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Supplementary information

Zwitterionic glutathione monoethyl ester as a new capping ligand for ultrasmall gold nanoparticles

Luiza Knittel†, Peter Schuck#, Christopher J. Ackerson§, Alioscka A. Sousa†,*

†Department of Biochemistry, Federal University of São Paulo, São Paulo, SP 04044, Brazil.

National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD 20892, USA.

§ Department of Chemistry, Colorado State University, Fort Collins 80521, USA.

MATERIALS AND METHODS

Reagents. $HAuCl_4 \cdot 3H_2O$, $NaBH_4$, glutathione, tiopronin, DMEM cell culture medium and fetal bovine serum (FBS) were purchased from Sigma-Aldrich; *p*MBA was purchased from TCI America; the dipeptide CG and glutathione monoethyl ester were acquired from Bachem; streptactin- and streptavidin-coated sepharose beads were purchased from GE Healthcare Life Sciences; the peptides ECGK-biotin and ECGGGWSHPQFEK were synthesized by EZBiolab (Carmel, IN, USA) and Proteimax (Cotia, SP, Brazil) respectively.

Nanoparticle synthesis. The synthesis of ~ 2 nm-diameter *p*MBA-coated gold nanoparticles (AuMBA) having a molecular formula of $Au_{144}(pMBA)_{60}$ has been detailed in a number of publications[1-2]. Briefly, 2 mmol of $HAuCl_4$ dissolved in 100 mL of methanol and 6.8 mmol of *p*MBA dissolved in 80 mL water (pH > 13) were mixed together and stirred for 2 days. After stirring, 50 mL of the product Au(I)-*p*MBA was added to a 270 mL/730 mL water/methanol mixture followed by addition of 3 mL of 0.25 M $NaBH_4$. This final mixture was then stirred for 18h and the product precipitated by adding 1 L of methanol and 200 mL of 2M ammonium acetate. After a few precipitation-wash cycles, the product was left to dry in air and resuspended in PBS. We note that the exact amount of $NaBH_4$ to use in the reaction should be determined empirically by finding the optimum $NaBH_4$:Au(I) molar ratio that leads to AuNPs of the right size and uniformity. Ligand exchange of AuMBA with CG, tiopronin, glutathione and glutathione monoethyl ester was performed as described previously for glutathione[3]. Briefly, ligand exchange was carried out in PBS using a 10:1 molar feed ratio of incoming:*p*MBA ligands. After reacting for 3h at room temperature, the AuNPs were induced to aggregate/precipitate by addition of an equal amount of ethanol and 20% by volume of 2 M ammonium acetate. The ligand-exchanged particles were purified by repeated centrifugation-wash cycles. After the final wash, the AuNPs were left to dry in air and resuspended in PBS.

Nanoparticle functionalization. ECGGGWSHPQFEK was added to a solution of AuMBA particles at a 2:1 or 5:1 molar feed ratio of incoming ligand:nanoparticle. After reacting for 2h at room temperature, the AuNPs were passivated with either glutathione or glutathione monoethyl ester by ligand exchange as described above. Incorporation of ECGK-biotin was identical except that a 10:1 molar feed ratio of incoming ligand:nanoparticle was used. The successful functionalization of AuGSH(neg) and AuGSH(zwt) was confirmed as described in Figs. S2, S3.

'Pull-down'-type binding assay. A small aliquot (10 μL) of either strep-tagged or biotinylated AuNPs was first diluted in 100 μL of pure FBS. The AuNPs in FBS were next added to an equal volume of streptactin-coated sepharose beads resulting in a final nanoparticle concentration of 2-4 μM. The concentration of FBS in the final mixture was higher than 50% because the beads (34 μm in diameter) exclude a large volume of solution. Pictures of solutions were taken after settling of the sepharose beads; UV-vis spectra were recorded from the supernatant using a Shimadzu UV-1800.

