Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supplementary Information

Siderocalin Fusion Proteins Enable a New $^{86}Y/^{90}Y$ Theranostic Approach

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General

All reagents and chemicals were purchased from commercial providers (Sigma Aldrich, VWR) and used without further purification. All aqueous solutions were prepared with deionized water (Millipore Milli-Q (18 MΩcm^{-1}) water purification system). Yttrium-86 was obtained from Brookhaven National Lab as $^{86}YCl_3$ (0.1 M HCl) through purchase from the National Isotope Development Center that is managed by the U.S. Department of Energy Isotope Program. Yttrium-90 was purchased as $^{90}YCl_3$ (0.04 M HCl) from Eckert & Ziegler.

Fusion Protein Production

For recombinant expression of fusion proteins, cDNAs encoding Trastuzumab Light Chain, Trastuzumab VH-CH1-Scn, (FAB) and Trastuzumab HC-Scn (IgG) were codon optimized for human cells (IDT), synthesized (IDT), and cloned into optimized lentiviral vectors incorporating fluorescent reporter proteins. Transductions and protein expression and purification were carried out as previously described[1]: HEK293 Freestyle cells (Invitrogen #R79007, RRID: CVCL_D6642) were grown in Freestyle 293 Expression media (Gibco #12338018) at 130 rpm, 37° C, 8% CO_2 in vented shake flasks. Cells were transduced, with near 100% efficiencies as judged by reporter fluorescence, at a density of 10^6 cells/mL in 10 mL Freestyle media. Cultures were grown to ~200 mL at which point comparative reduced/non-reduced SDS-PAGE gels were run with 20 uL 10x culture supernatant to determine expression levels and confirm proper folding. Cultures were expanded to 2L and harvested once cell density reached ~3.5 x 10^6 cells/mL. Cells were pelleted and supernatant was 0.65 micron filtered and supplemented with 150 mM NaCl before purification using HiTrap Protein L 1mL, (Cytiva #17547851) via AKTA Pure FPLC System (Cytiva). Filtered supernatant was flown over HiTrap Protein L column at 5mL/min and column was washed with 1xPBS. Protein was eluted with 0.1M Citrate pH 2.8 in to 1mL factions and immediately supplemented with 100uL of 1M Tris pH 8.0. Trastuzumab Fab-Scn and Trastuzumab IgG-Scn were further purified by preparative size-exclusion chromatography via A200 column (GE Health Sciences) in 1x PBS. Final purified proteins were analyzed by size-exclusion chromatography monodispersivity and endotoxin levels were measured with Endosafe Nexgen-PTS instrument (Charles River #PTS150K) via Endosafe LAL cartridges (Charles River) per the manufacturer's protocol. Fusion proteins were stored in 1X PBS at 10℃ until needed.

Construct sequences (*leader peptides in brown*; *Scn sequences in red*):

Trastuzumab heavy chain-Scn:

MDWTWRFLFVVAAATGVQSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR
QAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
SRWGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY
FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS
NTKVDKKVEPPKSCQDSTSDLIPAPPLSKVPLQQNFQDNQFQGKWYVVGLAGNAILRE
DKDPQKMYATIYELKEDKSYNVTSVLFRKKKCDYWIRTFVPGSQPGEFTLGNIKSYPGL
TSYLVRVVSTNYNQHAMVFFKKVSQNREYFKITLYGRTKELTSELKENFIRFSKSLGLP
ENHIVFPVPIDQCIDG

Trastuzumab V_H-C_H1-Scn:

MDWTWRFLFVVAAATGVQSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR
QAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
SRWGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY
FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS
NTKVDKKVEPPKSCQDSTSDLIPAPPLSKVPLQQNFQDNQFQGKWYVVGLAGNAILRE
DKDPQKMYATIYELKEDKSYNVTSVLFRKKKCDYWIRTFVPGSQPGEFTLGNIKSYPGL
TSYLVRVVSTNYNQHAMVFFKKVSQNREYFKITLYGRTKELTSELKENFIRFSKSLGLP
ENHIVFPVPIDQCIDG

Trastuzumab light chain:

MDMRVPAQLLGLLLLWLSGARCDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAW
YQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTT
PPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
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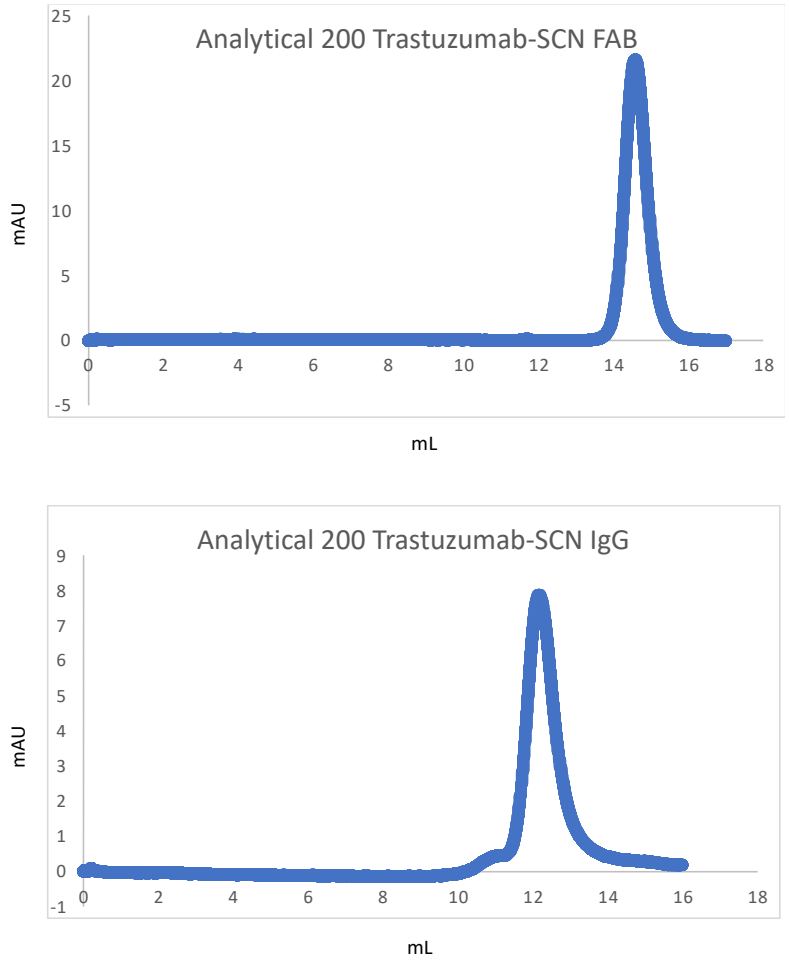

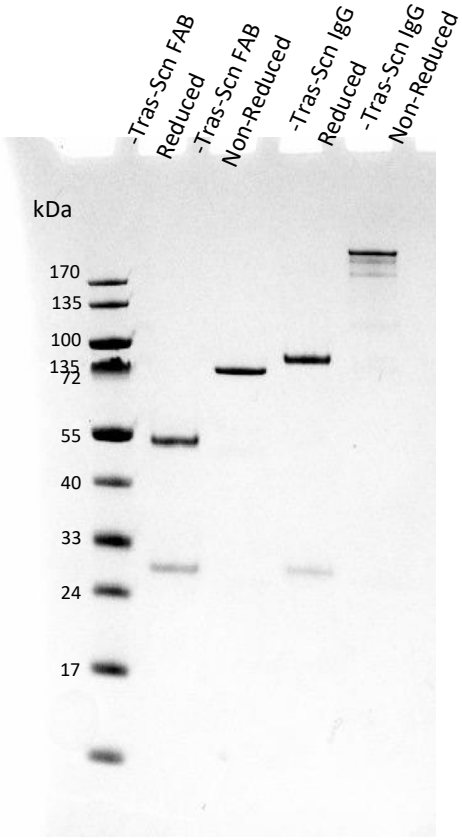

Figure S1. Trastuzumab Fab-Scn and Trastuzumab IgG-Scn purification by preparative size-exclusion chromatography via A200 column and corresponding PAGE analysis.

Fluorescence quenching experiments

Fluorescence quenching experiments were adapted from a previously reported procedure.[2] Equimolar amounts of YCl_3 or $ScCl_3$ (0.2 mM, 0.1 M HCl) and HOPO (0.2 mM, DMSO) were mixed and pH-adjusted to 7.4 using 0.1 M NaOH. The resulting metal complex M-HOPO (M = Y, Sc) was further diluted to 1 μM with 1X TBS buffer and stored in the dark at 10 °C. Recombinant wild-type Scn was freshly diluted from a 10 mg/mL stock (50 nM, 0.5 mL, 32 μg/mL ubiquitin, 1X TBS, pH 7.4) and then titrated with the M-HOPO complex stock solution in 5 μL increments, followed by gentle agitation and a 5-minute equilibrium period. Fluorescence quenching of Scn was measured after each titrant addition on a FluoTime 300 PicoQuant Fluorescence Spectrometer (settings: 8 nm excitation/emission slit band pass, λ_{exc} = 281 nm and λ_{em} = 338-345 nm, delta = 0.3 nm, integration time = 0.5 s). The narrow emission range was selected to minimize protein photobleaching. Fluorescence values were corrected for dilution upon addition of titrant. Fluorescence data were analyzed by nonlinear regression analysis of fluorescence response versus ligand concentration using DynaFit. The K_d values are the results of at least three independent titrations.

