Supporting Information

Surface Coverage-Regulated Cellular Interaction of Ultrasmall Luminescent Gold Nanoparticles

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Materials and Methods

Gold chloride trihydrate (HAuCl₄•3H₂O) and glutathione (GSH) were purchased from Sigma-Aldrich (St Louis, MO, USA). Cell-penetrating peptide, CRRRRRRRRR (CR₈, >95%), was obtained from Synpeptide Co., Ltd (Shanghai, China). LysoTracker™ Green DND-26 was purchased from Thermo Fisher (New Hampshire, USA). Phosphate buffer solutions were prepared according to the standard protocols and calibrated with SevenCompact™ pH/Ion Meter S220-Micro (METTLER TOLEDO, Switzerland). Other chemicals were obtained from Sigma-Aldrich and used directly. All glassware was cleaned with fresh aqua regia before use. Ultrapure water was achieved from a Pall water purification system (Cascada III.I, USA).

The luminescence spectra were collected using a LS-55 luminescence spectrophotometer (PerkinElmer, USA). The absorption spectra of gold nanoparticles (AuNPs) were obtained from a UV-Vis spectrophotometer (UV2600 Shimadzu, Japan). High resolution transmission electron microscopy (HRTEM) images were obtained with a JEOL JEM 2100F TEM (Japan) with an accelerating voltage of 200 kV. The zeta-potential values of the particles were measured using a Malvern Zetasizer (Malvern Nano-ZS). Elemental analyzer was carried out on a Vario EL cube (Elementar, Germany). All cell images were taken by a fully-motorized inverted microscope system (Olympus IX83-DSU, Japan, a spinning disk confocal technology) assembled with both Photometrics EMCCD (Evolve 512 Delta) and Retiga R1 CCD. The cell viability was assessed by MTT assay with an ELx800 Microplate Reader.
(BioTek Instruments Inc.). The in-vitro imaging for pH-responsive 09CR-AuNPs was collected with a UVP ChemStudio PLUS 815 imaging system (Analytikjena, USA).

**Synthesis of CR-AuNPs with Various Surface Coverage**

CR-AuNPs with different surface coverage were synthesized by a thermal reduction method with different ratio of ligand-to-Au but controlling the same ratio of GSH-to-CR₈. Briefly, 170 µL 1.0 M HAuCl₄ was added into 50 mL of 4.3 mM GSH and 0.14 mM CR₈ mixture solution under vigorous stirring (ligand-to-HAuCl₄ ratio = 1.3:1). Then, the mixture was heated in a 95°C oil bath to obtain highly 615 nm-emitting gold nanoparticles (13CR-AuNPs). The dual-emitting 09CR-AuNPs were synthesized with the same thermal reduction method as described above but using 50 mL of 3.0 mM GSH and 0.099 mM CR₈ mixture solution (ligand-to-HAuCl₄ ratio = 0.9:1). When the ligand concentration decreased to 2.3 mM GSH and 0.077 mM CR₈ (50 mL, ligand-to-HAuCl₄ ratio = 0.7:1), the 810 nm-emitting 07CR-AuNPs formed.

The synthesized CR-AuNPs were purified by centrifuging at 21000 g to remove the large aggregates produced in the reaction processes. The supernatant was further purified by adjusting the solution pH to ~4 and adding ethanol into the solution (ethanol-to-solution = 1:2 (v/v)), then centrifuging the solution at 4000 g for 10 min to discard the supernatant. The precipitates were then resuspended by adjusting the solution pH to 7 ~ 8, and then purified again with Amicon Ultra-4 centrifugal filter devices (10000 MWCO). As references, the GS-AuNPs with ligand-to-HAuCl₄ ratios
(0.7:1, 0.9:1 and 1.3:1) were synthesized with the similar thermal reduction method and purification processes.\textsuperscript{1,2} We further examined the purity of CR-AuNPs after purification by using NMR spectra. As shown in Figure S2, the free ligands, including GSH and CR\textsubscript{8}, showed obvious sharp \textsuperscript{1}H characteristic peaks. However, the CR-AuNPs displayed the negligible sharp \textsuperscript{1}H peaks on top of broadened nanoparticle signals,\textsuperscript{3} distinct from the previously reported AuNCs with physically adsorbed GS-SG,\textsuperscript{4} indicating that the surface ligands were bonding on the surface of the AuNPs instead of physically adsorbed and the free ligands were removed from samples.

