Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Supporting Information

Investigating the trans-membrane transport of HAIYPRH peptide-decorated nano-drugs

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Materials and methods

Materials

G5-PAMAM was purchased from the Weihai CY Dendrimer Technology Company. Holo-Tf was purchased from Sigma Alarich (St. Louis, MO). T7 with a cysteine on the N-terminal (Cys-T7) was purchased from Chinese Peptide Company (Hangzhou, China). 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 were cultured in Dulbecco's modified Eagle medium (DMEM, BI), and Vero cells were cultured in Minimum Eagle Medium (MEM, BI). Each type of medium was supplemented with 10% fetal bovine serum (FBS), penicillin (100 μg/mL), and streptomycin (100 μg/mL), and the cells were cultured at 37 °C in a humidified incubator containing 5% CO_2. The cells were subcultured until 75% of the petri dish was covered by cells. The cells were washed with PBS (phosphate buffer solution, 137 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) before using in force tracing experiments and nanoindentation experiments. The tests were carried out at 37 °C.

Synthesis and characterization of PAMAM-CPT-T7

G5-PAMAM aqueous solution and CPT were mixed and stirred overnight at room temperature at low speed, the sediment was centrifugally removed, the supernatant was retained, and a PAMAM-CPT solution was obtained. T7, NHS, and EDC were dissolved in aqueous solution and stirred at room temperature for 3 h at low speed to activate -COOH on the T7. Then, the mixture of PAMAM-CPT and activated T7 solution was stirred overnight at room temperature to obtain PAMAM-CPT-T7 by ultrafiltration purification. The resulting conjugates were purified through a 10 kD Molecular Weight Cutoff (MWCO) microcon centrifugal filter device to remove free T7. The prepared PAMAM-CPT-T7 was analyzed by UV-Vis spectroscopy. G5-

PAMAM contains 128 -N_2H on its surface, and all -N_2H on the PAMAM surface can bind to -COOH on T7 theoretically. The size of T7 is 2.16 nm, and the surface area of G5-PAMAM is ~90 nm^2. The G5-PAMAM surface is modified with a maximum of ~40 T7 theoretically. However, the surface of the PAMAM nanoparticle would not be occupied completely due to the steric hindrance. To exclude the influence of T7 density on the PAMAM surface in the experiment, we reacted PAMAM with an excess of T7 such that the density of T7 on the PAMAM surface may be similar.

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 50 μL of APTES (99%) and 20 μL of N, N-diisopropylethylamine (99%) were placed into a small container at the bottom of the desiccator, respectively, leaving the AFM tips exposed to APTES vapor overnight. Then, the silylated AFM tips were combined with heterobifunctional PEG (Acetal-PEG_{45}-NHS, 1 mg/mL) linker in chloroform 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, with conversion of the terminal acetal group into the aldehyde group, and the AFM tips were put into the mixture of PAMAM-CPT-T7 and 1 M $NaCNBH_3$ for 2 h. Finally, 5 mL of 1 M ethanolamine was added to the reaction solution to inactivate the unreacted aldehyde group. after functionalization, the AFM tips were washed with water for three times and protect from light stored at 4 °C in water until use.

Force tracing measurements

The force tracing technique were performed on AFM 5500 (Agilent Technologies, Chandler, AZ). The experiment was carried out at 37 ℃ serum-free medium controlled by temperature controller 325 (Agilent Technologies, Chandler, AZ). With the help of a CCD camera, the PAMAM-CPT-T7 functionalized AFM tip was located onto the cell membrane. The force-distance curves were obtained to find out the contact point between the PAMAM-CPT-T7 functionalized AFM tip and the cell membrane. Turn on the proportional-integral (PI) control system (P=0.001; I=0.001) and slowly bring

the AFM tip close to the cell membrane until the contact point is reached, the feedback system was closed. When the PAMAM-CPT-T7 was internalized by the cell, the AFM tip cantilever would bend downward to generate a force signal, 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 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. Before the force tracing test, the cells were incubated in serum-free medium and Tf-containing medium for a certain time, then the cells were rinsed three times with PBS, 2 mL of fresh serum-free medium without Tf was added, and the test was subsequently performed.