Table S1. K_d values from quenching experiments

	Kd	Error	n
Y-HOPO-Scn	2.447	0.620	3
Sc-HOPO-Scn	5.493	1.047	3

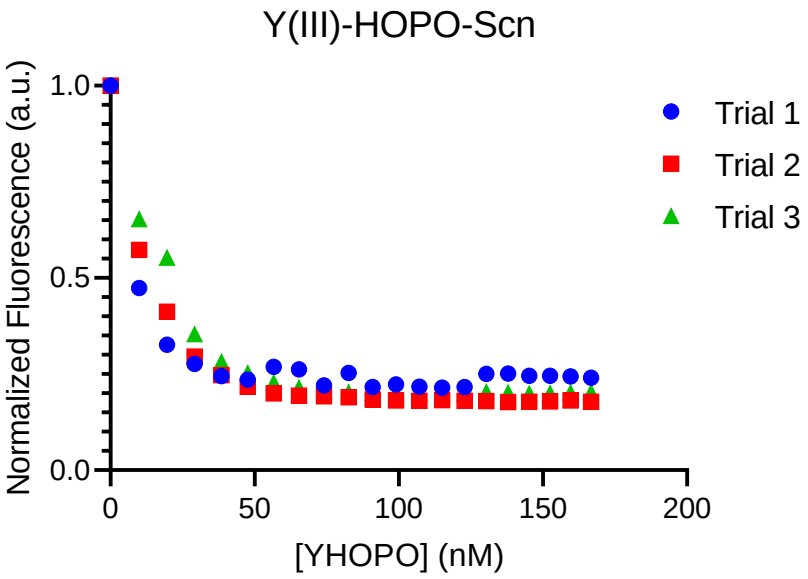

Figure S2. Fluorescence quenching (λ_{ex} = 281 nm, λ_{em} = 338-345 nm) of native siderocalin tryptophan residue emission as a function of Y-HOPO concentration. See curve fits in **Figure S4**.

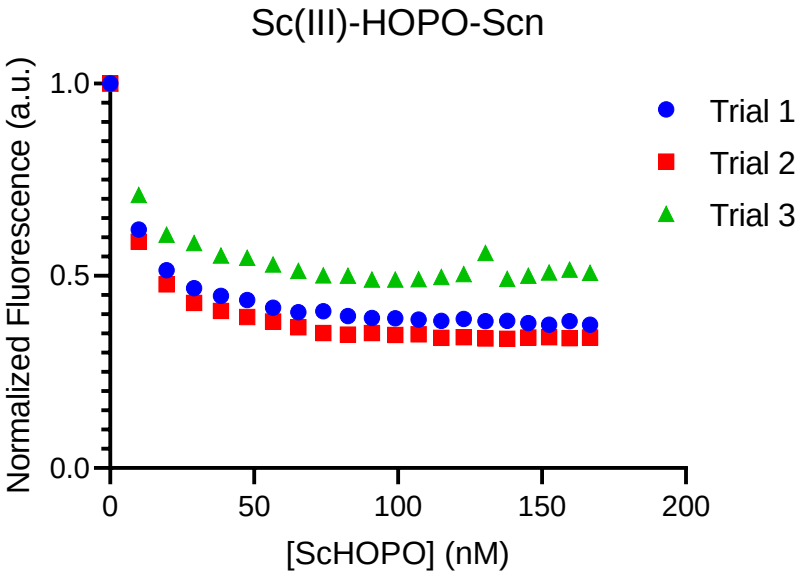

Figure S3. Fluorescence quenching (λ_{ex} = 281 nm, λ_{em} = 338-345 nm) of native siderocalin tryptophan residue emission as a function of Sc-HOPO concentration. See curve fits in **Figure S5**.

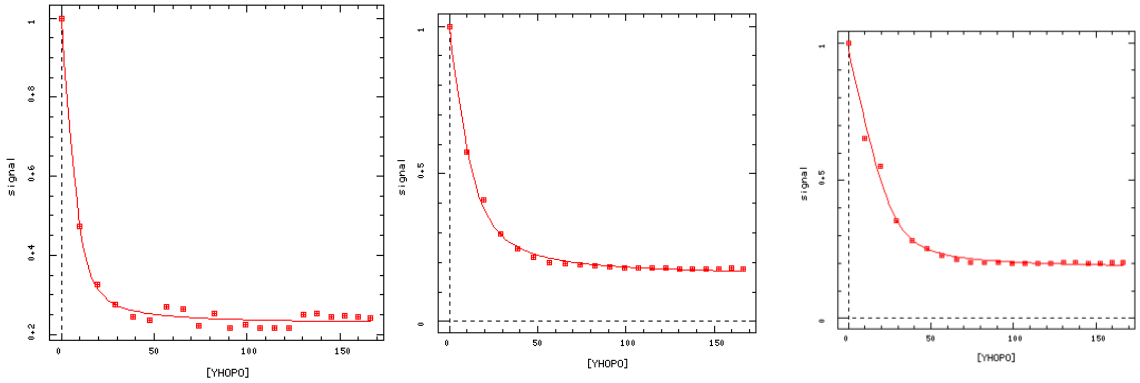

Figure S4. Fluorescence quenching curve fits Y-HOPO (n = 3) from DynaFit.

