Supporting Information for

Imidazolium-based mass tag for protein biomarkers detection by laser desorption ionization mass spectrometry

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This supporting information included the synthesis of imidazolium-based mass tags (IMTs) and nanoparticles, detection of cell surface proteins, LDI-MS spectra of Au-IMT1, fragment structures of IMT1, TEM image of Au@anti-CEA/IMT1 and CEA protein pre-incubation assay.

Experimental section

Chemicals and Reagents

Mass tag synthesis: Dichloromethane (AR), trichloromethane (AR), petroleum ether (AR), ethyl acetate (AR), methanol (AR), acetonitrile (AR), acetone (AR), and sodium sulphate (AR) were purchased from Tongguang Fine Chemicals Company (Beijing, China). Tosyl chloride (99%), tetraethylene glycol (99%), pentaethylene glycol (97%), triphenylmethanethiol (97%), 11-bromo-1-undecanol (98%), 4-dimethylaminopyridine (98%), lithium bromide (99%), trifluoroacetic acid (AR) were purchased from Aladdin Company (Shanghai, China). Triethylene (AR) glycol was purchased from China National Pharmaceutical Group Corporation (Beijing, China). Hexaethylene glycol (97%) was purchased from Bide Pharmatech Ltd. (Shanghai, China). Triisopropylsilane (97.5%) was purchased from J&K Scientific (Beijing, China).

Nanoparticle synthesis: Hydrogen tetrachloroaurate (III) hydrate was purchased from China National Pharmaceutical Group Corporation (Beijing, China). Citric acid trisodium salt (AR) was purchased from Xilong Chemical Co. Ltd. (Shantou, Guangdong, China).

Protein detection: Mouse monoclonal CA125 antibody (CA125) was purchased from Novus Biologicals, Inc. (Colorado, USA). Mouse monoclonal CEA antibody (CEA) was purchased from Fitzgerald, Inc. (Acton, MA, USA). Mouse monoclonal EpCAM antibody (EpCAM) was purchased from Proteintech Group, Inc. (Wuhan, Hubei, China). Acetonitrile (HPLC grade), methanol (HPLC grade), and hydrofluoric acid (HPLC grade) were purchased from Thermo Fisher Scientific (CA, USA). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, AR) was purchased from J&K Scientific Ltd. (Beijing, China). 4,7,10,13,16,19,22,25,32,35,38,41,44,47,50,53-Hexadecaoxa-28,29 dithiahexapentacontanedioic acid di-N-succinimidyl ester (NHS-PEG-S-S-PEG-NHS), (11-mercaptoundecyl) hexa (ethylene glycol) (HS-$(CH_2)_{11}(OC_2CH_2)_6OH$, MT), and sinapic acid were purchased from Sigma-Aldrich Co., LLC. (Shanghai, China). 1% Bovine Serum Albumin (BSA) was purchased from

Yuanye Bio-Tech. Co., Ltd (Shanghai, China). Phosphate Buffered Saline (PBS, 1.0 mmol/L, pH=7.4, 8.0 mmol/L Na_2HPO_4, 136.0 mmol/L NaCl, 2.0 mmol/L KH_2PO4, 2.6 mmol/L KCl) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Purified water was produced by Hangzhou Wahaha Group (Hangzhou, China).

