Light-Controlled Release of Therapeutic Proteins from Red Blood Cells

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Supporting Information

Synthesis of Peptides and Peptide-Cobalamin Constructs

Reagents for synthesis: All reagents and solvents were purchased from Sigma-Aldrich or Fisher Scientific except for 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU), 6-chloro-benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyClock), amino acids, NovaPeg Rink Amide Resin, which were obtained from NovaBiochem, ChemPep, or AAPPTEC.

Melittin (Mel): GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$
C_{18}-Mel: $CH_3(CH_2)_{16}CO$-GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$
BlockingSegment (BS): Ac-PEPAPEPEAEADAEADPEA
BS-Lys: Ac-PEPAPEPEAEADAEADPEA-K
BS-Cy5: Ac-PEPAPEPEAEADAEADPEA-K(Cy5)-K

Peptide Synthesis: General Methods. All peptides were synthesized using standard Fmoc peptide synthesis on Rink amide resin using a Prelude automatic peptide synthesizer from Protein Technologies [amino acids (5 eq), HATU (5 eq), DIPEA (10 eq), all in DMF]. Fmoc deprotection was performed using 20% piperidine in DMF, with 100 mM Oxyma added for all BS peptides synthesized to prevent racemization of aspartic acid side chains during deprotection. Melittin peptides were cleaved from resin using trifluoroacetic acid (TFA)/phenol/water/thioanisole/ethanedithiol (EDT) (82.5/5/5/5/2.5). BS peptides were cleaved from resin using TFA/water/triisopropylsilane (TIS) (95/2.5/2.5). Purity was confirmed with Agilent analytical LC chromatography as assessed by UV-Vis (220 nm for BS derivatives and 280 nm for Mel derivatives), fluorescence (Ex/Em 640/670 nm for BS-Cy5 and Ex/Em 280/350 nm for Mel derivatives) and mass spectrometry.

Synthesis of C_{18}-Mel
After synthesis of NH_2-GIGAVLKVLTTGLPALISWIKRKRQQ-resin via solid phase peptide synthesis, stearic acid (10 eq) was coupled to the N-terminus of the peptide using HATU (10 eq) and N,N-diisopropylethylamine (DIPEA, 20 eq) in DMF for 2 - 6 h. This reaction mixture was drained, rinsed with DMF, followed by an additional coupling of stearic acid. The peptide was cleaved from the resin using standard procedures (see **Peptide Synthesis: General Methods**). C_{18}-Mel was purified using a Biotage Flash Purification system on BioSnap Ultra C_{18} column (Biotage) with a 0.1% TFA water/isopropanol gradient. Exact mass calculated for $C_{149}H_{263}N_{39}O_{32}$: 3111.02, observed mass: 3112.4 (**Figure S1**).

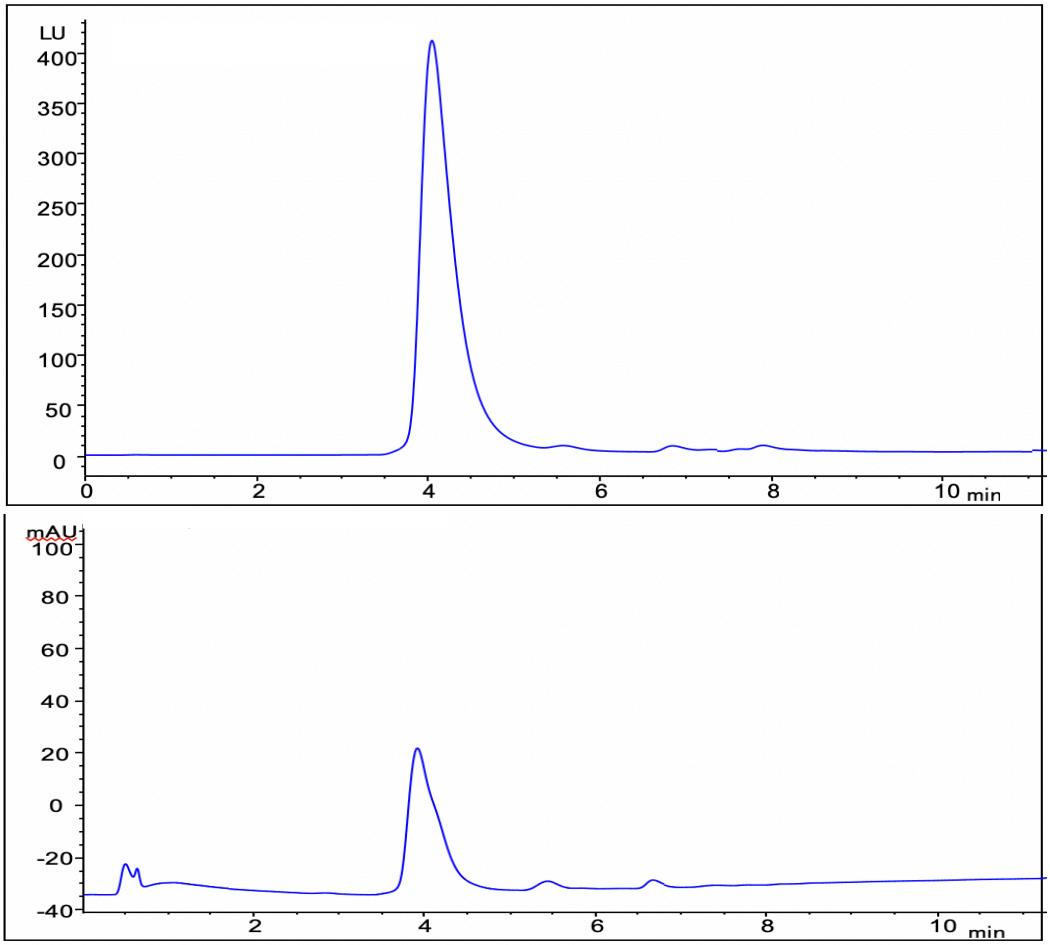


Figure S1: LC-MS chromatogram of purified C_{18}-Mel detected by fluorescent detector (top, λ_{ex} = 280 nm; λ_{em} = 350 nm) and by UV detector (bottom, monitored at 280 nm). Expected mass: 3111.02, observed mass: 3112.4.

Synthesis of BS-Lys

After synthesis of Fmoc-PEPAPEPEAEADAEADPEAK-resin, the terminal Fmoc was removed (see **Peptide Synthesis: General Methods**). The N-terminus was acetylated by adding acetic anhydride (10 eq), DIPEA (10 eq), and HATU (5 eq) in DMF. The peptide was cleaved from the resin using standard procedures (see **Peptide Synthesis: General Methods**). The peptide was purified using a Biotage Flash Purification system on BioSnap Ultra C_{18} column (Biotage) with a 0.1% TFA water/acetonitrile gradient. Exact mass calculated for $C_{89}H_{134}N_{22}O_{37}$: 2102.93, observed mass: 2102.2 (**Figure S2**).

