Supplementary Information

Simultaneous SERS detection and imaging of two biomarkers on cancer cell surface by self-assembly of branched DNA-gold nanoaggregates

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S1 Experimental section

S1.1 Reagents and apparatus

Reagents: All oligonucleotides used in the present study were synthesized and purified by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China), and the sequences were listed in Table S1. Hydrogen tetrachloroaurate(III) tetrhydrate ($HAuCl_4 \bullet 4H_2O$), trisodium citrate, were ordered from Sigma-Aldrich. Other chemicals employed were of analytical reagent grade and were used without further purification. Doubly distilled water was disinfected used throughout the experiments. The gold chip used for Raman detection was purchased from BioNavis Ltd.

Table S1. DNA Sequence Used in This Work

Name	Sequence(5' to 3')	Description
R_1	GCA GTT GAT CCT TTG GAT ACC CTG G CGT CTA ATA TTC AGC ATC	Recognition probe for MUC1
S_1	ATC CAA AGG CAT GTC AG GAT GCT GAA TAT TAG ACG	Linker strand for Raman probe 1
S_1'	SH-TTT GAT GCT GAA TAT TAG ACG	Linker strand for single gold nanoprobe 1
H_1	AGG CAT GTC CGC ATC CCA TTT TAC GAC ATG CCT TTG GAT TTT TTT-SH	Hairpin DNA for the formation of DNA-gold nanoaggregates (Raman probe 1)
H_2	GTC CGC ATC CAA AGG CGA TCG GAA GAT GCG GAC ATG CCT TTT TTT-SH	Hairpin DNA for Raman probe 1
H_3	ATC CAA AGG CAT GTC TCT TCT TAC CCT TTG GAT GCG GAC TTT	Hairpin DNA for Raman probe 1

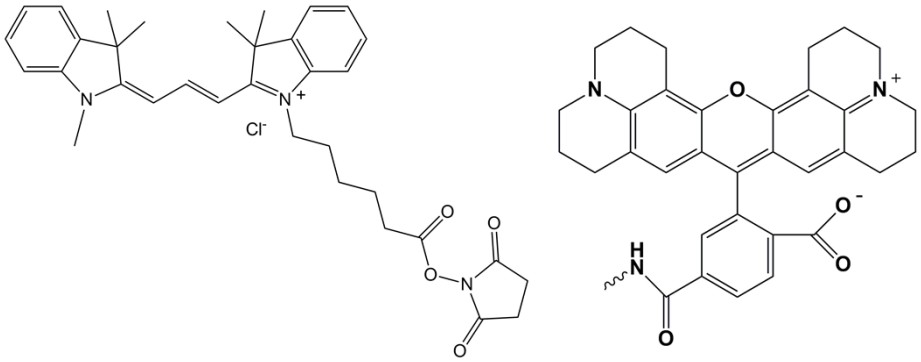
	TTT-SH	
R_2	GGT GGT GGT GGT TGT GGT GGT GGT GG CTA CCA CTT ATA ATC TTG	Recognition probe for nucleolin
R_2'	GCG CGT TGG CCG ACG CGC TTG TTT GG CTA CCA CTT ATA ATC TTG	Random probe for nucleolin
S_2	CCA CCA CAA GCG CGT AG CAA GAT TAT AAG TGG TAG	Linker strand for Raman probe 2
S_2'	SH-TTT CAA GAT TAT AAG TGG TAG	Linker strand for single gold nanoprobe 2
H_1'	CAA GCG CGT CGG CCA GCA GAC TAC ACG CGC TTG TGG TGG TTT TTT-SH	Hairpin DNA for the formation of DNA-gold nanoaggregates (Raman probe 2)
H_2'	CGT CGG CCA CCA CAA GGA GAC GAG TGG CCG ACG CGC TTG TTT TTT-SH	Hairpin DNA for Raman probe 2
H_3'	CCA CCA CAA GCG CGT GGA GAC TCC TTG TGG TGG CCG ACG TTT TTT-SH	Hairpin DNA for Raman probe 2
B_1	SH-TTT TTT CTA GTG ATG-Cy3	Cy3 modified bio-barcode DNA
B_2	SH-TTT CTA GTG ATG CGC-ROX	Rox modified bio-barcode DNA