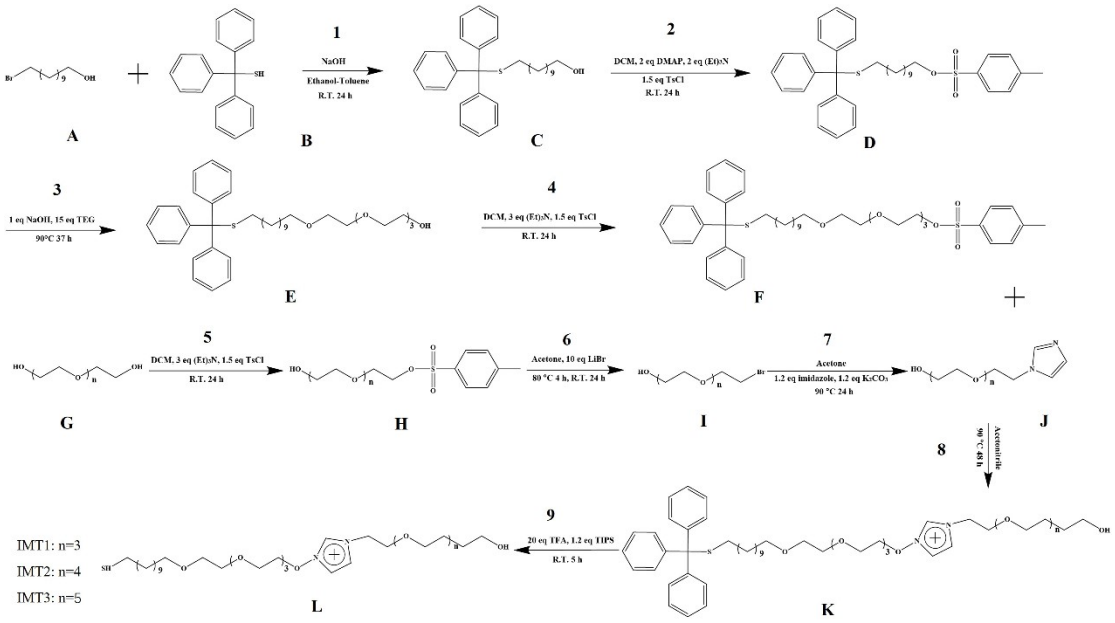
Cell culture: MCF-7 cells and RPMI-1640 Medium (+ 80 U/mL penicilin, + 0.08 mg/mL streptomycin) were purchased from Nanjing KeyGen Biotech. Co., Ltd (Nanjing, China). Fetal bovine serum (FBS) was purchased from Grand Island Biological Company (Grand Island, NY, USA). Accutase cell detachment solution (+ 0.5 mmol/L EDTA, + 3 mg/L phenol red) was purchased from Hyclone (Logan, UT, USA).

Apparatus

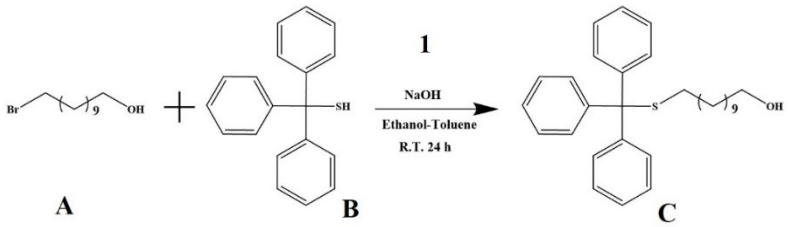
Most of the MS experiments and the immunoassays were carried out on Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) experiments (AB SCIEX 5800, Foster City, CA, USA) in reflection or linear mode. The laser intensity was set to be 6500 (instrument-specific units). Accurate m/z and molecular structures were confirmed with the help of Orbitrap MS (Q Exactive plus, Thermo Scientific, San Jose, CA, USA). The UV-vis absorption spectra were performed on Shimadzu UV-3600 plus spectrometer (Shimadzu, Japan) with a scanning range of 400 nm to 700 nm. Transmission electron microscopy (TEM) images was obtained on field-emission high resolution transmission electron microscope (Tecnai G2 F30 S-TWIN, USA) with accelerating voltage 300 kV. ^{1}H NMR spectra were measured on ARX400 nuclear magnetic resonance spectrometer (Bruker, Switzerland).

Synthesis of imidazole-based mass tags

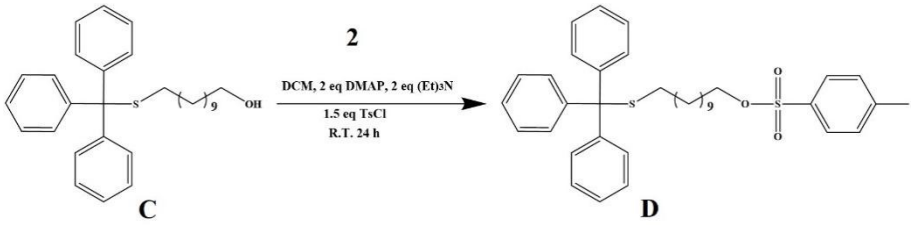
Imidazolium-based mass tags (IMTs) were prepared according to the reported literature with modifications [1,2].



