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Recording Force Events of Single Quantum-dot Endocytosis

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Control experiments

1. The details about the block experiments with cytochalasin B.

As well known, the cytoskeleton is involved in the process of endocytosis to induce the deformation of the plasma membrane, for internalization and the intracellular mobility of endocytic vesicles. Therefore, we tried to block the force signals by cytoskeleton inhibitor, cytochalasin B. After the HeLa cells were treated with 5 μg/mL cytochalasin B for 10 minutes at 37 °C, the force signal almost disappeared as shown in Figure 2e. The appearance probability of force signal “fi” or “fu” was only 5% or 7%, respectively. We obtained the histogram of uptaking and unbinding force value after blocking by cytochalasin B as shown in Figure 2i and 2j, which indicates the specific interaction events between the single quantum-dot (QD) and cell. This result demonstrates that the “fi” and “fu” force signals are specifically related to endocytosis. The further details about the HeLa cell before and after the treatment with cytochalasin B were studied using LSCM. In ESI Figure S1a, the cells are well distributed on the surface of glass slide, and the domains of red circles clearly show the tentacles of healthy HeLa cells. However, after the treatment with 5 μg/mL cytochalasin B for 10 minutes, the tentacles retract and the cells show round contours (ESI Figure S1b), which may be attributed to the depolymerization of essential proteins and the degradation of cytoskeletons.
ESI Figure S1. Optical images of HeLa cells before and after blocking with cytochalasin B. (a) Natural living HeLa cells. (b) HeLa cells treated by 5 μg/mL cytochalasin B for 10 minutes.

2. The details about the block experiments by extracting cholesterol.

Two main pathways of the endocytosis of nanoparticles, cholesterol-dependent and clathrin-dependent endocytoses, have been reported \(^1,3\). Lipid rafts, cell membrane microdomains enriched with sphingolipids and cholesterol, were reported to mediate endocytosis (i.e. cholesterol-dependent endocytosis), and its pathway of endocytosis is often confirmed through extracting cholesterol from cell membranes by methyl-β-cyclodextrin \(^3\). On the other hand, clathrin-mediated endocytosis is mainly inhibited by three effective reagents, hypertonic sucrose, chlorpromazine, and monodansylcadaverine \(^1\). In this work, we testified the pathway of the endocytosis of single QD by cholesterol depletion with methyl-β-cyclodextrin. Before the treatment, the typical uptake and unbinding force signals were clearly shown in Figure 2c. After the treatment with 5 mM methyl-β-cyclodextrin for 20 minutes at 37 °C, there was
scarce force signal observed in many thousands of force curves as shown in Figure 2f. After extracting cholesterols with methyl-β-cyclodextrin, the tentacles of cells contract and the contours show round. The red circles in ESI Figure S2a and 2b present the conspicuous comparison of typical changes. These changes indicate that the methyl-β-cyclodextrin has disturbed HeLa cell membrane microdomains, and would prevent the interaction events between QDs and cells. We also tried to use hypertonic sucrose to inhibit the endocytosis, and found that no inhibition happened. These results demonstrate that the specific interaction between single QD and HeLa cell, resulting in force signal in Figure 2c, would be lipid raft-mediated endocytosis rather than clathrin-mediated one.

ESI Figure S2. Optical images of HeLa cells before and after blocking with methyl-β-cyclodextrin. (a) Natural living HeLa cells. (b) HeLa cells treated by 5 mM methyl-β-cyclodextrin for 20 minutes.
3. Force spectroscopy performed by the clean tip at 37 °C, Poly (ethylene glycol) (PEG)-functionalized tip at 37 °C, and QD-modified tip at 4 °C on HeLa cells.