The free thiols were also measured by Ellman’s method,\textsuperscript{5} a typical colormetric method for detection of thiols. As shown in Figure S3, there were few free thiols in test sample of 09CR-AuNPs (25 nM, pink point). To confirm the purification of sample, we also determined the content of free thiols in high concentration stock sample of 09CR-AuNPs (17.9 μM, blue point). The 0.12 μM free thiols was found in this high concentration stock sample, which is negligible amount for surface coverage characterization and their applications. The above results from both NMR and colorimetry demonstrated that the free ligands after reaction were removed.

\textbf{Characterization of CR-AuNPs}

Chemical formula of CR-AuNPs can be expressed as Au\textsubscript{x}GS\textsubscript{y}CR\textsubscript{z}, here GS = GSH containing a deprotonated thiol group (C\textsubscript{10}H\textsubscript{16}N\textsubscript{3}O\textsubscript{6}S, 306 g/mol), CR = CR\textsubscript{8} containing a deprotonated thiol group (C\textsubscript{51}H\textsubscript{103}N\textsubscript{33}O\textsubscript{10}S, 1370 g/mol), x = number of Au atom, y = number of GS, and z = number of CR. The surface coverage of CR and
GS around AuNPs could be calculated with ligand-to-Au ratio of CR-AuNPs = (y+z)/x. All calculated parameters from element analyses were listed on Table S1 and S2.

**The Calculation of x**

For a spherical metal nanoparticle, the number of metal atom in this particle (N) can be calculated using following equation:

\[ R = r_s \cdot N^{1/3} \]

where \( R \) = particle radius, \( r_s \) = Wigner-Seitz radius.

For the 07CR-AuNP with average d = 1.96 nm (R = 0.98 nm; \( r_s \) = 0.145 nm for Au), \( x = N = 309 \).

For the 09CR-AuNP with average d = 1.91 nm (R = 0.955 nm; \( r_s \) = 0.145 nm for Au), \( x = N = 286 \).

For the 13CR-AuNP with average d = 1.98 nm (R = 0.99 nm; \( r_s \) = 0.145 nm for Au), \( x = N = 318 \).

**The Calculation of y and z**

Where Au = 197 g/mol, GS = C₁₀H₁₆N₃O₆S (306 g/mol), and CR = C₅₁H₁₀₃N₃₃O₁₀S (1370 g/mol). Then, y and z could be calculated from the results of element analyses according to following two equations:

\[ \frac{10 \times y \times 12 + 51 \times z \times 12}{x \times 197 + y \times 306 + z \times 1370} = C\% \] (for carbon content)
\[ \frac{3y \times 14 + 33z \times 14}{x \times 197 + y \times 306 + z \times 1370} = N\% \text{ (for nitrogen content)} \]

Similarly, the chemical formula of GS-AuNPs \((\text{Au}_x \text{GS}_y, \text{Au} = 197 \text{ g/mol, GS} = \text{C}_{10}\text{H}_{16}\text{N}_3\text{O}_6\text{S} (306 \text{ g/mol}))\) can be also obtained from the results of their corresponding element analyses according to their statistical average size and following equation:

\[ 12 \times 10 \times y / (N \times 197 + y \times 306) = C\% \text{ (for carbon content)} \]

The above calculation method was reported in our previous work.\(^1\) The relative molecular mass of the CR-AuNPs was calculated to be more than 86.7k, which was far over the measurement range of a conventional Electrospray Ionization Mass Spectrometer (ESI-MS), an efficient technique to determine the molecular formula of ultrasmall nanoclusters with low molecular weight.\(^6\)\(^8\)

**Gel Electrophoresis**

The GS-AuNPs and CR-AuNPs were diluted into 10 mM PB solution (pH 7.4) with 7.5% glycerol to a final volume of 20 µl. The resulting solution was analyzed by 1.5% agarose gel electrophoresis using the Gel Electrophoresis system (DYY-8C, China) with 6 V/cm, 20 mA for 30 min.