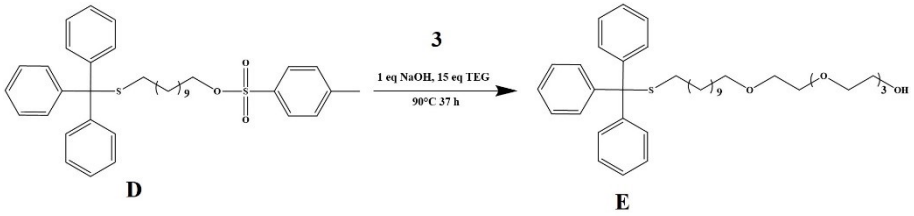
Scheme S1 The illustration of synthetic route of imidazole-based mass tags (IMT1, IMT2, and IMT3).



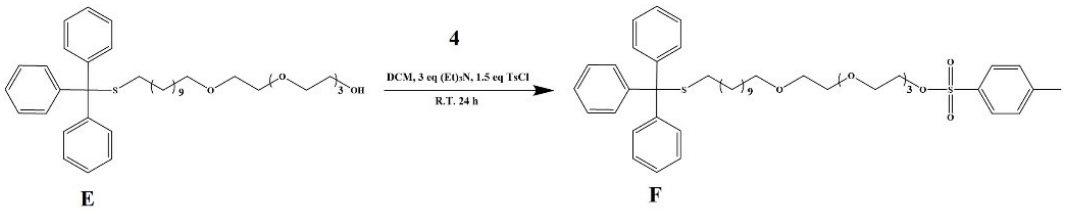
1: 11-Bromo-1-undecanol (**A**, 6.0 g) was dissolved in a solution of ethanol/toluene (1:1, 50 mL) followed by the addition of NaOH (1.43 g) in 15 mL of H_2O. Then triphenylmethanethiol (**B**, 7.92 g) in a solution of ethanol/toluene (1:1, 50 mL) was added to the 11-bromo-1-undecanol mixture. After reaction under stirring at room temperature for 24 h, the resulting reaction solution was poured into a saturated solution of $NaHCO_3$ and washed three times. Then, the organic layer was dried over anhydrous Na_2SO_4 and concentrated by evaporation. The residue was purified by silica gel chromatography with petroleum ether/ethyl acetate (9:1 and 4:1, v/v). The solvent was removed by a rotary evaporator to yield a colorless oil product **C** (Yield 90%).



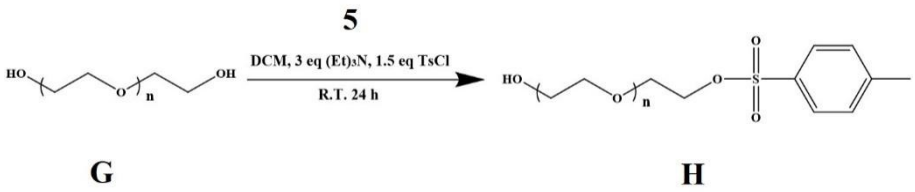
2: The product **C** (24 mmol/L) was dissolved in 100 mL of dichloromethane (DCM), and 4-dimethylaminopyridine (DMAP, 2.0 equiv) and triethylamine ($(Et)_3N$, 2.0 equiv) was added. The resulting solution was stirred at 0°C for 0.5 h. p-Toluenesulfonyl chloride (TsCl, 1.5 equiv) in 50 mL of DCM was added dropwise to the solution maintaining the temperature at 0°C. After 0.5 h, the reaction mixture was reacted for 24 h at room temperature. Then, the mixture was washed twice by 0.1 M HCl and three times by saturated solution of $NaHCO_3$. The organic layer was dried by anhydrous Na_2SO_4 and concentrated by evaporation. The obtained residue was purified by the column chromatography over silica gel using a mixture eluent of petroleum ether/ethyl acetate (7:1, v/v). The target product **D** was obtained as a colorless oil (Yield 90%).



