Paragraph 52 wherein the T cell activation assay comprises measuring T cell responses using a proliferation assay, *e.g.* [3H]-thymidine uptake.

54. The conjugation-competent polypeptide of Paragraph 52 or 53 wherein the polypeptide has less than 10% reactivity in the T cell proliferation assay, preferably less than 8, 6, 4, or 2 % reactivity, most preferably 0%.

55. The conjugation-competent polypeptide of any of Paragraphs 52 to 54 wherein the T cell activation assay comprises measuring T cell responses using a cytokine secretion assay, *e.g.* IL-2 ELISpot.

56. The conjugation-competent polypeptide of Paragraph 55 wherein the polypeptide has less than 10% reactivity in the cytokine secretion assay, preferably less than 8, 6, 4, or 2 % reactivity, most preferably 0%.

57. The conjugation-competent polypeptide of any of Paragraphs 49 to 56 wherein the polypeptide has less than 10% reactivity in a T cell proliferation assay and in a cytokine secretion assay.

58. The conjugation-competent polypeptide of any preceding Paragraph wherein the polypeptide does not stimulate an adverse antibody response in human.

59. A fusion polypeptide comprising a conjugation-competent polypeptide of any preceding Paragraph and a fusion partner polypeptide.

60. A polynucleotide which encodes the polypeptide of any of Paragraphs 1 to 59.

61. A plasmid comprising the polynucleotide of Paragraph 60.

62. A host cell comprising a polynucleotide of Paragraph 60 and/or a plasmid of Paragraph 61.

63. The host cell of Paragraph 62, which is a yeast cell, particularly a *Saccharomyces cerevisiae* cell.

64. A conjugate which comprises a bioactive compound, radiopharmaceutical or imaging agent, and a polypeptide according to any of Paragraphs 1 to 59, wherein the bioactive compound is radiopharmaceutical or imaging agent, linked to the polypeptide through a conjugation-competent cysteine residue of the polypeptide.

65. The conjugate of Paragraph 64 further comprising one or more (*e.g.* several) further bioactive compounds radiopharmaceuticals or imaging agents, each bioactive compound, radiopharmaceutical or imaging agent, being linked to the polypeptide through a conjugation-competent cysteine residue of the polypeptide.

66. A method of producing the polynucleotide of Paragraph 60 comprising:

(a) providing a nucleic acid molecule encoding a parent albumin or fragment thereof; and

(b) modifying the nucleic acid sequence of the nucleic acid molecule to encode a conjugation-competent polypeptide which is at least 70% identical to human albumin, particularly residues 1 to 585 of the mature human albumin polypeptide sequence of SEQ ID NO. 2, or a fragment thereof, wherein at least one position equivalent to a position selected from K93, E294, A226, E230, I271, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398, particularly from K93, E294, A226, E230, and I271, of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue.

67. A method of producing the polypeptide of any of Paragraphs 1 to 59, comprising:

(a) culturing the host cell of Paragraph 62 or 63 under conditions that allow expression of the polypeptide; and

(b) recovering the polypeptide from the host cell and/or from host cell growth medium.

68. The method of paragraph 67 in which the host cell exhibits enhanced chaperone activity.

69. The method of Paragraph 67 or 68 further comprising purifying the polypeptide obtained in step (b).

70. A method of producing the conjugate of Paragraph 64 or 65 which comprises linking a polypeptide of any one of Paragraphs 1 to 59, or produced by the method of any one of Paragraphs 67 to 69, to a bioactive compound, radiopharmaceutical or imaging agent, through a conjugation-competent cysteine residue of the polypeptide.

71. An associate comprising the conjugate of Paragraph 64 or 65 and a bioactive, therapeutic, prophylactic, diagnostic, imaging or other beneficial moiety.

72. A nanoparticle or a microparticle or a liposome comprising the polypeptide of any one of Paragraphs 1 to 59, the conjugate of Paragraph 64 or 65 or the associate of Paragraph 71.

73. A composition comprising the conjugate of Paragraph 64 or 65, the associate of Paragraph 71 or the nanoparticle or microparticle or liposome of Paragraph 72 and at least one (*e.g.* several) pharmaceutically acceptable carrier or diluent.

74. The conjugate of Paragraph 64 or 65, the associate of Paragraph 71, the nanoparticle or microparticle or liposome of Paragraph 72, or the composition of Paragraph 73, wherein the bioactive molecule, radiopharmaceutical or imaging agent, is selected from those described herein.

75. The conjugate of Paragraph 64, 65 or 74, or the associate of Paragraph 71, the nanoparticle or microparticle or liposome of Paragraph 72 for treatment of disease, treatment of illness and/or for diagnosis.

76. Use of a polypeptide as defined in any of Paragraphs 1 to 59 to increase half-life of a bioactive molecule, radiopharmaceutical or imaging agent.

The invention is further described by the following examples that should not be construed as limiting the scope of the invention.

## EXAMPLES

### Example 1: Preparation of variants.

### Preparation of specific HSA variant expression plasmids.

Methods for the expression of HSA variants were performed using several techniques, employing standard molecular biology techniques throughout, such as described in Sambrook, J. and D.W. Russell, 2001 (Molecular Cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y).

### Method 1.

Single amino acid mutations (K93C, A226C, E230C, I271C, E294C, and E358C) were introduced into the pDB5155 plasmid (encoding mutated C34A HSA, SEQ ID NO. 30) using a mutagenic forward primer and non-mutagenic reverse primer (Table 3). pDB5155, encoding a C34A mutant, based on the plasmid pDB5102 was made using a mutagenic forward primer and a non-mutagenic reverse primer (Table 3). pDB5102 is described in WO 2015/036579. Methylated template DNA was prepared by mixing about 1.7 µg of plasmid DNA with 5 µL 10x buffer (50 mM Tris-HCl mM β-mercaptoethanol, 10 mM EDTA pH 7.5 at 25°C - New England

Biolabs), 1 µL *dam* methyltransferase (New England Biolabs), 12.5 µL s-adenosylmethionine (New England Biolabs 80 µM final concentration) and water to 50 µl final volume and incubating at 37°C for 1.5 hours. Reaction mixtures were then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The relevant primers were employed in the PCR reaction (described in Tables 4 and 5) using *dam*-methylated pDB5102 as template and Q5 DNA polymerase (New England Biolabs). Amplification of the plasmid was confirmed by analysis of 5 µl of PCR product on a 1% TBE agarose gel. The remaining PCR product was supplemented with 5 µl buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 at 25°C - New England Biolabs) and 1 µl *Dpn*I enzyme, followed by incubation at 37°C for two hours. The reaction mixtures were then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. 1 µl of purified plasmid was transformed into *E. coli* 10-beta cells (New England Biolabs) and plated onto LB plates (5 g/L yeast extract, 10 g/L peptone from casein, 10 g/L NaCl, 12 g/L agar agar (Millers LB agar, Merck Millipore)) supplemented with 50 µg/mL ampicillin. Plasmids were isolated using a Qiagen Plasmid Plus Kit (Qiagen - according to manufacturer's instructions) and sequenced to confirm the presence of the desired mutation within the HSA sequence and the plasmid named pDB5155.

Methylated pDB5155 template DNA was prepared by mixing about 3.0 µg of plasmid DNA with 5 µL 10x buffer (50 mM Tris-HCl mM β-mercaptoethanol,10 mM EDTA pH 7.5 at 25°C - New England Biolabs), 1 µL *dam* methyltransferase (New England Biolabs), 12.5 µL 80 µM s-adenosylmethionine (New England Biolabs 80 µM final concentration) and water to 50 µl final volume and incubating at 37°C for two hours. Reaction mixtures were then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

The relevant primers were employed in the PCR reaction (described in Tables 4 and 5) using *dam*-methylated pDB5155 as template and Q5 DNA polymerase (New England Biolabs).

**Table 3. Oligonucleotides for mutagenic amplification with mutated codons underlined (R = reverse, F = Forward) and the resultant protein.**

| Oligo | Sequence (5' to 3') | SEQ ID NO. |
|---|---|---|
| C34A R | TTGTTGCAAGTATTGAGCGAAAGCGATCAAGACCAA | 31 |
| C34A F | TTCGCTCAATACTTGCAACAAGCTCCATTCGAAGATCACGTCAAG | 32 |
| L24C F | GAAGAAAACTTCAAGGCTTTGGTCTGTATCGCTTTCGCTCAATACTTGCA | 33 |
| F49C F | AGTTGGTCAACGAAGTTACCGAATGTGCTAAGACTTGTGTTGCTGACG | 34 |
| V54C F | GTTACCGAATTCGCTAAGACTTGTTGTGCTGACGAATCCGCGGAAAAC | 35 |

| Oligo | Sequence (5' to 3') | SEQ ID NO. |
|---|---|---|
| D56C F | GAATTCGCTAAGACTTGTGTTGCTTGTGAATCCGCGGAAAACTGTGACA | 36 |
| L66C F | CGCGGAAAACTGTGACAAGTCCTGTCACACCTTGTTCGGTGATAAGTT | 37 |
| A92C F | CGGTGAAATGGCTGACTGTTGTTGTAAGCAAGAACCAGAAAGAAACGAA | 38 |
| K93C F | GTGAAATGGCTGACTGTTGTGCTTGTCAAGAACCAGAAAGAAACGAATGT | 39 |
| Q94C F | AAATGGCTGACTGTTGTGCTAAGTGTGAACCAGAAAGAAACGAATGTTTC | 40 |
| E97C F | ACTGTTGTGCTAAGCAAGAACCATGTAGAAACGAATGTTTCTTGCAACAC | 41 |
| H128C F | TTGACGTCATGTGTACTGCTTTCTGTGACAACGAAGAAACCTTCTTGAAG | 42 |
| F156C F | ACTTCTACGCTCCAGAATTGTTGTGTTTCGCTAAGAGATACAAGGCTGC | 43 |
| A226C F | AGATTGTCTCAAAGATTCCCAAAGTGTGAATTCGCTGAAGTTTCTAAGTTG | 44 |
| E227C F | TGTCTCAAAGATTCCCAAAGGCTTGTTTCGCTGAAGTTTCTAAGTTGGTT | 45 |
| E230C F | GATTCCCAAAGGCTGAATTCGCTTGTGTTTCTAAGTTGGTTACTGACTTG | 46 |
| D237C F | GCTGAAGTTTCTAAGTTGGTTACTTGTTTGACTAAGGTTCACACTGAATGT | 47 |
| K240C F | TCTAAGTTGGTTACTGACTTGACTTGTGTTCACACTGAATGTTGTCACGG | 48 |
| D259C F | GGAATGTGCTGATGACAGAGCTTGTTTGGCTAAGTACATCTGTGAAAAC | 49 |
| K262C F | TGATGACAGAGCTGACTTGGCTTGTTACATCTGTGAAAACCAAGACTCT | 50 |
| N267C F | GACTTGGCTAAGTACATCTGTGAATGTCAAGACTCTATCTCTTCCAAGTTG | 51 |
| Q268C F | TTGGCTAAGTACATCTGTGAAAACTGTGACTCTATCTCTTCCAAGTTGAAG | 52 |
| I271C F | TACATCTGTGAAAACCAAGACTCTTGTTCTTCCAAGTTGAAGGAATGTTGT | 53 |
| L275C F | ACCAAGACTCTATCTCTTCCAAGTGTAAGGAATGTTGTGAAAAGCCATTG | 54 |
| E277C F | GACTCTATCTCTTCCAAGTTGAAGTGTTGTTGTGAAAAGCCATTGTTGGAA | 55 |
| L284C F | AAGGAATGTTGTGAAAAGCCATTGTGTGAAAAGTCTCACTGTATTGCTGAA | 56 |
| E294C F | AAGTCTCACTGTATTGCTGAAGTTTGTAACGATGAAATGCCAGCTGACTT | 57 |
| E311C F | CATCTTTGGCTGCTGACTTCGTTTGTTCTAAGGACGTTTGTAAGAACTAC | 58 |
| K317C F | TTCGTTGAATCTAAGGACGTTTGTTGTAACTACGCTGAAGCTAAGGACG | 59 |
| A322C F | GACGTTTGTAAGAACTACGCTGAATGTAAGGACGTCTTCTTGGGTATGTT | 60 |
| E333C F | GTCTTCTTGGGTATGTTCTTGTACTGTTACGCTAGAAGACACCCAGACT | 61 |

| Oligo | Sequence (5' to 3') | SEQ ID NO. |
|---|---|---|
| D340C F | CGAATACGCTAGAAGACACCCATGTTACTCCGTTGTCTTGTTGTTGAG | 62 |
| E354C F | TGTTGAGATTGGCTAAGACCTACTGTACTACCCTCGAGAAGTGTTGTG | 63 |
| E358C F | CTAAGACCTACGAAACTACCCTCTGTAAGTGTTGTGCTGCTGCTGACC | 64 |
| K359C F | GACCTACGAAACTACCCTCGAGTGTTGTTGTGCTGCTGCTGACCCA | 65 |
| A362C F | AAACTACCCTCGAGAAGTGTTGTTGTGCTGCTGACCCACACGAATGT | 66 |
| E382C F | TCGATGAATTCAAGCCATTGGTCTGTGAACCACAAAACTTGATCAAGCAA | 67 |
| L398C F | GCAAAACTGTGAATTGTTCGAACAATGTGGTGAATACAAGTTCCAAAACGC | 68 |
| L24C R | GACCAAAGCCTTGAAGTTTTCTTCACCCAAGTCCT | 69 |
| F49C R | TTCGGTAACTTCGTTGACCAACTTGACGTGATCTT | 70 |
| V54C R | ACAAGTCTTAGCGAATTCGGTAACTTCGTTGACCAA | 71 |
| D56C R | AGCAACACAAGTCTTAGCGAATTCGGTAACTTCGTT | 72 |
| L66C R | GGACTTGTCACAGTTTTCCGCGGATTCGTCAGC | 73 |
| A92C R | ACAACAGTCAGCCATTTCACCGTAGGTTTCTCTC | 74 |
| K93C R | AGCACAACAGTCAGCCATTTCACCGTAGGTTTCTC | 75 |
| Q94C R | CTTAGCACAACAGTCAGCCATTTCACCGTAGGTT | 76 |
| E97C R | TGGTTCTTGCTTAGCACAACAGTCAGCCATTTCAC | 77 |
| H128C R | GAAAGCAGTACACATGACGTCAACTTCTGGTCTAA | 78 |
| F156C R | CAACAATTCTGGAGCGTAGAAGTATGGGTGTCTTC | 79 |
| A226C R | CTTTGGGAATCTTTGAGACAATCTAGCGACAGCC | 80 |
| E227C R | AGCCTTTGGGAATCTTTGAGACAATCTAGCGACAG | 81 |
| E230C R | AGCGAATTCAGCCTTTGGGAATCTTTGAGACAATCT | 82 |
| D237C R | AGTAACCAACTTAGAAACTTCAGCGAATTCAGCCTT | 83 |
| K240C R | AGTCAAGTCAGTAACCAACTTAGAAACTTCAGCGAA | 84 |
| D259C R | AGCTCTGTCATCAGCACATTCCAACAAGTCACCG | 85 |
| K262C R | AGCCAAGTCAGCTCTGTCATCAGCACATTCCAAC | 86 |
| N267C R | TTCACAGATGTACTTAGCCAAGTCAGCTCTGTCATC | 87 |
| Q268C R | GTTTTCACAGATGTACTTAGCCAAGTCAGCTCTGT | 88 |
| I271C R | AGAGTCTTGGTTTTCACAGATGTACTTAGCCAAGTC | 89 |
| L275C R | CTTGGAAGAGATAGAGTCTTGGTTTTCACAGATGTA | 90 |
| E277C R | CTTCAACTTGGAAGAGATAGAGTCTTGGTTTTCACAG | 91 |
| L284C R | CAATGGCTTTTCACAACATTCCTTCAACTTGGAAGA | 92 |
| E294C R | AACTTCAGCAATACAGTGAGACTTTTCCAACAATGG | 93 |

| Oligo | Sequence (5' to 3') | SEQ ID NO. |
|---|---|---|
| E311C R | AACGAAGTCAGCAGCCAAAGATGGCAAGTCAGCT | 94 |
| K317C R | ACAAACGTCCTTAGATTCAACGAAGTCAGCAGCC | 95 |
| A322C R | TTCAGCGTAGTTCTTACAAACGTCCTTAGATTCAACG | 96 |
| E333C R | GTACAAGAACATACCCAAGAAGACGTCCTTAGCTTC | 97 |
| D340C R | TGGGTGTCTTCTAGCGTATTCGTACAAGAACATAC | 98 |
| E354C R | GTAGGTCTTAGCCAATCTCAACAACAAGACAACGG | 99 |
| E358C R | GAGGGTAGTTTCGTAGGTCTTAGCCAATCTCAACA | 100 |
| K359C R | CTCGAGGGTAGTTTCGTAGGTCTTAGCCAATCTC | 101 |
| A362C R | ACAACACTTCTCGAGGGTAGTTTCGTAGGTCTTAG | 102 |
| E382C R | GACCAATGGCTTGAATTCATCGAAAACCTTAGCGT | 103 |
| L398C R | TTGTTCGAACAATTCACAGTTTTGCTTGATCAAGTTTTG | 104 |

**Table 4. PCR reaction components**

| | | | | |
|---|---|---|---|---|
| Template (5ng/µL) | 1 µL | | Forward primer (10 µM) | 2.5 µL |
| 5x buffer | 10 µL | | Reverse primer (10 µM) | 2.5 µL |
| dNTP (2.5 mM) | 1 µL | | Q5 polymerase | 0.5 µL |
| Sterile water | 32.5 µL | | | |

**Table 5: PCR reaction conditions**

| Temperature | Cycle Length | Number of cycles |
|---|---|---|
| 98 °C | 2 min | 1 |
| 98 °C | 10 sec | 30 |
| 60°C | 30 sec | |
| 72 °C | 5 min | |
| 72 °C | 7 min | 1 |

Amplification of the plasmid was confirmed by analysis of 5 µl of PCR product on a 1% TBE agarose gel. The remaining PCR product was supplemented with 4 µl buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 at 25°C - New England Biolabs) and 1 µl *Dpn*I enzyme, followed by incubation at 37°C for one hour. The reaction mixtures were then purified using a QIAquick 96 PCR purification kit (Qiagen) according to the manufacturer's instructions. 2 µl of purified plasmid was transformed into competent *E. coli* DH5-alpha cells and grown in a 96 deep well block in 1.2 mL LB media (1%

w/v bacteriological tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl) supplemented with 50 µg/mL ampicillin to repair nicks in the DNA backbone. Plasmids were isolated using a QiaPrep 96 turbo miniprep kit (Qiagen - according to manufacturer's instructions). The thio-albumin constructs are detailed in Table 6.

Plasmid DNA was prepared for transformation into *S. cerevisiae* as described in WO 2015/036579 (incorporated herein by reference), Method 4, except that 9723bp *Acc*65I-*Bam*HI fragment from pDB4164 was used as the gapped vector fragment instead of the 9721bp fragment from pDB3936, which has two additional bases GC next to the *BamHI* site to create a *NotI* restriction site **GC**GGCCGC (additional bases in **bold**). pDB3936 is described in WO 2011/124718 (incorporated herein by reference). pDB4164 also differs from pDB3936 in containing a 1368bp sequence between the *Acc*65I and *Bam*HI sites containing an apramycin resistance selectable marker which was excised by the *Acc*65I and *Bam*HI digestion and was not used in the gap-repair transformation. The host strain for the constructs was *S.* cerevisiae BXP10 $cir^0$ (WO 2015/036759, incorporated herein by reference). Transformed cells were grown as single colonies on selective agar plates (BMMD+CSM-Leu or BMMD) from which isolated colonies were patched out, also on selective agar plates, for the preparation of cryopreserved yeast stocks and samples for analysis. Cryopreserved stocks were made from 5 mL of a 48 hour BMMD+CSM-Leu shake flask culture mixed with an equal volume of 40% [w/v] trehalose and 1 mL aliquots transferred to cryovials for storage at -80$^0$C. 0.5 mL BMMD in 48-well microtitre plate wells was inoculated with yeast from the patch plates and grown for 4-days at 30°C with shaking as described in WO 2015/036579, Method 4 (incorporated herein by reference). Shake flask cultures were inoculated from trehalose stocks. Purification of these variants from shake flask was performed as described in WO 2012/150319 (incorporated herein by reference).

Preparation of the expression plasmids for the L24C, F49C, V54C, D56C, L66C, A92C, Q94C, E97C, H128C, F156C, E227C, D237C, K240C, D259C, K262C, N267C, Q268C, L275C, E277C, L284C, E311C, K317C, A322C, E333C, D340C, E354C, K359C, A362C, E382C, and L398C (all in C34A background) was slightly different to that described above:

Single amino acid mutations were introduced into the pDB5155 plasmid (encoding mutated C34A HSA, SEQ ID NO. 30) using a mutagenic forward primer and non-mutagenic reverse primer (Table 3).

Methylated template DNA was prepared by mixing about 2.5 µg of plasmid DNA with 5 µL 10x buffer (50 mM Tris-HCl mM β-mercaptoethanol, 10 mM EDTA pH 7.5 at 25°C - New England Biolabs), 1 µL *dam* methyltransferase (New England Biolabs), 12.5 µL 80 µM s-adenosylmethionine (New England Biolabs 80 µM final concentration) and water to 50 µl final volume and incubating at 37°C for one hour. Reaction mixtures were then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

The relevant primers were employed in the PCR reaction (described in Tables 4 and 5, above) using *dam*-methylated pDB5155 as template and Q5 DNA polymerase (New England Biolabs).

Amplification of the plasmid was confirmed by analysis of 5 µl of PCR product on a 1% TBE agarose gel. The remaining PCR product was supplemented with 4 µl buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 at 25°C - New England Biolabs) and 1 µl *Dpn*I enzyme, followed by incubation at 37°C for one hour. The reaction mixtures were then purified using a QIAquick 96 PCR purification kit (Qiagen) according to the manufacturer's instructions. 1 µl of purified plasmid was transformed into competent *E. coli* DH5-alpha cells and grown in a 96 deep well block in 1.2 mL LB media (1% w/v bacteriological tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl) supplemented with 50 µg/mL ampicillin to repair nicks in the DNA backbone. Plasmids were isolated using a QiaPrep 96 turbo miniprep kit (Qiagen - according to manufacturer's instructions). The thio-albumin constructs are detailed in Table 6.

Plasmid DNA was prepared for transformation into *S. cerevisiae* as described in WO 2015/036579, Method 4 (incorporated herein by reference). The host strain for the constructs was *S. cerevisiae* DYB7 (Payne *et al.* (2008) Applied and Environmental Microbiology Vol 74(24):7759-7766). The yeast microtitre plate growth diverged from the method as described in WO 2015/036579 in that transformations were performed in duplicate and the initial growth was for two days. Stocks were produced from the two days growth by transfer of 50 µl culture to a fresh microtitre plate containing 50 µl 40% (w/v) trehalose. 50 µl of the two day culture was also added to a fresh microtitre plate containing 450 µL of BMMD+CSM-leu and incubated at 30°C with shaking (200 rpm, 2.5 cm orbit at in a sealed chamber at 100% humidity in an Eppendorf Innova 44 incubated shaker) for a further four days. Culture supernatants were harvested by centrifugation at 3000 rpm for 5 minutes and 375 µl of supernatant was transferred to a fresh 48-well microtitre plate.

**Production of expression plasmid and yeast stocks.**

Preparation of the expression plasmids and transformation of *S. cerevisiae* was performed as described in WO 2011/051489 and WO 2012/150319 (incorporated herein by reference) by the 48-hour stocking method, using equal volumes of culture and trehalose. The host strain for the constructs was *S. cerevisiae* BXP10 $Cir^0$ (WO 2015/036759, incorporated herein by reference). Purification of variants from shake flask was performed as described in WO 2012/150319 unless otherwise stated.

The resultant albumin variants are summarized in Table 6.

**Table 6**

| Albumin variant | SEQ ID NO. |
|---|---|
| C34A | 30 |
| C34A+L24C | 105 |
| C34A+F49C | 106 |
| C34A+V54C | 107 |
| C34A+D56C | 108 |
| C34A+L66C | 109 |
| C34A+A92C | 110 |
| C34A+K93C | 111 |
| C34A+Q94C | 112 |
| C34A+E97C | 113 |
| C34A+H128C | 114 |
| C34A+F156C | 115 |
| C34A+A226C | 116 |
| C34A+E227C | 117 |
| C34A+E230C | 118 |
| C34A+D237C | 119 |
| C34A+K240C | 120 |
| C34A+D259C | 121 |

| Albumin variant | SEQ ID NO. |
|---|---|
| C34A+K262C | 122 |
| C34A+N267C | 123 |
| C34A+Q268C | 124 |
| C34A+I271C | 125 |
| C34A+L275C | 126 |
| C34A+E277C | 127 |
| C34A+L284C | 128 |
| C34A+E294C | 129 |
| C34A+E311C | 130 |
| C34A+K317C | 131 |
| C34A+A322C | 132 |
| C34A+E333C | 133 |
| C34A+D340C | 134 |
| C34A+E354C | 135 |
| C34A+E358C | 136 |
| C34A+K359C | 137 |
| C34A+A362C | 138 |
| C34A+E382C | 139 |
| C34A+L398C | 140 |

**Example 2. Thiol determination of DTNB incubated thio-albumin variants**

The free thiol content of thiol albumin variants was determined at small scale using microtitre plate (MTP) grown cultures. The tested thiol albumin variants included the C34A substitution, and thus should lack the thiol group of native albumin. As such, they were each expected to have only one free thiol.

The number of free thiols on a protein can be determined spectrophotometrically using Ellman's reagent. Ellman's reagent (5'5'-dithio-bis(2-nitrobenzoic acid) (DTNB)) is an aromatic disulphide which reacts with thiol groups to form a mixed disulphide of the protein and one mole of 5-thio-2-nitrobenzoic acid (TNB) (per mole of protein sulfhydryl group). This reaction also results in a yellow colour from free TNB being released in solution. Alternatively the number of free thiols on a protein can be determined using mass spectrometric analysis of protein sample treated with DTNB reagent. 5-thio-2-nitrobenzoic acid (TNB) has a molecular weight of 199 Da, thus an increase in mass of 197 Da (TNB minus $H_2$ lost during disulphide bond formation with

the free thiol group on the test protein) indicates the presence of one free thiol group on the protein sample.

4 µl Buffer 2 (4 mg/mL DTNB, 500 mM sodium phosphate, pH 7.0) was added to 200 µL of the test protein culture sample in a 96-well MTP format. The preparation was allowed to incubate for 25 minutes at ambient temperature (20±5 °C) to allow TNB labelling. Protein intact mass was determined by UltraPerformance Liquid Chromatography Mass Spectrometry (UPLC-MS). UPLC separation was carried out on 10 µL of sample using a Waters Acquity on a BEH 50 × 2.1mm ACQUITY BEH 1.7 µm 300Å C4 column and a 5 min analytical gradient of buffer A 0.1% formic acid and Buffer B 100% acetonitrile 0.1% formic acid. Eluted proteins were directly introduced to a Bruker MicrOTOF II mass spectrometer *via* an Electrospray Ionisation (ESI) source. All instrument control and sample tables were controlled using BioPharma Compass™. All data were manually processed over the leading edge of the protein peak between 2.9-3.0 minutes in Data Analysis. This included spectral smoothing using a Gauss smoothing algorithm set at 0.0765 Da and a baseline correction setting of 0.8 flatness. Deconvoluted intact mass spectra were obtained using the Max. Entropy algorithm, all methods and parameters were set within BioPharma Compass™.

