Supporting Information

Process characterization of epithelial-mesenchymal transition in alveolar epithelial type II cells using surface-enhanced Raman scattering spectroscopy

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S1. Materials and methods

1.1 Materials

Chloroauric acid terahydrate ($HAuCl_4 \cdot 4H_2O$), trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) and hydroquinone were all purchased from Sunshine Biotechnology Ltd. (Nanjing, China). The cell culture media and supplements were purchased from Alfa Aesar (USA). N-hydrox-ysuccinimide (NHS) and 1-ethyl-3-[3-dimethy-laminopropyl] carbodiimide hydrochloride (EDC) were purchased from Sangon Biotech Co. Ltd. (China). HS-PEG-COOH (molecular weight 3 kDa) was purchased from ToYong Biotechnology Ltd. (Shanghai, China). The cell counting kit (CCK-8) purchased from Sigma-Aldrich, was stored at 4 °C. TAT-peptides were ordered from GL Biochem Ltd. (Shanghai, China). All glassware was cleaned with aqua regia and then rinsed thoroughly with deionized water. Water used in the experiments was ultrapure deionized water (resistance, 18 MΩ cm^{-1}).

1.2 Synthesis of AuNSs

AuNSs were synthesized through a seed-mediated growth approach. Gold seeds were prepared through a modified turkevich method. 300 μL $HAuCl_4$ (100 mM) solution was put into a flask with 30 mL of deionized water under vigorous stirring and heated to boil. As soon as the solution was boiling, 900 μL of 1% sodium citrate aqueous solution was added and kept at boiling until the solution became wine red in color. The solution was then cooled under stirring to obtain gold seeds. The seeds were used within 1 day after preparation.

For a typical synthesis of AuNSs, 25 μL of $HAuCl_4$ (100 mM) was added into 10

mL of deionized water under vigorous stirring, next 50 μL of gold seeds, 22 μL of 1 % sodium citrate and 1 mL of hydroquinone (30 mM) were added into the reaction system one by one. The solution was kept under stirring at room temperature for 30 minutes to obtain AuNSs.

1.3 Preparation of SERS probes

SH-PEG-COOH was used as the capping materials in the process of preparation of SERS probes. It could couple to the AuNSs by the chemical bonds sulfhydryl group (-SH), meanwhile SH-PEG-COOH also played the role of conjugation agent in connecting AuNSs with TAT molecules. EDC and NHS acted as the role of coupling agents, the carboxy group (-COOH) of PEG would be activated by adding EDC and NHS into the colloid solution, the TAT molecules could be conjugated to the surface of PEG-stabilized AuNSs. Firstly, 100 μL of HS-PEG-COOH (10 mM) solution was added to 10 mL of freshly synthesized AuNSs solution and gentle stirring for 1 h at room temperature. Then, 10 μL of 100 mM EDC and 15 μL of 100 mM NHS were added into the PEG-stabilized AuNSs solution and allowed to react for 1 h. Finally, 50 μL of TAT molecules were mixed and stirred for another 4 h. The excess reagents were removed by centrifugation three times at 7000 rpm for 10 min. In our experiment, SERS probe preparation mainly refer to the previous reports.[1,2]

1.4 Cell lines and culture

The alveolar epithelial type II (ATII cells) was obtained from Yili Bio-technology Co. Ltd. (Shanghai, China). The 3T3-NIH cell line was purchased from American Type Culture Collection. Both cells were cultured in high-glucose Dulbecco's Modified

Eagle Medium (DMEM) containing 10% FBS and maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C. All of the cells were passaged 1:2 or 1:3 using 0.25% trypsin when they reached 70-90 % confluence. To induce differentiation of ATII cells into fibroblasts, ATII cells were incubated with BLM (25 μM) and as-prepared SERS probes. After 7 and 14 days, the medium was removed and cells were harvested for SERS analysis.

