SUPPLEMENTAL MATERIAL

Multimodal Imaging of the Receptor for Advanced Glycation End-products with Molecularly Targeted Nanoparticles

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SUPPLEMENTAL INFORMATION

CHEMICAL SYNTHESIS

Chemicals

Human serum albumin (HSA, fraction V), ethylenediamine-core PAMAM G4 dendrimer, glyoxylic acid, $NaCNBH_3$, and anhydrous DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Sulfosuccinimidyl (Sulfo-NHS) acetate and PEGylated bis(sulfosuccinimidyl)suberate (Bis(NHS)PEG_5) were purchased from Thermo Scientific (Waltham, MA). 2-(4-isothiocyanatobenzyl)-NOTA (p-SCN-Bn-NOTA) was purchased from Macrocyclics (Plano, TX). Rhodamine (NHS ester) was purchased from Lumiprobe (Hunt Valley, MA).

Synthesis of G4-$[Ac]_{32}$.

Five hundred milligrams of ethylene-diamine-core PAMAM generation 4 dendrimer were desiccated in a 100 mL round-bottom flask and subsequently dissolved in 100 mM sodium bicarbonate buffer (pH 9.0) to a final concentration of 10 mg/mL with magnetic stirring. To this dendrimer solution, 36 mol equiv of sulfosuccinimidyl acetate were added as a solid and allowed to dissolve. The pH of the reaction mixture was immediately adjusted to 8.5 with 1 N NaOH and the reaction was allowed to proceed for 2 h at 25 °C. The partially acetylated PAMAM (G4) product was purified by ultrafiltration with deionized water using 10K MWCO Amicon Ultra-15 filters (Millipore) and lyophilized to obtain a white crystalline solid (yield: 97%). The product (**Figure S1A**) was characterized by proton nuclear magnetic resonance (1H NMR) spectroscopy (D_2O, 400 MHz); δ= 1.91 (105H, $COCH_3$), 2.28-2.42 (248H, C-CH_2-CONH), 2.50-2.61 (124H, N-CH_2CH_2NH), 2.68-2.80 (248H, N-CH_2CH_2CO), 3.14-3.36 (318H, CONH-CH_2). Extent of surface acetylation was determined according to the method of Majoros et al.[3]

Synthesis of G4-$[Ac]_{32}$-$[NOTA]_{16}$.

Six hundred milligrams of G4-$[Ac]_{32}$ were added to a 100-mL round-bottom flask and subsequently dissolved in 100 mM sodium bicarbonate buffer (pH 9.0) to a final concentration of 10 mg/mL with magnetic stirring. To the dendrimer solution, 16 mol equiv of SCN-Bz-NOTA dissolved in anhydrous DMSO, were added. The pH of the reaction mixture was immediately adjusted to 8.5 with 1N NaOH, and the reaction proceeded for 18 hr at 25 °C in the absence of light. The product was purified by ultrafiltration with deionized water using 10K MWCO Amicon Ultra-15 filters (Millipore), lyophilized and stored at -80°C. The product (**Figure S1B**) was characterized by proton nuclear magnetic resonance (1H NMR) spectroscopy (D_2O, 400 MHz).

Synthesis of G4-$[Ac]_{32}$-$[NOTA]_{16}$-$[Rho]_{8}$.

To render dendrimer nanoparticle multimodal, G4-$[Ac]_{32}$-$[NOTA]_{16}$ was dissolved in 100 mM sodium bicarbonate buffer (pH 8.5) to a final concentration of 10 mg/mL with magnetic stirring and incubated with 8 mol equiv of rhodamine NHS ester for 1 hr at 25 °C in the absence of light. The product was purified by ultrafiltration with deionized water using 10K MWCO Amicon Ultra-15 filters (Millipore), lyophilized and stored in dark at -80°C. The product (**Figure S1C**) was characterized by proton nuclear magnetic resonance (1H NMR) spectroscopy (D_2O, 400 MHz).

Synthesis of G4-$[Ac]_{32}$-$[NOTA]_{16}$-$[Rho]_{8}$-$[PEG_5\text{-}X]_{8}$.