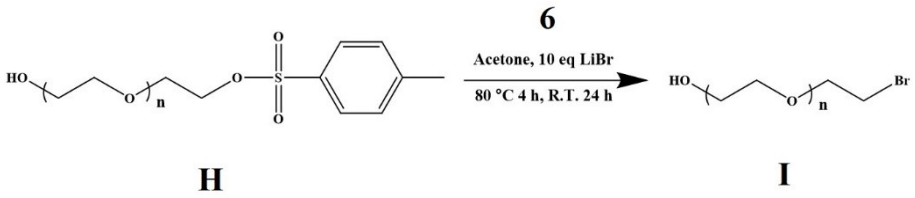
3: NaOH (0.8 g) was dissolved in 1 mL of H_2O, followed by the addition of 50 mL of tetraethylene glycol (TEG, 1.5 equiv). After the mixture was stirred for 1 h at 90°C, the color of the solution changed from colorless to yellow. Subsequently, the product **D** (19 mmol/L) in TEG was added to the reaction mixture and the solution was heated to reflux for 36 h at 90°C. After cooling to the room temperature, the mixture was extracted with petroleum ether/ethyl acetate (4:1, v/v) for six times, and then organic layer was dried over anhydrous Na_2SO_4. The solvent was removed by a rotary evaporator and the residue was purified by the column chromatography with petroleum ether/ethyl acetate (2:1 and 1:1, v/v) to yield a yellow oil product **E** (Yield 60%).



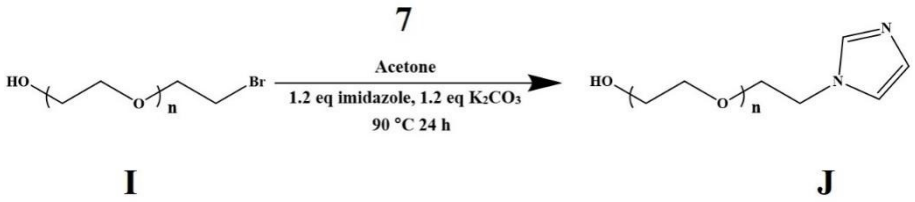
4: A mixture of product **E** (11 mmol/L) in DCM and $(Et)_3N$ (3.0 equiv) was stirred at 0°C for 0.5 h, then TsCl (1.5 equiv) in 50 mL of DCM was injected drop by drop to the mixture solution maintaining the temperature at 0°C for 0.5 h and room temperature for 24 h. The reaction mixture was monitored by TLC, washed three times by saturated $NaHCO_3$, dried over anhydrous Na_2SO_4 and concentrated in a vacuum. The crude product was purified on a silica gel column (etroleum ether/ethyl acetate, 1:1 and 1:2, v/v) to give a yellow oil product **F** (Yield 90%).



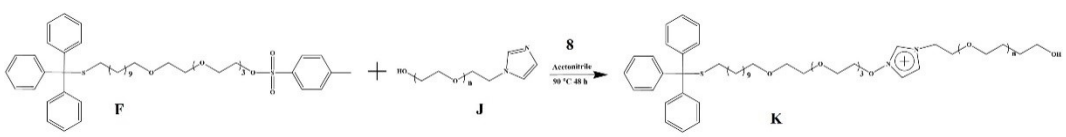
5: To a solution of triethylene glycol (or TEG, pentaethylene glycol, 20 mmol/L) in DCM (100 mL), $(Et)_3N$ (3.0 equiv) was added and the reaction mixture was stirred at 0°C for 0.5 h. TsCl (1.5 equiv) in 50 mL of DCM was added dropwise under continuous stirring in ice bath for 0.5 h, followed by the reaction of 24 h at room temperature. The mixture was stirred for 24 h at room temperature. The reaction was checked by TLC. The mixture solution was washed by saturated $NaHCO_3$ (three times), dried over Na_2SO_4, evaporated, and purified to give a colorless oil product **H** by the column chromatography using a mixture eluent of petroleum ether/ethyl acetate (1:1 and 0:1, v/v) (Yield 80%).



6: To a solution of product **H** (16 mmol/L) in dry acetone (100 mL), lithium bromide (10.0 equiv) was added and the reaction mixture was heated at reflux for 4 h at 80°C. After cooling to room temperature, the mixture was concentrated by evaporation and the crude product was precipitated in trichloromethane (50 mL). The white solid was filtered and the resulting solution washed twice with H_2O, and dried over Na_2SO_4 and reduced pressure to give the product **I** as a pale yellow solid (Yield 80%).