The results of the above thiol analysis of the thio-albumin samples are summarised in Table 7. An increase in mass of 197 Da upon DTNB incubation is predicted to be indicative of the presence of one free thiol group on the protein in the sample. A mass increase of 197±15 Da as actually measured by MS was taken as indicative of the correct mass. All variants successfully bound a molecule of TNB.

**Table 7: Mass Spectrometry DTNB thiol screening results**

| **Variant description (all C34A)** | **Molecular weight (Da)** | | | |
|---|---|---|---|---|
| | **Variant** | **Post DTNB treatment** | | |
| | **Theoretical** | **Theoretical** | **Actual measured** | **Difference (Actual minus theoretical)** |
| L24C | 66397 | 66594 | 66599 | 5 |
| F49C | 66363 | 66560 | 66568 | 8 |
| V54C | 66411 | 66608 | 66613 | 5 |
| D56C | 66395 | 66592 | 66600 | 8 |
| L66C | 66397 | 66594 | 66599 | 5 |
| A92C | 66439 | 66636 | 66641 | 5 |
| K93C | 66382 | 66579 | 66588 | 9 |
| Q94C | 66382 | 66579 | 66581 | 2 |
| E97C | 66381 | 66578 | 66580 | 2 |

| Variant description (all C34A) | Molecular weight (Da) | | | |
|---|---|---|---|---|
| | Variant | Post DTNB treatment | | |
| | Theoretical | Theoretical | Actual measured | Difference (Actual minus theoretical) |
| H128C | 66373 | 66570 | 66572 | 2 |
| F156C | 66363 | 66560 | 66564 | 4 |
| A226C | 66439 | 66636 | 66637 | 1 |
| E227C | 66381 | 66578 | 66584 | 6 |
| E230C | 66381 | 66578 | 66582 | 4 |
| D237C | 66395 | 66592 | 66593 | 1 |
| K240C | 66382 | 66579 | 66584 | 5 |
| D259C | 66395 | 66592 | 66594 | 2 |
| K262C | 66382 | 66579 | 66584 | 5 |
| N267C | 66396 | 66593 | 66592 | -1 |
| Q268C | 66382 | 66579 | 66584 | 5 |
| I271C | 66397 | 66594 | 66596 | 2 |
| L275C | 66397 | 66594 | 66597 | 3 |
| E277C | 66381 | 66578 | 66583 | 5 |
| L284C | 66397 | 66594 | 66592 | -2 |
| E294C | 66381 | 66578 | 66581 | 3 |
| E311C | 66381 | 66578 | 66589 | 11 |
| K317C | 66382 | 66579 | 66582 | 3 |
| A322C | 66439 | 66636 | 66640 | 4 |
| E333C | 66381 | 66578 | 66582 | 4 |
| D340C | 66395 | 66592 | 66602 | 10 |
| E354C | 66381 | 66578 | 66583 | 5 |
| E358C | 66381 | 66578 | 66583 | 5 |
| K359C | 66382 | 66579 | 66583 | 4 |
| A362C | 66439 | 66636 | 66641 | 5 |
| E382C | 66381 | 66578 | 66586 | 8 |
| L398C | 66397 | 66594 | 66597 | 3 |

**Example 3. Aggregation screening of thio-albumin variants**

Variants were tested for tendency to remain as a monomer in solution. Each variant has a single free thiol group. Therefore, they were tested in comparison with wild-type HSA, which also has a single free thiol group.

Shake flask culturing of *S. cerevisiae* and purification was performed as described in WO 2012/150319 (incorporated herein by reference) with the following modifications. BMMS media (10 mL) was inoculated with *S. cerevisiae* and grown for 2 days at 30°C with orbital shaking at 200 rpm. An aliquot of each starter culture (5 mL) was used to inoculate 2 × 200 mL BMMS media and grown for 5 days at 30°C with orbital shaking at 200 rpm. Cells were harvested by filtration through a 0.2 µm vacuum filter membrane (Nalgene Sterile Top Filter) and the supernatant retained for purification.

A single step chromatography procedure was used to prepare purified material from the thio-albumin variants. The purification step used a column (bed volume approximately 2 mL) packed with AlbuPure® matrix (ProMetic BioSciences Ltd, Cambridge UK or Albumedix Ltd (formerly Novozymes Biopharma UK Ltd)). This was equilibrated with 50 mM sodium acetate, pH 5.3, and loaded with neat shake flask culture supernatants, at approximately pH 5.5 - 6.5, to approximately 20 mg protein/mL matrix. The column was washed with approximately 10 column volumes each of 50 mM sodium acetate, pH 5.3, and 50 mM ammonium acetate, pH 8.0, respectively. Bound protein was eluted using approximately 10 column volumes of 50 mM ammonium acetate, 10 mM octanoate, pH 7.0. The flow rate throughout was 240 cm/h using an AKTA Explorer system (GE Healthcare). Eluate samples were approximately 20 mL in volume. The concentration and percentage monomer of the eluate samples was determined by Gel Permeation High Pressure Liquid Chromatography (GP-HPLC). Protein concentrations were determined using a LC2010 HPLC system (Shimadzu) equipped with UV detection under Shimadzu VP7.3 client server software control. Injections of 25 µL were made onto a 7.8 mm internal diameter x 300 mm length TSK G3000SWXL column (Tosoh Bioscience), with a 6.0 mm internal diameter x 40 mm length TSK SW guard column (Tosoh Bioscience). Samples were chromatographed in 25 mM sodium phosphate, 100 mM sodium sulphate, 0.05% (w/v) sodium azide, pH 7.0 at 1 $mL.min^{-1}$, with a run time of 15 minutes. Samples were quantified by UV detection at 280 nm, by peak area, relative to a recombinant human albumin standard of known concentration (10 mg/mL).

The samples were reanalysed to determine the change in percentage monomer post seven weeks storage at 2-8°C, and post 6 months storage at 2-8°C. The percentage monomer (in brackets) was determined for each sample relative to its wild type control under the same storage conditions. The results are summarised in Table 8A. Final eluate concentrations were in the range of 0.6-1.2 mg/mL, resulting in 12-24 mg protein recovered post purification. All variants had a monomer percentage equivalent to or higher than that of the wild type control at T=0, which had a monomer percentage of 87%. The variants maintained their monomeric protein percentage over 7 weeks' storage at 2-8°C, with no significant evidence of aggregation propensity during 6 months storage at 2-8°C observed for at least four variants.

**Table 8A: GPHPLC aggregation screening results**

| Sample | GPHPLC conc. (mg/mL) | % Monomer | | | Δ% Monomer | |
|---|---|---|---|---|---|---|
| | | T=0 | T=7 week | T=6 month | 0-7 week | 0-6 month |
| WT albumin control | 1.1 | 87 (100) | 88 (100) | 89 (100) | 1 | 2 |
| C34A+K93C | 0.7 | 91 (105) | 92 (105) | 92 (103) | 1 | 1 |
| C34A+A226C | 1.1 | 93 (107) | 93 (106) | 93 (105) | 0 | 0 |
| C34A+E230C | 0.6 | 90 (103) | 91 (103) | ND (ND) | 1 | ND |
| C34A+I271C | 1.2 | 91 (105) | 91 (103) | 91 (102) | 0 | 0 |
| C34A+E294C | 0.9 | 96 (110) | 96 (109) | 96 (108) | 0 | 0 |
| C34A+E358C | 1.0 | 89 (102) | 83 (94) | 80 (90) | -6 | -9 |

ND: Not determined

Further variants were analysed using the method previously described in Example 3, or alternatively using an Agilent 1260 isocratic UHPLC (Ultra-High Performance Liquid Chromatography) instrument. For the UHPLC method, injections of 4 µL were made onto a 4.6 mm id × 150 mm length BEH 200Å, 1.7 µm column (Waters), using the mobile phase described in Example 3, at 0.5 $mL.min^{-1}$, with a run time of 5 minutes. Samples were quantified by UV detection at 280 nm, by peak height relative to a recombinant human albumin standard of known concentration (10 mg/mL).

The samples were reanalysed post eight weeks storage at 2-8°C, and post 4 months storage at 2-8°C to determine the change in percentage monomer. The percentage monomer (in brackets) was determined for each sample relative to its wild type control under the same storage conditions. The results are summarised in Table 8B. Final eluate concentrations were in the range of 0.1-1.0 mg/mL, resulting in 2-20 mg protein recovered post purification. The majority of variants had a monomer percentage equivalent to or higher than that of the wild type control at T=0, which had a monomer percentage of 86%. These variants maintained their monomeric protein over 8 weeks' storage at 2-8°C, with no significant evidence of aggregation propensity during 4 months storage at 2-8°C observed. However, it was evident that variants C34A+L66C, C34A+E277C, and C34A+E311C had a relatively low percentage monomer at T=0, and consequently had a propensity to form aggregates.

**Table 8B: GPHPLC aggregation screening results**

| Sample | GPHPLC conc. (mg/mL) | % Monomer | | | Δ% Monomer | |
|---|---|---|---|---|---|---|
| | | T=0 | T=8 week | T=4 month | 0-8 week | 0-4 month |
| WT albumin control | 0.6 | 86 (100) | 88 (100) | 87 (100) | 2 | 1 |
| C34A+L24C | 0.7 | 94 (109) | 96 (109) | 97 (112) | 2 | 3 |
| C34A+F49C | 0.5 | 94 (109) | 95 (108) | 94 (108) | 1 | 0 |
| C34A+V54C | 0.5 | 93 (108) | 94 (107) | 93 (107) | 1 | 0 |
| C34A+D56C | 0.3 | 85 (99) | 77 (88) | 75 (86) | -8 | -10 |
| C34A+L66C | 0.2 | 7 (8) | 12 (14) | 6 (7) | 5 | -1 |
| C34A+A92C | 0.9 | 93 (108) | 94 (107) | 94 (108) | 1 | 1 |
| C34A+Q94C | 0.1 | 95 (111) | 96 (109) | 95 (109) | 1 | 0 |
| C34A+E97C | 0.5 | 88 (102) | 85 (97) | 85 (98) | -3 | -3 |
| C34A+H128C | 0.6 | 92 (107) | 93 (106) | 93 (107) | 1 | 1 |
| C34A+F156C | 1.0 | 92 (107) | 94 (107) | 94 (108) | 2 | 2 |
| C34A+E227C | 0.5 | 86 (100) | 88 (100) | 88 (101) | 2 | 2 |
| C34A+D237C | 0.5 | 93 (108) | 95 (108) | 94 (108) | 2 | 1 |
| C34A+K240C | 0.6 | 93 (108) | 94 (107) | 94 (108) | 1 | 1 |
| C34A+D259C | 0.5 | 93 (108) | 95 (108) | 94 (108) | 2 | 1 |
| C34A+K262C | 0.6 | 92 (107) | 93 (106) | 93 (107) | 1 | 1 |
| C34A+N267C | 0.6 | 94 (109) | 95 (108) | 95 (109) | 1 | 1 |
| C34A+Q268C | 0.8 | 95 (111) | 96 (109) | 96 (110) | 1 | 1 |
| C34A+L275C | 0.5 | 94 (109) | 95 (108) | 94 (108) | 1 | 0 |
| C34A+E277C | 0.7 | 65 (76) | 60 (68) | 59 (68) | -5 | -6 |
| C34A+L284C | 0.7 | 92 (107) | 94 (107) | 94 (108) | 2 | 2 |
| C34A+E311C | 0.7 | 54 (63) | 48 (55) | 46 (53) | -6 | -8 |
| C34A+K317C | 0.6 | 83 (97) | 82 (93) | 82 (94) | -1 | -1 |
| C34A+A322C | 0.8 | 81 (94) | 84 (96) | 83 (95) | 3 | 2 |
| C34A+E333C | 0.3 | 94 (109) | 97 (110) | 95 (109) | 3 | 1 |
| C34A+D340C | 0.6 | 93 (108) | 94 (107) | 94 (108) | 1 | 1 |
| C34A+E354C | 0.7 | 89 (104) | 90 (102) | 90 (103) | 1 | 1 |
| C34A+K359C | 0.6 | 86 (100) | 87 (99) | 87 (100) | 1 | 1 |
| C34A+A362C | 0.6 | 89 (104) | 89 (101) | 88 (101) | 0 | -1 |
| C34A+E382C | 0.6 | 86 (100) | 84 (96) | 84 (97) | -2 | -2 |
| C34A+L398C | 0.7 | 90 (105) | 92 (105) | 87 (100) | 2 | -3 |

**Example 4. Conjugation efficiency and controlled hydrolysis of thio-albumin variants**

Thio-albumin variants from Example 3 were conjugated with biotin (Thermo Scientific, EZ-Link Maleimide-PEG2-Biotin) using a 3.2 fold molar excess of maleimide-PEG2-biotin to protein. A reaction schematic is shown in Figure 4. The thio-albumin AlbuPure® eluates were diluted with phosphate buffered saline (PBS buffer), pH 7.4 to give 10mL solutions at 0.3 mg/mL (45.15 nmol) and conjugated as described below Table 9A.

The MS spectrum for the thio-albumin variant C34A+A226C indicated that no conjugation had occurred post an overnight incubation with maleimide-PEG2-biotin. The results are summarised in Table 9A. The MS spectra for the thio-albumin variants C34A+E230C, and C34A+I271C indicated that conjugation had occurred post an overnight incubation, giving approximately 72% or 72% monoconjugate respectively (*i.e.* the same level of monoconjugate) when comparing the relative peak heights of conjugated and unconjugated species. The MS spectrum for C34A+I271C is shown in Figure 5A. The MS spectrum for thio-albumin variant C34A+K93C shown in Figure 5B, exhibited a single species at 66908 Da indicating the correct molecular weight for the thio-albumin variant plus a single addition of maleimide-PEG2-biotin (+525 Da). This confirmed the variant had a single free thiol available for conjugation. Comparable results were obtained for thio-albumin variants C34A+E294C and C34A+E358C.

**Table 9A: Conjugation efficiency results**

| Sample Description | Reference Mr unconjugated (Da) | Theoretical conjugate mass (Da) | Conjugate intact mass result (Da) | % conjugation |
|---|---|---|---|---|
| WT control | 66439 | 66964 | * | * |
| C34A+K93C | 66382 | 66907 | 66908 | 100 |
| C34A+A226C | 66439 | 66964 | 66440 | 0 |
| C34A+E230C | 66381 | 66906 | 66908 | 72 |
| C34A+I271C | 66397 | 66922 | 66924 | 72 |
| C34A+E294C | 66381 | 66906 | 66909 | >95 |
| C34A+E358C | 66381 | 66906 | 66909 | 100 |

* WT control sample failed to inject on MS during sequence run.

Further variants were analysed and the results are shown in Table 9B. For samples C34A+L66C and C34A+Q94C the protein concentrations were low, hence 10 mL solutions at

0.15 mg/mL (22.58 nmol) were used. Stock solutions of 2 mg/mL biotin were prepared by the addition of 5 x 200 µL aliquots of PBS buffer, pH 7.4, to each of two 2 mg pre-weighed EZ-Link micotubes, the vials were rinsed to maximise recovery of the lyophilised product. The two 1 mL volumes were pooled into a 7mL container with a lid. From the biotin stock solution, 38 µL (144.5 nmol) was added to the 10 mL albumin samples to give approximately a 3.2-fold molar excess of biotin over albumin. However, for the C34A+L66C and C34A+Q94C samples only 19 µL biotin was added to maintain a 3.2 fold excess of maleimide-PEG2-biotin to protein. Samples were gently mixed and incubated at ambient temperature overnight. Post incubation, the samples were subjected to mass spectrometry to determine the intact protein mass post conjugation according to the method described in Example 2, but using a 15 minute analytical gradient, and processing data for the protein peak between approximately 7 and 10 minutes. The MS spectra results summarised in Table 9B indicated that thio-albumin variants C34A+L66C, C34A+A92C, C34A+Q94C, C34A+D259C, C34A+L275C, and C34A+L284C did not conjugate post an overnight incubation with maleimide-PEG2-biotin. The MS spectra for the WT control, and the thio-albumin variants C34A+L24C, C34A+V54C, C34A+H128C, C34A+E227C, C34A+K240C, C34A+K262C, C34A+Q268C, C34A+E277C, C34A+K317C, C34A+A322C, C34A+K359C and C34A+A362C indicated 90% conjugation or greater with maleimide-PEG2-biotin.

**Table 9B: Conjugation efficiency results**

| Sample Description | Reference Mr unconjugated (Da) | Theoretical conjugate mass (Da) | Conjugate intact mass result (Da) | % conjugation |
|---|---|---|---|---|
| WT control | 66439 | 66964 | 66966 | 93 |
| C34A+L24C | 66397 | 66922 | 66924 | 96 |
| C34A+F49C | 66363 | 66888 | 66889 | 84 |
| C34A+V54C | 66411 | 66936 | 66938 | 100 |
| C34A+D56C | 66395 | 66920 | 66922 | 79 |
| C34A+L66C | 66397 | 66922 | 66400 | 0 |
| C34A+A92C | 66439 | 66964 | 66407 | 0 |
| C34A+Q94C | 66382 | 66907 | 66409 | 0 |
| C34A+E97C | 66381 | 66906 | 66907 | 9 |
| C34A+H128C | 66373 | 66898 | 66899 | 100 |
| C34A+F156C | 66363 | 66888 | 66890 | 76 |
| C34A+E227C | 66381 | 66906 | 66907 | 95 |
| C34A+D237C | 66395 | 66920 | 66921 | 73 |
| C34A+K240C | 66382 | 66907 | 66908 | 100 |

| Sample Description | Reference Mr unconjugated (Da) | Theoretical conjugate mass (Da) | Conjugate intact mass result (Da) | % conjugation |
|---|---|---|---|---|
| C34A+D259C | 66395 | 66920 | 67424 | 0 |
| C34A+K262C | 66382 | 66907 | 66908 | 100 |
| C34A+N267C | 66396 | 66921 | 66922 | 47 |
| C34A+Q268C | 66382 | 66907 | 66908 | 92 |
| C34A+L275C | 66397 | 66922 | 66897 | 0 |
| C34A+E277C | 66381 | 66906 | 66908 | 90 |
| C34A+L284C | 66397 | 66922 | 67427 | 0 |
| C34A+E311C | 66381 | 66906 | 66909 | 76 |
| C34A+K317C | 66382 | 66907 | 66909 | 91 |
| C34A+A322C | 66439 | 66964 | 66965 | 94 |
| C34A+E333C | 66381 | 66906 | 66907 | 83 |
| C34A+D340C | 66395 | 66920 | 66923 | 12 |
| C34A+E354C | 66381 | 66906 | 66908 | 32 |
| C34A+K359C | 66382 | 66907 | 66908 | 95 |
| C34A+A362C | 66439 | 66964 | 66966 | 94 |
| C34A+E382C | 66381 | 66906 | 66909 | 83 |
| C34A+L398C | 66397 | 66922 | 66925 | 36 |

The stability of maleimide conjugate bonds is not robust. The succinimide can revert back to maleimide and free thiol *via* a retro-Michael pathway (Figure 4). Thus, highly undesirably, the released maleimide may react with other thiol reactive species and the released thiol may react with other compounds *in vivo*. To avoid retro-Michael reactivity, the succinimide may be hydrolysed to succinic acid, effectively taking on $H_2O$ (+18 Da) and locking the conjugate to be thiol-stable. The property of thiol-stability by hydrolysis is desirable as it would ensure that there was no unwanted thiol transfer taking place in various environments *in vivo*. Therefore, controlled hydrolysis of the succinimide was performed by increasing the pH and temperature. Post conjugation the samples were transferred to Vivaspin 20 centrifugal concentrators (Sartorius) and balanced with PBS buffer pH 7.4. The samples were centrifuged at 4,500 x g for 15 minutes to reduce the volume to approximately 200 µL. A diafiltration cup was fitted to the Vivaspin 20 vessels and subsequently filled with 15mL of PBS buffer pH 9.0. The samples were centrifuged at 4,500 x g for 15 minutes a second time. A further 15 mL PBS buffer pH 9.0 was added and the samples centrifuged a third time to ensure that all the free maleimide-PEG2-biotin was removed from solution. The remaining retentate was removed and made up to a final volume of 10 mL with PBS buffer pH 9.0 (*i.e.* assuming no losses then to a

concentration of 0.3 mg/mL). The samples were incubated at 37°C for at least 24 hours for controlled hydrolysis to occur to determine the stability of the thio ether conjugate bond. The results are summarised in Table 10.

The yield of the hydrolysed thiol stable wild type control conjugate was in the order of 53%, likely due to the competing retro-Michael deconjugation during hydrolysis (Figure 6A). Also observed was an average conjugate mass shift of +14 Da indicating that partial hydrolysis had occurred. It was apparent that the thio-albumin variants that had the highest conjugation efficiency also had improved conjugate stability upon controlled hydrolysis. Specifically the reaction favoured the hydrolysis of the succinimide rather than the retro-Michael deconjugation pathway. An example of C34A+E294C is shown in Figure 6B indicating no conjugate losses following incubation at pH 9.0, 37°C. Comparable results were obtained for thio-albumin variants C34A+K93C, C34A+E294C and C34A+E358C with no significant losses during controlled hydrolysis.

**Table 10: Controlled hydrolysis stability results**

| Sample Description | Reference Mr unconjugated (Da) | Theoretical conjugate mass (Da) | Conjugate intact mass result (Da) | Conjugate mass increase (Da) | % conjugation post hydrolysis |
|---|---|---|---|---|---|
| WT control | 66439 | 66964 | 66978 | 14 | 53 |
| C34A+K93C | 66382 | 66907 | 66911 | 4 | 100 |
| C34A+A226C | 66439 | 66964 | 66441 | 2 | 0 |
| C34A+E230C | 66381 | 66906 | 66926 | 20 | 63 |
| C34A+I271C | 66397 | 66922 | 66939 | 6 | 61 |
| C34A+E294C | 66381 | 66906 | 66926 | 20 | 100 |
| C34A+E358C | 66381 | 66906 | 66927 | 21 | 100 |

The combined aggregation results and conjugation results are summarised together in Table 11. It was apparent that the variants C34A+K93C and C34A+E294C had improved aggregation profiles compared to wild type albumin, conjugated to a high percentage with maleimide-PEG2-biotin, and had minimal loss of conjugate following controlled hydrolysis at pH 9.0, 37°C. These variants were selected for further evaluation.

**Table 11: Thio-albumin variant aggregation screen and conjugation results summary**

| Sample Description | Improved aggregation profile | Conjugation efficiency >95% | No losses during controlled hydrolysis | Variant selected |
|---|---|---|---|---|
| WT control | | | | |
| C34A+K93C | ✓ | ✓ | ✓ | ✓ |
| C34A+A226C | ✓ | | | |
| C34A+E230C | ✓ | | | |
| C34A+I271C | ✓ | | | |
| C34A+E294C | ✓ | ✓ | ✓ | ✓ |
| C34A+E358C | | ✓ | ✓ | |

**Example 5: Combination variants**

**Method 2.**

Combination variants (Table 12) were produced to combine the mutations K93C and E294C described both with and without the HSA C34A mutation. Briefly, plasmids comprising the individual mutations were prepared, and the mutations combined by restriction enzyme digestion and ligation.

1 µl of purified plasmid produced in Method 1 corresponding to the mutations K93C or E294C was transformed into *E. coli* NEB 5-alpha (New England Biolabs) and plated onto LB plates (as described above) supplemented with 50 µg/mL ampicillin. Plasmids were isolated using a Qiagen Plasmid Plus Kit (Qiagen - according to manufacturer's instructions) and sequenced to confirm the presence of the desired mutation within the HSA sequence. These plasmids were named pDB5623 (C34A + K93C) and pDB5624 (C34A + E294C).

A fragment was removed from plasmid pDB5624 using the *Nhe*I and *Sph*I restriction sites and was purified using a QIAquick Gel Extraction Kit (Qiagen) and ligated into pDB5623 digested with the same enzymes to produce construct pDB5625. pDB5626 and pDB5627 were constructed by insertion of the fragment produced by digestion of pDB5102 with *Sac*II and *Pst*I restriction enzymes into similarly digested pDB5623 and pDB5624. pDB5102 is described in WO 2015/036579 (incorporated herein by reference). The ligated plasmids were all transformed into *E. coli* NEB 5-alpha and plated onto LB plates (as described above) supplemented with 50 µg/mL ampicillin. Plasmids were isolated using a Qiagen Plasmid Plus Kit (Qiagen - according to manufacturer's instructions) and sequenced to confirm the presence of the desired mutation within the HSA sequence.

To produce pDB5628 a fragment was removed from plasmid pDB5102 using the *Sac*II and *Pst*I restriction sites and was purified using a QIAquick Gel Extraction Kit (Qiagen) and ligated into pDB5625 digested with the same enzymes. The ligated plasmids were all transformed into *E. coli* NEB 5-alpha and plated onto LB plates supplemented with 50 µg/mL

ampicillin. Plasmids were isolated using a Qiagen Plasmid Plus Kit (Qiagen - according to manufacturer's instructions) and sequenced to confirm the presence of the desired mutation within the HSA sequence.

**Table 12: Summary information for combination variants**

| Variant | Number of thiols | Plasmid | Protein SEQ ID NO. |
|---|---|---|---|
| C34A+K93C | 1 | pDB5623 | 111 |
| C34A+E294C | 1 | pDB5624 | 129 |
| C34A+K93C+E294C | 2 | pDB5625 | 141 |
| K93C | 2 | pDB5626 | 142 |
| E294C | 2 | pDB5627 | 143 |
| K93C+E294C | 3 | pDB5628 | 144 |

**Production of expression plasmid and yeast stocks.**

Preparation of the expression plasmids and transformation of *S. cerevisiae* was performed as described in WO 2012/150319 by the 48-hour stocking method (incorporated herein by reference). The host strain for the constructs was *S. cerevisiae* BXP10 $Cir^0$ (WO 2015/036579, incorporated herein by reference). Purification of variants from shake flask was performed as described in WO 2012/150319 (incorporated herein by reference) unless otherwise stated.

**Example 6. Production, purification and conjugation of thio-albumin variants**

Cryopreserved yeast stocks each in 1 mL aliquots were inoculated into separate shake flasks containing 100 mL BMMS growth medium (yeast nitrogen base without amino acids or $(NH_4)_2SO_4$, Difco 1.7 g/L; citric acid monohydrate 6.09 g/L; $Na_2HPO_4.2H_2O$ 25.27 g/L; $(NH_4)_2SO_4$ 5.0 g/L; pH 6.5±0.2; sucrose added to 20 g/L). Cells were transferred from the shake flask to the fermenter (10 L working volume, Sartorius Biostat C 10-3 fermenter) when the concentration of cells in the shake flask reached 0.8–1.2 mg/mL achieving a cell inoculum concentration of ≥10 mg/L (greater than or equal to 10 mg/L) in the fermenter.

The thio-albumin variants were produced by axenic culture of each of the yeast strains in high cell density (HCD) fed-batch fermentation. The aim of the fermentation was to achieve maximum biomass and productivity by controlling feed rate addition so that formation of by-products such as ethanol and acetate were avoided. Further details of the fermentation process are described in WO 96/37515 (incorporated herein by reference). The temperature and pH were controlled at 30°C and pH 6.2 respectively. Culture supernatant was harvested by centrifugation using a Sorvall RC 3C centrifuge (DuPont) to provide materials for immediate purification and the remaining materials were frozen (-20°C) for storage, before being thawed for subsequent purifications. Final product concentrations were determined by GP-HPLC using

a LC2010 HPLC system (Shimadzu) equipped with UV detection under Shimadzu VP7.3 client server software control as described in Example 3. Table 13 provides the yields of each thio-albumin variant (in mg/mL culture supernatant) and shows that high product titres of greater than 1 mg/mL culture supernatant were obtained in all cases.