Nanoindentation experiments

Force-distance curves were obtained directly from the cells using Agilent 5500 AFM instruments (Agilent Technologies, Chandler, AZ.) to obtain cell stiffness before and after nano-drugs treatment. 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. The AFM Tip applied a compression force orthogonal to the cell at a speed of 2 μm/s with a sweep range of 2 μm. The number of cells tested under each condition is about 15, and ~6000 force-indentation curves is obtained for each set.

Blocking experiments

A549 cells were incubated with clathrin-mediated endocytosis pathway inhibitor Chlorpromazine (CPZ, final concentration of 28 μM) at 37 ℃ for 20 min. The cells were incubated with Filipin (final concentration of 8 μM), which inhibited clathrin-mediated endocytosis, for 30 min. We treated the cells with EIPA (final concentration of 33 mM), a macropinocytosis pathway inhibitor, at 37 °C for 40 min. A549 cells were incubated with a final concentration of 50 μM ferristatin, an inhibitor that specifically inhibited TfR receptor, for 1 h.

Fluorescence labeling and imaging

Before the fluorescence imaging, A549 and Vero cells were cultured on glass

dishes for at least 24 h. PAMAM-CPT-T7 was reacted with excess Cy5 for 3 h, and the obtained mixture was purified with a 1 kDa Ultrafiltration centrifugal tube to obtain Cy5-labeled PAMAM-CPT-T7. Tf with different concentrations (final concentration of 15 μM, 25 μM and 35 μM) was added to incubate the cells for 1 h, and Tf (final concentration of 25 μM) was incubated with cells for 10 min, 1 h and 2 h, respectively, then co-incubated with Cy5-labeled PAMAM-CPT-T7 at 37 °C for 30 min. The cells were washed three times with PBS before fluorescence imaging. For blocking experiments, we added EIPA, CPZ, Filipin, and ferristatin 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 intensity is calculated using the Image J software.

Young's modulus calculation

Young's modulus (E) of cells was calculated from the force-distance curves using Hertz model. The Hertz model requires that the sample surface are continuous, frictionless and small deformations. Although the cells do not correspond the requirements, the elastic information of the cells obtained using the Hertz model is still useful. The Young's modulus was calculated 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 tip radius, and δ is the half-opening angle of the sharp tip. The cells are considered linear, elastic, isotropic, incompressible, small strain values, and the Poisson ratio is 0.5.

Calculation of displacement

The displacement and average velocity during the PAMAM-CPT-T7 endocytosis process were also calculated. PAMAM-CPT-T7 is functionalized on the AFM tip with PEG linker (length of about 20 nm), and the functionalized AFM tip contacts with the cell surface. PAMAM-CPT-T7 is endocytosed by the cell, resulting in downward

bending of the AFM tip cantilever and stretching of the PEG linker. Therefore, the PAMAM-CPT-T7 displacement D is equal to the bending distance d of the AFM tip cantilever and the stretching length Q of the PEG linker, as shown in equation (2).

$$D = d + Q \tag{2}$$

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:

$$\frac{FL_p}{k_BT} = \frac{1}{4}\left(1 - \frac{Q}{L_0} + \frac{F}{k_0}\right)^{-2} - \frac{1}{4} + \frac{Q}{L_0} - \frac{F}{k_0} \tag{3}$$

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 length of PEG linker, and L_0 is the contour length. Referring to the literature,[1] 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:

$$F = k \times d \tag{4}$$

where 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 (2)-(4), the relationship between displacement D and entry force was obtained.

