

Title: Molecularly Engineered Nanobodies for Tunable Pharmacokinetics and Drug Delivery

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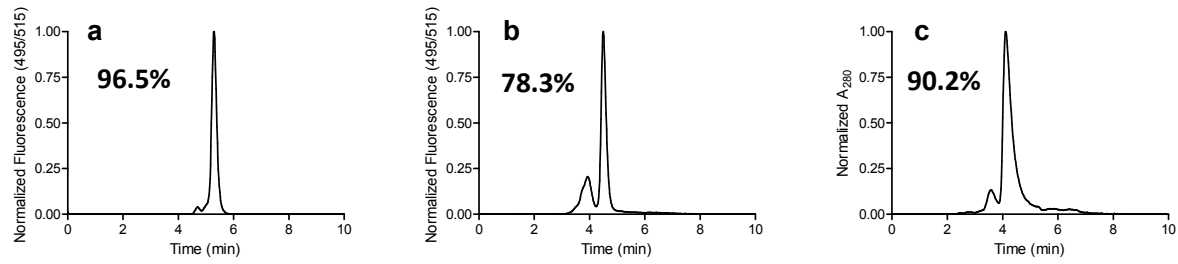


Figure S1: Size exclusion HPLC trace for fluorescently labeled (a) VCAMElid, (b) BiVCAMElid, and (c) VCAM/ALB8. Values represent % monomer content. All proteins were purified to >90% purity prior to further use.

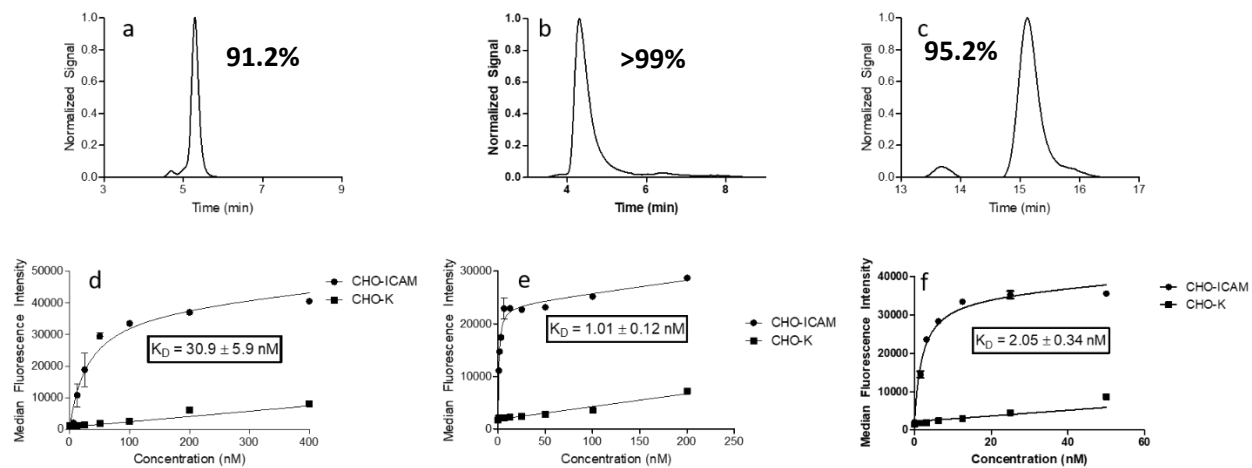


Figure S2: In vitro characterization of untargeted control nanobody and engineered variants. Size exclusion HPLC trace for (a) monovalent, (b) bivalent, and (c) albumin-binding control nanobodies. Binding of control nanobodies to cells transfected to express human intercellular adhesion molecule-1: (d) monovalent, (e) bivalent, (f) albumin-binding.

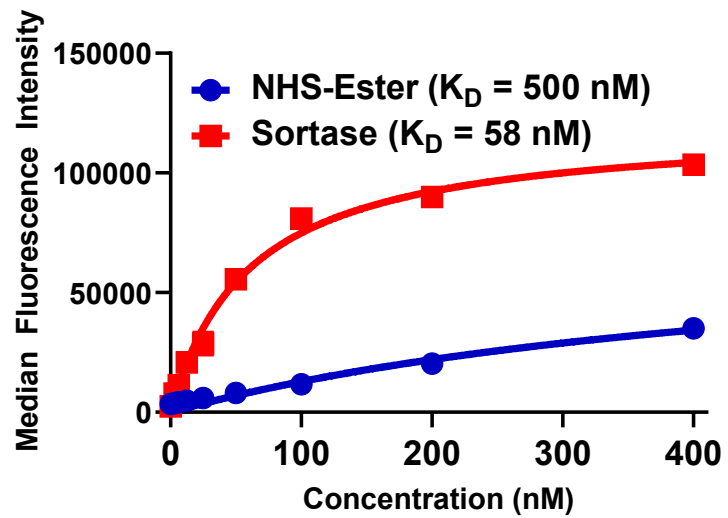


Figure S3: Comparative binding of VCAMelid to mVCAM-1 following labeling at the C-terminus and on free amines.