**Table 13: Thio-albumin variant protein concentration by GP-HPLC**

| Sample Description | Number of thiols | Concentration by GPHPLC (mg/mL) | SEQ ID NO. |
|---|---|---|---|
| C34A+K93C | 1 | 3.1 | 111 |
| C34A+E294C | 1 | 4.6 | 129 |
| C34A+K93C+E294C | 2 | 2.3 | 141 |
| K93C | 2 | 1.8 | 142 |
| E294C | 2 | 3.9 | 143 |
| K93C+E294C | 3 | 1.6 | 144 |

The variants were purified at scale by a two-step chromatography process. The first purification step was using AlbuPure® chromatography as previously described in Example 3 but washing the column with approximately 4 column volumes of 50 mM sodium acetate, pH 5.3, 10 column volumes of 50 mM sodium phosphate, pH 8.0, and 10 column volumes of 50 mM ammonium acetate pH 8.0 respectively. Bound protein was eluted using between 1 and 3 column volumes of 50 mM ammonium acetate, 10 mM sodium octanoate, pH 7.0. The AlbuPure® eluates were then further purified using ion exchange chromatography *via* DE-FF as described in Evans *et al*. (2010), Protein Expression and Purification Volume 73, Issue 2, Pages 113-124. Post purification, the DE-FF eluate samples were concentrated and buffer exchanged by ultrafiltration/diafiltration using 10,000 molecular weight cut-off Vivacell 100 centrifugal concentrators (Sartorius). The samples were centrifuged at 2,000 x g for 30 minutes (multiple times) to reduce the volume to below 10 mL before diafiltration against 10 volumes of 25 mM sodium phosphate, 215 mM sodium chloride, pH 6.5. Post diafiltration, sample concentrations were in the range of 124 to 177 mg/mL. The samples were diluted to a final formulation concentration of 50 mg/mL in 25 mM sodium phosphate, 215 mM sodium chloride, pH 6.5.

The thio-albumin variants were conjugated with maleimide-PEG2-biotin as described in Example 4, but with a 3.2-fold molar excess of biotin over the free thiol content (number of free thiols). Due to some variants having multiple free thiol sites available for conjugation, the expected molecular weights for all biotin conjugation permutations are summarised in Table 14. The variants with two or three thiol groups increased by 2 x 525 Da, and 3 x 525 Da respectively. The relative peak heights of each peak species were used to calculate the percentage of target conjugate, *i.e.* the correct percentage of a single, double or triple biotin labelled thio-albumin variant. The K93C+E294C variant had a total of 3 free thiol residues, the

MS spectrum for this variant is shown in Figure 7A. It was evident from the single peak species on the MS spectrum that the variant has successfully conjugated 3 moles of maleimide-PEG2-biotin per mole protein, as indicated by a mass increase of 1575 Da (3 x 525 Da) to 67968 Da (Table 15). The samples were incubated at 37°C, pH 9, for at least 24 hours for controlled hydrolysis to occur to determine the stability of the thio ether conjugate bond as previously described in Example 4. The results are summarised in Table 15. The yield of the hydrolysed thiol stable K93C+E294C conjugate was in the order of 20% triple conjugate, due to the competing retro-Michael deconjugation of the C34 conjugate during hydrolysis (Figure 7B). The main species was now a hydrolysed thiol stable double conjugate with a mass of 67476 Da indicating that hydrolysis had occurred to the double conjugate species. It was evident that the variants containing a cysteine at position C34 had significant deconjugation during hydrolysis compared to the variants with a C34A mutation. The double thiol variant C34A+K93C+E294C was 62% double conjugated pre hydrolysis and 56% post hydrolysis. An observed peak species with a mass 66443 Da confirmed that hydrolysis had occurred with minimal conjugate loss (Figure 7C) compared to the K93C+E294C conjugate which contained a cysteine at C34 (Figure 7B) highlighting that the K93C and E294C variants had improved conjugate stability when using a maleimide linker.

**Table 14: Expected molecular weights post conjugation and hydrolysis**

| Sample | No. thiols | Free Mr | Single conjugate Mr | | Double conjugate Mr | | Triple conjugate Mr | |
|---|---|---|---|---|---|---|---|---|
| | | | + biotin (525 Da) | Hydro-lysed | + 2x biotin | Hydro-lysed | + 3x biotin | Hydro-lysed |
| C34A+ K93C | 1 | 66382 | 66907 | 66925 | n/a | n/a | n/a | n/a |
| C34A+ E294C | 1 | 66381 | 66906 | 66924 | n/a | n/a | n/a | n/a |
| C34A+ K93C+ E294C | 2 | 66356 | 66881 | 66899 | 67406 | 67442 | n/a | n/a |
| K93C | 2 | 66414 | 66939 | 66957 | 67464 | 67500 | n/a | n/a |
| E294C | 2 | 66413 | 66938 | 66956 | 67463 | 67499 | n/a | n/a |
| K93C+ E294C | 3 | 66388 | 66913 | 66931 | 67438 | 67474 | 67963 | 68017 |

n/a: not applicable

**Table 15: Conjugation efficiency and controlled hydrolysis results**

| Sample Description | Number of thiols | Post conjugation | | Post hydrolysis | |
|---|---|---|---|---|---|
| | | Conjugate intact mass result (Da) | % target conjugate | Conjugate intact mass result (Da) | % target conjugate |
| C34A+K93C | 1 | 66910 | 68 | 66927 | 70 |
| C34A+E294C | 1 | 66908 | 52 | 66927 | 46 |
| C34A+K93C+ E294C | 2 | 67410 | 62 | 67443 | 56 |
| K93C | 2 | 67467 | 99 | 67502 | 36 |
| E294C | 2 | 67467 | 87 | 67501 | 40 |
| K93C+E294C | 3 | 67968 | 98 | 68020 | 20 |

The formulated samples were subjected to a six month stability assessment at 2-8°C by GPHPLC, using the method described in Example 3. The percentage monomer (in brackets) was determined for each sample relative to its wild type control under the same storage conditions. The percentage monomer results are summarized in Table 16, and indicated that aggregation levels were within acceptable limits when the albumin variants were formulated at 50 mg/mL and stored for six months at 2-8°C.

**Table 16: GPHPLC protein stability assessment at 50mg/mL, post storage at 2-8°C**

| Sample Description | Number of thiols | % Monomer at | | | | | Δ% Monomer 0-6 month | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|---|---|
| | | T = 0 | T = 1m | T = 2m | T = 3m | T = 6m | | |
| WT control | 1 | 93.8 (100) | 94.5 (100) | 94.5 (100) | 94.4 (100) | 94.9 (100) | 1.1 | 2 |
| C34A+K93C | 1 | 92.1 (98) | 91.2 (97) | 90.2 (95) | 89.3 (95) | 88.1 (93) | -4.0 | 111 |
| C34A+E294C | 1 | 92.4 (99) | 92.7 (98) | 91.6 (97) | 91.0 (96) | 90.8 (96) | -1.6 | 129 |
| C34A+K93C+ E294C | 2 | 84.4 (90) | 81.8 (87) | 79.7 (84) | 78.4 (83) | 75.9 (80) | -8.5 | 141 |
| K93C | 2 | 88.2 (94) | 86.9 (92) | 85.5 (91) | 84.9 (90) | 84.2 (89) | -4.0 | 142 |

| Sample Description | Number of thiols | % Monomer at | | | | | Δ% Monomer 0-6 month | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|---|---|
| | | T = 0 | T = 1m | T = 2m | T = 3m | T = 6m | | |
| E294C | 2 | 89.3 (95) | 90.2 (95) | 89.7 (95) | 89.0 (94) | 88.9 (94) | -0.4 | 143 |
| K93C+E294C | 3 | 82.1 (88) | 79.8 (84) | 78.4 (83) | 77.3 (82) | 76.8 (81) | -5.3 | 144 |

m = month

**Example 7: Combination variants having altered FcRn binding**

HSA having the K573P substitution, as described in WO 2011/051489 (incorporated herein by reference), has a higher affinity for FcRn than does wild type HSA. Constructs were produced to combine the mutations in the variants described in Table 12 with the HSA K573P mutation from plasmid pDB4673.

**Method 3.**

A fragment was removed from plasmids pDB5623, 5624, 5625, 5626, 5627 and 5628 using the *Pst*I and *Xho*I restriction sites and was purified using a QIAquick Gel Extraction Kit (Qiagen) and ligated into pDB4673 digested with the same enzymes to produce constructs pDB5704, 5707, 5710, 5713, 5716 and 5719 (Table 17). The ligated plasmids were all transformed into *E. coli* NEB 5-alpha (New England Biolabs) and plated onto LB plates supplemented with 50 µg/mL ampicillin. Plasmids were isolated using a Qiagen Plasmid Plus Kit (Qiagen - according to manufacturer's instructions) and sequenced to confirm the presence of the desired mutations within the HSA sequence.

**Table 17: Summary information for variants having altered FcRn binding**

| Variant | Plasmid | Protein SEQ ID NO. |
|---|---|---|
| K573P | pDB4673 | 145 |
| C34A + K93C + K573P | pDB5704 | 146 |
| C34A + E294C + K573P | pDB5707 | 147 |
| C34A + K93C + E294C + K573P | pDB5710 | 148 |
| K93C + K573P | pDB5713 | 149 |
| E294C + K573P | pDB5716 | 150 |
| K93C + E294C + K573P | pDB5719 | 151 |

**Production of expression plasmid and yeast stocks.**

Preparation of the expression plasmids and transformation of *S. cerevisiae* was performed as described in WO 2012/150319 by the 48-hour stocking method (incorporated herein by reference). The host strain for the constructs was *S. cerevisiae* BXP10 $Cir^0$ (WO 2015/036579, incorporated herein by reference)). Purification of variants from shake flask was performed as described in WO 2012/150319 (incorporated herein by reference) unless otherwise stated.

**Example 8. Aggregation screening of combination variants having altered FcRn binding**

Shake flask culturing of *S. cerevisiae* and purification was performed as described in Example 3. A single step AlbuPure chromatography procedure was used to prepare purified material from 6 variants as described in Example 3. Post purification the 20 mL eluates were concentrated to less than 200 µL using Vivaspin centrifugal concentrators as described in Example 4. Post concentration the samples were buffer exchanged by the addition of 10 mL of 25 mM sodium phosphate, 215 mM sodium chloride, pH 6.5 and the samples centrifuged as before. The final volumes recovered were between 75 µL and 200 µL. The concentration and percentage monomer of the eluate samples was determined by Gel Permeation High Pressure Liquid Chromatography (GP-HPLC) as described in Example 3. The results are summarised in Table 18. Final product concentrations were in the range of 47 to 154 mg/mL. A typical wild type albumin control in Example 4 resulted in a monomer percentage of 87% at 1.1 mg/mL (Table 8A). All variants analysed had monomer percentages equal to or greater than 87% even at significantly higher protein concentrations. This indicated that all variants had minimal propensity to aggregate.

**Table 18: GPHPLC aggregation screening results**

| Sample description | GPHPLC monomer concentration (mg/mL) | % Monomer at T=0 |
|---|---|---|
| C34A+K93C+K573P | 48.2 | 91.8 |
| C34A+E294C+K573P | 153.5 | 90.8 |
| C34A+K93C+E294C+K573P | 98.8 | 88.4 |
| K93C+K573P | 101.7 | 86.9 |
| E294C+K573P | 115.5 | 87.6 |
| K93C+E294C+K573P | 46.5 | 91.2 |

**Example 9. Conjugation efficiency and controlled hydrolysis of combination variants having altered FcRn binding**

The thio-albumin combination variants (Table 17) were conjugated with a 3.2 fold excess of maleimide-PEG2-biotin as described in Example 6. Due to some variants having multiple

free thiol sites available for conjugation, the expected molecular weights for all biotin conjugation permutations are summarised in Table 19.

**Table 19: Expected molecular weights of albumin variants post conjugation and hydrolysis**

| Sample | No. thiols | Free Mr | Single conjugate Mr | | Double conjugate Mr | | Triple conjugate Mr | |
|---|---|---|---|---|---|---|---|---|
| | | | + biotin (525 Da) | Hydro-lysed | + 2x biotin | Hydro-lysed | + 3x biotin | Hydro-lysed |
| C34A+ K93C+ K573P | 1 | 66351 | 66876 | 66894 | n/a | n/a | n/a | n/a |
| C34A+E294C + K573P | 1 | 66350 | 66875 | 66893 | n/a | n/a | n/a | n/a |
| C34A+ K93C+ E294C+573P | 2 | 66325 | 66850 | 66868 | 67375 | 67411 | n/a | n/a |
| K93C+ K573P | 2 | 66383 | 66908 | 66926 | 67433 | 67469 | n/a | n/a |
| E294C+ K573P | 2 | 66382 | 66907 | 66925 | 67432 | 67468 | n/a | n/a |
| K93C+E294C +K573P | 3 | 66357 | 66882 | 66900 | 67407 | 67443 | 67932 | 67986 |

n/a: not applicable

The molecular weight of the variants with two or three thiol groups increased by 2 x 525 Da, and 3 x 525 Da respectively. The relative peak heights of each peak species were used to calculate the percentage of target conjugate, *i.e.* the percentage of a single, double or triple biotin labelled thio- albumin variant. The K93C+E294C+K573P variant had a total of 3 free thiol residues (the third thiol being provided by native Cys34); the MS spectrum for this variant is shown in Figure 8A. It was evident from the single peak species on the MS spectrum that the variant has successfully conjugated with 3 moles of maleimide-PEG2-biotin per mole protein, as indicated by a mass increase of 1575 Da (3 x 525 Da) to 67940.8 Da. The samples were incubated at 37°C, pH 9, for at least 18 hours for controlled hydrolysis to occur to determine the stability of the thio ether conjugate bond as previously described in Example 4. The results are summarized in Table 20.

The yield of the hydrolysed thiol stable K93C+E294C+K573P conjugate was in the order of 23% triple conjugate, likely due to the competing retro-Michael deconjugation of the C34 conjugate during hydrolysis (Figure 8B). The main species was now a hydrolysed thiol stable

double conjugate with a mass of 67447.3 Da indicating that hydrolysis had occurred to this double conjugate species. It was evident that the variants containing a cysteine at position C34 underwent more pronounced deconjugation during hydrolysis compared to the variants with a C34A mutation.

**Table 20: Conjugation efficiency and controlled hydrolysis results**

| Sample Description | Number of thiols | Post conjugation | | Post hydrolysis | |
|---|---|---|---|---|---|
| | | Conjugate intact mass result (Da) | % target conjugate | Conjugate intact mass result (Da) | % target conjugate |
| C34A+K93C+ K573P | 1 | 66878 | 100 | 66896 | 100 |
| C34A+E294C+ K573P | 1 | 66880 | 85 | 66897 | 89 |
| C34A+K93C+ E294C+ K573P | 2 | 67382 | 90 | * | * |
| K93C+K573P | 2 | 67438 | 100 | 67473 | 32 |
| E294C+ K573P | 2 | 67441 | 100 | 67474 | 25 |
| K93C+E294C+ K573P | 3 | 67941 | 100 | 67989 | 23 |

*low intensity MS spectrum, unable to accurately quantify data

**Example 10. Surface Plasmon Resonance (SPR) analysis of combination variants having altered FcRn binding, pre and post conjugation with maleimide-PEG2-biotin**

Thio-albumin combination variants detailed in Tables 12 and 17 were produced by fed-batch fermentation and purified according to Example 6. Post purification, the samples were concentrated and the buffer was exchanged against a minimum of 7 continuous volumes of 25 mM sodium phosphate, 215 mM sodium chloride, pH 6.5 using 10,000 molecular weight cut-off Centramate Tangential Flow Filtration Membrane cassettes (PALL) before final formulation at 20 mg/mL in buffer (25 mM sodium phosphate, 215 mM sodium chloride, pH 6.5). Subsequently, a size exclusion chromatography step (Sephacryl® S200, GE Healthcare) was performed. For each sample 25 mL was split equally between two Vivaspin 20 centrifugal concentrators and centrifuged at 4,500 x g for two 20 minute time periods to reduce the total volume to 5 mL. The concentrated material was loaded onto a 483 mL S200 column and the monomer peak collected to generate monomeric protein at greater than 98% for FcRn binding analysis by SPR. Post purification, eluates were diluted to 5 mg/mL (± 5%). The binding affinity of each variant for the human FcRn receptor was determined both pre and post conjugation with

maleimide-PEG2-biotin. Variants were conjugated with a 3.2 fold excess of maleimide-PEG2-biotin as described in Example 6. The percentage conjugation was determined by MS as described in Example 2, but using a 15 minute analytical gradient, and processing data for the protein peak between approximately 7 and 10 minutes. The results are shown in Table 21 and indicated all samples had conjugated to varying extent, depending on the number of thiols.

**Table 21: Conjugation efficiency for samples for SPR**

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 0 | 100 | n/a | n/a | 2 |
| C34A+K93C | 1 | 0 | 100 | n/a | n/a | 111 |
| C34A+E294C | 1 | 74 | 26 | n/a | n/a | 129 |
| C34A+K93C+ E294C | 2 | 0 | 74 | 26 | n/a | 141 |
| K93C | 2 | 0 | 26 | 74 | n/a | 142 |
| E294C | 2 | 0 | 81 | 19 | n/a | 143 |
| K93C+E294C | 3 | 0 | 0 | 80 | 20 | 144 |
| K573P | 1 | 8 | 93 | n/a | n/a | 145 |
| C34A+K93C +K573P | 1 | 0 | 100 | n/a | n/a | 146 |
| C34A+E294C +K573P | 1 | 20 | 80 | n/a | n/a | 147 |
| C34A+K93C+ E294C+K573P | 2 | 0 | 10 | 90 | n/a | 148 |
| K93C+K573P | 2 | 0 | 0 | 100 | n/a | 149 |
| E294C+K573P | 2 | 0 | 18 | 82 | n/a | 150 |
| K93C+E294C +K573P | 3 | 0 | 0 | 40 | 60 | 151 |

n/a: not applicable

SPR analyses were carried out using a Biacore 3000 instrument (GE Healthcare). Flow cells of CM5 sensor chips were coupled with soluble human FcRn (1200-1600 RU) using amine coupling chemistry as described in the protocol provided by the manufacturer (GE Healthcare). The coupling was performed by injecting 5 µg/mL of the protein in 10 mM sodium acetate pH 4.5 (GE healthcare). Phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005% Tween 20) at pH 5.5 was used as running buffer and dilution buffer. Regeneration of the surfaces were

performed using injections of HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) at pH 7.4 (GE Healthcare). Post immobilisation, the chip was left to stabilise with a constant flow (5 µL/min) of running buffer. Chip surface was conditioned by injecting 3x injections of running buffer followed by 3x injections of regeneration buffer. Surfaces were checked for activity with native sequence HSA control. For determination of binding kinetics, serial dilutions of albumin variants (10-0 µM) were injected over immobilized receptor at a constant flow rate (30 µL/min) at 25 °C. In all analyses, data were zero adjusted and the reference cell subtracted. Data evaluations were performed using BIAevaluation 4.1 software (BIAcore AB). The results pre and post conjugation are shown in Tables 22 and 23 respectively.

The thio-albumin variants screened over human FcRn bound to the receptor in a reversible, pH-dependent manner.

The thio-albumin variants in a wild type background (*i.e.* the only amino acid alterations were those that were introduced to affect the number of conjugatable cysteine residues) gave a similar fold increase in binding affinity to FcRn compared to the wild type control (SEQ ID NO. 2) both pre and post conjugation. The thio-albumin variants which also included a K573P mutation (to increase the affinity of the albumin variant to FcRn) maintained their increase in FcRn affinity, compared to the K573P control (SEQ ID NO. 145), pre and post conjugation indicating that neither the change in conjugatable cysteine residues nor the conjugation partner had an observable influence on the binding affinity of the albumin variant to FcRn.

**Table 22: FcRn affinity for variants pre conjugation**

| Sample Description | Number of thiols | Ka ($10^3$/Ms) | Kd ($10^3$/Ms) | KD (µM) | Fold > WT | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 7.38 | 54.0 | 7.32 | n/a | 2 |
| C34A+K93C | 1 | 12.4 | 44.9 | 3.62 | 2.02 | 111 |
| C34A+E294C | 1 | 6.13 | 46.7 | 7.61 | 0.96 | 129 |
| C34A+K93C+E294C | 2 | 10.41 | 39.5 | 3.79 | 1.93 | 141 |
| K93C | 2 | 8.19 | 43.45 | 5.30 | 1.38 | 142 |
| E294C | 2 | 5.0 | 46.3 | 9.25 | 0.79 | 143 |
| K93C+E294C | 3 | 7.65 | 37.9 | 4.95 | 1.48 | 144 |
| K573P | 1 | 5.70 | 3.76 | 0.66 | 11.10 | 145 |
| C34A+K93C+K573P | 1 | 8.06 | 3.83 | 0.48 | 15.25 | 146 |
| C34A+E294C+K573P | 1 | 6.07 | 3.96 | 0.65 | 11.26 | 147 |

| Sample Description | Number of thiols | Ka ($10^3$/Ms) | Kd ($10^3$/Ms) | KD (μM) | Fold > WT | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| C34A+K93C+ E294C+ K573P | 2 | 8.11 | 3.67 | 0.45 | 16.27 | 148 |
| K93C+K573P | 2 | 8.37 | 4.07 | 0.48 | 15.25 | 149 |
| E294C+K573P | 2 | 5.65 | 4.17 | 0.74 | 9.89 | 150 |
| K93C+E294C+K573P | 3 | 6.8 | 3.82 | 0.56 | 13.07 | 151 |

n/a: not applicable

Fold > WT = KD (μM) WT control / KD (μM) variant

**Table 23: FcRn affinity for samples post conjugation with maleimide-PEG2-biotin**

| Sample Description | Number of thiols | Ka ($10^3$/Ms) | Kd ($10^3$/Ms) | KD (μM) | Fold > WT | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 9.26 | 25.4 | 2.74 | n/a | 2 |
| C34A+K93C | 1 | 12.95 | 19.85 | 1.53 | 1.79 | 111 |
| C34A+E294C | 1 | 8.07 | 20.15 | 2.50 | 1.09 | 129 |
| C34A+K93C+E294C | 2 | 11.55 | 17.4 | 1.51 | 1.80 | 141 |
| K93C | 2 | 10.2 | 21.0 | 2.06 | 1.33 | 142 |
| E294C | 2 | 9.38 | 19.9 | 2.12 | 1.29 | 143 |
| K93C+E294C | 3 | 11.8 | 19.3 | 1.63 | 1.68 | 144 |
| K573P | 1 | 9.25 | 3.12 | 0.337 | 8.13 | 145 |
| C34A+K93C+K573P | 1 | 14.35 | 2.97 | 0.207 | 13.24 | 146 |
| C34A+E294C+K573P | 1 | 10.42 | 2.97 | 0.285 | 9.61 | 147 |
| C34A+K93C+E294C+ K573P | 2 | 13.7 | 2.71 | 0.198 | 13.84 | 148 |
| K93C+K573P | 2 | 13.7 | 2.93 | 0.214 | 12.80 | 149 |
| E294C+K573P | 2 | 11.4 | 3.22 | 0.283 | 9.68 | 150 |
| K93C+E294C+K573P | 3 | 13.05 | 3.31 | 0.254 | 10.79 | 151 |

n/a: not applicable

Fold > WT = KD (μM) WT control / KD (μM) variant

**Example 11. Aggregation analysis of combination variants having altered FcRn binding**

The thio-albumin combination variants formulated at 5 mg/mL in Example 10 were analysed for their tendency to remain as a monomer in solution. WT HSA, the variant K573P and the variant C34A+L302C were prepared as described in Example 10 and included as

controls. The free thiol content for each variant was determined at T=0 and following 3 months storage at 5°C by mass spectrometric analysis of protein sample treated with DTNB reagent, similar to the method of Example 2. For this example, 80 µL of each variant sample was diluted with 920 µL of buffer 1 (100 mM Tris-HCl, 10 mM EDTA, pH 8.0). To each variant sample, 50 µL of buffer 2 (4 mg/mL DTNB, 500 mM sodium phosphate, pH 7.0) was added. The resultant preparation incubated for at least 25 minutes at ambient temperature (20±5 °C) to allow TNB labelling. Post incubation, the samples were subjected to mass spectrometry to determine the intact protein mass post conjugation as per the method described in Example 2, but using a 15 minute analytical gradient, and processing data for the protein peak between approximately 7 and 10 minutes. The results are summarised in Table 24A and Table 24B. An increase in mass of 197 Da upon DTNB incubation is indicative of the presence of one free thiol group on the protein in the sample. An increase of 394 Da and 591 Da is indicative of two or three free thiol groups respectively. All samples had high levels of free thiol at the start of the stability study, and the majority maintained a high free thiol level following 3 months storage at 5°C.

**Table 24A: Mass Spectrometry DTNB free thiol results**

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 0 | 91 | 0 | 9 | 2 |
| C34A+L302C | 1 | 0 | 100 | 0 | 0 | 152 |
| C34A+K93C | 1 | 0 | 91 | 0 | 9 | 111 |
| C34A+E294C | 1 | 6 | 94 | 0 | 0 | 129 |
| C34A+K93C+E294C | 2 | 0 | 39 | 61 | 0 | 141 |
| K93C | 2 | 16 | 0 | 84 | 0 | 142 |
| E294C | 2 | 0 | 28 | 84 | 0 | 143 |
| K93C+E294C | 3 | 0 | 0 | 51 | 49 | 144 |
| K573P | 1 | 0 | 91 | 0 | 9 | 145 |
| C34A+K93C+K573P | 1 | 0 | 93 | 0 | 7 | 146 |
| C34A+E294C+K573P | 1 | 7 | 87 | 0 | 6 | 147 |
| C34A+K93C+E294C+K573P | 2 | 0 | 5 | 89 | 0 | 148 |
| K93C+K573P | 2 | 0 | 0 | 92 | 0 | 149 |
| E294C+K573P | 2 | 0 | 7 | 87 | 0 | 150 |
| K93C+E294C+K573P | 3 | 0 | 0 | 22 | 78 | 151 |

**Table 24B: Mass Spectrometry DTNB free thiol results, post storage at 5°C**

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 0 | 94 | 6 | 0 | 2 |
| C34A+L302C | 1 | 0 | 100 | 0 | 0 | 152 |
| C34A+K93C | 1 | 0 | 95 | 0 | 5 | 111 |
| C34A+E294C | 1 | 55 | 45 | 0 | 0 | 129 |
| C34A+K93C+E294C | 2 | 0 | 54 | 46 | 0 | 141 |
| K93C | 2 | 23 | 0 | 77 | 0 | 142 |
| E294C | 2 | 0 | 50 | 50 | 0 | 143 |
| K93C+E294C | 3 | 0 | 0 | 73 | 27 | 144 |
| K573P | 1 | 0 | 94 | 0 | 6 | 145 |
| C34A+K93C+K573P | 1 | 0 | 95 | 0 | 5 | 146 |
| C34A+E294C+K573P | 1 | 14 | 86 | 0 | 0 | 147 |
| C34A+K93C+E294C+K573P | 2 | 0 | 10 | 90 | 0 | 148 |
| K93C+K573P | 2 | 0 | 0 | 94 | 0 | 149 |
| E294C+K573P | 2 | 0 | 13 | 87 | 0 | 150 |
| K93C+E294C+K573P | 3 | 0 | 0 | 32 | 68 | 151 |

Samples were stored for 3 months at 5°C and 40°C and the aggregation profile determined at various time points by GPHPLC as described in Example 3. The percentage monomer (in brackets) was determined for each sample relative to its wild type control under the same storage conditions. The results for 5°C and 40°C are provided in Tables 25 and 26 respectively. All variants had a monomer greater than 98% at T=0. The majority of thio-albumin variants maintained a monomeric protein percentage equal to or greater than 97% during 3 month's storage at 5°C. Relative to the other variants analysed, variant C34A+L302C was more prone to aggregation. It was also evident that the majority of thio-albumin variants maintained a monomeric protein percentage equal to or greater than 80% during 3 months storage at 40°C, even those containing two or three thiol residues. However, it was evident that variant C34A+L302C was more prone to aggregation with a monomer percentage of 73.2% following 3 months storage at 40°C.