First, HSA-CML or HSA alone (10 mg/mL) was dissolved in PBS (pH 7.4) and incubated with 20-fold excess of Bis(NHS)PEG_5 for 1 hr at 25 °C to crosslink PEG to N-terminus of HSA-CML or HSA alone. After the reaction was completed, the product was purified using 10K MWCO Amicon Ultra-15 filters (Millipore) and lyophilized. Next, G4-$[Ac]_{32}$-$[NOTA]_{16}$-$[Rho]_8$ was dissolved in 100 mM sodium bicarbonate buffer (pH 9.0) to a final concentration of 10 mg/mL with magnetic stirring. To the dendrimer solution, 8 mol equiv of NHS-PEG_5-HSA-CML (for RAGE-targeted nanoparticle) or NHS-PEG_5-HSA (for non-targeted control) was added and incubated for 1 hr at 25 °C. The final product was purified using 100K MWCO Amicon Ultra-15 filters (Millipore) and lyophilized.

Chemical modification of HSA (synthesis of CML)

Carboxymethyllysine (CML) modified HSA was prepared as described previously.[1] Briefly, 100 mg/mL of HSA (87.3 mM lysine equivalents) was dissolved in 10 mL of 0.2 M sodium phosphate buffer (pH 7.8). After adding 87.3 mM glyoxylic acid and 261.9 mM $NaCNBH_3$, the solution was stirred for 24 hr at 37 °C protected from light, followed by two-times dialysis against sodium phosphate buffer (10 mM, pH 7.0) and once against sodium chloride (100 mM). To prepare CML samples with different levels of modification (0 – 100%) of lysine groups, HSA solution (100 mg/mL) was incubated in the same way as described above except for various concentrations of glyoxylic acid and 3x molar excess of $NaCNBH_3$. CML solutions were purified and concentrated using 10K MWCO Amicon Ultra-4 filters (Millipore) by centrifugation (4000 g for 40 min). Protein concentrations were determined by bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) using HSA standards. To confirm the amount of modified lysine residues we used 2,4,6-trinitrobenzenesulfonic acid (TNBS) and method published previously[2] to determine the difference in free amino groups between modified and unmodified protein preparations (**Figure S2)**. For subsequent experiments, CML samples were lyophilized and stored at -80 °C.

