Cholesterol Functionalization of Gold Nanoparticles Enhances Photo-Activation of Neural Activity

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I. Additional Experimental Methods

Synthesis of 20 nm AuNPs. AuNPs with 20 nm in diameter were synthesized directly in aqueous phase by using seed growth method in the presence of citric acid and ascorbic acid. For 10 nm seed AuNPs, 200 μL (2.0×10^{-5} mol) of 100 mM tetrachloroauric (III) acid ($HAuCl_4 \cdot 3H_2O$) aqueous stock solution and 200 μL (2.0×10^{-5} mol) of 100 mM of citric acid aqueous solution were added into the 50 mL of deionized water and the reaction mixture was vigorously stirred at room temperature for 5 min. Then, 200 μL (4.0×10^{-5} mol) of 200 mM L-ascorbic acid aqueous solution was added into the reaction mixture, followed by vigorous stirring for next 30 minutes. Next, the growth solution was prepared with 0.4 mM HAuCl4 and 0.8 mM sodium citrate in 50 mL of deionized water. To synthesize 20 nm AuNPs, the 7 mL of seed NPs was added to the growth solution followed by adding L-ascorbic acid (2 mM as final concentration). The reaction mixture was stirred for next 3 hrs in room temperature and kept without stirring for additional 24 hrs for the complete reaction AuNPs and deactivation of ascorbic acid. The reaction of AuNPs was confirmed by the red shift of surface plasmon band peak and the decrease of the ascorbic acid and aurate peaks near UV region (< 300 nm) using UV-vis spectroscopy. The final sizes were confirmed by TEM and DLS measurement.

Ligand Exchange of AuNPs with TA-PEG-COOH, AuNP-COOH. For the ligand exchange reaction, the pre-synthesized of large AuNPs were added to the excessive amount of TA-PEG-COOH (MW of PEG ~ 600) based on previous paper. {E.OH. ACS Nano2011} Briefly, 10 mL of as-synthesized citrate-modified AuNPs were mixed with 25 μL of 100 mM stock TA-PEG-COOH aqueous stock solution. The solution was stirred for 8 hours with pH 8 adjusted by adding NaOH and the dispersion was purified from free ligands by three cycles of centrifugation using a centrifugal membrane filtration device (10-30 K molecular weight cut-off, Millipore Corporation, Billerica, MA) and deionized water.

UV-Vis Spectroscopy. Electronic absorption spectra were recorded using a Shimadzu UV-1800 UV-vis spectrophotometer to monitor the changes of spectra before and after biomolecule conjugation on AuNP-COOH.

Transmission Electron Microscopy and Energy-dispersive X-ray spectroscopy: Structural characterization and element analysis of the as-prepared NPs were carried out using a JEOL 2200-FX analytical high-resolution transmission electron microscope with a 200 kV accelerating voltage. Samples for transmission electron microscopy (TEM) were prepared by spreading a drop (5 ~ 10 μl) of the filtered NPs dispersion (filtered by using 0.25 μm syringe filters (Millipore)) onto the ultrathin carbon/holey support film on a 300 mesh Au grid (Tedpella, Inc.) and letting it dry. The concentration of NPs in the deionized water used was ~ 10 nM for 20 nm. Individual particle sizes were measured using a Gatan Digital Micrograph (Pleasanton, CA); average sizes along with standard deviations were extracted from analysis of at least 50-100 nanoparticles.

Dynamic Light Scattering. Dynamic light scattering (DLS) measurements were carried out using ZetaSizer NanoSeries equipped with a HeNe laser source (λ = 633 nm) (Malvern Instruments Ltd, Worcestershire, UK) and analyzed using Dispersion Technology Software (DTS, Malvern Instruments Ltd, Worcestershire, UK). Similar concentration of TEM samples were loaded into disposable cells, and data were collected at 25°C. All the samples were prepared in 0.1×PBS buffer pH 7.4. For each sample, the autocorrelation function was the average of five runs of 10 seconds each and then repeated about three to six times. CONTIN analysis was then used to number or intensity mode *versus* hydrodynamic size profiles for the dispersions studied.