**Table 25: GPHPLC protein stability assessment at 5 mg/mL, post storage at 5°C**

| Sample Description | Number of thiols | % Monomer at | | | | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| | | T = 0 | T = 1 month | T = 2 month | T = 3 month | |
| WT control | 1 | 99.8 (100) | 99.3 (100) | 99.7 (100) | 99.7 (100) | 2 |
| C34A+L302C | 1 | 98.3 (99) | 95.5 (96) | 94.9 (95) | 95.0 (95) | 152 |
| C34A+K93C | 1 | 99.5 (100) | 99.2 (100) | 98.9 (99) | 98.7 (99) | 111 |
| C34A+E294C | 1 | 99.5 (100) | 99.4 (100) | 99.2 (100) | 99.2 (100) | 129 |
| C34A+K93C+E294C | 2 | 99.1 (99) | 98.5 (99) | 97.9 (98) | 97.5 (98) | 141 |
| K93C | 2 | 99.5 (100) | 99.3 (100) | 99.0 (99) | 98.8 (99) | 142 |
| E294C | 2 | 99.6 (100) | 99.4 (100) | 99.2 (100) | 99.1 (99) | 143 |
| K93C+E294C | 3 | 99.2 (99) | 98.8 (100) | 98.3 (99) | 97.9 (98) | 144 |
| K573P | 1 | 99.7 (100) | 99.7 (100) | 99.6 (100) | 98.5 (99) | 145 |
| C34A+K93C+ K573P | 1 | 99.6 (100) | 99.2 (100) | 99.0 (99) | 98.6 (99) | 146 |
| C34A+E294C+ K573P | 1 | 99.4 (100) | 99.1 (100) | 98.8 (99) | 98.5 (99) | 147 |
| C34A+K93C+E294C+K573P | 2 | 99.3 (100) | 98.5 (99) | 97.7 (98) | 96.9 (97) | 148 |
| K93C+K573P | 2 | 99.8 (100) | 99.7 (100) | 99.6 (100) | 98.5 (100) | 149 |
| E294C+K573P | 2 | 99.7 (100) | 98.9 (99) | 99.0 (99) | 98.8 (99) | 150 |
| K93C+E294C+ K573P | 3 | 99.5 (100) | 99.1 (100) | 98.8 (99) | 98.5 (99) | 151 |

**Table 26: GPHPLC protein stability assessment at 5 mg/mL, post storage at 40°C**

| Sample Description | Number of thiols | % Monomer at | | | | | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|---|
| | | T = 0 | T = 0.5 month | T = 1 month | T = 2 month | T = 3 month | |
| WT control | 1 | 99.8 (100) | 99.4 (100) | 99.3 (100) | 99.0 (100) | 97.6 (100) | 2 |
| C34A+ L302C | 1 | 98.3 (99) | 87.6 (88) | 80.9 (82) | 75.6 (76) | 73.2 (75) | 152 |
| C34A+K93C | 1 | 99.5 (100) | 96.1 (97) | 93.4 (94) | 90.3 (91) | 86.6 (89) | 111 |
| C34A+ E294C | 1 | 99.5 (100) | 98.6 (99) | 96.6 (97) | 96.0 (97) | 95.4 (98) | 129 |
| C34A+ K93C+ E294C | 2 | 99.1 (99) | 93.8 (94) | 89.3 (90) | 85.1 (86) | 80.4 (82) | 141 |
| K93C | 2 | 99.5 (100) | 96.6 (97) | 94.5 (95) | 90.4 (91) | 88.8 (91) | 142 |
| E294C | 2 | 99.6 (100) | 98.1 (99) | 95.7 (96) | 95.1 (96) | 94.2 (97) | 143 |
| K93C+ E294C | 3 | 99.2 (99) | 93.9 (95) | 89.3 (90) | 82.9 (84) | 80.0 (82) | 144 |
| K573P | 1 | 99.7 (100) | 99.3 (100) | 99.0 (100) | 98.7 (100) | 97.4 (100) | 145 |
| C34A+ K93C+ K573P | 1 | 99.6 (100) | 95.5 (96) | 93.0 (94) | 89.5 (90) | 86.5 (89) | 146 |
| C34A+ E294C+ K573P | 1 | 99.4 (100) | 97.3 (98) | 95.9 (97) | 95.0 (96) | 94.4 (97) | 147 |
| C34A+ K93C+ E294C+ K573P | 2 | 99.3 (100) | 91.8 (92) | 88.3 (89) | 82.7 (84) | 80.9 (83) | 148 |
| K93C+ K573P | 2 | 99.8 (100) | 98.1 (99) | 96.7 (97) | 94.5 (96) | 92.6 (95) | 149 |
| E294C+ K573P | 2 | 99.7 (100) | 97.5 (98) | 95.8 (97) | 94.5 (96) | 94.4 (97) | 150 |
| K93C+ E294C+ K573P | 3 | 99.5 (100) | 94.9 (96) | 92.0 (93) | 88.7 (90) | 84.1 (86) | 151 |

**Example 12. Conjugation of combination variants having altered FcRn binding, with fluorescent probes**

Thio-albumin combination variants formulated at 5 mg/mL in Example 10, following 6 weeks storage at 2-8°C, were conjugated using a 3-fold excess of Alexa Fluor® 488-PEG4-Lys(monobromomaleimide)-NH2 dye (Figure 9A) or 5-carboxyfluorescein-PEG4-Lys(monobromomaleimide)-NH2 dye (Figure 10A) (Almac Group Ltd., UK, custom synthesis). Variants were diluted with PBS buffer, pH 7.4 to give 1 mL solutions at 1 mg/mL (15.05 nmol). A 1 mg/mL stock solution of Alexa Fluor® 488-PEG4-Lys(monobromomaleimide)-NH2 dye was prepared by reconstituting 1.6 mg material with 1.6 mL PBS buffer pH 7.4. From the Alexa Fluor® 488-PEG4-Lys(monobromomaleimide)-NH2 dye stock solution, 51.5 µL (45.15 nmol) was added to the single thiol variants, 103 µL (90.3 nmol) dye stock solution was added to the double thiol variants, and 154.5 µL (135.3 nmol) dye stock solution was added to the triple thiol variants to give a threefold excess of Alexa Fluor® 488-PEG4-Lys(monobromomaleimide)-NH2 dye over the number of free thiols. A 0.5 mg/mL stock solution of 5-carboxyfluorescein-PEG4-Lys(monobromomaleimide)-NH2 dye was prepared by reconstituting 1.7 mg material with 1.7 mL dimethyl sulfoxide (DMSO) and 1.7 mL PBS pH 7.4 buffer. From the 5-carboxyfluorescein-PEG4-Lys(monobromomaleimide)-NH2 dye stock solution 44.3 µL (45.15 nmol) was added to the single thiol variants, 88.6 µL (90.3 nmol) dye stock solution was added to the double thiol variants, and 132.9 µL (135.3 nmol) dye stock solution was added to the triple thiol variants to give a threefold excess of 5-carboxyfluorescein-PEG4-Lys(monobromomaleimide)-NH2 dye over the number of free thiols. Samples were gently mixed and incubated at ambient temperature overnight in the dark. Post incubation the samples were analysed by mass spectrometry to determine the intact protein mass post conjugation as per the MS method described in Example 2, but using a 15 minute analytical gradient, and processing data for the protein peak between approximately 7 and 10 minutes. The results are summarised in Table 27 and Table 28.

The MS spectrum for the altered FcRn binding variant K573P shown in Figure 9B, exhibited a single species at 67468.5 Da indicating the correct molecular weight for a K573P variant plus a single addition of Alexa Fluor® 488-PEG4-Lys(monobromomaleimide)-NH2 dye (+1058 Da). This confirmed the variant had a single free thiol located at Cys34 available for conjugation. The thio-albumin variant K93C+E294C+K573P shown in Figure 9C indicated that conjugation had occurred post an overnight incubation, giving approximately 42% diconjugate and 58% triconjugate species respectively, when comparing the relative peak heights of conjugated species. It was evident that the main peak species had increased by approximately 3174 Da (3 x 1058 Da) to 69536 Da. This indicated the variant had three free thiols available for conjugation.

The MS spectrum for the altered FcRn binding variant K573P shown in Figure 10B, exhibited a single species at 67310.6 Da indicating the correct molecular weight for a K573P

variant plus a single addition of 5-carboxyfluorescein-PEG4-Lys(monobromomaleimide)-NH2 dye (+901 Da). The thio-albumin variant C34A+K93C+E294C+K573P shown in Figure 10C indicated that conjugation had occurred post an overnight incubation, giving approximately 9% monoconjugate and 91% diconjugate species respectively, when comparing the relative peak heights of conjugated species. It was evident that the main peak species had increased by approximately 1802 Da (2 x 901 Da) to 68129.7 Da. This indicated the variant had two free thiols available for conjugation.

**Table 27: Conjugation efficiency results of thio-albumin variants with Alexa Fluor® 488-PEG4-Lys(monobromomaleimide)-NH2 dye**

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 0 | 93 | n/a | n/a | 2 |
| C34A+K93C | 1 | 0 | 100 | n/a | n/a | 111 |
| C34A+E294C | 1 | 100 | 0 | n/a | n/a | 129 |
| C34A+K93C+E294C | 2 | * | * | * | n/a | 141 |
| K93C | 2 | 17 | 0 | 83 | n/a | 142 |
| E294C | 2 | 0 | 89 | 11 | n/a | 143 |
| K93C+E294C | 3 | * | * | * | * | 144 |
| K573P | 1 | 0 | 100 | n/a | n/a | 145 |
| C34A+K93C+K573P | 1 | 0 | 100 | n/a | n/a | 146 |
| C34A+E294C+K573P | 1 | 8 | 92 | n/a | n/a | 147 |
| C34A+K93C+ E294C+ K573P | 2 | 0 | 7 | 93 | n/a | 148 |
| K93C+K573P | 2 | 0 | 0 | 91 | n/a | 149 |
| E294C+ K573P | 2 | 0 | 0 | 100 | n/a | 150 |
| K93C+E294C+K573P | 3 | 0 | 0 | 42 | 58 | 151 |

n/a: not applicable

*low intensity MS spectrum, unable to accurately quantify data

**Table 28: Conjugation efficiency results of thio-albumin variants with 5-carboxyfluorescein-PEG4-Lys(Bromomaleimide)-NH2 dye**

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 0 | 96 | n/a | n/a | 2 |

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| C34A+K93C | 1 | 0 | 100 | n/a | n/a | 111 |
| C34A+E294C | 1 | 100 | 0 | n/a | n/a | 129 |
| C34A+K93C+E294C | 2 | * | * | * | n/a | 141 |
| K93C | 2 | 30 | 0 | 70 | n/a | 142 |
| E294C | 2 | * | * | * | n/a | 143 |
| K93C+E294C | 3 | * | * | * | | 144 |
| K573P | 1 | 0 | 100 | n/a | n/a | 145 |
| C34A+K93C+ K573P | 1 | 0 | 100 | n/a | n/a | 146 |
| C34A+E294C+K573P | 1 | 18 | 82 | n/a | n/a | 147 |
| C34A+K93C+E294C+ K573P | 2 | 0 | 9 | 91 | n/a | 148 |
| K93C+K573P | 2 | 1 | 0 | 99 | n/a | 149 |
| E294C+ K573P | 2 | * | * | * | n/a | 150 |
| K93C+E294C+K573P | 3 | * | * | * | * | 151 |

n/a: not applicable

*low intensity MS spectrum, unable to accurately quantify data

**Example 13. Conjugation of combination variants having altered FcRn binding, with paclitaxel**

Thio-albumin combination variants formulated at 5 mg/mL in Example 10, following 3 months storage at 2-8°C, were conjugated using a 1.5 fold excess of paclitaxel which was *via* an ester group activated with a monobromomaleimide moiety, as shown in Figure 11A, resulting in the molecule monobromomaleimide-paclitaxel (Almac Group Ltd., UK custom synthesis). Variants were diluted with PBS buffer, pH 7.4 to give 1 mL solutions at 1 mg/mL (15.05 nmol). A 2 mg/mL stock solution of monobromomaleimide-paclitaxel was prepared by reconstituting 6.6 mg material with 3.3 mL DMSO. From the monobromomaleimide- paclitaxel stock solution, 12.24 µL (22.58 nmol) was added to the single thiol variants, 24.47 µL (45.15 nmol) stock solution was added to the double thiol variants, and 36.71µL (67.73 nmol) stock solution was added to the triple thiol variants to give a threefold excess of monobromomaleimide-paclitaxel over the number of free thiols. Samples were gently mixed and incubated at ambient temperature overnight. Post incubation the samples were subjected to mass spectrometry to determine the intact protein mass post conjugation as per the MS method described in Example 2, but using a 15 minute analytical gradient, and processing data for the protein peak between approximately 7 and 10 minutes. The results are summarised in Table 29.

The MS spectrum for the altered FcRn binding variant K573P shown in Figure 11B indicated that conjugation had occurred post an overnight incubation, giving approximately 77% monoconjugated and 23% unconjugated species respectively, when comparing the relative peak heights of the protein species. It was evident that the main peak species at 67412.2 Da had increased by approximately 1004 Da due to a single addition of monobromomaleimide-paclitaxel. The MS spectrum for the thio-albumin variant K93C+E294C+K573P shown in Figure 11C indicated that conjugation had occurred post an overnight incubation, giving approximately 6% monoconjugate, approximately 60% diconjugate and 30% triconjugate species respectively, when comparing the relative peak heights of conjugated species. It was evident that the main peak species had increased by approximately 2008 Da (2 x 1004 Da) to 68364.2 Da, with a 69383.7 Da species indicative of a 3012 Da triple addition.

**Table 29: Conjugation efficiency results of thio-albumin variants with monobromomaleimide-paclitaxel**

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 24 | 76 | n/a | n/a | 2 |
| C34A+K93C | 1 | 50 | 50 | n/a | n/a | 111 |
| C34A+E294C | 1 | 100 | 0 | n/a | n/a | 129 |
| C34A+K93C+E294C | 2 | * | * | * | n/a | 141 |
| K93C | 2 | 30 | 26 | 44 | n/a | 142 |
| E294C | 2 | 0 | 100 | 0 | n/a | 143 |
| K93C+E294C | 3 | * | * | * | 0 | 144 |
| K573P | 1 | 23 | 77 | n/a | n/a | 145 |
| C34A+K93C+K573P | 1 | 59 | 41 | n/a | n/a | 146 |
| C34A+E294C+K573P | 1 | 34 | 66 | n/a | n/a | 147 |
| C34A+K93C+E294C+ K573P | 2 | 10 | 50 | 40 | n/a | 148 |
| K93C+K573P | 2 | 8 | 40 | 52 | n/a | 149 |
| E294C+ K573P | 2 | 0 | 18 | 68 | n/a | 150 |
| K93C+E294C+K573P | 3 | 0 | 6 | 60 | 30 | 151 |

n/a: not applicable

*low intensity MS spectrum, unable to accurately quantify data

**Example 14. Conjugation of combination variants having altered FcRn binding, with exenatide peptide**

Thio-albumin combination variants formulated at 5 mg/mL in Example 10, following 3 months storage at 2-8°C, were conjugated using a 1.5 fold excess of monobromomaleimide-PEG2-exenatide peptide as shown in Figure 12A (Almac Group Ltd., UK, custom synthesis). Variants were diluted with PBS buffer, pH 7.4 to give 1 mL solutions at 1 mg/mL (15.05 nmol). A 5 mg/mL stock solution of monobromomaleimide-PEG2-exenatide peptide was prepared by reconstituting 5 mg material with 1 mL PBS buffer pH 7.4. From the monobromomaleimide-PEG2-exenatide peptide stock solution, 21.17 µL (22.58 nmol) was added to the single thiol variants, 42.35 µL (45.15 nmol) peptide stock solution was added to the double thiol variants, and 63.52µL (67.73 nmol) peptide stock solution was added to the triple thiol variants to give a threefold excess of monobromomaleimide-PEG2-exenatide peptide over the number of free thiols. Samples were gently mixed and incubated at ambient temperature overnight. Post incubation the samples were subjected to mass spectrometry to determine the intact protein mass post conjugation as per the MS method described in Example 2, but using a 15 minute analytical gradient, and processing data for the protein peak between approximately 7 and 10 minutes. The results are summarised in Table 30.

The MS spectrum for the altered FcRn binding variant K573P shown in Figure 12B indicated that conjugation had occurred post an overnight incubation, giving approximately 33% monoconjugate and 67% unconjugated species respectively, when comparing the relative peak heights of protein species. It was evident that the main peak species at 66409.2 Da was unconjugated K573P variant. The second species had increased by approximately 4609 Da due to a single addition of monobromomaleimide-PEG2-exenatide peptide. The thio-albumin variant C34A+K93C+E294C+K573P shown in Figure 12C indicated that conjugation had occurred post an overnight incubation, giving approximately 33% diconjugate species, approximately 45% monoconjugate species, and approximately 22% unconjugated species respectively, when comparing the relative peak heights of protein species. It was evident that the main peak species had increased by approximately 4609 Da to 70941.7 Da, with a 75557.3 Da species indicative of a 9218 Da addition representing a double conjugation of monobromomaleimide-PEG2-exenatide peptide.

**Table 30: Conjugation efficiency results of thio-albumin variants with exenatide peptide**

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 71 | 29 | n/a | n/a | 2 |
| C34A+K93C | 1 | 74 | 26 | n/a | n/a | 111 |
| C34A+E294C | 1 | 100 | 0 | n/a | n/a | 129 |

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| C34A+K93C+E294C | 2 | * | * | * | n/a | 141 |
| K93C | 2 | 79 | 0 | 21 | n/a | 142 |
| E294C | 2 | * | * | * | n/a | 143 |
| K93C+E294C | 3 | * | * | * | * | 144 |
| K573P | 1 | 67 | 33 | n/a | n/a | 145 |
| C34A+K93C+K573P | 1 | 74 | 26 | n/a | n/a | 146 |
| C34A+E294C+ K573P | 1 | 51 | 49 | n/a | n/a | 147 |
| C34A+K93C+E294C+ K573P | 2 | 22 | 45 | 33 | n/a | 148 |
| K93C+K573P | 2 | 60 | 0 | 39 | n/a | 149 |
| E294C+K573P | 2 | 21 | 33 | 47 | n/a | 150 |
| K93C+E294C+K573P | 3 | * | * | * | * | 151 |

n/a: not applicable

*low intensity MS spectrum, unable to accurately quantify data

**Example 15. Conjugation of combination variants having altered FcRn binding affinity, with FLAG peptide**

Thio-albumin combination variants formulated at 5 mg/mL in Example 10, following 3 months storage at 2-8°C, were conjugated using a 1.5 fold excess of maleimide-propyl-FLAG peptide as shown in Figure 13A (Peptide Protein Research Ltd., UK, custom synthesis). Variants were diluted with PBS buffer, pH 7.4 to give 1 mL solutions at 1 mg/mL (15.05 nmol). A 1 mg/mL stock solution of maleimide-propyl-FLAG peptide was prepared by reconstituting 5.4 mg material with 5.4 mL PBS buffer pH 7.4. From the maleimide-propyl-FLAG peptide stock solution, 26.28 µL (22.58 nmol) was added to the single thiol variants, 52.56 µL (45.15 nmol) peptide stock solution was added to the double thiol variants, and 78.84 µL (67.73 nmol) peptide stock solution was added to the triple thiol variants to give a threefold excess of maleimide-propyl-FLAG peptide over the number of free thiols. Samples were gently mixed and incubated at ambient temperature overnight. Post incubation the samples were subjected to mass spectrometry to determine the intact protein mass post conjugation as per the MS method described in Example 2 but using a 15 minute analytical gradient, and processing data for the protein peak between approximately 7 and 10 minutes. The results are summarised in Table 31.

The MS spectrum for the altered FcRn binding variant K573P shown in Figure 13B indicated that conjugation had occurred post an overnight incubation, giving approximately 29%

monoconjugate and 71% unconjugated species respectively, when comparing the relative peak heights of protein species. It was evident that the main peak species at 66409.1 Da was unconjugated K573P variant. The second most abundant peak species had increased by approximately 1164 Da due to a single addition of maleimide-propyl-FLAG peptide. The MS spectrum for the thio-albumin variant K93C+E294C+K573P shown in Figure 13C indicated that conjugation had occurred post an overnight incubation, giving approximately 29% triconjugate species, approximately 50% diconjugate species, approximately 20% monoconjugate species, and approximately 2% unconjugated species respectively, when comparing the relative peak heights of the protein species. It was evident that the main peak species had increased by approximately 2328 Da to 68685.5 Da, with a 69850.5 Da species indicative of a 3492 Da addition representing a triple conjugation of maleimide-propyl-FLAG peptide.

**Table 31: Conjugation efficiency results of albumin variants with FLAG peptide**

| Sample Description | Number of thiols | Unconjugated % | Mono-conjugate % | Di-conjugate % | Tri-conjugate % | Protein SEQ ID NO. |
|---|---|---|---|---|---|---|
| WT control | 1 | 73 | 27 | n/a | n/a | 2 |
| C34A+K93C | 1 | 48 | 52 | n/a | n/a | 111 |
| C34A+E294C | 1 | 80 | 20 | n/a | n/a | 129 |
| C34A+K93C+E294C | 2 | 12 | 77 | 10 | n/a | 141 |
| K93C | 2 | 45 | 30 | 25 | n/a | 142 |
| E294C | 2 | 26 | 63 | 11 | n/a | 143 |
| K93C+E294C | 3 | * | * | * | * | 144 |
| K573P | 1 | 71 | 29 | n/a | n/a | 145 |
| C34A+K93C+K573P | 1 | 47 | 53 | n/a | n/a | 146 |
| C34A+E294C+K573P | 1 | 22 | 78 | n/a | n/a | 147 |
| C34A+K93C+E294C+K573P | 2 | 5 | 34 | 61 | n/a | 148 |
| K93C+K573P | 2 | 23 | 50 | 27 | n/a | 149 |
| E294C+ K573P | 2 | 10 | 51 | 39 | n/a | 150 |
| K93C+E294C+K573P | 3 | 2 | 20 | 50 | 29 | 151 |

n/a: not applicable

*low intensity MS spectrum, unable to accurately quantify data

**Example 16. Immunogenicity assessment of thio-albumin variants using EpiScreen™ time course T cell assay**

Thio-albumin variants K93C (SEQ ID NO. 142) and E294C (SEQ ID NO. 143) were prepared as described in Example 10 along with a wild type albumin control (SEQ ID NO. 2). In

contrast to Example 10, the size exclusion chromatography eluates were diluted to 4 mg/mL (± 5%). Albumin test samples were assessed for their ability to induce CD4+ T cell responses using the EpiScreen™ time course T cell assay (Abzena, Cambridge UK). Briefly, the EpiScreen™ assay was carried out as follows: peripheral blood mononuclear cells from a cohort of 50 healthy donors representing the European and North American population (based on HLA allotypes) were incubated with the test samples. T cell responses were measured using proliferation assays ([3H]-Thymidine uptake) and cytokine secretion assays (IL-2 ELISpot).

The frequency of positive responses in the proliferation assay were low for all samples (ranges from 0% to 8%) and no positive responses were observed in the IL-2 ELISpot assay suggesting a low risk of clinical immunogenicity for all three samples.

**CLAIMS**

1. A conjugation-competent polypeptide comprising an amino acid sequence which is at least 70% identical to human albumin (SEQ ID NO. 2), or a fragment thereof;

wherein at least one (*e.g.* several) position equivalent to a position selected from K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, L284, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue; and

wherein the conjugation-competent polypeptide has a tendency to exist as a monomer in solution which is at least 70% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution.

2. The conjugation-competent polypeptide of Claim 1, wherein the polypeptide comprises one or more (*e.g.* several) of:

substitution of an amino acid, other than cysteine, with a cysteine at a position corresponding to a position equivalent to any of residues K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO. 2; and/or

insertion of a cysteine at a position adjacent the N- or C- side of an amino acid corresponding to a position equivalent to any of residues K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO. 2.

3. The conjugation-competent polypeptide of Claim 1 or 2 wherein two, three, four, five or more (*e.g.* several) positions equivalent to positions selected from K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO. 2 comprise a conjugation-competent cysteine residue.

4. The conjugation-competent polypeptide of any preceding claim, wherein the polypeptide has a tendency to exist as a monomer in solution which is at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution.

5. The conjugation-competent polypeptide of any preceding claim, wherein at a position equivalent to position 34 of SEQ ID NO. 2 there is a conjugation-competent cysteine.

6. The conjugation-competent polypeptide of any of claims 1 to 4, wherein at a position equivalent to position 34 of SEQ ID NO. 2 there is not a conjugation-competent cysteine.

7. The conjugation-competent polypeptide of any preceding claim in which the polypeptide comprises two or more (several) conjugation-competent cysteine residues, wherein, when the polypeptide is folded, there is a distance of at least 5 Å between at least two of the conjugation-competent cysteine residues.

8. The conjugation-competent polypeptide of any preceding claim, wherein the polypeptide comprises substitution of an amino acid, other than cysteine, with a cysteine at one or both positions corresponding to a position equivalent to residues K93 or E294 of SEQ ID NO. 2.

9. The conjugation-competent polypeptide of any preceding claim which is capable of forming a conjugate with maleimide-polyethylenglycol2-biotin at a conjugation efficiency of at least 95%, suitably wherein the conjugate is at least 95% stable upon controlled hydrolysis.

10. A conjugation-competent polypeptide comprising an amino acid sequence which is at least 70% identical to human albumin (SEQ ID NO. 2), or a fragment thereof;

wherein at least one (*e.g.* several) position equivalent to a position selected from K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, L66, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, E277, L284, E311, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue; and

comprising at least one (*e.g.* several) further modification compared to SEQ ID NO. 2, such as a further modification which causes the polypeptide to have at least one (*e.g.* several) further conjugation-competent cysteine, or alters the binding affinity of the polypeptide for FcRn, or alters the plasma half-life of the polypeptide.

11. The conjugation-competent polypeptide of Claim 10 wherein the at least one (*e.g.* several) further modification comprises at least one (*e.g.* several) further conjugation-competent cysteine as defined in any one of Claims 1, 2, 3 or 8.

12. The conjugation-competent polypeptide of any preceding claim wherein at least one (*e.g.* several) position equivalent to a position selected from D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371,

N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2 comprises a conjugation-competent cysteine.