Table S2. DNA Sequence Used for fluorescence probes

Name	Sequence(5' to 3')	Description
H_1	AGG CAT GTC CGC ATC CCA TTT TAC GAC ATG CCT TTG GAT TTT TTT-Cy3	Hairpin DNA for the formation of fluorescence probe 1
H_2	GTC CGC ATC CAA AGG CGA TCG GAA GAT GCG GAC ATG CCT	Hairpin DNA for fluorescence probe 1

	TTT TTT-Cy3	
H_3	ATC CAA AGG CAT GTC TCT TCT TAC CCT TTG GAT GCG GAC TTT TTT-Cy3	Hairpin DNA for fluorescence probe 1
H_1'	CAA GCG CGT CGG CCA GCA GAC TAC ACG CGC TTG TGG TGG TTT TTT-Rox	Hairpin DNA for the formation of fluorescence probe 2
H_2'	CGT CGG CCA CCA CAA GGA GAC GAG TGG CCG ACG CGC TTG TTT TTT- Rox	Hairpin DNA for fluorescence probe 2
H_3'	CCA CCA CAA GCG CGT GGA GAC TCC TTG TGG TGG CCG ACG TTT TTT- Rox	Hairpin DNA for fluorescence probe 2

The structure of Cy3 and Rox



Apparatus: Field emission scanning electron microscope images were taken with JSM-7500F instrument (Hitachi). Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out on the Tanon EPS-300 power supply (Tanon Science & Technology Co., Ltd., Shanghai, China). UV–vis absorption spectra were obtained with a Cary 50 UV–vis–NIR spectrophotometer (Varian). Fluorescence measurements

were carried out at a F4500 fluorometer. Raman measurement was performed on an inVia Raman Microscope (Renishaw, England). Line mapping was performed with a Streamline Raman mapping system. Transmission electron microscopy (TEM) imaging was taken with JEM-2000EX/ASID2 instrument (Hitachi, Japan). Confocal fluorescence microscope (CFM) images were captured with a Leica TCS SP5 II confocal laser scanning microscope equipped with an Andor EMCCD camera (Germany).

S1.2 Preparation of AuNPs

Gold nanoparticles (AuNPs) were prepared by reduction of tetrachloroauric acid ($HAuCl_4$) with trisodium citrate following the previously reported method [1]. Briefly, after boiling the $HAuCl_4$ solution (0.01%, 100 mL) under vigorous stirring, 1.5 mL of 1% trisodium citrate was added dropwise to the solution and stirred for 20 min at the boiling point. When the color of the solution was turned from gray yellow to deep red, it could indicate the formation of AuNPs. With continuous stirring, the solution was cooled to room temperature and the obtained gold colloidal solutions were stored in brown glass at 4 °C until use.

S1.3 Immobilization of haripin DNA and bio-barcode DNA on the surface of AuNPs

The haripin DNA and the Raman dye molecules modified bio-barcode DNA were immobilezed on the surface of AuNPs following the below procedures. (Taton et al., 2000). 10 μL of 1.0×10^{-7} M hairpin DNA (5'-thiol) and 50 μL of 1.0×10^{-6} M bio-barcode DNA (5'-thiol, 3'-Rox or Cy3) were added to freshly prepared AuNPs (1

mL), and shaken gently at 37 °C for 24 h. After that, the solution was aged in salts (0.05 M NaCl, 200 μL) for 6 h and in salts (0.1 M NaCl, 200 μL) for 6 h, respectively. The obtained solution was centrifuged for 30 min at 10,000 rpm to remove the excess reagents, the red precipitate was washed and centrifuged repeatedly for three times. The resulting hairpin DNA-barcode-Au nanoprobe was dispersed into a phosphate buffer solution (PBS, 0.1 M, pH 7.0) and stored at 4 °C for further use.

