Supporting information

Direct cytosol delivery of mRNA by micron-sized co-assembly with designer oligopeptides

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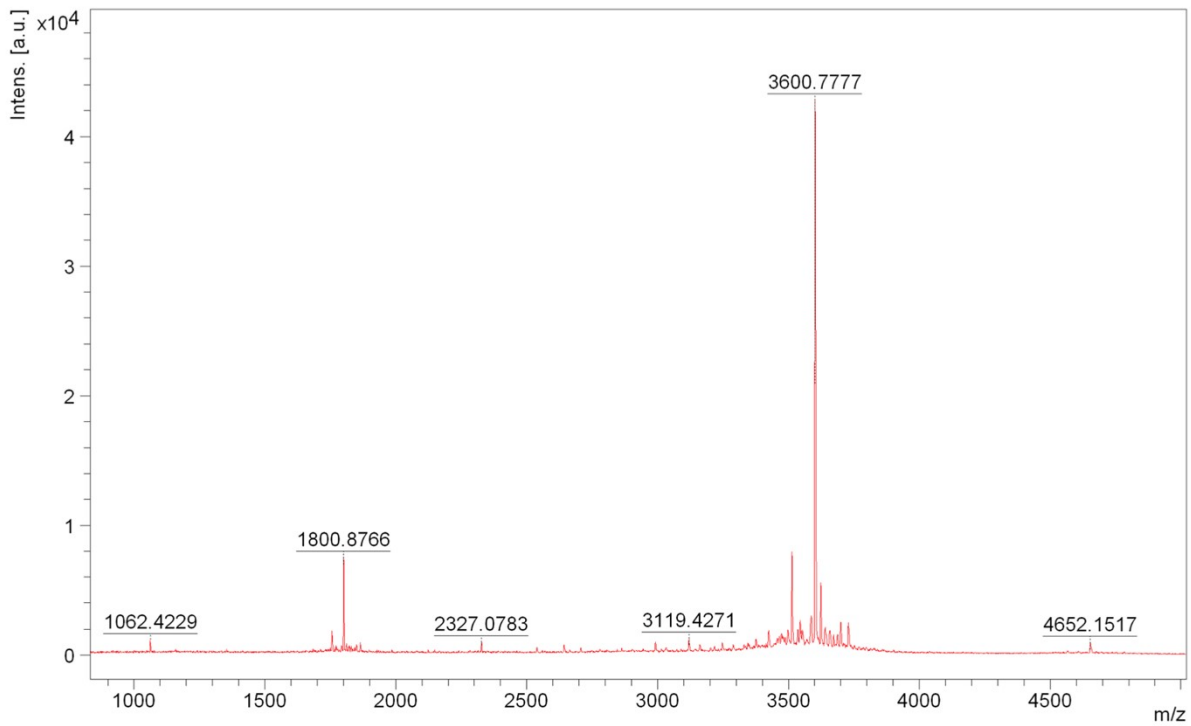


Figure S0. MALDI-TOF spectrum of pepMAX, with observed mass, 3600.7 [calculated for $(M+H)^+$, 3601.4].

