A Dual Signal Amplification Nanosensor Based on SERS Technology for Detection of Tumor-Related DNA

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1. Material and methods

SERS measurement. A microscopic Raman instrument (Renishaw) with a 633 nm laser, a 50× objective lens and a Peltier cooled CCD was used for Raman spectral measurement. Before each measurement, the instrument was calibrated using a silicon wafer with a Raman peak at 520 cm^{-1}. After that, each spectrum was obtained with 10 s inquisition time and then undergo process of baseline correction using a polynomial multipoint fitting function and curve fitting function provided by WiRE 4.3 software. The final intensities of Raman peaks were used for spectral analysis, such as quantitative analysis.

Preparation of $^{Os\text{-}S}$CO. 0.19 1 g (0.20 mmol) of (p-benzenethiolato)-decarbonyl-hydrido-triosmium and 250 mL of cyclohexane were mixed into 500 mL round bottom neck flask. The solution is purged with carbon monoxide for 15 min. The solution is then irradiated for 2.5 hour by using an external high-pressure mercury lamp. After removal of solvent in vacuum, the yellow residue is chromatographed on silica thin layer chromatography (TLC) plates using hexane-CH_2C1,(85: 15) as eluent. The product, is at R, = 0.17. The IR CO absorptions (hexane): 2109 (very weak), 2075 (strong), 2028 (strong), 2012 (weak).

Preparation of $^{Re\text{-}S}$CO. $Re_2(CO)_8(MeCN)_2$ (120 mg, 0.177 mmol) and 2 equivalents (0.354 mmol) of benzene-1,4-dithiol were dissolved in 20 ml of toluene. Subsequently the reaction mixture was heated to 80 ^{0}C. All the starting material reacts within 30 min as shown by the disappearance of the characteristic IR absorption of $Re_2(CO)_8(MeCN)_2$ at 2069 cm^{-1}. The solution was cooled to room temperature and after solvent removal the crude material was purified by TLC using the following solvent mixtures: CH_2Cl_2-hexane (1: 7). The IR CO absorptions (hexane): 2106 (weak), 2094 (medium), 2025 (very strong), 1969 (medium).

Fabrication of nanopillar substrate and $^{Os\text{-}S}$CO embedded substrate. Four inches p-type single side polished <100> wafers were used. Etching is conducted in an Advanced Silicon Etcher. Nanopillars were formed using a ion etching method. After step, the nanopillars were exposed to argon plasma for 1 min to completely remove the etchant gas residuals and the decomposition products. A chromium adhesion layer is evaporated onto the Si nanopillar structure. Finally, gold was deposited on the heads of the nanopillars by electron beam evaporation.

For SERS measurement of $^{Os\text{-}S}$CO and $^{Re\text{-}S}$CO, the substrate was repeatedly cleaned with absolute ethanol. The substrate was incubated into prepared 1 mL of ethanolic solution of $^{Os\text{-}S}$CO and $^{Re\text{-}S}$CO (100 μM) for 1 hour. After that, the substrate was rinsed with ethanol (1 mL x 3) sequentially before Raman measurement. For $^{Os\text{-}S}$CO embedded substrate, substrate was incubated with $^{Os\text{-}S}$CO (100 μM) for 24 hours. Finally, the substrates were coated with Au (99.999% purity, JEOL) at various thicknesses by sputtering technique (JEOL, JFC-1600 Auto fine coater). Each metal layer was deposited at a rate of 1.33nm/s.

Electromagnetic Simulation. To simulate the plasmonic properties of the gold nanopillars, we used an RF extended module under COMSOL Multiphysics. The desired size and 3D shape were drawn in draw mode using the Cartesian coordinate system. The boundary conditions and perfectly matched layer were also defined in draw mode. Simulation duration for a single nanoparticle took about 4 hours.