A series of control experiments were also performed to verify the interaction events between single QD and the cell. We first applied a clean AFM tip to engage force spectroscopy on Hela cells at 37 °C, and found that there was no force signal observed in many thousands of force curves as shown in ESI Figure S3a. Furthermore, we used PEG functionalized AFM tip to perform force spectroscopy at 37 °C, and no force signal was observed in many thousands of force curves too, as shown in ESI Figure S3b. The control experiments clearly indicate that the force signals in Figure 2c originated from the interactions between the HeLa cell and QD attached to the AFM tip, rather than the penetration of AFM tip or the interference of other molecules on the AFM tip. To further rule out that the force signals might be related to other membrane activities. We engaged the force spectroscopy with QD-tethered tips on HeLa cell at 4 °C, and no force signal was observed due to the low activity of cell membranes at low temperature\(^3\) (ESI Figure S3c), which provides further evidences for the specific interactions between QD on the tip and cell membranes during endocytosis. These results demonstrate that the method in our experiments is successful to investigate the interaction force between single QD and the living cell.
ESI Figure S3. Control experiments. (a) A typical force curve in many thousands of force curves by a clean AFM tip on the HeLa cell at 37 °C. (b) A typical force curve in many thousands of force curves by a PEG attached AFM tip on the HeLa cell at 37 °C. (c) A typical force curve in many thousands of force curves performed by the QD-tethered tip on the HeLa cell at 4 °C.
The schematic process of endocytosis of single QD during engaging the force spectroscopy

Endocytosis is a cell-type and cargo-specific process by which the materials from the external environment are internalized\textsuperscript{4,5}. In this work, we recorded the events of single QD’s endocytosis by force spectroscopy, and successfully inhibited these events through cytoskeleton degradation and cholesterol extraction from cell membranes. Therefore, we deduce that the interactions between single QD and the HeLa cell would be lipid raft-mediated endocytosis. Based on these results, we drew a possible scheme for the endocytosis of single QD during engaging the force spectroscopy (ESI Figure S4). The AFM tip tethered with QDs first moved towards the surface of cell membranes along the arrow direction (ESI Figure S4a). As the QD on the AFM tip encountered cell membranes (ESI Figure S4b), the QD with positive charges would bind to the negatively charged cell membranes because of the electrostatic force or possible receptor interactions. During the process of the cell uptaking the QD, the AFM cantilever tethered with the QD bent downwards (ESI Figure S4c), which resulted in the uptaking force signal observed in the force curve (Figure 2c). After the endocytosis event, the AFM tip pulled the QD from the cell during the retrace process in the force spectroscopy, as shown in ESI Figure S4d. As the AFM tip withdrew away, the QD departed from the cell membranes (ESI Figure S4e), which induced the unbinding force signal observed in the force curve (Figure 2c).
ESI Figure S4. The possible scheme of the endocytosis of single QD attached on the AFM tip. (a) The AFM tip tethered with the QD moves towards the cell membrane surface. (b) The AFM tip touches on the cell membranes. (c) The cell membranes uptake the QD. (d) The AFM tip withdraws from the cell. (e) The QD departs from the cell membranes.

From the slope of the force curves, we could calculate that the maximum contact time of the AFM tip on cell membranes in the approaching process can be up to about 0.4 s, which is sufficient for QD uptake into the cell via the cell membranes (thickness is about 15 nm). According to the previous report, the average speed of QD transport in early endocytosis is about 1.6 µm/s. Because of the limitation of spatial resolution, only the time for clathrin-mediated endocytosis that is related to large particle endocytosis was detected by fluorescence microscope until now, and the time for lipid raft-mediated endocytosis that is related to the small particle endocytosis has not been clearly reported. It has been reported that the wrapping time of receptor-mediated endocytosis is about 2 s for 50 nm nanoparticle, and the mobility of nanoparticle
varies in accord with particle size. Therefore, we assume that there is enough time for the tiny QD (4 nm) uptake into the cell during engaging the force spectroscopy.