S1.4 Preparation of the Raman probes

For preparing the DNA-gold nanoaggregates, three hairpin DNA-Au nanoprobes and the linker DNA (S_1 OR S_2) were mixed together and incubated at 20 °C for 1 h. Then the mixture was centrifuged for 30 min at 10,000 rpm to remove the excess reagents and the precipitate was washed repeatedly for three times. The resulting Raman probes was dispersed into a buffer solution (0.1 M PBS, pH 7.0) and stored at 4 °C for cancer cell detection.

S1.5 Cultivation of the cancer cells

The MCF-7 cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 IU/mL penicillin-streptomycin in a CO_2 environment (95% air and 5% CO_2) at 37 °C. After growing to 90% confluence, the cells were digested with trypsin and 1.0 mL suspension dispersed in RPMI 1640 cell media buffer was centrifuged at 3000 rpm for 5 min, then it was washed with PBS (0.1 M, pH 7.0) for three times and resuspended in 50 μL PBS. The cell number was counted on a hemocytometer prior to each experiment.

S1.6 Incubation of the cancer cells with the prepared Raman probes

The suspended cells were used for incubation with recognition probes (R_1 and R_2) at room temperature. After 30 min, the prepared Raman probes were added and incubated with the cells for 1 h. Nonspecific binding Raman probes were washed out using PBS and the remaining probes were selectively attached to the biomarkers of cancer cell surface for Raman measurement.

S1.7 SERS Measurements

10 μL of MCF-7 cell suspension was casted on gold chip and the Raman spectra were obtained by using a Renisaw in Via Raman spectrometer at an excitation laser of 633 nm. A microscope equipped with a 50 × long working distance objective was used to focus the incident excitation laser. The laser power was 25 mW, and the acquisition time was limited to 10 s for all samples. Line mapping was performed with a Streamline Raman mapping system (Renishaw, U.K.) and SERS maps were prepared by integrating the area under the characteristic peaks. The Raman spectra and images were calibrated with WiRE Raman Software (Version 3.3).

S2 Characterization of Raman dyes modified AuNPs

The prepared AuNPs, Rox-modified DNA, Cy3-modified DNA and the hairpin DNA-barcode-Au nanoprobes were characterized by UV-visible spectra. As could be seen from Figure S1 (A), curve a exhibited the characteristic absorbance of AuNPs at ~520 nm. Curve b showed the characteristic absorbance of DNA at ~260 nm and the spectral property of rhodamine dye at 500-600 nm consistent with the literature[2]. Curve C exhibited both the characteristic absorbance of Rox-DNA and AuNPs,

indicating the successful conjugation of Rox-DNA with AuNPs. The results of Figure S1 (B) indicated that Cy3-DNA was also conjugated with AuNPs.

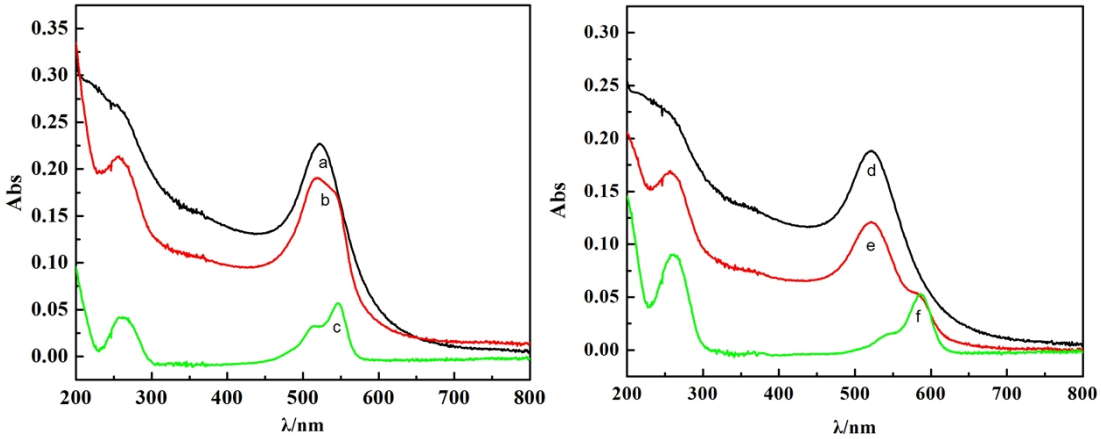


Figure S1. UV spectra of AuNPs (a), Cy3 modified Barcode DNA (b), Cy3-DNA-AuNPs (c), AuNPs (d), Rox modified Barcode DNA (e), Rox-DNA-AuNPs (f).