Preparation of DNR-SiO_2@Au-$^{Re\text{-}S}$CO. SiO_2@Au was purchased from Nanoseedz (Hong Kong). 10 mL of SiO_2@Au (25 OD) was centrifuged and its pellet was collected. Pellet was then dispersed in 5 mL of DMSO, and 50 μL of APTES was then added. The mixture was allowed to react for 20 hours, and then was collected by centrifugation and washed with ethanol twice, and amine-functionalized SiO_2@Au pellet was collected. Pellet was dispersed in THF (10 mL), succinic anhydride (42 mg, 0.417 mmol) was added as a 1 mM solution in DMSO. The reaction was allowed to stir

at room temperature. After 20 h, the carboxyl-functionalized SiO_2@Au was recovered by centrifugation at 5000 rpm for 10 min. The conjugation of daunorubicin with carboxyl was followed by reported procedure. Daunorubicin conjugated to carboxyl-functionalized SiO_2@Au, we used EDC/NHS coupling that can bind covalently with COOH- SiO_2@Au and amine groups of DNR. A 0.1 M EDC/NHS solution (1-ethyl-3-(3-dimethylaminopropyl)- carbodiimide/N-hydroxysulfosuccinimide (Sulfo-NHS), and 80 μL of 10 mM daunorubicin were mixed with 10 mL of the carboxyl-functionalized SiO_2@Au solution for 24 hr. Finally, the DNR-SiO_2@Au were washed with DI water twice, and pellet was collected. For loading $^{Re\text{-}S}$CO, pellet (600 μL) was dispersed in 400 μL ethanoic solution of $^{Re\text{-}S}$CO (1 M) for 24 h. Pellet was collected by centrifugation at 2000 rpm and washed with water (2 mL) three times. After that DNR-SiO_2@Au-$^{Re\text{-}S}$CO was dispersed in buffer and keep in 4^{o}C. Please note that the ratio of water to ethanol is important. the high ratio of ethanol will reduce the loading of $^{Re\text{-}S}$CO to silica.

The $^{Re\text{-}S}$CO loading content and entrapment efficiency were calculated by the following equations: Loading content = (concentration of $^{Re\text{-}S}$CO – remaining $^{Re\text{-}S}$CO in solution)/concentration of SiO_2@Au. The heating process from SiO_2@Au was monitored with IR camera (FLIR, A325sc). In the release experiment, 1.0 mL of SiO_2@Au-$^{Re\text{-}S}$CO solution was agitated and irradiated with 808 nm laser light (2 W/cm^2). The mixture was centrifuged at one hour intervals. The supernatant was collected and the same volume of fresh buffers was added back to the residual mixture. The amount of released $^{Re\text{-}S}$CO in the surfactant was determined by Raman measurement at 2113 cm^{-1} on nanopillar substrate.

Preparation of DNR-$^{Re\text{-}S}$CO Nanotag. 10 μM (1 μL) of freshly prepared solutions of $^{Re\text{-}S}$CO in ethanol were mixed with 60-nm gold colloids (2.6×10^{10} particles/mL, BBInternational UK) in ethanol. After a 2-day incubation period, the excess metal carbonyl was removed by centrifugation (10,000 rpm, 2 min). We quantified the number of metal carbonyls bound on the nanoparticles by using ICP-MS

to measure the concentration of rhenium elements from unreacted metal carbonyls left behind in the process (supernatant). The unreacted amount was used to substrate the original amount of metal carbonyls. The amount was divided by the number of gold nanoparticles, in which we determined based on the peak optical density and BBInternational UK product data sheet. The number of metal carbonyls per gold nanoparticles was ~216 ± 42. For the conjugation of NRD to nanotag, DNR was first incubated with thiol-PEG (2K)-NHS for overnight in 1.5 to 1 ratio via the interaction between amine and NHS functional group. The excess of DNR will be removed via dialysis in water for 12 hours. The nanotag was then incubated with 10 μM (1 μL) of thiol-PEG (2K)-DNR for 2 hours. The excess thiol-PEG (2K)-DNR was removed by centrifugation (10,000 rpm, 2 min).