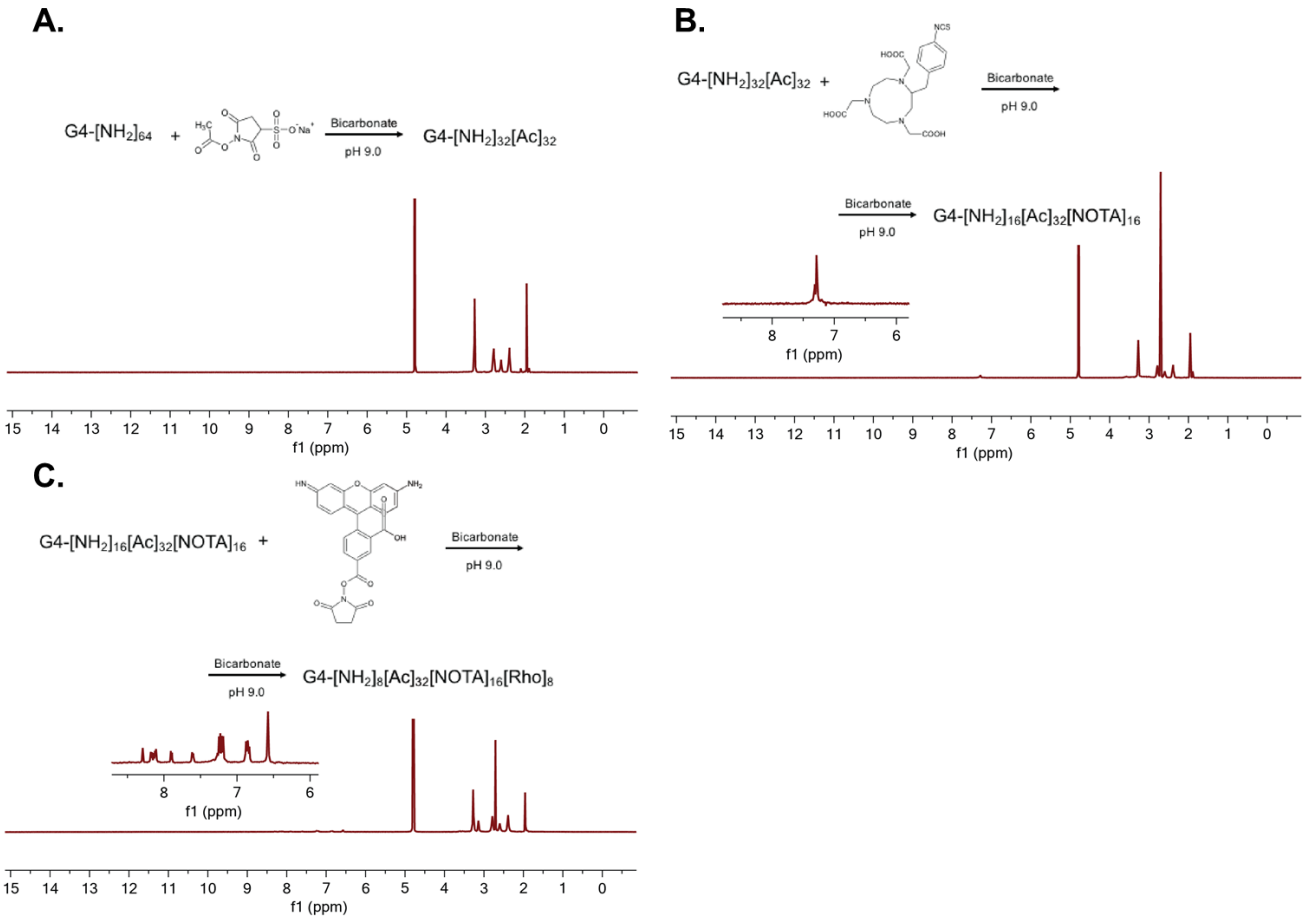

Figure S1. Schematic of the synthesis of multimodal RAGE-targeted G4-dendrimer nanoparticle with ^{1}H NMR characterization of the synthetic route. The final step (not shown) involved targeting at RAGE by conjugating the well-characterized RAGE ligand, carboxymethyl-lysine (CML) modified human serum albumin (HSA) or HSA only (for non-targeted control nanoparticle) via PEG_5 linker. 1H NMR spectra were acquired at 400 MHz for 20 mg/mL solutions of products from intermediate synthesis steps.

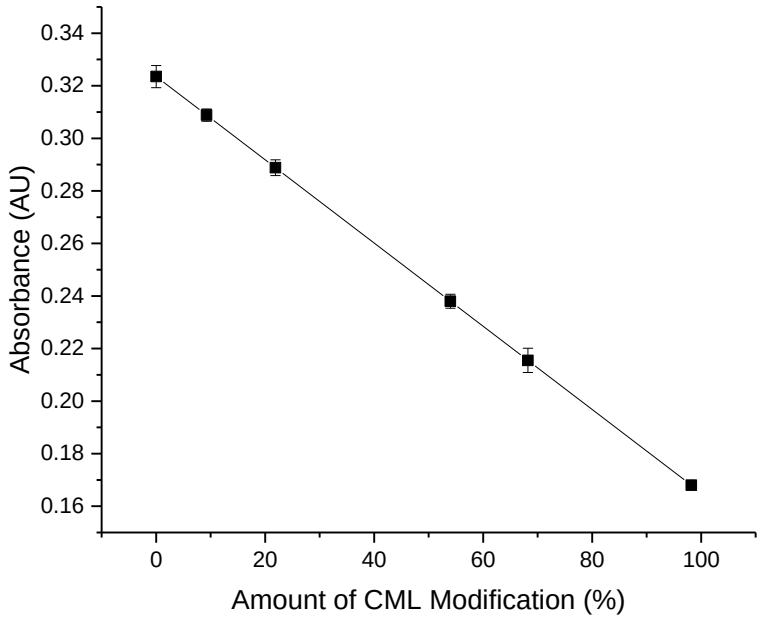

Figure S2. Absorbance readings detect amount free amine groups and confirm CML modification of HSA protein.