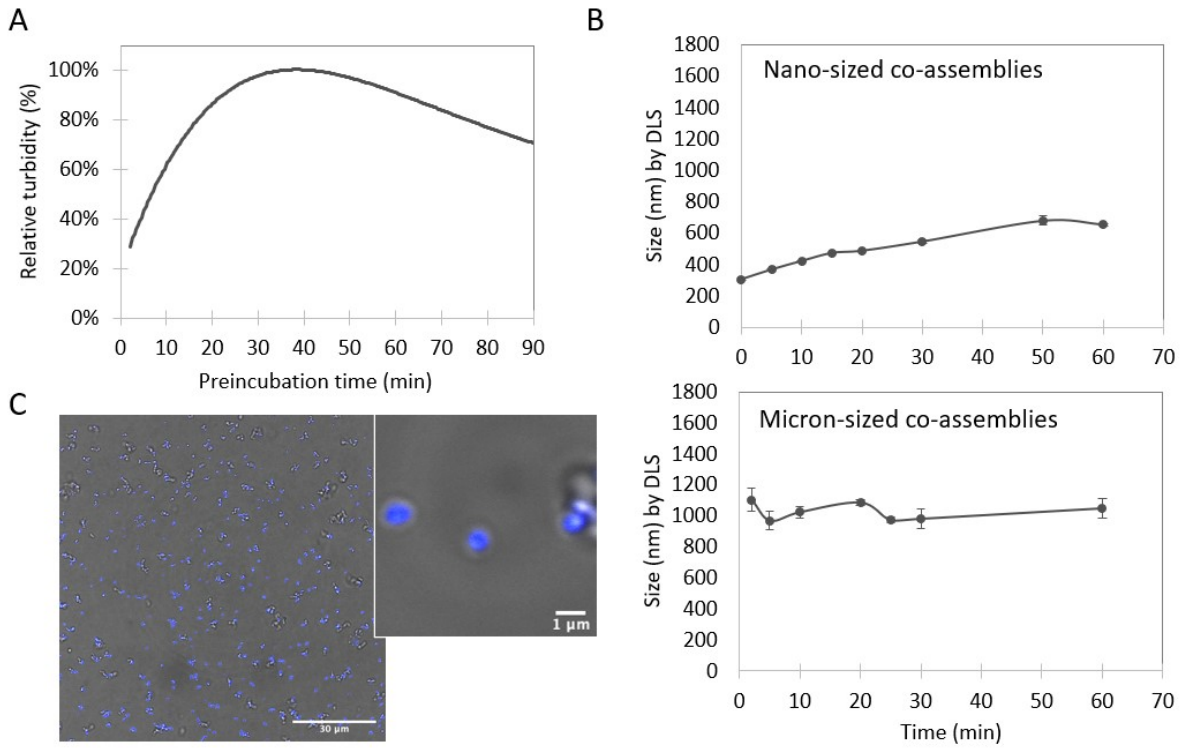


Figure S1. (A) Relative turbidity measurements of co-assemblies pre-incubated with 250mM NaCl for different time intervals. (B) Hydrodynamic size of nano-sized/micron-sized co-assemblies formed without/with 250mM NaCl for 30min. Then, the samples were diluted 40-fold in DMEM medium and observed for 60 minutes. (Data shown are mean ± SD, n ≥ 3) (C) Confocal microscope images of the co-assemblies (pre-incubated with 250mM NaCl for 30min), with mRNA labeled by Cy5 (blue). (N/P=4, 0.1mg/ml mRNA)

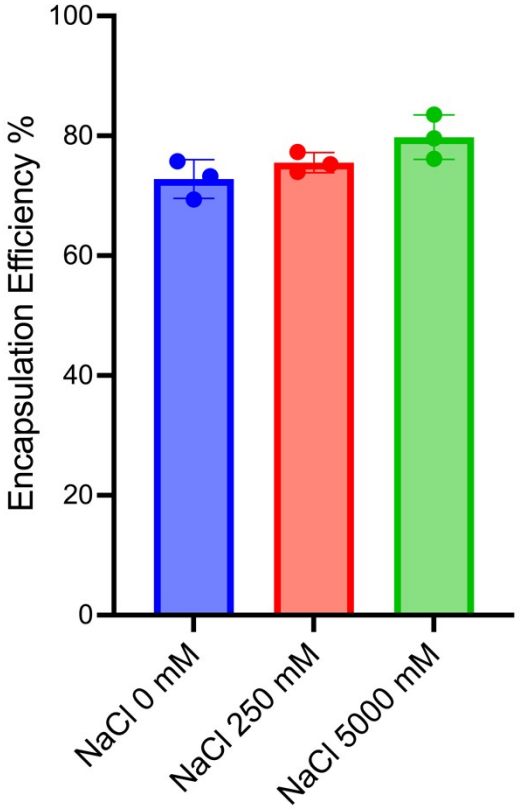


Figure S2. RiboGreen assay results indicating the encapsulation efficiency of mRNA in PepMAX2 co-assemblies were evaluated at three different NaCl concentrations: 0 mM, 250 mM, and 5000 mM. (n=3)