13. The conjugation-competent polypeptide of any preceding claim in which the polypeptide comprises one or more (*e.g.* several) of:

substitution of an amino acid, other than cysteine, with a cysteine at a position corresponding to a position equivalent to any of residues D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371, N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2; and/or

insertion of a cysteine at a position adjacent the N- or C- side of an amino acid corresponding to a position equivalent to any of residues D1, A2, H3, S5, A55, S58, C75, T76, T79, E82, T83, E86, C91, D121, V122, C124, T125, D129, C169, C177, A229, T236, E266, D269, S270, S273, S304, K313, D314, C316, N318, A320, C361, A364, C369, A371, N386, Q390, Q397, S435, T478, T496, A504, E505, T506, T508, D549, C558, D562, C567, A581, L585 and A578 of SEQ ID NO. 2; and/or

deletion or substitution of a cysteine at a position corresponding to any of C360, C316, C75, C168, C558, C361, C91, C124, C169 and C567 of SEQ ID NO. 2 so as to generate a conjugation competent cysteine at any of C369, C361, C91, C177, C567, C316, C75, C169, C124 and C558; and/or

addition of a cysteine to the N- side of the N-terminal residue of an albumin sequence or to the C- side of the C-terminal residue of an albumin sequence.

14. The conjugation-competent polypeptide of any preceding claim in which the polypeptide comprises conjugation-competent cysteines located at: (a) A2 + L585, (b) A2 + A364 + D562 + L585C, (c) A2 and adjacent the C-side of the C-terminus of the albumin (d) T79 + A364; (e) A364 + D1; (f) T79 + D562 + A364; (g) D562 + A364 + D1; (h) T79 + D562 + A364 + A504; (i) T79 + D562 + A364 + L585; (j) T79 + D562 + A364 + D1; (k) T79 + D562 + A364 +L585 + D1; (l) E86 + D562 + A364 +A504 + A2; (m) S270 + A581; (n) S270 + D129; (o) S270 + A581 + E82; (p) S270 + A581 + D129; (q) S270 + A581 + E82 + D129; (r) S270 + A581 + E82 + D129 + Q397; (s) C369 + C177; (t) A364 + A581; (u) T79 + A364 + A581; (v) A364 + A581 + D129; (w) A364 + C177; (x) D562 + C369; (y) D129 + C369; (z) A581 + C369; or (aa) D562 + D129 + C369.

15. The conjugation-competent polypeptide of any preceding claim which comprises or consists of albumin domain III or a variant thereof and at least one (*e.g.* several) additional albumin domain or fragment thereof, such as a second albumin domain III or a variant thereof.

16. The conjugation-competent polypeptide of any preceding claim which comprises or consists of at least one (*e.g.* several) albumin domain III or variant or fragment thereof wherein at least one (*e.g.* several) albumin domain III comprises one or more (*e.g.* several) substitutions in positions corresponding to the positions in SEQ ID NO. 2 selected among: 573, 500, 550, 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 574, 575, 577, 578, 579, 580, 581, 582 and 584.

17. The conjugation-competent polypeptide of Claim 16, wherein the one or more (*e.g.* several) substitutions in positions corresponding to the positions in SEQ ID NO. 2 is selected among: K573Y, W, P, H, F, V, I, T, N, S, G, M, C, A, E, Q, R, L, D, K500E, G, D, A, S, C, P, H, F, N, W, T, M, Y, V, Q, L, I, R, Q417A, H440A, H464Q, E492G, D494N,Q,A, E495Q,A, T496A, D494E+Q417H, D494N+T496A, E492G+V493P, P499A, E501A,Q, N503H,K, H510Q, H535Q, K536A, P537A, K538A, K541G,D, D550E,N, E492G+K573P,A, or E492G/N503H/K573P.

18. The conjugation-competent polypeptide of any preceding claim wherein the polypeptide comprises alterations at two or more (several) positions selected from positions corresponding to positions (a) 492 and 580; (b) 492 and 574; (c) 492 and 550; (d) 550 and 573; (e) 550 and 574; (f) 550 and 580 in SEQ ID NO. 2.

19. The conjugation-competent polypeptide of any preceding claim comprising:

(i) an N-terminal region comprising a first albumin which is a human albumin variant, in which the N-terminal of the first albumin comprises all amino acids of the human albumin variant except the C-terminal 2 to 30 amino acids; and

(ii) a C-terminal region of a second albumin, which is selected from macaque albumin, mouse albumin, rabbit albumin, sheep albumin, human albumin, goat albumin, chimpanzee albumin, hamster albumin, guinea pig albumin, rat albumin, cow albumin, horse albumin, donkey albumin, dog albumin, chicken albumin, or pig albumin, or a variant thereof, in which the C-terminal of the second albumin or albumin variant comprises the C-terminal 2 to 30 amino acids of the second albumin or albumin variant;

wherein the polypeptide has (i) an altered plasma half-life compared with the human albumin variant and/or (ii) an altered binding affinity to FcRn compared with the human albumin variant.

20. The conjugation-competent polypeptide of any preceding claim comprising one or more (*e.g.* several) alterations in Domain I of the mature human albumin polypeptide sequence of SEQ ID NO. 2; and one or more (*e.g.* several) alterations in Domain III of the mature human

albumin polypeptide sequence of SEQ ID NO. 2, wherein the one or more (*e.g.* several) alterations cause the polypeptide to have an altered binding affinity to FcRn.

21. The conjugation-competent polypeptide of Claim 20 wherein the alteration(s) in Domain I are selected from positions corresponding to any of positions 78 to 120 of SEQ ID NO. 2, such as any of positions 78 to 88 and/or from any of 105 to 120; and the alteration(s) in Domain III are selected from positions corresponding to any of positions 425, 505, 510, 512, 524, 527, 531, 534, 569, 573, 575 of SEQ ID NO. 2.

22. The conjugation-competent polypeptide of Claim 21 wherein the alteration at the position corresponding to positions 78 to 120 or 425, 505, 510, 512, 524, 527, 531, 534, 569, 573, and/or 575 of SEQ ID NO. 2 is a substitution; and the alteration is optionally a substitution selected from (i) 83N, K or S; (ii) 111D, G, H, R, Q or E; or (iii) 573P, Y, W, H, F, T, I or V.

23. The conjugation-competent polypeptide of any preceding claim comprising one or more (*e.g.* several) alterations in Domain II of the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions 349, 342, 381, 345, 384, 198, 206, 340, 341, 343, 344, 352, 382, 348, and/or 383 in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

24. The conjugation-competent polypeptide of Claim 23 wherein the alteration at the position corresponding to position 349, 342, 381, 345, 384, 198, 206, 340, 341, 343, 344, 352, 382, 348, and/or 383 is a substitution; and the alteration is optionally a substitution selected from (i) 349F, W, Y, H, P, K or Q, preferably F; (ii) 342Y, W, F, H, T, N, Q, A, C, I, L, P, V, preferably Y; (iii) 381G or A, preferably G; or (iv) 345E, H, I or Q.

25. The conjugation-competent polypeptide of any preceding claim comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V418, T420, V424, E505, V547, K573 in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

26. The conjugation-competent polypeptide of any preceding claim comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V381, preferably V381N or Q; E383, preferably E383A, G, I, L, or V; N391, preferably N391A, G, I, L or V; Y401

preferably Y401D or E; K402, preferably K402A, G, I, L, or V; L407, preferably L407F, N, Q, W, or Y; Y411, preferably Y411Q, or N; K413, preferably K413C, S, or T; K414, preferably K414S or T; V415C, preferably V415C, S, or T; Q416, preferably Q416H or P; V424, preferably V424A, G, I, L, N, or Q; V426D, preferably V426D, E, H, or P; G434, preferably G434C, S, or T; E442, preferably E442K or R; R445, preferably R445F, W or Y; P447, preferably P447S or T; E450, preferably E450D or E; S454, preferably S454C, M or T; V455, preferably V455N or Q; V456, preferably V456N or Q; L457, preferably L457F, W or Y; Q459, preferably Q459K or R; L463, preferably L463N or Q; E495, preferably E495D; T506, preferably T506F, W or Y; T508, preferably T508K, R, or S; F509, preferably F509C, I, L, M, V, W or Y; A511, preferably A511F, W, or Y; D512, preferably D512F, W or Y; T515, preferably T515C, H, N, P, Q or S; L516, preferably L516F, S, T, W or Y; S517, preferably S517C, F, M, T, W or Y; K519, preferably K519A, G, I, L, or V; R521, preferably R521F, W or Y; I523, preferably I523A, D, E, F, G, K, L, N, Q, R, V, W or Y; K524, preferably K524A, G, I, L or V; K525, preferably K525A, G, I, L or V; Q526, preferably Q526C, M, S, T or Y; T527, preferably T527F, W or Y; E531, preferably E531A, G, I, L or V; H535, preferably H535D, E or P; K538, preferably K538F, W or Y; A539, preferably A539I, L or V; K541, preferably, K541F, W or Y; K557, preferably K557A, G, I, L or V; A561, preferably A561F, W or Y; T566, preferably T566F, W or Y; A569, preferably A569H or P in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

27. The conjugation-competent polypeptide of any preceding claim comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions V547, preferably V457A; K573, preferably K573P or Y; I523, preferably I523A or G; T527, preferably T527M; K500, preferably K500A; or E505, preferably E505Q in SEQ ID NO. 2; wherein the one or more (*e.g.* several) alterations causes the conjugation-competent polypeptides to have (i) an altered plasma half-life and/or (ii) an altered binding affinity to FcRn.

28. The conjugation-competent polypeptide of any preceding claim comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions 573, 523, 527 or 505 of SEQ ID NO. 2, preferably K573Y; I523G; I523A; T527M; E505Q; or K573P.

29. The conjugation-competent polypeptide of claim 28 comprising one or more (*e.g.* several) alterations in the mature human albumin polypeptide sequence of SEQ ID NO. 2 selected from the group consisting of positions corresponding to positions K573Y and I523G; K573Y, I523G and T527M; K573Y, E505Q and T527M; K573Y and T527M; K573P and I523G;

K573P, I523G and T527M; K573P, E505Q and T527M; K573P and T527M; V547A; V547A and K573P; V547A, E505Q, K573P and T527M; or K500A and H510Q.

30. The conjugation-competent polypeptide of any one of Claims 10 to 29 wherein the conjugation-competent polypeptide has a tendency to exist as a monomer in solution which is at least 70% of the tendency of the polypeptide of SEQ ID NO. 2 to exist as a monomer in solution, and optionally at least 75%, at least 80%, at least 90%, at least 95% or at least 100%.

31. The conjugation-competent polypeptide of any preceding claim, in which the polypeptide has at least 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.2, 99.4, 99.6, 99.8% sequence identity to SEQ ID NO. 2.

32. The conjugation-competent polypeptide of any preceding claim wherein, when the polypeptide is folded, there are at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and preferably all 17 of the native disulphide bonds of the polypeptide of SEQ ID NO. 2.

33. The conjugation-competent polypeptide of any preceding claim in which the polypeptide further comprises a further linker to which a bioactive compound, radiopharmaceutical or imaging agent may be linked.

34. The conjugation-competent polypeptide of any preceding claim wherein the alteration(s) to provide a conjugation competent cysteine residue(s) does not adversely affect the immunogenicity of the polypeptide in human.

35. A fusion polypeptide comprising a conjugation-competent polypeptide of any preceding claim and a fusion partner polypeptide.

36. A polynucleotide which encodes the polypeptide of any of Claims 1 to 35.

37. A plasmid comprising the polynucleotide of Claim 36.

38. A host cell comprising a polynucleotide of Claim 36 and/or a plasmid of Claim 37.

39. The host cell of claim 38, which is a yeast cell, particularly a *Saccharomyces cerevisiae* cell.

40. A conjugate which comprises (i) a bioactive compound, radiopharmaceutical or imaging agent and (ii) a polypeptide according to any of Claims 1 to 35, wherein the bioactive

compound, radiopharmaceutical or imaging agent is linked to the polypeptide through a conjugation-competent cysteine residue of the polypeptide.

41. The conjugate of Claim 40 further comprising one or more (*e.g.* several) further bioactive compounds radiopharmaceuticals or imaging agents, each bioactive compound, radiopharmaceutical or imaging agent being linked to the polypeptide through a conjugation-competent cysteine residue of the polypeptide.

42. A method of producing the polynucleotide of Claim 36 comprising:

providing a nucleic acid molecule encoding a parent albumin or fragment thereof; and

modifying the nucleic acid sequence of the nucleic acid molecule to encode a conjugation-competent polypeptide which is at least 70% identical to human albumin, particularly residues 1 to 585 of the mature human albumin polypeptide sequence of SEQ ID NO. 2, or a fragment thereof, wherein at least one position equivalent to a position selected from K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, L284, K317, A322, E333, D340, E354, K359, A362, E382, L398 of SEQ ID NO. 2 comprises a conjugation-competent cysteine residue.

43. A method of producing the polypeptide of any of Claims 1 to 35, comprising:

(a) culturing the host cell of Claim 38 or 39 under conditions that allow expression of the polypeptide; and

(b) recovering the polypeptide from the host cell and/or from host cell growth medium.

44. The method of Claim 43 further comprising purifying the polypeptide obtained in step (b).

45. A method of producing the conjugate of Claim 40 or 41 which comprises linking a polypeptide of any one of Claims 1 to 35, or produced by the method of any one of Claims 43 or 44, to a bioactive compound, radiopharmaceutical or imaging agent through a conjugation-competent cysteine residue of the polypeptide.

46. An associate comprising the conjugate of Claim 40 or 41 and a bioactive, therapeutic, prophylactic, diagnostic, imaging or other beneficial moiety.

47. A nanoparticle or a microparticle or a liposome comprising the polypeptide of any one of Claims 1 to 35, the conjugate of Claim 40 or 41 or the associate of Claim 46.

48. A composition comprising the conjugate of Claim 40 or 41, the associate of Claim 46 or the nanoparticle or microparticle or liposome of Claim 47 and at least one (*e.g.* several) pharmaceutically acceptable carrier or diluent.

49. The conjugate of Claim 40 or 41, the associate of Claim 46, the nanoparticle or microparticle or liposome of Claim 47, or the composition of Claim 48, wherein the bioactive molecule, radiopharmaceutical or imaging agent is selected from:
(i) therapeutic compounds, such as: 4-1BB ligand, 5-helix, A human C-C chemokine, A human L105 chemokine, A human L105 chemokine designated huL105_3, A monokine induced by gamma-interferon (MIG), A partial CXCR4B protein, A platelet basic protein (PBP), α1-antitrypsin, ACRP-30 Homologue, Complement Component C1q C, Adenoid-expressed chemokine (ADEC), aFGF, FGF-1, AGF, AGF Protein, albumin, an etoposide, angiostatin, Anthrax vaccine, Antibodies specific for collapsin, antistasin, Anti-TGF beta family antibodies, antithrombin III, APM-1, ACRP-30, Famoxin, apo-lipoprotein species, Arylsulfatase B, b57 Protein, BCMA, Beta-thromboglobulin protein (beta-TG), bFGF, FGF2, Blood coagulation factors, BMP Processing Enzyme Furin, BMP-10, BMP-12, BMP-15, BMP-17, BMP-18, BMP-2B, BMP-4, BMP-5, BMP-6, BMP-9, Bone Morphogenic Protein-2, calcitonin, Calpain-10a, Calpain-10b, Calpain-10c, Cancer Vaccine, Carboxypeptidase, C-C chemokine, MCP2, CCR5 variant, CCR7, CCR7, CD11a Mab, CD137, 4-1BB Receptor Protein, CD20 Mab, CD27, CD27L, CD30, CD30 ligand, CD33 immunotoxin, CD40, CD40L, CD52 Mab, Cerebus Protein, Chemokine Eotaxin, Chemokine hIL-8, Chemokine hMCP1, Chemokine hMCP1a, Chemokine hMCP1b, Chemokine hMCP2, Chemokine hMCP3, Chemokine hSDF1b, Chemokine MCP-4, chemokine TECK and TECK variant, Chemokine-like protein IL-8M1 Full-Length and Mature, Chemokine-like protein IL-8M10 Full-Length and Mature, Chemokine-like protein IL-8M3, Chemokine-like protein IL-8M8 Full-Length and Mature, Chemokine-like protein IL-8M9 Full-Length and Mature, Chemokine-like protein PF4-414 Full-Length and Mature, Chemokine-like protein PF4-426 Full-Length and Mature, Chemokine-like protein PF4-M2 Full-Length and Mature, Cholera vaccine, Chondromodulin-like protein, c-kit ligand, SCF, Mast cell growth factor, MGF, Fibrosarcoma-derived stem cell factor, CNTF and fragment thereof (such as CNTFAx15`(Axokine™)), coagulation factors in both pre and active forms, collagens, Complement C5 Mab, Connective tissue activating protein-III, CTAA16.88 Mab, CTAP-III, CTLA4-Ig, CTLA-8, CXC3, CXC chemokine receptor 3, cyanovirin-N, Darbepoetin, designated exodus, designated huL105_7, DIL-40, Dnase, EDAR, EGF Receptor Mab, ENA-78, Endostatin, Eotaxin, Epithelial neutrophil activating protein-78, EPO receptor, EPOR, erythropoietin (EPO) and EPO mimics, Eutropin, Exodus protein, Factor IX, Factor VII, Factor VIII, Factor X and Factor XIII, FAS Ligand Inhibitory Protein (DcR3), FasL, FGF, FGF-12, Fibroblast growth factor homologous factor-1, FGF-15, FGF-16, FGF-18, FGF-3, INT-2, FGF-4, gelonin, HST-1, HBGF-4, FGF-5, FGF-6, Heparin binding secreted transforming factor-2, FGF-

8, FGF-9, Glia activating factor, fibrinogen, flt-1, flt-3 ligand, Follicle stimulating hormone Alpha subunit, Follicle stimulating hormone Beta subunit, Follitropin, Fractalkine, fragment. myofibrillar protein Troponin I, FSH, Galactosidase, Galectin-4, G-CSF, GDF-1, Gene therapy, Glioma-derived growth factor, glucagon, glucagon-like peptides, Glucocerebrosidase, glucose oxidase, Glucosidase, Glycodelin-A, Progesterone-associated endometrial protein, GM-CSF, gonadotropin, Granulocyte chemotactic protein-2 (GCP-2), Granulocyte-macrophage colony stimulating factor, growth hormone, Growth related oncogene-alpha (GRO-alpha), Growth related oncogene-beta (GRO-beta), Growth related oncogene-gamma (GRO-gamma), hAPO-4, TROY, hCG, Hepatitus B surface Antigen, Hepatitus B Vaccine, HER2 Receptor Mab, hirudin, HIV gp120, HIV gp41, HIV Inhibitor Peptide, HIV Inhibitor Peptide, HIV Inhibitor Peptide, HIV protease inhibiting peptides, HIV-1 protease inhibitors, HPV vaccine, Human 6CKine protein, Human Act-2 protein, Human adipogenesis inhibitory factor, human B cell stimulating factor-2 receptor, Human beta-chemokine H1305 (MCP-2), Human C-C chemokine DGWCC, Human CC chemokine ELC protein, Human CC type chemokine interleukin C, Human CCC3 protein, Human CCF18 chemokine, Human CC-type chemokine protein designated SLC (secondary lymphoid chemokine), Human chemokine beta-8 short forms, Human chemokine C10, Human chemokine CC-2, Human chemokine CC-3, Human chemokine CCR-2, Human chemokine Ckbeta-7, Human chemokine ENA-78, Human chemokine eotaxin, Human chemokine GRO alpha, Human chemokine GROalpha, Human chemokine GRObeta, Human chemokine HCC-1, Human chemokine HCC-1, Human chemokine I-309, Human chemokine IP-10, Human chemokine L105_3, Human chemokine L105_7, Human chemokine MIG, Human chemokine MIG-beta protein, Human chemokine MIP-1alpha, Human chemokine MIP1beta, Human chemokine MIP-3alpha, Human chemokine MIP-3beta, Human chemokine PF4, Human chemokine protein 331D5, Human chemokine protein 61164, Human chemokine receptor CXCR3, Human chemokine SDF1alpha, Human chemokine SDF1beta, Human chemokine ZSIG-35, Human Chr19Kine protein, Human CKbeta-9, Human CX3C 111 amino acid chemokine, Human DNAX interleukin-40, Human DVic-1 C-C chemokine, Human EDIRF I protein sequence, Human EDIRF II protein sequence, Human eosinocyte CC type chemokine eotaxin, Human eosinophil-expressed chemokine (EEC), Human fast twitch skeletal muscle troponin C, Human fast twitch skeletal muscle troponin I, Human fast twitch skeletal muscle Troponin subunit C, Human fast twitch skeletal muscle Troponin subunit I Protein, Human fast twitch skeletal muscle Troponin subunit T, Human fast twitch skeletal muscle troponin T, Human foetal spleen expressed chemokine, FSEC, Human GM-CSF receptor, Human gro-alpha chemokine, Human gro-beta chemokine, Human gro-gamma chemokine, Human IL-16 protein, Human IL-1RD10 protein sequence, Human IL-1RD9, Human IL-5 receptor alpha chain, Human IL-6 receptor, Human IL-8 receptor protein hIL8RA, Human IL-8 receptor protein hIL8RB, Human IL-9 receptor protein, Human IL-9 receptor protein variant #3, Human IL-9 receptor protein variant fragment, Human IL-9 receptor protein variant fragment #3, Human interleukin 1

delta, Human interleukin 10, Human interleukin 18, Human interleukin 18 derivatives, Human interleukin-1 beta precursor, Human interleukin-1 beta precursor, Human interleukin-1 receptor accessory protein, Human interleukin-1 receptor antagonist beta, Human interleukin-1 type-3 receptor, Human interleukin-10 (precursor), Human interleukin-11 receptor, Human interleukin-12 40 kD subunit, Human interleukin-12 beta-1 receptor, Human interleukin-12 beta-2 receptor, Human interleukin-12 p35 protein, Human interleukin-12 p40 protein, Human interleukin-12 receptor, Human interleukin-13 alpha receptor, Human interleukin-13 beta receptor, Human interleukin-15, Human interleukin-15 receptor from clone P1, Human interleukin-17 receptor, Human interleukin-18 protein (IL-18), Human interleukin-3, human interleukin-3 receptor, Human interleukin-3 variant, Human interleukin-4 receptor, Human interleukin-5, Human interleukin-6, Human interleukin-7, Human interleukin-7, Human interleukin-8 (IL-8), Human intracellular IL-1 receptor antagonist, Human IP-10 and HIV-1 gp120 hypervariable region fusion protein, Human IP-10 and human Muc-1 core epitope (VNT) fusion protein, human liver and activation regulated chemokine (LARC), Human Lkn-1 Full-Length and Mature protein, Human mammary associated chemokine (MACK) protein Full-Length and Mature, Human mature chemokine Ckbeta-7, Human mature gro-alpha, Human mature gro-gamma polypeptide used to treat sepsis, Human MCP-3 and human Muc-1 core epitope (VNT) fusion protein, Human MI10 protein, Human MI1A protein, Human monocyte chemoattractant factor hMCP-1, Human monocyte chemoattractant factor hMCP-3, Human monocyte chemotactic proprotein (MCPP) sequence, Human neurotactin chemokine like domain, Human non-ELR CXC chemokine H174, Human non-ELR CXC chemokine IP10, Human non-ELR CXC chemokine Mig, Human PAI-1 mutants, Human protein with IL-16 activity, Human protein with IL-16 activity, Human secondary lymphoid chemokine (SLC), Human SISD protein, Human STCP-1, Human stromal cell-derived chemokine, SDF-1, Human T cell mixed lymphocyte reaction expressed chemokine (TMEC), Human thymus and activation regulated cytokine (TARC), Human thymus expressed, Human TNF-alpha, Human TNF-beta (LT-alpha), Human type CC chemokine eotaxin 3 protein sequence, Human type II interleukin-1 receptor, Human wild-type interleukin-4 (hIL-4) protein, Human ZCHEMO-8 protein, Humanized Anti-VEGF Antibodies, and fragments thereof, Humanized Anti-VEGF Antibodies, and fragments thereof, Hyaluronidase, ICE 10 kD subunit, ICE 20 kD subunit, ICE 22 kD subunit, Iduronate-2-sulfatase, Iduronidase, IL-1 alpha, IL-1 beta, IL-1 inhibitor (IL-1i), IL-1 mature, IL-10 receptor, IL-11, IL-11, IL-12 p40 subunit, IL-13, IL-14, IL-15, IL-15 receptor, IL-17, IL-17 receptor, receptor, IL-19, IL-1i fragments, IL1-receptor antagonist, IL-21 (TIF), IL-3 containing fusion protein, IL-3 mutant proteins, IL-3 variants, IL-4, IL-4 muteins, IL-4 mutein Y124G, IL-4 mutein Y124X, IL-5, IL-5 muteins, Il-5 receptor, IL-6, Il-6 receptor, IL-7 receptor clone, IL-8 receptor, IL-9 mature protein variant (Met117 version), immunoglobulins or immunoglobulin-based molecules or fragment of either (*e.g.* a Small Modular ImmunoPharmaceutical™ ("SMIP") or dAb, Fab' fragments, F(ab')2, scAb, scFv or scFv fragment), including but not limited to plasminogen, Influenza Vaccine, Inhibin alpha,

Inhibin beta, insulin, insulin-like growth factor, Integrin Mab, inter-alpha trypsin inhibitor, inter-alpha trypsin inhibitor, Interferon gamma-inducible protein (IP-10), interferons (such as interferon alpha species and sub-species, interferon beta species and sub-species, interferon gamma species and sub-species), interleukin 6, interleukin 8 (IL-8) receptor, interleukin 8 receptor B, interleukin-1alpha, interleukin-2 receptor associated protein p43, interleukin-3, interleukin-4 muteins, interleukin-8 (IL-8) protein, interleukin-9, interleukin-9 (IL-9) mature protein (Thr117 version), interleukins (such as IL10, IL11 and IL2), Japanese encephalitis vaccine, Kalikrein Inhibitor, Keratinocyte growth factor, Kunitz domain protein (such as aprotinin, amyloid precursor protein and those described in WO 03/066824, with or without albumin fusions), LACI, lactoferrin, Latent TGF-beta binding protein II, leptin, Liver expressed chemokine-1 (LVEC-1), Liver expressed chemokine-2 (LVEC-2), LT-alpha, LT-beta, Luteinization Hormone, Lyme Vaccine, Lymphotactin, Macrophage derived chemokine analogue MDC (n+1), Macrophage derived chemokine analogue MDC-eyfy, Macrophage derived chemokine analogue MDC-yl, Macrophage-derived chemokine (MDC), Maspin, Protease Inhibitor 5, MCP-1 receptor, MCP-1a, MCP-1b, MCP-3, MCP-4 receptor, M-CSF, Melanoma inhibiting protein, Membrane-bound proteins, Met117 human interleukin 9, MIP-3 alpha, MIP-3 beta, MIP-Gamma, MIRAP, Modified Rantes, monoclonal antibody, MP52, Mutant interleukin 6 S176R, myofibrillar contractile protein Troponin I, Natriuretic Peptide, Nerve Growth Factor-beta, Nerve Growth Factor-beta2, Neuropilin-1, Neuropilin-2, Neurotactin, Neurotrophin-3, Neurotrophin-4, Neurotrophin-4a, Neurotrophin-4b, Neurotrophin-4c, Neurotrophin-4d, Neutrophil activating peptide-2 (NAP-2), NOGO-66 Receptor, NOGO-A, NOGO-B, NOGO-C, Novel beta-chemokine designated PTEC, N-terminal modified chemokine GroHEK/hSDF-1alpha, N-terminal modified chemokine GroHEK/hSDF-1beta, N-terminal modified chemokine met-hSDF-1 alpha, N-terminal modified chemokine met-hSDF-1 beta, OPGL, Osteogenic Protein-1 (OP-1), BMP-7, Osteogenic Protein-2, OX40, ACT-4, OX40L, Oxytocin (Neurophysin I), parathyroid hormone, Patched, Patched-2, PDGF-D, Pertussis toxoid, Pituitary expressed chemokine (PGEC), Placental Growth Factor, Placental Growth Factor-2, Plasminogen Activator Inhibitor-1 (PAI-1), Plasminogen Activator Inhibitor-2 (PAI-2), Platelet derived growth factor, Platelet derived growth factor Bv-sis, Platelet derived growth factor precursor A, Platelet derived growth factor precursor B, Platelet Mab, platelet-derived endothelial cell growth factor (PD-ECGF), Platelet-Derived Growth Factor A chain, Platelet-Derived Growth Factor B chain, polypeptide used to treat sepsis, Preproapolipoprotein “milano” variant, Preproapolipoprotein “paris” variant, pre-thrombin, Primate CC chemokine "ILINCK", Primate CXC chemokine "IBICK", proinsulin, Prolactin, Prolactin2, prosaptide, Protease inhibitor peptides, Protein C, Protein S, pro-thrombin, prourokinase, RANTES, RANTES 8-68, RANTES 9-68, RANTES peptide, RANTES receptor, Recombinant interleukin-16, Resistin, restrictocin, Retroviral protease inhibitors, ricin, Rotavirus Vaccine, RSV Mab, saporin, sarcin, Secreted and Transmembrane polypeptides, serum cholinesterase, serum protein (such as a blood clotting

factor), Soluble BMP Receptor Kinase Protein-3, Soluble VEGF Receptor, Stem Cell Inhibitory Factor, Straphylococcus Vaccine, Stromal Derived Factor-1 alpha, Stromal Derived Factor-1 beta, Substance P (tachykinin), T1249 peptide, T20 peptide, T4 Endonuclease, TACI, Tarc, TGF-beta 1, TGF-beta 2, Thr117 human interleukin 9, thrombin, thrombopoietin, thrombopoietin derivative 1, thrombopoietin derivative 2, thrombopoietin derivative 3, thrombopoietin derivative 4, thrombopoietin derivative 5, thrombopoietin derivative 6, thrombopoietin derivative 7, Thymus expressed chemokine (TECK), Thyroid stimulating Hormone, tick anticoagulant peptide, Tim-1 protein, TNF-alpha precursor, TNF-R, TNF-RII, TNF p75 Receptor, Death Receptor, tissue plasminogen activator (tPA), transferrin, transforming growth factor beta, Troponin peptides, Truncated monocyte chemotactic protein 2 (6-76), Truncated RANTES protein (3-68), tumour necrosis factor, Urate Oxidase, urokinase, Vasopressin (Neurophysin II), VEGF R-3, flt-4, VEGF Receptor, KDR, flk-1, VEGF-110, VEGF-121, VEGF-138, VEGF-145, VEGF-162, VEGF-165, VEGF-182, VEGF-189, VEGF-206, VEGF-D, VEGF-E, VEGF-X, von Willebrand's factor, Wild type monocyte chemotactic protein 2, ZTGF-beta 9;