The fluorescence changes of Rox-modified DNA and Cy3-modified DNA after being used to incorporate with AuNPs were also investigated. From the results of Figure S2, it could be seen that the fluorescence intensity of the supernatant was dramatically decreased, indicating that lots of the Rox-DNA and Cy3-DNA were conjugated with AuNPs.

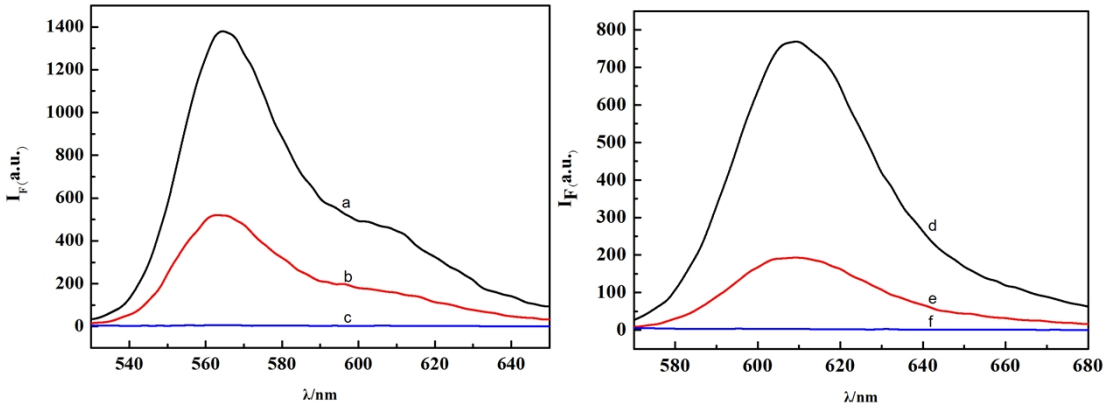


Figure S2. Fluorescence spectra of Cy3 modified Barcode DNA (a), the supernatant separated from Cy3-DNA-AuNPs (b), PBS (c). Rox modified Barcode DNA (d), the supernatant separated from Rox-DNA-AuNPs (e), PBS (f).

S3 Optimization of the ratio of barcode DNA to haripin DNA

The Raman intensity is influenced by the proportion of the barcode DNA and the hairpin DNA immobized on the surface of AuNPs. Figure S3 showed the variance of Raman intensity with the proportion of the barcode DNA and the hairpin probe. It could be seen that the Raman intensity increased upon raising the proportion from 10:1 to 50:1, and then it tended to level off. Thus the ratio of 50:1 was selected for the subsequent assays.