Preparation of AuNP-PEG-Chol Conjugates. AuNP-COOH were covalently conjugated to HCl.NH_2-PEG_{2000}-Chol and HCl.NH_2-PEG_{2000}-FITC *via* carbodiimide chemistry. A stock solution of Chol-PEG-NH_2.HCl (10 mM) or HCl.NH_2-PEG_{2000}-FITC (10 mM) was prepared in water. A stock solution containing NHSS (25 mM) or EDC (500 mM) was prepared in PBS (10x, pH 7.0) buffer. An aliquot (10 μL) of the freshly prepared stock solution of NHSS and EDC was immediately added to 1.0 mL of 34 nM AuNP-COOH in PBS (10x, pH 7.0) buffer and stirred for 5 min. For conjugation of HCl.NH_2-PEG_{2000}-Chol, an aliquot (10 μL) of stock solution of HCl.NH_2-PEG_{2000}-Chol was added to this mixture and stirred for 2 h. For conjugation of HCl.NH_2-PEG_{2000}-Chol and HCl.NH_2-PEG_{2000}-FITC to same AuNP-COOH, an aliquot (7.0 μL) of stock solution of HCl.NH_2-PEG_{2000}-Chol and another aliquot (3.0 μL) of stock solution of HCl.NH_2-PEG_{2000}-FITC were added to this mixture and stirred for 2 h. The reaction mixture was briefly centrifuged and the supernatant was subjected to size exclusion chromatography using a PD-10 column equilibrated with DPBS (0.1X). The AuNP band was collected and analyzed by agarose (1%) gel electrophoresis. The as-synthesized AuNP conjugates (AuNP-PEG-Chol and AuNP-PEG-Chol-FITC) were characterized for their particle concentration, size and charge. Particle size and distribution were measured by dynamic light scattering (DLS) as described above. Zeta-potential was measured on a ZetaSizer NanoSeries equipped with a HeNe laser source (λ = 633 nm) (Malvern Instruments Ltd.) and an avalanche photodiode for detection. For each analysis at least six measurements were performed and the data is represented as average value ± SEM.

Cell Culture, Cellular Staining and Imaging for AuNP-PEG-Chol/FITC. HEK 293T/17 cells (ATCC, Manassas, VA) were cultured at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Cells were cultured in complete growth medium defined as follows: Dulbecco's Modified Eagle's Medium (DMEM, purchased from ATCC) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (ATCC) and 1% antibiotic/antimycotic (Sigma). Cells were cultured in T25 flasks and a subculture was performed every 3–4 days. Cellular NP delivery experiments were performed using cells between passages 5 and 15. Cells were seeded to 35 mm petri dish with 14 mm glass bottom insert (#1.0 cover glass, MatTek Corp., MA, USA) at a density of ~7 x10^4 cells/mL (3 mL/well). Dishes were coated with fibronectin (20 μg/mL) in DPBS before adding the cell suspension. Stock solutions of AuNP-PEG-Chol-FITC (18 nM in DPBS) was diluted to 3 nM in DMEM-HEPES and incubated on cell monolayers 20 min at 37°C. The solution was removed and cell monolayers were washed with LCIS. The cell monolayer was imaged in LCIS on confocal laser scanning microscopy (CLSM) using a Nikon A1RSi confocal microscope equipped with a 488 nm argon laser with fluorescence detection channels set to 500-550 nm (green) with dichroic mirrors at 405/488/561/640 nm. All images were collected using a Plan Apo 60X objective.