Scanning transmission electron microscopy (STEM) imaging. High–angle annular dark–field (HAADF) STEM images of AuNPs were recorded in a 300 kV Tecnai TF30 transmission electron microscope (FEI Company) equipped with a Schottky field emission gun and a model–3000 HAADF detector (Fischione Instruments). Image quantification was carried out as described previously[4]. Briefly, in HAADF STEM imaging of ultrasmall AuNPs, the intensity at each pixel is proportional to the projected mass of Au at that pixel (after background subtraction). Taking AuMBA as a calibration standard (144 Au atoms; 2 nm diameter), we computed the distribution of nanoparticle diameters (d) for AuGSH(zwt) according to the equation $d = 2(N/144)^{1/3}$, where N was the measured number of Au atoms/particle in the STEM images.

Analytical ultracentrifugation. Sedimentation velocity experiments were carried out in an Optima XL-I analytical ultracentrifuge equipped with GUI version 5.7 and firmware version

5.06 (Beckman Coulter) using standard methods[5]. AuNPs were characterized by AUC in pure PBS for measurement of size and uniformity, in PBS supplemented with 25% cell culture medium for assessment of aggregation, and in PBS supplemented with 10% FBS for assessment of aggregation and serum protein binding. Samples were pre–incubated in the different media for 3h at room temperature prior to measurements. The rotor speed was set to 25,000 rpm and absorbance scans were recorded at 520 nm. Data were analyzed in the software SEDFIT with a c(s) sedimentation coefficient distribution[6]. Sedimentation coefficients were corrected for relative viscosity of the different solutions[4].

Inductively coupled plasma mass spectrometry (*ICP-MS) analysis.* RAW 264.7 macrophages were seeded in a 24 well plate and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C and in a 5% CO_2 atmosphere. After 24h, cells were washed 3 times with PBS and incubated with either 2 μM AuGSH(neg) or AuGSH(zwt) in PBS for 2h. Next, cells were washed 3 times with PBS to remove excess nanoparticles; this was followed by short treatment with a I_2/KI mixture (0.25 mM I2; 0.12 M KI; 30 seconds) to dissolve extracellular particles strongly adhered to the plate surface and extracellular components[7]. Cells were washed one last time with PBS and digested with 2 mL of aqua regia for 6h. The cell digest was diluted to 10 mL with ultra pure water and analyzed by ICP-MS (Midwest Laboratories, Omaha, NE, USA) for quantification of the amount of Au.

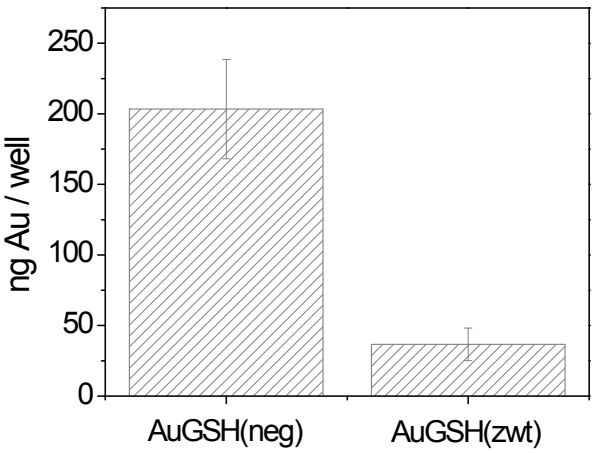

Figure S1. ICP-MS analysis of the amount of internalized Au by RAW 264.7 macrophages.