1.5 CCK-8 viability assays

CCK-8 was used to investigate the biological effect of TAT-functionalized AuNSs on the viability of the ATII cells in terms of cell viability, 1.0×10^5 cells were seeded into each well of a 96-well plate. After cell adhesion is verified, cells were incubated with various concentrations of SERS probes (0, 0.5, 2.5, 5.0, 12.5, 25 μg/mL) for 24 h. Then, the 10 μL CCK8 solution was added to 90 μL of culture medium. The cells were subsequently incubated for 24 h at 37 °C and the attenuance was measured at 450 nm.

1.6 Immunofluorescent staining and western blot assay

The immunofluorescence analysis was performed as described.[3] The following primary antibodies were employed: Rabbit anti-Ecadherin and rabbit anti-α-smooth muscle actin (SMA) (all from Abcam Inc. Cambridge, MA). Alexa Fluor 594 or 488-conjugated goat anti-rabbit IgG (Invitrogen) was used as the secondary antibody. Nuclei were stained with 1 μg/mL DAPI (Sigma).

For Western blotting analysis, the whole cell lysates (20 μg protein) were separated using SDS/10% PAGE and electrophoretically transferred to a PVDF

membrane (Roche) by standard procedures. After the membranes had been blocked, primary antibodies were added to the membranes followed by incubation at 4 °C for 12 h. After three washes in PBST, the membranes were incubated with the secondary antibody at 37 °C for 1 h. The primary antibodies employed were: rabbit anti-E-cadherin (ab40772), rabbit anti-α-smooth muscle actin (SMA) (ab5694), rabbit anti-vimentin (ab92547), mouse-anti-CK-18 (ab668), rabbit-anti-collagen (ab34710) and mouse anti-β-actin (ab8226). Horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (BA1051 and BA1055, Boster, Wuhan, China) was used as the secondary antibody. The dilution concentration was used as recommended. Immunoreactive protein bands were detected using an Odyssey Scanning System (LI-COR, Inc., Lincoln, NE).

1.7 Characterization

The morphology of the as-prepared AuNSs was characterized with a Zeiss ULTRA-plus scanning electron microscope (SEM) and a Philips TECNAI 10 transmission electron microscope (TEM). UV-vis absorption spectra of samples were obtained with a Shimadzu UV3600 UV-Vis-NIR spectrum-photometer. Fourier Transform Infrared spectroscopy (FT-IR) measurements were made on a FT-IR Spectrometer TENSOR 27. The LSPR scatting spectra of nanoparticles were measured using a micro spectrometer with a grating. The TEM observation of AuNSs inside cells were performed by a JEM-2100HR transmission electron microscopy. The fluorescent images were captured using a confocal fluorescence microscope. Raman measurements were performed with a 785 nm laser and the laser power was adjust to

5 mW. The corresponding laser was focused onto the sample using a 50× long working distance objective. SERS spectra were collected in static mode and acquisition time was 10 seconds over a wavenumber range of 600-1700 cm^{-1} for cells. In order to obtain spatial-averaged SERS spectral information of individual cell, the laser spot was scanned across nearly the whole cell during SERS acquisition. The measured SERS spectra of 20 cells were used to obtain an average SERS spectrum for each cell.

1.8 Statistical analysis

The data were shown as means ± SD. Statistical analyses were carried out using the SPSS software for windows version 11.0 (SPSS Inc, Chicago, IL, USA). Values at ** $P<0.05$ were considered as statistically significant.

S2. Results and discussion

2.1 Characterization of SERS probes

Fig. S1A showed the UV-vis absorption spectra of synthetic progress of SERS probes. The spectrum of the AuNSs showed a maximum absorption at 645 nm due to surface plasmon resonance. The spectrum of AuNSs and PEG-stabilized AuNSs looked almost identical. After being conjugated the TAT molecules, which absorbed on the surfaces of PEG-stabilized AuNSs through stable amide bonds, the intensity of maximum absorption peak reduced a little and the absorption peak had a red shift of ~11 nm. We recorded the FT-IR spectra of AuNSs as well as its conjugated forms (Fig. S1B). The broadened peak at 3200-3400 cm^{-1} was attributed to the presence of -