DNA detection. $^{Os\text{-}S}CO$ embedded nanopillar substrate was first functionalized with thiol functionalized streptavidin (Nanocs). 1 mg of thiol functionalized streptavidin dissolved in 2 mL of water in a 2.5 mL eppendorf tube. Substrate (0.3 x 0.3 cm) was incubated in the solution for 24 hour. Substrate was then washed with water (5 mL) for three times and dried under nitrogen gas flow. Standard target DNA sequence which was prepared from amine reactive biotin (NHS-Biotin) and terminal NH_2 in the DNA sequence. For DNA test, we prepared 5 mL of hybridization mixture (2x standard saline citrate, 50% deionized formamide, 10% dextran sulfate, 10% Denhardt's solution, 0.5% Tween 20, 0.25 mg/mL salmon sperm DNA) containing the biotinylated EBV DNA specific from Bam HI (W) V region of EBV genome (Enzo Diagnostic, Inc.). The mixture as denatured simultaneously on a 95 ^{o}C heating for 12 minutes and cooling rapidly. After hybridization at 37 ^{o}C, the substrate was incubated with substrate for 1 hour. The substrate was then washed with saline sodium citrate buffer for three times and incubated with DNR-SiO_2@Au-$^{Re\text{-}S}CO$ solution (20 OD of nanorod) for 30 minute at room temperature. After washing with SSC, the substrate was dried under nitrogen flow. SERS measurement was done before NIR (808 nm; 2 W/m^2) irradiation applied as for monitoring the release process. NIR was applied to the

substrate (the light spot fully cover the substrate, 0.3 cm) for 1 hour. Specificity study was performed with 100 ng/mL of non-complementary and base mismatch.

BamHI (W) V is tandemly repeated (7 to 12 times) in the viral genome and widely used for hybridization detection of EBV. The sequence (5'-3') is BiotinATCCCCACCGGCCCTTCTCTCTGTCCCCCTGCTCCTCTCCAACCTTCG CTCCACCCTAGACCCCAG

Complementary sequence:
TAGGGGTGGCCGGGAAGAGAGACAGGGGGACGAGGAGAGGTTGGAAGC GAGGTGGGATCTGGGGTC

Single base mismatch sequence:
TAG**T**GGTGGCCGGGAAGAGAGACAGGGGGACGAGGAGAGGTTGGAAGC GAGGTGGGATCTGGGGTC

Double base mismatch sequence:
TAGGGGTGGCC**T**G**T**AAGAGAGACAGGGGGACGAGGAGAGGTTGGAAGCG AGGTGGGATCTGGGGTC

Quadruple base mismatch sequence:
TAGGGGTG**A**CCGGGAAGAGAGACAGGG**T**GACGAGGAGAGG**G**TGGAAGC GAGGTGGG**G**TCTGGGGTC

2. Supporting figures

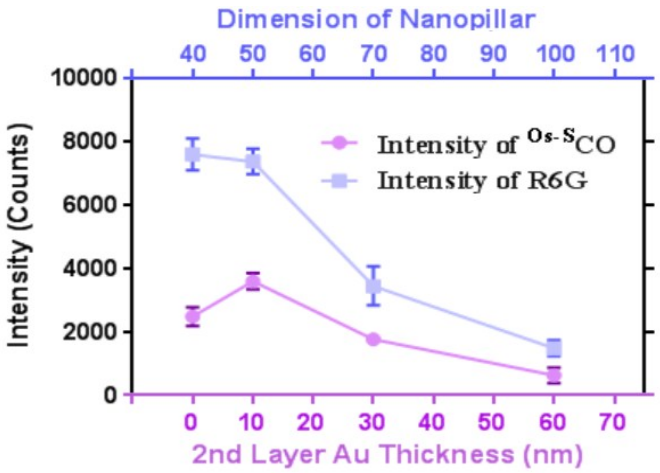

Figure S1. Calculated near-field electromagnetic field distribution of the Au-$^{Os\text{-}S}$CO-Au nanopillar with different 2nd Au layer thickness.

