Supporting information

for

Plasmonic enhancement of two-photon excitation fluorescence by colloidal assemblies of very small AuNPs templated on M13 phage

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SUPPORTING METHODS

p3 cloning of RGD-bearing peptide. The display of GRGDSP peptide on p3 protein was accomplished using Ph.D. Peptide Display Cloning System (New England Biolabs Inc., Ontario). Oligonucleotide including the GRGDSP sequence (synthesized by IDT, Integrated DNA Technologies, Inc.) (Table 2.2.1) was annealed to the extension primer (Table 2.2.1) and then extended as a duplex using Klenow fragment (New England Biolabs, Ontario). The extended duplex and M12KE vector were digested with Acc65I and EagI restriction enzymes (New England

Biolabs, Ontario) according to the instructions of the manufacturer, and then digestion products were gel purified. The extended duplex was purified on an 8% non-denaturing polyacrylamide gel and extracted from the gel using a QIAEX II Gel Extraction Kit (QIAGEN, Ontario). The digested M13KE vector was agarose-gel purified with QIAquick Gel Extraction Kit (QIAGEN, Ontario). The purified vector and DNA duplex were ligated using T4 DNA ligase (New England Biolabs, Ontario) at 16 °C overnight and the ligation mix was transformed into XL10-Gold ultracompetent cells (Agilent, ON). After overnight incubation of the transformed cells on LB/IPTG/X-gal plates (LB agar; IPTG, isopropyl-β-D-thiogalactoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside) at 37 °C, blue plaques were picked and analyzed for the presence of the insert. PCR analysis was performed by amplification of the M13KE DNA region, which includes the insert using designed primer sequences (Table S1). Positive phage plagues were sent to *the Plate-forme d'Analyses Génomiques* (Université Laval) for sequence analysis.

p8 Cloning of Gold Binding Peptide. Recombinant M13 phage displaying G**RGD**SP peptide on the p3 minor coat protein were engineered to display a VSGSSPDS gold binding peptide on the major coat protein p8. The insert was positioned at the N-terminus of the wild type p8 protein by creating a PstI restriction site (CTGCAG) in this region. This was achieved by mutation of the nucleic acid base at position 1372 of M13 plasmid from T to A. The existing PstI site of M13 plasmid at position 6245 was deleted by mutating A to T at position 6249, as well. The site-directed mutagenesis at these positions was accomplished by using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (QIAGEN, Ontario). The phage DNA for mutagenesis was prepared by propagating M13 phage. After propagation, the phage particles were collected by centrifugation and dsDNA inside the pellet was purified using QIAprep Spin Miniprep Kit (QIAGEN, Ontario).

For mutagenesis, purified dsDNA was amplified using designed primer sequences (IDT, Integrated DNA Technologies, Inc.) (Table S2) and mutant strands were synthesized. After the reaction, the parent DNA template was digested with DpnI endonuclease at 37 °C for 5 minutes. The remaining mutated ssDNA was transformed into XL10-Gold ultracompetent cells and incubated overnight at 37 °C on LB/IPTG/X-gal plates. The mutant closed circle ssDNA was converted into duplex form *in vivo* and propagated as phage. Following the incubation, the blue plaques on LB/IPTG/X-gal plates including mutated phage DNA were picked up and propagated. The mutated regions of propagated phage DNA were amplified by PCR using the designed primer sequences (IDT, Integrated DNA Technologies, Inc.) (Table S2) and sent to the *Plate-forme d'Analyses Génomiques* (Université Laval) for DNA sequence analysis.

The gold-binding peptide was inserted into modified M13 phage using a PstI restriction site. The insert was positioned between the first and the fifth amino acids of the p8 protein by replacing residues 2–4 from (Alanine1 - Glutamic acid2 - Glycine3 - Aspartic acid4 - Aspartic acid5) to (Alanine1 – insert - Aspartic acid5). Two primer sequences were designed: a forward primer including the insert sequence and a reverse primer to linearize the vector (Table S2). M13 phage with an engineered PstI restriction site was propagated and viral DNA was purified using QIAprep Spin Miniprep Kit (QIAGEN, Ontario). To incorporate the insert, PCR was performed using Phusion® High-Fidelity DNA Polymerase (New England Biolab, Ontario), the two primers, and the mutated M13 plasmid as DNA template according to the manufacturer's instructions (New England Biolab, Ontario). The obtained product was gel purified (1% agarose in TAE buffer, 45 minutes, 80 mV), extracted using QIAEX II Gel Extraction Kit (QIAGEN, Ontario), digested with PstI enzyme (New England Biolabs, Ontario) at 37 °C overnight and recircularized with T4 DNA Ligase (New England Biolabs, Ontario) by incubating overnight at 16 °C. The ligation mixture