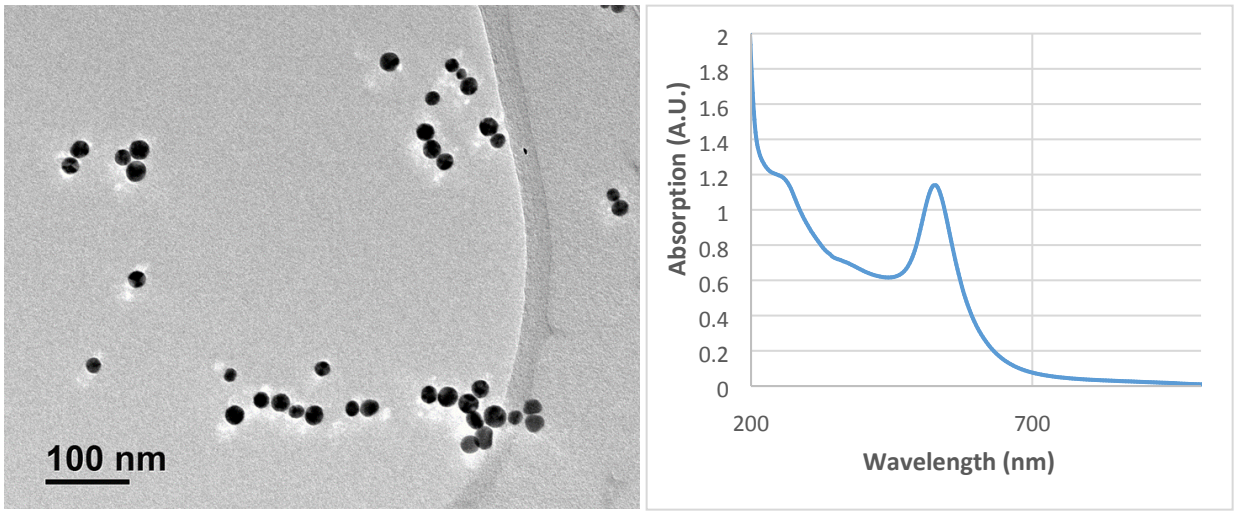

Figure S1. TEM and spectroscopic analysis of AuNPs used in this study. (A) TEM image of 20 nm AuNP-COOH (average size 20 ± 3.0 nm). (B) UV-vis spectroscopy of 20 nm AuNP-COOH.

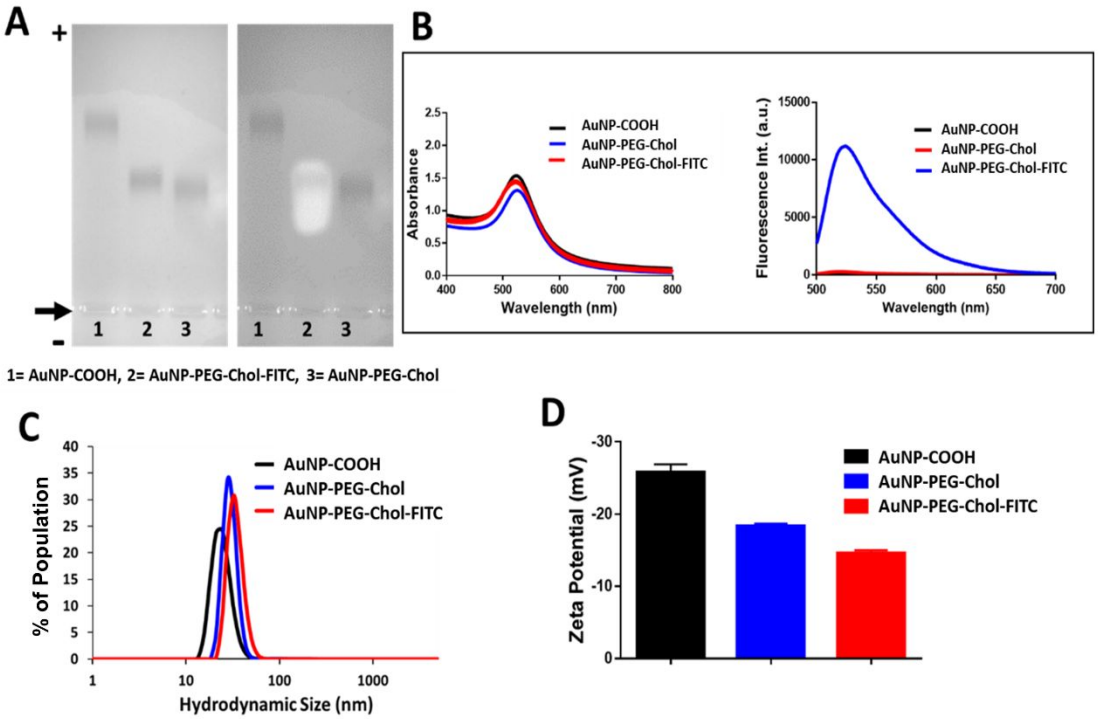

Figure S2. Physicochemical characterization of the AuNP-COOH before and after conjugation with NH_2-PEG_{2000}-Chol *via* EDC coupling. (A) Gel electrophoresis analysis of AuNPs in 1% agarose gel. The arrow on the left represents the line of wells where samples were loaded. The gel to the right was collected in fluorescence mode to visualize the FITC signal in lane 2. (B) Absorption and fluorescence emission spectra of AuNPs in 0.1x PBS (pH 7.4). Fluorescence spectra were collected by exciting the samples at 488 nm. (C) DLS size distribution data for the AuNPs before and after the PEG-Chol conjugation. (D) Zeta potential of the AuNPs before and after the PEG-Chol conjugation.

