**Supporting Information for:**

# One-step Gene Delivery into Cytoplasm in a Fusion-Dependent Manner

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## Materials and mehtods

Materials.

1,2-dioleoyl-snglycero-3-phosphocholine (DOPC, Avanti Polar Lipids), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG) purchased form Xi"an ruixi Biological Technology Co.,Ltd. Fluorescein isothiocyanate (FITC), Octadecyl rhodamine B (R18), Dicyclohexylcarbodiimide (DCC), poly-l-lysine (PLL, Mn=27400), and 4-dimethylaminopyridine (DMAP) purchased from Sigma-Aldrich.

Dulbecco's modified Eagle's medium (DMEM), Opti-MEM and bovine serum (FBS) were purchased from Invitrogen Corporation (Carlsbad, CA). Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI), and 3,3-dioctadecyloxacarbocyanine, perchlorate (DiO) were purchased from Sigma-Aldrich. LysoTracker Red was obtained from Invitrogen. EGFP-N1 pDNA encoding EGFP (Clontech, Palo Alto, CA, USA), pGL3.0 (Promega, Madison, WI, USA) and RFP (as a gift from Beijing University) were amplified in the Escherichia coliDH-5a, isolated

and purified by PureYield_ Plasmid Maxiprep System (Promega, Madison, WI, USA). The absorbance at the wavelength of 260 and 280 nm was measured by UV Spectrophotometer (Lambda 950 UV/VIS/NIR spectrophotometer, Perkin Elmer, MA, USA) to confirm the purity and concentration of plasmid DNA

Characterization

The size and size distribution (PDI) of micelles were performed using a laser particle size analyzer (zetasizer Nano, Malvern, UK) at a wavelength of 633 nm with a constant angle of 173$^{o}$. The diameter of micelles was received from the average of three measurement results. Morphologies of micelles were observed under a Hitachi H600 transmission electron microscopy (TEM) system at operated voltage of 75 kV. For TEM measurement, the sample was prepared by adding a drop of micelles solution onto the copper grid, and then the sample was air-dried and measured at room temperature.

Synthesis of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-Arg-Arg-Arg-Arg (DSPE-4A)

The DSPE-4A was prepared by standard Fmocsolid-phase peptide synthesis (SPPS) using CLEAR Amide Resin and the corresponding N-Fmoc-protected amino acids with side chains properly protected. 20% piperidine in anhydrous N, N'-dimethylformamide (DMF) was used during the deprotection of the Fmoc group, then the next Fmoc-protected amino acid was coupled to the free amino group using O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronilum hexafluorophosphate (HATU) as the coupling reagent. To

couple DSPE to the peptide (Fmoc-Lys(Mtt)-Arg-Arg-Arg-Arg-NH2 ), 3% trifluoroacetic acid (TFA) in dichloromethane (DCM) was used during the deprotection of the Mtt group and then oxalic acid was coupled to the free amino group of Lys using HATU as the coupling reagent. In the last coupling step, DSPE was used to cap the side chain carboxyl group of the oxalic acid in the peptide (Fmoc-Lys(PA)-Arg-Arg-Arg-Arg-NH-OA ). After the last coupling step, excess reagents were removed by washing five times using DMF, followed by washing five times using dichloromethane (DCM). The peptide derivatives were cleaved from the resin by reagent A (containing TFA, 1,2-ethanedithiol, thioanisole andanisole) and the mixture was stirred at room temperature, filtered and distilled under reduced pressure. The resulting precipitate was dissolved in acetonitrile for HPLC purification.

Preparation of F-MEND

Procedure for the preparation of DNA loaded F-MEND is comprised of three steps as follows (i) DNA condensation with poly-L-lysine (PLL) (PLL/DNA): DNA and PLL were dissolved with 10 mM HEPES buffer (pH 7.4). To condense the plasmid DNA, the DNA solution (0.1 mg/ml) was mixed with a PLL solution (0.1 mg/ml) under vortexing at room temperature. The DNA content of suspension of PLL/DNA complex (DPC) prepared at a nitrogen/phosphate (N/P) ratio of 2.5 was 0.05 mg/ml. (ii) Hydration of the lipid film: After the condensation of DNA, 0.25 ml of DPC suspension was added to the lipid film, formed by the evaporation of a chloroform solution of 137.5 nmol lipid, followed by incubation for 10 min to hydrate lipid. The

final concentration of lipid was 0.55 mM. (iii) Sonication for the packaging of the condensed DNA: To coat DPC with lipids, the glass tube was then sonicated for about 1 min in a bath-type sonicator (125 W, Branson Ultrasonics, Danbury, CT).