The scheme only indicates the possible mechanism of the endocytosis of single nanoparticle within a few nanometers size. We also studied the endocytosis process using gold nanoparticles with the diameter of 4 nm, 12 nm, and 17 nm, respectively, and the preliminary data shows the similar process as the QDs (not shown). Meanwhile, we found that the sizes of nanoparticles affected the force of endocytosis, which will be reported in a separate work. To study the effects of surface characteristics of nanoparticles, we tried to detect the endocytosis of QDs with –COOH groups on the surface, and found that no endocytosis activity was observed. Therefore, the positive charges on the surface of nanoparticles are necessary for the efficient endocytosis due to the electrostatic attraction with the negatively charged cell membranes.
The force curve of the endocytosis event during the tip contact with the cell membranes

In some case that the QD-modified AFM tip located on the top position of the cells during the force-distance cycle, the AFM tip stayed on the cell membranes all the while, which produced the force curve as shown in ESI Figure S5. The slow slope of the force curve indicates that the AFM tip still on the cell membranes, however the force signals “fi” and “fu” indicate the occurring of endocytosis, this type force curve further demonstrates that the endocytosis indeed can be fulfilled during the force-distance cycle process.

ESI Figure S5. The force curve by a QD-modified AFM tip on the living cell. “fu” appeared while the AFM tip still contacted with the cell membranes.
**Experimental Section**

**Cell Culture:** Parental HeLa cells were obtained from Shanghai Institute of Biological Sciences. APTES (Aminopropyltriethoxysilane) functionalized glass slides were prepared as previously reported. The cells were cultured on APTES-glass slides in DMEM (Dulbecco’s modified Eagle’s medium) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO₂. Usually, the cells need to be cultured for 2 or 3 days to achieve 75% confluence on the glass slide. Before performing force spectroscopy experiments, the HeLa cells were rinsed with fresh DMEM for three times to remove the metabolite of cells.

**Conjugation of QDs on AFM Tips:** Water-soluble, mercaptoethylamine capped CdTe QDs were obtained following the previous synthetic routes. The mercaptoethylamine capping would also decrease the cytotoxicity of QDs that is partly from the Cd²⁺. Functionalization of AFM tips (Microlever, Veeco, Santa Barbara, CA, coated for MacMode AFM by Agilent Technologies, Chandler, AZ) with APTES was just as preparing APTES-glass slides described above. The tips were cleaned with O₃ for 20 minutes to get rid of any organic contamination, and then placed in a Petri dish at the bottom of a dessicator and modified with APTES. Subsequently, Aldehyde-PEG-NHS linker was conjugated in triethylamine and CHCl₃ as described. Then the AFM cantilevers were immersed in a 100 μL phosphate buffer solution (PBS) drop containing 0.6 μM mercaptoethylamine capped QDs with the diameter of 4 nm for functionalization. In addition, 2 μL 1M NaCNBH₃ solution was added to the drop and mixed carefully with pipette. After the functionalization, 5
μL 1M ethanomine solution was added to the solution drop in order to passivate unreacted aldehyde groups. Finally, the modified AFM tips were rinsed with PBS for three times and stored at 4 °C until use.

**AFM Imaging and Force Curve Measurements:** Topography and force curve measurements were all acquired using AFM 5500 (Agilent Technologies, Chandler, AZ) in DMEM at 37 °C. The blocking experiments were performed by the addition of 5 μg/mL cytochalasin B and 5 mM methyl-β-cyclodextrin, respectively. Several thousand force curves were recorded at different positions on the cells. The deflection sensitivity of the photo-detector was determined by the slope of the force-distance curves taken on the bare surface of mica. The AFM cantilevers were calibrated by a reference cantilever (CLFC, Veeco, Santa Barbara, CA) as described 14.

**Fluorescence Microscopy Imaging:** HeLa cells were incubated in DMEM with QDs at 37 °C for 1h, stained with DiI for 30 minutes, and washed prior to imaging by a Leica SP2 laser scanning confocal microscope. To excite the QD and DiI, the UV laser line of 405 nm and He/Ne laser line of 543 nm were used, respectively. Band-pass filters were set up, such as 580–620 nm for the DiI emission signal, and 555-620 nm for the QD emission signal. In the sequential multiple cellular stain, DiI were excited with the 543 nm laser line and the QD with the 405 nm laser line. The emission signals passed through the 580-620 nm and 555-620 nm filters, respectively. All images were captured with a 100x/1.4 oil DIC objective and collected in multichannel mode.
References