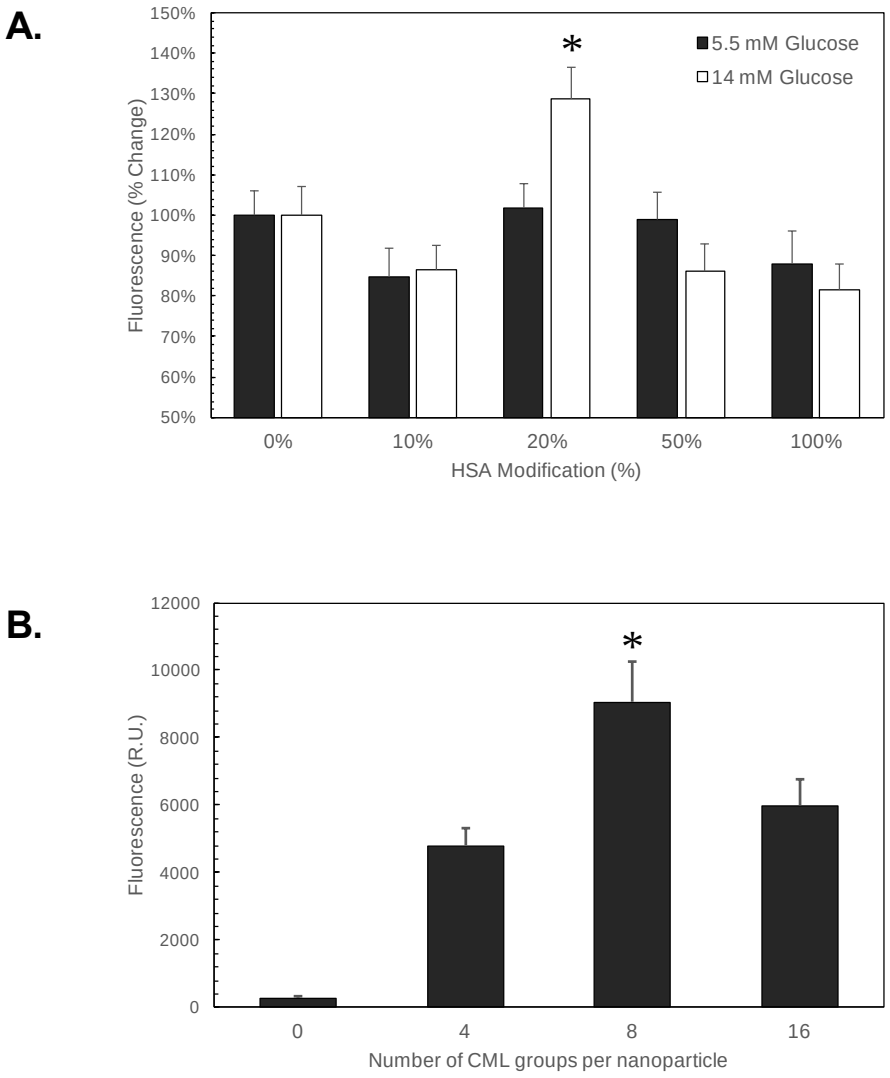

Figure S3. Optimization of G4-CML binding properties to HUVECs by varying the level of the modification of albumin lysine groups (A) and number of modified carboxy-methyl-lysine albumin molecules conjugated to G4 dendrimer (B).