Agarose gel electrophoresis

To assess DNA-binding ability of the NPs, agarose gel electrophoresis was used. The binary and ternary complexes were prepared as described above. Complexes (containing 0.5mg DNA) were mixed with 4mL loading buffer and loaded into a 0.8 wt % agarose gel containing 0.5 mg/mL ethidium bromide. Electrophoresis was run in 1×TAE buffer at 10 mA for 30 min. DNA retardation was analyzed using UV illuminator to show the band of the DNA.

Transfection in vitro

For transfection experiments, plasmid were used as model gene to evaluate the transfection efficiency of ternary complexes. Hela cells were seeded in the 24-well plate at a density of $5\times10^4$ cells/well and incubated in 10 % FBS of DMEM containing 100 U/mL penicillin, and 100 mg/mL streptomycin for 20 h. Then, the medium was removed and replaced by Opti-MEM before transfection.

Cell viability

The cytotoxicity of F-MEND and F-MEND/(PLL/DNA) complexes was evaluated by MTT assay. Hela cells were seeded at $5 \times 10^3$ cells per well in 96-well plates and subsequently treated with different amounts of F-MEND (10 μg, 20 μg, 40 μg for each well) in a plate and F-MEND/(PLL/DNA) (F-MEND :10 μg, 20 μg, 40 μg with 0.2 ug DNA) in another plate, respectively. Each sample was conducted in five

parallel groups. After 24 h incubation at 37 °C in 5% $CO_2$, the solution was replaced by 100 μL MTT (0.5 mg $mL^{-1}$) diluted by DMEM for each well and incubated for 4 h under the same conditions. Then the MTT solution was exchanged by 150 μL of DMSO for each well to dissolve the formazan crystals. After shaking for 30 seconds, the absorbance of each well at 570 nm was measured using an Infinite M200.

Table S1 The composition of F-MEND lipid

| F-MEND | Lipid composition (mg ) | | | | Size (nm) | Zeta |
|---|---|---|---|---|---|---|
| | DSPE-4A | DOPC | DSPE-PEG | Chol | | |
| 1 | 4 | 3 | 0.8 | 0.8 | 147 | 16 |
| 2 | 4 | 3 | 1.6 | 0.8 | 152 | 15 |
| 3 | 4 | 3 | 2.4 | 0.8 | 150 | 11 |
| 4 | 8 | 1.5 | 0.8 | 0.8 | 153 | 13 |
| 5 | 8 | 1.5 | 1.6 | 0.8 | 149 | 14 |
| 6 | 8 | 1.5 | 2.4 | 0.8 | 154 | 15 |

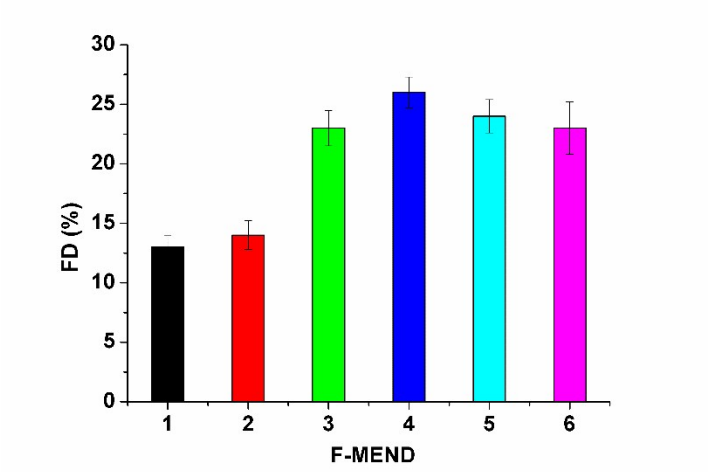


**Fig. S1** Membrane fusogenicities of liposomal formulations based on fluorescence dequenching (FD) of HeLa cells treated with liposomes loaded with self-quenching lipid dye R18. Results are presented as a percentage of R18 FD over the maximum fluorescence intensity of R18 added to the cells. Error bars represent ±SD (n=3).