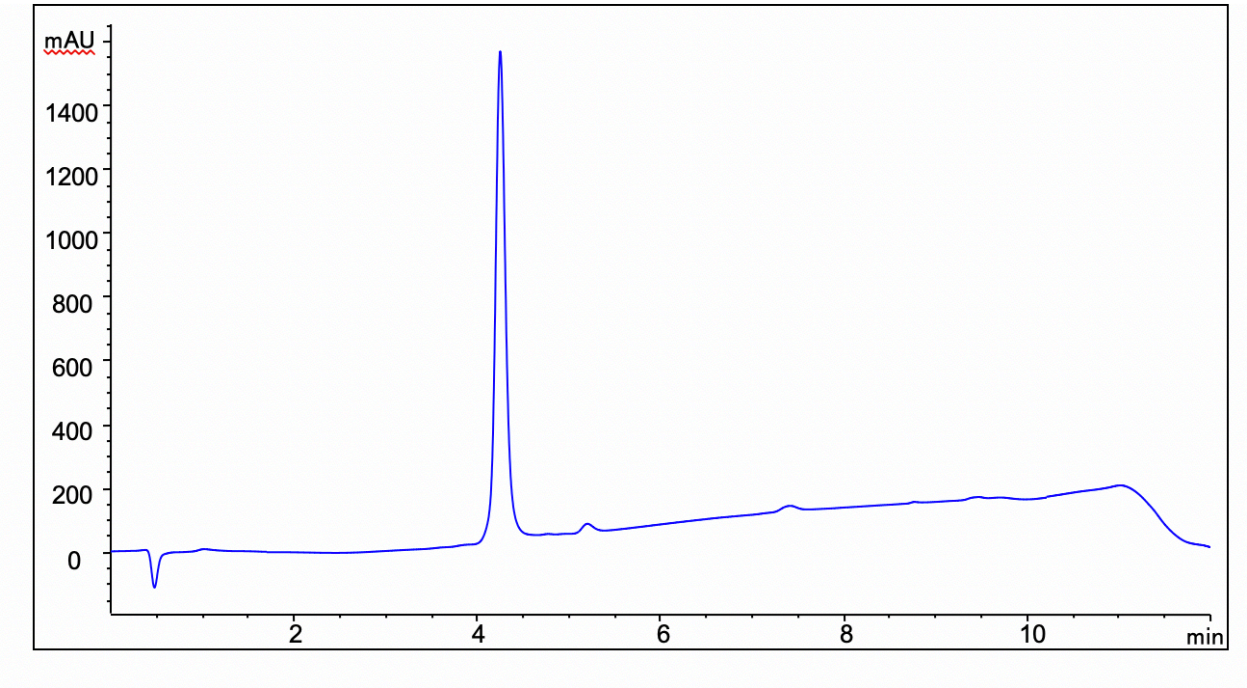


Figure S2: LC-MS chromatogram of purified BS-Lys detected by UV detector (monitored at 220 nm). Expected mass: 2102.93, observed mass: 2102.2.

Synthesis of BS-Cy5

After synthesis of Fmoc-PEPAPEPEAEADAEADPEA-K(methyltrityl)-K-resin, the N-terminus Fmoc was removed (see **Peptide Synthesis: General Methods**). The N-terminus was acetylated by adding acetic anhydride (10 eq), DIPEA (10 eq), and HATU (5 eq) in DMF. The Lys(methyltrityl) residue was deprotected by first rinsing the resin with dichloromethane (DCM) followed by the addition of DCM/hexafluoro-2-propanol (HFIP)/trifluoroethane (TFE)/triethylsilane (TES) (6.5/2/1/0.5) 2x for 1 h. The resin was then sequentially rinsed with DMF, 250 mM DIPEA in DMF, and DMF. Cy5 (3 eq) was activated with PyClock (10 eq) and DIPEA (20 eq) and coupled to the resin-bound peptide at room temperature in DMF overnight. The peptide was cleaved from the resin using standard procedures (see **Peptide Synthesis: General Methods**) and purified using the Biotage Flash Purification system on a BioSnap Ultra C_{18} column (Biotage) with a 0.1% TFA water/acetonitrile gradient. Exact mass calculated for $C_{127}H_{183}N_{26}O_{39}$: 2696.31, observed mass: 2696.4 (**Figure S3**).

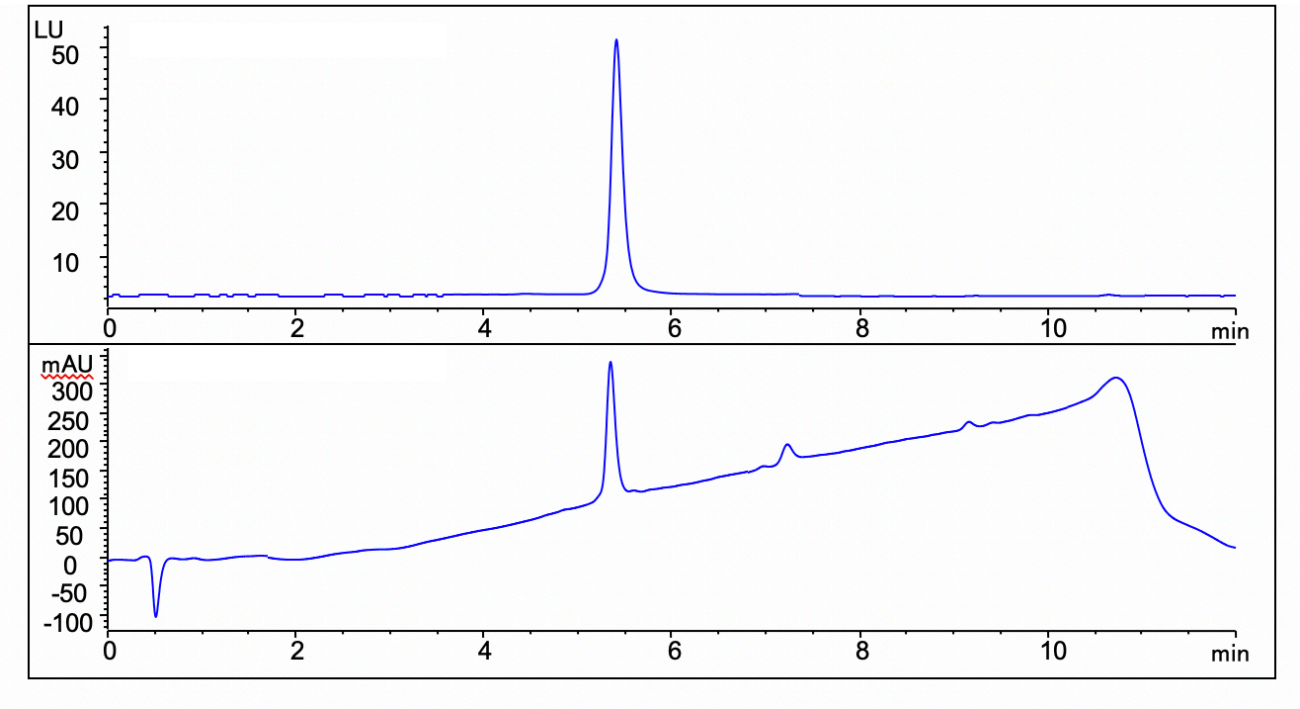
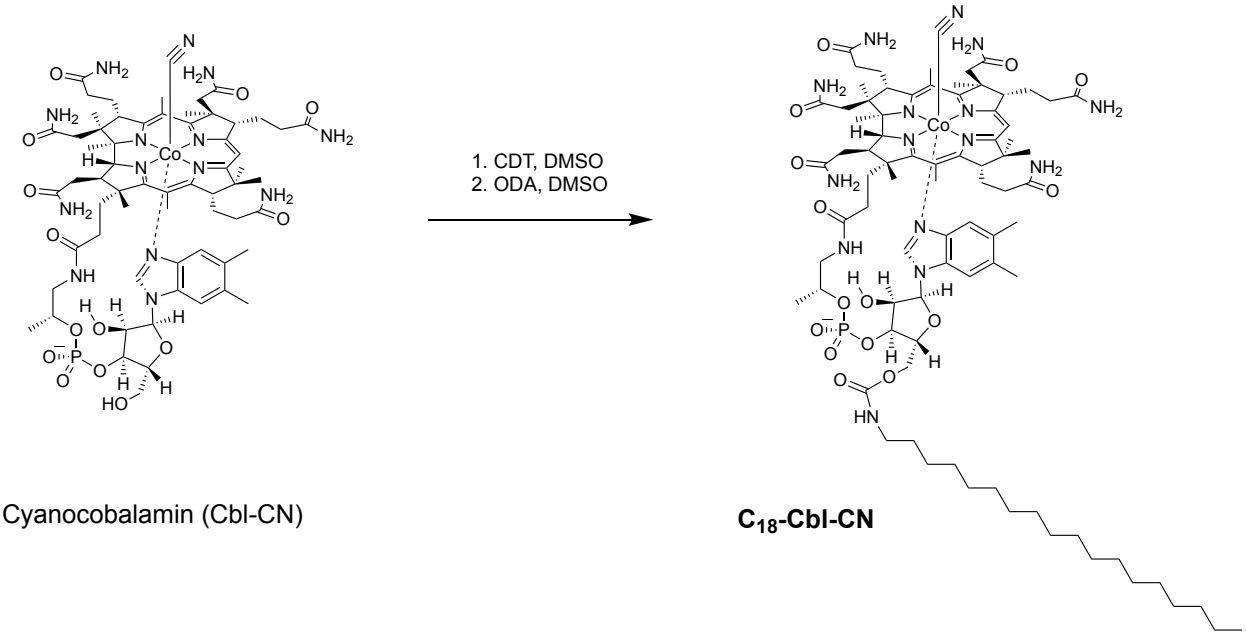


Figure S3: LC-MS chromatogram of purified BS-Cy5 detected by fluorescent detector (top, λ_{ex} = 640 nm; λ_{em} = 670 nm) and by UV detector (bottom, monitored at 220 nm). Expected mass: 2696.31, observed mass: 2696.4**.**

Synthesis of Cobalamin Derivatives: The synthesis of all cobalamin (Cbl) derivatives were performed following the protocols outlined in Smith et al (2014).

