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Supporting Information

Toxicity and Cellular Uptake of Gold Nanorods in Vascular Endothelium and Smooth Muscles of Isolated Rat Blood Vessel: Importance of Surface Modification

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Chemicals and Materials. Chloroauric acid ($HAuCl_4.3H_2O$, 99.9%), sodium borohydride ($NaBH_4$, 99%), silver nitrate ($AgNO_3$, 99+%), ascorbic acid (99+%), poly(allylamine hydrochloride), (PAH, MW ~ 15000 g/mole) and poly(acrylic acid, sodium salt) (PAA, MW ~15000 g/mole), Cetyltrimethylammonium bromide (CTAB, 99%, Sigma Ultra), and ionomycin were obtained from Sigma-Aldrich Chemicals and used as received. All solutions were prepared with 18 MΩ Milli-Q water (Millipore). Glassware was cleaned with aqua regia and rinsed thoroughly with 18 MΩ Milli-Q water. Dialysis cassettes (slide-A-lyzer dialysis cassettes, cutoff MW=3,500 or 10,000 Dalton) were purchased from Thermo Scientific. m-PEG-Thiol (MW 5000) was purchased from Lysan Bio Inc. Bovine aortic endothelial cells (BAEC) and Endothelial Growth Medium were obtained from Cell Applications (San Diego, CA). WST-1 kit was purchased from Roche Applied Science (Mannheim, Germany). LIVE/DEAD viability assay kit was purchased form Molecular probes (Eugene, OR) contains two dyes: Ethidium homodimer

and calcein-AM. Total protein assay kit (D_c Bio-Rad protein assay) was purchased from Bio-Rad Laboratories, Hercules, CA.

Instrumentations. Absorption spectra for gold nanorods and absorbance values for the WST-1 assay were collected on a PowerWave X52 microplate reader (Bio-tek, Winooski, VT). Transmission electron microscopy (TEM) data for gold nanoparticles were obtained with a JEM 1230 transmission electron microscope (JEOL USA Inc., Peabody, MA) at 110 kV. TEM grids were prepared by drop-casting the purified gold nanorod solution (7 μL) on the TEM grids and drying them in atmosphere. Zeta potential measurements and dynamic light scattering analysis were performed on a Brookhaven Zeta PALS instrument at 25 ^{o}C. An ultracentrifuge (model J2-21, Beckman instruments) was used in gold nanorod synthesis and purification. Vascular function tests (myography) were carried out using aortic rings that mounted in an oxygenated wire myograph chamber (Danish Myo Technology, Multi Myograph model 610 M). Nitric oxide (NO) analysis was done using NO analyzer (Sievers, Boulder, CO). Fluorescence images were taken using a Zeiss LSM 510 Axioplan 2 Confocal Laser Scanning Microscope with an IR-Achroplan water dipping 40x objective.

CTAB-capped gold nanorod (CTAB-GNRs) synthesis. CTAB-capped gold nanorods were synthesized using the wet chemical seed-mediated method as previously described.[1] The synthesis starts with preparing gold seed solution, which was used to prepare gold nanorods.

A solution of 2.5 x 10^{-4} M $HAuCl_4$ was prepared in 0.1 M CTAB. $NaBH_4$ (600 μL, 10 mM) at 0 ^oC was added to the gold/CTAB solution (10 mL) with vigorous stirring for 10 min. The resulting seed nanoparticles (~3 nm) were used in the synthesis of gold nanorods by seeding growth solution which is prepared by mixing the following aqueous solutions in a 125 mL conical flask: CTAB solution (95 mL, 0.1 M), silver nitrate solution (0.8 mL, 10 mM; aspect ratio of the gold nanorod is controlled by varying silver nitrate concentration), chloroauric acid (5 mL, 10 mM), ascorbic acid solution (0.55 mL, 0.1 M). Finally seed solution (0.12 mL) was added and the entire solution was mixed and left undisturbed overnight (14-16 hours) for gold nanorods growth. The brown-colored gold nanorod solution was purified by centrifugation to remove excess CTAB (twice at 14000 rpm, 3 min each). Attempts to remove CTAB by dialysis led to irreversible nanoparticle aggregation, unsuitable for the present work.[2]