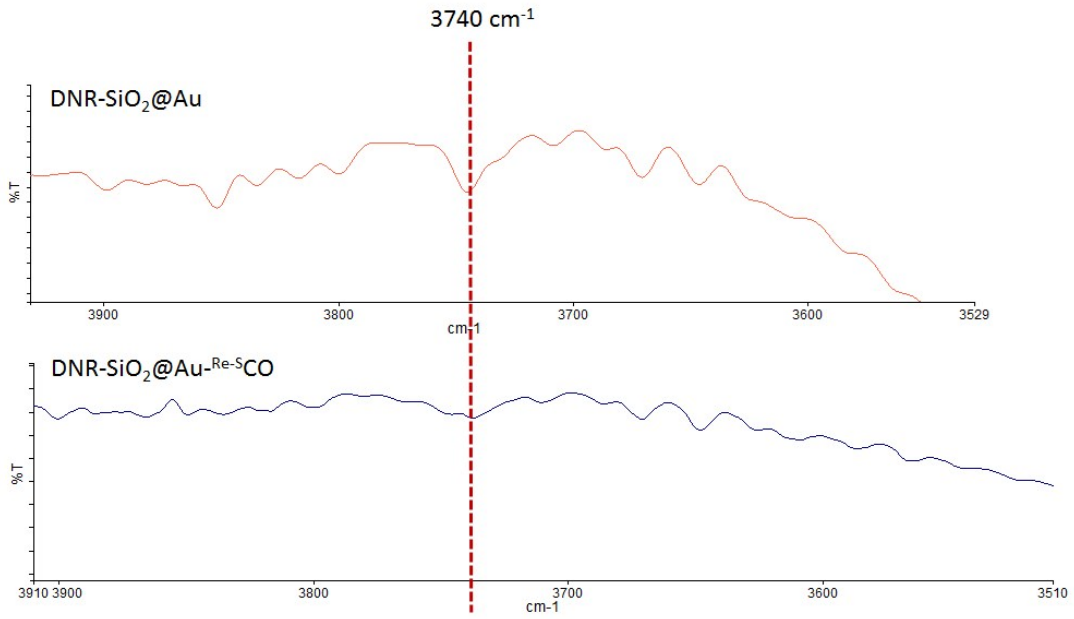

Figure S2. A broad absorption between 3800-3300 cm^{-1} is observed. The 3740 cm^{-1} is due to the stretching mode of free silanols and a broad absorption at lower wavenumbers, extending to 3300 cm^{-1} which is due to hydrogen bonding between silanols.

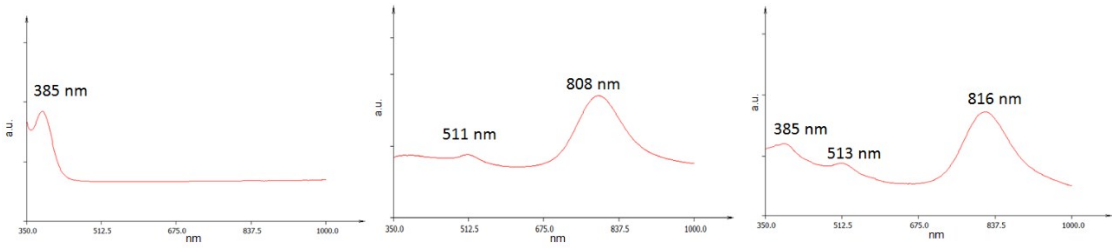

Figure S3. Two typical Au plasmon resonances of SiO_2@Au in the transverse band (520 nm) and the longitudinal band (~800 nm) and absorption of $^{Re\text{-}S}CO$.

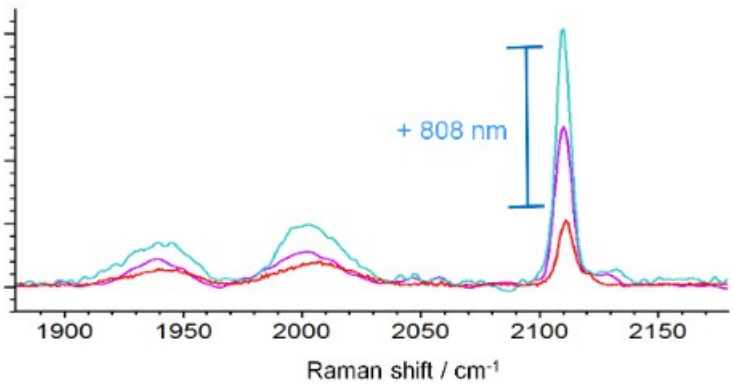

Figure S4. Spectra of $^{Re\text{-}S}CO$ release induced by irradiation with an 808 nm laser.