was transformed into XL10-Gold ultracompetent cells (Agilent, Ontario) and incubated on LB/IPTG/X-gal plates at 37 °C overnight. The blue plaques were picked up and their plasmids were amplified as previously described. After purification with QIAprep Spin Miniprep Kit (QIAGEN, Ontario), DNA samples were sent to the *Plate-forme d'Analyses Génomiques* of Laval University for DNA sequencing.

BSPP-passivated AuNPs. Size distribution analysis of TEM images demonstrated that the synthesis of AuNPs resulted in 3 and 13 nm particles (Figure S1B and Figure S1D). AuNPs purchased from Tedpella Inc. were also characterized by TEM and possessed ~9 nm diameters (Figure S1C). UV-Vis absorption spectra of colloidal AuNPs were also recorded and size information extracted from SPR peak position was in agreement with the measured particle diameters obtained by TEM (Figure S1A). The SPR band of AuNPs, which is around 520 nm, is affected by particle size as the plasmon wavelength maximum shifts to the longer wavelengths with increasing particle size [1]. A similar trend was also observed in our measurement: the smallest plasmon wavelength maximum was recorded for 3 nm AuNPs whereas 13 nm AuNPs had the highest value.

Assessment of Integrin Binding Affinity of M13-templated Gold Assemblies. Phage ELISA (**E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay) was performed to determine the integrin affinity of RGD bearing M13-templated gold assemblies. In this assay, the wells of a microplate were coated with 200 µL of human integrin alpha V beta 1 heterodimer protein (Acrobiosystems Inc., Newark, US) dissolved in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) at a concentration of 14 µg/mL. After incubating the microplate overnight

at 4 °C, the unbound protein was removed and the wells were incubated with 200 µL of blocking buffer (3% bovine serum albumin (BSA) in PBS) for 2 hours at room temperature. Meanwhile, the phage-templated gold assemblies composed of 3 nm, 9 nm and 13 nm AuNPs and their corresponding control reaction solutions without phage templates were prepared. The samples were diluted with an equal volume of blocking buffer and incubated for 20 min before their addition to the wells. After removal of the blocking buffer, 200 µL of the diluted samples were added to the integrin-coated wells and incubated for 2 hours at room temperature. The wells were extensively washed with washing buffer (0.05% Tween 20 in PBS) and 200 µL of horseradish peroxidase conjugated anti-M13 monoclonal antibody (Fisher Scientific, Ontario) in blocking buffer was added to label the bound phages inside the wells. The microplate was incubated for 1 hour at room temperature and unbound anti-M13 antibodies were removed by washing six times with washing buffer. Binding of anti-M13 antibodies to recombinant M13 phages inside the wells was detected by adding 200 µL of TMB (3,3',5,5'-tetramethylbenzidine) substrate (Invitrogen Inc.) and recording the enzymatic reaction with absorbance measurements at 450 nm. The enzymatic reaction was stopped after 45 min by adding 100 µL of stopping reagent (Invitrogen Inc.) and the absorbance measurements were performed using microplate reader (Cytation 5 imaging reader, Biotek, US). As seen in Figure S3B, formation of blue color was observed in all wells containing M13–AuNP assemblies. On the other hand, the wells incubated with only AuNPs did not show any color formation. The absorbance values of the samples in each well were plotted as seen in Figure S3A. The results of ELISA showed that AuNPs assembly along the p8 major coat protein of M13 phage did not sterically interfere with the specific binding ability of RGD peptide to integrin protein. As a result, M13–AuNP assemblies were able to recognize and bind to the integrin protein coated on the wells.

SUPPORTING RESULTS

Table S1. Oligonucleotide sequences for M13 phage p3 cloning.

