Electronic Supporting Information (ESI)

Exploring the trans-membrane dynamic mechanism of single polyamidoamine nano-drug by "force tracing" technique

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Experimental section:

1. Encapsulation of Methotrexate (MTX)

MTX was purchased from the Aladdin Industrial Corporation. G8-Polyamindoamine dendrimers (G8-PAMAM, methanol solution, 40 wt %) were purchased from Weihai CY Dendrimer Technology Company.

The reaction required the amount of substance in according to the molar ration: n (MTX)/n (G8-PAMAM) = 50/1. So, MTX (1 mg, 0.0022 mmol) was dissolved in 1 mL deionized water at a concentration of 1 mg/mL, G8-PAMAM was added to the solution. Reaction system was stirred for 12 h at speed of 500 r/min away from light with nitrogen protection under room temperature[1]. After that, the products solution was filtrated through an ultrafiltration tube (10 K, PALL Corporation) to remove the free MTX.

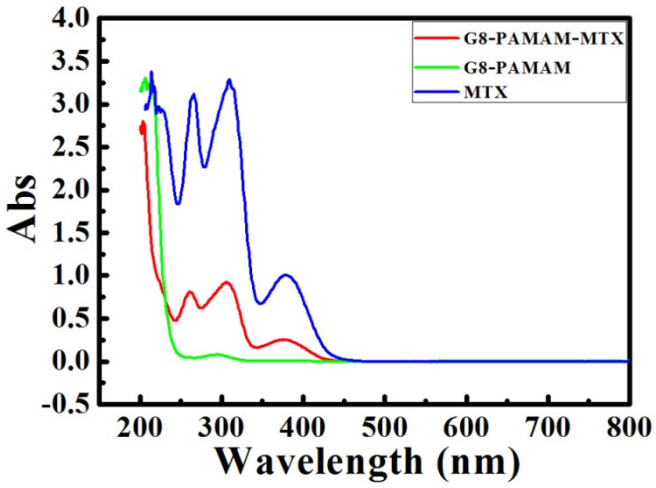

Fig. S1. UV-Vis Spectra of G8-PAMAM nanoparticles (green), MTX (blue), and G8-PAMAM-MTX nano-drug (red).

Thus-prepared G8-PAMAM-MTX nano-drugs were characterized by UV-Vis spectra (Varian Cary 50), the results are shown in Fig. S1. The UV-Vis absorption peaks of MTX at λ=370 nm, 302 nm, 265 nm, and 205 nm (blue). The UV-Vis absorption band of G8-PAMAM is from 200 nm to 310 nm, and the characteristic peak is λ=279 nm (green), these signals are consistent with the characteristics of PAMAM[2]. It is clearly shown that the UV-Vis spectra of G8-PAMAM-MTX nano-drug contain the characteristic peaks of MTX and G8-PAMAM. The results demonstrate that MTX molecules have been encapsulated into the G8-PAMAM internal cavity.

2. Conjugation of MTX with G4-PAMAM

EDC (1-(3-Dimethylaminopropyl)-3-ethylcarbodiimidehydrochloride) was purchased from TCI Company. HOBt (1-Hydroxybenzotriazole) and DIPEA (N-Ethyldiisopropylamine) were purchased from J&K Scientific.

First of all, MTX (1mg, 0.0022 mmol) was dissolved in DMSO (1 mL), then EDC (0.63 mg, 0.0033 mmol) and HOBt (0.5 mg, 0.0033 mmol) were added into the DMSO solution and stirred for 0.5 h, subsequently the G4-PAMAM (0.88×10^{-4} mmol) and DIPEA (0.56 mg, 0.0044 mmol) were added to the DMSO solution. The reaction system was stirred with the speed of 500 r/min at 35 ℃ for 72 h in the dark under nitrogen protection[3, 4]. After reaction the solvent was removed by lyophilization, the dried product was dissolved in deionized water and purified by sephadex G-25 (GE Healthcare), obtaining the target product of G4-PAMAM-MTX nano-drug.