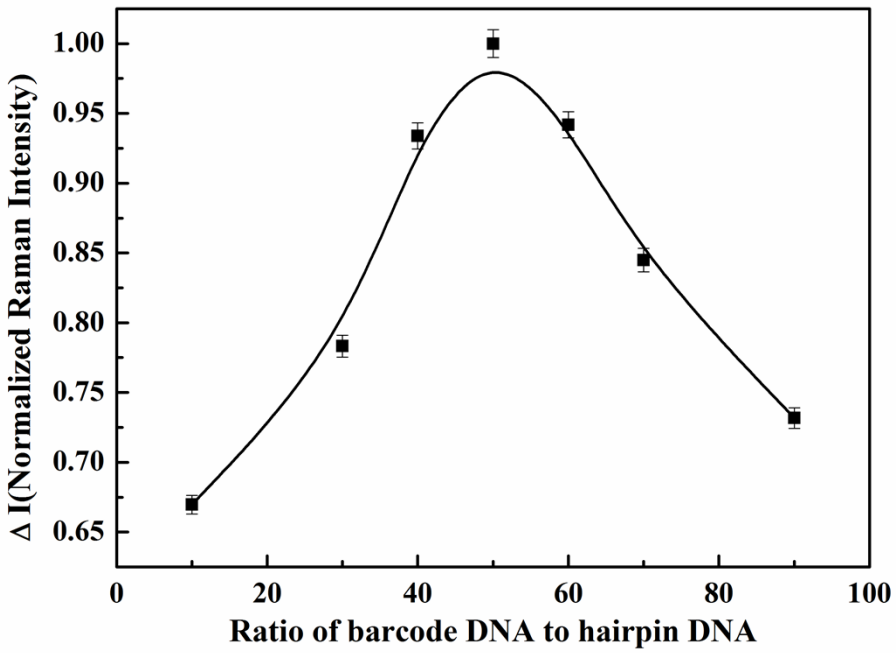


Figure S3. Raman signals for different ratio of barcode DNA to hairpin DNA.

S4 Optimization of the reaction time

For self-assembly of the branched DNA-gold nanoaggregates, the reaction time is an important influencing factor. The final SERS signals were dependent on the amount of the AuNPs in the prepared Raman probes. In order to obtain higher sensitivity, the time of the self-assembly was investigated and Figure S4 showed the changes of Raman signals generated by performing the experiment at different time intervals. The results showed that the Raman intensities increased rapidly with the

increase of reaction time to 60 min, and a plateau effect was reached after this time. Therefore, the reaction time was controlled at 60 min all through the experiment.

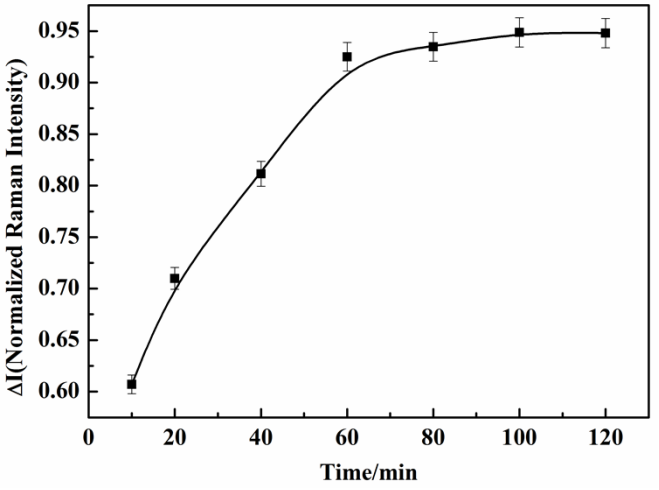
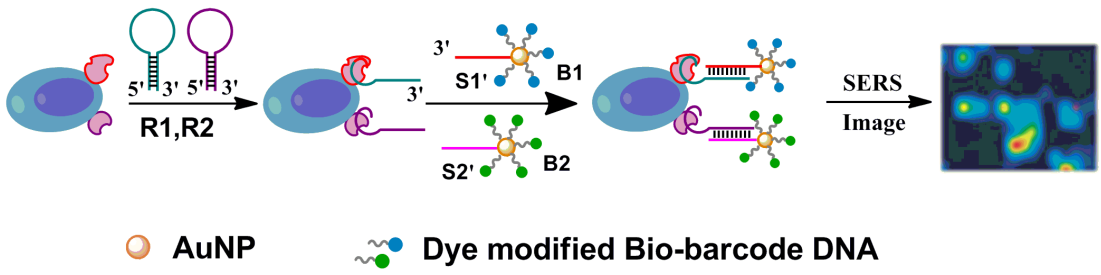


Figure S5. Influence of the reaction time on the Raman intensity.

S5 Verification of the amplification effect of the branched DNA-gold nano-aggregates

To verify the amplification effect of the branched DNA-gold nanoaggregates for SERS detection, another experiment was performed using single gold nanoprobe. The principle was shown in Scheme S1 and the single gold nanoprobe was prepared by immobilizing the linker strand (S_1' or S_2') and the dye modified bio-barcode DNA (B_1 or B_2) on the surface of AuNPs. Then MUC1 mucin and nucleolin distributed on the surface of MCF-7 cells were detected using this method and the results were shown in Figure S6. Curve a and curve b were the Raman spectra from two random points of the cell surface using single gold nanoprobe, in which the SERS signals were very low. However, the signals were significantly enhanced by using of the branched DNA-gold nanoaggregates for MCF-7 cell detection as shown in curve c and curve d, demonstrating the effective signal enhancement of our proposed platform.