Synthesis of C_{18}-Cbl-CN

Carbonylditriazole (CDT, 5 eq, 303 mg, 1.85 mmol) was added to cyanocobalamin (Cbl-CN, 500 mg, 0.37 mmol) dissolved in 5 mL anhydrous dimethyl sulfoxide (DMSO). The solution was mixed for 5 h, cold ether:DCM (3:2) was added and the mixture was sonicated for ~1 min. The reaction mixture was centrifuged, the supernatant was decanted, and the precipitate was washed with ether 3x and dried completely. Octadecylamine (ODA, 1 g, 10 eq) was dissolved in 25 mL anhydrous DMSO and added to the dried precipitate. After mixing the reaction overnight, 50 mL of ether was added. The mixture was then spun down and the supernatant was removed. The remaining precipitate was washed with ether 3x and dried. C_{18}-Cbl-CN was purified using the Biotage Flash Purification system on a UltraSnap C_{18} column (Biotage) with a 0.1% TFA water/methanol (MeOH) gradient. Exact mass calculated for $C_{82}H_{125}CoN_{15}O_{15}P$: 1649.85, observed mass: 1649.6 (**Figure S4**).



Cyanocobalamin (Cbl-CN)

C_{18}-Cbl-CN

Scheme S1: Synthesis of C_{18}-Cbl-CN.

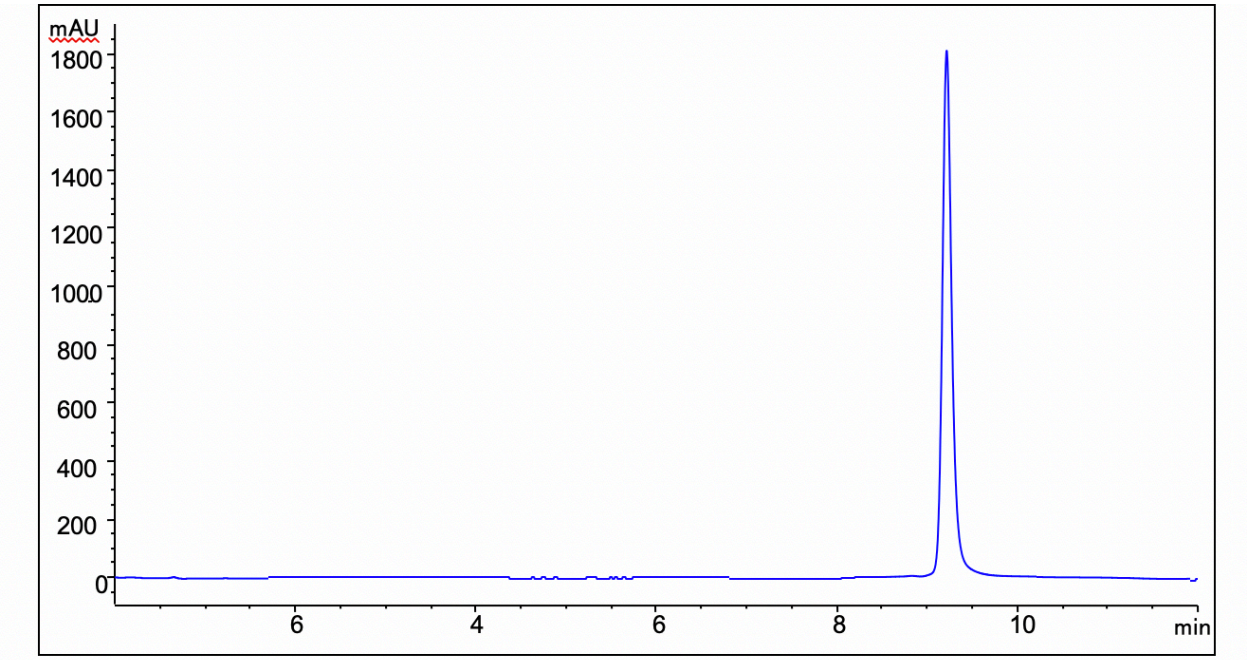
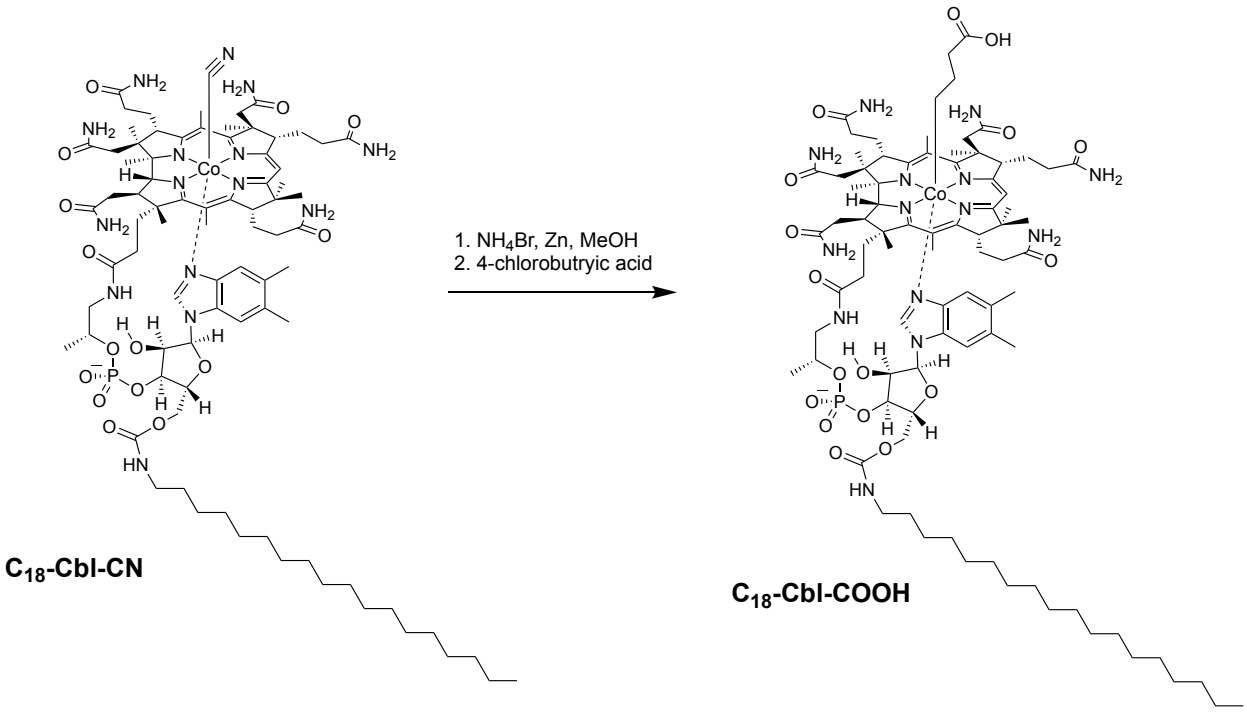


Figure S4: LC-MS chromatogram of purified C_{18}-Cbl-CN detected by UV detector (monitored at 357 nm). Expected mass: 1649.85, observed mass: 1649.6.