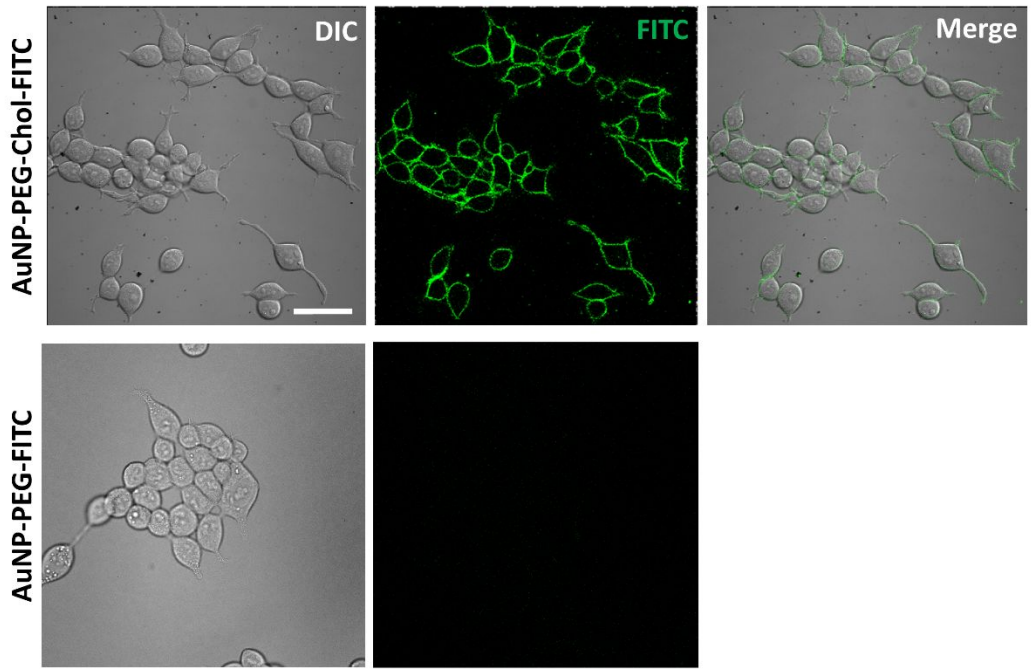

Figure S3. Confocal microscopy imaging of membrane labeling of AuNP-PEG-Chol-FITC in HEK293T/17 cells. Shown are DIC and confocal fluorescence images of live cells stained with PEG_{2000}-Chol-FITC**.** The cells were incubated with ~3 nM AuNP for 20 min at 37 °C and washed prior to imaging. Scale bar, 50 μm. Top row shows cells incubated with AuNP-PEG-Chol-FITC conjugates while the bottom row shows cells incubated with AuNP-PEG-FITC conjugates.

$$E_{Th} = a \times D^b$$

Figure S4. Relation of laser power and pulse duration to total energy. E_{Th} is the energy necessary to depolarize the cell membrane to its threshold for AP firing, a is an independent term that relates to the number of AuNPs and their average distance to the membrane, D is the duration of the laser pulse and b is the power law that governs the relationship.