The prepared PAMAM-CPT-T7 nanodrugs were characterized by UV-Vis spectroscopy, as shown in Fig S1. The characteristic peak of T7-Cy5 (red) is 650 nm (Cy5) and 280 nm (T7),[2] G5-PAMAM is 278 nm (blue),[3] CPT is 365 nm (green).[4] PAMAM-CPT-T7 nano-drug (black) contains the characteristic peaks of T7-Cy5, CPT, and G5-PAMAM. The results showed that PAMAM-CPT-T7 nano-drugs were synthesized successfully.

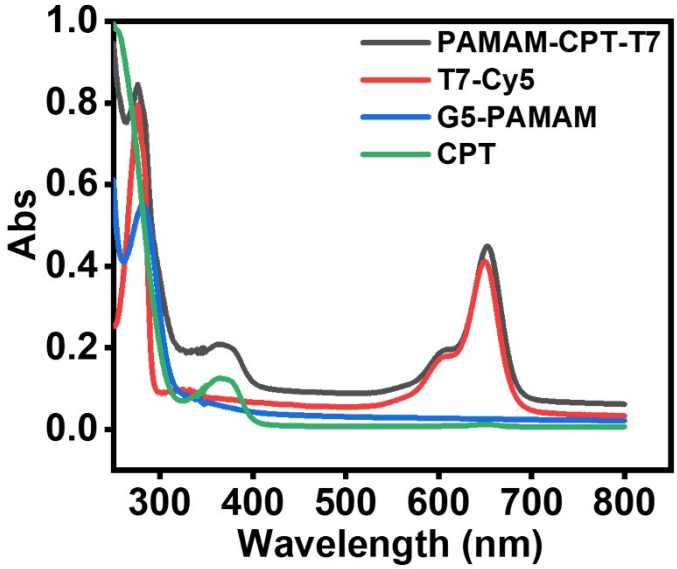

Fig. S1 UV-Vis Spectra of PAMAM-CPT-T7 (black), T7-Cy5 (red), G5-PAMAM (blue), and CPT (green).

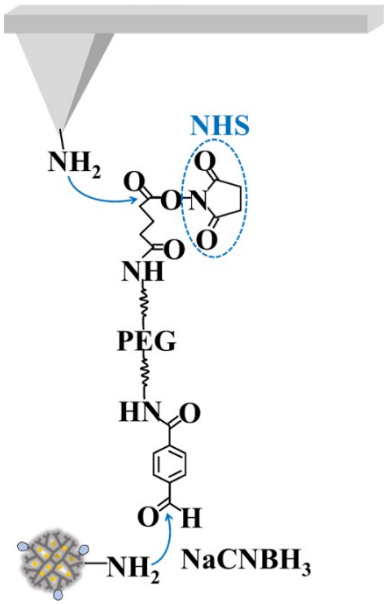

Fig. S2 PAMAM-CPT-T7 is attached to the AFM tip via a heterobifunctional PEG linker.

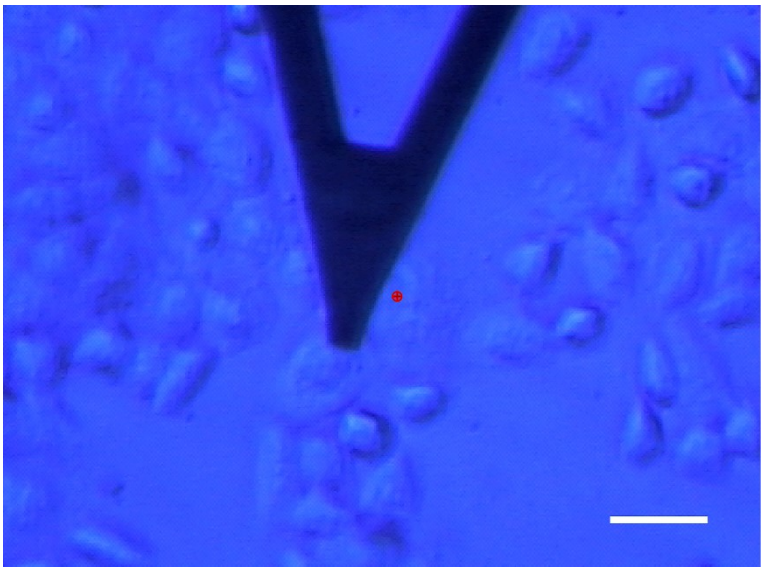

Fig. S3 The optical image of the AFM tip cantilever locating above the living A549 cell. (Scale bar: 60 μm).