Synthesis of C_{18}-Cbl-COOH

500 mg (0.30 mmol) of **C_{18}-Cbl-CN** (see **Synthesis of C_{18}-Cbl-CN**) was dissolved in 15 mL MeOH with NH_4Br (750 mg, 5% w/v) and purged with N_2. Zn (980 mg, 50 eq, 15 mmol) was added, the solution was sonicated for ~1 min, and then mixed for 30 min with protection from light. 4-chlorobutryic acid (150 µL, 5 eq, 1.5 mmol) was added directly to the solution and mixed for an additional 4 h in the dark. Zn was decanted from reaction mixture and **C_{18}-Cbl-COOH** was then purified using the Biotage Flash Purification system on a UltraSnap C_{18} column (Biotage) with a 0.1% TFA water/MeOH gradient. Exact Mass Calculated for $C_{85}H_{132}CoN_{14}O_{17}P$: 1710.90, observed mass: 1709.6 (**Figure S5**).



Scheme S2: Synthesis of C_{18}-Cbl-COOH.

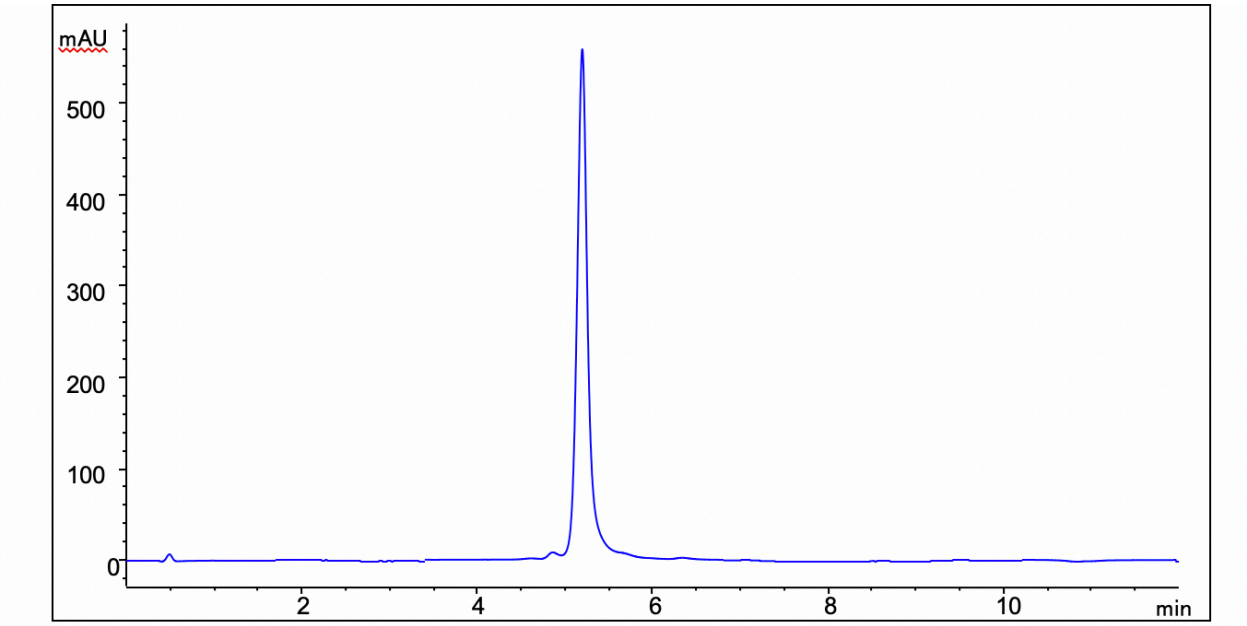
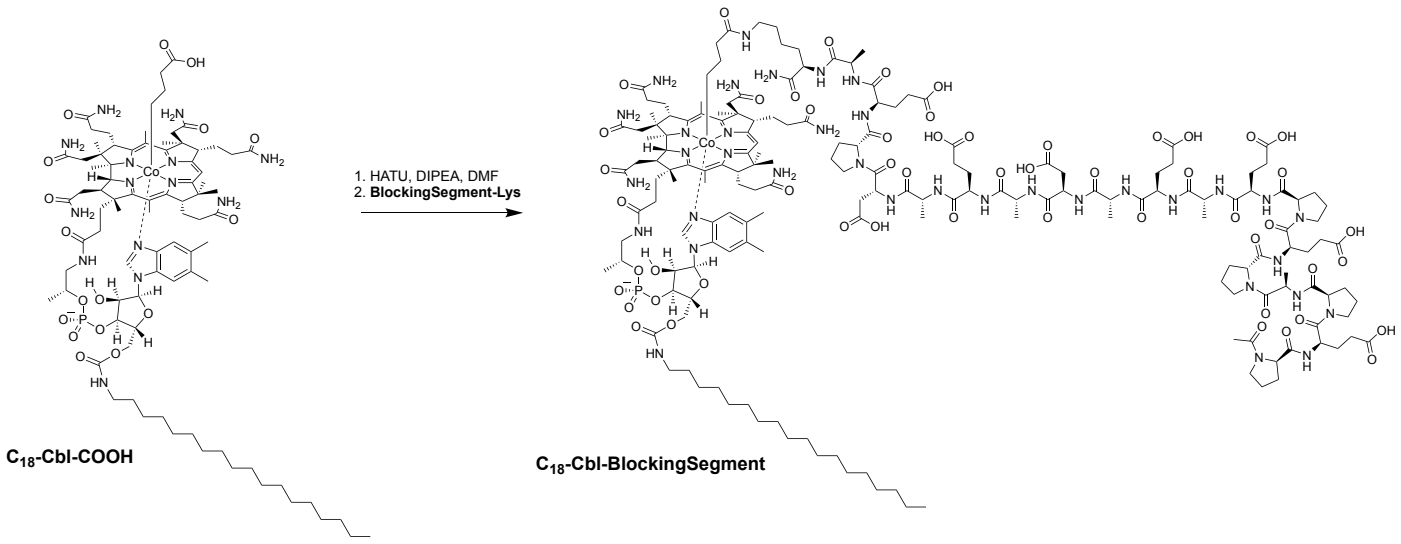


Figure S5: LC-MS chromatogram of purified C_{18}-Cbl-COOH detected by UV detector (monitored at 357 nm). Expected mass: 1710.9, observed mass: 1709.6.

Synthesis of C_{18}-Cbl-BS

The following reaction was protected from light due to the light sensitivity of the Co-C bond. 96 uL of 104 mM C_{18}-Cbl-COOH (see **Synthesis of C_{18}-Cbl-COOH**) in DMF (0.010 mmol) was added to 115 uL of 87 mM HATU in DMF (0.010 mmol, 1 eq) and 35 µL of pure DIPEA (0.2 mmol, 20 eq). After 5 min in the dark, 500 µL of 23 mM BS-Lys (see **Synthesis of BS-Lys**) in DMF (0.012 mmol, 1.2 eq) was added. After 30 min, ether was added to reaction and resulting precipitate was washed with ether twice, dried, and subsequently purified using Biotage Flash Purification system on a BioSnap Ultra C_{18} column (Biotage) with a 0.1% TFA water/acetonitrile gradient. Exact mass calculated for $C_{174}H_{264}CoN_{36}O_{53}P$: 3795.81, observed mass: 3794.1 (**Figure S6**).



Scheme S3: Synthesis of C_{18}-Cbl-BS.

