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Supplementary Information

Evaluating the therapeutic efficacy of nano-drugs targeting epidermal growth factor receptor

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Materials and methods

Materials

G7-PAMAM was purchased from the Weihai CY Dendrimer Technology Company. EGF (molecular weight: 6.2 kDa, sequence: NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWW ELR) was purchased from peprotech Biotechnology Co. Ltd. GE11 (YHWYGYTPQNVI) was purchased from Sangon Biotech. 1- (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Aladdin. Other related reagents (analytical grade) were purchased from sigma Aldrich.

Cell culture

Human non-small cell lung cancer cells (A549) and African green monkey kidney cells (Vero) were purchased from Shanghai Academy of biological sciences. A549 cells and Vero cells were cultured in DMEM (dulbecco's modified eagle's) and MEM (minimum essential medium), respectively, which contains 10% fetal bovine serum (FBS), 100% fetal bovine serum, 100 μg/ml streptomycin, 100 μg/ml penicillin, and BIOMYC-3 antibiotic solution. They were cultured in a constant temperature incubator at 37 ℃ and 5% CO_2. Before the force tracing experiment, the cells were washed with PBS (phosphate buffer solution, 1000 mL PBS containing 0.2 g KH_2PO_4, 0.2 g KCl, 3.5 g $Na_2HPO_4 \cdot 12H_2O$, 8.0 g NaCl) for three times. Then 2 mL serum-free DMEM and MEM was added into the culture dishes and used for force tracing experiments (the cells were kept at 37 ℃).

Synthesis of PAMAM-CPT-EGF and PAMAM-CPT-GE11

G7-PAMAM aqueous solution (0.2 mg/μL) and CPT (2.4 mg) were stirred overnight with low speed at room temperature. The precipitate was removed by centrifugation and the supernatant was retained to obtain PAMAM-CPT. EGF, NHS, and EDC were dissolved in and stirred with low speed at room temperature for 3 h to active EGF. Then, the mixture of PAMAM-CPT and activated EGF (10 μM) solution was stirred overnight at room temperature to obtain PAMAM-CPT-EGF by ultrafiltration purification. PAMAM-CPT-GE11 was prepared by the same method.

AFM Tip modification

The AFM tips (MSCT, D-tip with the normal spring constant of 0.03 N/m, Bruker, USA) were cleaned with the piranha solution (H_2SO_4: 30% H_2O_2, 3:1, v/v) and ultraviolet light, and the AFM tips were incubated with 50 μL of 3-aminopropyltriethoxysilane (APTES) and 20 μL of N, N-diisopropylethylamine (99%) by vapour deposition method. Then, the silylated AFM tips were combined with heterobifunctional PEG (Acetal-PEG_{45}-NHS, 1 mg/mL) linker in toluene and 0.5% triethylamine (v/v) solution for 2 h. The PEG-modified AFM tips were then immersed in 1% citric acid solution for 10 min to carboxy aldehyde activation. Subsequently, the AFM tips were immersed in the mixture of PAMAM-CPT-EGF or PAMAM-CPT-GE11 and 1 M $NaCNBH_3$ for 2 h. Finally, 5 μL of 1 M ethanolamine was added to the reaction solution to inactivate the unreacted aldehyde group. After reaction for

15 min, the tips were washed with PBS solution three times, and stored at 4 °C until use.

Force tracing measurements

Force tracing measurements were performed using Agilent AFM 5500 (Agilent Technologies, USA). Before force tracing measurements, 2 mL DMEM or MEM medium was added to cell culture dishes (A549 cells or Vero cells). In order to ensure that the experiment was carried out on the cell membrane, a CCD camera was used to help locate the modified AFM tip on the cell membrane. Force tracing experiments were performed on the relatively flat region of cells, which can avoid large fluctuations from cell to collect force-time curves with low noise and clear force-time signals. The typical force-distance curves were obtained to find out the contact point. In order to reach the contact point, we slowly approached the AFM tip onto the cell membrane by turn on the proportional-integral (PI) control system (P=0.001; I=0.001). When the nano-drug modified on the AFM tip was internalized by the cells, the AFM tip cantilever would bend downward, and the deflection of the AFM tip cantilever will be recorded by a 16-bit DA/AD card (PCI-6361e, National Instruments, controlled by LabVIEW) to generate a force-time curve. The collected force-time curves were analyzed by LabVIEW software. The 20 kS/s sampling rate (20 k data points per second) of data acquisition was applied, and the high frequency electronic noise was filtered by a 100 low pass filter. Usually, we locate the nano-drug modified AFM tip on one position of cell membrane to continuously record the deflection-time curve (which could be converted into force-time curve) for 20 min, and the data are divided into several files according to time (one file including 12 s). After ~20 min detecting, the AFM tip will be retracted and changed to another region. For each set of data, around 4000 force curves were obtained from 10-15 cells at different positions.