Oligonucleotide	Sequence (5'- 3')
M13KE Forward	TGT ACC GTA ACA CTG AGT TTC
M13KE Reverse	ATT CAC CTC GAA AGC AAG CTG A
GRGDSP	CAT GTT TCG GCC GAG CCG CCG CCC GGG CTG TCA CCA CGG CCA GAG TGA GAA TAG AAA GGT ACC CGG G
Extension Primer	CAT GCC CGG GTA CCT TTC TAT TCT C

Table S2. Oligonucleotide sequences for M13 phage p8 cloning.

Oligonucleotide	Sequence (5'- 3')
Primer for Mutation at 1372	GCT GTC TTT CGC TGC AGA GGG TGA CGA TCC
Forward Primer for Verification of Mutation 1372	TTC TTA AAC AGC TTG ATA CCG ATA G
Reverse Primer for Verification of Mutation 1372	TGT TTC GCG CTT GGT ATA ATC G
Primer for Mutation at 6249	GCT TGC ATG CCT GCT GGT CCT CGA ATT CAC
Forward Primer for Verification of Mutation 6249	GCC ATT CGC CAT TCA GGC TG
Reverse Primer for Verification of Mutation 6249	TGA GTT AGC TCA CTC ATT AGG CAC
Forward Primer for p8 Cloning	CCT CTG CAG CGA AAG ACA GCA TCG G
Reverse Primer for p8 Cloning	ATA TAT CTG CAG TGT CGG GTA GTA GTC CGG ATT CGG ATC CCG CAA AAG CGG CCT TTA ACT CCC
Forward Primer for Verification of p8 Insert	TTC TTA AAC AGC TTG ATA CCG ATA G
Reverse Primer for Verification of p8 Insert	TGT TTC GCG CTT GGT ATA ATC G

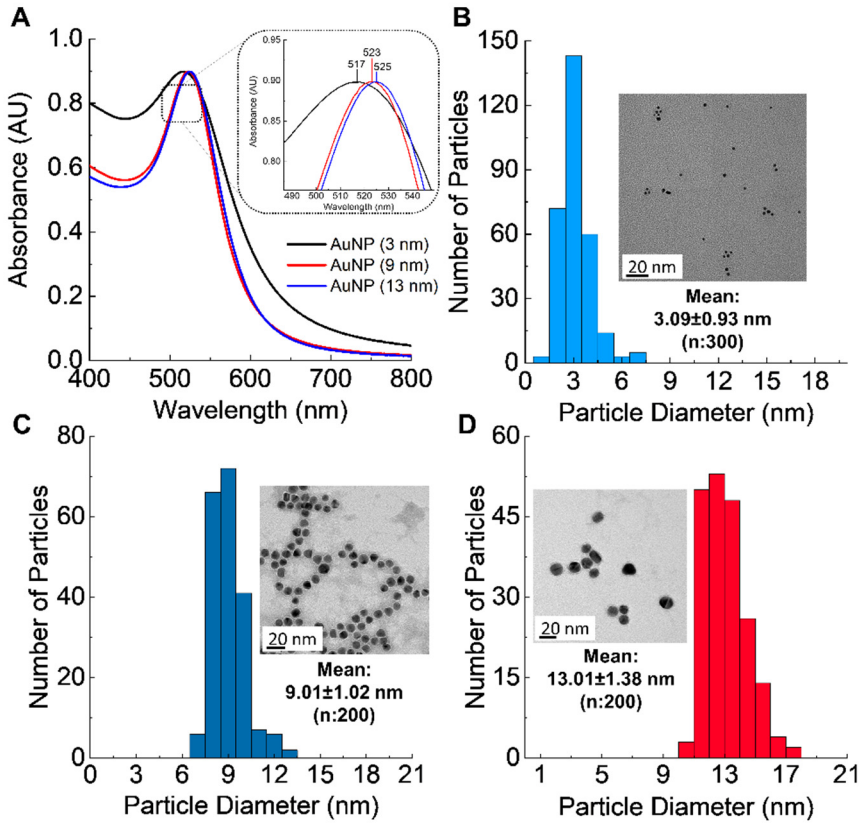

Figure S1. A) UV-Vis absorption spectra of BSPP-passivated AuNPs with diameters of 3 nm (black line), 9 nm (red line), and 13 nm (blue line). TEM images and corresponding size distribution histograms of BSPP-passivated AuNPs with 3 nm (B), 9 nm (C), and 13 nm (D) diameters.

