Supplementary Figures and Tables

A new class of recombinant human albumin with multiple surface thiols exhibit stable conjugation, and enhanced FcRn binding and blood circulatory half-life

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Running title: *Engineered albumin with enhanced drug-carrier potential*

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Supplementary Table 1. List of primers used in the generation of single-thiol variants. All variants are with a C34A background.

Variant	Forward Primer 5'- 3'	Reverse Primer 5'- 3'
L24C	GAAGAAAACTTCAAGGCTTTGGTCTGTATCGCTTTC GCTCAATACTTGCA	GACCAAAGCCTTGAAGTTTTCTTCACCCAAGTCCT
F49C	AGTTGGTCAACGAAGTTACCGAATGTGCTAAGACTT GTGTTGCTGACG	TTCGGTAACTTCGTTGACCAACTTGACGTGATCTT
V54C	GTTACCGAATTCGCTAAGACTTGTTGTGCTGACGAA TCCGCGGAAAAC	ACAAGTCTTAGCGAATTCGGTAACTTCGTTGACCAA
D56C	GAATTCGCTAAGACTTGTGTTGCTTGTGAATCCGCG GAAAACTGTGACA	AGCAACACAAGTCTTAGCGAATTCGGTAACTTCGTT
L66C	CGCGGAAAACTGTGACAAGTCCTGTCACACCTTGTT CGGTGATAAGTT	GGACTTGTCACAGTTTTCCGCGGATTCGTCAGC
A92C	CGGTGAAATGGCTGACTGTTGTTGTAAGCAAGAACC AGAAAGAAACGAA	ACAACAGTCAGCCATTTCACCGTAGGTTTCTCTC
K93C	GTGAAATGGCTGACTGTTGTGCTTGTCAAGAACCAG AAAGAAACGAATGT	AGCACAACAGTCAGCCATTTCACCGTAGGTTTCTC
Q94C	AAATGGCTGACTGTTGTGCTAAGTGTGAACCAGAAA GAAACGAATGTTTC	CTTAGCACAACAGTCAGCCATTTCACCGTAGGTT
E97C	ACTGTTGTGCTAAGCAAGAACCATGTAGAAACGAAT GTTTCTTGCAACAC	TGGTTCTTGCTTAGCACAACAGTCAGCCATTTCAC
H128C	TTGACGTCATGTGTACTGCTTTCTGTGACAACGAAG AAACCTTCTTGAAG	GAAAGCAGTACACATGACGTCAACTTCTGGTCTAA
F156C	ACTTCTACGCTCCAGAATTGTTGTGTTTCGCTAAGA GATACAAGGCTGC	CAACAATTCTGGAGCGTAGAAGTATGGGTGTCTTC
A226C	AGATTGTCTCAAAGATTCCCAAAGTGTGAATTCGCT GAAGTTTCTAAGTTG	CTTTGGGAATCTTTGAGACAATCTAGCGACAGCC
E227C	TGTCTCAAAGATTCCCAAAGGCTTGTTTCGCTGAAG TTTCTAAGTTGGTT	AGCCTTTGGGAATCTTTGAGACAATCTAGCGACAG
E230C	GATTCCCAAAGGCTGAATTCGCTTGTGTTTCTAAGTT GGTTACTGACTTG	AGCGAATTCAGCCTTTGGGAATCTTTGAGACAATCT
D237C	GCTGAAGTTTCTAAGTTGGTTACTTGTTTGACTAAG GTTCACACTGAATGT	AGTAACCAACTTAGAAACTTCAGCGAATTCAGCCTT
K240C	TCTAAGTTGGTTACTGACTTGACTTGTGTTCACACTG AATGTTGTCACGG	AGTCAAGTCAGTAACCAACTTAGAAACTTCAGCGAA
D259C	GGAATGTGCTGATGACAGAGCTTGTTTGGCTAAGTA CATCTGTGAAAAC	AGCTCTGTCATCAGCACATTCCAACAAGTCACCG
K262C	TGATGACAGAGCTGACTTGGCTTGTTACATCTGTGA AAACCAAGACTCT	AGCCAAGTCAGCTCTGTCATCAGCACATTCCAAC
N267C	GACTTGGCTAAGTACATCTGTGAATGTCAAGACTCT ATCTCTTCCAAGTTG	TTCACAGATGTACTTAGCCAAGTCAGCTCTGTCATC
Q268C	TTGGCTAAGTACATCTGTGAAAACTGTGACTCTATC TCTTCCAAGTTGAAG	GTTTTCACAGATGTACTTAGCCAAGTCAGCTCTGT
I271C	TACATCTGTGAAAACCAAGACTCTTGTTCTTCCAAG TTGAAGGAATGTTGT	AGAGTCTTGGTTTTCACAGATGTACTTAGCCAAGTC
L275C	ACCAAGACTCTATCTCTTCCAAGTGTAAGGAATGTT	CTTGGAAGAGATAGAGTCTTGGTTTTCACAGATGTA