**The pH-Related Spectra Responses**

The emissions at 615 nm and 810 nm of dual-emitting 09CR-AuNPs showed a ratiometric responses to pH changes (Figure 1E and F), whereas the invariant
absorption spectra (Figure S8A). With the pH values increased from 4.2 to 10.8, the intensity of the 615 nm emission increased largely (~4 fold) while the 810 nm emission intensity decreased gradually (~1.5 fold). Furthermore, the ratio of the intensities of the 810 nm to 615 nm emissions were a weak acidic-sensitive and increased by a factor of 1.2 (about 60% of change) in the physiological pH range (7.4-5.0), which would benefit to the determination of the pH value in the physiological environment. These ratiometric pH responses could be visualized by an in-vitro microscopy (Figure S8B). Similar to the observations of fluorescent spectra, the changes of ambient pH value from 4.1 to 7.4 gave rise to significant increase of emission intensities at 615 nm and slight decrease of emission intensities at 810 nm. As a result, we acquired a distinct ratiometric fluorescent images of dual-emitting 09CR-AuNPs over pH range from 4.1 to 7.4, demonstrating our dual-emitting 09CR-AuNPs is capable of the efficient identification of ambient pH. The good reversibility of ratiometric pH responses of the dual-emitting 09CR-AuNPs indicated they are very stable over this pH range (Figure S8C). The ratiometric pH responses of the dual-emitting 09CR-AuNPs were also stable in saline solution and little affected by the ionic strength of the solution (Figure S8D). Differentiating from dual-emitting AuNPs, the luminescence of single-emitting AuNPs showed lower pH dependency (Figure S9), and the ratio values of $I_{615\text{ nm}}/I_{810\text{ nm}}$ or $I_{810\text{ nm}}/I_{615\text{ nm}}$ from the mixture of 07CR-AuNPs and 13CR-AuNPs showed negligible changes in different pH conditions from 4.5 to 10.7 (Figure S10). These results demonstrated that the
ratiometric pH response was originated from a single particle of dual-emitting 09CR-AuNP containing two coupled 615 nm and 810 emission centers.

**In-vitro pH-Dependent Ratiometric Imaging**

The fluorescent images of CR-AuNPs in DPBS with different pH values were taken by an UVP ChemStudio PLUS 815 imaging system (Analytikjena, USA) with an excited light source at 365 nm and treated with an analytical software (Olympus cellSens) to obtain the final pH-responsive ratiometric images (Figure S8B, 810 nm to 615 nm, red channel to green channel).

**Cell Culture**

HeLa cell line was friendly provided from professor Zhili Rong of the Southern Medical University (Guangzhou, China). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cells were maintained in 5% CO₂ at 37 °C and a humidified atmosphere.

**The Cytotoxicity of CR-AuNPs**

The cell viabilities of HeLa cells treated with various CR-AuNPs with different concentrations were evaluated by MTT assay. HeLa cells were seed in 96-well plates (Coster, USA) with density of 1×10⁴ cells per well. After incubating for 12 h, the medium was replaced by medium containing CR-AuNPs with different concentrations from 0.001 to 1000 nM. The cells were cultured for another 24 h, and then 20 uL of
freshly prepared MTT (5 mg/mL) solution was added into each well. After incubation for 4 h, the solution of each well was removed carefully, and 150 μL DMSO was added into each well. Then, the plate was gently shaken for 10 min to dissolve produced precipitates, and the absorbance of MTT at 490 nm (630 nm as reference) was monitored by a microplate reader. Cell viabilities were shown by the ratios of absorbance of the cells with treatments of CR-AuNPs to that of the cells incubated with medium only (Figure S11). Once the incubation concentration was larger than 500 nM, the 07CR-AuNPs might largely influence the cell viabilities as shown in Figure S11. Therefore, the CR-AuNPs with concentration of 500 nM was chosen as incubation concentration to interact with cells for the following experiments.

**Cell Imaging**

The HeLa cell lines were seeded in glassy bottom cell culture dishes at a density of ~1×10^5 cells per dish and incubated for 12 h to obtain the adherent cells for subsequent imaging.