OH groups on the surface of PEG-stabilized AuNSs.[4,5] The two prominent and sharp peaks at about 1649 cm^{-1} and 1553 cm^{-1}, assigned to the C=O stretching (amide I) and the asymmetric stretching mode in the CO-NH of TAT-functionalized AuNSs.[6,7] Furthermore, the SERS spectra were measured to identify the functional groups of samples (Fig. S1C). The appearance of characteristic bands at 1553 cm^{-1} could be ascribed to amide II bands of TAT-functionalized AuNSs.[8] It suggested that the TAT molecules were successfully conjugated to the surface of AuNSs. Fig. S1D showed the LSPR scattering spectra of AuNSs, PEG-stabilized AuNSs and TAT-functionalized AuNSs. Compared with the scattering spectrum of AuNSs, the scattering peak (λmax) had a red-shift to longer wavelengths after being conjugated with TAT molecules. The TAT conjugation process may lead to spectral red-shift due to the increase of refractive index (RI) on the plasmonic structure surface.[9]

2.2 Stability measurements

Since TAT-functionalized AuNSs were to be exploited as intracellular SERS probes, they should retain optical properties in the physiological medium. The stability of SERS probes was checked via a reliable aggregation test. This was done by mixed with different concentrations of NaCl solution.[10] Fig. S2B and S2C showed the UV-vis spectra of PEG-stabilized AuNSs and TAT-functionalized AuNSs from the aggregation test. No significant changes in the spectral shape and the maximum absorption peak were observed, which meant that the SH-PEG-COOH anchored on the AuNSs surfaces, providing effective stabilization against aggregation.[11] The UV-vis absorption spectra of the AuNSs reduced greatly (Fig. S2A), and it suggested that

there were little partial aggregation of AuNSs. We further tested the stability of the AuNSs, PEG-stabilized AuNSs and TAT-functionalized AuNSs when placing them in PBS solution (pH=7.4) for several days at room temperature. It was found that the UV-vis spectra of PEG-stabilized AuNSs (Fig. S2E) and TAT-functionalized AuNSs (Fig. S2F) displayed few changes after five days. These results confirmed that the TAT-functionalized SERS probes were reliable for applications in physiological media.

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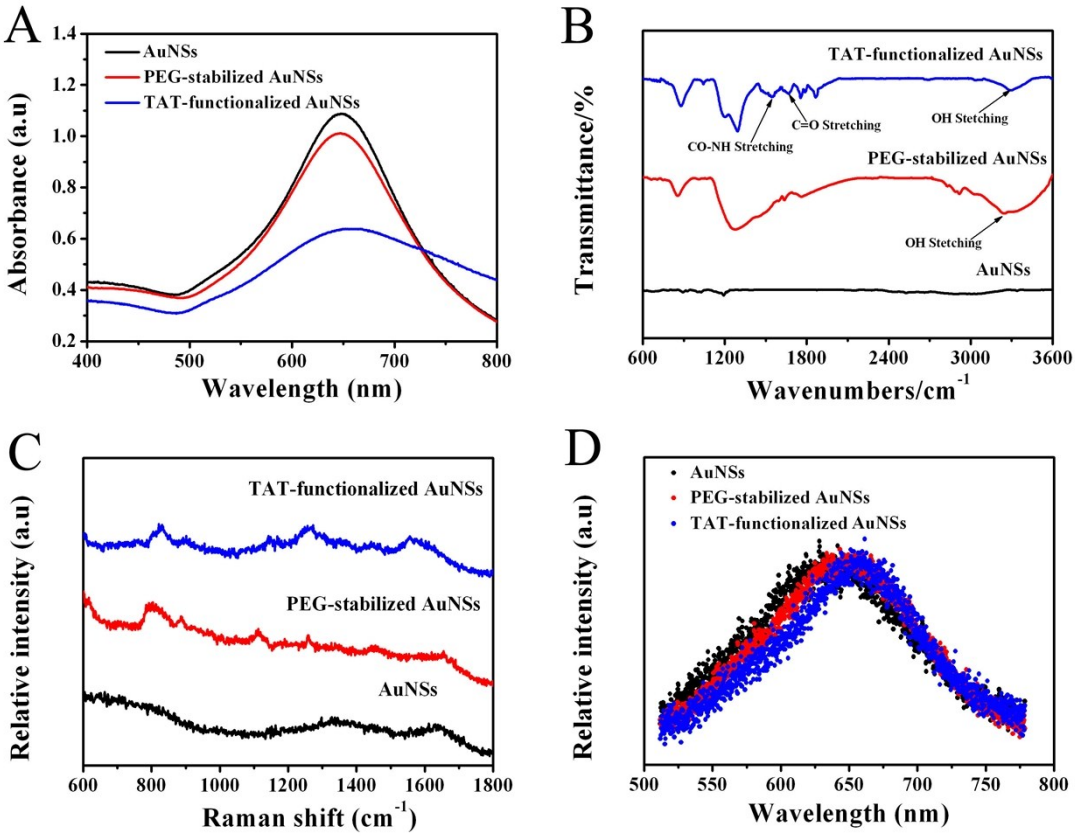