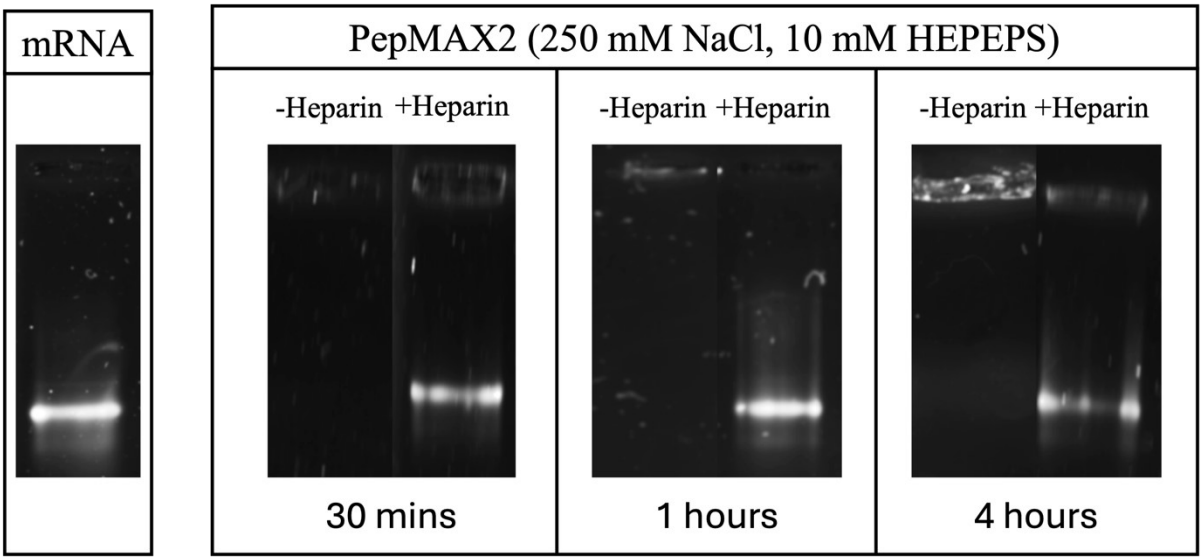


Figure S3. Gel electrophoresis analysis of mRNA release from microparticles. The gel shows the presence of mRNA bands at various time points (30 min, 1 hour, and 4 hours) in the heparin release medium, indicating the successful release of mRNA. No bands were observed in the HEPES condition, suggesting that HEPES does not facilitate mRNA release under the tested conditions. Each lane contains 100 ng of mRNA. (n=3)

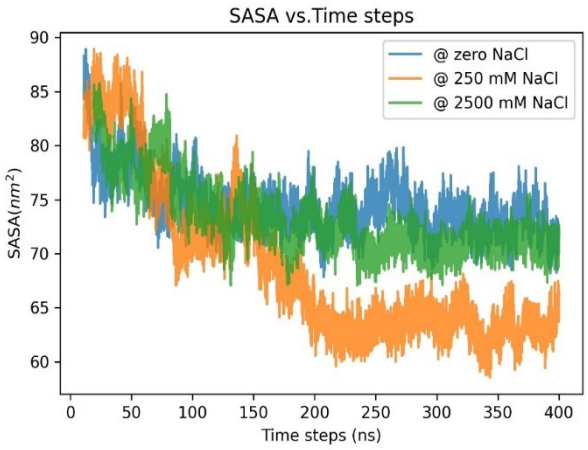


Figure S4. Solvent accessible surface area (SASA) as a function of time estimated using 400ns all atomistic MD simulation trajectories performed at 298 K temperature at three different salt conditions (0 mM (blue),250 mM (orange) and 2500 mM (green)) (A). SASA evolution for PepMAX2 and model mRNA at three different salt concentrations.

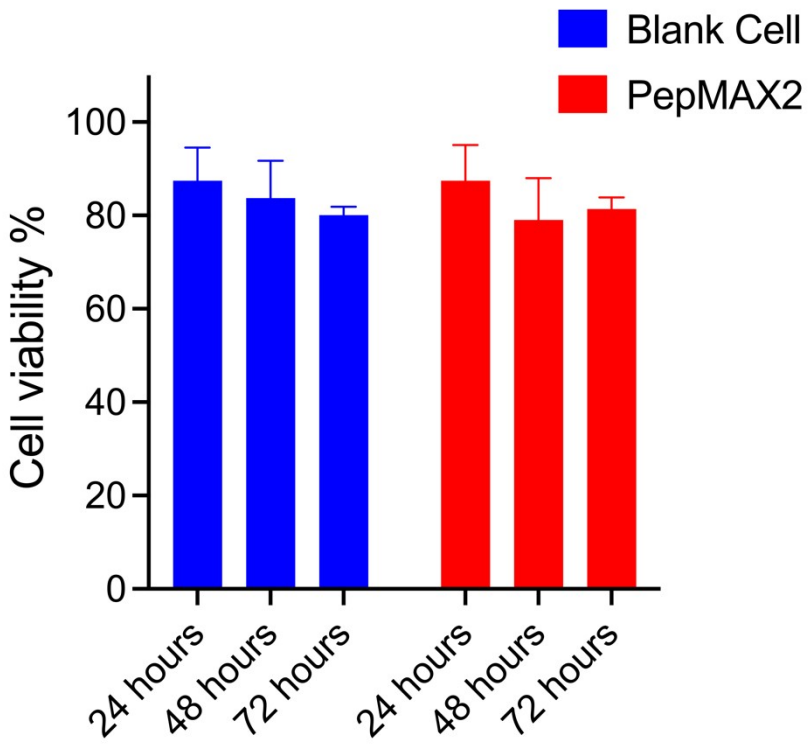


Figure S5. MTT assay was conducted on HeLa cells using PepMAX2 under optimal conditions (250 mM salt for 30 minutes) at various time points post-transfection, specifically at 24, 48, and 72 hours. The positive control consisted of untransfected HeLa cells in Opti-MEM/2.5% FBS, while a negative control of blank medium and DMSO was included to eliminate background signals.