Blocking experiments

In blocking experiments, free EGF and GE11 peptides (both at a final concentration of 50 ng/mL) were coincubated with A549 cells for 30 min. Chlorpromazine (CPZ) is an inhibitor that inhibits clathrin-mediated endocytosis by preventing clathrin-coated pits from being assembled on the plasma membrane. A549 cells were incubated with CPZ (final concentration of 10 μg/mL) at 37 ℃ for 20 min. Cholesterol is more important in vesicle/raft-dependent endocytosis, and Filipin is a commonly used inhibitor.[1] It can deplete cholesterol in the plasma membrane and inhibit the transport of cholesterol through the caveolin-dependent transport pathway. Therefore, the cells were incubated with Filipin with final concentration of 5 μg/mL for 30 min. In addition, macropinocytosis is also an important way of cell entry. EIPA (5-(N-ethyl-N-isopropyl), Amiloride) can interfere with cell membrane Na^+/H^+ ATP enzyme, thereby inhibiting the cell's macropinocytosis.[2] We treated the cells with EIPA (final concentration 10 mg/mL) at 37 °C for 40 min. Genistein is an inhibitor

that specifically inhibits the EGFR receptor tyrosine kinase.[3] We incubated Genistein (final concentration 50 μM) with A549 cells for 30 min.

Fluorescence labeling and imaging

Before the fluorescence imaging, A549 and Vero cells were cultured on glass dishes for at least 24 h. PAMAM-CPT-EGF, PAMAM-CPT-GE11 and PAMAM-CPT were reacted with excess Cy5 for 3 h, and the obtained mixture was purified with a 3 kDa Ultrafiltration centrifugal tube to obtain Cy5-labeled PAMAM-CPT, PAMAM-CPT-EGF, and PAMAM-CPT-GE11. The cells were incubated with Cy5-labeled PAMAM-CPT, PAMAM-CPT-EGF, and PAMAM-CPT-GE11 for 30 min at 37 °C, respectively, then the cells were washed three times with PBS before fluorescence imaging. For blocking experiments, we added EIPA, CPZ, Filipin, and Genistein respectively to incubate with A549 cells (the above reagent concentration and the incubation time with the cells are the same as in the blocking experiments of force tracing), rinse 3 times with PBS before the fluorescence imaging. For Vero cells labeling, the same method was used. Fluorescence imaging was performed on a fluorescence microscope (Nikon-Ti-S). Cy5 was excited with a 625 nm He-Ne laser. The fluorescence images were quantified by ImageJ to obtain the fluorescence number, which represents the total number of fluorescent cells under the same cell density in petridish, and at least three independent experiments were performed for each condition.

Nanoindentation experiments

Agilent AFM 5500 (Agilent Technologies, Chandler, AZ) was used to determine the cell stiffness before and after nano-drug treatment directly under physiological conditions. The microsphere of polystyrene with a diameter of approximate 10 μm is glued to the AFM tip cantilever (D-tip) and the spring constant is measured as K=0.03917 N/m. Force-distance curves were obtained directly from cells cultured in petri dishes at a speed of 2 μm/s with a scanning range of 2 μm and the same movement distance (500 nm) of piezoceramics after contact with the cell membrane. The number of cells tested in each condition is about 15, and ~6000 force-distance curves is obtained for each set by at least three independent experiment.

Young's modulus calculation

Young's modulus (E) of cells was calculated from the force-distance curves using the Hertz model.[4] The Hertz model requires some hypothesis that the surfaces are continuous and frictionless and small deformations. Although, these assumptions do not correspond completely to reality in cells, the Hertz model is still useful for achieving information about cell elasticity. The Young's modulus was calculated according by the following equation:

$$F = \frac{4}{3}\frac{E}{(1-\vartheta^2)}\sqrt{R}\delta^{\frac{3}{2}} \quad (1)$$

Here, F is the loading force, E is the Young' s modulus, R is the Microsphere radius, and δ is the indentation depth. The cells are considered linear, elastic, isotropic,

incompressible, and having small strain values, so the Poisson ratio is 0.5.