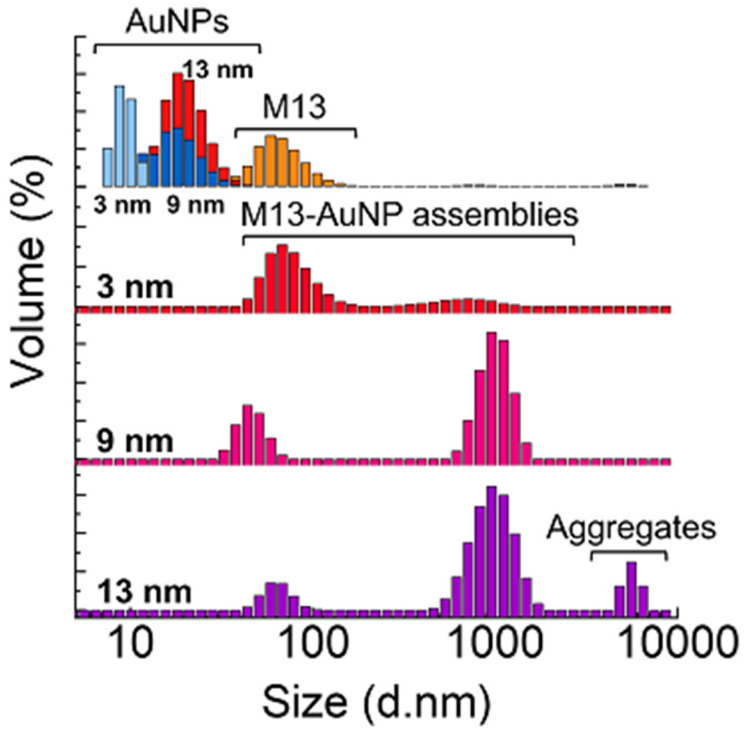

Figure S2. DLS histograms showing the size distribution of AuNPs, M13 phage, and M13–AuNP assemblies. The histogram in red represents M13–AuNP assemblies prepared using 3 nm AuNPs whereas the histograms in pink and in purple represent M13–AuNP assemblies composed of 9 nm and 13 nm AuNPs, respectively.

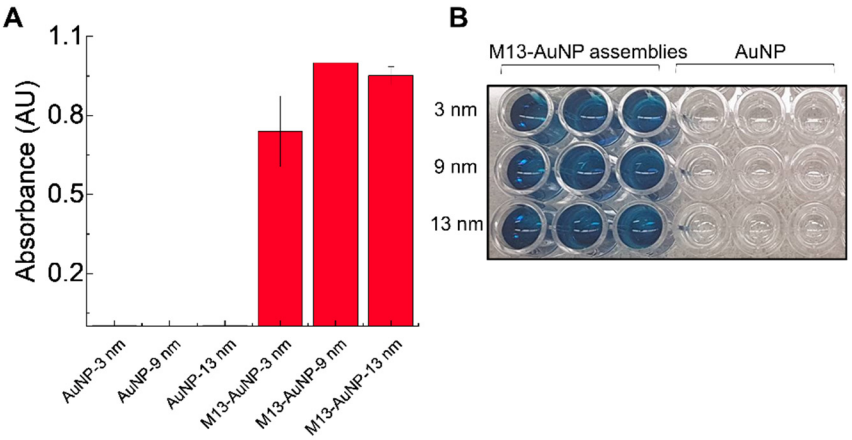

Figure S3. A) Reactivity of RGD bearing M13–AuNP assemblies and colloidal AuNPs with human integrin protein. **B**) ELISA plate showing the color development due to the binding of

integrin to RGD bearing M13-AuNP assemblies. The wells in the first line of ELISA plate contain the samples which are prepared using 3 nm AuNPs whereas the samples in the second line and the third line are composed of 9 nm and 13 nm AuNPs, respectively.