	GTGAAAAGCCATTG	
E277C	GACTCTATCTCTTCCAAGTTGAAGTGTTGTTGTGAAAAGCCATTGTTGGAA	CTTCAACTTGGAAGAGATAGAGTCTTGGTTTTCACAG
L284C	AAGGAATGTTGTGAAAAGCCATTGTGTGAAAAGTCTCACTGTATTGCTGAA	CAATGGCTTTTCACAACATTCCTTCAACTTGGAAGA
E294C	AAGTCTCACTGTATTGCTGAAGTTTGTAACGATGAAATGCCAGCTGACTT	AACTTCAGCAATACAGTGAGACTTTTCCAACAATGG
E311C	CATCTTTGGCTGCTGACTTCGTTTGTTCTAAGGACGTTTGTAAGAACTAC	AACGAAGTCAGCAGCCAAAGATGGCAAGTCAGCT
K317C	GACGTTTGTAAGAACTACGCTGAATGTAAGGACGTCTTCTTGGGTATGTT	ACAAACGTCCTTAGATTCAACGAAGTCAGCAGCC
A322C	GTCTTCTTGGGTATGTTCTTGTACTGTTACGCTAGAAGACACCCAGACT	TTCAGCGTAGTTCTTACAAACGTCCTTAGATTCAACG
E333C	CGAATACGCTAGAAGACACCCATGTTACTCCGTTGTCTTGTTGTTGAG	GTACAAGAACATACCCAAGAAGACGTCCTTAGCTTC
D340C	TGTTGAGATTGGCTAAGACCTACTGTACTACCCTCGAGAAGTGTTGTG	TGGGTGTCTTCTAGCGTATTCGTACAAGAACATAC
E354C	GACCTACGAAACTACCCTCGAGTGTTGTTGTGCTGCTGCTGACCCA	GTAGGTCTTAGCCAATCTCAACAACAAGACAACGG
E358C	AAACTACCCTCGAGAAGTGTTGTTGTGCTGCTGACCCACACGAATGT	GAGGGTAGTTTCGTAGGTCTTAGCCAATCTCAACA
K359C	TCGATGAATTCAAGCCATTGGTCTGTGAACCACAAAACTTGATCAAGCAA	CTCGAGGGTAGTTTCGTAGGTCTTAGCCAATCTC
A362C	GCAAAACTGTGAATTGTTCGAACAATGTGGTGAATACAAGTTCCAAAACGC	ACAACACTTCTCGAGGGTAGTTTCGTAGGTCTTAG
E382C	GTTACCGAATTCGCTAAGACTTGTTGTGCTGACGAATCCGCGGAAAAC	GACCAATGGCTTGAATTCATCGAAAACCTTAGCGT
L398C	GAATTCGCTAAGACTTGTGTTGCTTGTGAATCCGCGGAAAACTGTGACA	TTGTTCGAACAATTCACAGTTTTGCTTGATCAAGTTTTG

Supplementary Table 2. Free thiol profile detection by DTNB addition (+197±15 Da increase in mass) of the new mutations in the thiol variants. Predicted molecular mass post DTNB treatment for conjugation efficiency measurements using MS spectra. Difference shows actual measured mass minus theoretical mass (Da). All variants are with a C34A background.