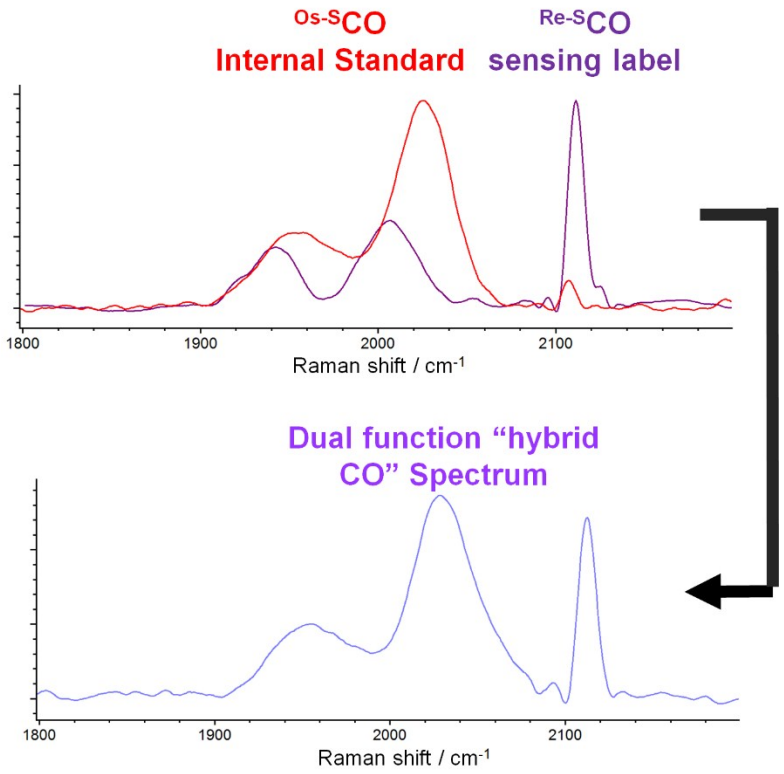

Figure S5. Overlapping SERS spectra of Os-SCO and Re-SCO used to produce a dual functional "hybrid CO" spectrum.

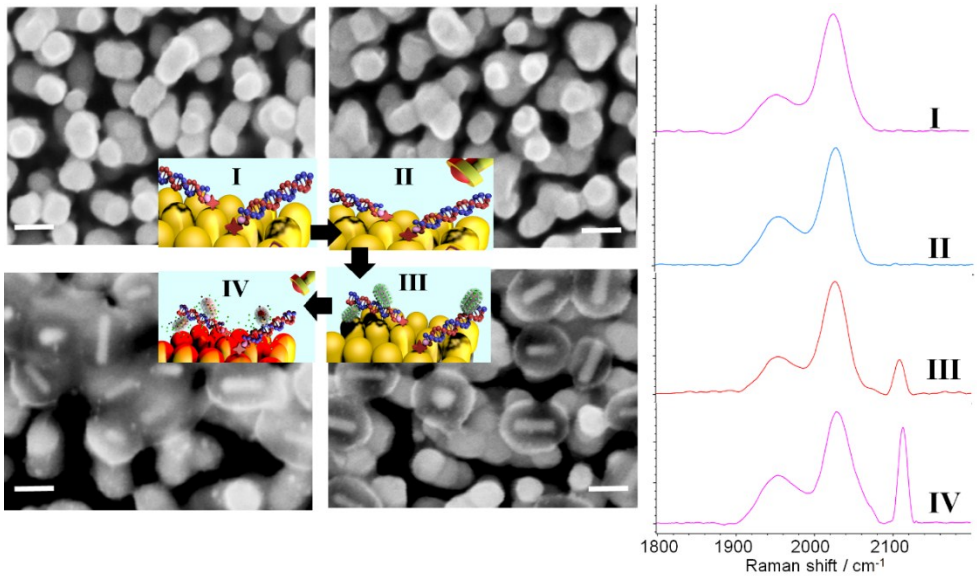

Figure S6. Mechanism detection and the generation of hybrid spectrum.