Calculation of displacement

The displacement and average velocity of endocytosis of PAMAM-CPT-EGF and PAMAM-CPT-GE11 were calculated. As shown in Fig. S14a, PAMAM-CPT-EGF is modified on the AFM tip by a PEG linker (length of about 20 nm), and the modified AFM tip contacts the cell surface. Subsequently, PAMAM-CPT-EGF is endocytosed by the cell, resulting in downward bending of the AFM tip cantilever and stretching of the PEG linker. Therefore, the PAMAM-CPT-EGF displacement D is equal to the bending distance d of the AFM tip cantilever and the stretching length Q of PEG linker, as shown in equation (1).

$$D = d + Q \tag{1}$$

The force-dependent stretching behavior of PEG linker can be described by the extended worm-like chain (WLC) model, which is described by the following equation:

$$\frac{FL_p}{k_BT} = \frac{1}{4}\left(1 - \frac{Q}{L_0} + \frac{F}{k_0}\right)^{-2} - \frac{1}{4} + \frac{Q}{L_0} - \frac{F}{k_0} \tag{2}$$

In the equation, k_B presents the Boltzmann constant, T is the absolute temperature, L_p is the persistence length, k_0 is the enthalpic correction, Q is the extension of PEG linker, and L_0 is the contour length. Referring to the literature, the persistence length L_p is 3.8±0.02 Å, and the enthalpic correction k_0 is 1561±33 pN. The PEG unit length is 4.2 Å and the terminus is 5.25 Å, the total estimated contour length L_0 of PEG (45 unit) we used is approximately 194 Å.

According to Hooke's law, the bending distance d of AFM tip cantilever can be calculated by the following equation:

$$F = k \times d \tag{3}$$

In the equation, F is the force of a single nano-drug entry cell measured from the force tracing curve, and k is the spring constant of the AFM tip cantilever. Based on equations (1), (2), and (3), the relationship between displacement D and entry force was obtained.

The prepared PAMAM-CPT-EGF and PAMAM-CPT-GE11 nano-drugs were characterized using UV-Vis spectroscopy, as shown in Figure S1. The characteristic peak wavelength for CPT is 365 nm (red), G7-PAMAM is 278 nm (blue), EGF-Cy5 and GE11-Cy5 (green) are 650 nm (Cy5) and 280nm (EGF/GE11). Both PAMAM-CPT-EGF and PAMAM-CPT-GE11 contained the characteristic peaks mentioned above. The results indicate that PAMAM-CPT-EGF and PAMAM-CPT-GE11 nano-drugs have been successfully synthesized

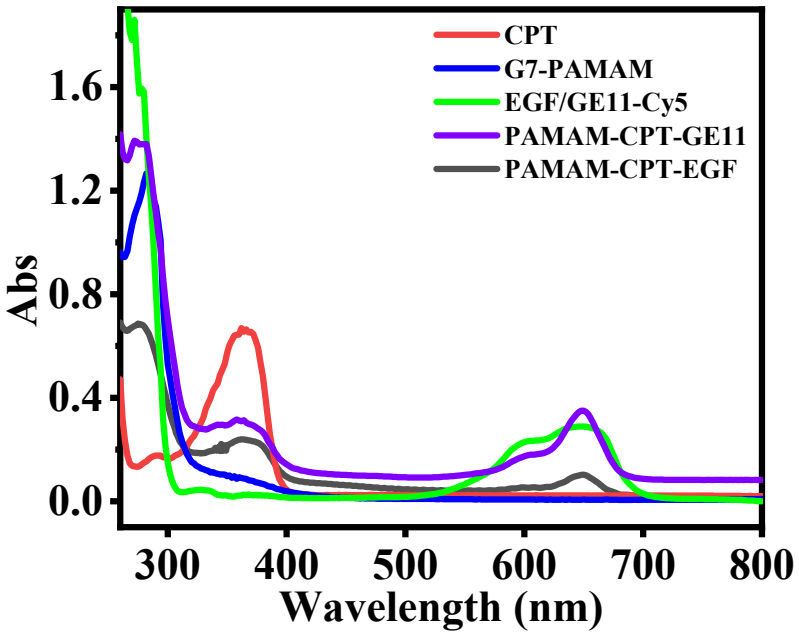

Fig. S1 UV-Vis Spectra of EGF/GE11-Cy5 (green), CPT (red), G7-PAMAM (blue), PAMAM-CPT-EGF (black), and PAMAM-CPT-GE11 (purple).

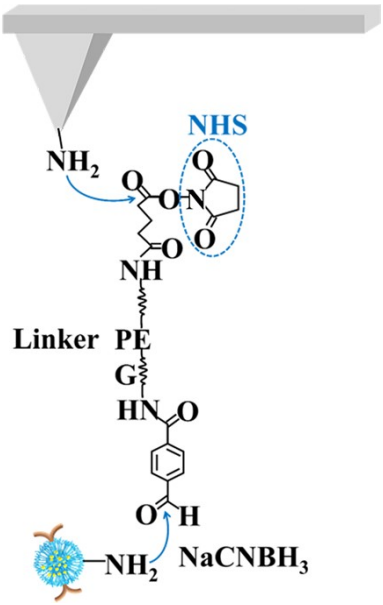

Fig. S2 PAMAM-CPT-EGF is attached to the AFM tip via a heteobifunctional PEG linker.