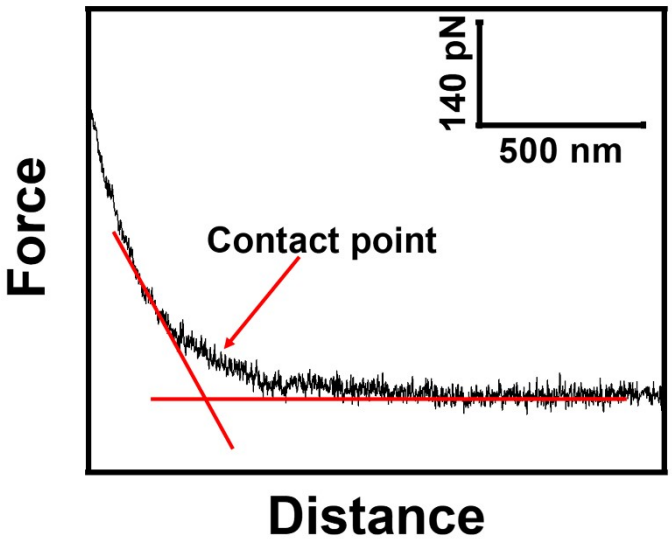

Fig. S4 The contact point between the PAMAM-CPT-T7 modified AFM tip and the cell surface. The contact point is the intersection of the slope (red line) and the flat part in the force-distance curve, indicating by the red arrow.

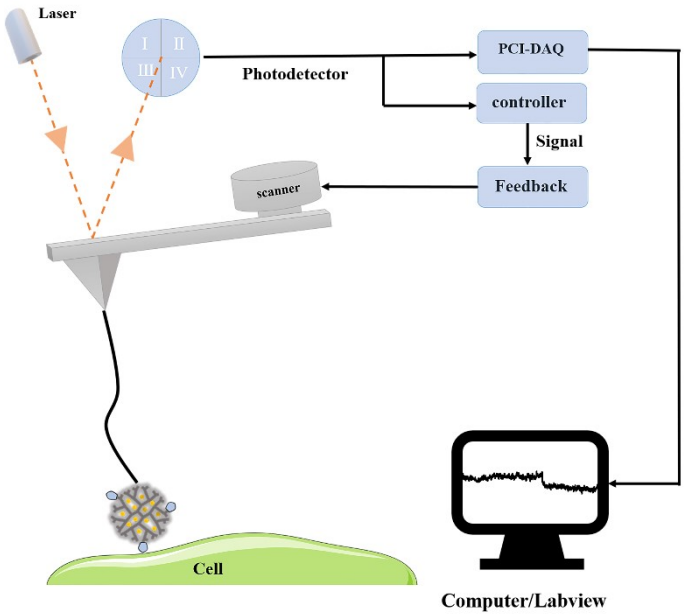

Fig. S5 Schematic diagram of the force tracing technique workflow using for detecting the cellular uptake of PAMAM-CPT-T7.