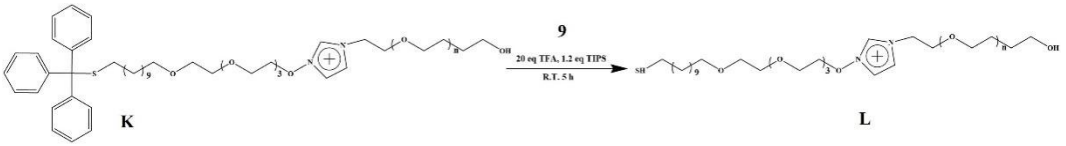


7: In this step, the mixture of K_2CO_3 (6.6 mmol/L) and imidazole (6.6 mmol/L) was added to the solution of product **I** (5 mmol/L) in dry acetone (100 mL), and the mixture was heated at reflux for 24 h at 90°C. The completed reaction mixture was concentrated under reduced pressure and the residue was dissolved in 100 mL of H_2O with the aid of sonication. Afterward, the aqueous layer was extracted by ethyl acetate for three times, and organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography with DCM/methanol (9:1, v/v) as an eluent. The target product **J** was obtained as a colorless oil (Yield 40%).



8: The product **J** (2 mmol/L) was dissolved in 10 mL of acetonitrile followed by the addition of product **F** (2 mmol/L) in 10 mL of acetonitrile. The reaction occurred at

reflux at 90°C for 48 h. The solvent was dried by a rotary evaporator and purified by column chromatography with DCM/methanol (9:1 and 8:1, v/v) as an eluent to yield a white solid product **K** (Yield 50%).



9: The product **K** (2 mmol/L) was dissolved in 10 mL of DCM and a solution of trifluoroacetic acid (TFA, 20.0 equiv) was added under N_2, causing the color of the solution to change to yellow. Afterward the triisopropylsilane (TIPS) was added and the reaction mixture was stirred for 5 h at room temperature. The solvent and TFA was removed using a rotavapor. The residue was dissolved in 10 mL of H_2O with support of sonication and further extracted with DCM/methanol (4:1, v/v) for three times. The solvent was dried in a high vacuum system to yield a yellow oil product **L**.

IMT1 (n=3)：HRMS-ESI (m/z): $[M]^+$ calculated for $C_{30}H_{59}N_2O_8S^+$, 607.3987, found, 607.3981. ^{1}H NMR (500 MHz, D_2O/MeOD-d_4): δ 1.26-1.53 (m, 16H), δ 1.57-1.59 (m, 4H), δ 2.36 (s, H), δ 2.51-2.53 (t, 2H), δ 3.43-3.50 (t, 2H), δ 3.61-3.69 (m, 22H), δ 3.87-3.89 (t, 4H), δ4.39-4.41 (t, 4H)，δ7.29-7.30 (br, 1H), δ7.59 (s, 1H), δ7.64-7.66 (br, 1H), δ8.89 (s, 1H).

IMT2 (n=4)：HRMS-ESI (m/z): $[M]^+$ calculated for $C_{32}H_{63}N_2O_9S^+$, 651.4249, found, 651.4243. ^{1}H NMR (500 MHz, D_2O/MeOD-d_4): δ 1.26-1.35 (m, 16H), δ 1.55-1.58 (m, 4H), δ 2.37 (s, H), δ 2.51-2.53 (t, 2H), δ 3.48-3.50 (t, 2H), δ 3.61-3.69 (m, 26H), δ 3.87-3.89 (t, 4H), δ 4.40-4.42 (t, 4H), δ 7.31-7.32 (br, 1H), δ 7.59 (s, 1H), δ 7.65-7.66 (br, 1H), δ 8.88 (s, 1H).

IMT3 (n=5)：HRMS-ESI (m/z): $[M]^+$ calculated for $C_{34}H_{67}N_2O_{10}S^+$, 695.4511, found, 695.4503. ^{1}H NMR (500 MHz, D_2O/MeOD-d_4): δ 1.27-1.40 (m, 16H), δ 1.54-1.60 (m, 4H), δ 2.37 (s, H), δ 2.50-2.52 (t,2H), δ 3.48-3.50 (t, 2H), δ 3.59-3.64 (m, 30H), δ 3.86-3.91 (t, 4H), δ 4.41-4.43 (t, 4H), δ 7.29-7.30 (br, 1H), δ 7.61 (s, 1H), δ 7.65-7.67 (br, 1H), δ 8.91 (s, 1H).