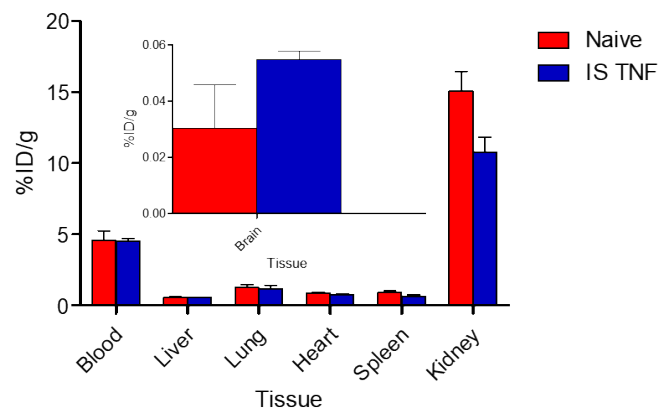


Figure S4: In vivo behavior of ^{125}I -labeled SOD-azide in naïve and TNF- α -injured mice. (a) Whole body biodistribution of SOD and (b) brain uptake of SOD.

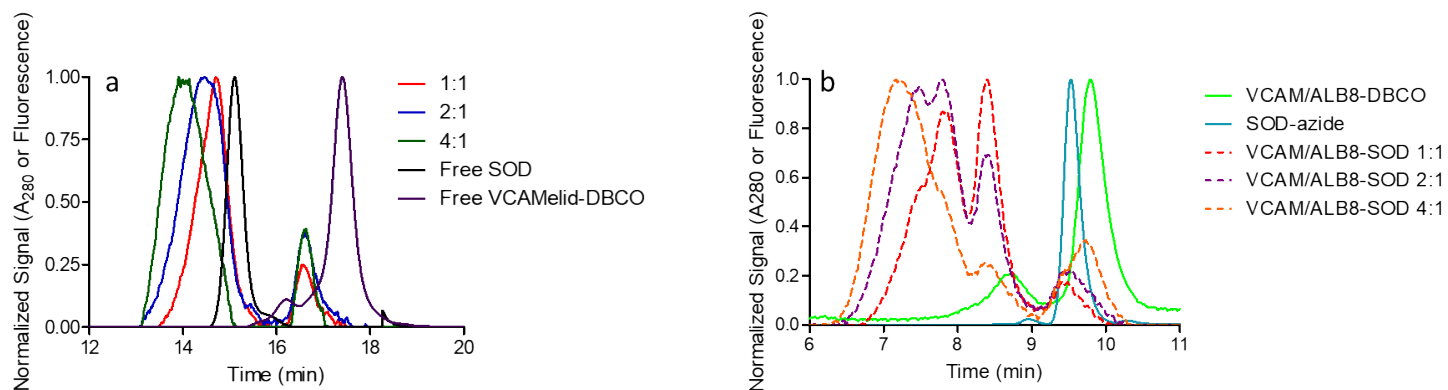


Figure S5: Size exclusion HPLC trace for (a) VCAMelid-SOD conjugates at a variety of reaction ratios and (b) VCAM/ALB8-SOD conjugates. The fraction of VCAMelid conjugated to SOD was 86.3% (1:1), 86.6% (2:1), and 88.0% (4:1). The fraction of VCAM/ALB8 conjugated to SOD was 90.9% (1:1), 89.8% (2:1), and 84.0% (4:1).