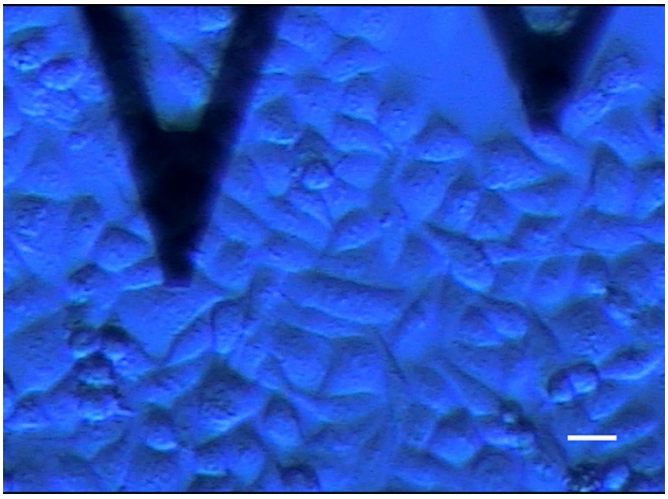

Fig. S3 The optical image of the AFM tip cantilever locating above the living A549 cell. (Scale bar: 60 μm).

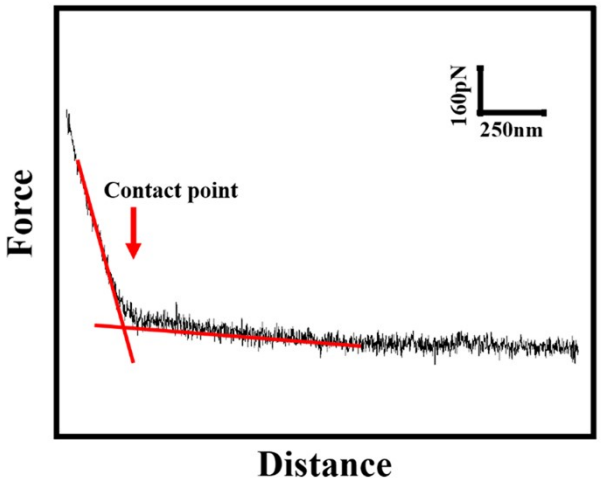

Fig. S4 The contact point between the PAMAM-CPT-EGF modified AFM tip and the cell surface. The contact point is the intersection of the slope (red line) and the flat part in the force-distance curve, indicating by the red arrow.

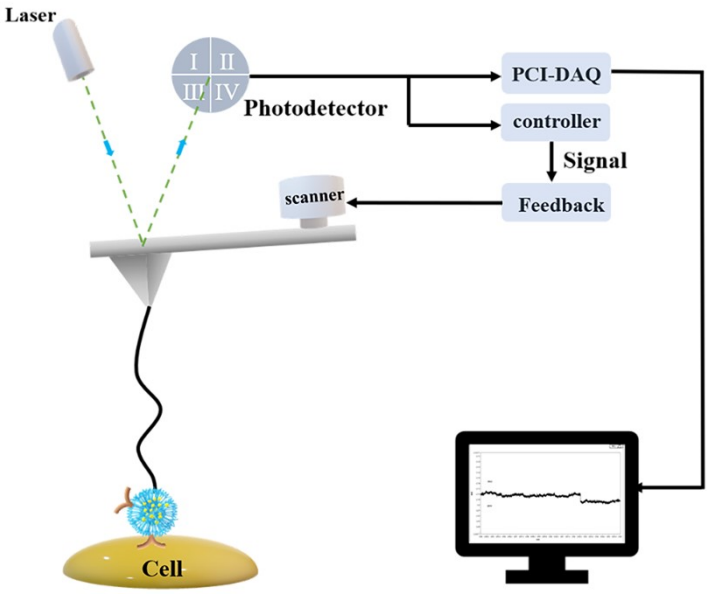

Fig. S5 Schematic diagram of the force tracing technique workflow using for detecting the cellular uptake of PAMAM-CPT-EGF.