**pH-Dependent Membrane-Binding Imaging** was executed in different pH conditions (7.4, 6.8, 6.3 and 5.8) to elaborate the pH-dependent interactions between CR-AuNPs (615 nm-emission, dual-emission and 810 nm-emission) and cellular membrane. Briefly, the adherent cells were gently washed for three times by using DPBS with pH values at 7.4. For blank, DPBS solution with pH values at 7.4 was added into dish, and the cell images were collected for cellular background subtraction. For experimental group, the 500 nM CR-AuNPs was added to dish
containing DPBS solution with pH values at 7.4 to start their interaction with cells and incubated for 10 min at room temperature, then removed incubated solution and gently washed for twice by using DPBS with pH values at 7.4 to end their interaction with cells. The fluorescent living cell images of these treated cells were collected and used to analyze the interactions between CR-AuNPs and cellular membrane. The membrane-binding images in the other pH conditions were achieved with similar operations by changing the DPBS with other pH values (6.8, 6.3 and 5.8). As comparison, the pH-dependent interactions between GS-AuNPs (615 nm-emission, dual-emission and 810 nm-emission) and cellular membrane were also displayed by similarly membrane-binding imaging. The images were taken by a microscopy system (Olympus, Japan) with a R1 CCD through 100X oil-immersion objective under metal halide lamp excitation (green channel for 615 nm-emitting imaging, Ex: 460-500 nm, Em: 575-625 nm, exposure time: 5 s; and red channel for 810 nm-emitting imaging, Ex: 355-375 nm, Em: 765-855 nm, exposure time: 5 s). The both ratiometric images of \( R_{615/810} \) nm and \( R_{810/615} \) nm were obtained by combining the two single-channel images and converting with ratio analyses by an analytical software (Olympus cellSens). For statistical intensities or ratios, 5 points on membrane were chosen randomly, and thus their average intensities or ratio values were obtained.

**Time-Dependent Cellular Interaction Imaging:** The adherent cells were gently washed for three times with DPBS. For blank, DPBS solution with pH values at 7.4 was added into dish, and the cellular fluorescent images were collected for fluorescent background subtraction. For experimental group, the CR-AuNPs (500 nM) was added
into each dish containing cell culture medium to start their interaction with cells. After incubation for 1, 3, or 6 h, incubated solution with CR-AuNPs was removed and cells were gently washed for three times with DPBS to end their interaction with cells, the live cell imaging was achieved to observe the interaction of CR-AuNPs with cells. The images were taken by a microscopy system (Olympus, Japan) with an EMCCD through a 100X oil-immersion objective under Xe-lamp excitation (green channel for 615 nm-emitting imaging, Ex: 460-500 nm, Em: 575-625 nm, exposure time: 0.2 s; and red channel for 810 nm-emitting imaging, Ex: 355-375 nm, Em: 765-855 nm, exposure time: 0.5 s). The ratiometric images (810 nm to 615 nm, red channel to green channel) were obtained by combining the two single-channel images and converting with ratio analyses by an analytical software (Olympus cellSens). For statistical percentages of the luminescence intensities around cellular membrane, the scanned layers (n=5, z axis) were chosen to calculate the intensities of whole cell and intramembrane, then the intensities around cellular membrane could be collected by using the intensities of whole cell decreased the intensities of intramembrane, thus percentages of intensities around cellular membrane were obtained by using intensities around cellular membrane to divide that of the whole cell.

**Endocytosis Imaging:** The adherent cells were gently washed for three times with DPBS. For colocalization imaging, the 500 nM CR-AuNPs or GS-AuNPs with Lysotracker Green were respectively added to dish containing cell culture medium to start their interaction with cells. After incubation for 6 h, incubated solution with CR-AuNPs was removed and cells were gently washed for three times with DPBS to
end their interaction with cells, the living cell imaging was executed to observe the colocalization of CR-AuNPs or GS-AuNPs with Lysotracker Green. The images were taken by a microscopy system (Olympus, Japan) with an EMCCD through a 100X oil-immersion objective under Xe-lamp excitation (blue channel for Lysotracker Green, Ex: 460-500 nm, Em: 505-555nm, exposure time: 0.1 s; green channel for 615 nm-emitting imaging, Ex: 460-500 nm, Em: 575-625 nm, exposure time: 0.2 s; and red channel for 810 nm-emitting imaging, Ex: 355-375 nm, Em: 765-855 nm, exposure time: 0.5 s). The colocalization percentages between AuNPs and Lysotracker Green were exhibited by their colocalization coefficient R. The ratiometric images (810 nm to 615 nm, red channel to green channel) were obtained by combining the two single-channel images and converting with ratio analyses by an analytical software (Olympus cellSens). Similar to the above statistical processes, the luminescent intensities of cell in scanned layers (n=5, z axis) were collected to analyze the percentages of statistical ratio values larger than 3 in the ratiometric 3D-images, as well as the percentages of the colocalization between AuNPs and Lysotracker Green.

