CTAB-coated gold nanorods elicit allergic response through degranulation and cell death in human basophils

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Methods and Materials

Reagents

Propidium iodide (PI) and fMLP were purchased from Sigma-Aldrich, USA. Calcein-AM and fluo-4-AM were obtained from Invitrogen, USA. Annexin-V-Red Fluorescent Protein (RFP) was synthesised in house and purified by nickel affinity column.

Preparation of the CTAB-coated and PEG-coated gold nanorods

Detailed preparation methods for the CTAB- and PEG-coated Au-NRs were described in our previous report. ¹ Briefly, the CTAB-coated Au-NRs were grown by a seed-mediated method in an aqueous environment. The seed solution was prepared by adding NaBH₄ into the mixture of HAuCl₄ and CTAB, and was then kept at room

temperature for 2 h. The growth solution was prepared by sequentially adding HAuCl₄, AgNO₃, HCl and ascorbic acid into CTAB, followed by gentle mixing. After the addition of the seed solution into the growth solution, the mixture was gently mixed and incubated at room temperature overnight. The CTAB-coated Au-NRs were collected by centrifugation (6300 g, 10 min) and resuspended in deionized water after incubation. Concentration was determined by the extinction spectrum measurements as described previously. ^{2,3}

The PEG-coated Au-NRs were directly prepared from the CTAB-coated NRs through an ion exchange process. The CTAB-coating was replaced with excess mPEG-SH (methoxy-PEG-thiol, MW: 5000, Rapp Polymere) to ensure complete ion exchange. Excess mPEG-SH was removed by centrifugation (6300 g, 10 min). After resuspension of the NRs in deionized water, the concentration of the PEG-coated NRs was determined by extinction spectral measurements.

Cell culture and preparation

Human basophils KU812 (American Type Culture Collection, USA) were cultured at a cell density of 1×10^5 /ml in RPMI 1640 culture medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA) at 37 °C and 5% CO₂. Cells were passaged twice a week.

Alamar blue assay

For the cell viability assay, basophils (1 x 10^6 /ml) were treated with different agents at 37 °C, 5% CO₂. After treatment, the cells were incubated with the alamar blue reagent (Invitrogen, USA) according to the protocol suggested by the company for 4 h at 37 °C, 5% CO₂. Fluorescence was measured with a fluorescence plate reader (Tecan) (excitation: 485 nm, emission: 595 nm). Positive control was achieved by adding 1 μ M staurosporine (STS) to cells to induce apoptosis.

Calcein leakage assay, propidium iodide, fluo-4 and annexin-V-RFP staining assay

Basophils were loaded with calcein-AM (2 μ M) (Invitrogen) or fluo-4-AM (1 μ M) (Invitrogen) for 10-30 min in dark before treatments. The plasma membrane is permeable to calcein-AM or fluo-4-AM. Inside the cells, the non-fluorescent calcein-AM or fluo-4-AM is converted into the polar calcein or fluo-4 which cannot pass through the plasma membrane and hence remains intracellularly. For the PI assay, PI (2 μ g/ml) (Sigma-Aldrich) was added to the cells for 10 min in dark after treatment. PI can pass through the damaged plasma membrane but not the intact membrane in healthy cells. ⁴ Hence, the cells at the late apoptotic phase with damaged plasma membranes (loss of membrane integrity) show low calcein green and high PI red fluorescence. For the annexin-V binding assay, cells were labelled with annexin-V-RFP (red fluorescent protein) for 20 min at room temperature after treatment. Annexin-V-RFP is able to label the phosphatiylserine being externalised on the outer leaflet of plasma membrane during apoptosis for the determination of loss of membrane asymmetry. Fluo-4 is able to report an increase in the intracellular Ca²⁺ level of the KU-812 cells after treatment.

After washing, flow cytometry analysis was performed on a FACSCanto flow cytometer (BD Biosciences), using WinMDI software for data acquisition and analysis. Green and red fluorescence were determined after excitation at 488 nm from a minimum of 10,000 cells. In the supplementary figures, the numbers in each flow cytometric histogram show the % of total cell population in the selected region (M1 or M2).

Histamine release assay

Histamine released during allergic degranulation of basophils was measured by histamine ELISA kit (Immuno-Biological Laboratories). Basophils $(1 \times 10^6/ml)$ were treated with different agents for 20 min at 37 °C, 5% CO₂. After treatments, cell supernatants were submitted to ELISA according to the protocol suggested by the

company. Total release was achieved by adding Triton X-100 (0.1%, v/v) into wells to lyse all basophils.