Scheme S1. Schematic diagram for simultaneous detection of two biomarkers on the cancer cell surface using Raman dyes-modified AuNPs as Raman probes.

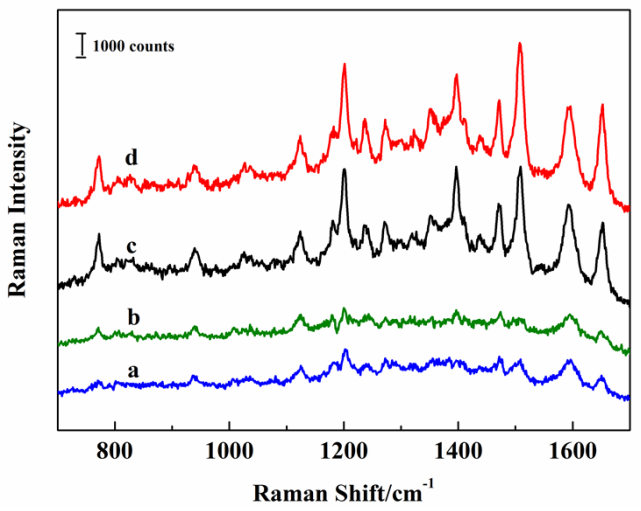


Figure S6. SERS spectra of the MCF-7 cells incubated with (a), (b) the single gold nanoprobe; (c), (d) the branched DNA-gold nanoaggregates.

S6 Effect of the temperature for preparing the DNA-gold nanoaggregates

The experimental temperature was set at 37 °C (suitable for cancer cell culture) and 20 °C (room tempetature) for the assembly of three hairpin DNA species. From the polyacrylamide gel electrophoresis results of Figure S7, a high molecular weight product was formed at 20 °C (lane 3) and several misty fragments appeared in lane 4 when the experimental temperature was set at 37 °C. The results indicated that lower temperature was more favorable to the assembly of the hairpin

DNA species. Considering the reaction time and efficiency, the experimental temperature for preparing the DNA-gold nanoaggregates was set at 20 °C.

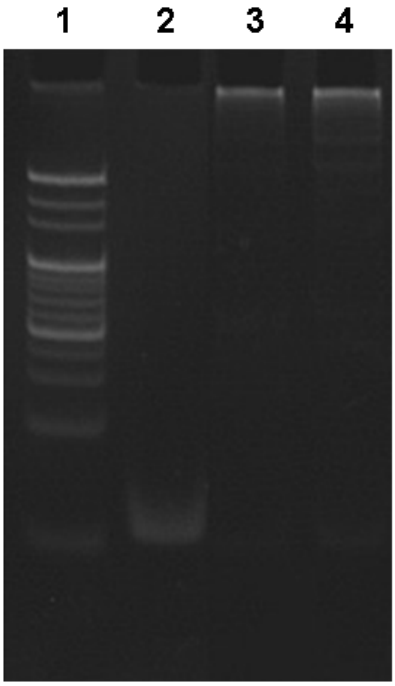


Figure S7. Polyacrylamide gel electrophoresis of the assembly of hairpin DNA species. Lane 1: DNA marker; Lane 2: 1.0×10^{-6} M of S1; Lane 3: the procuct after incubating S1, H1, H2 and H3 at 20 °C for 1 h; Lane 4: the procuct after incubating S1, H1, H2 and H3 at 37 °C for 1 h.