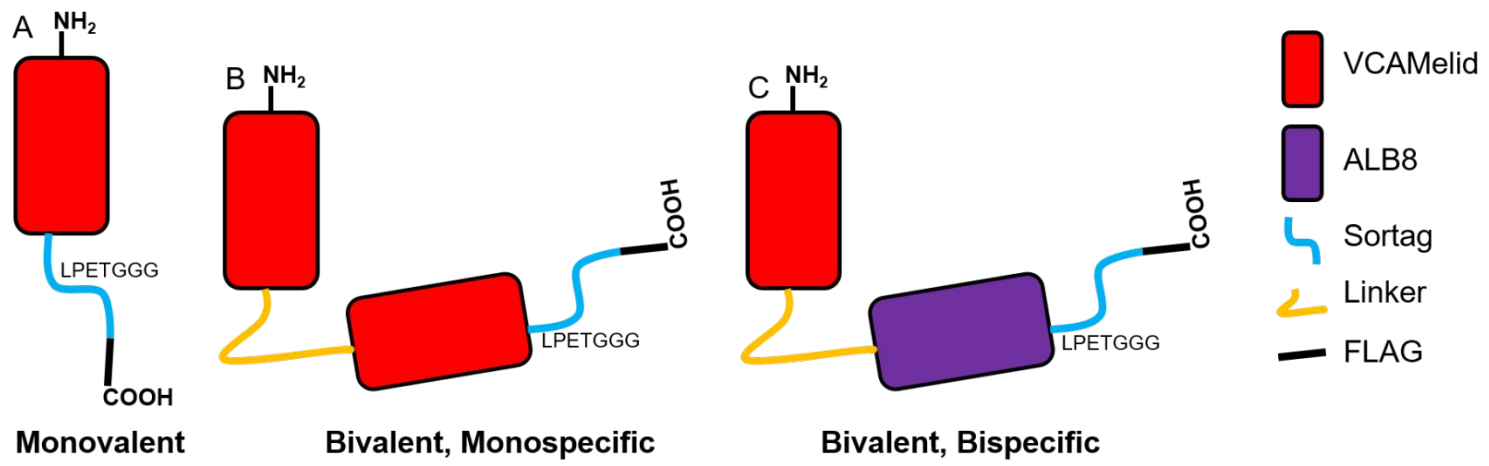


Figure S6: Nanobody constructs used in this study. A: Monovalent, B: Bivalent, C: Bispecific

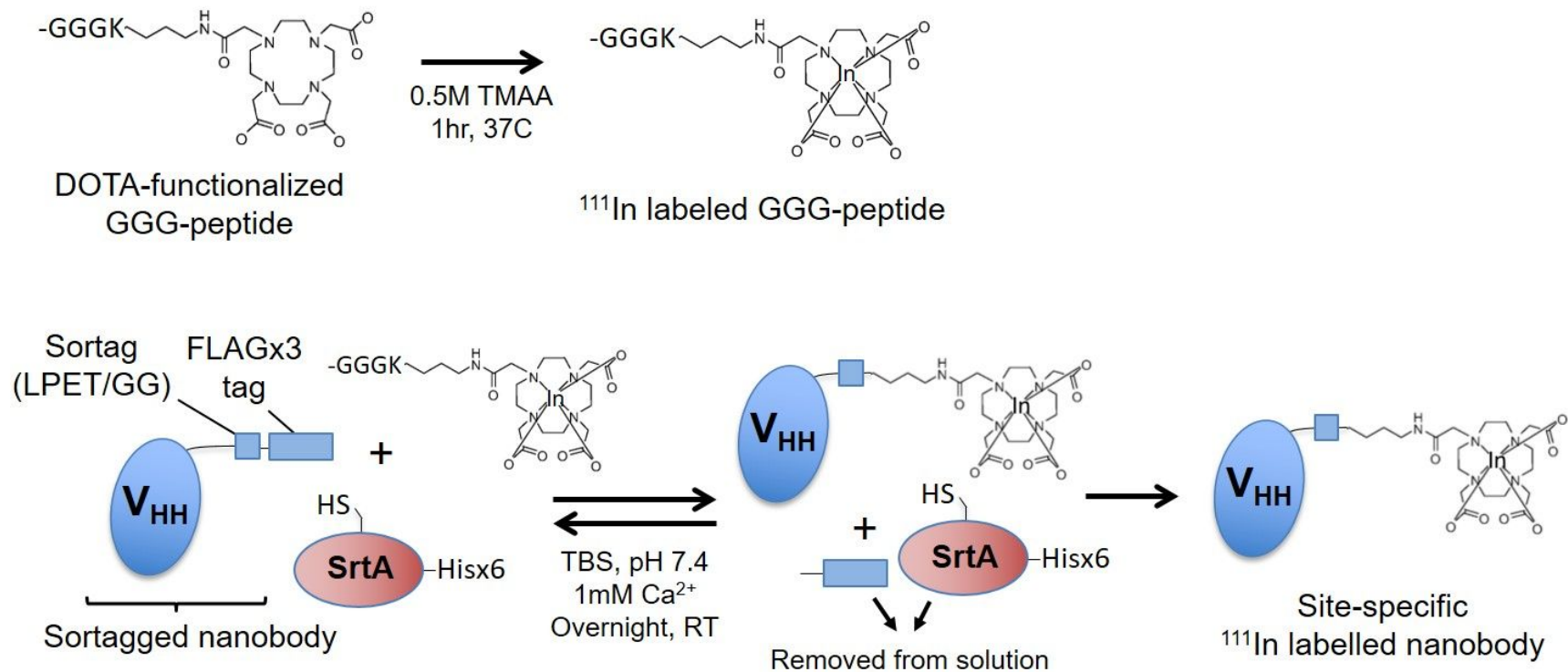


Figure S7: Reaction scheme for Sortase A-based radiolabeling of nanobodies (Nb). DOTA-functionalized GGG-peptide was labeled with ^{111}In by incubation in metal-free, 0.5M TMAA, pH 4.5, for 1 hour at 37°C. Nb (40 μM), Sortase A (40 μM), and radiolabeled peptide (100 μM) were incubated in Tris-buffered saline (TBS), pH 7.4, containing 1 mM CaCl_2 overnight at room temperature. Post-reaction, Sortase A was removed from solution using Ni-NTA agarose beads and unreacted peptide was removed via desalting.