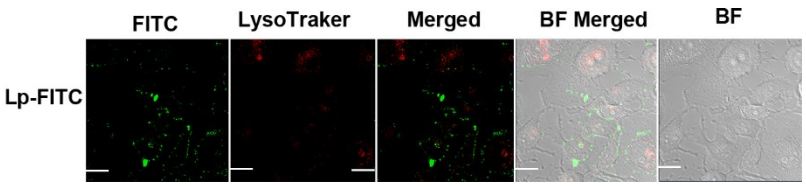


**Fig. S2** Confocal microscopic images of cancer cells treated with FITC labeled F-MEND lipid for 2 h. Scale bar indicates 20 μm.

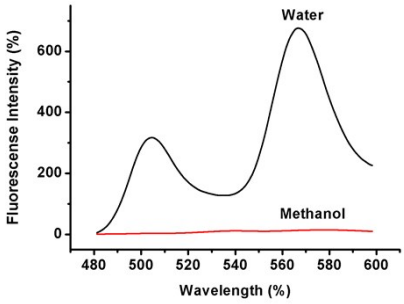


**Fig. S3** The fluorescence intensity of DiO and DiI in F-MEND.

The nanoparticles entry inside the cell through a process termed “endocytosis”. Endocytosis can also be classified into two broad categories, phagocytosis and pinocytosis. Phagocytosis is typically restricted to specialized mammalian cells (macrophages, neutrophils, monocytes and dendritic cells), whereas pinocytosis occurs in all cells. In the approach pinocytosis is classified as clathrin dependent endocytosis (also known as clathrin mediated endocytosis (CME)) and clathrin-independent endocytosis. The clathrin-independent pathways are further classified as caveolae-mediated endocytosis, clathrin and caveolae independent endocytosis and micropinocytosis. In the manuscript, chlorpromazine for clathrin mediated endocytosis, methyl-b-cyclodextran for cavelolae mediated endocytosis, cytochalasin D for caveolae and micropinocytosis mediated endocytosis, and chloroquine for the

disrupting endososmes and lysosomes and N-ethylmaleimide eliminating membrane protein functions by alkylating sulfhydryl. As shown in supporting information Fig S4, almost every endocytotic inhibitor exerted a slight effect on the internalization of F-MEND lipid, indicating that the cellular internalization of F-MEND lipid involves nonspecific extensive endocysibe endocytotic pathways[1-3].

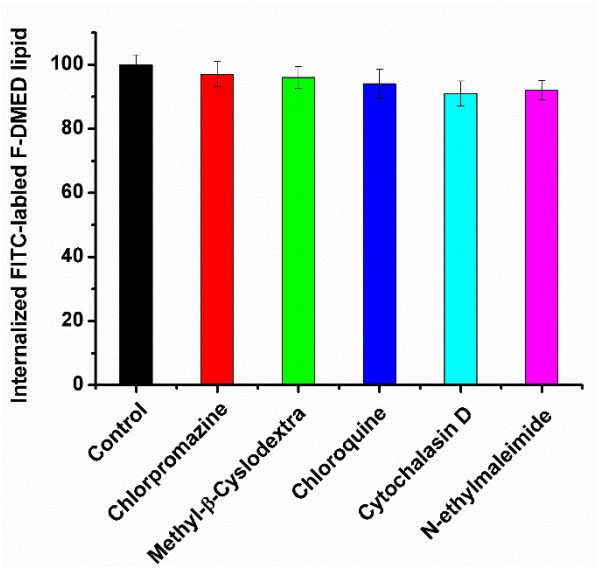


**Fig. S4** Effects of endocytotic inhibitors on cellular internalization of F-MEND lipid. Hela cells were pre-treated with various inhibitors, rinsed, and then incubated with 200 μM FITC-labeled F-MEND lipid for 2 h. Internalized F-MEND lipid were detected by flow cytometry after extracting cell-membrane associated F-MEND lipid using 2% BSA for 20 min at 4 °C. Cells not treated with inhibitors were used as controls. Error bars represent ±SD (n=3).

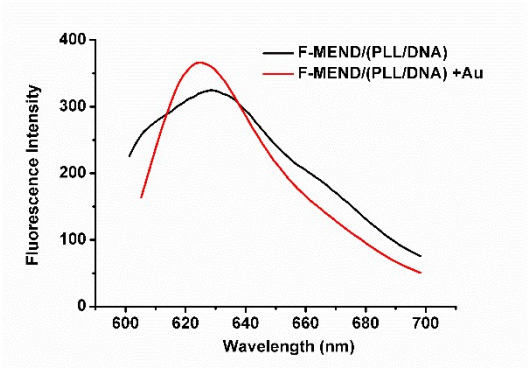


**Fig. S5** The fluorescence spectra of F-MEND/(PLL/DNA) after addition of different volumes of the AuNPs solution (1 mg/ml).