Fig. S1 (A) UV-vis absorption spectra of AuNSs (black curve), PEG-stabilized AuNSs (red curve), and TAT-functionalized AuNSs (blue curve). (B) The FT-IR spectra of AuNSs (black curve), PEG-stabilized AuNSs (red curve), and TAT-functionalized AuNSs (blue curve). (C) SERS spectra obtained from AuNSs (black curve), PEG-stabilized AuNSs (red curve), and TAT-functionalized AuNSs (blue curve). (D) The LSPR scattering spectra of AuNSs (black curve), PEG-stabilized AuNSs (red curve), and TAT-functionalized AuNSs (blue curve) under dark-field microscopy.

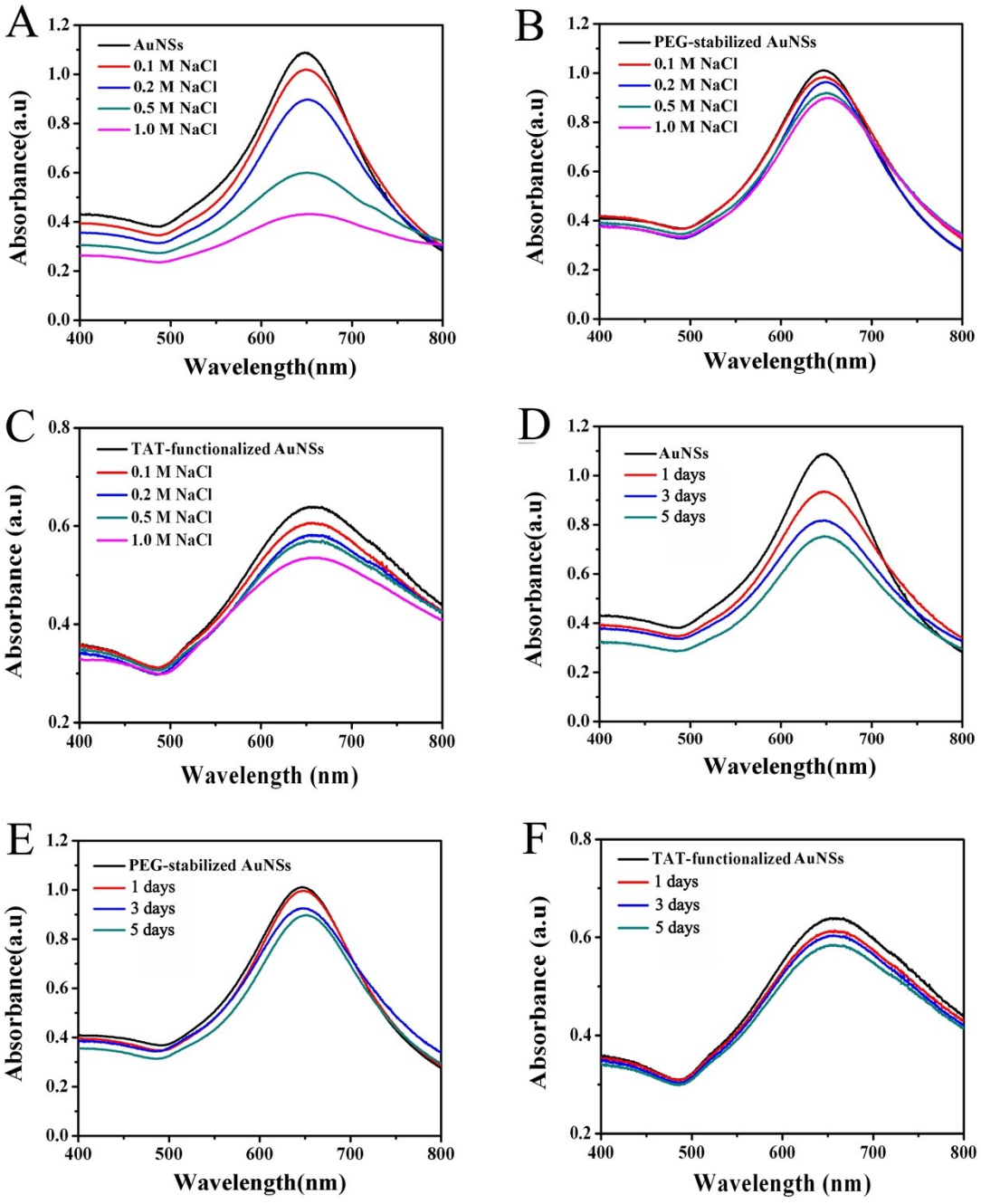

Fig. S2 Stability analysis of SERS probes. UV-vis absorption spectra of (A) AuNSs, (B) BSA-stabilized AuNSs and (C) TAT-functionalized AuNSs after mixing with different concentrations of NaCl, respectively. UV-vis spectra were recorded of (D) AuNSs, (E) BSA-stabilized AuNSs and (F) TAT-functionalized AuNSs after mixing with PBS and for periods of several days of storage at room temperature, respectively.

Table 1 Raman band assignments

Raman shift (cm^{-1})	Band assignment
624	C–C twisting mode of phenylalanine
652	C–C twisting mode of tyrosine
674	C–S stretching mode of cytosine
720	C-S (protein), A (ring breathing mode of DNA/RNA bases)
756	symmetric breathing of tryptophan