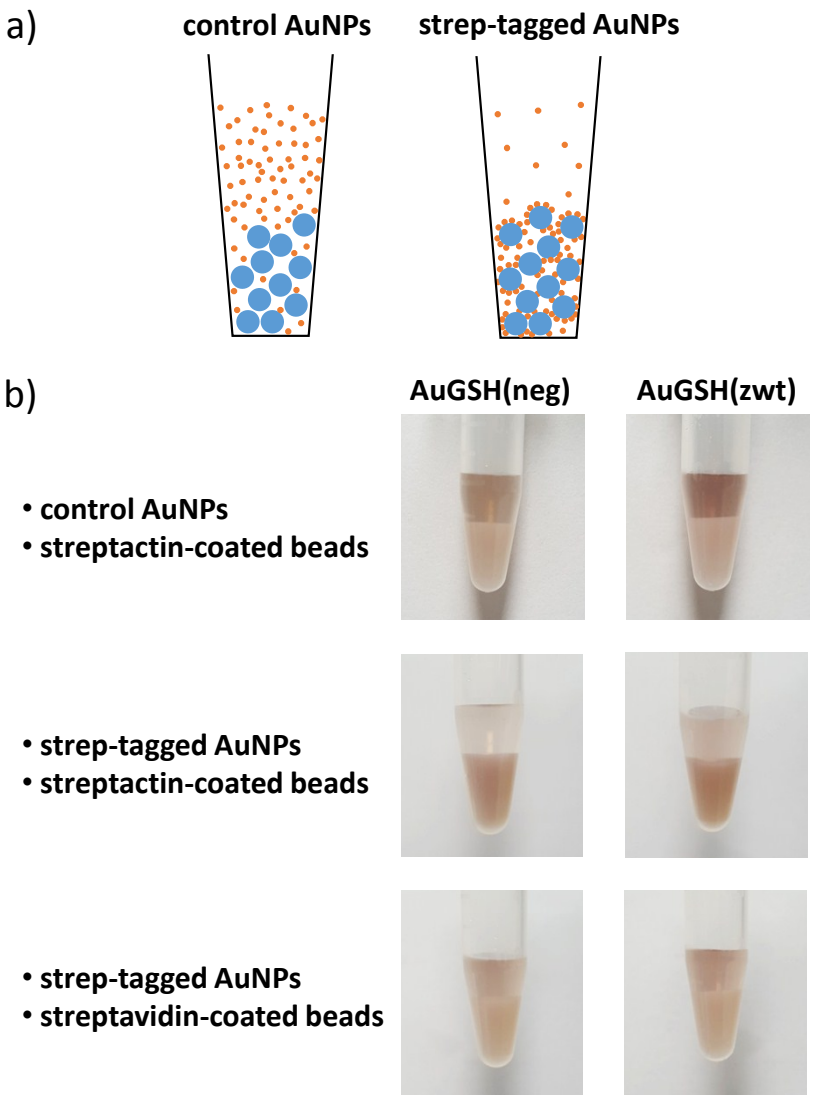

Figure S2. Binding of strep-tagged AuNPs to streptactin-coated beads in PBS. (a) Schematic illustration of binding experiment. Non-functionalized (control) and strep-tagged AuNPs are incubated with streptactin-coated sepharose beads in PBS. Control AuNPs do not bind streptactin and remain mostly in the supernatant after settling of the beads. Strep-tagged AuNPs bind streptactin and settle down with the beads. AuNPs are represented as orange spheres and beads as blue spheres. **(b)** (Top row) Control AuNPs in PBS were mixed with streptactin-coated sepharose beads. After settling of the beads, solutions showed a nanoparticle-rich (brownish) supernatant phase. (Middle row) Strep-tagged AuNPs in PBS were mixed with streptactin-coated sepharose beads. AuNPs bound streptactin leaving a supernatant phase depleted of nanoparticles. (Bottom row) Additional evidence for the binding specificity of strep-tagged AuNPs to streptactin-coated beads was obtained by replacing streptactin for streptavidin. Due to the much lower binding affinity of strep-tag II for streptavidin (~ 70 μM) relative to streptactin (~ 1 μM)[8], strep-tagged AuNPs did not efficiently bind streptavidin-coated beads thus resulting in a brownish supernatant phase.