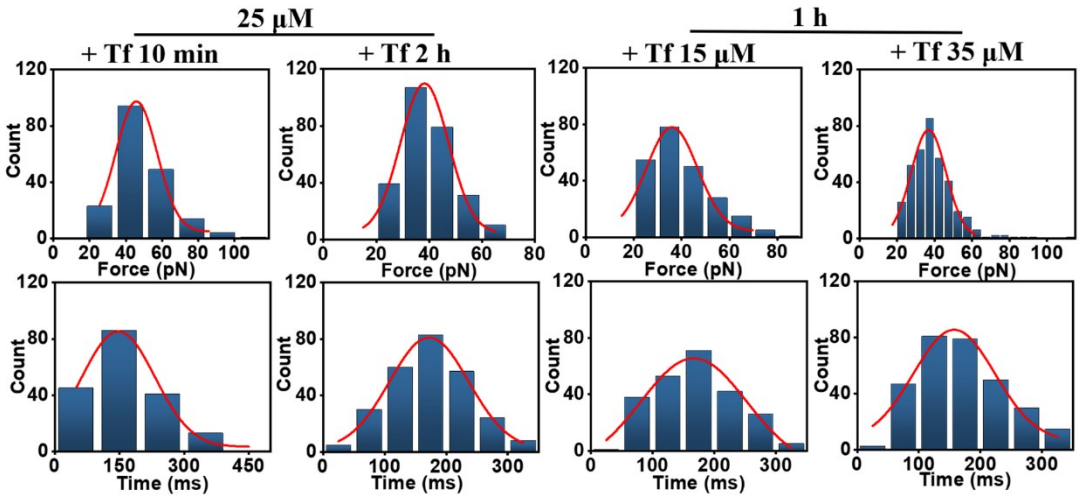

Fig. S6 The force and duration distribution of PAMAM-CPT-T7 entering cells after the A549 cells were co-incubated with Tf at a final concentration of 25 μM for 10 min and 2 h, or the A549 cells were co-incubated with Tf at a final concentration of 15 μM and 35 μM for 1 h.