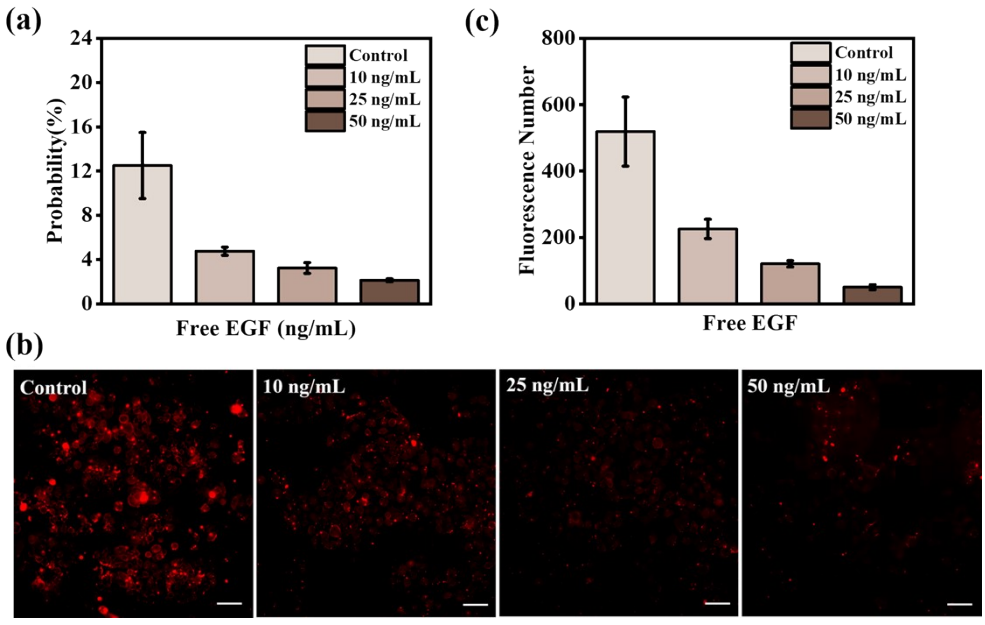

Fig. S6 The concentration-dependent behavior of EGF inhibiting PAMAM-CPT-EGF entry cell. (a) The probability of observed force-time signal in force-time curves before (Control) and after inhibition with 10, 25, 50 ng/mL free EGF. (b) Fluorescence images for A549 cells uptake PAMAM-CPT-EGF before (Control) and after inhibition with 10, 25, 50 ng/mL free EGF. Red fluorescence represents the Cy5-labeled PAMAM-CPT-EGF nano-drug. (Scale bar: 100 μm) (c) Statistics analysis of fluorescence count corresponding to fluorescence images. The results are the mean value from three independent experiments (mean value ± standard deviation).

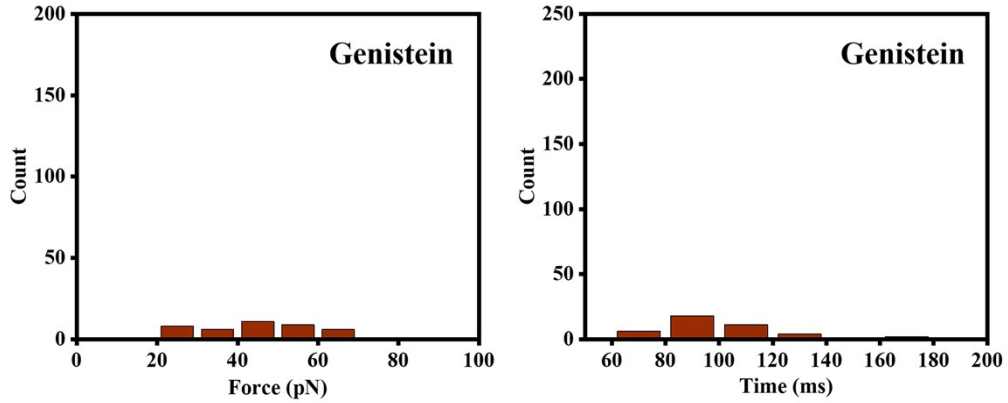

Fig. S7 Force and duration distribution of PAMAM-CPT-EGF entry cell after blocking with genistein.