(ii) chemotherapy drugs, such as: 13-cis-Retinoic Acid, 2-CdA, 2-Chlorodeoxyadenosine, 5-Azacitidine, 5-Fluorouracil, 5-FU, 6-Mercaptopurine, 6-MP, 6-TG, 6-Thioguanine, Abraxane, Accutane®, Actinomycin-D, Adriamycin®, Adrucil®, Agrylin®, Ala-Cort®, Aldesleukin, Alemtuzumab, ALIMTA, Alitretinoin, Alkaban-AQ®, Alkeran®, All-transretinoic Acid, Alpha Interferon, Altretamine, Amethopterin, Amifostine, Aminoglutethimide, Anagrelide, Anandron®, Anastrozole, Arabinosylcytosine, Ara-C, Aranesp®, Aredia®, Arimidex®, Aromasin®, Arranon®, Arsenic Trioxide, Asparaginase, ATRA, Avastin®, Azacitidine, BCG, BCNU, Bevacizumab, Bexarotene, BEXXAR®, Bicalutamide, BiCNU, Blenoxane®, Bleomycin, Bortezomib, Busulfan, Busulfex®, C225, Calcium Leucovorin, Campath®, Camptosar®, Camptothecin-11, Capecitabine, Carac™, Carboplatin, Carmustine, Carmustine Wafer, Casodex®, CC-5013, CCNU, CDDP, CeeNU, Cerubidine®, Cetuximab, Chlorambucil, Cisplatin, Citrovorum Factor, Cladribine, Cortisone, Cosmegen®, CPT-11, Cyclophosphamide, Cytadren®, Cytarabine, Cytarabine Liposomal, Cytosar-U®, Cytoxan®, Dacarbazine, Dacogen, Dactinomycin, Darbepoetin Alfa, Dasatinib, Daunomycin, Daunorubicin, Daunorubicin Hydrochloride, Daunorubicin Liposomal, DaunoXome®, Decadron, Decitabine, Delta-Cortef®, Deltasone®, Denileukin diftitox, DepoCyt™, Dexamethasone, Dexamethasone acetate, Dexamethasone Sodium Phosphate, Dexasone, Dexrazoxane, DHAD, DIC, Diodex, Docetaxel, Doxil®, Doxorubicin, Doxorubicin liposomal, Droxia™, DTIC, DTIC-Dome®, Duralone®, Efudex®, Eligard™, Ellence™, Eloxatin™, Elspar®, Emcyt®, Epirubicin, Epoetin alfa, Erbitux™, Erlotinib, Erwinia L-asparaginase, Estramustine, Ethyol, Etopophos®, Etoposide, Etoposide Phosphate, Eulexin®, Evista®, Exemestane, Fareston®, Faslodex®, Femara®, Filgrastim, Floxuridine, Fludara®, Fludarabine, Fluoroplex®, Fluorouracil, Fluoxymesterone, Flutamide, Folinic Acid, FUDR®, Fulvestrant, G-CSF, Gefitinib, Gemcitabine, Gemtuzumab ozogamicin, Gemzar®, Gleevec™, Gliadel® Wafer, GM-CSF, Goserelin, Granulocyte-Colony Stimulating Factor, Granulocyte Macrophage Colony Stimulating

Factor, Halotestin®, Herceptin®, Hexadrol, Hexalen®, Hexamethylmelamine, HMM, Hycamtin®, Hydrea®, Hydrocort Acetate®, Hydrocortisone, Hydrocortisone Sodium Phosphate, Hydrocortisone Sodium Succinate, Hydrocortone Phosphate, Hydroxyurea, Ibritumomab, Ibritumomab Tiuxetan, Idamycin®, Idarubicin, Ifex®, IFN-alpha, Ifosfamide, IL-11, IL-2, Imatinib mesylate, Imidazole Carboxamide, Interferon alfa, Interferon Alfa-2b (PEG Conjugate), interleukin-2, interleukin-11, Intron A® (interferon alfa-2b), Iressa®, Irinotecan, Isotretinoin, Kidrolase®, Lanacort®, Lapatinib, L-asparaginase, LCR, Lenalidomide, Letrozole, Leucovorin, Leukeran, Leukine™, Leuprolide, Leurocristine, Leustatin™, Liposomal Ara-C, Liquid Pred®, Lomustine, L-PAM, L-Sarcolysin, Lupron®, Lupron Depot®, Matulane®, Maxidex, Mechlorethamine, Mechlorethamine Hydrochloride, Medralone®, Medrol®, Megace®, Megestrol, Megestrol Acetate, Melphalan, Mercaptopurine, Mesna, Mesnex™, Methotrexate, Methotrexate Sodium, Methylprednisolone, Meticorten®, Mitomycin, Mitomycin-C, Mitoxantrone, M-Prednisol®, MTC, MTX, Mustargen®, Mustine, Mutamycin®, Myleran®, Mylocel™, Mylotarg®, Navelbine®, Nelarabine, Neosar®, Neulasta™, Neumega®, Neupogen®, Nexavar®, Nilandron®, Nilutamide, Nipent®, Nitrogen Mustard, Novaldex®, Novantrone®, Octreotide, Octreotide acetate, Oncospar®, Oncovin®, Ontak®, Onxal™, Oprevelkin, Orapred®, Orasone®, Oxaliplatin, Paclitaxel, Paclitaxel Protein-bound, Pamidronate, Panitumumab, Panretin®, Paraplatin®, Pediapred®, PEG Interferon, Pegaspargase, Pegfilgrastim, PEG-INTRON™, PEG-L-asparaginase, PEMETREXED, Pentostatin, Phenylalanine Mustard, Platinol®, Platinol-AQ®, Prednisolone, Prednisone, Prelone®, Procarbazine, PROCRIT®, Proleukin®, Prolifeprospan 20 with Carmustine Implant, Purinethol®, Raloxifene, Revlimid®, Rheumatrex®, Rituxan®, Rituximab, Roferon-A® (Interferon Alfa-2a), Rubex®, Rubidomycin hydrochloride, Sandostatin®, Sandostatin LAR®, Sargramostim, Solu-Cortef®, Solu-Medrol®, Sorafenib, SPRYCEL™, STI-571, Streptozocin, SU11248, Sunitinib, Sutent®, Tamoxifen, Tarceva®, Targretin®, Taxol®, Taxotere®, Temodar®, Temozolomide, Teniposide, TESPA, Thalidomide, Thalomid®, TheraCys®, Thioguanine, Thioguanine Tabloid®, Thiophosphoamide, Thioplex®, Thiotepa, TICE®, Toposar®, Topotecan, Toremifene, Tositumomab, Trastuzumab, Tretinoin, Trexall™, Trisenox®, TSPA, TYKERB®, VCR, Vectibix™, Velban®, Velcade®, VePesid®, Vesanoid®, Viadur™, Vidaza®, Vinblastine, Vinblastine Sulfate, Vincasar Pfs®, Vincristine, Vinorelbine, Vinorelbine tartrate, VLB, VM-26, Vorinostat, VP-16, Vumon®, Xeloda®, Zanosar®, Zevalin™, Zinecard®, Zoladex®, Zoledronic acid, Zolinza, Zometa®;

(iii) radiopharmaceuticals such as: Carbon-11, Carbon-14, Chromium-51, Cobalt-57, Cobalt-58, Erbium-169, Fluorine-18, Gallium-67, Gold-198, Indium-111, Indium-113m, Iodine-123, Iodine-125, Iodine-131, Iron-59, Krypton-81m, Nitrogen-13, Oxygen-15, Phosphorous-32, Rhenium-186, Rubidium-82, Samarium-153, Selenium-75, Strontium-89, Technetium-99m, Thallium-201, Tritium, Xenon-127, Xenon-133, Yttrium-90, and

(iv) Imaging agents, such as: Gadolinium, magnetite, manganese, technetium, I125, I131, P32, Tl201, Iopamidol, PET-FDG.

50. The conjugate of Claim 40, 41 or 49, or the associate of Claim 46, the nanoparticle or microparticle or liposome of Claim 47 for treatment of disease, treatment of illness and/or for diagnosis.

5

51. Use of a polypeptide as defined in any of Claims 1 to 35 to increase the half-life of a molecule such as a bioactive agent, an imaging agent, a diagnostic agent, a contrast agent or a therapeutic compound.

**Figure 1**

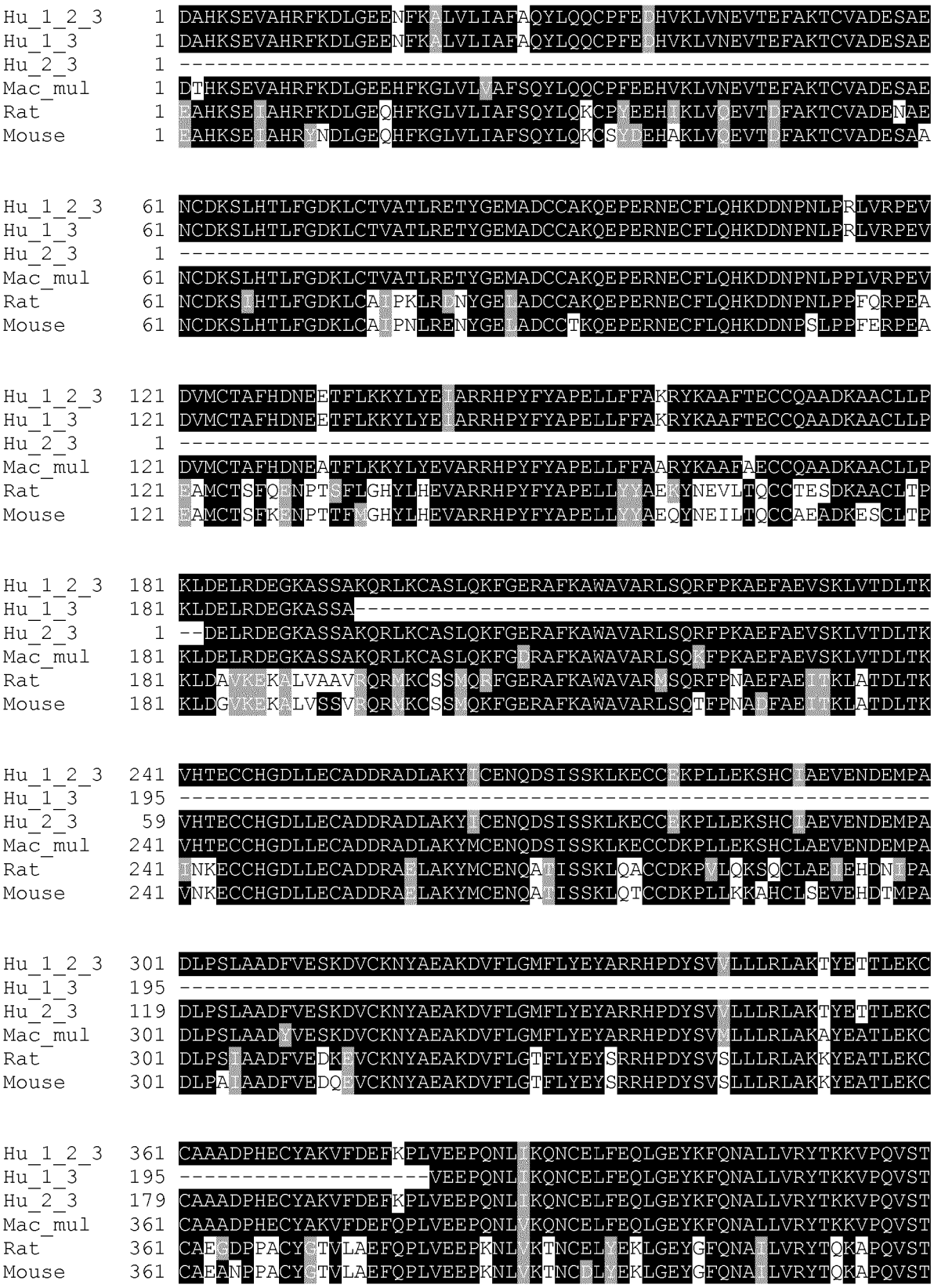
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Figure 1 (continued)

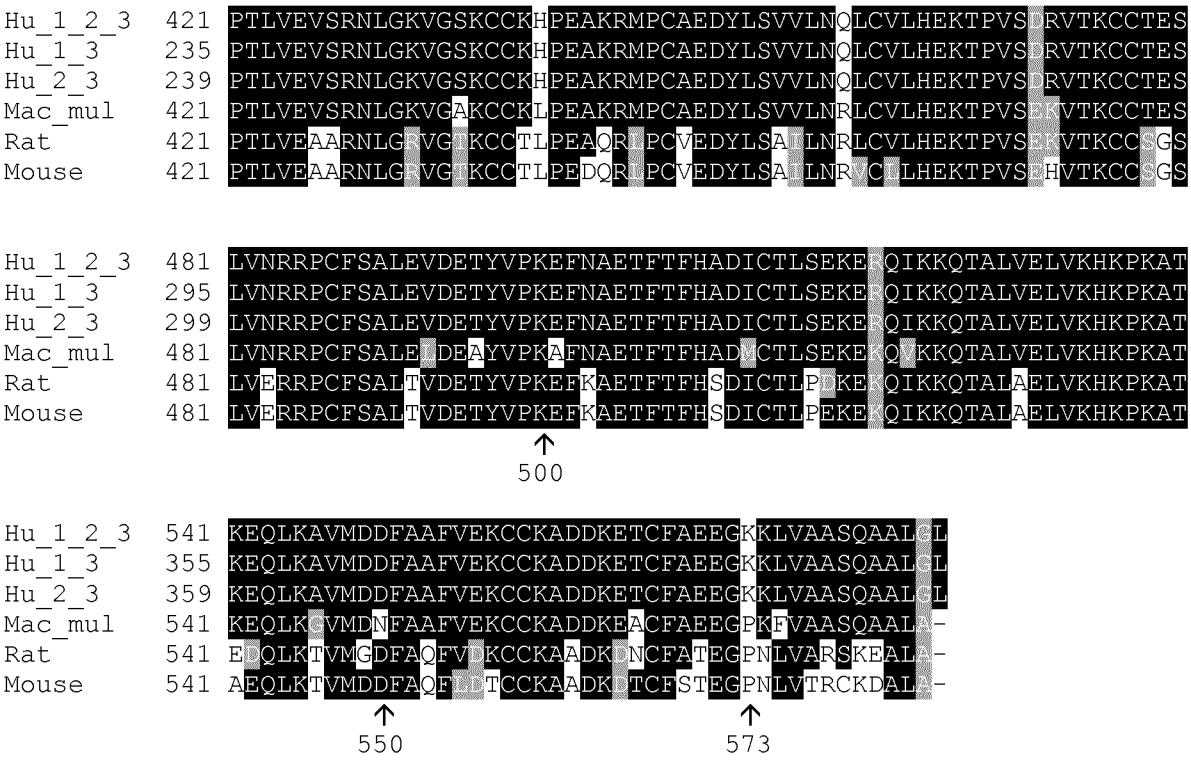
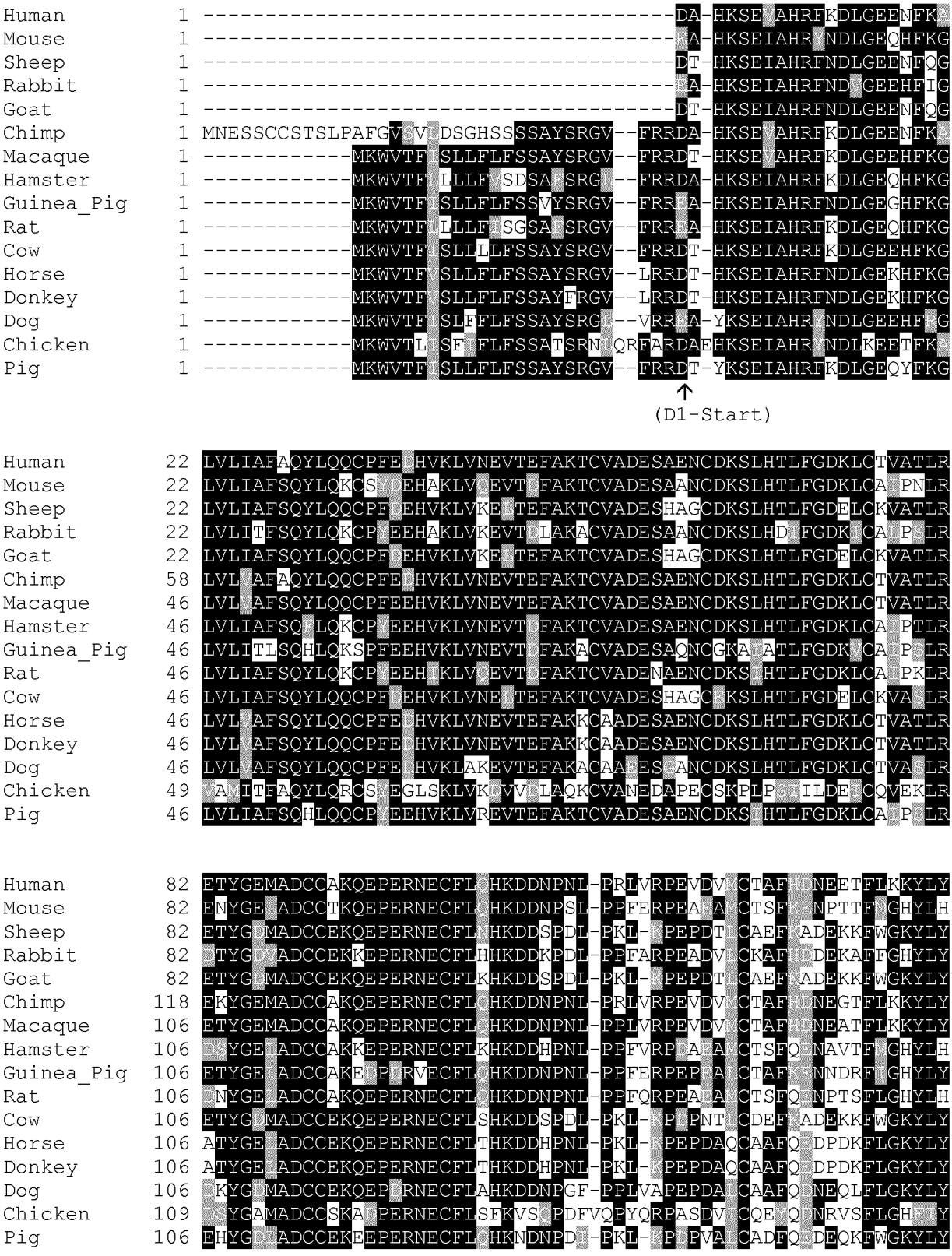


**Figure 2**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Dog 1
Chicken 1
Pig 1
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Rabbit 22
Goat 22
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Rabbit 82
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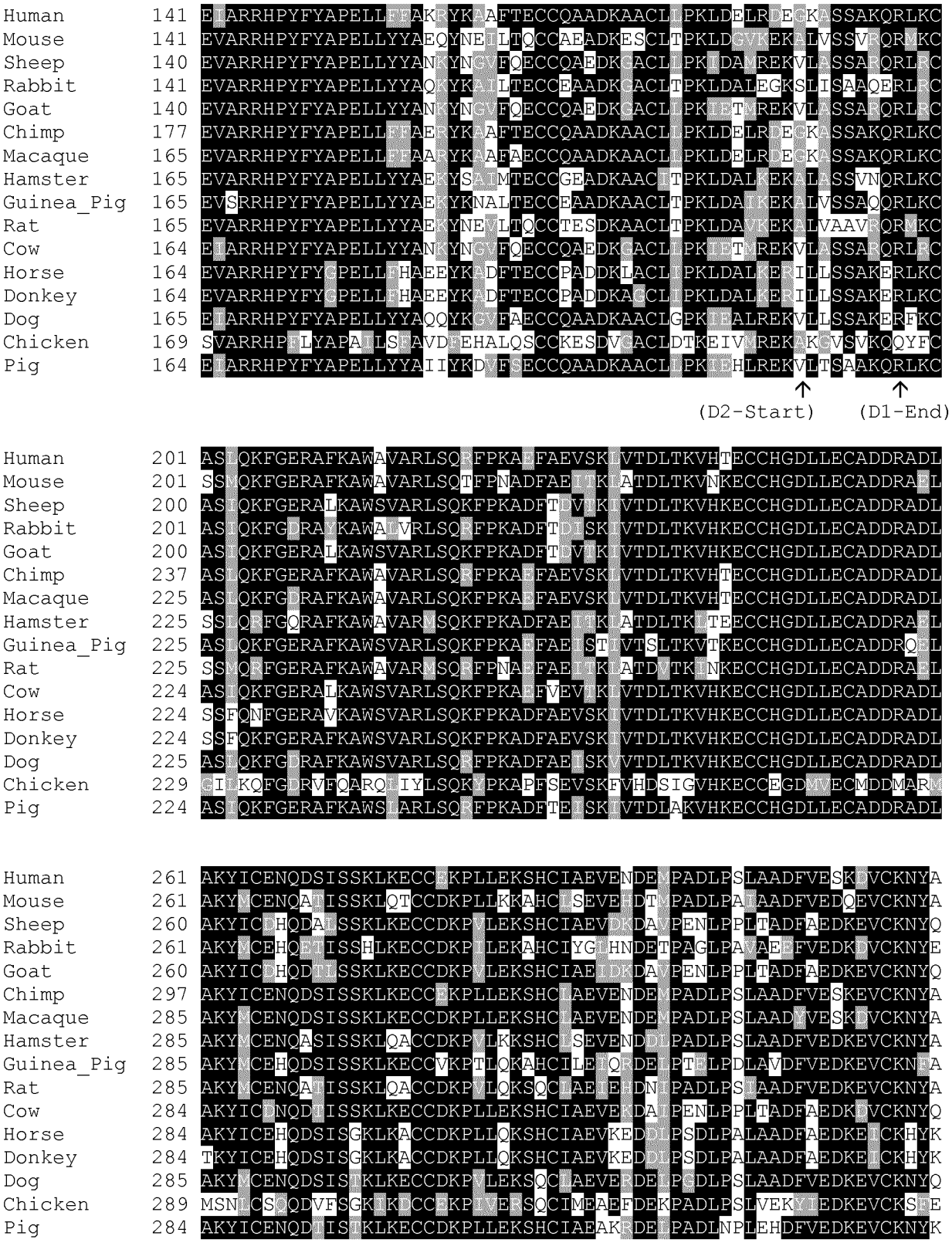
Figure 2 (continued)



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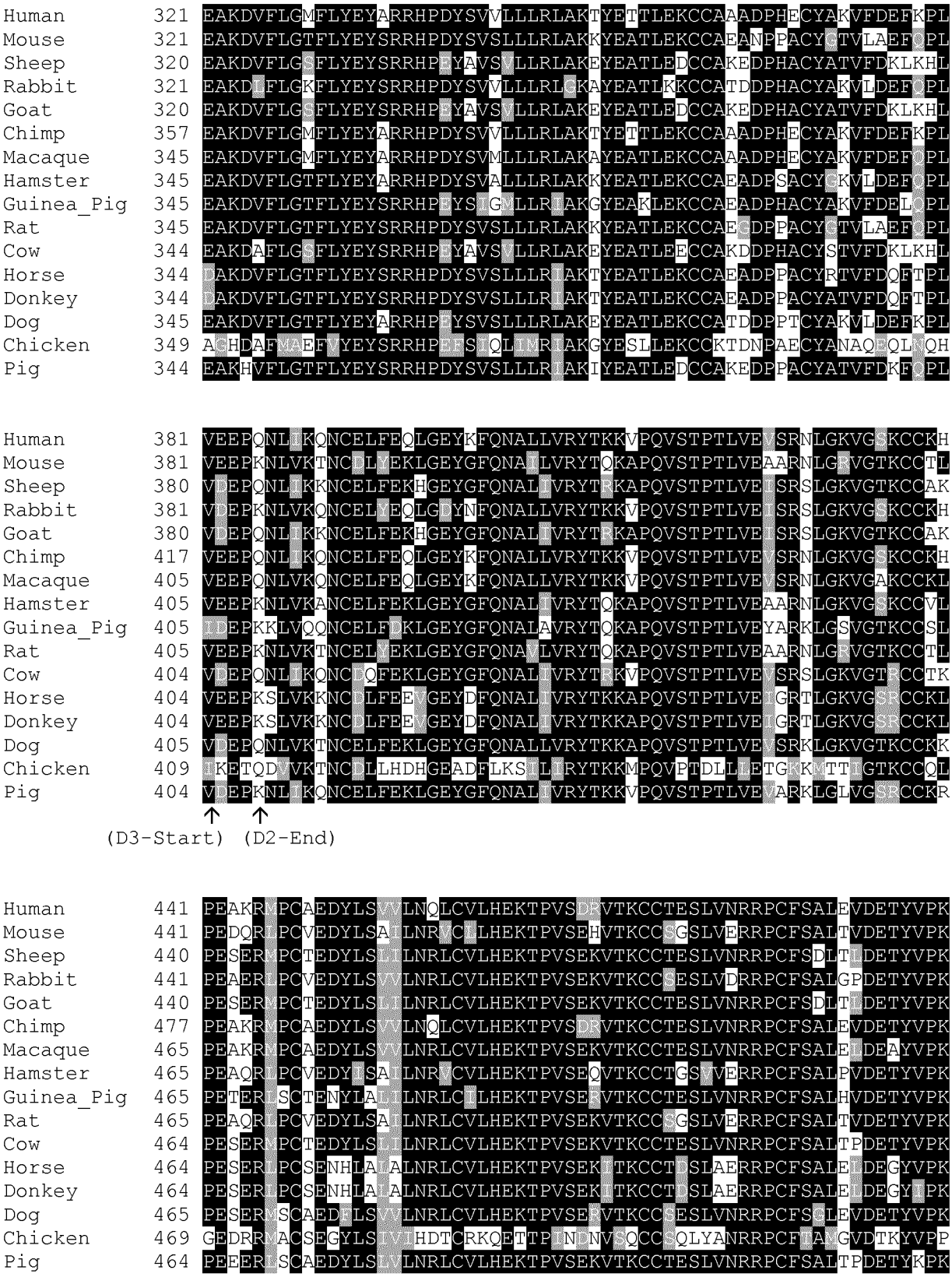