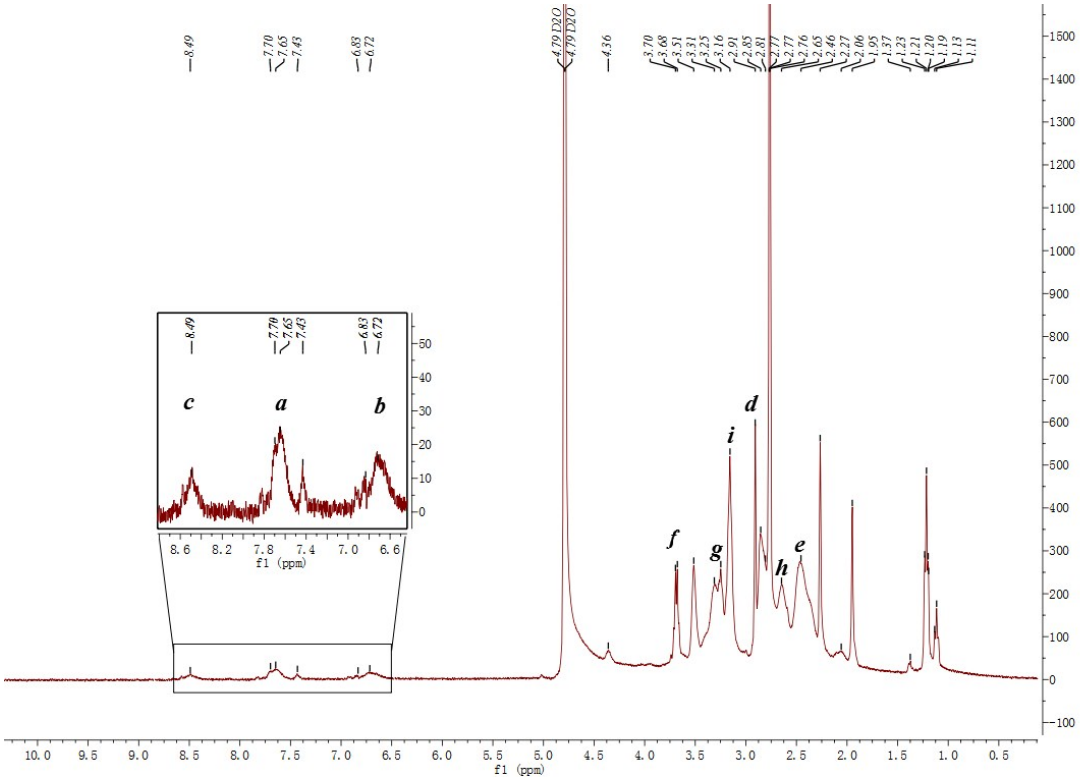

Fig. S2. The ^{1}H-NMR spectroscopy of G4-PAMAM-MTX nano-drugs.

G4-PAMAM-MTX nano-drugs were characterized by ^{1}H-NMR spectroscopy (Bruker 400 MHZ), which was performed using D_2O as solvent at 400 MHz, as shown in Fig. S2, the ^{1}H-NMR spectrum of the G4-PAMAM-MTX nano-drug contains characteristic signals of both MTX and G4-PAMAM. H***a***-H***d***, including δ=7.65-7.70 ppm (d, H***a*** of *p*-benzene), δ=6.72-6.84 ppm (d, H***b*** of *P*-benzene), δ=8.49 ppm (s, H***c*** of 2-pyrazine), and δ=2.91 ppm (s, H***d*** of methyl) are the characteristic signals of MTX[5], and the molecular structure details of the H***a***-H***d*** is shown in Fig. S3. The G4-PAMAM repeating unit structure characteristic signals are represented as H***e***-H***i***: δ=2.27 ppm (br, NCH_2CH_2N), δ=2.46 ppm (br, H***e***), δ=2.65 ppm (br, H***h***), δ=3.16-3.31 ppm (m, H***g***, H***i***), and δ=3.65 ppm (m, H***f***), the detail molecule structures are shown in Fig. S3. Therefore, the results indicated that the MTX had interacted with the G4-PAMAM, and the G4-PAMAM-MTX nano-drugs were formed by conjugation[4, 6].