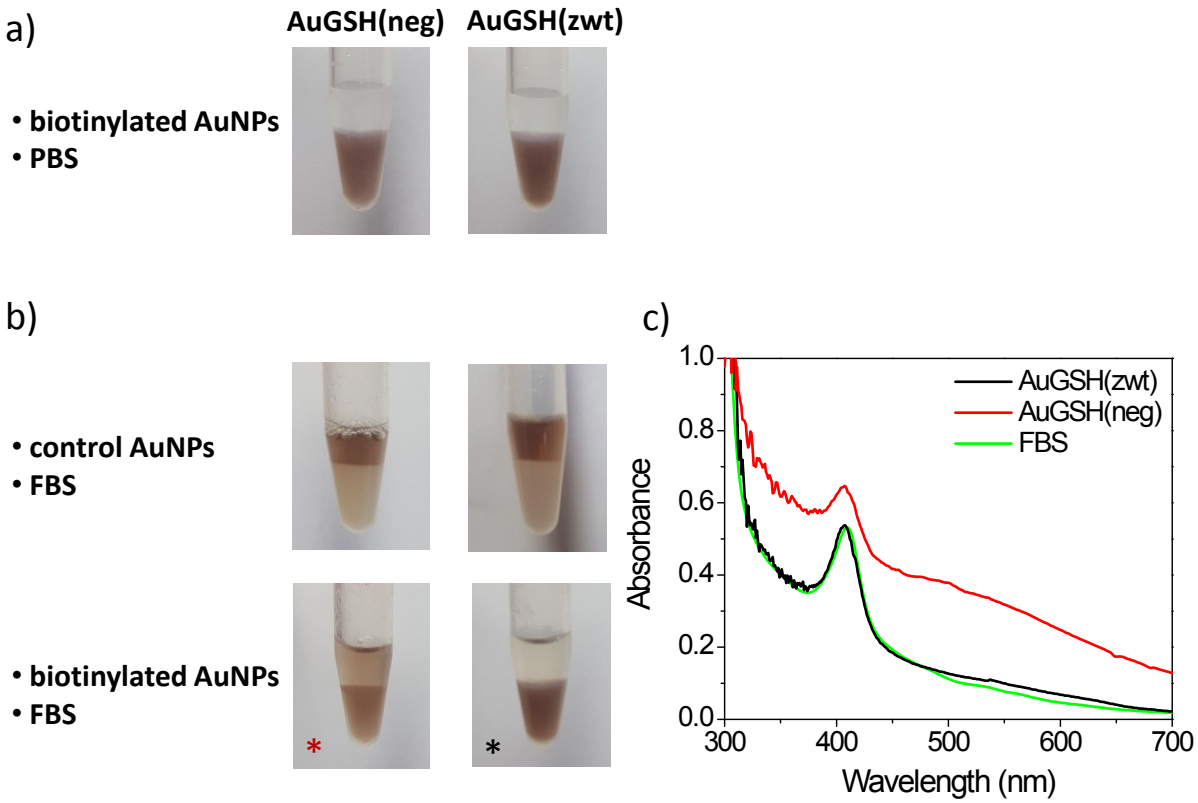

Figure S3. Binding of biotinylated AuNPs to streptactin-coated beads in serum. (a) Biotinylation of AuNPs was first confirmed by incubation with streptactin-coated sepharose beads in PBS. Upon binding to streptactin, AuNPs settled with the beads leaving a clear supernatant. **(b)** (Top row) Non-functionalized (control) AuNPs in FBS did not bind streptactin remaining mostly in the supernatant after settling of the beads (same figure as Fig. 5b, top row). (Bottom row) Relative to strep-tagged AuGSH(neg) (Fig. 5b, bottom row), biotinylated AuGSH(neg) interacted with streptactin much more efficiently. Biotinylated AuGSH(zwt) bound streptactin completely as apparent from the clear supernatant phase. **(c)** UV-vis spectra of supernatant of solutions marked with an asterisk in (b). Supernatant of AuGSH(neg) was diluted twice in PBS before analysis by UV-vis. Spectrum in green was recorded from a control sample of FBS (diluted 1:1 in PBS). Peak at 400 nm corresponds to FBS components. Overlapping UV-vis spectra of FBS control and AuGSH(zwt) indicates that binding of biotinylated AuGSH(zwt) to streptactin was complete.

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