PAA-coated gold nanorods (PAA-GNRs).[3] Layer-by-layer electrostatic assembly coating was used to coat charged CTAB-GNRs with negatively charged polyelectrolytes (poly(acrylic acid, sodium salt), PAA, MW ~15000 g/mole). To the purified CTAB-capped gold nanorod solution (1 mL), PAA solution (200μL, 10 mg/mL prepared in 10 mM NaCl solution) and NaCl solution (100 μL, 10 mM) were added simultaneously. The resulting solution was mixed gently for 2 hours to allow for complete polymer coating. To get rid of excess PAA polymer after coating, the coated gold nanorod solution was centrifuged for 3 min at 14000 rpm, two times. The pellet was re-suspended in DI water for further study.

PAH-coated gold nanorods (PAH-GNRs).[3] To the purified PAA-coated gold nanorod solution (1 mL), PAH solution (200 μL, 10 mg/mL prepared in 10 mM NaCl solution) and NaCl solution (100 μL, 10 mM) were added simultaneously. The resulting solution was mixed gently for 2 hours to allow for complete polymer coating. To get rid of the excess PAH polymer after coating, the coated gold nanorod solution was centrifuged for 3 min at 14000 rpm, two times. The pellet was re-suspended in DI water for further study.

Synthesis and characterization of polyethylene glycol-capped gold nanorods (PEG-GNRs).[4] CTAB-Capped gold nanorods were prepared as described above (100 mL). To get rid of excess CTAB, gold nanorod solutions were centrifuged at 9000 rpm for 30 minutes and supernatant solutions were aspirated. Residual pellets were collected (without addition of water, total volume of collected pellets were 0.4-0.5 mL). The gold nanorod pellets solution (highly concentrated) was added drop-wise to 2 mL of aqueous m-PEG-Thiol solution (20 mg/mL, average M.W= 5000 Da) and the resulted solution was immediately placed in a 1.0 mL cellulose membrane dialysis cassette (cutoff M.W. 3500 Da) against 4.0 L of DI water for 72 hours. Dialysis using cutoff M.W. 3500 Da allows to get rid of excess CTAB but not PEG polymers (M.W 5000 Da) and thus promote thiolated-PEG to chemisorb onto the surface of gold nanorods (displacing the CTAB bilayer by forming strong S-Au bond).[4] The content of the dialysis cassette was transferred to a 1.0 mL cellulose membrane dialysis cassette (cutoff M.W. 10,000Da) against 4.0 L of DI water for 48 hours to get rid of excess PEG.

PEGylation reaction was confirmed by the flip in zeta potential from highly positive value for CTAB-GNRs to very low negative value for PEG-GNRs, which indicate the displacement of cationic CTAB molecules by neutral PEG molecules. It is worth to note that neutral PEG-GNRs were stable in aqueous media against aggregation due to the presence of dense shell of hydrophilic PEG molecules, which provide hydration shell (steric stabilization).[5] The displacement was confirmed also by stability evaluation of CTAB- and PEG-GNRs in organic solvent (ethanol) as described below.

Stability of gold nanorods against aggregation in ethanol. Gold nanorods (1.0 mL, 1 nM as gold rods) from aqueous solution were centrifuged at 9000 rcf for 20 minutes. The supernatant solutions were removed and 0.5 mL ethanol was added to the gold nanorod pellets. Repeated mixing using pipette was used to suspend the pellet in ethanol followed by sonication for 30 seconds. Immediately, UV-Vis spectra were obtained for gold nanorods in ethanol and compared to the spectra in water. Broadening of plasmon peaks and increase in hydrodynamic diameters were used to judge on nanorods aggregation as reported previously (Figure S3).[6]

CTAB-GNRs are known to aggregate in organic solvents due the desorption of CTAB molecules from the bilayer to the organic bulk. However, PEGylation of nanoparticles are known to enhance their stability in both aqueous and various organic solvents. UV-Vis spectra of CTAB-GNRs in ethanol exhibited severe broadening to transverse and longitudinal plasmon peaks due to nanorod aggregation and plasmon coupling (Figure S3A).[6] However, UV-Vis spectra of PEG-GNRs in ethanol have typical plasmon peaks shape without broadening (Figure S3A). Red-shift of longitudinal

plasmon peak of PEG-GNRs from 750 nm in water to 764 nm in ethanol ($\Delta \lambda_{max}$= 16 nm) is due to the change in the refractive index of the solvent (Figure S3A). The increase in the hydrodynamic diameter of CTAB-GNRs and not PEG-GNRs, as measured by Dynamic light scattering (DLS), confirmed the aggregation of CTAB-GNRs but not PEG-GNRs upon transfer rods from water to ethanol (Figure S3B). Collectively, Zeta potential analysis and stability in ethanol support the displacement of CTAB by PEG molecules at the surface of gold nanorods.