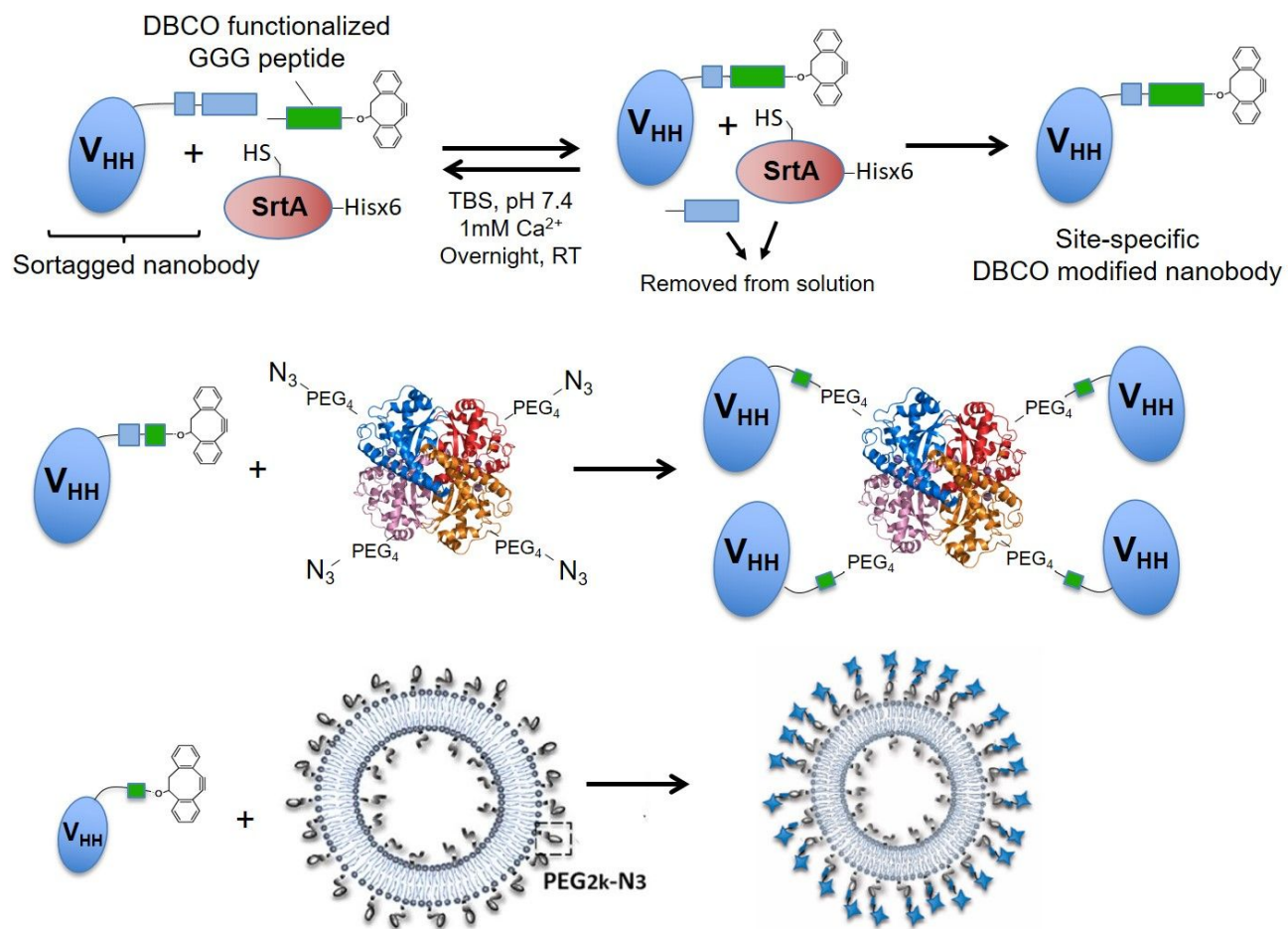


Figure S8: Reaction scheme for conjugation of nanobody (Nb). Upper panel: Nb are functionalized with DBCO using Sortase A (SrtA)-mediated transpeptidation. Middle panel: Conjugation of DBCO-modified Nb to azide-modified SOD-1 using strain-promoted alkyne-azide cycloaddition (SPAAC). Lower panel: Conjugation of DBCO-modified Nb to azide-containing liposomes using SPAAC.