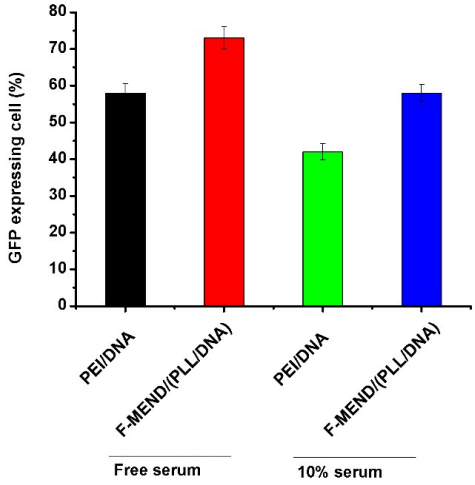


**Fig. S6** The transfection efficiencies of F-MEND/(PLL/DNA) were determined by flow cytometer at 48 h in Hela cells. Error bars represent ±SD (n=3).

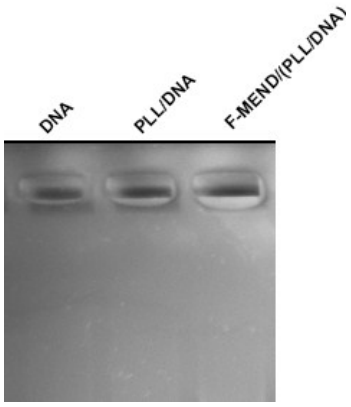


**Fig. S7** Effect of DNase I on pDNA, PLL/DNA and F-MEND/(PLL/DNA) with different incubation times was estimated by agarose gel electrophoresis.

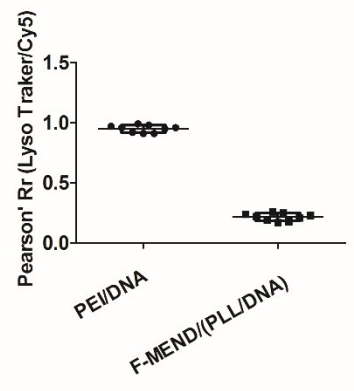


**Fig. S8** Statistics of co-located ratio of Cy5-DNA with lysosome was carried by counting pixels of red (signals of Cy5) and green (signals of lysosome).

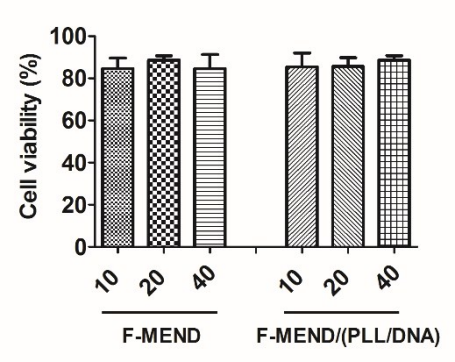


**Fig. S9** The in vitro cytotoxicity of F-MEND and F-MEND/(PLL/DNA) (F-MEND: 10 μg, 20 μg, 40 μg, and F-MEND/(PLL/DNA): 10 μg, 20 μg, 40 μg with 0.5 μg DNA) were evaluated by MTT assay in Hela cells. Error bars represent ±SD (n=3).

Table S2 Stability of F-MEND lipid

| F-MEND | Size (nm)[a] | PDI [a] | Size (nm)[b] | PDI[b] |
|---|---|---|---|---|
| 1 | 148 ± 9 | 0.11 | 155 ± 13 | 0.12 |
| 2 | 153 ± 10 | 0.10 | 157 ± 12 | 0.12 |
| 3 | 149 ± 9 | 0.10 | 156 ± 11 | 0.11 |
| 4 | 151 ± 11 | 0.11 | 158 ± 10 | 0.13 |

[a]Determined using Laser particle size analyzer at 25°C in PBS (10 mM, pH 7.4).
[b]Determined using Laser particle size analyzer at 25°C in PBS (10 mM, pH 7.4) containing 10% (v/v) FBS .

**References**


1. I. A. Khalil, K. Kogure, H. Akita and H. Harashima, *Pharmacological reviews*, 2006, **58**, 32-45.
2. G. Sahay, D. Y. Alakhova and A. V. Kabanov, *Journal of controlled release*, 2010, **145**, 182-195.
3. J. Wang, Y. Wang and W. Liang, *Journal of controlled release*, 2012, **160**, 637-651.