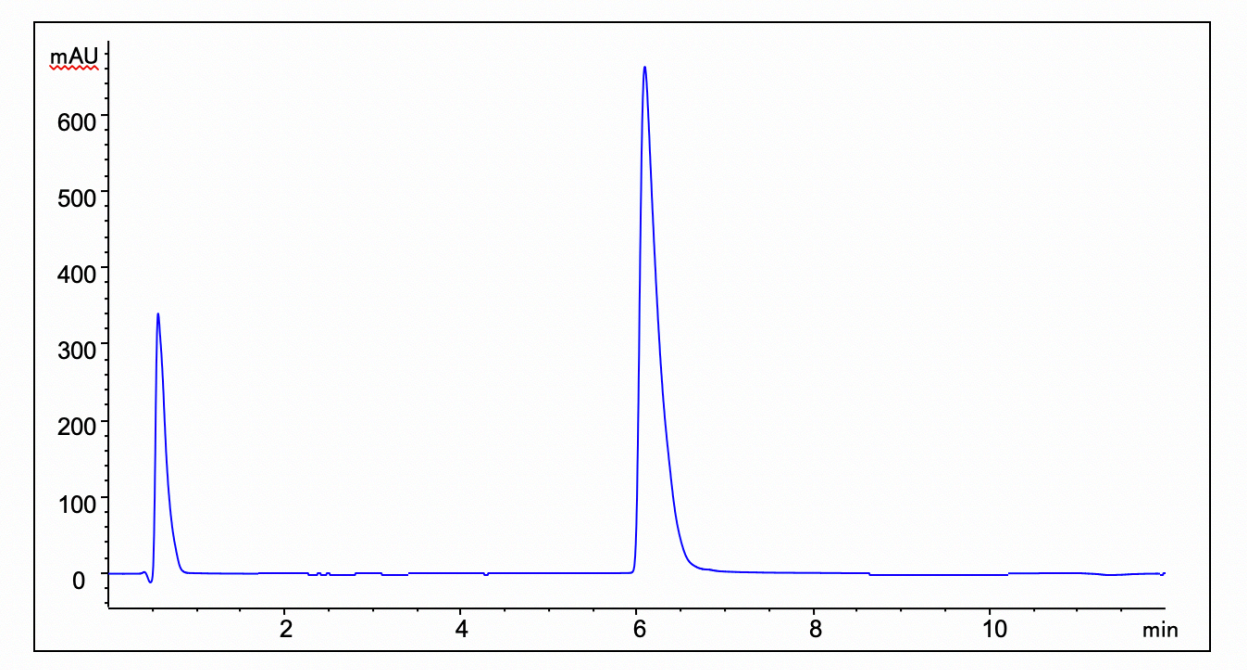
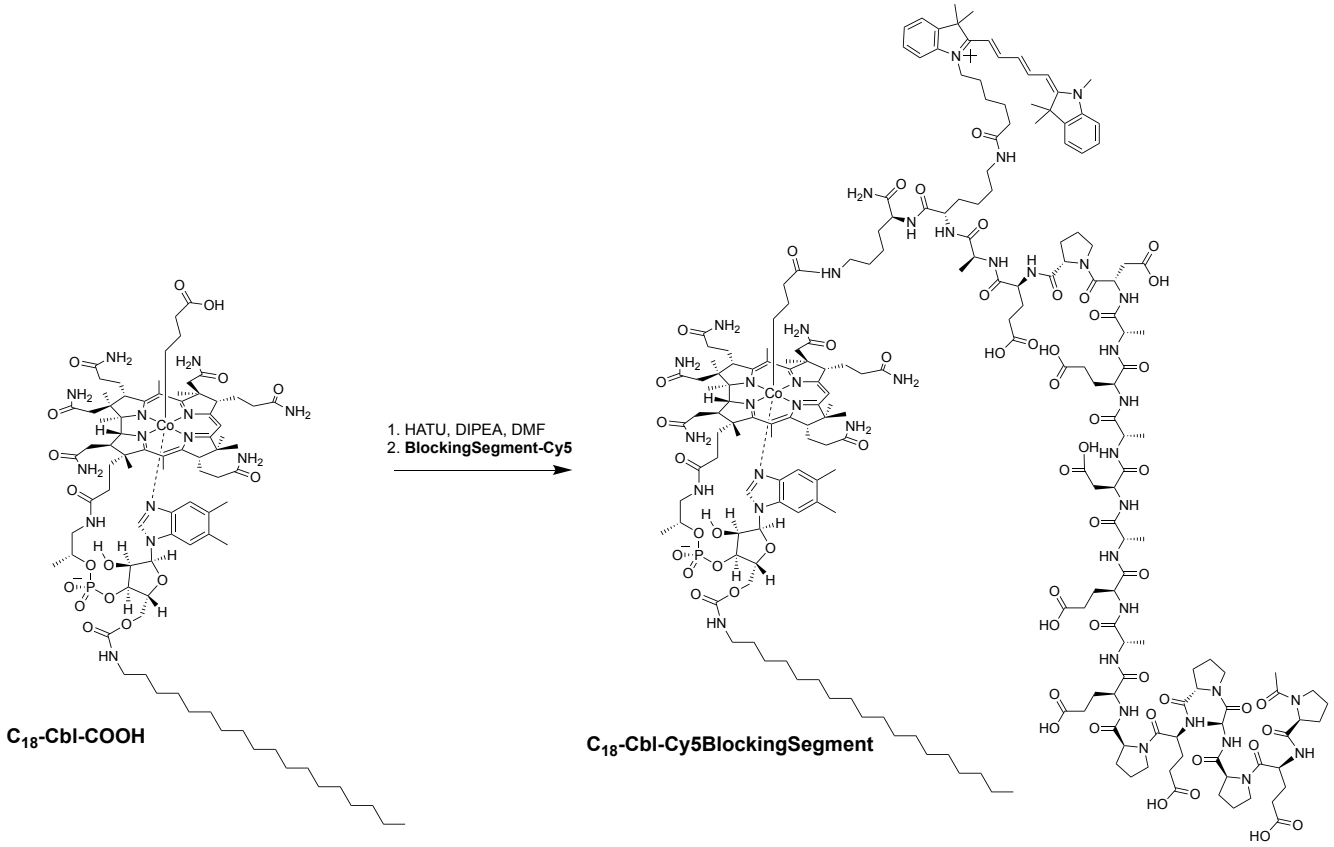


Figure S6: LC-MS chromatogram of purified C_{18}-Cbl-BS detected by UV detector (monitored at 357 nm, solvent peak at 0.3 min). Expected mass: 3795.81, observed mass: 3794.1.

Synthesis of C_{18}-Cbl-Cy5BS

The following reaction was protected from light due to the light sensitivity of the Co-C bond. 23 µL of 73 mM **C_{18}-Cbl-COOH** (see **Synthesis of C_{18}-Cbl-COOH**) in DMF (0.0017 mmol) was added to 35 µL of 49 mM HATU in DMF (0.0017 mmol, 1 eq) and 340 µL of 100 mM DIPEA in DMF (0.034 mmol, 20 eq). After 5 min in the dark, 1.9 mL of 1.3 mM BS-Cy5 (see **Synthesis of BS-Cy5**) in DMF (0.0026 mmol, 1.5 eq) was added. After 30 min, ether was added to reaction and resulting precipitate was washed with ether twice and dried. C_{18}-Cbl-Cy5BS was purified using the Biotage Flash Purification system on a BioSnap Ultra C_{18} column (Biotage) with a 0.1% TFA water/acetonitrile gradient. Exact mass calculated for $C_{212}H_{313}CoN_{40}O_{55}P$: 4389.2, observed mass: 4390.4 (**Figure S7**).



Scheme S4: Synthesis of C_{18}-Cbl-Cy5BS.