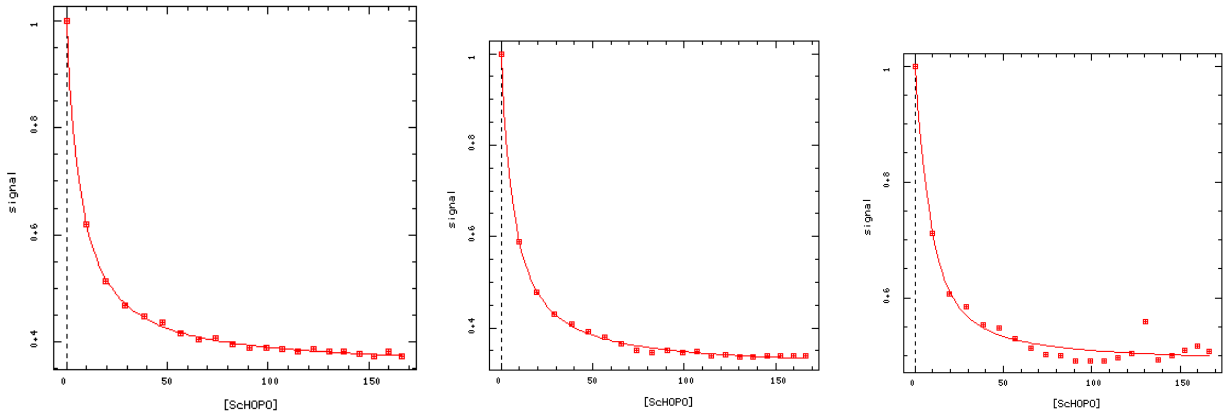

Figure S5. Fluorescence quenching curve fits Sc-HOPO (n = 3) from DynaFit

Cell Culture and Animal Model

All procedures and protocols used in small animal PET imaging and biodistribution studies were reviewed and approved by the Institutional Animal Care and Use Committee at Lawrence Berkeley National Laboratory. Experiments were performed in compliance with guidelines from the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) in AAALAC accredited facilities. Animals were housed in ventilated cages, given food and water *ad libitum*, and allowed to acclimate for roughly one week prior to treatment. SKOV-3 cells were used as received from ATCC. SKOV-3 cells were cultured in McCoy's 5A medium with 10% FBS (v/v) at 37 ℃ and 5% CO_2. Female NODSCID mice (10 weeks, Charles River Laboratories) were inoculated in the lower flank with a 1:1 mixture of SKOV-3 cells (~10^6 cells) in Matrigel. Tumors were measured with digital calipers and volume (V) was calculated as [$V = L \times (W/2)^2$] where L and W are the length and width of the tumor in mm. Biodistribution studies with ^{90}Y commenced once tumors were palpable (~30 mm^3), and PET imaging studies with ^{86}Y were conducted once the tumors measured ~50 mm^3.

Radiolabeling and TLC

Ligand radiolabeling was achieved by adding ^{90}Y (0.04 M HCl) or ^{86}Y (0.1 M HCl) to 3,4,3-LI-HOPO dissolved in DMSO at 100:1 metal:ligand ratio and incubating at room temperature for 5 minutes. Solutions were further diluted with 1X DPBS and a pH of 7.4 was confirmed by spotting onto pH paper. Subsequently, the ^{86}Y-HOPO or ^{90}Y-HOPO solution was added to either wildtype Scn (200:1 protein:metal) or the fusion protein (100:1 protein:metal) in a PBS matrix and then incubated at room temperature for 5 minutes. Activity was measured *in vivo* by whole-body monitoring via Capintec-CRC-15r system. The radiochemical purities of radiolabeled antibody drug conjugate solutions were determined by radio-iTLC (Bioscan System 200 Imaging Scanner), using Supelco iTLC-SG glass plates (Sigma Aldrich) and 20 mM EDTA at pH 5.5 as the mobile phase. Radiochemical purities were determined by drawing two regions of interest (using Bioscan System 200 Imaging Scanner software) over the Gaussian peak at the origin (which contains $^{90/86}$Y-HOPO) and the region outside the origin peak (which constitutes free $^{90/86}$Y).

Table S2. Radiochemical purities (%) for Y-HOPO-Protein constructs.

^{90}Y or ^{86}Y (Time)	Scn	Fab	IgG
^{86}Y Day 0	64	95	55
^{90}Y Day 0	86	87	86
^{90}Y Day 1	86	87	84
^{90}Y Day 3	85	85	87

[a] Conditions: Mobile phase = 20 mM EDTA, pH 5.5.