786	DNA and phosphodiester bands DNA
818	PO^{2-}stretch nucleic acids
852	tyrosine
867	ribose vibration, one of the distinct RNA modes
874	hydroxyproline, tryptophan
923	glycogen and lactic acid
941	skeletal modes (polysaccharides, amylopectin)
1006	phenylalanine
1017	symmetric ring breathing of phenylalanine
1033	CH_2CH_3 bending modes of phospholipids
1066	proline
1095	lipid, phosphodioxy group (PO_2^- in nucleic acids)
1128	C–N stretching (proteins), C-O stretching (carbohydrates)
1171	(CH) phenylalanine, tyrosine
1222	amide III (β sheet structure), T,A (DNA/RNA)
1249	amide III
1279	amide III (a-helix), triglycerides (fatty acids)
1296	CH_2 deformation (protein assignment)
1306	C–N stretching aromatic amines
1336	δ□ (CH_3) δ (CH_2) twisting, collagen (protein assignment)
1363	tryptophan
1395	C=O symmetric stretch, CH_2 deformation
1420	CH_2 scissoring vibration (lipid band)
1438	CH_2 scissoring
1449	C=N stretching
1462	δCH_2, Disaccharides, sucrose
1491	C–H bending in amino radical cations
1523	C=C carotenoid
1544	amide II
1553	amide II
1586	C=C olefinic stretch
1608	tyrosine, phenylalanine ring vibration
1617	C=C bending in phenylalanine and tyrosine
1646	amide I, α-helix in protein
1659	amide I band (protein band)
1683	ν (C=O) of phospholipids