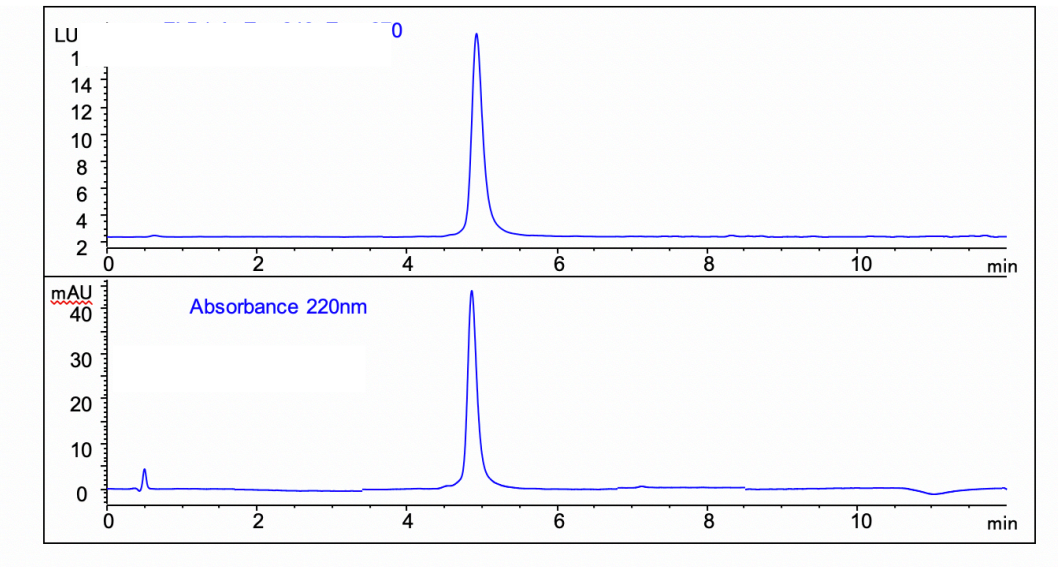


Figure S7: LC-MS chromatogram of purified C_{18}-Cbl-Cy5BS detected by fluorescent detector (top, λ_{ex} = 640 nm; λ_{em} = 670 nm) and by UV-Vis detector (bottom, monitored at 357 nm). Expected mass: 4389.2, observed mass: 4390.4.

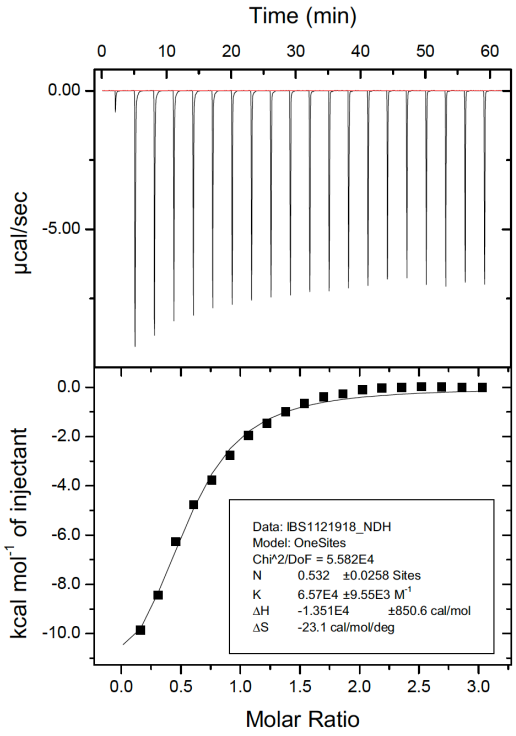


Figure S8: Isothermal calorimetry (ITC) data using the Mel peptide as the macromolecule in the cell and the BS peptide as the ligand in the syringe. The inverse of K was used to derive the K_D of 15.2 ± 2.6 µM.

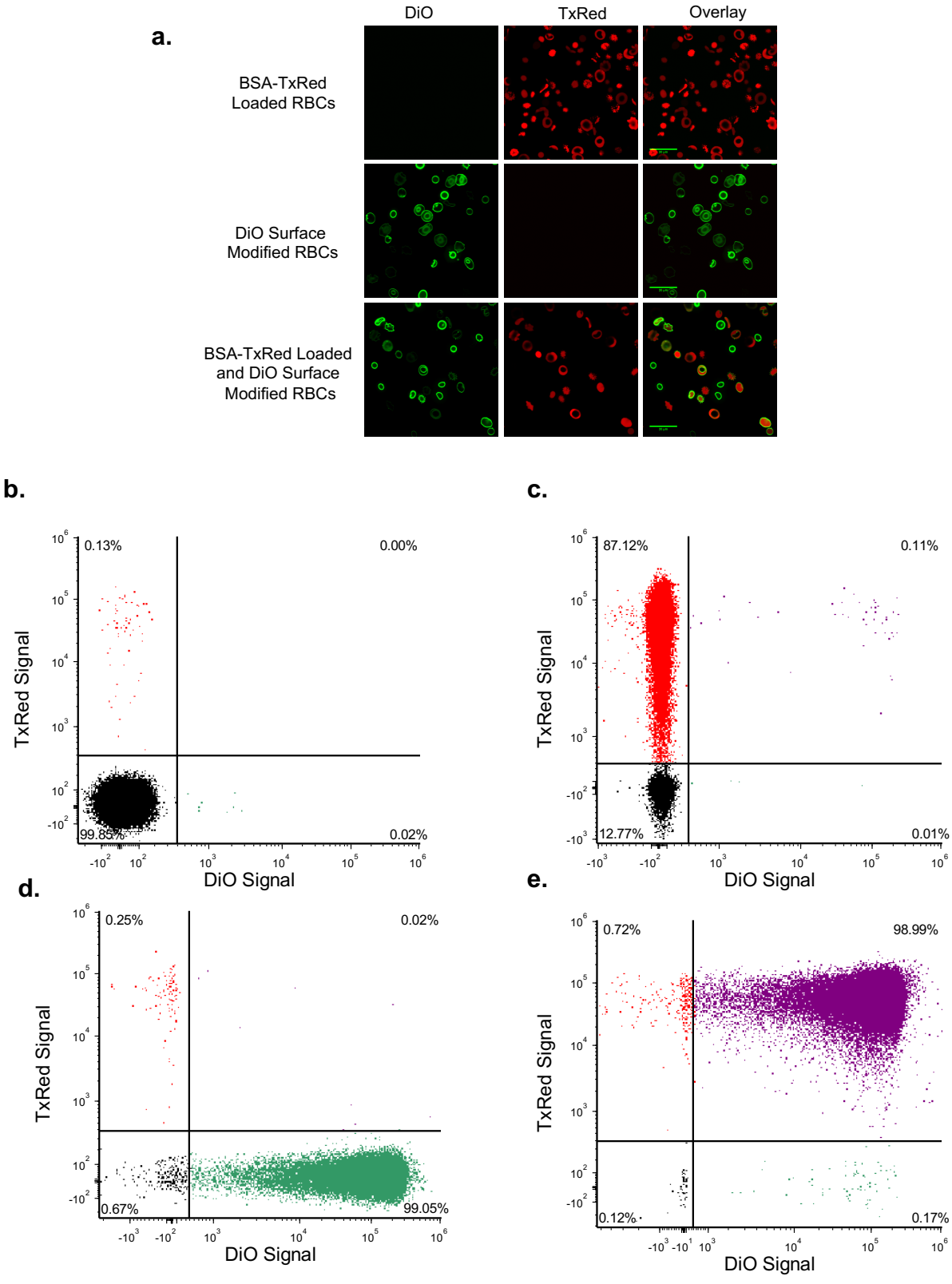

a.
DiO
TxRed
Overlay
BSA-TxRed Loaded RBCs
DiO Surface Modified RBCs
BSA-TxRed Loaded and DiO Surface Modified RBCs
b.
0.13%
0.00%
99.85%
0.02%
TxRed Signal
DiO Signal
c.
87.12%
0.11%
12.77%
0.01%
TxRed Signal
DiO Signal
d.
0.25%
0.02%
0.67%
99.05%
TxRed Signal
DiO Signal
e.
0.72%
98.99%
0.12%
0.17%
TxRed Signal
DiO Signal

Figure S9. a. Single confocal microscopy slice of human RBCs internally loaded with BSA-TxRed (top row), surface modified with the membrane labeling DiO dye (middle row), or both (bottom row). The overlay demonstrates the successful internal loading of the BSA-TxRed using the dialysis method described. **b**. Flow cytometry scatter plot of human RBCs that have been internally mock loaded with buffer, **c.** BSA-Texas Red, **d.** surface modified with DiO, **e.** internally loaded with BSA-Texas Red and surface modified with DiO.