β -hexosaminidase release assay

We adopted the enzymatic activity assay described before. ⁵ To evaluate β -hexosaminidase released during allergic degranulation, the enzyme activity was measured using 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide (MUG) as the substrate of the enzyme. In brief, basophils (1 x 10⁶/ml) were treated with different agents for 20 min. After treatments and centrifufation, 25 µl of the cell supernatant was added with 100 µl of 1.2 mM MUG in 0.05 M sodium acetate buffer (pH 4.4), and the solution was incubated at 37 °C, 5% CO₂ for 2 h. During the enzymatic reaction, methylumbelliferrone, one of the products from MUG, gives significant fluorescence at 450 nm upon excitation at 360 nm. Fluorescence was measured with a fluorescence plate reader (Tecan) for the release of β -hexosaminidase (excitation: 360 nm, emission: 450 nm). Total release was achieved by adding Triton X-100 (0.1%, v/v) to the cells.

Statistical analysis

The differences between the results of experimental and control treatments were analyzed for statistical significance by Student's *t*-test. Each experiment was repeated for three times, each with three determinations. The trend of response from different batches of cells was similar if not identical. All data points in the bar chart are mean \pm SD (standard deviation) from three different sets of experiments executed at different dates.

Reference

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Figure S1. Physical and chemical properties of the NRs. **a**) Schematic diagrams showing the surface modification of the CTAB- and PEG-coated Au-NRs. **b**) High-resolution transmission electron microscopic image of the NRs shows the uniform rod-shape of the Au-NRs with a dimension of 15 nm x 64 nm. The scale bar shows 100 nm. **c**) Extinction spectra of the CTAB- and PEG-coated Au-NRs with a peak absorption at 790 nm and 802 nm respectively.



Figure S2. Effects of FBS on the cytotoxicity in KU812 cells. Basophils (1×10^6 /ml) were treated with the CTAB- (**a**, **b**) or PEG-coated Au-NRs (**c**, **d**) at the concentration as indicated at 37 °C and 5% CO₂ for 4 (**a**, **c**) or 24 h (**b**, **d**). The cell viability was then determined by alamar blue assay (square: 0% FBS; rhombus: 5% FBS; triangle: 10% FBS; circle: 20% FBS). Results are mean ± SD (n=3).





b)



Figure S3. Membrane permeabilization induced by the CTAB-, but not PEG-coated Au-NRs in KU812 cells. Basophils (1 x 10⁶/ml) were loaded with calcein-AM (2 μ M) and treated with the CTAB-, PEG-coated Au-NRs or STS in the calcein leakage assay (**a**), or stained with PI (2 μ g/ml) after treatments (**b**) at the concentration as indicated at 37 °C and 5 % CO₂ for 4 or 24 h as indicated. After washing, the cells were submitted to flow cytometric analysis for fluorescence measurement. In the histograms, the x-axis represents green fluorescence for calcein or red fluorescence for PI. Figure on top shows the % of the total cell population in the selected region. Results are mean ± SD (n=3). a)



CTAB-NR

PEG-NR

b)



Figure S4. CTAB-coated, but not PEG-coated Au-NRs induce PS externalization of plasma membrane in KU812 cells. Basophils $(1 \times 10^6/\text{ml})$ were treated with the CTAB-, the PEG-coated Au-NRs or STS at the concentrations as indicated at 37 °C and 5 % CO₂ for 4 (**a**) or 24 h (**b**) and then labeled with annexin-V-RFP and. After washing, the cells were submitted to flow cytometric analysis for fluorescence measurement. In the histograms, the x-axis represents the red fluorescence for annexin-V-RFP. Figure on top shows the % of the total cell population in the selected region. Results are mean ± SD (n=3).

a)



b)



Figure S5. CTAB-coated, but not PEG-coated Au-NRs increase intracellular calcium in KU812 cells. Basophils (1 x 10⁶/ml) were loaded with fluo-4-AM (1 μ M) and treated with the CTAB-, PEG-coated Au-NRs or ionomycin at the concentration as indicated at 37 °C and 5 % CO₂ for 4 (**a**) or 24 h (**b**). After washing, the cells were submitted to flow

cytometric analysis for fluorescence measurement. In the histograms, the x-axis represents green fluorescence for fluo-4. Figure on top shows the % of the total cell population in the selected region. Results are mean \pm SD (n=3).