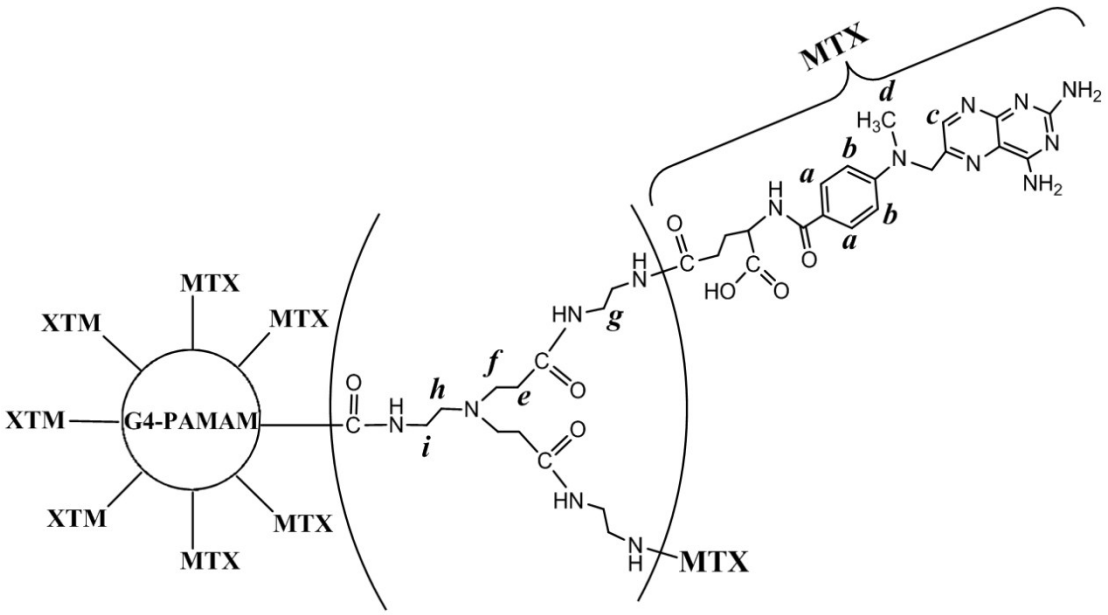

Fig. S3. The chemical structure of G4-PAMAM-MTX nano-drug. The bracket represents the terminal structure of the G4-PAMAM repeating unit, and ***a-i*** indicate different positions of the H.

3. Cell culture:

African green monkey kidney (Vero) cells were purchased from the Shanghai Institutes of Biological Sciences. The cells were maintained in a 5% CO_2 and 95% air environment at 37 ℃, supplemented with Minimum Eagle Medium (MEM, GIBCO) with 10% Fetal Bovine Serum (FBS, GIBCO), penicillin (100 international units mL^{-1}), and streptomycin (100 μg mL^{-1}). The cells were sub-cultured for 2 or 3 days when cells covered 75% of Petri dish area. The adherent Vero cells were washed with PBS (phosphate buffer solution) for three times and serum-free medium one time to remove cell debris and unattached cells before using in the "force tracing" experiments.

4. Modification of AFM probes with G8-PAMAM-MTX/G4-PAMAM-MTX NPs:

Firstly, AFM tips (MSCT, Veeco, Santa Barbara, CA) were soaking in freshly prepared Piranha solution (H_2SO_4:30% H_2O=3:1, v/v) for 1 h. Then the tips were purged with O_3 flow for 20 min to further remove the organic pollution on the tips after sequential cleaning with water, ethanol, and dried by argon flow. Subsequently, tips were modified with 3-aminopropyltriethoxysilane (APTES) using vapor phase deposition method for 80 min as previously described[7], combined with the heterobifunctional PEG (benzaldehyde-PEG76-NHS, FW~3962, SensoPath Technologies, Bozeman, 1 $mgmL^{-1}$) in methylbenzene with 0.5% triethylamine (v/v) for 2 h. Then, the AFM tips were conjugated with G8-PAMAM-MTX or G4-

PAMAM-MTX nano-drugs in PBS solution with the presence of 0.01 M $NaCNBH_3$ for 1 h after drying by argon flow. To deactivate the unreacted aldehyde groups, 5 μL of 1 M ethanolamine was added into the working solution and reacted for 10 min. Finally, the tips were washed by PBS for three times and stored at 4 ℃ until use.

5. The technology of single molecular "force tracing" based on AFM:

The experiments were carried out at 37 ℃ by temperature control 325 (Agilent Technologies, Chandler, AZ). After the Petri dish was washed by PBS, 2 mL MEM were added into Petri dish. The sensitivity of photo-detector and the spring constant of AFM tip were set right as reported previously[8]. To perform the "force tracing" test, AFM tip modified with PAMAM-MTX nano-drug was rightly located onto the dense Vero cells monitoring by CCD camera, which could ensure that the experiments were accomplished on the surface of cell membranes, as shown in Fig. S4.