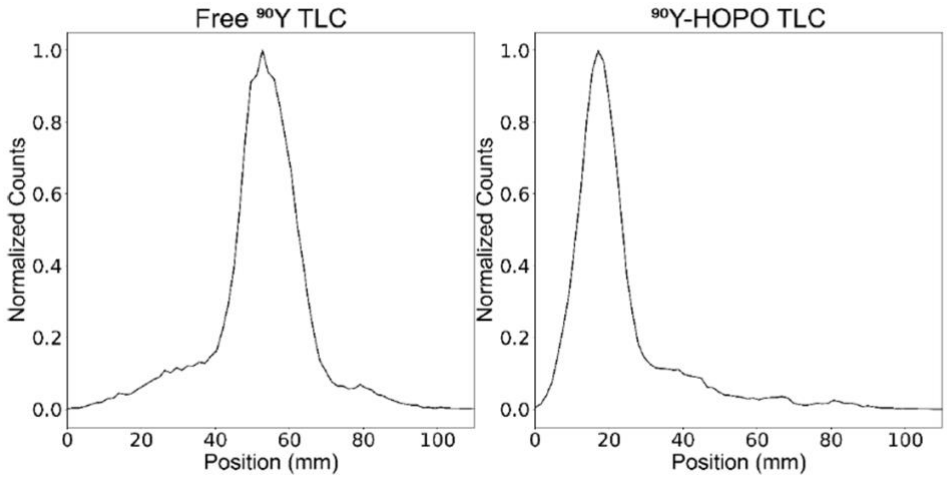

Figure S6. RadioTLC chromatograms for free ^{90}Y (left) and ^{90}Y-HOPO (right).

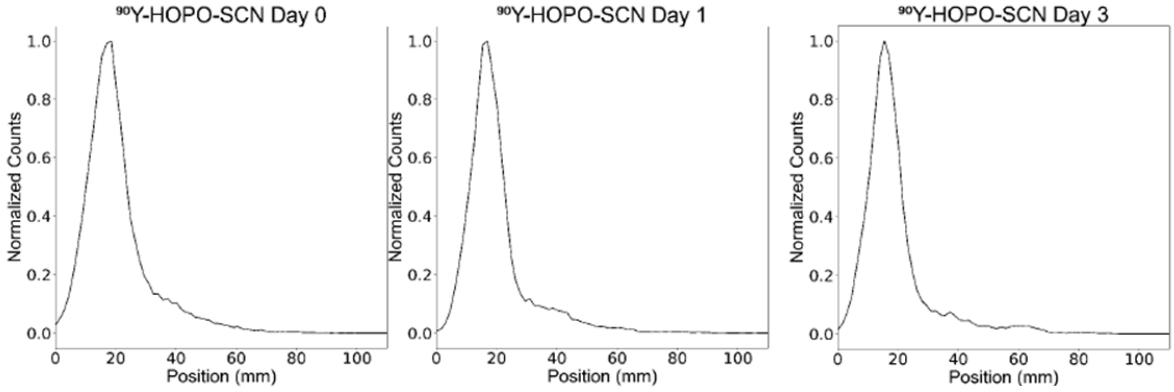

Figure S7. RadioTLC chromatograms for ^{90}Y-HOPO-Scn at day 0, 1, and 3.

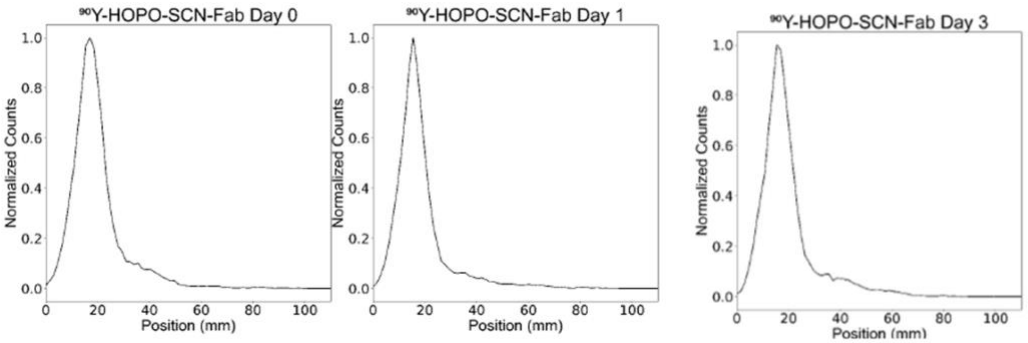

Figure S8. RadioTLC chromatograms for ^{90}Y-HOPO-Fab at day 0, 1, and 3.