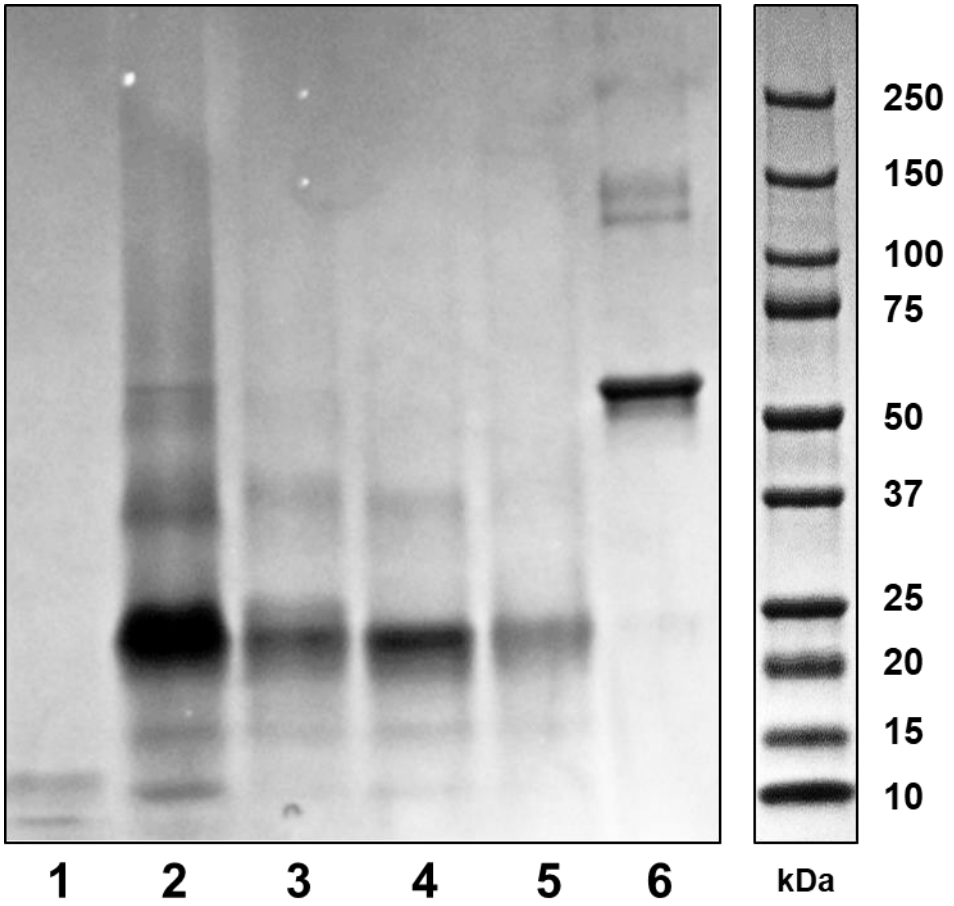

Figure S4. SDS-PAGE gel of the intermediate products of G4-Ac-NOTA-Rho-PEG_4-CML synthesis. Lanes from left to right represents (1) G4 PAMAM dendrimer, (2) G4-Ac, (3) G4-Ac-NOTA, (4) G4-Ac-NOTA-Rho, (5) G4-Ac-NOTA-Rho-PEG_4 and the final product (6) G4-Ac-NOTA-Rho-PEG_4-CML. Final construct (6) shows high molecular weight band (targeted nanoparticle construct) while a low molecular weight band near 53 kDa is due to unbound CML protein that was not filtered from the solution, but is prior to all experiments.