Protein Analysis. To evaluate the adsorption of protein from incubation media to the surface of gold nanorods, we measured the loss of total protein after mixing the incubation media with GNRs followed by centrifugation. Microcentrifuge tubes (1.5 mL, Low Binding Corning Microcentrifuge Tubes) were treated with M199 for 12 hours to saturate protein adsorption to the wall of centrifuge tubes followed by washing with DI water and drying at room temperature. Fresh M199 media (100 μL contains 1.2 mg/mL FBS) were added. Microcentrifuge tubes then received either 100 μL gold nanorod solution or vehicle (controls) and incubated for 8 hours at 37^{o}C followed by centrifugation to remove nanorods (10,000 rcf for 10 minutes). The used M199 media was phenol-red free to avoid interference with the Bio-Rad protein assay. Total protein was estimated in supernatant solution using D_c Bio-Rad protein assay (with bovine serum albumin as a protein standard), as per the manufacturer instructions. The loss of protein in supernatant solutions was assumed to be due to the adsorption to the surface of gold nanorods (Figure S6).

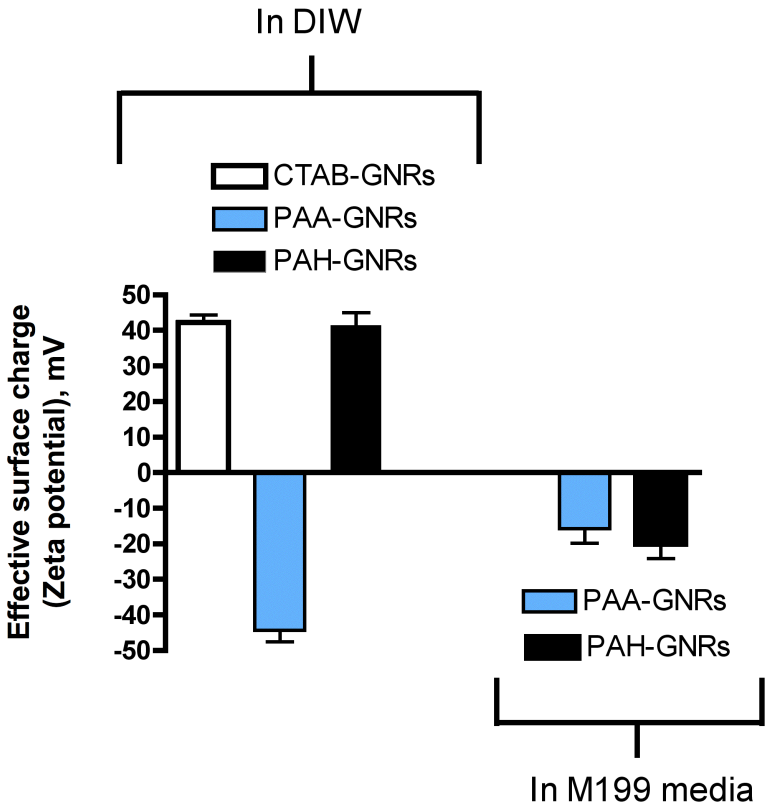

Figure S1. Effective surface charge (zeta potential) of gold nanorods with different surface chemistry in water (DIW) or in M199 media containing 10% fetal bovine serum. PAA-GNRs are prepared by coating CTAB-GNRs, where PAH-GNRs are prepared by coating PAA-GNRs. Nanorods were mixed with M199 media for 8 hours at 37^{o}C followed by purification (centrifugation) and suspending in water prior to zeta potential measurements. CTAB: Cetyltrimethylammonium bromide; poly(acrylic acid, sodium salt); PAH: Poly(allylamine hydrochloride).