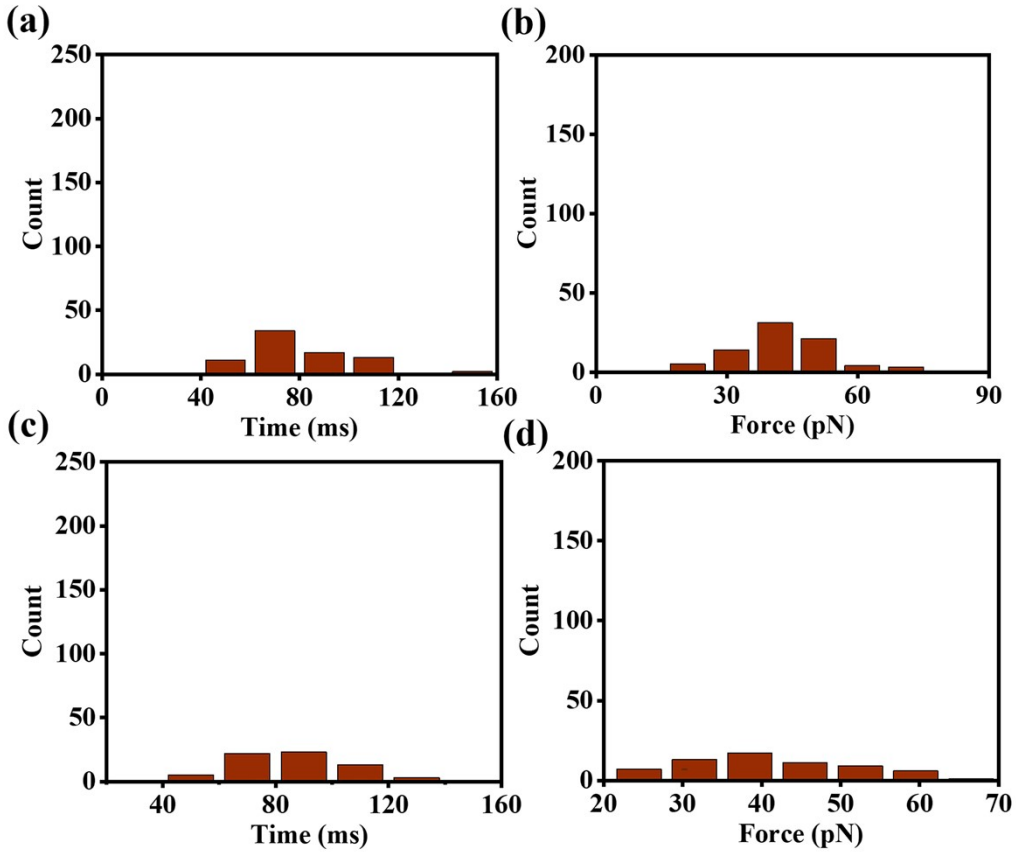

Fig. S8 The dynamic parameters of a single nano-drug entry cancer and normal cells. (a, b) Duration and force distribution of PAMAM-CPT entry A549 cell. (c, d) Duration and force distribution of PAMAM-CPT-EGF entry Vero cell.

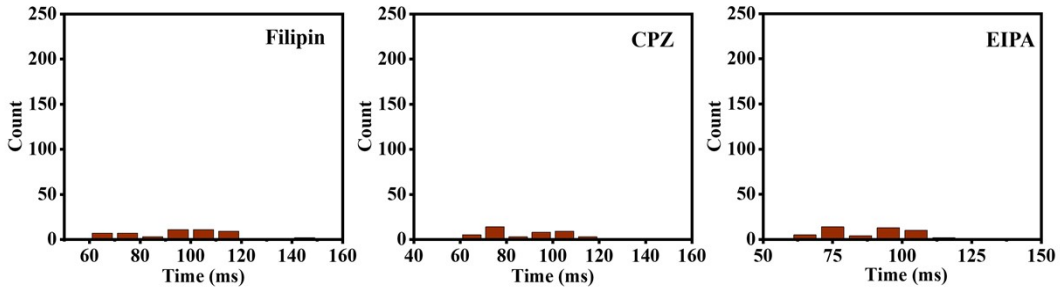

Fig. S9 Duration distribution after blocking with Filipin, CPZ, and EIPA.

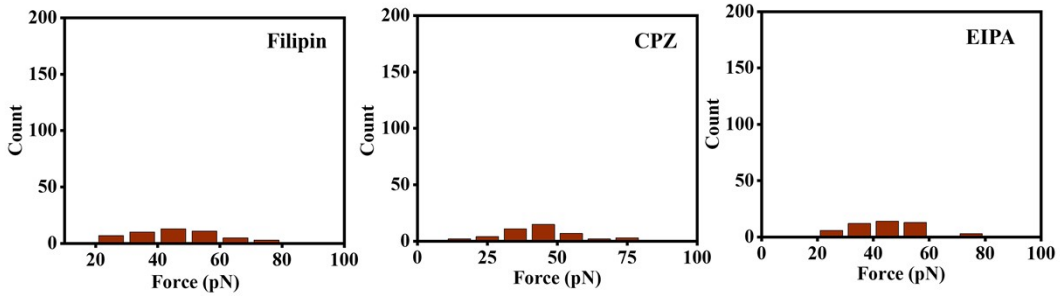

Fig. S10 Force distribution after blocking with Filipin, CPZ, and EIPA.