Variant	Theoretical Mass	Mass After DTNB Treatment		
		Theoretical	Actual Measured	Difference
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
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

Variant	Theoretical Mass	Mass After DTNB Treatment		
		Theoretical	Actual Measured	Difference
E382C	66381	66578	66586	8
L398C	66397	66594	66597	3

Supplementary Table 3. GP-HPLC aggregation detection of the monomer stability of rHSA thiol variants over 6 months at 2-8°C. Bracketed values relative to WT rHSA control. ND: not determined.

Sample	**GPHPLC Conc. (mg/mL)**	**% Monomer**			**Δ% Monomer**	
		T=0	**T=7 week**	**T=6 month**	**0-7 week**	**0-6 months**
WT rHSA	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

Supplementary Table 4. UHPLC aggregation detection of the monomer stability of rHSA thiol variants over 4 months at 2-8°C. Bracketed values relative to WT rHSA control.

Sample	**UHPLC conc. (mg/mL)**	**% Monomer**			**Δ% Monomer**	
		T=0	**T=8 week**	**T=4 month**	**0-8 week**	**0-4 months**
WT rHSA	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

Sample	**UHPLC conc. (mg/mL)**	**% Monomer**			**Δ% Monomer**	
		T=0	**T=8 week**	**T=4 month**	**0-8 week**	**0-4 months**
C34A+L398C	0.7	90 (105)	92 (105)	87 (100)	2	-3

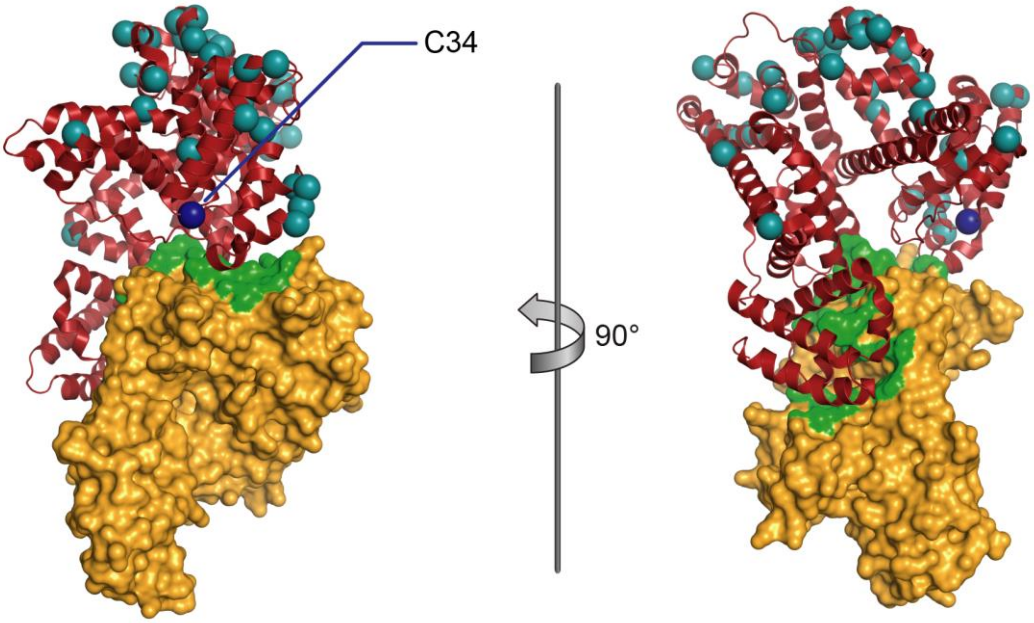
Supplementary Table 5. Conjugation efficiency of maleimide-PEG2-biotin (+525Da) to rHSA thiol variants presented as percentage of the conjugation measured by the weight increase by mass spectrometry.