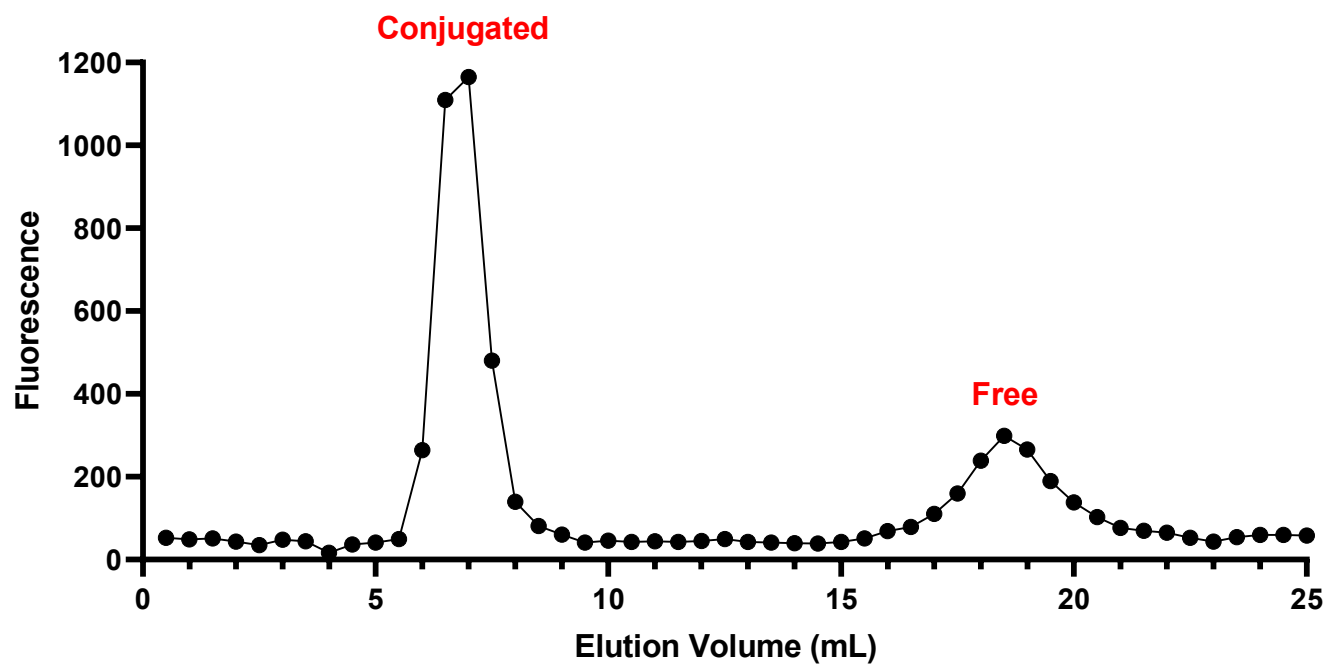


Figure S9: Efficiency of conjugation of VCAMelid-DBCO to azide liposomes. Fluorescence was monitored at each 0.5 mL elution fraction collected from the Sepharaose CL-4B column. From AUC analysis of the elution profile, 65.6% of added nanobodies were conjugated to the liposomes.

Table S1: Tissue pharmacokinetics of VCAMelid, BiVCAMelid, and VCAM/ALB8. Values are displayed as mean (SEM).

Tissue	Molecule	20 minutes	2 hours	6 hours	24 hours
Blood	VCAMelid	2.26 (0.11)	1.06 (0.07)	0.139 (0.019)	
	BiVCAMelid	4.98 (0.42)	3.24 (0.20)	0.614 (0.039)	
	VCAM/ALB8	32.9 (0.5)	22.5 (1.5)	14.5 (1.0)	5.15 (0.15)
Lung	VCAMelid	2.79 (0.20)	2.92 (0.28)	1.38 (0.14)	
	BiVCAMelid	3.80 (0.34)	4.56 (2.47)	2.34 (0.10)	
	VCAM/ALB8	6.21 (0.59)	5.54 (0.13)	7.10 (0.59)	6.26 (0.40)
Liver	VCAMelid	2.29 (0.22)	2.56 (0.69)	1.46 (0.12)	
	BiVCAMelid	4.88 (0.69)	2.73 (0.60)	3.34 (0.36)	
	VCAM/ALB8	13.9 (1.6)	12.5 (1.8)	18.2 (1.6)	12.5 (1.1)
Heart	VCAMelid	1.50 (0.08)	0.818 (0.196)	0.308 (0.115)	
	BiVCAMelid	2.95 (0.34)	0.839 (0.111)	0.847 (0.152)	
	VCAM/ALB8	4.19 (0.08)	3.88 (0.02)	4.54 (0.54)	2.74 (0.03)
Kidney	VCAMelid	155 (12)	214 (13)	195 (53)	
	BiVCAMelid	163 (24)	206 (14)	260 (19)	
	VCAM/ALB8	32.5 (0.8)	34.8 (1.8)	48.4 (10.0)	41.9 (2.4)
Spleen	VCAMelid	25.0 (0.9)	15.4 (1.7)	12.2 (1.7)	
	BiVCAMelid	48.0 (5.5)	16.5 (1.0)	17.5 (4.4)	
	VCAM/ALB8	90.5 (8.6)	101 (2)	79.4 (8.1)	115 (6)
Brain	VCAMelid	0.781 (0.106)	0.467 (0.116)	0.260 (0.056)	
	BiVCAMelid	1.56 (0.22)	0.595 (0.248)	0.168 (0.062)	
	VCAM/ALB8	2.90 (0.18)	2.06 (0.16)	1.62 (0.15)	1.43 (0.14)

Table S2: Whole-body biodistribution of VCAMelid-SOD and VCAM/ALB8-SOD. Values are displayed as mean (SEM).