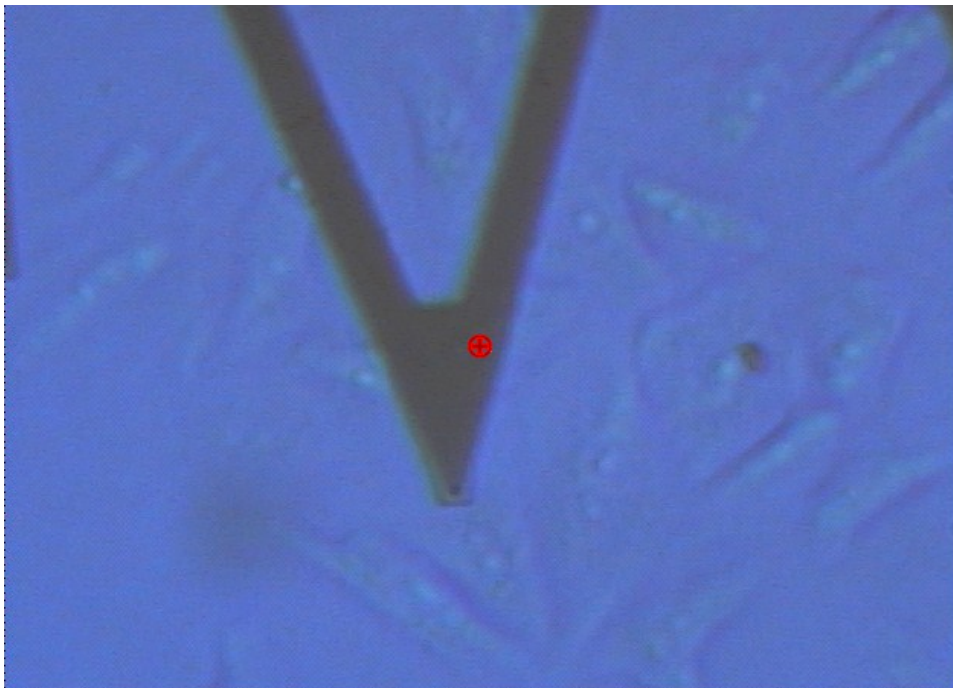

Fig. S4. AFM tip was rightly located onto the dense Vero cell membranes.

In order to find out the contact point between nano-drug attached on AFM tip and living cell membranes, the AFM tip was engaged to the cell surface and performed force-distance measurements. The contact point is the intersection of flat part and slope in the force-distance curve. Subsequently, we moved the AFM tip to the contact point slowly by opening the

proportional-integral (PI) control system (P = 0.001; I = 0.001; the error signal between the set point and the deflection of the cantilever is 2.0 V). Then the feedback system was turned off when the nano-drug attached on AFM tip was just on the contact point. The cantilever would bend downwards once the G8-PAMAM-MTX/G4-PAMAM-MTX was uptake by Vero cells, and the deflection of the cantilever was recorded by a 16-bit DA/AD card (PCI-6361e, National Instruments), which was controlled by LabVIEW software. During the experiments, thousands of force curves were collected at different position of different cells and analyzed by MATLAB 7.9 (Math Works Inc, Natick, MA, USA).

Comparison of endocytosis forces of G4-PAMAM-MTX and G8-PAMAM-MTX nano-drugs is shown in Fig. S5. The difference between the two sets of data can be demonstrated, even it is not distinct due to the conjugated MTX molecules on G4-PAMAM dendrimer terminal (enlarging the particle size).

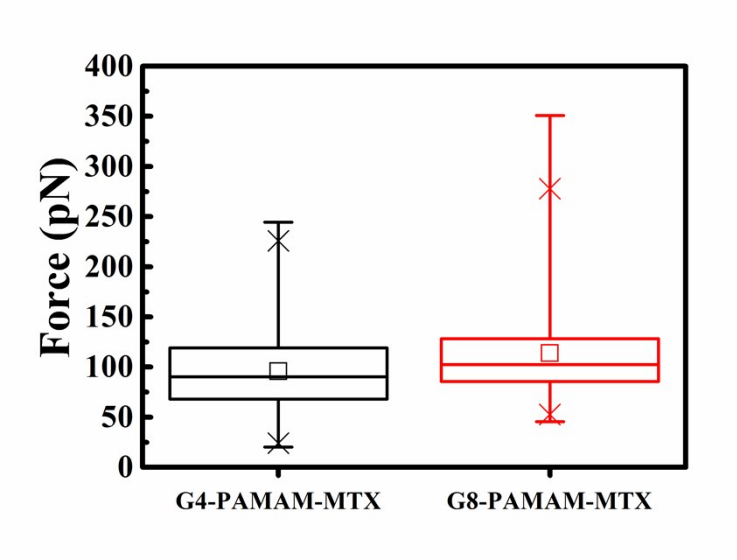

Fig. S5. The box plot graph of endocytosis forces for G4-PAMAM-MTX (black) and G8-PAMAM-MTX (red) nano-drug. The boxes mean the main distribution (50%) of force value with the vertical lines through boxes representing the overall distribution, and the horizontal lines in every box represent the median, the meaning of □ is the mean value, and × is the 1% to 99% data range.

6. Block and control experiments:

In block experiments, Vero cells were pre-incubated with cytochalasin B (final concentration 2 μg mL^{-1}) for 20 min, chlorpromazine (CPZ, ultimate concentration 10 μg mL^{-1}) for 30 min,

filipin (final concentration 5 μg mL^{-1}) for 30 min, methyl-β-cyclodextrin (M-β-CD, 5 mM) for 10 min, 5-(N-Ethyl-N-isopropyl) Amiloride (EIPA) (60 μM) for 1 h, and nystatin (0.03mM) for 30 min, respectively. In control experiments, without any modification tip and tip modified with PEG linker were used to perform "force tracing" experiments on normal cells, the results showed that the force signals really resulted from the cellular uptake of G8-PAMAM-MTX NPs rather than other events.

References

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