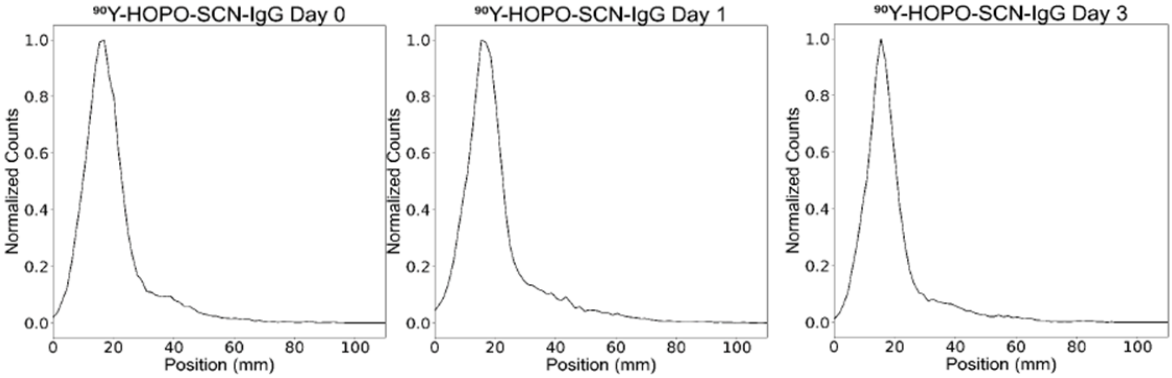

Figure S9. RadioTLC chromatograms for free ^{90}Y-HOPO-IgG at day 0, 1, and 3.

PET imaging and analysis

Mice (n=3) were injected intraperitoneally (100 µCi, 0.2 mL) and subsequently imaged at 5, 25, and 50 hours p.i. on a Concorde microPET R4, which supports a transaxial resolution of 1.66mm FWHM, in the head-first supine position. An energy window of 350–650keV and a coincidence timing window of 6 ns were used during image acquisition. The counting rates in the processed images were converted to standard uptake values (SUV) by using a system calibration factor obtained by imaging a cylinder filled with ^{86}Y. The reported images are coronal maximum intensity projection images. Images were analyzed using AMIDE v1.0.5.

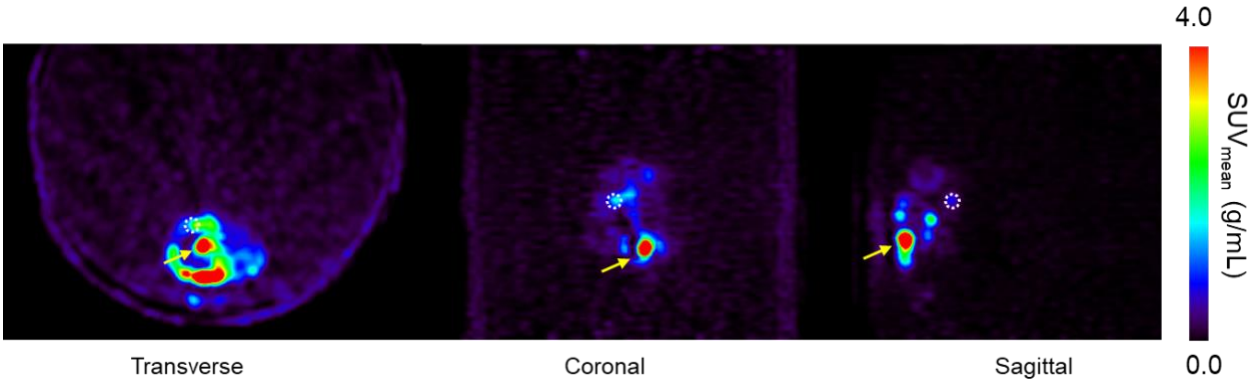

Figure S10. MIP images of ^{86}Y-HOPO-IgG at 5 hours p.i. through the transverse, coronal, and sagittal planes. White dashed circle = tumor, yellow arrow = bladder. 5 mm slice. Note change in scale bar.