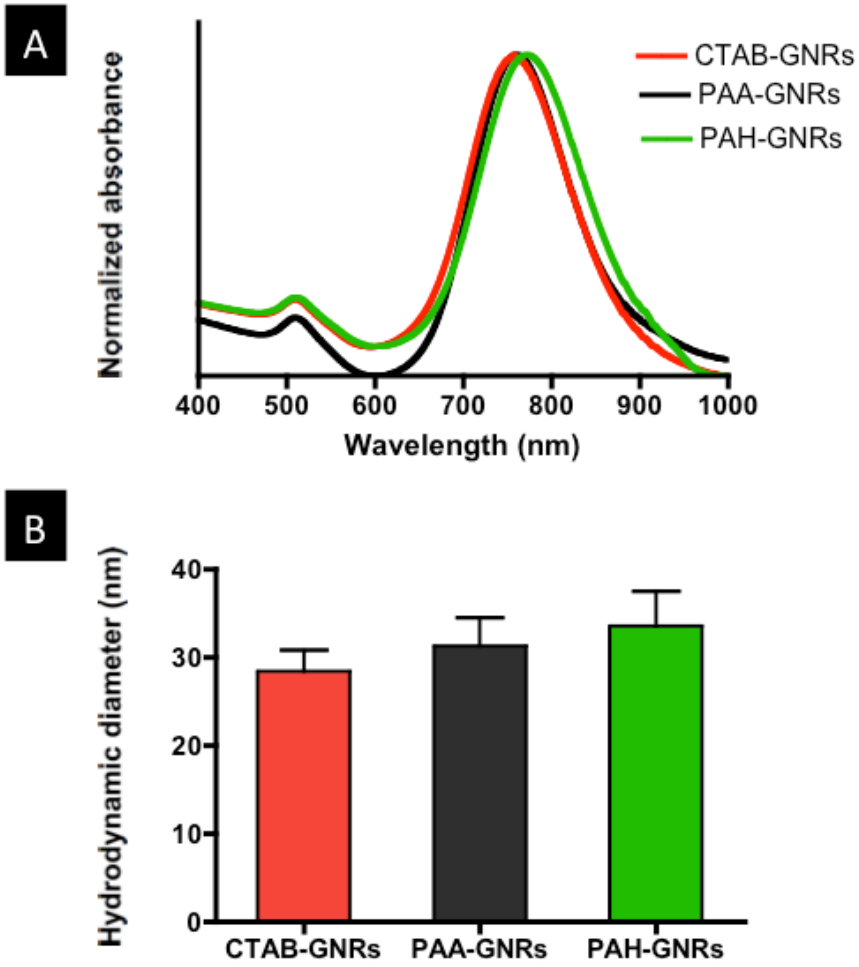

Figure S2. A) Normalized UV-Vis absorbance spectra of CTAB-capped (red), PAA-coated (black) and PAH-coated (green) gold nanorods. After coating, transverse and longitudinal plasmon peaks maintained their typical shape without broadening, which indicates that nanorods are not aggregating upon coating. Small red shift after PAH-coating could be due to change in dielectric constant at the surface of gold nanorods. B) Hydrodynamic diameter of gold nanorods after coating with PAA and PAH as measured using dynamic light scattering technique. CTAB: Cetyltrimethylammonium bromide; PAA: poly(acrylic acid, sodium salt); PAH: Poly(allylamine hydrochloride).