Sample Description	Reference MW Unconjugated (Da)	Theoretical Conjugate mass (Da)	Conjugate Intact Mass (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+K93C	66382	66907	66908	100
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+A226C	66439	66964	66440	0
C34A+E227C	66381	66906	66907	95
C34A+E230C	66381	66906	66908	72
C34A+D237C	66395	66920	66921	73
C34A+K240C	66382	66907	66908	100
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+I271C	66397	66922	66924	72
C34A+L275C	66397	66922	66897	0
C34A+E277C	66381	66906	66908	90
C34A+L284C	66397	66922	67427	0
C34A+E294C	66381	66906	66909	>95
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+E358C	66381	66906	66909	100
C34A+K359C	66382	66907	66908	95
C34A+A362C	66439	66964	66966	94

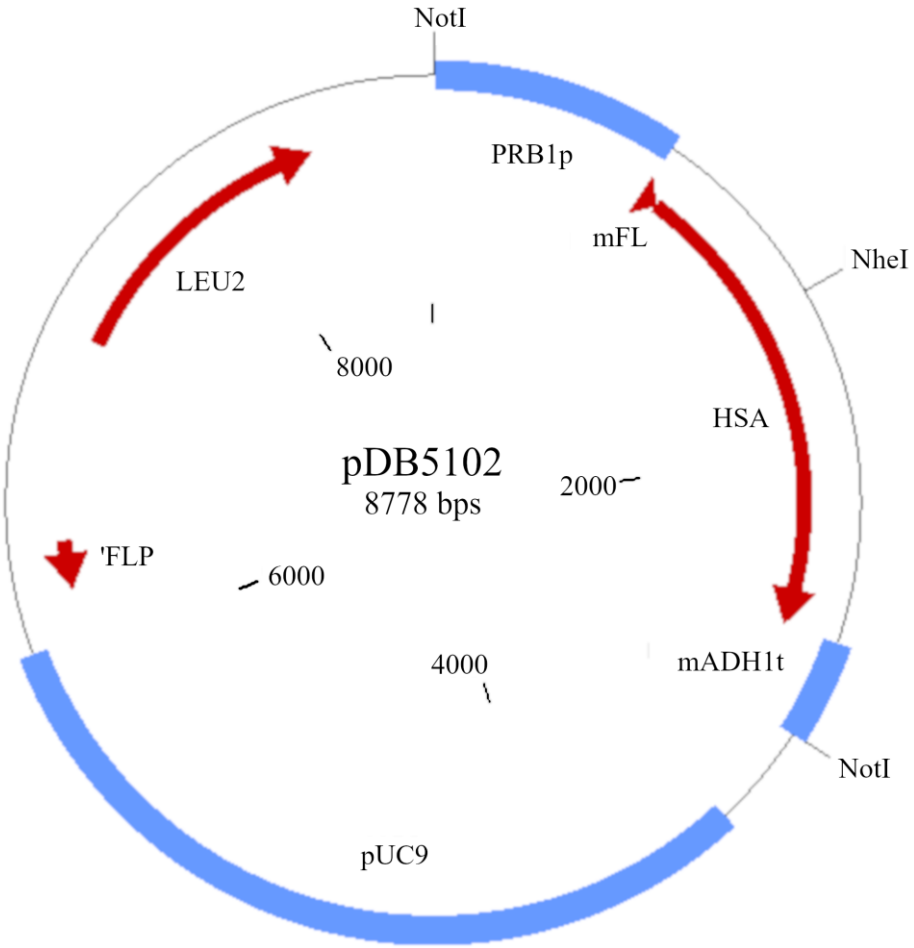
Sample Description	Reference MW Unconjugated (Da)	Theoretical Conjugate mass (Da)	Conjugate Intact Mass (Da)	% Conjugation
C34A+E382C	66381	66906	66909	83
C34A+L398C	66397	66922	66925	36