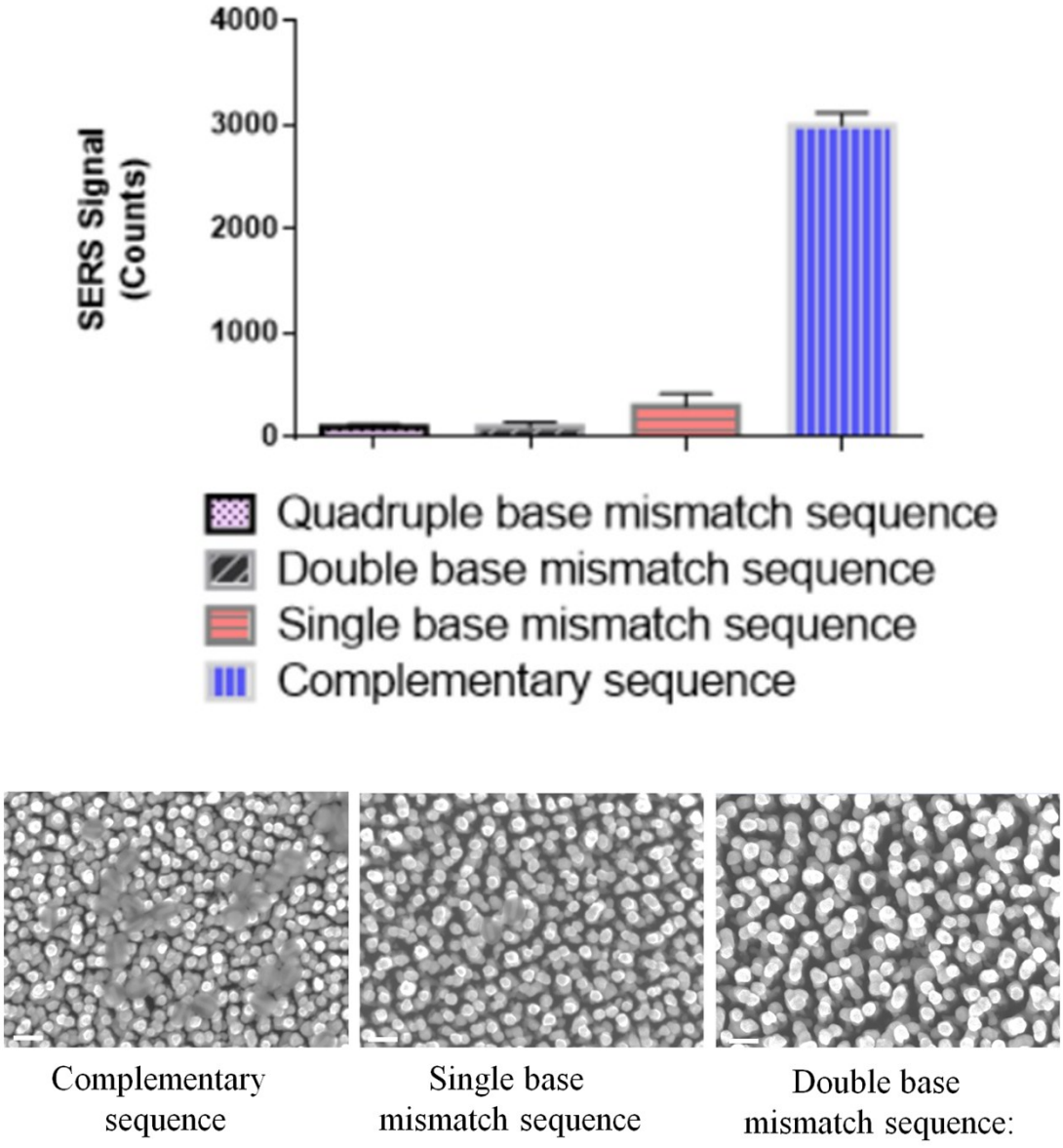

Figure S7. SERS response showing the selectivity of the nanosensor with 1 pM concentration of DNA and SEM images of specific study with different mismatch sequence and complementary sequence.