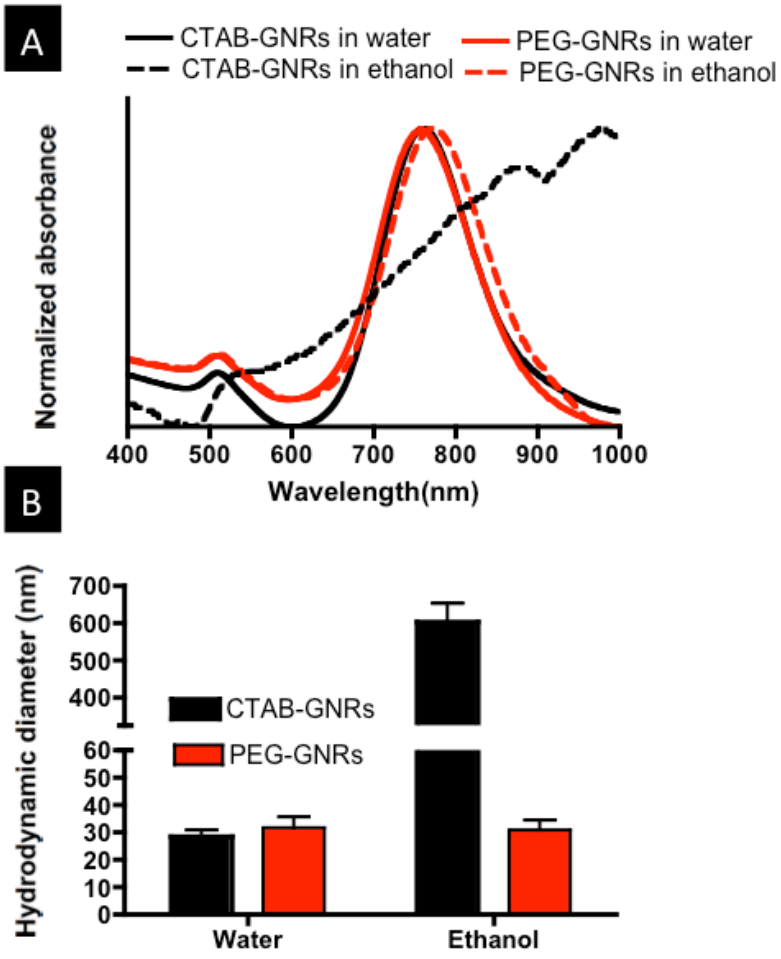

Figure S3. A) Stability evaluation of CTAB-capped gold nanorods (CTAB-GNRs) versus PEG-capped gold nanorods (PEG-GNRs) as judged by monitoring the broadening of the plasmon longitudinal peak as a sensitive tool to follow nanorods aggregation. Transferring CTAB-GNRs from water to ethanol resulted in a significant broadening and red shift of the plasmon transverse and longitudinal peaks as a result of nanorods aggregation (compare solid-black line with dashed-black line). Transferring PEG-GNRs from water to ethanol did not result in a significant broadening of the plasmon peaks, which indicates nanorods stability against aggregation (compare solid-red line with dashed-red line). Red-shift of the longitudinal plasmon peak for PEG-GNRs upon transferring from water into ethanol is due to change in dielectric constant of the solvent. B) Hydrodynamic diameter (D_H) of CTAB-GNRs (black columns) versus PEG-GNRs (red columns) in both water and ethanol. Transfer from water to ethanol resulted in aggregation of CTAB-GNRs but not PEG-GNRs as evident from the significant increase of D_H for CTAB-GNRs but not for PEG-GNRs in ethanol.