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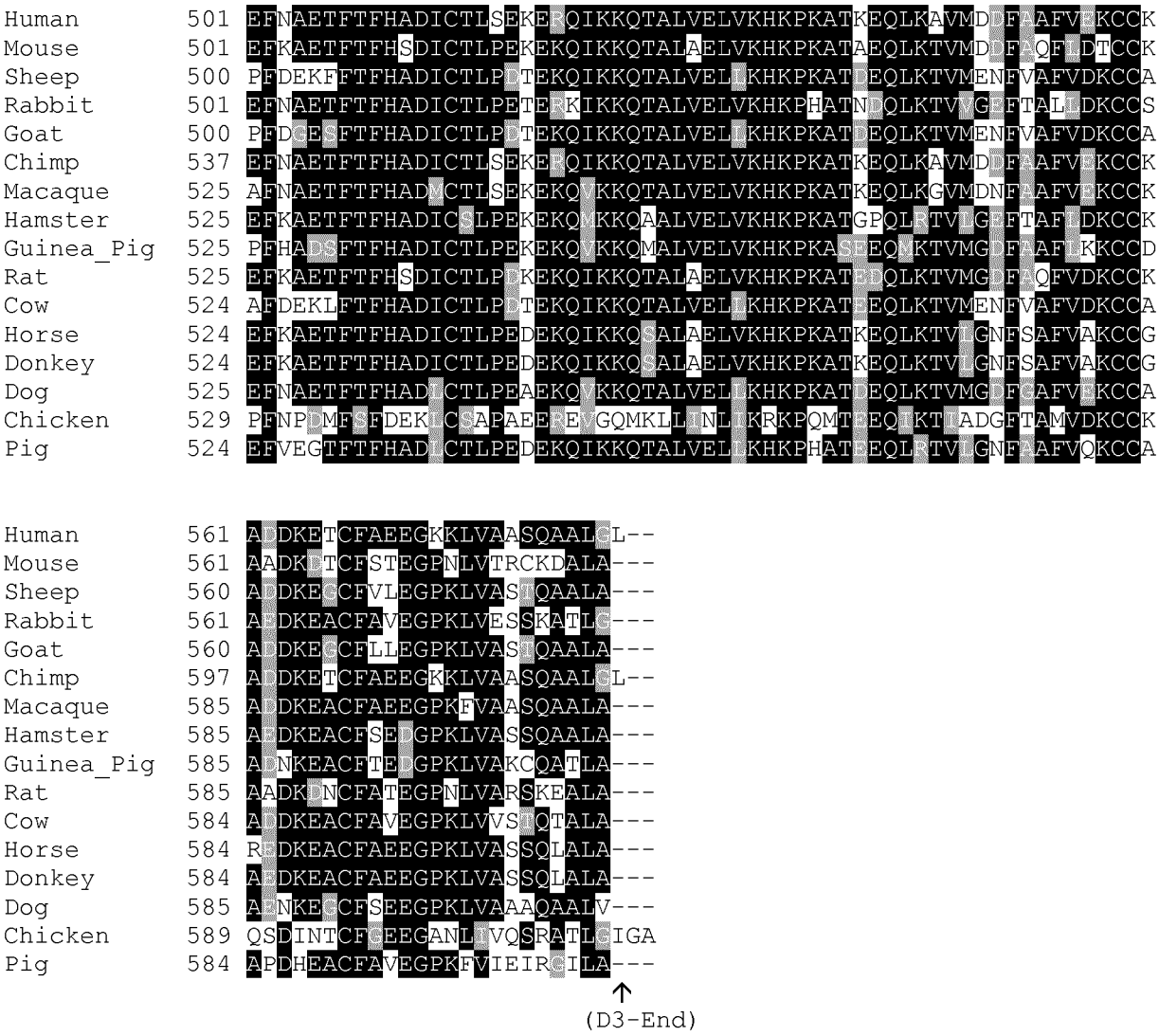

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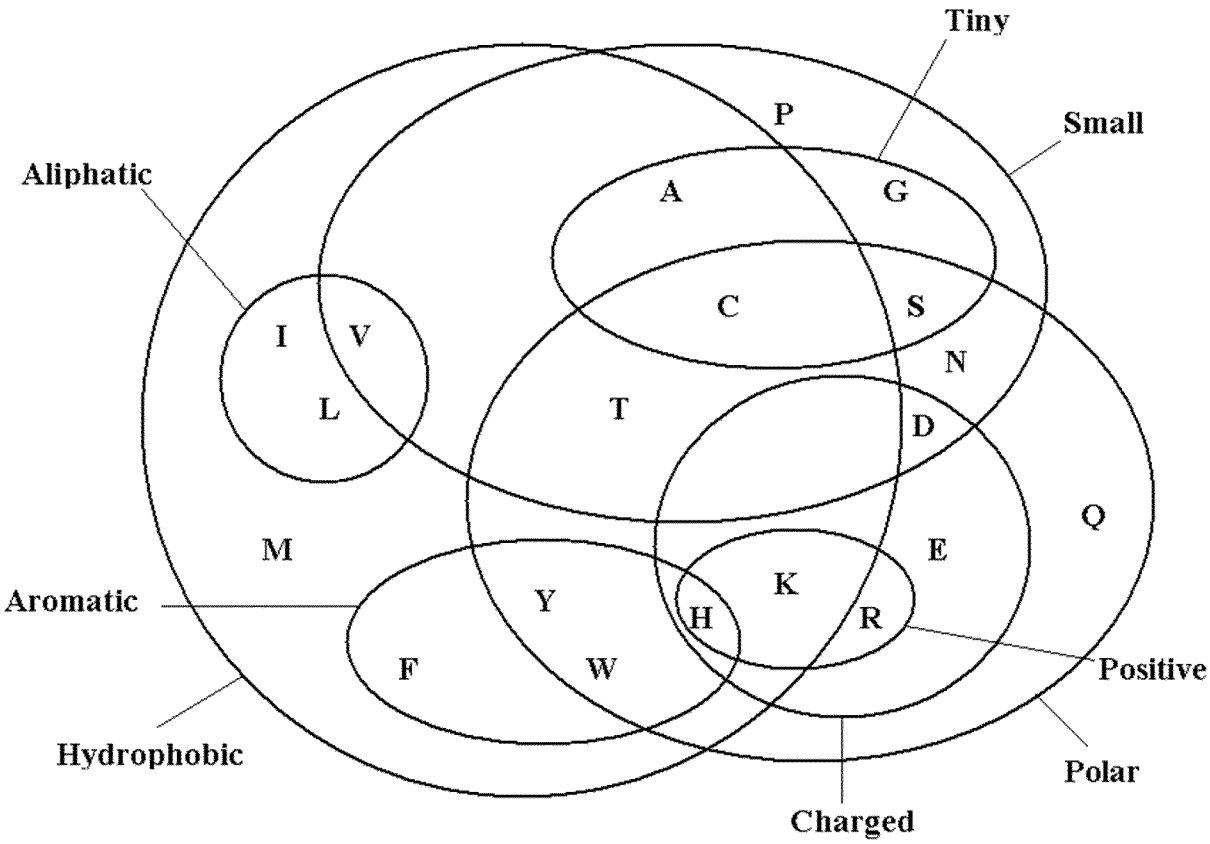
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Polar
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Figure 4

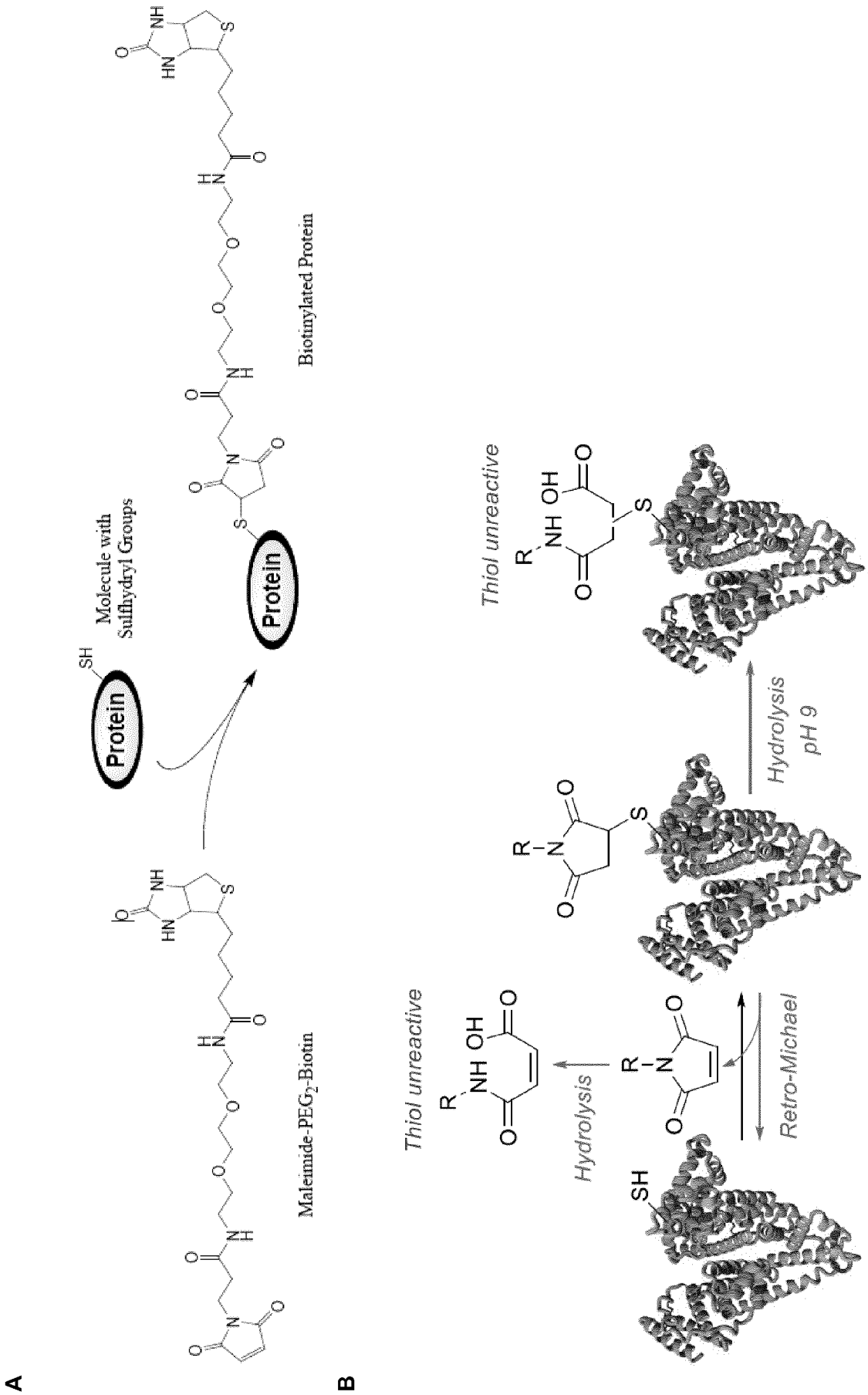
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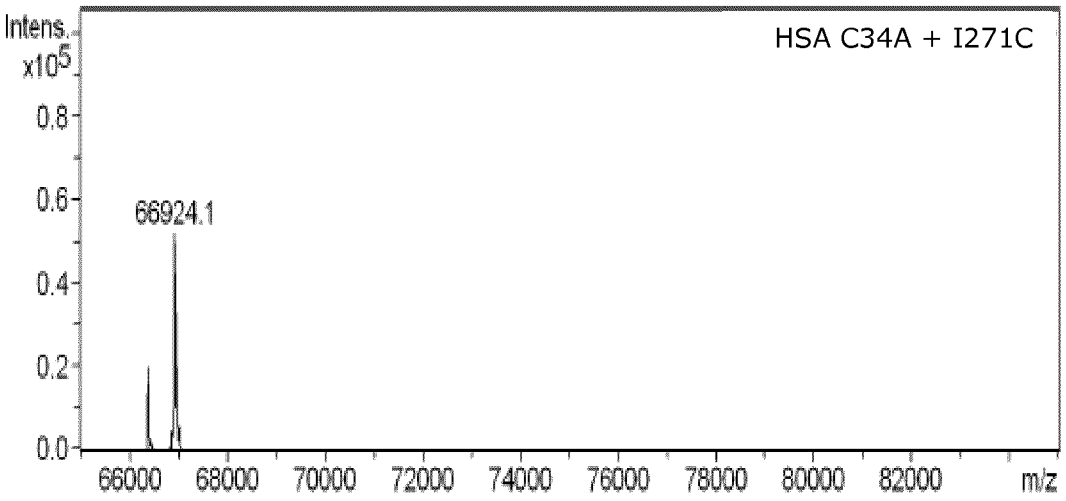
**Figure 5**

**A**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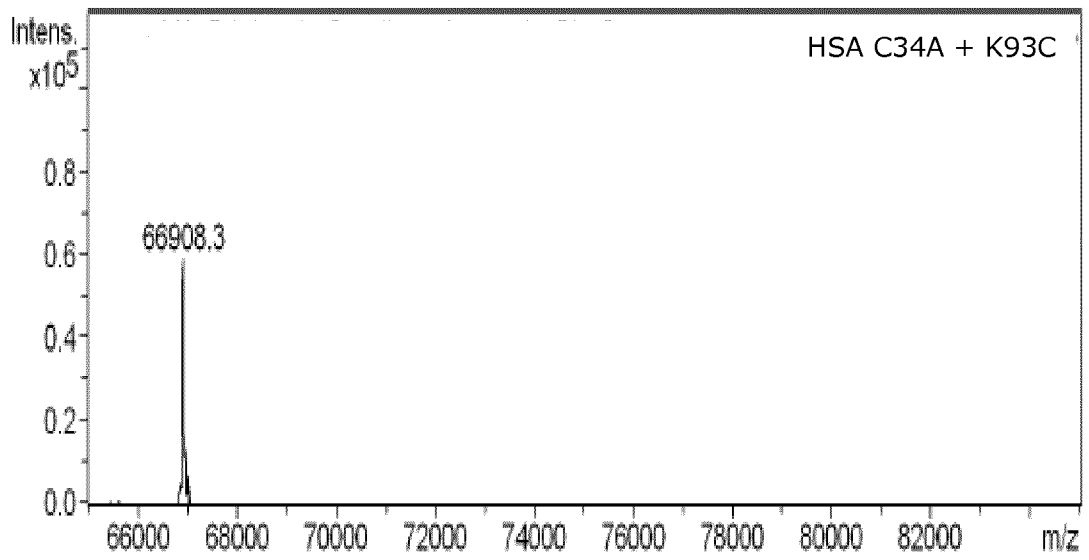
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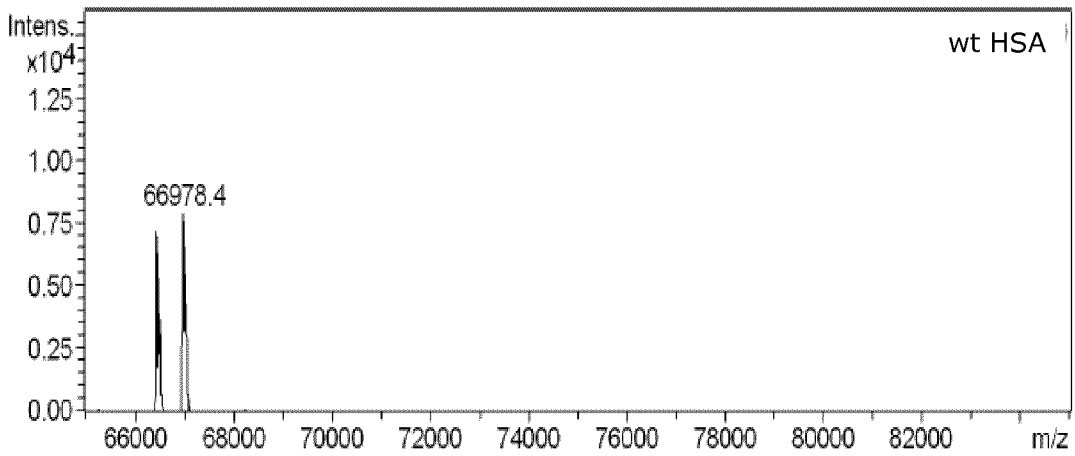
**Figure 6**

**A**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0.75
0.50
0.25
0.00
66978.4
wt HSA
66000
68000
70000
72000
74000
76000
78000
80000
82000
m/z

**B**

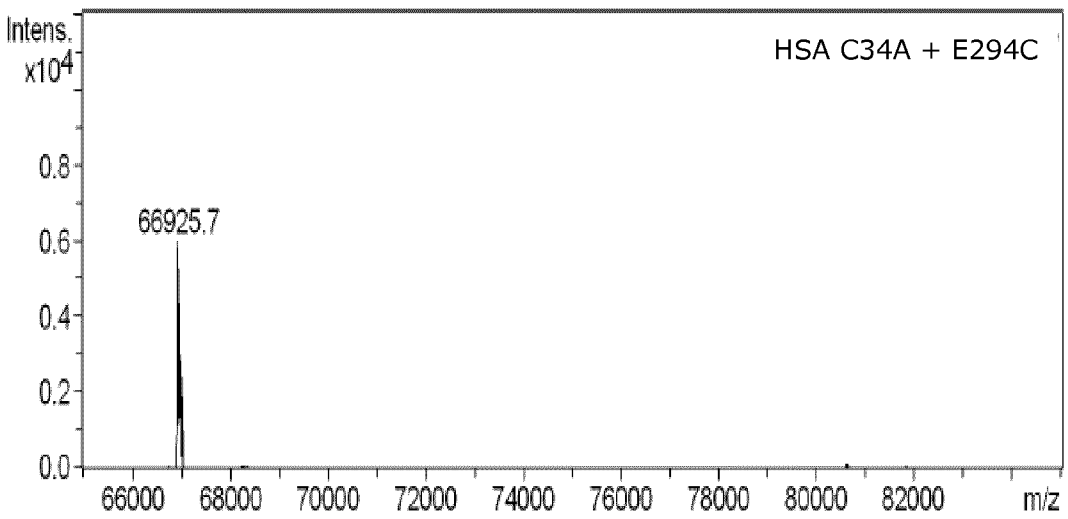
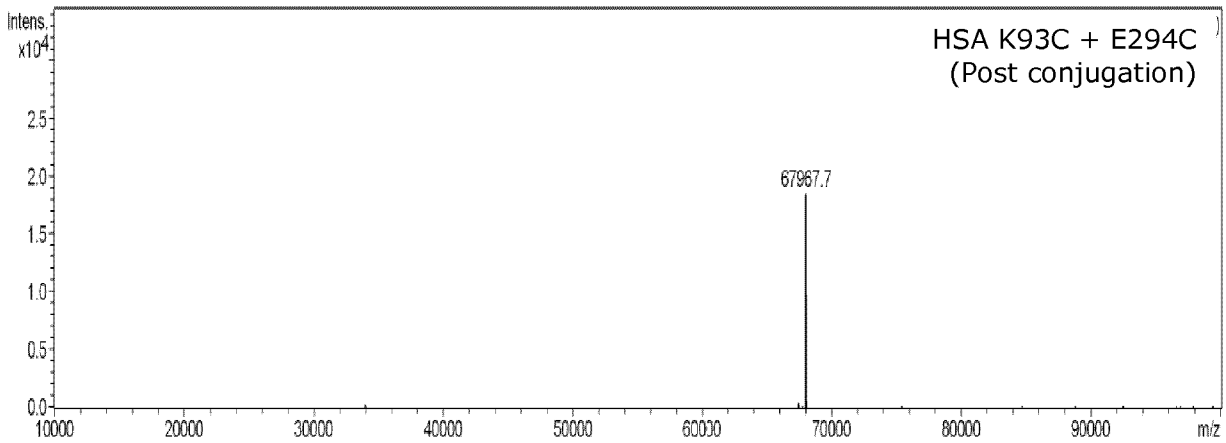
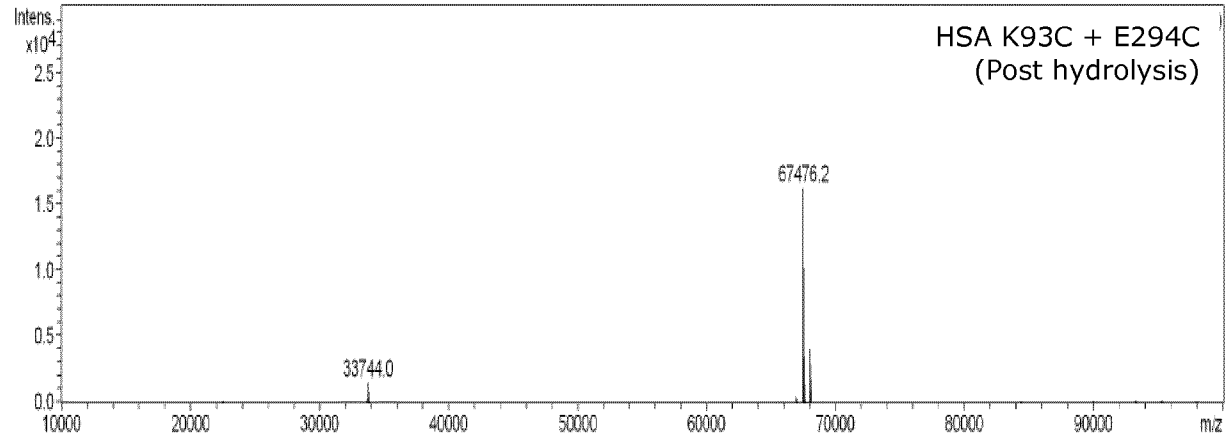

Intens.
x10^4
0.8
0.6
0.4
0.2
0.0
66925.7
HSA C34A + E294C
66000
68000
70000
72000
74000
76000
78000
80000
82000
m/z