Tissue	Condition	SOD	Control-SOD	VCAMelid-SOD	Control/ALB8-SOD	VCAM/ALB8-SOD
Blood	Naïve	4.61 (0.63)	7.57 (0.30)	4.12 (0.13)	38.1 (1.3)	31.4 (2.9)
	Injured	4.54 (0.17)	12.2 (0.3)	4.36 (0.14)	44.3 (1.4)	24.3 (0.9)
Lung	Naïve	1.28 (0.16)	2.60 (0.67)	2.82 (0.06)	3.77 (1.14)	4.40 (1.08)
	Injured	1.19 (0.23)	1.94 (0.46)	2.79 (0.30)	5.41 (3.80)	6.53 (0.45)
Liver	Naïve	0.586 (0.040)	19.1 (0.8)	17.8 (0.6)	7.81 (0.27)	6.30 (0.38)
	Injured	0.565 (0.033)	21.7 (1.1)	14.8 (0.9)	10.2 (0.1)	11.3 (0.8)
Heart	Naïve	0.895 (0.054)	1.44 (0.16)	1.41 (0.11)	2.11 (0.25)	2.31 (0.30)
	Injured	0.764 (0.070)	1.42 (0.05)	1.55 (0.07)	1.50 (0.14)	1.98 (0.07)
Kidney	Naïve	15.1 (1.4)	34.1 (1.6)	27.3 (0.8)	3.56 (0.30)	8.98 (0.84)
	Injured	10.8 (1.1)	35.8 (3.3)	24.1 (1.2)	6.63 (0.56)	8.89 (0.29)
Spleen	Naïve	0.919 (0.136)	11.4 (0.5)	31.1 (2.1)	5.00 (0.46)	40.3 (2.2)
	Injured	0.660 (0.088)	10.5 (0.5)	28.1 (1.5)	7.75 (0.04)	44.1 (2.5)
Brain	Naïve	0.030 (0.016)	0.071 (0.004)	0.328 (0.029)	0.126 (0.024)	0.341 (0.005)
	Injured	0.055 (0.003)	0.102 (0.005)	1.28 (0.10)	0.166 (0.001)	1.90 (0.07)
Thyroid (%ID)	Naïve	0.422 (0.009)	2.94 (0.29)	2.29 (0.25)	0.255 (0.037)	0.178 (0.016)
	Injured	0.348 (0.039)	2.73 (0.71)	2.44 (0.35)	0.135 (0.041)	0.226 (0.040)

Table S3: Whole-body biodistribution of VCAMElid and VCAM/ALB8-targeted liposomes. Values are displayed as mean (SEM).

Tissue	Condition	Untargeted	VCAM	VCAM/ALB8
Blood	Naïve	27.7 (0.9)	15.8 (5.6)	19.8 (2.6)
	Injured	17.7 (3.4)	15.7 (5.2)	19.2 (3.2)
Lung	Naïve	12.1 (1.1)	3.27 (0.30)	13.0 (2.1)
	Injured	10.5 (1.2)	4.24 (1.29)	16.7 (1.3)
Liver	Naïve	25.4 (2.6)	17.6 (3.1)	13.3 (2.7)
	Injured	31.5 (3.0)	30.3 (8.0)	26.3 (1.0)
Heart	Naïve	1.50 (0.15)	0.678 (0.196)	0.996 (0.138)
	Injured	1.15 (0.32)	1.19 (0.34)	1.52 (0.20)
Kidney	Naïve	3.45 (0.30)	1.86 (0.48)	2.57 (0.33)
	Injured	2.39 (0.15)	3.05 (0.89)	4.14 (0.39)
Spleen	Naïve	94.3 (13.9)	104 (16)	109 (17)
	Injured	63.3 (2.9)	141 (34)	108 (9)
Brain	Naïve	0.264 (0.007)	0.132 (0.011)	0.278 (0.026)
	Injured	0.427 (0.035)	1.04 (0.25)	1.12 (0.23)

Table S4: Tissue-to-blood ratios and immunospecificity indexes of VCAMelid.