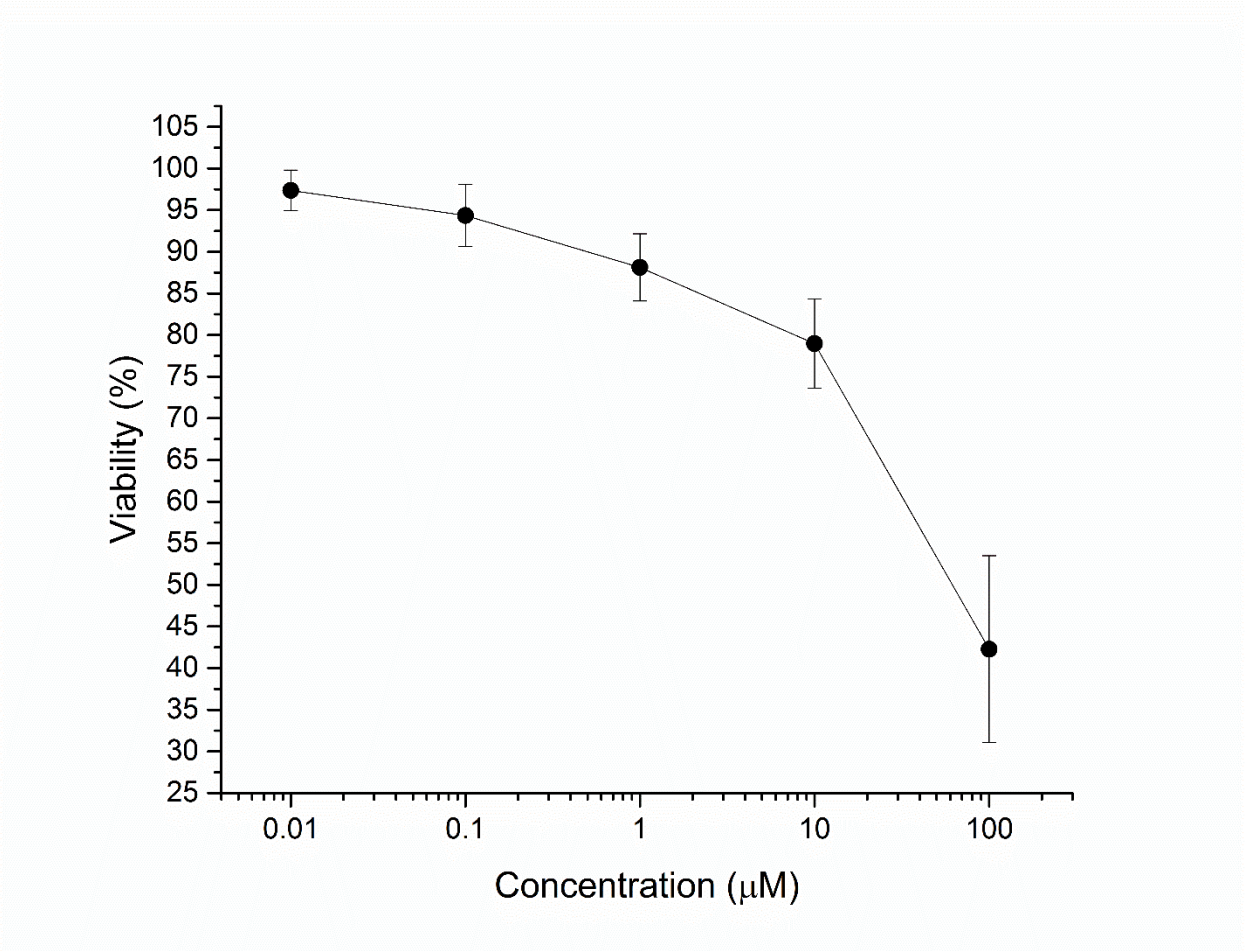

Figure S5. Trypan Blue viability assay to assess toxicity of G4-Ac-NOTA-Rho-PEG_4-CML probe. Probe demonstrates noticeable toxicity between 10-100 μM (well above concentrations used for imaging or cell studies).