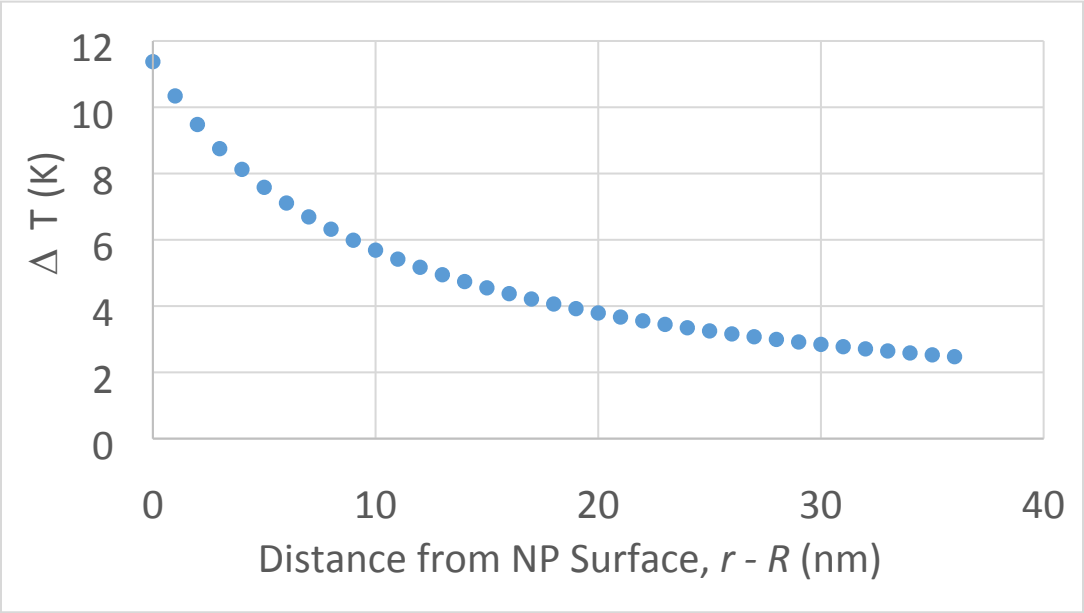

Figure S5. The estimated temperature change as a function of distance from the surface of the AuNP. This was based on the temperature enhancement equation in main document. Experimental conditions used for this calculation were as follows: average laser power = 114 mW, focused spot size ~ 5 μm. The temperature increase just at the plasma membrane surface (~6 nm from the surface of the AuNP core) will be ~ 7 ºC based on this estimation. *r* is the distance from the center of the AuNP and *R* is the radius of the AuNP.

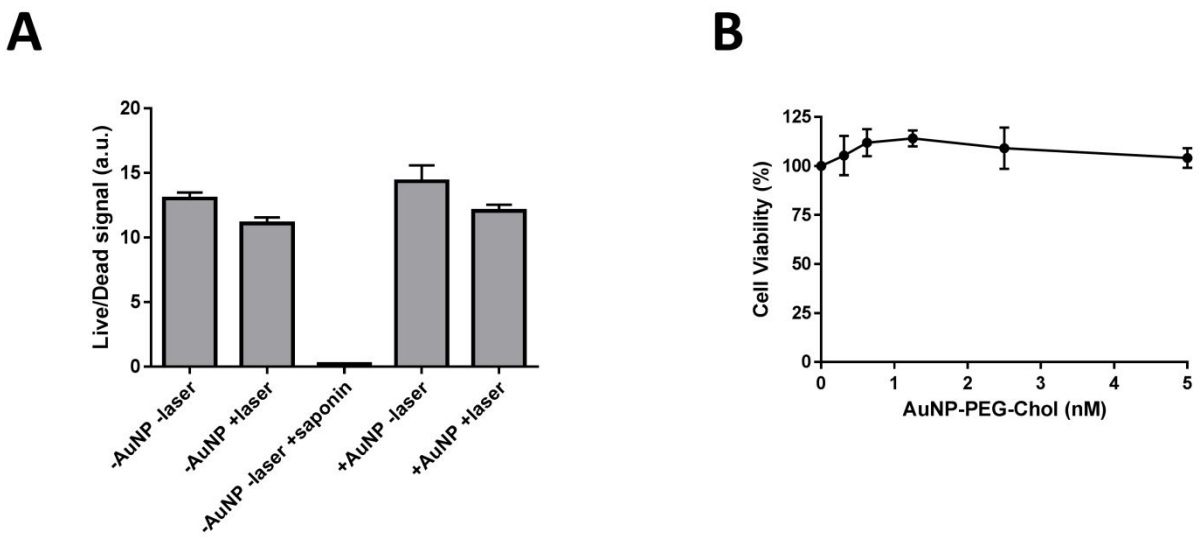

Figure S6. Cytotoxicity of AuNPs in DRG neurons and HEK 293T/17 cells. (A) Live/dead analysis (calcein-AM (live) and ethidium homodimer-1 (dead)) of DRG neurons labeled with AuNPs (1 nM) and photoexcited with laser pulse of 0.5 msec (225 mW). Controls included DRG neurons alone (-AuNP/-laser), neurons with no AuNPs exposed to laser (-AuNP/+laser), and neurons labeled with AuNPs with no photoexcitation (+AuNP/-laser). Saponin-treated cells were used as a control for neuronal cell necrosis. (B) Cellular proliferation assay in HEK 293T/17 cells incubated for 1 h with varying concentrations of AuNPs as indicated followed by the removal of the NPs and subsequent culture of the cells in complete medium for 72 h. Cell viability was determined by formation of a formazan product from a tetrazolium substrate by viable cells and is expressed as percent control of untreated cells.