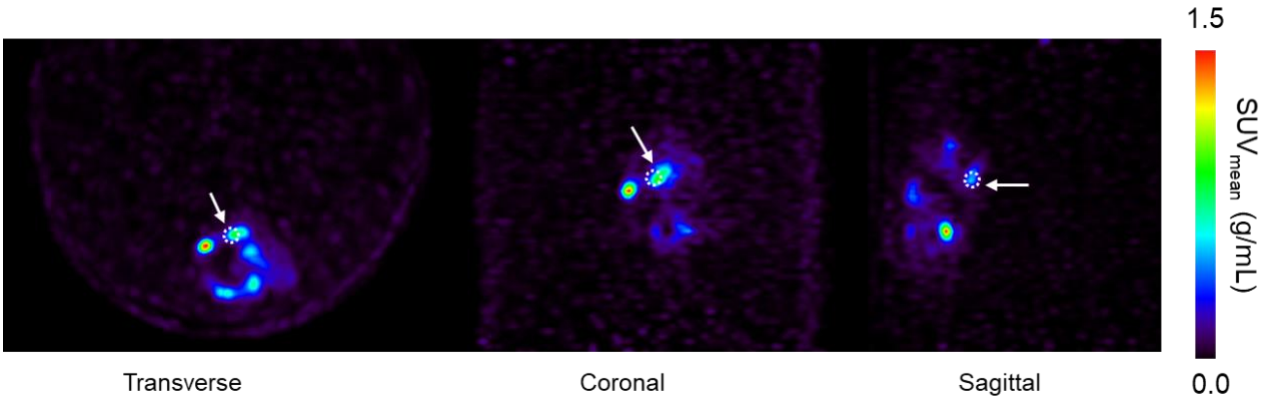

Figure S11. MIP images of ^{86}Y-HOPO-IgG at 5 hours p.i. through the transverse, coronal, and sagittal planes. White dashed circle = tumor. 5 mm slice. Note change in scale bar.

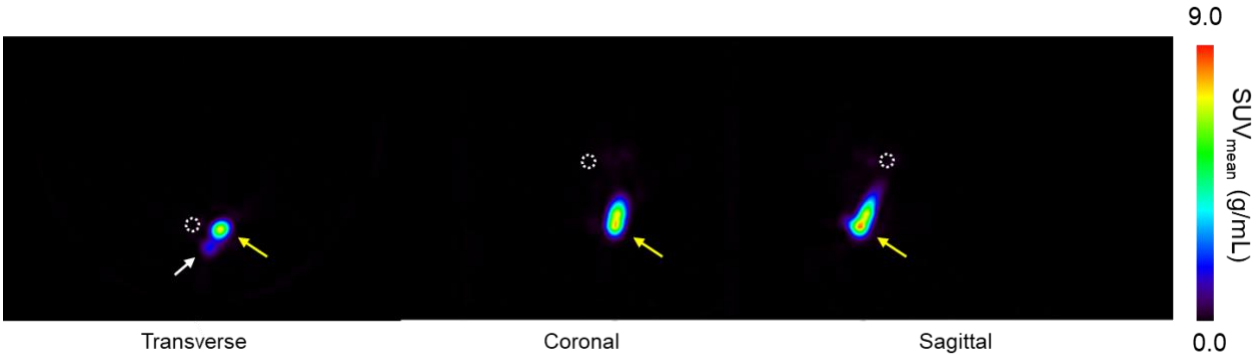

Figure S12. MIP images of ^{86}Y-HOPO-Scn at 5 hours p.i. through the transverse, coronal, and sagittal planes. White dashed circle = approximate tumor location, white arrow = bladder, yellow arrow = right kidney. 5 mm slice.

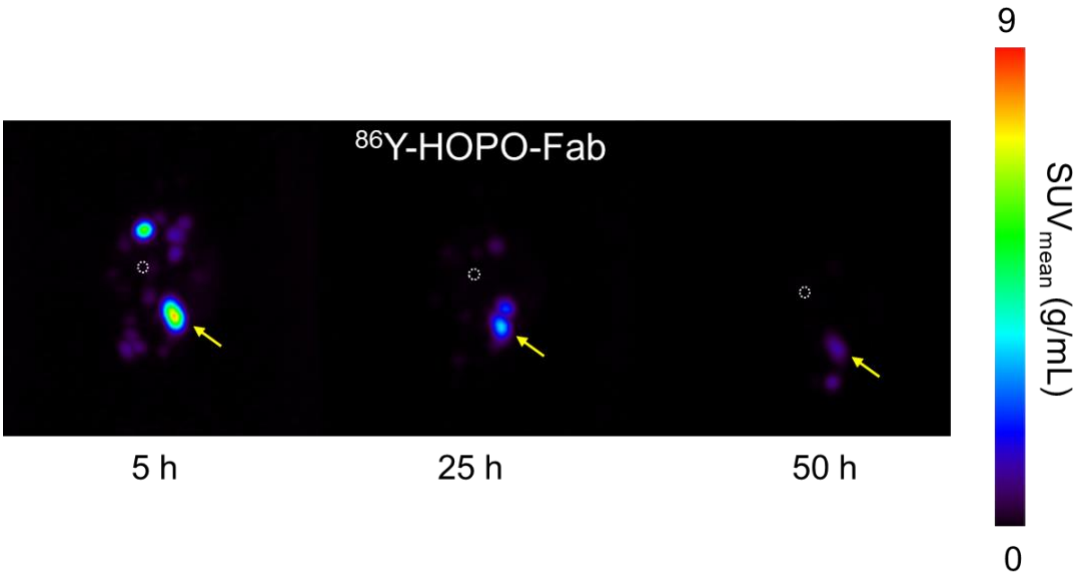

Figure S13. MIP coronal images of ^{86}Y-HOPO-Fab at 5, 25 and 50 hours p.i. White dashed circle = approximate tumor location, yellow arrow = right kidney.10 mm slice.