Concentration of	Intensity
1000	3000
1000	3100
1000	3312
1000	2980
1000	2987
800	2500
800	2312
800	2411
800	2245
800	2233
500	1500
500	1432
500	1344
500	1654
500	1622
200	800
200	760
200	654
200	986
200	811
100	400
100	342
100	321
100	564
100	532

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99104676
R Square	0.98217369
Adjusted R Square	0.98139863
Standard Error	136.256862
Observations	25

ANOVA

	df	*SS*	*MS*	*F*	*ignificance F*
Regression	1	23527265.39	23527265	1267.228	1.28E-21
Residual	23	427016.4456	18565.93		
Total	24	23954281.84			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*pper 95.0%*
Intercept	161.070748	49.49947357	3.253989	0.003496	58.67329	263.4682	58.67329	263.4682
X Variable 1	2.82886395	0.079466616	35.59814	1.28E-21	2.664475	2.993253	2.664475	2.993253

LOD 57.74

LOD = (3.3*49.49)/2.8

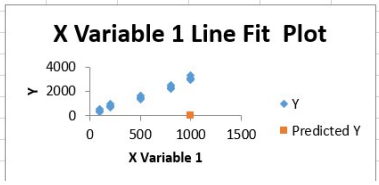

Figure 8. Determination of limit of detection.

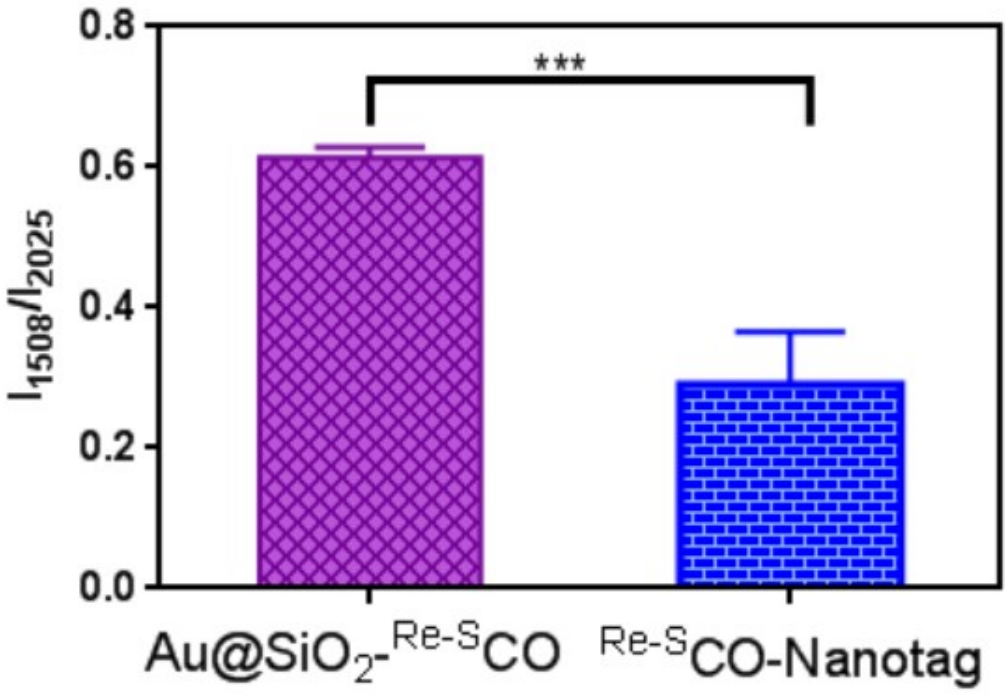

Figure S9. Comparison of DNR-Au@SiO-$^{Re\text{-}S}$CO with the SERS nanotag (DNR-$^{Re\text{-}S}$CO nanotag) method.

Table S1. Recovery study of SERS assay.

	Spiked Concentration (nM)	Detected Concentration (nM)	Recovery (%)
PBS Buffer	1.5	1.35 ± 0.12	90
Plasma	1.5	1.31 ± 0.30	87