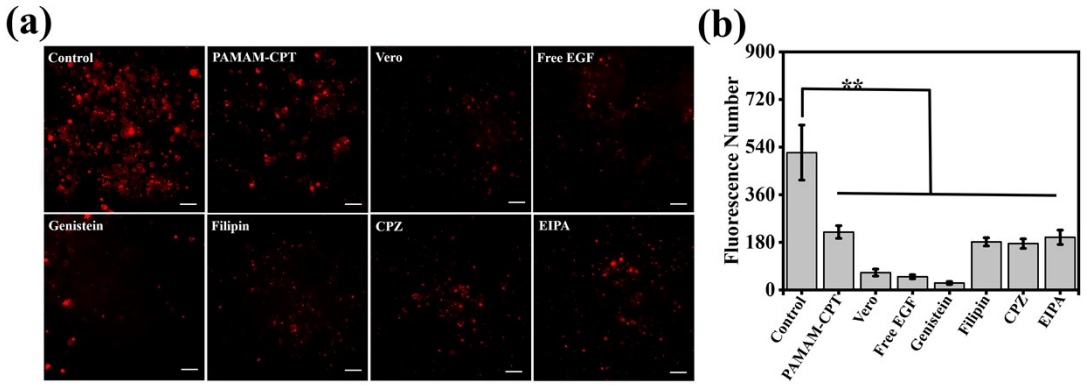

Fig. S11 Fluorescence imaging of nano-drugs entry A549 and Vero cells. (a) Fluorescence images for A549 cells coincubated with PAMAM-CPT-EGF before (Control) and after inhibition with CPZ, EIPA, filipin, free EGF, A549 cells coincubated with PAMAM-CPT, and Vero cells coincubated with PAMAM-CPT-EGF. Red fluorescence represents the Cy5-labeled nano-drugs. (Scale bar: 100 μm) (b) Statistics analysis of fluorescence count corresponding to fluorescence images. Two-tailed Student's t-test: ** $P<0.01$.

The observed red fluorescence count (reflects the amount of cells uptake nano-drugs) is 519±104 according to statistics analysis.[5] After addition free EGF (final concentration 50 ng/mL), the fluorescence count decreases to 50±8. When we

coincubated genistein (50 μM) with A549 cells for 30 min, the count of fluorescence decreases to 26±6. Meanwhile, the fluorescence count for PAMAM-CPT-EGF coincubated Vero cells and PAMAM-CPT coincubated A549 cells is 66±13 and 219±24 respectively. A549 cells were pretreated with filipin, CPZ and EIPA, then coincubated with Cy5-labeled PAMAM-CPT-EGF. The fluorescence count significantly reduces to 182±16, 175±18, and 199±27 respectively.

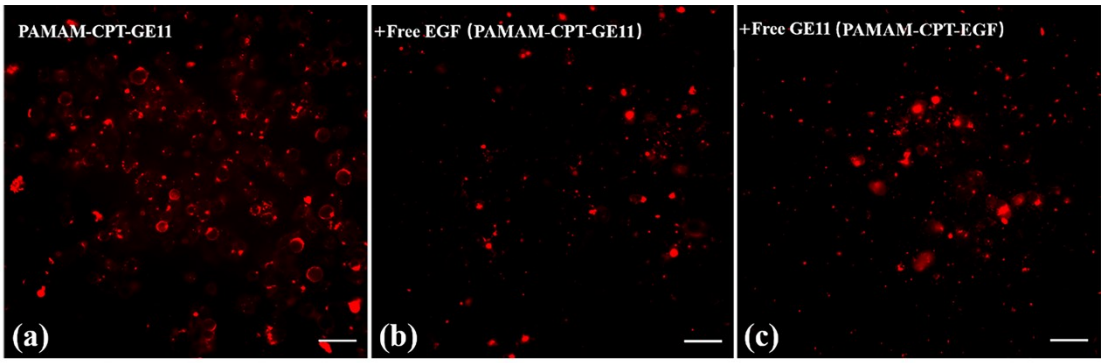

Fig. S12 Fluorescence imaging of nano-drugs entry A549 cells. (a, b) Fluorescence images of A549 cells uptake PAMAM-CPT-GE11 before (Control) and after blocking with free EGF. (c) Fluorescence images of A549 cells uptake PAMAM-CPT-EGF after blocking with free GE11. (Scale bar: 100 μm).