Biodistribution and organ analysis

For biodistribution studies, mice (n=3) were administered 45 µCi (1.7 MBq, 0.2 mL) of ^{90}Y-HOPO-IgG, ^{90}Y-HOPO-Fab, and non-targeting ^{90}Y-HOPO-Scn in addition to PBS as a control. Mice were euthanized 24 h p.i. and organs of interest (liver, kidney, spleen, heart, lung, tumor, and remaining abdominal cavity [ART]) were harvested for analysis. All organ samples were dried at 100 °C for 24 hours, ashed at 575 °C for 24 hours, and then the ashes dissolved in concentrated nitric acid. The dissolved organs were mixed with 10 mL of Ultima Gold LLT (Perkin Elmer, Shelton) for a by liquid scintillation counting (Packard Tri-Carb model B4430, Perkin Elmer) using a window of 50 keV-2000 keV with the results being reported in terms of %ID/g.

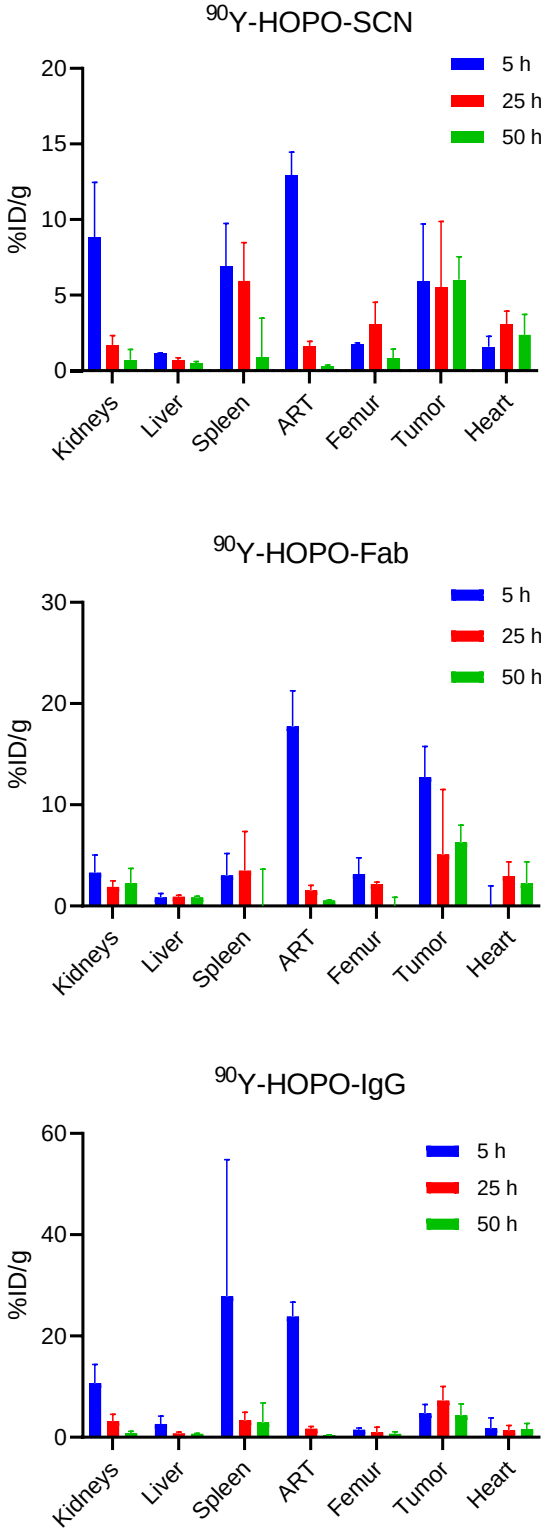

Figure S14. Biodistribution (given as %ID/g) at 5, 25 and 50 hours p.i. of ^{90}Y-labeled Scn, Fus, and IgG constructs. ART = remaining abdominal tissue.

References

(1) Bandaranayake, A. D.; Correnti, C.; Ryu, B. Y.; Brault, M.; Strong, R. K.; Rawlings, D. J. Daedalus: a robust, turnkey platform for rapid production of decigram quantities of active recombinant proteins in human cell lines using novel lentiviral vectors. *Nucleic Acids Research* **2011**, *39* (21), e143-e143. DOI: 10.1093/nar/gkr706 (acccessed 4/1/2023).
(2) Abergel, R. J.; Clifton, M. C.; Pizarro, J. C.; Warner, J. A.; Shuh, D. K.; Strong, R. K.; Raymond, K. N. The Siderocalin/Enterobactin Interaction: A Link between Mammalian Immunity and Bacterial Iron Transport1. *Journal of the American Chemical Society* **2008**, *130* (34), 11524-11534. DOI: 10.1021/ja803524w.