For exploration of endocytosis pathway, the endocytic inhibitors\(^9\) including chlorpromazine (ZPC, clathrin inhibitor), methyl-\(\beta\)-cyclodextrin (CD, caveolae inhibitor), amiloride (AML, actin inhibitor), and sodium azide (NaN\(_3\), energy distraction) were respectively added into incubated medium for 1 h. And then, the incubating solution was replaced by the medium with CR-AuNPs. After incubation for another 6 h, cells were gently washed for three times with DPBS. The images
were taken by a microscopy system (Olympus, Japan) with an EMCCD through a 40X oil-immersion objective under Xe-lamp excitation (green channel for 615 nm-emitting imaging, Ex: 460-500 nm, Em: 575-625 nm, exposure time: 0.1 s; and red channel for 810 nm-emitting imaging, Ex: 355-375 nm, Em: 765-855 nm, exposure time: 0.2 s). The ratiometric images (810 nm to 615 nm, red channel to green channel) were obtained by combining the two single-channel images and converting with ratio analyses by an analytical software (Olympus cellSens). The luminescence intensities of 5 cells in the scanned layers (n=5, z axis) were collected to analyze inhibiting effect of various endocytosis inhibitors.
Figure S1. The absorption, excitation and emission spectra of 07CR-AuNPs (A), 09CR-AuNPs (B) and 13CR-AuNPs (C), and their corresponding HRTEM images and core-size distributions of 07CR-AuNPs (D, 1.96 ± 0.53 nm), 09CR-AuNPs (E, 1.91 ± 0.50 nm) and 13CR-AuNPs (F, 1.98 ± 0.51 nm).

Figure S2. $^1$H NMR spectra of GSH (A), CR$_8$ (B), 09GS-AuNPs (C) and 09CR-AuNPs (D).
Figure S3. UV-Vis spectra of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) with different concentration of GSH from blank to 48.8 μM (A), their linear relationship of absorbance to the concentration of GSH (B) and concentration of samples: test sample of 09CR-AuNPs (pink), high concentration stock sample of 09CR-AuNPs (blue).

Table 1. The structure parameters of CR-AuNPs obtained from the results of element analyses.

| Materials  | C%   | N%   | x   | y   | z   | Surface coverage (%) |
|------------|------|------|-----|-----|-----|-----------------------|
| 07CR-AuNPs | 12.71| 4.97 | 309 | 85.2| 1.88| 28.2                  |
|            | 13.05| 4.92 | 309 | 92.2| 1.29| 30.6                  |
|            | 12.70| 4.96 | 309 | 85.2| 1.86| 28.2                  |
| 09CR-AuNPs | 13.87| 5.38 | 286 | 90.8| 1.84| 32.4                  |
|            | 13.75| 5.32 | 286 | 89.8| 1.77| 32.0                  |
|            | 13.79| 5.32 | 286 | 90.5| 1.72| 32.2                  |
| 13CR-AuNPs | 18.22| 7.71 | 318 | 142.8| 6.18| 46.9                  |
|            | 18.23| 7.71 | 318 | 143.1| 6.16| 46.9                  |
|            | 18.17| 7.71 | 318 | 141.6| 6.24| 46.5                  |
Figure S4. The absorption, excitation and emission spectra of 07GS-AuNPs (A), 09GS-AuNPs (B) and 13GS-AuNPs (C), and their corresponding HRTEM images and core-size distributions of 07GS-AuNPs (D, 2.16±0.55 nm), 09GS-AuNPs (E, 1.69±0.38 nm) and 13GS-AuNPs (F, 1.90±0.28 nm).