Tissue	Condition	Tissue/Blood Untargeted	Tissue/Blood VCAMelid	Immunospecificity Index
Lung	Naïve	0.897 (0.100)	1.13 (0.12)	1.26 (0.13)
	IV LPS	1.01 (0.21)	1.35 (0.04)	1.34 (0.04)
	IS TNF- α	2.61 (0.53)	1.23 (0.09)	0.472 (0.034)
Liver	Naïve	0.550 (0.041)	0.422 (0.019)	0.767 (0.035)
	IV LPS	0.568 (0.109)	1.88 (0.12)	3.31 (0.21)
	IS TNF- α	1.12 (0.13)	1.01 (0.10)	0.906 (0.089)
Heart	Naïve	0.262 (0.039)	0.540 (0.042)	2.06 (0.16)
	IV LPS	0.511 (0.059)	0.882 (0.044)	1.73 (0.09)
	IS TNF- α	0.801 (0.274)	0.663 (0.035)	0.828 (0.044)
Kidney	Naïve	37.4 (1.2)	67.7 (1.2)	1.81 (0.03)
	IV LPS	16.6 (1.2)	24.0 (1.0)	1.44 (0.06)
	IS TNF- α	74.8 (14.7)	68.4 (5.2)	0.915 (0.069)
Spleen	Naïve	0.738 (0.078)	10.3 (0.6)	13.9 (0.8)
	IV LPS	0.514 (0.135)	4.25 (0.20)	8.27 (0.38)
	IS TNF- α	1.27 (0.05)	11.0 (0.4)	8.69 (0.33)
Brain	Naïve	0.018 (0.006)	0.071 (0.004)	3.93 (0.22)
	IV LPS	N.M.	N.M.	N.M.
	IS TNF- α	0.066 (0.004)	0.345 (0.047)	5.20 (0.71)

Table S5: Tissue-to-blood ratios and immunospecificity indexes of BiVCAMelid.

Tissue	Condition	Tissue/Blood Untargeted	Tissue/Blood BiVCAMelid	Immunospecificity Index
Lung	Naïve	0.572 (0.065)	1.10 (0.15)	1.93 (0.27)
	IS TNF- α	0.438 (0.052)	0.762 (0.068)	1.74 (0.16)
Liver	Naïve	0.180 (0.022)	1.02 (0.15)	5.64 (0.85)
	IS TNF- α	0.303 (0.021)	0.980 (0.139)	3.23 (0.46)
Heart	Naïve	0.329 (0.028)	0.631 (0.071)	1.92 (0.22)
	IS TNF- α	0.322 (0.014)	0.592 (0.068)	1.84 (0.21)
Kidney	Naïve	53.3 (2.5)	27.9 (1.1)	0.524 (0.020)
	IS TNF- α	59.9 (8.5)	32.7 (4.8)	0.546 (0.081)
Spleen	Naïve	0.537 (0.021)	13.3 (0.6)	24.7 (1.2)
	IS TNF- α	0.605 (0.088)	9.65 (1.11)	15.9 (1.8)
Brain	Naïve	0.058 (0.005)	0.116 (0.008)	2.02 (0.14)
	IS TNF- α	0.083 (0.008)	0.313 (0.045)	3.76 (0.54)

Table S6: Tissue-to-blood ratios and immunospecificity indexes of VCAM/ALB8.

Tissue	Condition	Tissue/Blood Untargeted	Tissue/Blood VCAM/ALB8	Immunospecificity Index
Lung	Naïve	0.052 (0.009)	0.114 (0.025)	2.19 (0.48)
	IS TNF- α	0.052 (0.011)	0.189 (0.018)	3.64 (0.34)
Liver	Naïve	0.028 (0.004)	0.179 (0.012)	6.31 (0.42)
	IS TNF- α	0.063 (0.016)	0.424 (0.048)	6.70 (0.76)
Heart	Naïve	0.070 (0.008)	0.105 (0.002)	1.49 (0.03)
	IS TNF- α	0.038 (0.003)	0.127 (0.002)	3.34 (0.06)
Kidney	Naïve	0.558 (0.044)	0.619 (0.079)	1.11 (0.14)
	IS TNF- α	0.499 (0.030)	0.989 (0.024)	1.98 (0.05)
Spleen	Naïve	0.093 (0.011)	2.36 (0.127)	25.4 (1.4)
	IS TNF- α	0.130 (0.011)	2.76 (0.261)	21.1 (2.0)
Brain	Naïve	0.005 (0.001)	0.016 (0.001)	1.78 (0.12)
	IS TNF- α	0.004 (0.001)	0.088 (0.005)	5.27 (0.75)

Table S7: Tissue-to-blood ratios and immunospecificity indexes of VCAMelid-SOD conjugates.