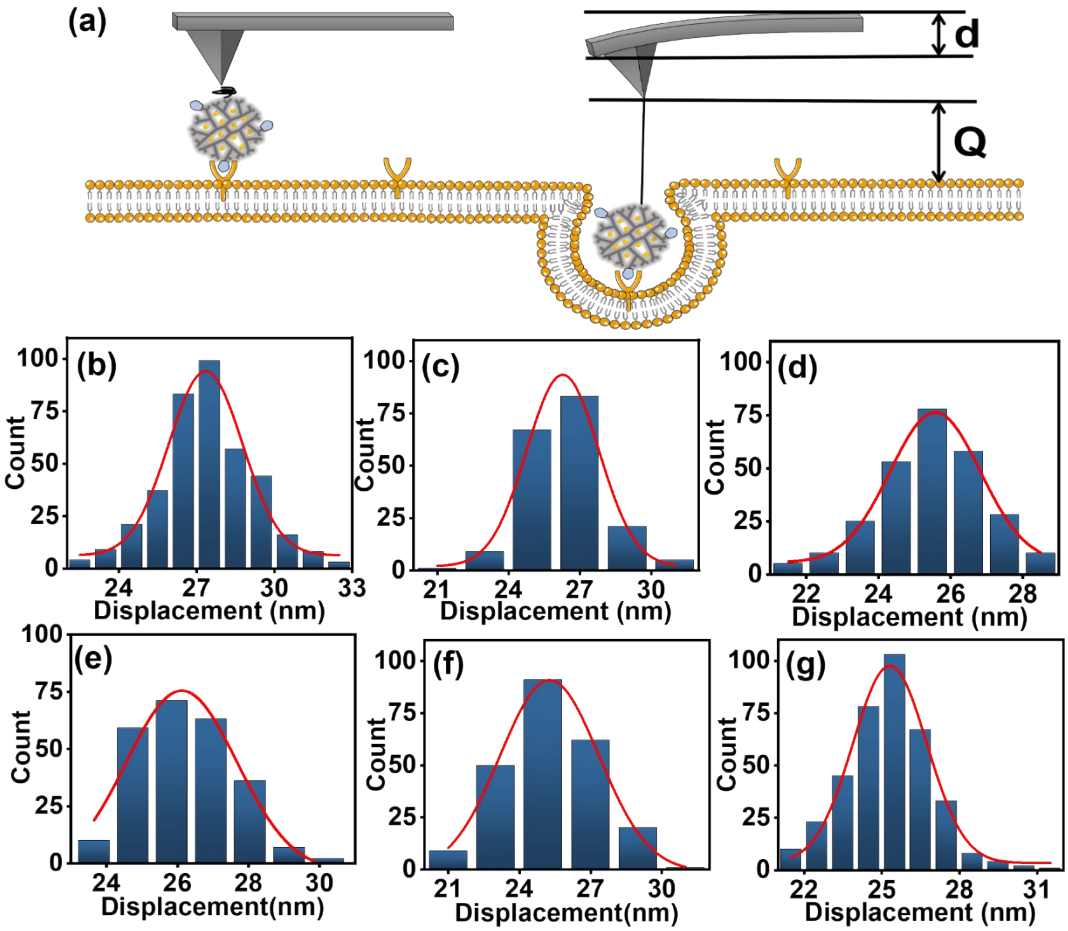

Fig. S7 The displacement of nano-drug entry cell. (a) The displacement diagram during nano-drug entry cell. (b) Displacement distribution of PAMAM-CPT-T7 entry A549 cell before co-incubation with free Tf, which is in the range of 22.17-32.71 nm with an average value of 27.40±1.78 nm. (n≈400). (c) Displacement distribution of PAMAM-CPT-T7 entry A549 cell after co-incubation with 25 μM Tf for 10 min, which is in the range of 21.33-31.61 nm with an average value of 26.45±1.66 nm. (n≈200). (d) Displacement distribution of PAMAM-CPT-T7 entry A549 cell after co-incubation with 25 μM Tf for 2 h, which is in the range of 21.46-28.79 nm with an average value of 25.48±1.46 nm. (n≈250) (e) Displacement distribution of PAMAM-CPT-T7 entry A549 cell after co-incubation with 25 μM Tf for 1 h, which is in the range of 24.04-30.57 nm with an average value of 26.24±1.33 nm. (n≈250). (f) Displacement distribution of PAMAM-CPT-T7 entry A549 cell after co-incubation with 15 μM Tf for 1 h, which is in the range of 21.40-30.12 nm with an average value of 25.35±1.83

nm. (n≈250). (g) Displacement distribution of PAMAM-CPT-T7 entry A549 cell after co-incubation with 35 μM Tf for 1 h, which is in the range of 21.46-31.93 nm with an average value of 25.26±1.65 nm. (n≈400).

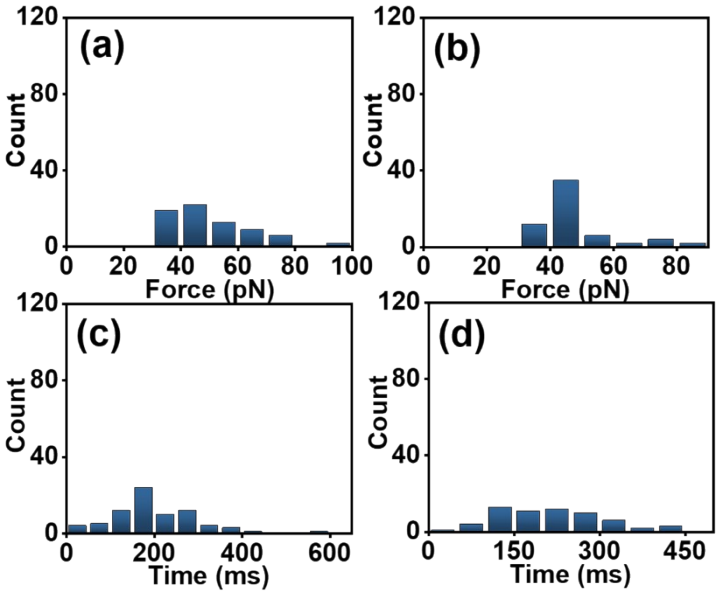

Fig. S8 Force and duration distribution of PAMAM-CPT-T7 entry Vero cell. (a, b) Force distribution for cellular uptake of PAMAM-CPT-T7 before and after coincubation with free Tf. (c, d) Duration distribution for cellular uptake of PAMAM-CPT-T7 before and after coincubation with free Tf.