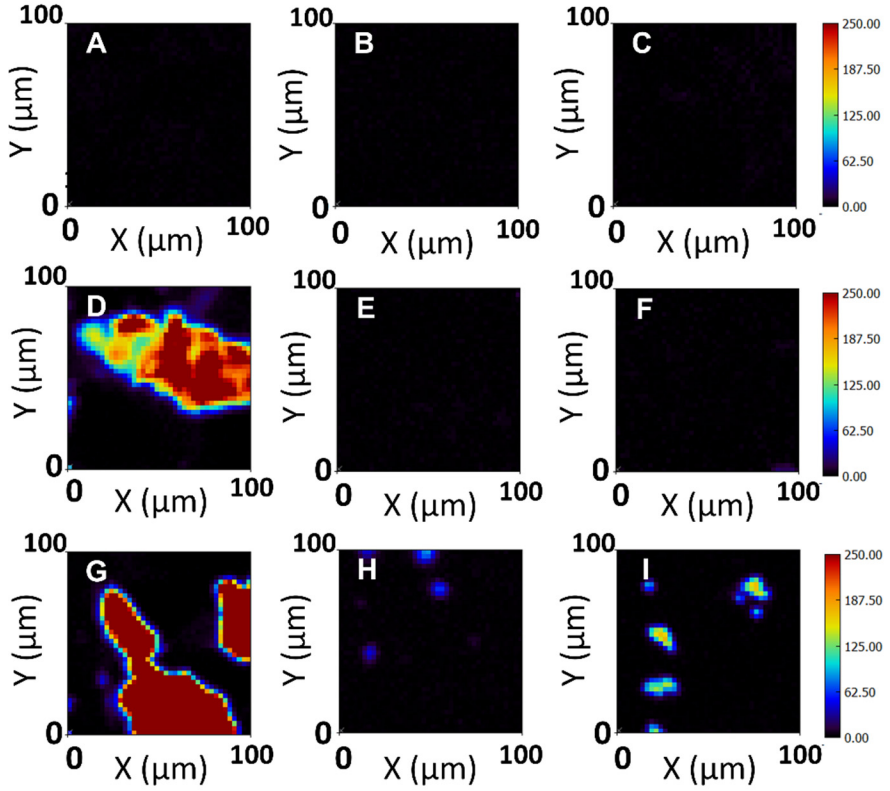

Figure S4. Representative SERS maps constructed from the area of peak at 1085 cm^{-1} for M13–AuNP assemblies prepared from A-B-C) 3-nm, D-E-F) 9-nm, G-H-I) 13-nm AuNPs.

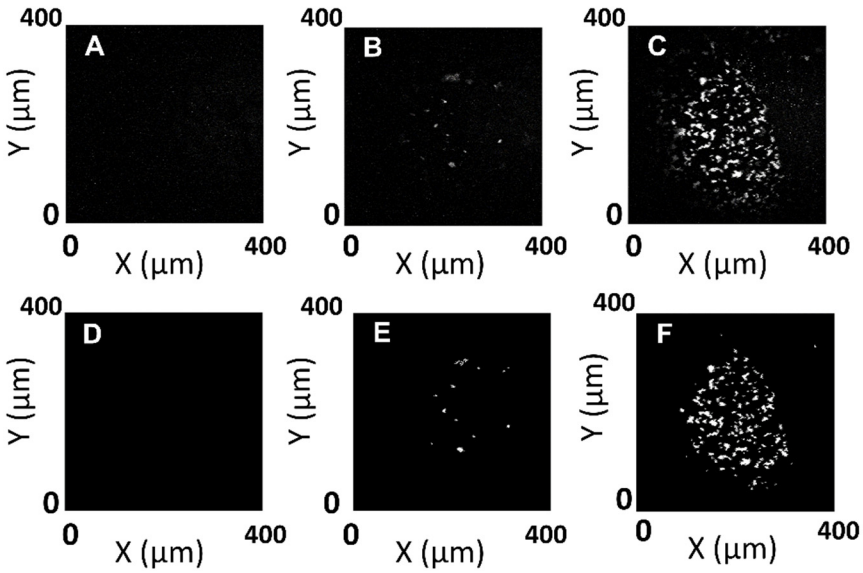

Figure S5. Representative 'original' 2PEF images of M13–AuNP assemblies prepared with A) 3-nm, B) 9-nm, and C) 13-nm AuNPs. Processed 2PEF images of M13–AuNP assemblies prepared with D) 3-nm, E) 9-nm, and F) 13-nm AuNPs.

REFERENCES

(1) Huang, X.; El-Sayed, M. A. Gold nanoparticles: Optical properties and implementations in cancer diagnosis and photothermal therapy. *J. Adv. Res.* **2010,** *1* (1), 13-28, DOI: https://doi.org/10.1016/j.jare.2010.02.002.