S7 TEM images of H_1-B_1-AuNPs and the DNA-gold nanoaggregates

For further demonstrating the formation of DNA-gold nanoaggregates, transmission electron microscopy (TEM) imaging was performed in the revised ESI and the results were shown in Figure S8. In this experiment, AuNPs were functionalized with hairpin DNA (H_1, H_2 or H3) and Cy3 modified bio-barcode DNA (B1), which were marked as H_1-B_1-AuNPs, H_2-B_2-AuNPs and H_3-B_3-AuNPs

respectively. Figure S8 (A) was the TEM image of H_1-B_1-AuNPs, which could be seen that the AuNPs were dispersed well. However, the AuNPs aggregated when three kinds of hairpin DNA-barcode-AuNPs were mixed with the linker DNA (S1) as could be seen from Figure S8 (B) and (C). The concentration of H_1-B_1-AuNPs was identical with the total concentration of H_1-B_1-AuNPs, H_2-B_2-AuNPs and H_3-B_3-AuNPs, thus it could be convinced that the formation of DNA-gold nanoaggregates was due to the hybridization of three hairpin DNA species.

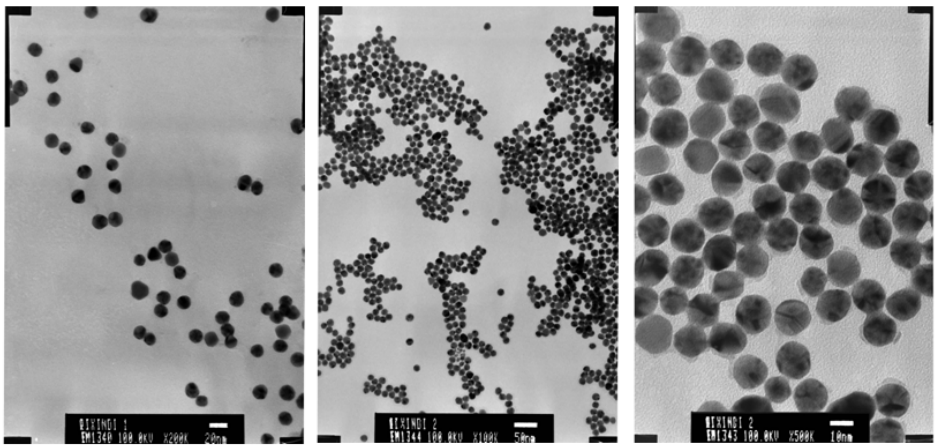


Figure S8. (A) TEM image of H_1-B_1-AuNPs. (B), (C) TEM images of the fabricated DNA-gold nanoaggregates after mixing the linker DNA (S1), H_1-B_1-AuNPs, H_2-B_2-AuNPs and H_3-B_3-AuNPs for 1 h at 20 °C.

S8 SERS images of human oral epithelial cell

For testing the specificity of this method, human oral epithelial cells were used as control cells and incubated with the recognition probes of MUC1 mucin and nucleolin (R1, R2) and two Raman probes for Raman mapping image. The results of SRES images were shown in Figure S9. No Raman signal was observed from human oral epithelial cells, which indicated that the nanoaggregates could not be

bound onto the surface of human oral epithelial cells, because both MUC1 and nucleolin are negative expressed.