Table 2. The structure parameters of GS-AuNPs obtained from the results of element analyses.

| Materials   | C%    | x    | y    | Surface coverage (%) |
|-------------|-------|------|------|----------------------|
| 07GS-AuNPs  | 11.55 | 413  | 111  | 26.9                 |
|             | 11.68 | 413  | 113  | 27.3                 |
|             | 11.70 | 413  | 113  | 27.4                 |
| 09GS-AuNPs  | 13.49 | 198  | 66.8 | 33.7                 |
|             | 13.51 | 198  | 67.0 | 33.8                 |
|             | 13.53 | 198  | 67.1 | 33.9                 |
| 13GS-AuNPs  | 17.20 | 281  | 141  | 50.3                 |
|             | 17.08 | 281  | 140  | 49.7                 |
|             | 17.13 | 281  | 140  | 50.0                 |
Figure S5. The surface coverage of GS-AuNPs with different ligand intensities calculated from the results of element analyses.

Figure S6. The zeta-potentials of 09GS-AuNPs and 09CR-AuNPs in 10 mM phosphate buffer solution with different pH values (7.4, 6.8, 6.3, 5.8 and 5.3).

Figure S7. The image for gel electrophoresis of 07GS-AuNPs, 07CR-AuNPs, 09GS-AuNPs, 09CR-AuNPs, 13GS-AuNPs and 13CR-AuNPs from 1 to 6 under ultraviolet irradiation (λ=365 nm).
Figure S8. (A) The UV-vis spectra of dual-emitting 09CR-AuNPs at different pH values from 4.19 to 10.77. (B) The pH-responsive luminescent images of dual-emitting 09CR-AuNPs at 615 nm and 810 nm in DPBS with different pH values under the excited light with λ=365 nm, their merged and ratiometric images (numerator: 810 nm, denominator: 615 nm) were also shown. (C) The reversibility of ratiometric emissions of dual-emitting 09CR-AuNPs at pH values between 7.5 and 5.5. (D) The effect of ionic strength on the ratio of emissions from dual-emitting 09CR-AuNPs in the DPBS solution with additional NaCl solution from 0.001 to 300 mM at both pH 5.5 and 7.4. Some HCl or NaOH (5 M) was added into DPBS to obtain the DPBS with different pH values under the monitor of a SevenCompact™ pH/Ion Meter S220-Micro (METTLER TOLEDO, Switzerland).
**Figure S9.** The pH-dependent fluorescent spectra (A, C) and intensities (B, D) of 07CR-AuNPs (A, B) and 13CR-AuNPs (C, D) at different pH values from 4.5 to 10.8.

**Figure S10.** The fluorescent spectra of mixture of 07CR-AuNPs and 13CR-AuNPs at different pH values (from 4.5 to 10.7), and their intensity ratio values of 810 nm and 615 nm emissions.

**Figure S11.** (A) The cell viabilities of HeLa cells treated with 07CR-AuNPs (A), 09CR-AuNPs (B) and 13CR-AuNPs (C) with different concentrations (1 to 12: 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 nM).
Figure S12. The fluorescent images of living cells after treatment with 07GS-AuNPs (A, left), 13GS-AuNPs (A, right) and 09GS-AuNPs (B) in DPBS with different pH of 7.4, 6.8, 6.3 and 5.8, and their statistical intensities (C) and ratios (F). The red channel for 810 nm, and green channel for 615 nm. Scale bar: 10 μm. Due to the very weak signals of cells in pH 7.4 and 6.8 (Figure S9B), the statistical ratio of $R_{615/810}$ nm and $R_{810/615}$ nm might have high data error.
Figure S13. The time-dependent fluorescent images of living cells after treatment with 07GS-AuNPs (A), 09GS-AuNPs (B) and 13GS-AuNPs (C). The red channel for 810 nm, and green channel for 615 nm. Scale bar: 10 μm.

Figure S14. The statistical fluorescent intensities of endocytosis pathway images (Figure 5) without or with treatment inhibitors.

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