Supplementary Table 6. Average K_D *(µM) of single-thiol rHSA variants for binding to mouse FcRn or human FcRn at pH 5.5 or 7.0. Biolayer inteferometric measurements were done in triplicates using the Octet RED96 system (PALL/Fortebio). NB: No binding, PF: poor fit/low binding.*

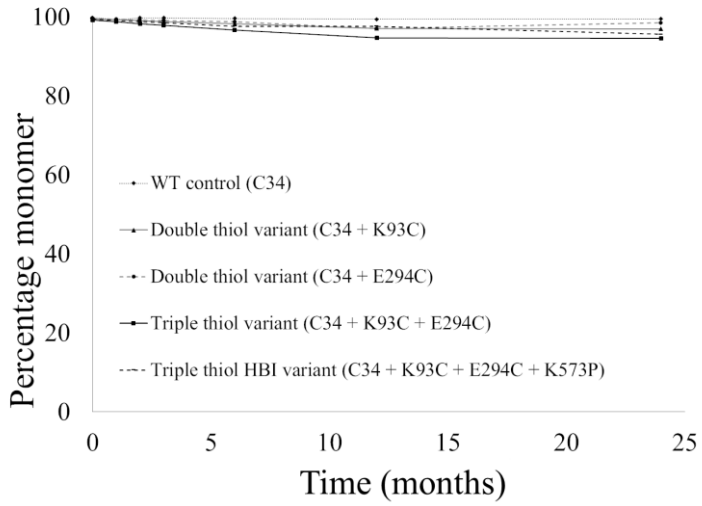
Albumin	**mFcRn**		**hFcRn**	
	pH 5.5 (µM)	**pH 7.0 (µM)**	**pH 5.5 (µM)**	**pH 7.0 (µM)**
WT	9.369	NB	0.443	PF
HBI	1.045	NB	0.027	2616
HBII	0.587	NB	0.008	446.4



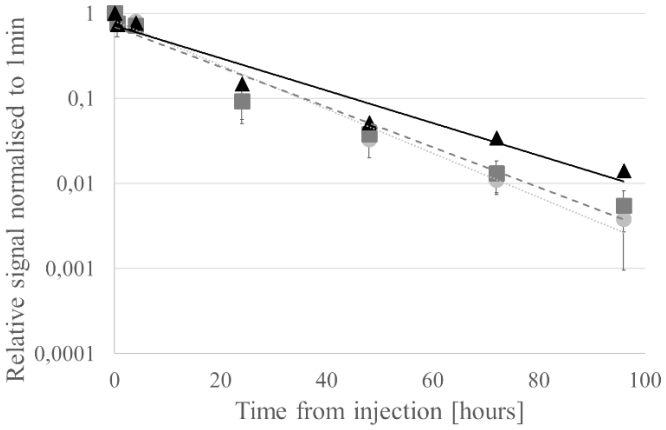
Supplementary Figure 1. Crystal structure of human serum albumin in complex with the FcRn receptor. The beta-2-microglobulin FcRn/complex is shown in gold and human serum albumin in red. The location of the natural occurring cysteine (Cys34) in dark blue and the positions were cysteines with reactive thiol groups have been inserted (detailed in supplementary table 2) are shown in teal. Interface residues within a distance of 5 Å from albumin are marked in green on the surface representation of FcRn receptor. Modified from pdb 4n0f.



Supplementary Figure 2. Plasmid map of pDB5102. The map shows the main features of the DNA used to construct the albumin expression cassettes for the variant proteins.



Supplementary Figure 3. Stability of thiol rHSA variants during long term storage at 5 °C, maintaining >95% monomer over 24 months.



Supplementary Figure 4. Relative fluorescent signal in serum samples 1 min to 96 hours (entire period) after injection of triple-thiol rHSA variants bearing 3xAF680 (● NB, ■ WT, ▲ HBII, n=7) with fitted exponential curves.