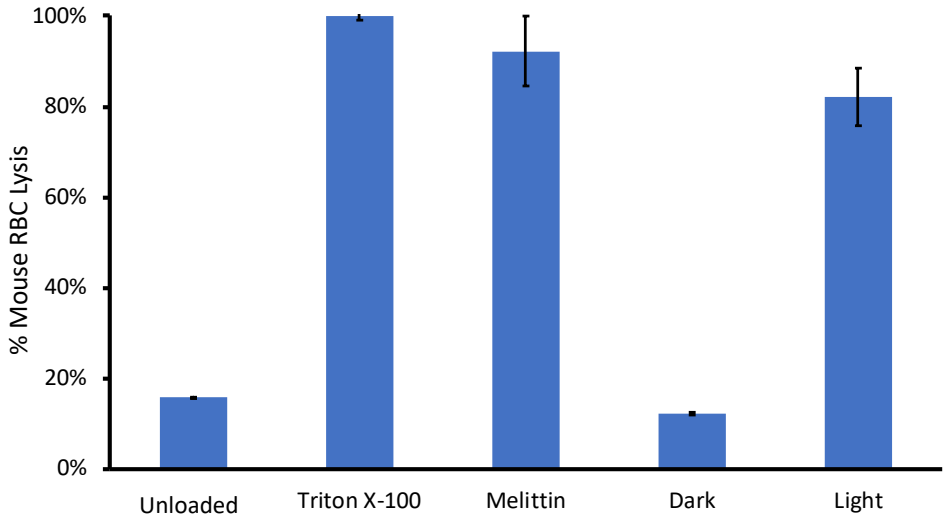


Figure S10. Mouse RBC (mRBC) lysis as a function of various conditions. BSA-TexasRed is embedded within the interior of RBCs. Following exposure of the mRBCs to various conditions, the vessels are centrifuged and Texas Red fluorescence in the supernatant is taken as a measure of hemolysis. **Unloaded**: Minimal lysis is observed with mRBCs lacking a surface-anchored photolytic trigger (Unloaded) that were illuminated with 525 nm LEDs. **Triton X-100**-treated RBCs with surface-anchored C_{18}-Cbl-BS/C_{18}-Mel is used as the 100% lysis control. **Dark or Light** mRBCs are cells containing surface-anchored C_{18}-Cbl-BS/C_{18}-Mel and exposed to the dark (minimal lysis) or 525 nm LEDs.

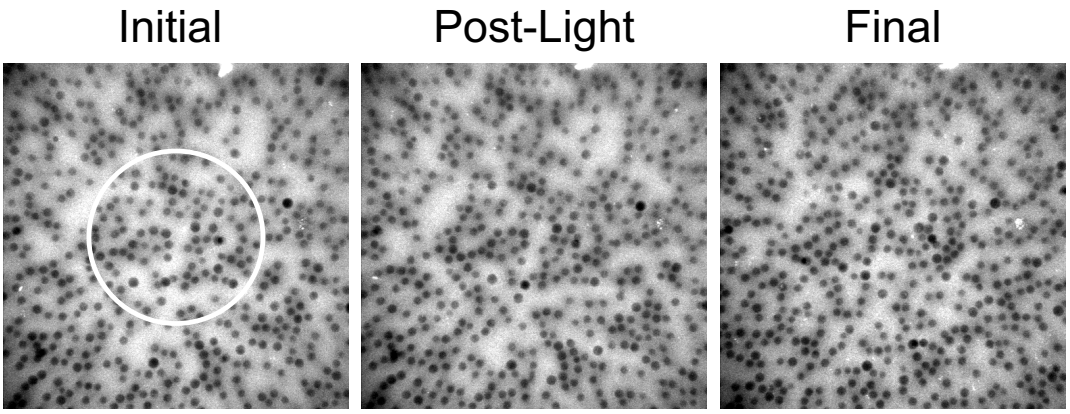
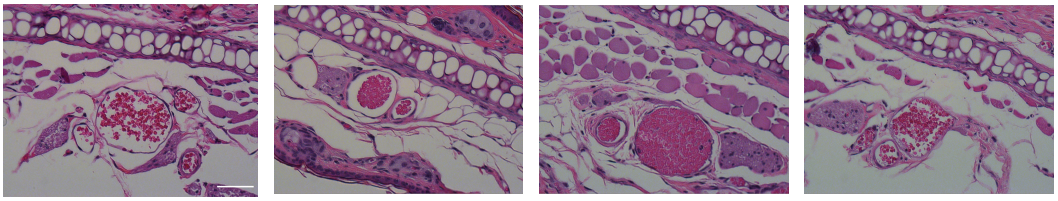
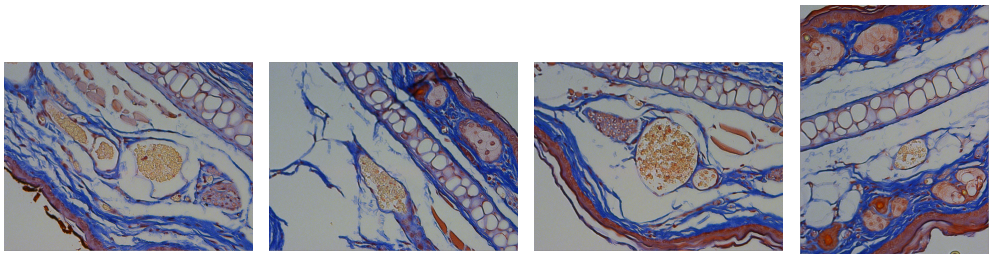


Figure S11. RBCs internally loaded with thrombin and surface modified with **only** C_{18}-Cbl-BS were incubated with (Alexa Fluor 647)-fibrinogen. The region of illumination is highlighted prior to (left), during (middle), and 210 s following (right) 515 nm exposure. Thrombin-loaded RBCs that were only surface modified with C_{18}-Cbl-BS show no change in fluorescence after photolysis.

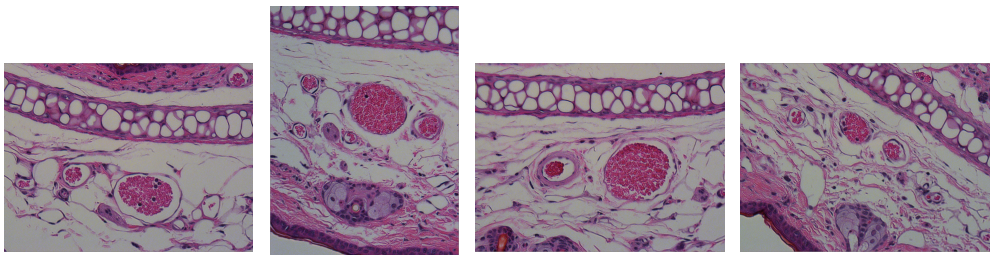
a.



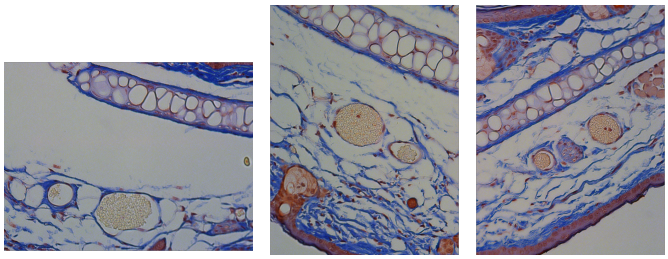
b.



c.



d.