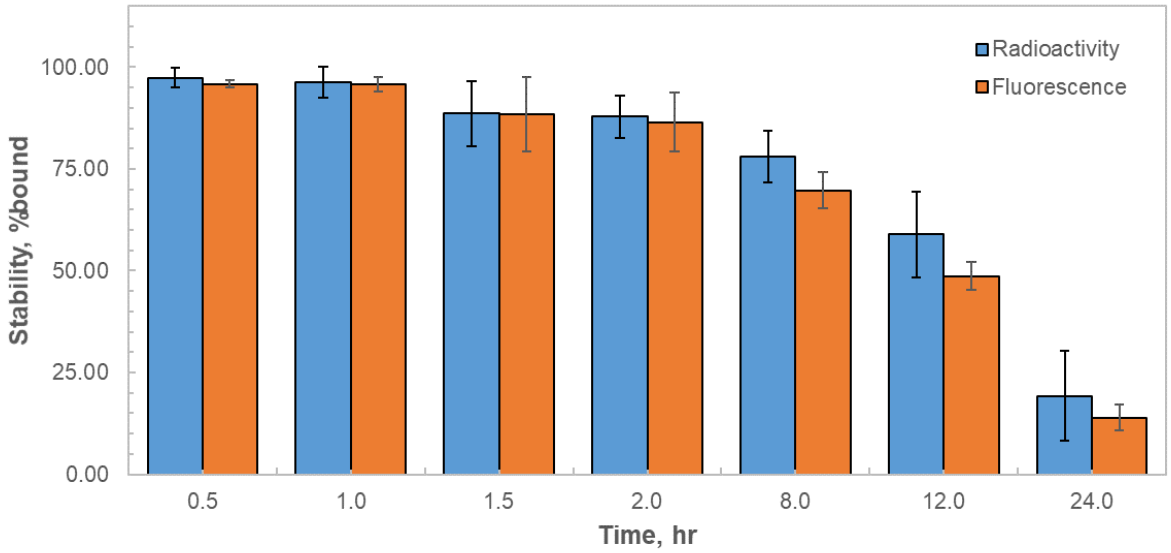

Figure S6. Stability of ^{64}Cu-Rho-G4-CML was assessed by incubating probe in plasma. ^{64}Cu-Rho-G4-CML remained stable through the imaging time-frame (1 hour) and remained constant up until ~12 hours, after which there was a significant decrease in stability.

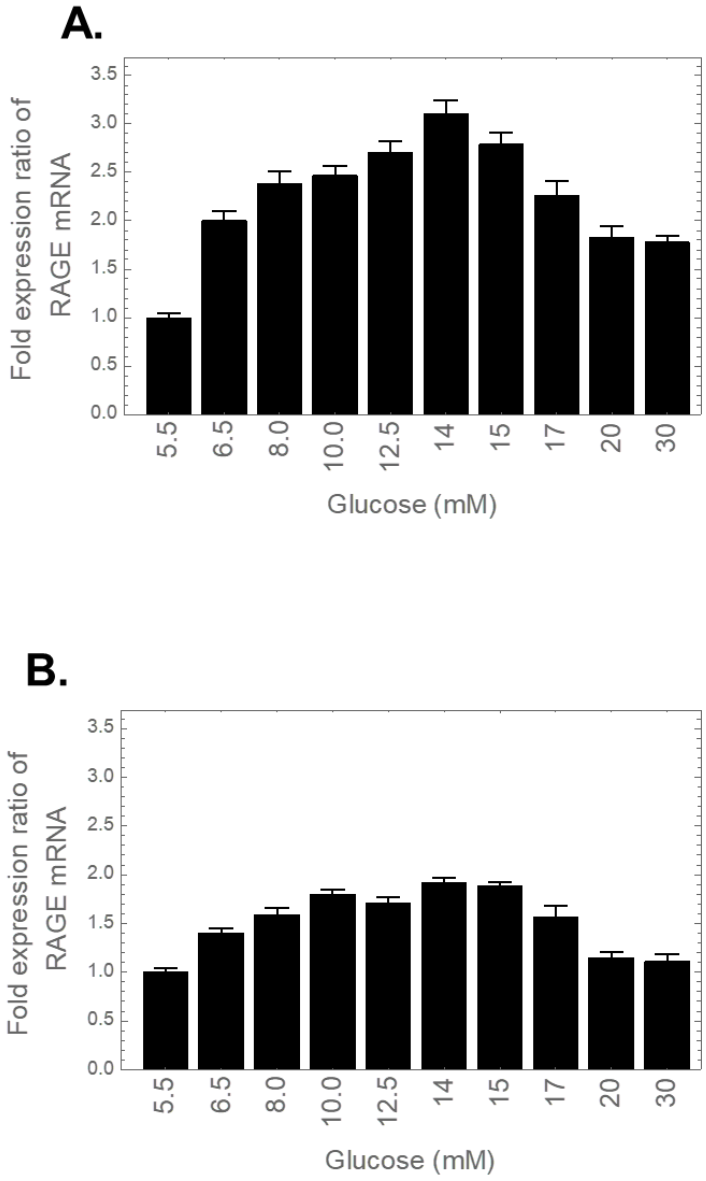

Figure S7. RAGE mRNA levels in HUVECs cultured in various glucose concentrations (5.5 mM – 30 mM) for the duration of 12 hours (A) and 24 hours (B). Incubation of HUVECs between 12-14 hours in diabetic-like environment with 14 mM glucose induced the highest RAGE mRNA levels.

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(2) Habeeb, A. F. S. A. Determination of Free Amino Groups in Proteins by Trinitrobenzenesulfonic Acid. *Anal. Biochem.* **1966**, *14*, 328–336.

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