Synthesis and Functionalization of Nanoparticles

AuNPs were synthesized according to the method of Frens.[3] Briefly, 100 mL 1 mmol/L $HAuCl_4$ in water was heated to boiling followed by adding 10 mL 38.8 mmol/L sodium citrate solution, boiling was continued for another 10 min with color changed into a purplish red. Then, heating source was removed and the reaction continued with stirring for 15 min. The size and concentration of AuNPs were determined by UV-Vis spectroscopy using the method of Haiss et al.[4] AuNPs with a diameter of ~14 nm and concentration of 10 nmol/L were synthesized by this procedure.

The functionalization of AuNPs was conducted as follow: Different antibodies (CA125, CEA and EpCAM) reacted with NHS-PEG-S-S-PEG-NHS linker in a 2: 1 molar ratio in 10 mmol/L HEPES at room temperature for 12 h. At the same time, IMTs were added into 1 mL of 1 nmol/L AuNPs solution and reacted for 12 h. Then, the reacted antibody-PEG-SH was added to 1 mL of different IMTs-modified AuNPs solution for 12 h to obtain the probes. Different amounts of AuNPs, antibodies, and IMTs were optimized to get the best ratio of 1: 50: 20000. Afterwards, the probes solution was centrifuged (6000 rpm, 30 min) and washed three times by water, then the obtained probes were resuspended in 1 mmol/L PBS buffer and stored at 4℃.

Cell Culture

MCF-7 cells (human breast adenocarcinoma) were cultured in RPMI-1640 medium (+80 U/mL penicillin, +0.08 mg/mL streptomycin) supplied with 10% FBS at 37°C in an atmosphere of 5% CO_2. During logarithmic growth period, the cells were harvested using Accutase solution within 2 min at 37°C. After cells were collected by centrifugation (1000 rpm, 4 min), they were resuspended in PBS to obtain a uniform cell suspension. The cell count was measured using a cell counter (Petroff-Hausser, USA). The obtained cell suspension was diluted with PBS to a series of concentrations for further use.

Target Recognition and Detection of Cell Surface Proteins

For cell labeling, 100 μL cell suspension with a concentration of 5×10^6 cells/mL was dispersed into 0.1% BSA solution. Then, 10 μL of the probe (final concentration of 340 pmol/L) was added and incubated with the cells at 37°C in the dark for 20 min.

The labeled cell suspension was washed and centrifuged (2000 rpm, 2 min), three times with PBS buffer and one time with water, and then suspended in 50% methanol aqueous solution. The supernatant of the last washing was checked by MS to ensure the complete cleaning of the unbound IMTs. The cell count was measured using a cell counter before MS detection. The dissociation of probes under different laser energy was explored. With the increase of laser energy, the characteristic ion signal was significantly enhanced, but accompanied by more -CH_2- fragmentation peaks. Taken together, 6500 laser energy was selected for this experiment. Au-S-$(CH_2)_{11}(OC_2CH_2)_6OH$ (Au-MT) (m/z = 781.5), a commercially available molecule with the same partial structure as IMTs, was selected as internal standard to correct MS signal. To confirm the specificity of the IMTs-based probes, a protein pre-incubation assay was conducted. CEA modified probes were pre-incubated with different amounts of CEA protein (0 ug, 0.01 ug, 0.05 ug, and 1 ug) respectively at 37°C for 20 min. After the removal of excess protein by washing, the conjugates were incubated with MCF-7 cells and detected following above steps.