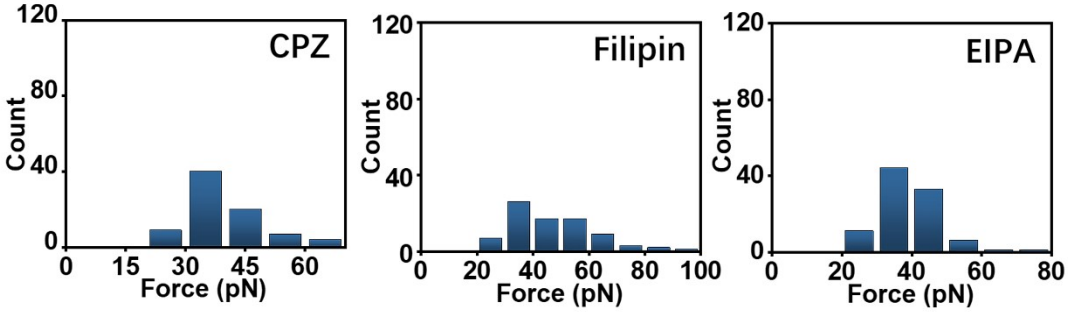

Fig. S9 Force distribution after blocking with CPZ, Filipin, and EIPA.

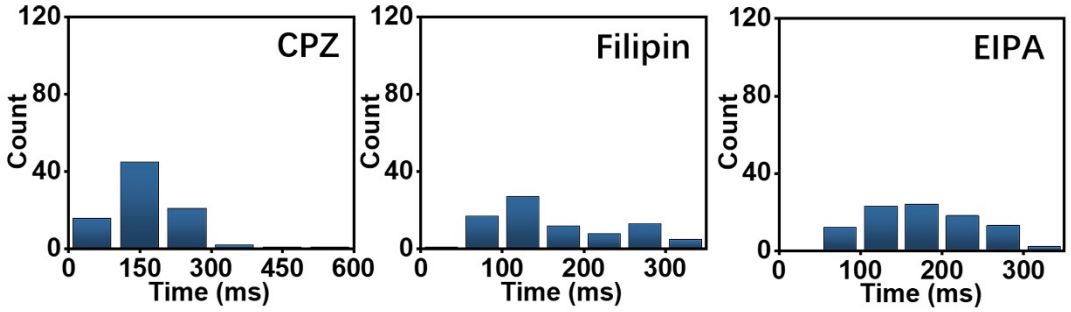

Fig. S10 Duration distribution after blocking with CPZ, Filipin, and EIPA.

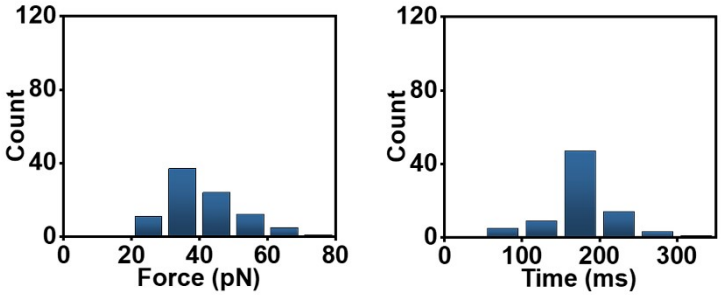

Fig. S11 Force and duration distribution of PAMAM-CPT-T7 entry cell after blocking with ferristatin.

References

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