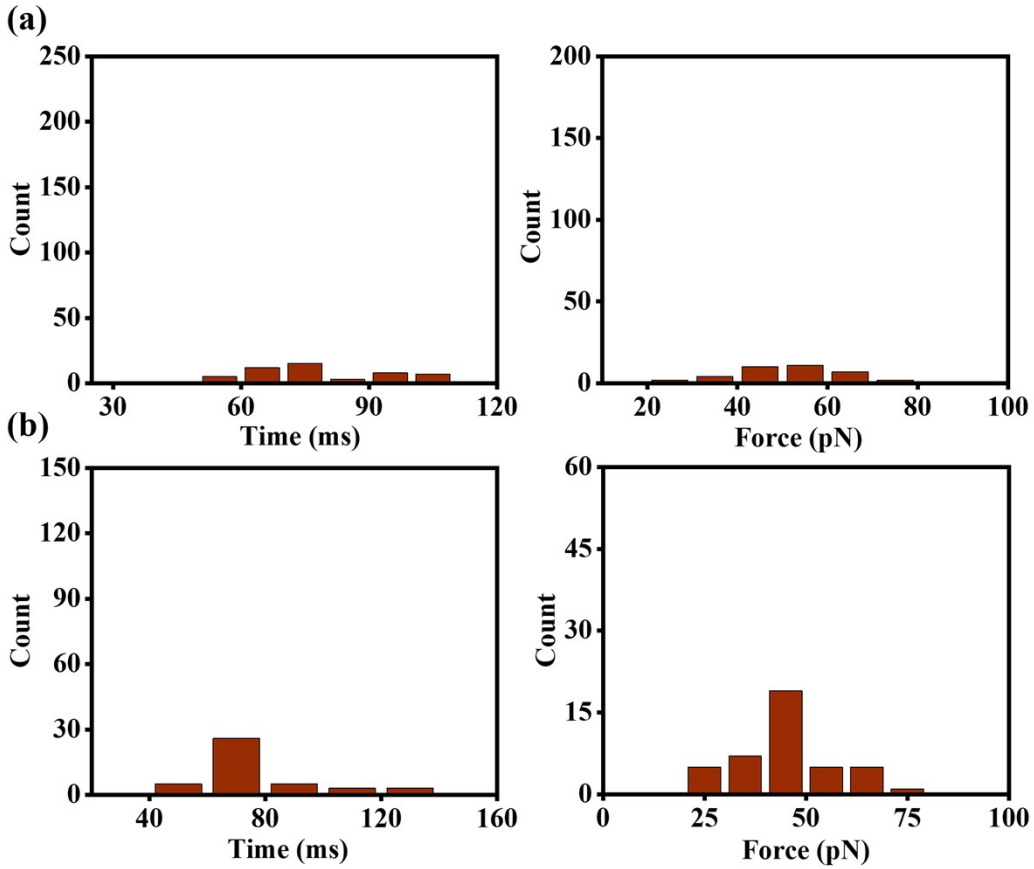

Fig. S13 Force and duration distribution after cross-blocking. (a) Distribution of force and duration of PAMAM-CPT-EGF entry A549 cell blocked by free GE11 peptide. (b) Force and duration distribution of PAMAM-CPT-GE11 entry A549 cell blocked by free EGF.

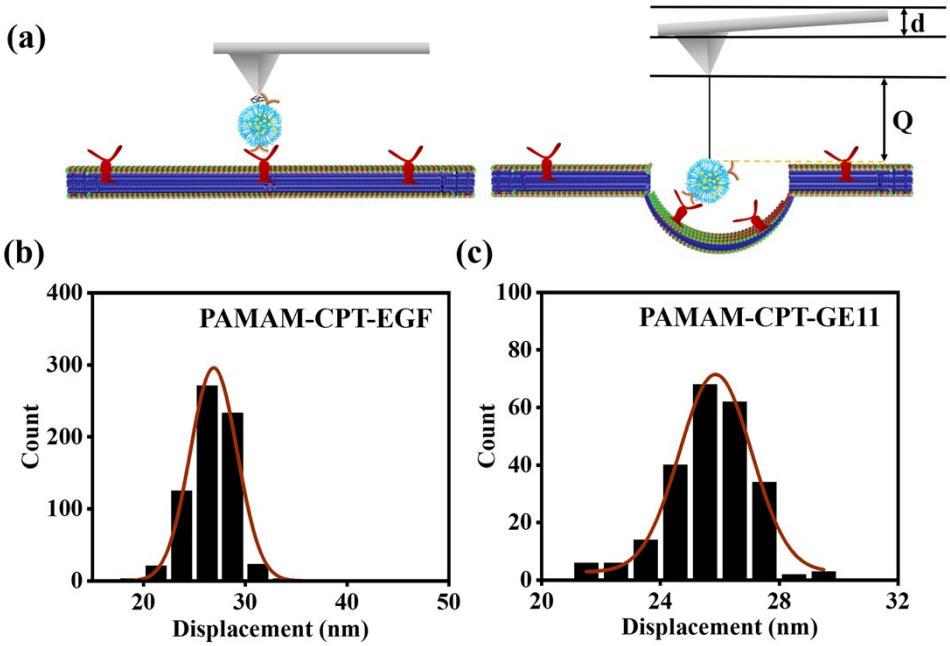

Fig. S14 The displacement of nano-drug entry cell. (a) The displacement diagram during nano-drug entry cell. (b) Displacement distribution of PAMAM-CPT-EGF entry A549 cell, which is in the range of 18.0-38.0 nm with an average value of 26.6±3.2 nm. (n≈450). (c) Displacement distribution of PAMAM-CPT-GE11 entry A549 cell, which is in the range of 21.5-29.7 nm with a mean value of 25.7±1.4 nm. (n≈200).

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