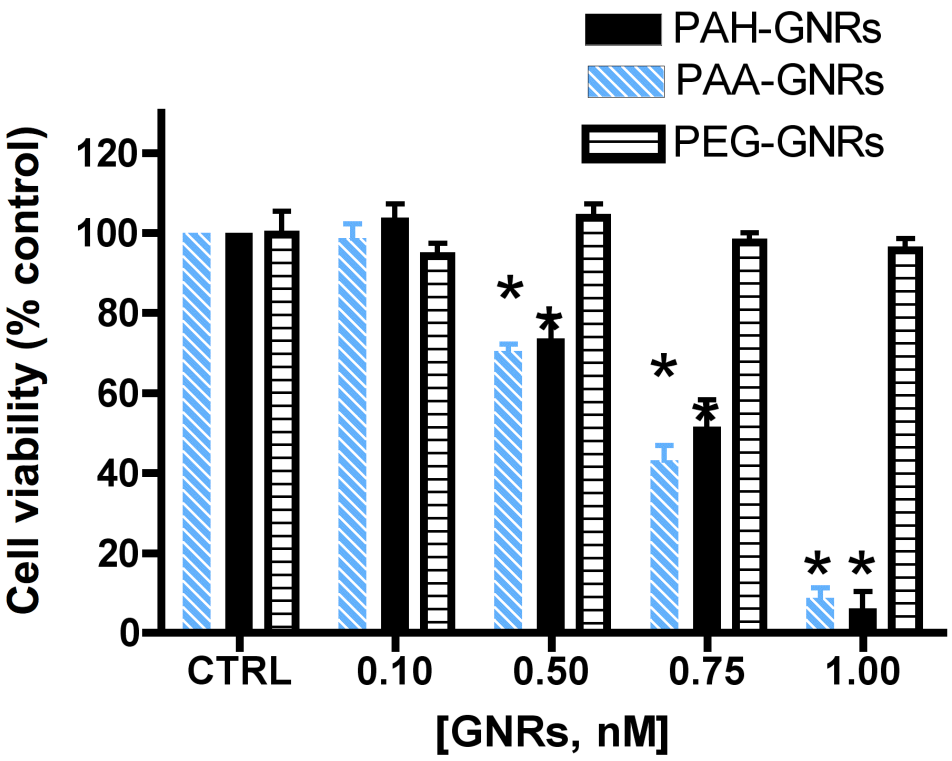

Figure S4. Cell viability of BAEC as function of increasing concentration of PAA-GNRs, PAH-GNRs, or PEG-GNRs. n=6 in each group, *indicates statistical difference from control (CTRL) group ($p<0.05$). BAEC: Bovine aortic endothelial cells, PAA: poly(acrylic acid, sodium salt); PAH: Poly(allylamine hydrochloride); PEG: Polyethylene glycol.