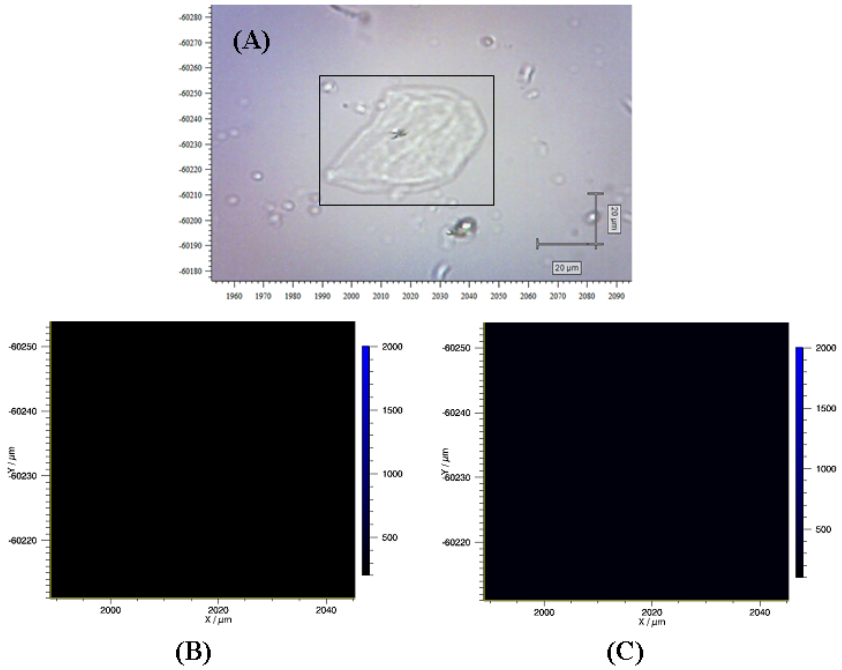


Figure S9. (A) Optical image of single human oral epithelial cell; (B) Raman mapping image prepared by integrating the area under the peak of 1586 cm^{-1} for Cy3; (C) Raman mapping image prepared by integrating the area under the peak of 1499 cm^{-1} for Rox.

S9 Fluorescence images of MCF-7 cells

For performing fluorescence images of MCF-7 cells, the fluorescence probes were prepared by self-assembly of Cy3 or Rox-modified hairpin probes and the recognition probes were not changed. The cells were examined by Confocal fluorescence microscope (CFM) with 514 nm excitation and the results were shown in Figure S10. The Cy3 signal was displayed in green with 540-580 nm emission and the Rox signal was displayed in red with 600-640 nm emission. The imaging results also indicated that the distribution of MUC1 mucin and nucleolin

could be observed and two biomarkers could be simultaneously detected.

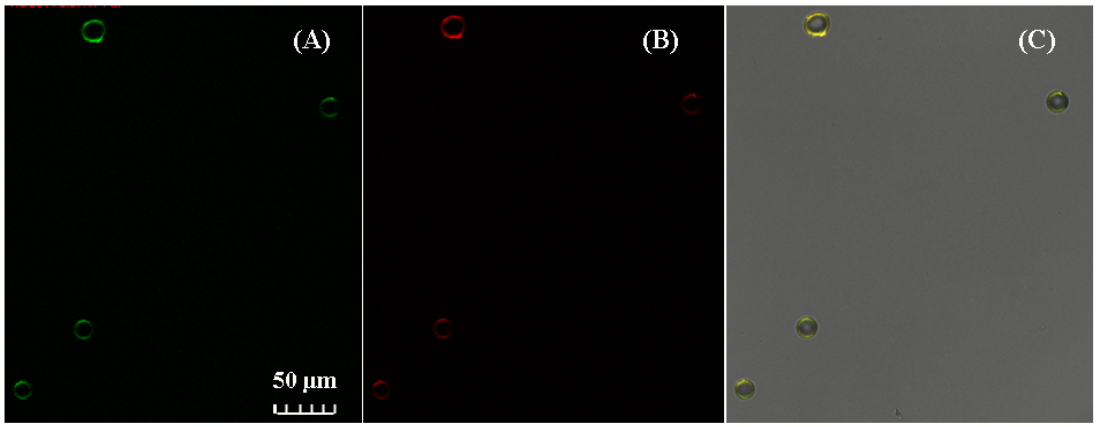


Figure S10. Confocal laser scanning microscopy images of MCF-7 cancer cells. (A) Fuorescence image of MCF-7 cells recorded by Cy3 with 514 nm excitation and 540-580 nm emission; (B) F uorescence image of MCF-7cells recorded by Rox with 514 nm excitation and 600-640 nm emission; (C) Merged image of A, B and bright-field image of MCF-7 cells.

References

1 K. C. Grabar, P. C. Smith, M. D. Musick, J. A. Davis, D. G. Walter, M. A. Jackson, A. P. Guthrie, M. J. Natan, *J. Am. Chem. Soc*., **1996**. 118, 1148.

2 X. Xue, F. Wang, J. Zhou, F. Chen, Y. Li, J. Zhao, *J. Agric. Food Chem.*, **2009**, **57**, 4500.