Tissue	Condition	Tissue/Blood Untargeted	Tissue/Blood VCAMelid-SOD	Immunospecificity Index
Lung	Naïve	0.343 (0.088)	0.685 (0.015)	2.00 (0.04)
	IS TNF- α	0.159 (0.038)	0.640 (0.068)	4.01 (0.43)
Liver	Naïve	2.53 (0.11)	4.31 (0.14)	1.71 (0.05)
	IS TNF- α	1.78 (0.09)	3.39 (0.20)	1.90 (0.11)
Heart	Naïve	0.190 (0.021)	0.342 (0.027)	1.80 (0.14)
	IS TNF- α	0.117 (0.004)	0.355 (0.016)	3.04 (0.14)
Kidney	Naïve	4.50 (0.22)	6.62 (0.20)	1.47 (0.05)
	IS TNF- α	2.94 (0.27)	5.51 (0.28)	1.87 (0.10)
Spleen	Naïve	1.51 (0.06)	7.54 (0.51)	5.01 (0.34)
	IS TNF- α	0.865 (0.039)	6.44 (0.34)	7.45 (0.39)
Brain	Naïve	0.009 (0.001)	0.080 (0.007)	8.45 (0.75)
	IS TNF- α	0.008 (0.001)	0.294 (0.023)	35.1 (2.8)

Table S8: Tissue-to-blood ratios and immunospecificity indexes of VCAM/ALB8-SOD conjugates.

Tissue	Condition	Tissue/Blood Untargeted	Tissue/Blood VCAM/ALB8-SOD	Immunospecificity Index
Lung	Naïve	0.099 (0.030)	0.140 (0.034)	1.42 (0.35)
	IS TNF- α	0.122 (0.086)	0.269 (0.018)	2.20 (0.15)
Liver	Naïve	0.205 (0.007)	0.201 (0.012)	0.978 (0.059)
	IS TNF- α	0.229 (0.001)	0.466 (0.035)	2.04 (0.15)
Heart	Naïve	0.055 (0.007)	0.074 (0.010)	1.33 (0.17)
	IS TNF- α	0.034 (0.003)	0.081 (0.003)	2.41 (0.09)
Kidney	Naïve	0.093 (0.008)	0.286 (0.027)	3.07 (0.29)
	IS TNF- α	0.150 (0.013)	0.366 (0.012)	2.45 (0.08)
Spleen	Naïve	0.131 (0.012)	1.28 (0.07)	9.77 (0.52)
	IS TNF- α	0.175 (0.001)	1.81 (0.11)	10.4 (0.6)
Brain	Naïve	0.003 (0.001)	0.011 (0.001)	3.29 (0.05)
	IS TNF- α	0.004 (0.001)	0.078 (0.003)	20.9 (0.7)

Table S9: Tissue-to blood ratios and immunospecificity indexes of camelid-targeted liposomes.

Tissue	Condition	Tissue/Blood Untargeted Lipos	Tissue/Blood VCAMelid Lipo	Tissue/Blood VCAM/ALB8 Lipos	ISI VCAMelid Lipo	ISI VCAM/ALB8 Lipos
Lung	Naïve	0.436 (0.039)	0.207 (0.019)	0.655 (0.107)	0.475 (0.043)	1.50 (0.25)
	IS TNF- α	0.595 (0.067)	0.270 (0.082)	0.870 (0.068)	0.453 (0.138)	1.46 (0.11)
Liver	Naïve	0.916 (0.092)	1.12 (0.20)	0.672 (0.136)	1.22 (0.21)	0.734 (0.148)
	IS TNF- α	1.79 (0.17)	1.93 (0.51)	1.37 (0.05)	1.08 (0.28)	0.768 (0.029)
Heart	Naïve	0.054 (0.005)	0.043 (0.012)	0.050 (0.007)	0.795 (0.230)	0.931 (0.129)
	IS TNF- α	0.065 (0.018)	0.076 (0.022)	0.079 (0.010)	1.17 (0.33)	1.22 (0.16)
Kidney	Naïve	0.124 (0.011)	0.118 (0.030)	0.130 (0.017)	0.948 (0.242)	1.05 (0.14)
	IS TNF- α	0.135 (0.008)	0.194 (0.056)	0.216 (0.020)	1.44 (0.42)	1.60 (0.15)
Spleen	Naïve	3.40 (0.50)	6.56 (1.03)	5.50 (0.88)	1.93 (0.30)	1.62 (0.26)
	IS TNF- α	3.59 (0.16)	8.97 (2.18)	5.64 (0.49)	2.50 (0.61)	1.57 (0.14)
Brain	Naïve	0.010 (0.001)	0.008 (0.001)	0.014 (0.001)	0.874 (0.071)	1.47 (0.14)
	IS TNF- α	0.024 (0.002)	0.066 (0.016)	0.060 (0.012)	2.75 (0.66)	2.49 (0.50)

Table S10: Size and polydispersity index (PDI) of camelid-targeted liposomes

Liposome	Size (nm)	PDI
Control	151.1	0.036
VCAMelid	158.1	0.104
VCAM/ALB8	156.2	0.087