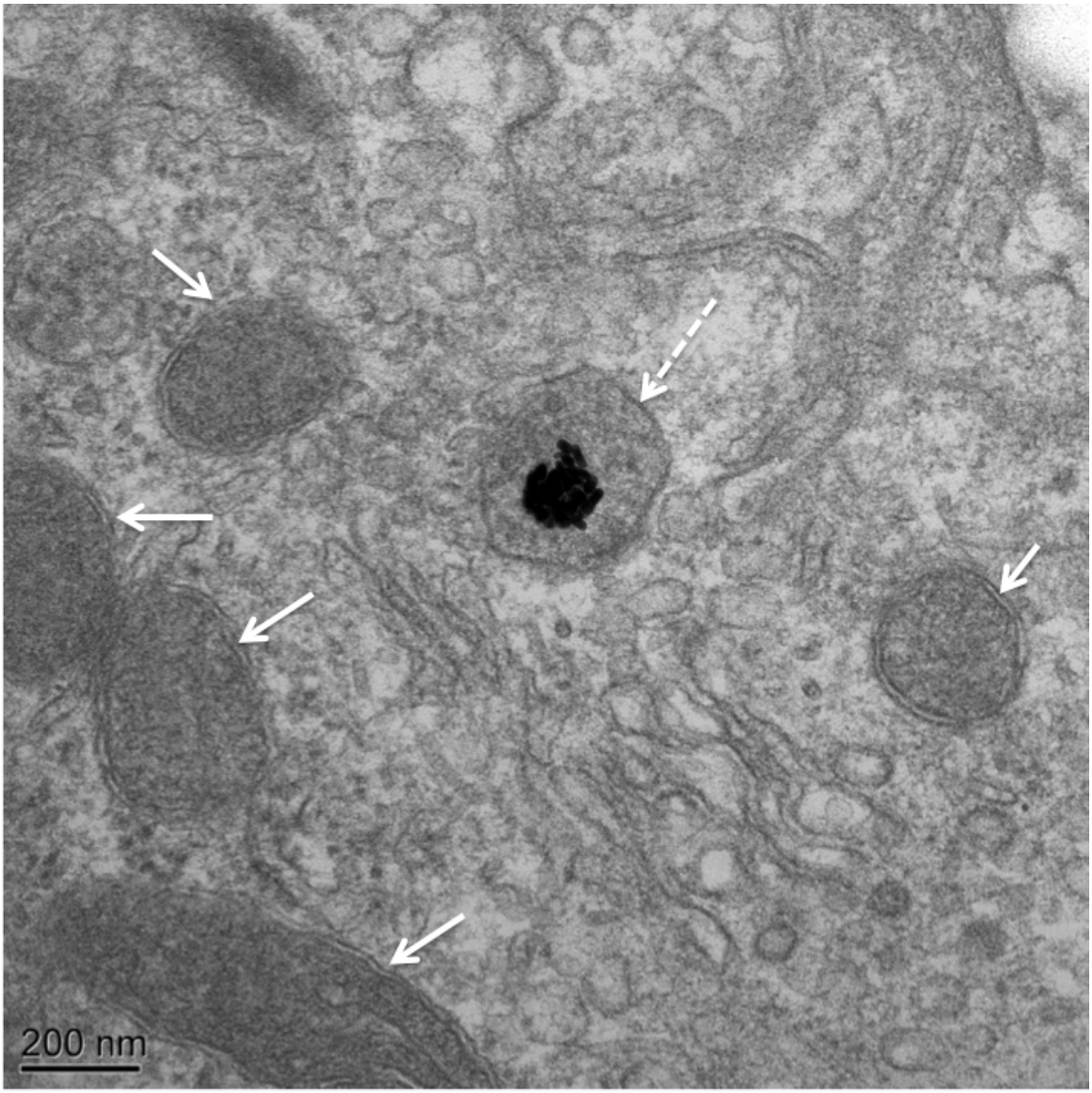

Figure S5. Transmission electron microscope image of endothelial cell in aortic ring exposed to PAH-GNRs (0.1 nM for 8 hours). Gold nanorods were found as clusters in endocytic vesicle in the cytoplasm. Note that the vesicle containing the nanorods is a single-membrane vesicle (a characteristic of endocytic vesicle, dashed-white arrow), where other intercellular organelles are double-membrane compartments (solid-white arrows) Scale bar= 200 nm.

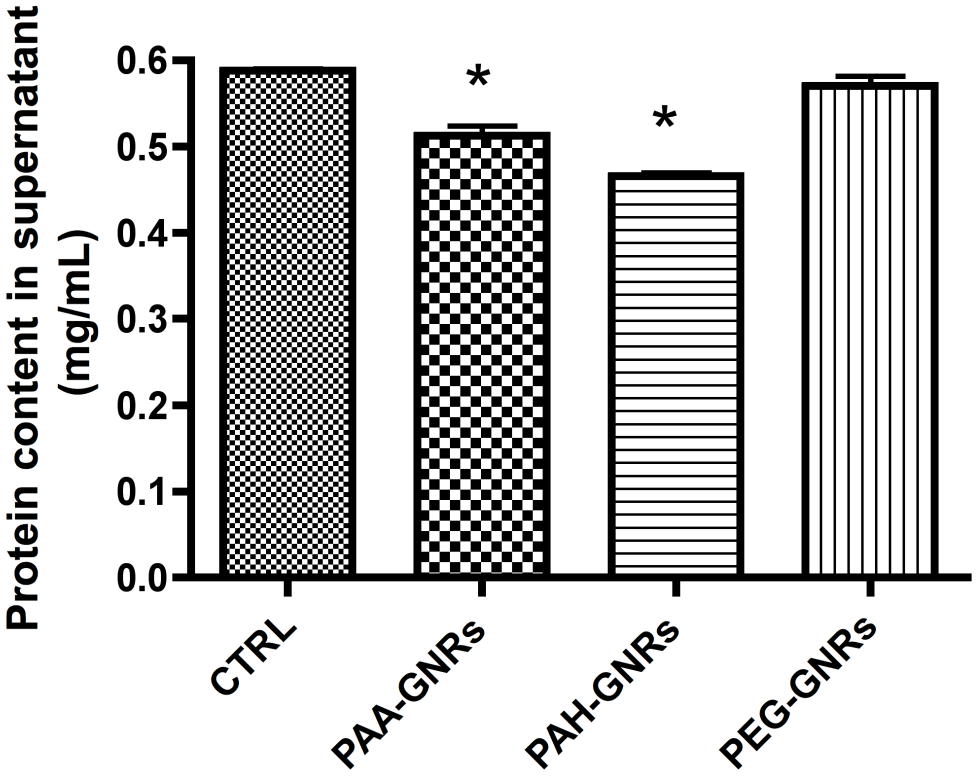

Figure S6. Total protein content in the supernatant of mixture of gold nanorods and M199 media contains 10% fetal bovine serum (FBS). Assay was performed using a D_c Bio-Rad protein assay with bovine serum albumin as a protein standard. 100 μL gold nanorods or vehicle solutions were mixed with 100 μL M199 media (contains 1.2 mg/mL FBS) for 8 hrs at 37^{o}C followed by centrifugation to remove nanorods (10,000 rcf for 10 minutes). Control group (CTRL) is vehicle without nanorods. * Indicates statistical difference from control (CTRL) group ($p<0.05$).

References (Supporting Information)

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