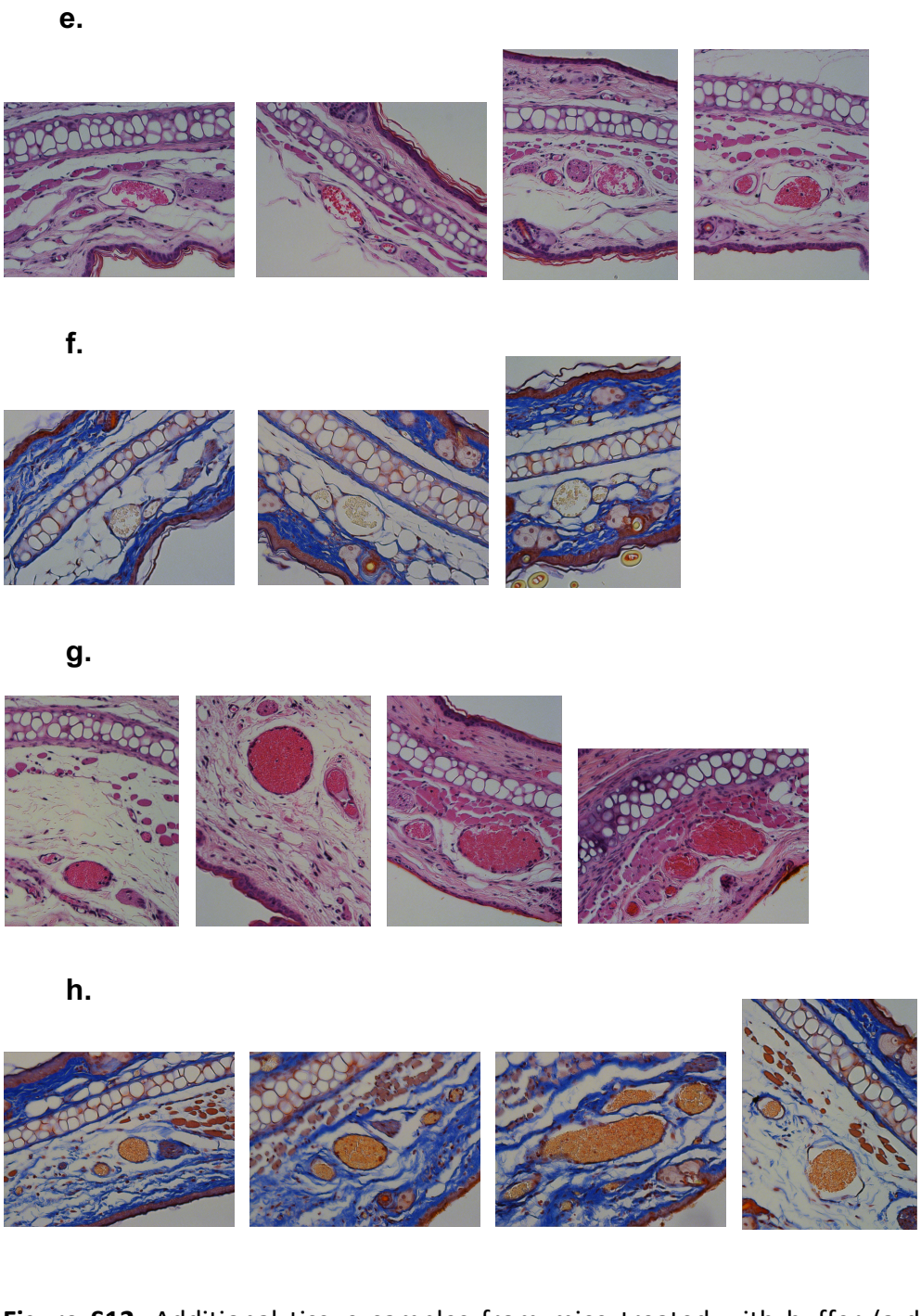


Figure S12. Additional tissue samples from mice treated with buffer (a-d) or thrombin (e-h) internally loaded murine RBCs surface modified with C_{18}-Mel/C_{18}-Cbl-BS. Murine RBCs internally loaded with buffer or thrombin and surface modified with C_{18}-Mel/C_{18}-Cbl-BS were tail vein injected into healthy FVB mice (n = 4 for each experimental condition). A 1 mm^2 region of one ear from each mouse was illuminated (561 nm) under a confocal microscope, the mice euthanized, and both "dark" (a, b, e, f) and "light" (c, d, g, h) ears harvested. 4 µm cross-sections of the fixed tissues were stained with H&E (a, c, e, g) and Martius Scarlet Blue dyes (b, d, f, h). Scale bar in **12.a** = 50 µm.

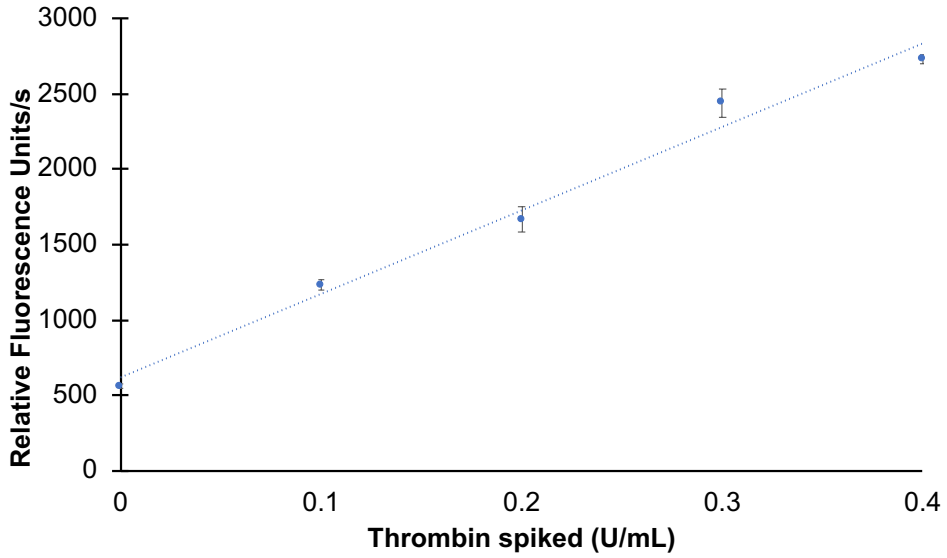


Figure S13. Standard addition plot to determine thrombin activity using peptide substrate that was hydrolyzed by thrombin in the sample and spiked thrombin standards that resulted in an increase of fluorescence (λ_{ex} = 370 nm; λ_{em} = 450 nm) over time, y=5544.8x + 615.68, R^2=0.99.

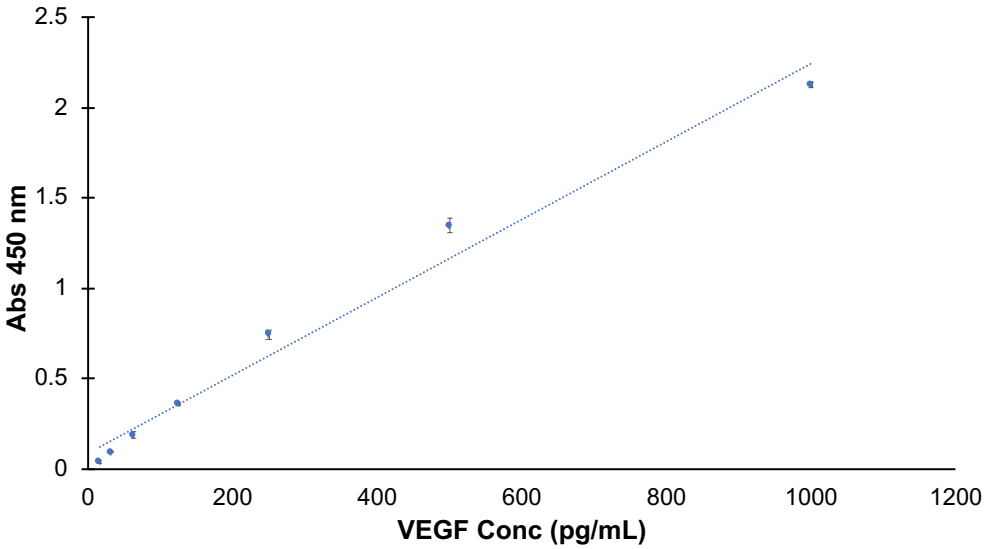


Figure S14. Standard curve of VEGF concentrations from ELISA with absorbance measured at 450 nm, y=0.0022x + 0.09, R^2=0.98.