**Figure 7**

**A**


Intens.
x10^4
HSA K93C + E294C
(Post conjugation)
67967.7
m/z

**B**


Intens.
x10^4
HSA K93C + E294C
(Post hydrolysis)
67476.2
33744.0
m/z

**C**

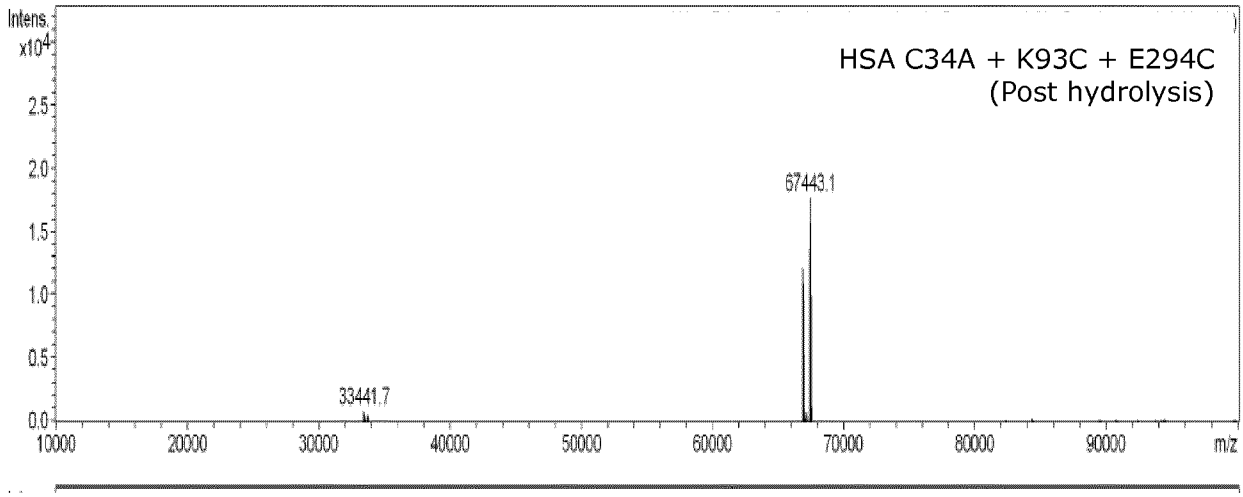

Intens.
x10^4
HSA C34A + K93C + E294C
(Post hydrolysis)
67443.1
33441.7
m/z

**Figure 8**

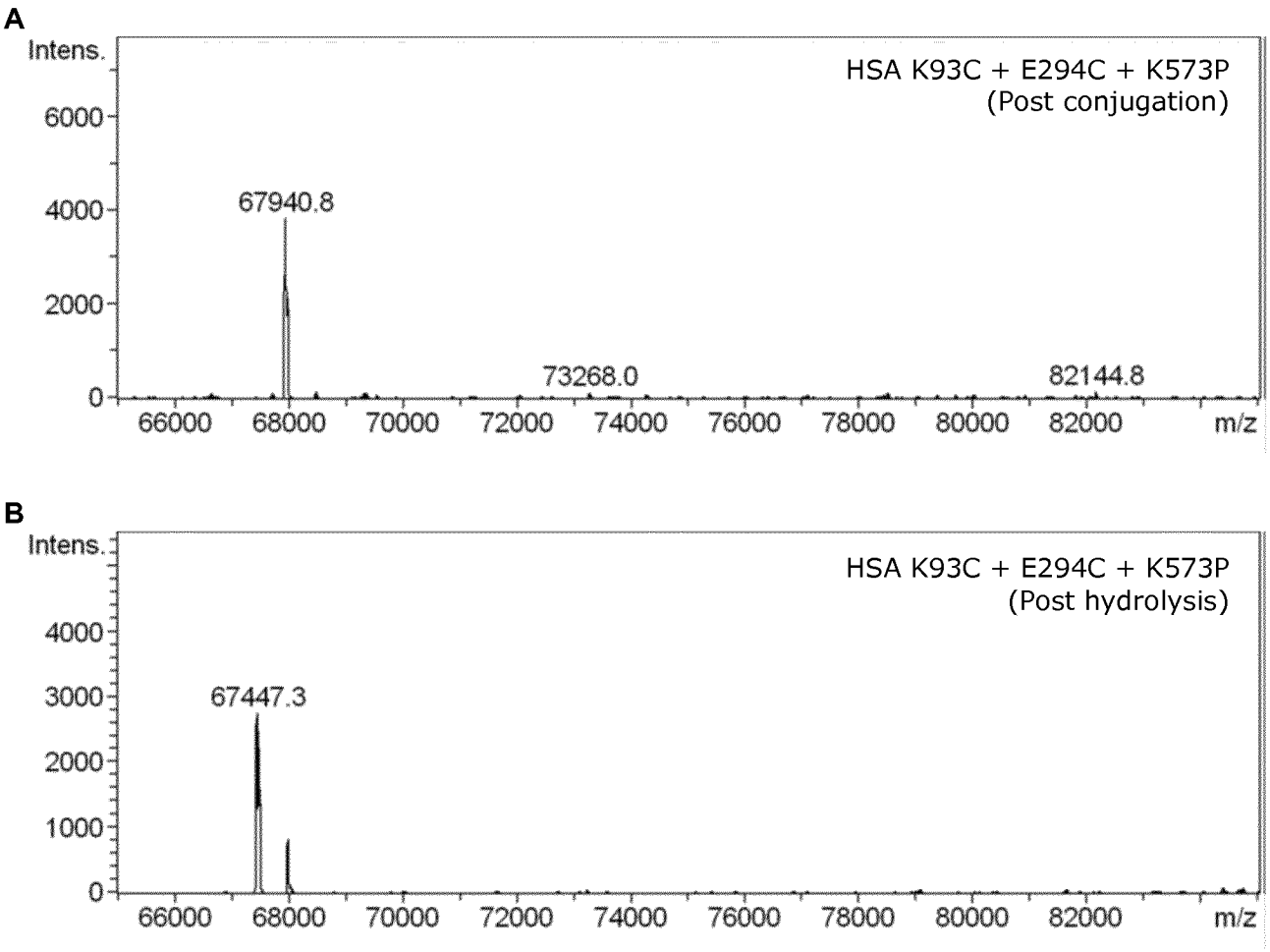
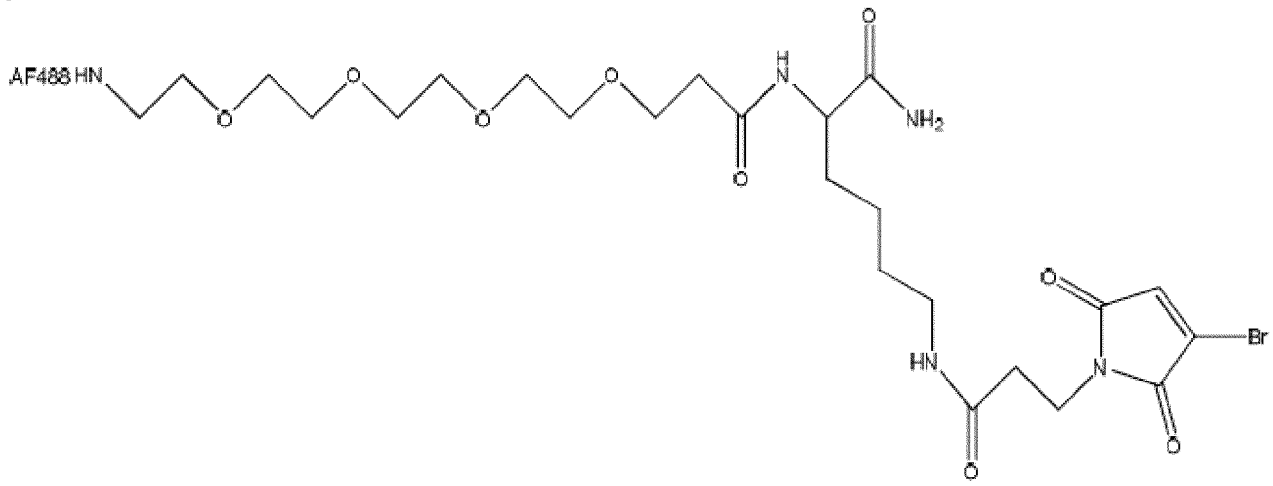
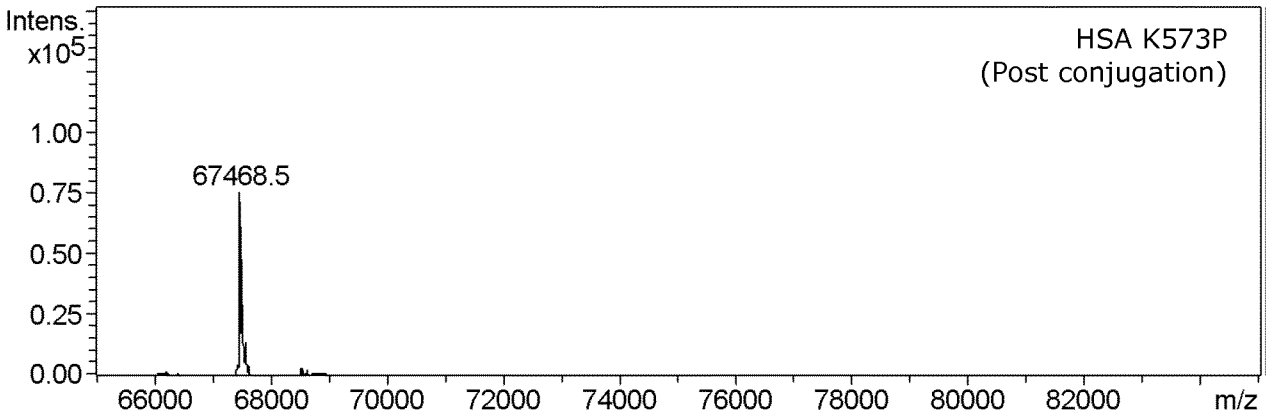

A
Intens.
HSA K93C + E294C + K573P
(Post conjugation)
6000
4000
2000
0
67940.8
73268.0
82144.8
66000
68000
70000
72000
74000
76000
78000
80000
82000
m/z
B
Intens.
HSA K93C + E294C + K573P
(Post hydrolysis)
4000
3000
2000
1000
0
67447.3
66000
68000
70000
72000
74000
76000
78000
80000
82000
m/z

**Figure 9**

**A**


AF488 HN
O
O
O
O
H
N
O
NH2
HN
O
O
N
O
Br

**B**


Intens.
x10^5
1.00
0.75
0.50
0.25
0.00
67468.5
HSA K573P
(Post conjugation)
66000
68000
70000
72000
74000
76000
78000
80000
82000
m/z

**C**

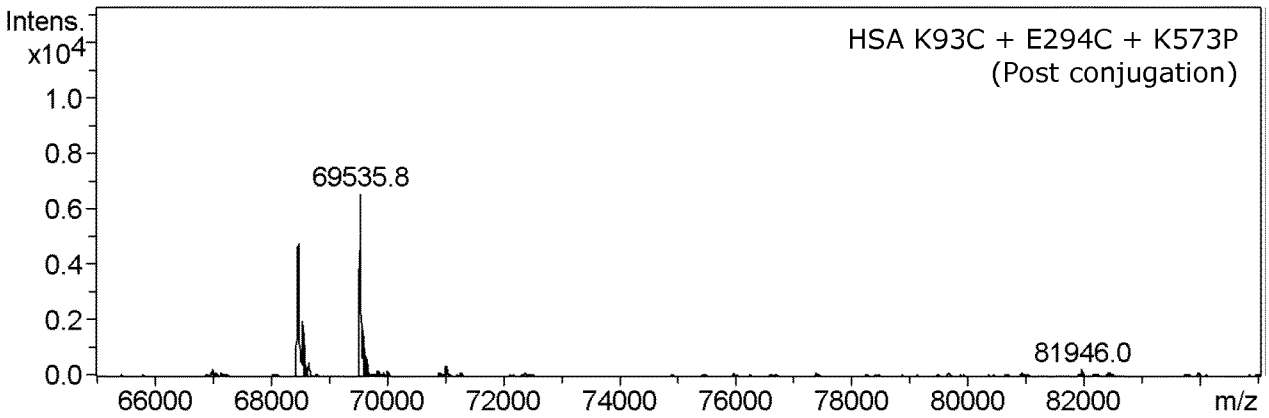
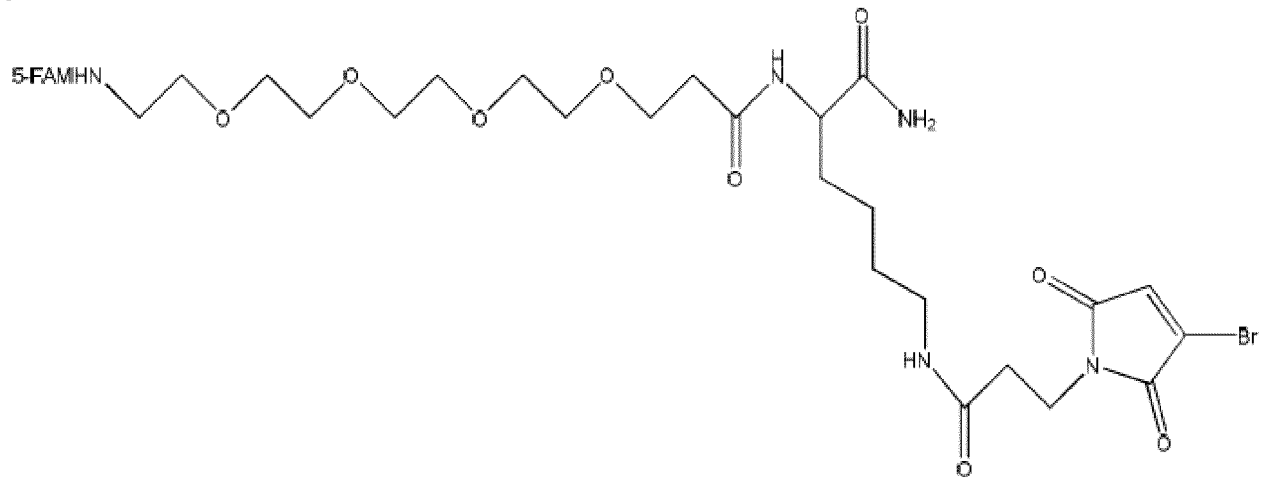
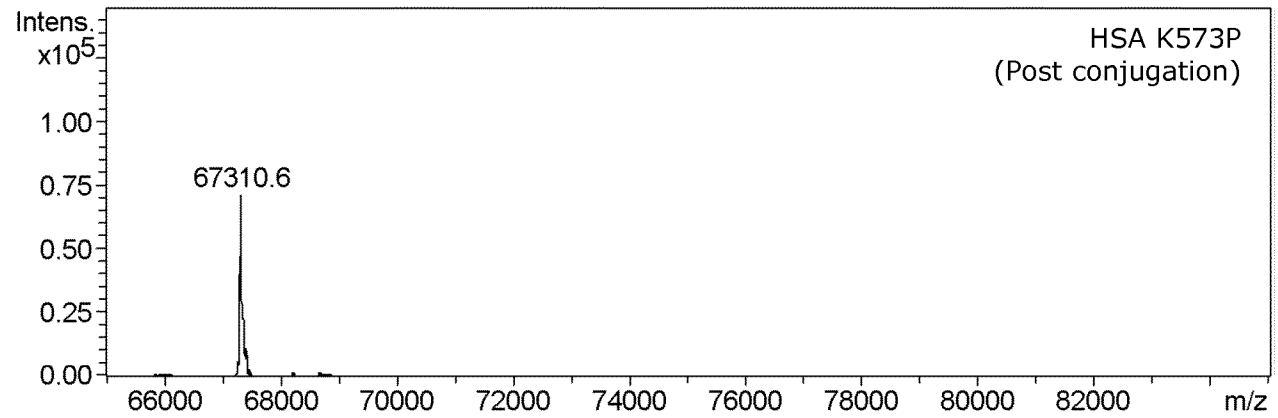

Intens.
x10^4
1.0
0.8
0.6
0.4
0.2
0.0
69535.8
81946.0
HSA K93C + E294C + K573P
(Post conjugation)
66000
68000
70000
72000
74000
76000
78000
80000
82000
m/z

**Figure 10**

**A**




**B**




**C**

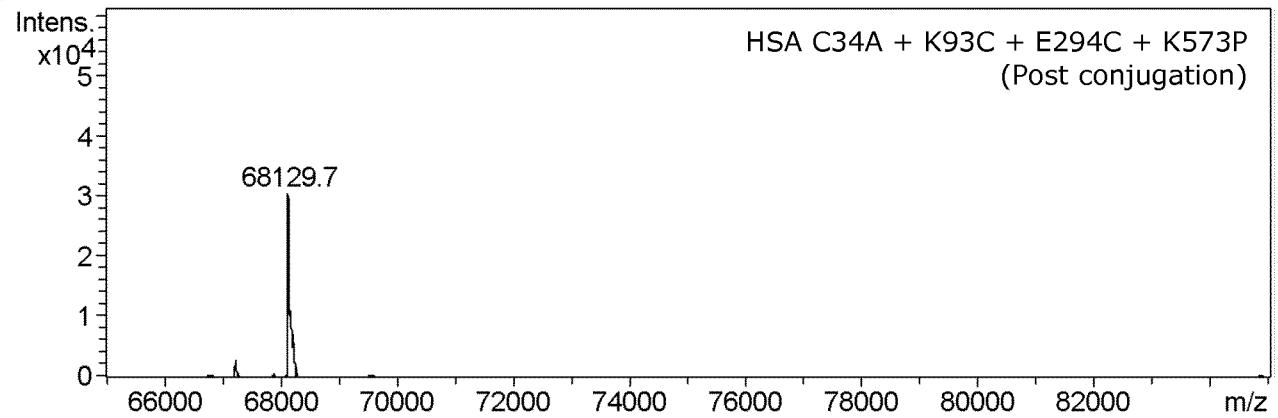
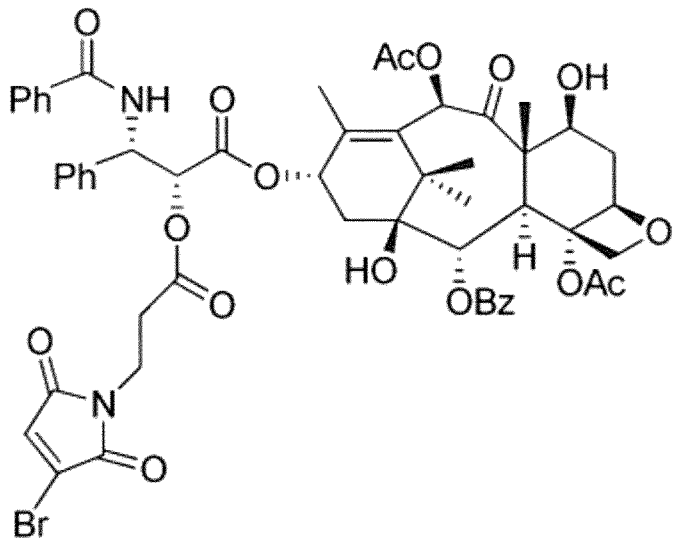
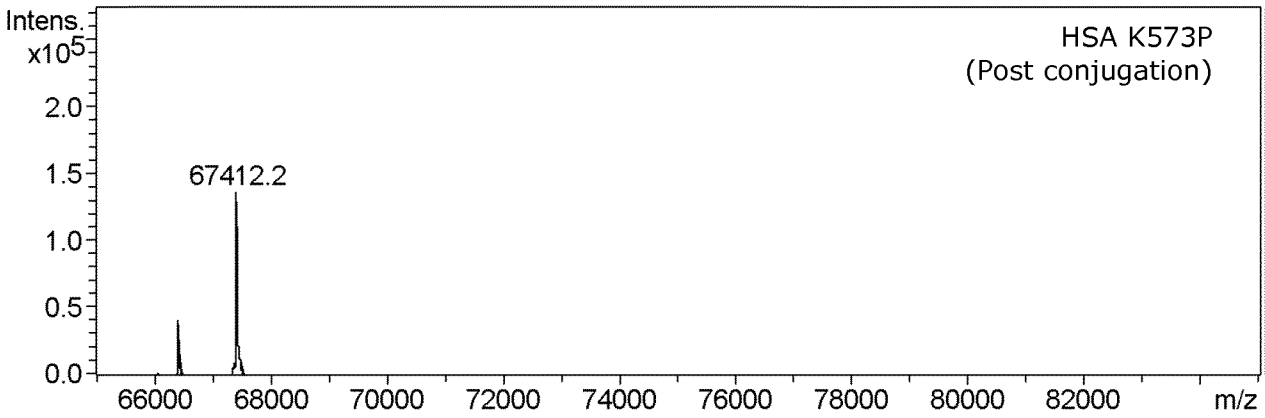


**Figure 11**

**A**




**B**




**C**

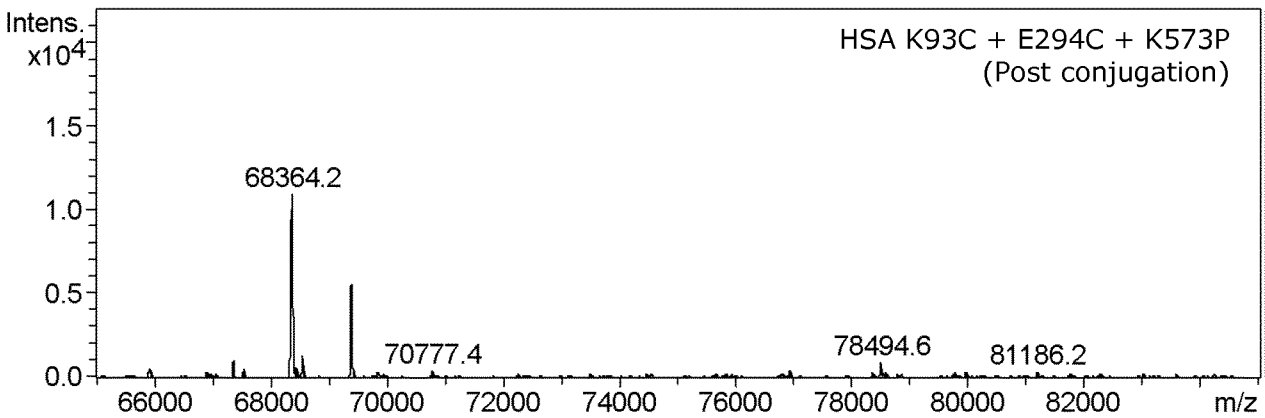
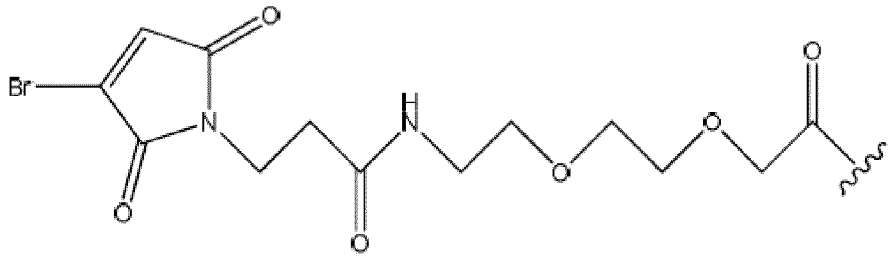


**Figure 12**

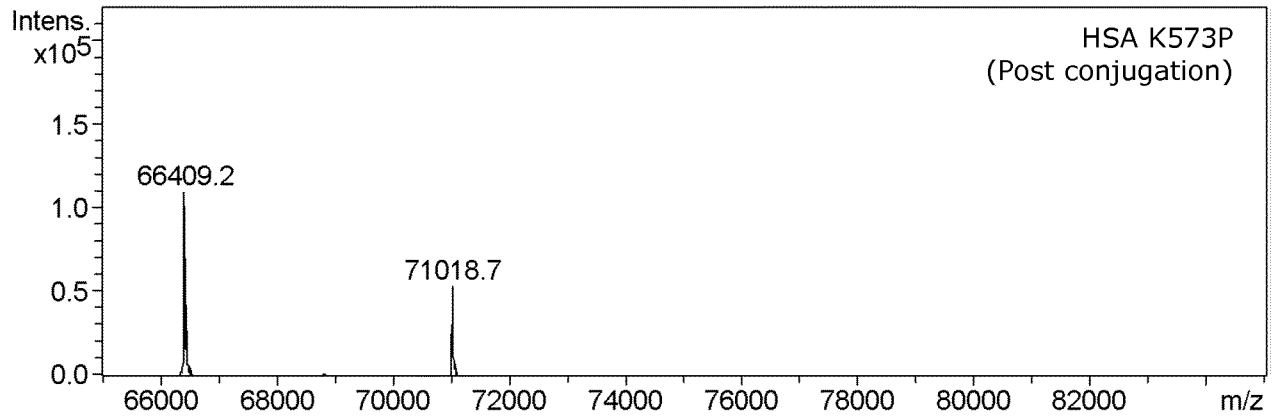
**A**

$NH_2$-HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPSK(Bromomaleimide-PEG2)–$NH_2$

Where bromomaleimide-PEG2 =




**B**




**C**

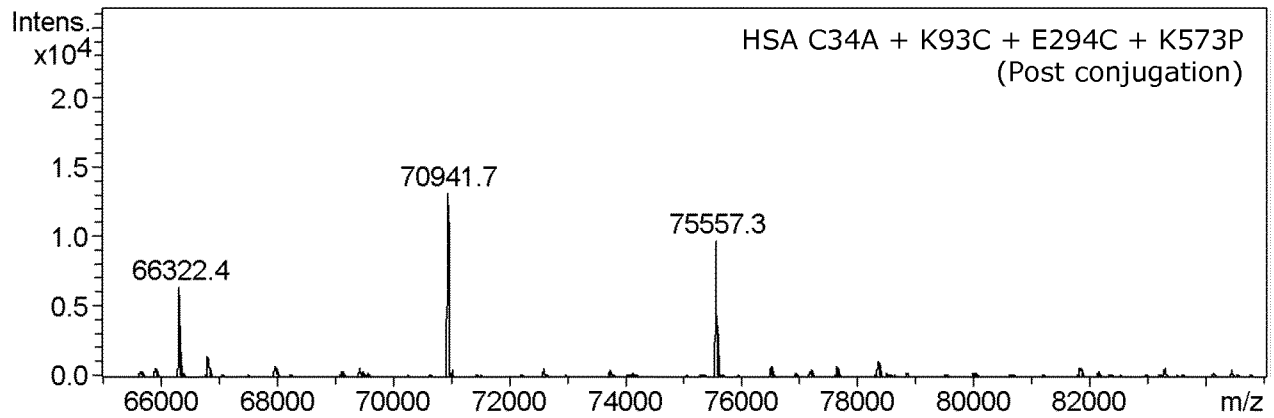
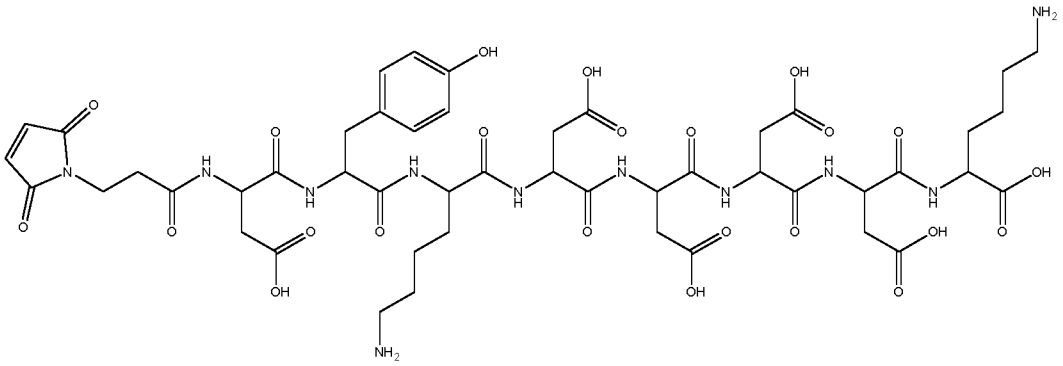
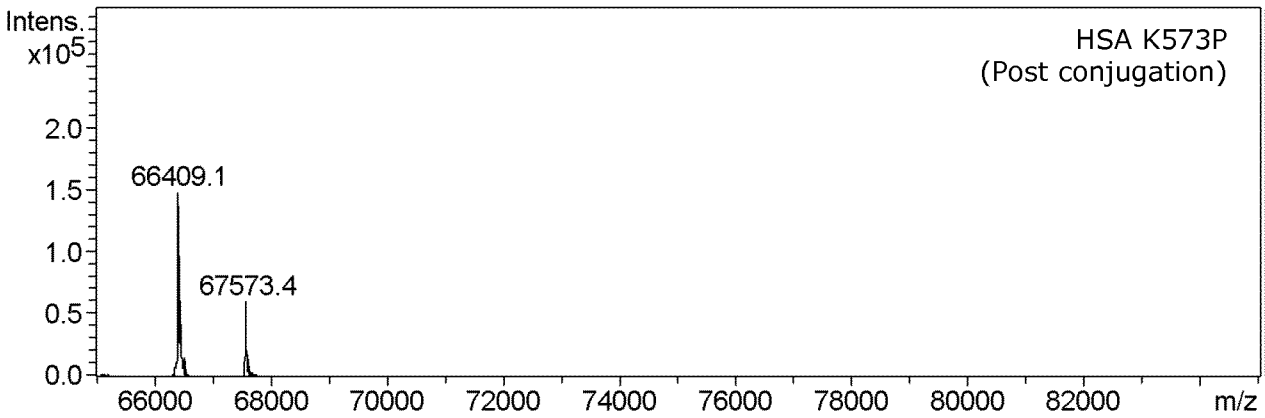


**Figure 13**

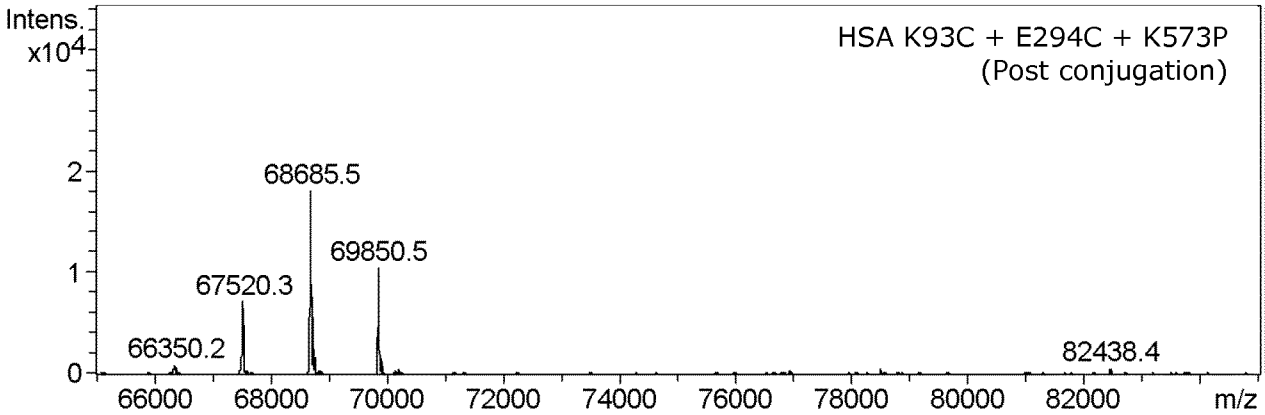
**A**



**B**




**C**



# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/069748

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/765
ADD.

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2010/092135 A2 (NOVOZYMES BIOPHARMA UK LTD [GB]; NOVOZYMES AS [DK]; FINNIS CHRISTOPHER) 19 August 2010 (2010-08-19) cited in the application | 1-5,7-51 |
| A | page 17, line 8 - page 18, line 4 | 6 |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 October 2016 | 24/10/2016 |

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

1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/069748

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
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| | | EP 2396347 A2 | 21-12-2011 |
| | | JP 5936112 B2 | 15-06-2016 |
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