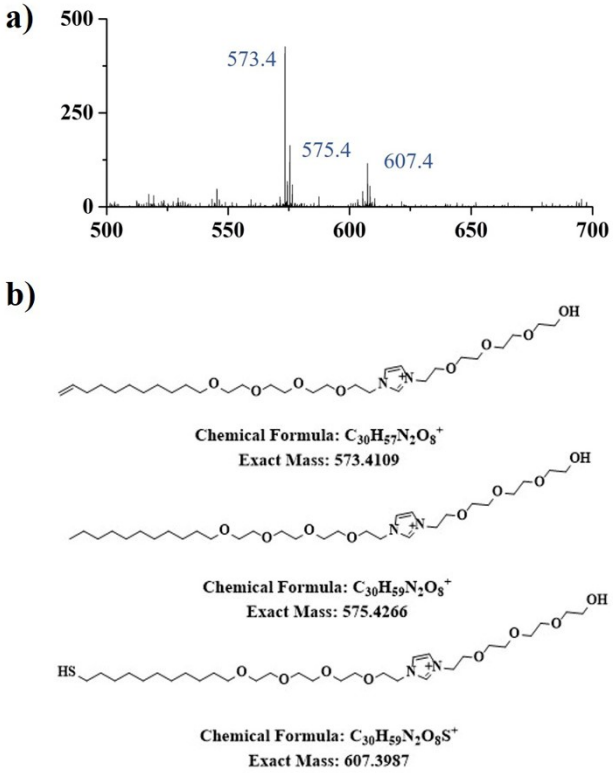


Fig. S1 a) LDI-MS spectrum of Au-IMT1. b) The fragment structures of IMT1.

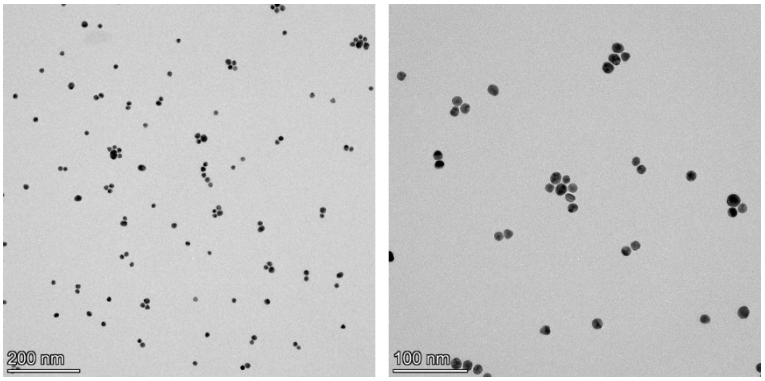


Fig. S2 TEM image of Au@anti-CEA/IMT1.

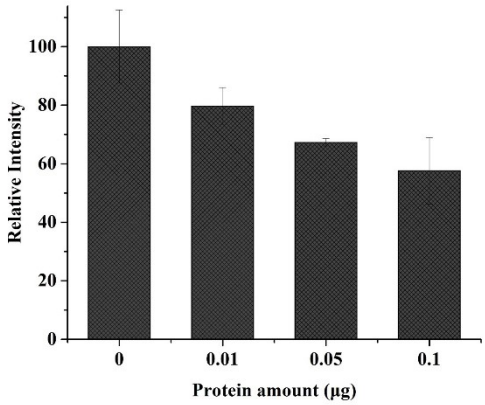


Fig. S3 CEA protein pre-incubation assay (IMTs-based probes were pre-incubated with varied concentrations of CEA protein before being labeled on the cell surface. Y-axis is the relative MS signal intensity of IMTs).

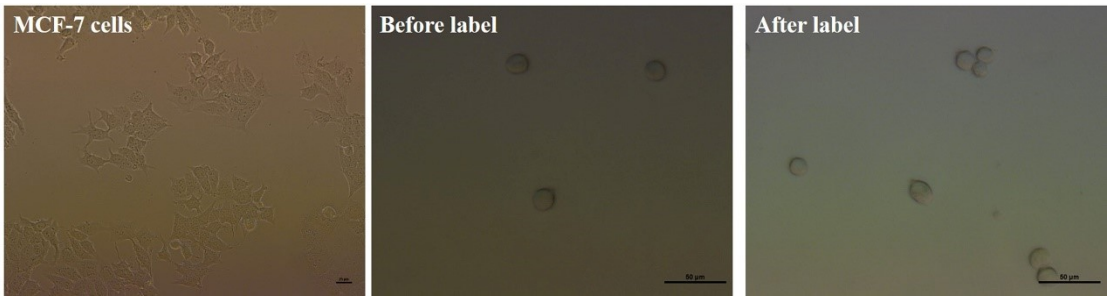


Fig S4. The optical imaging of MCF-7 cells before and after label.

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