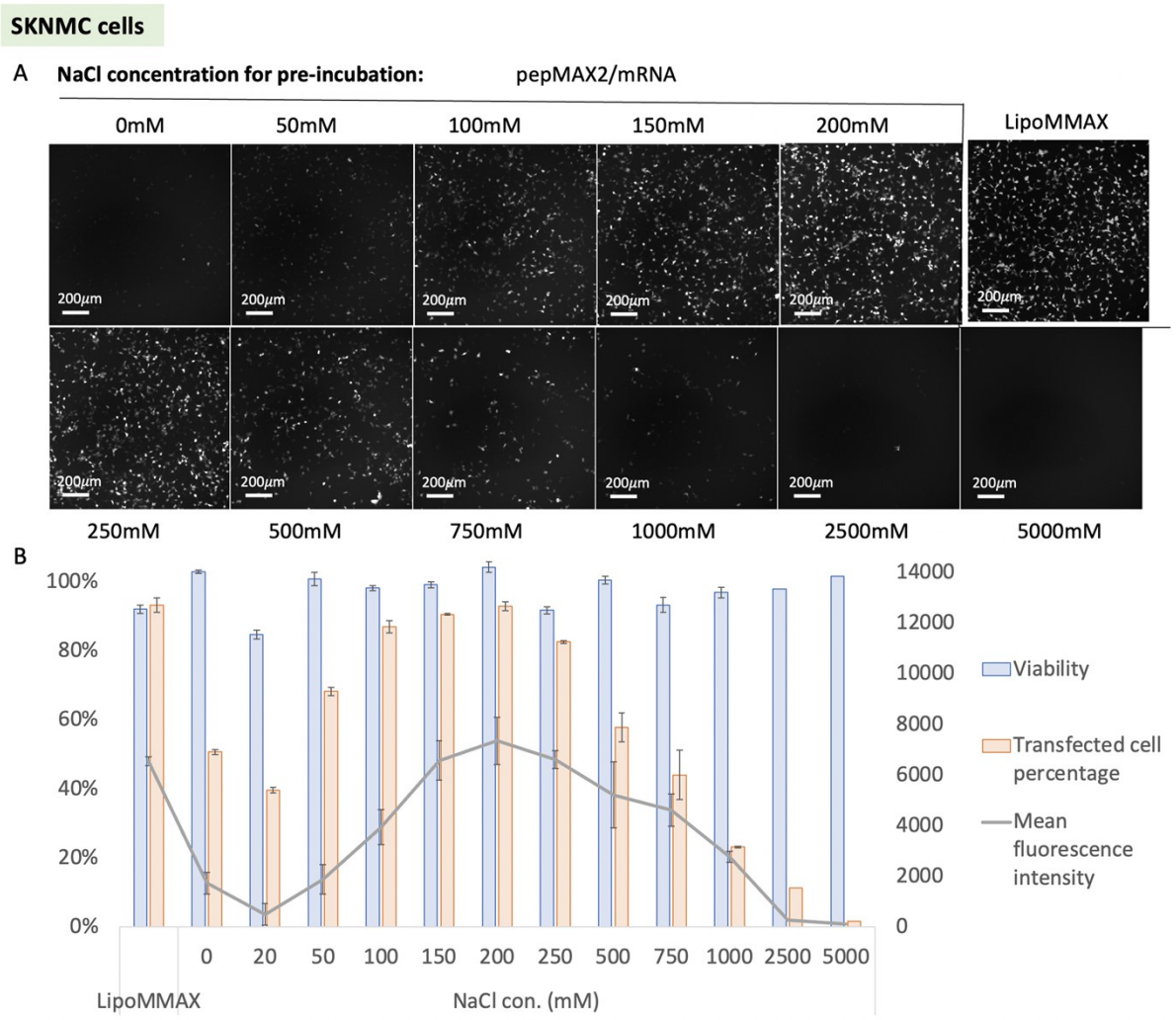


Figure S6. Transfection efficacy of pepMAX2/mRNA on SKNMC cells. Similarly, co-assemblies were pre-incubated for 30min in different concentrated NaCl solution. The N/P ratio were kept the same at 4, with peptide concentration at 2.5μM for 100ng loading. The transfection efficiency was observed by (A) fluorescent microscope and quantified by (B) flow cytometry, with cell viability, percentage of cells transfected and mean fluorescence intensity (MFI) of the positive cells. LipoMMAX served as a control with the same mRNA loading as in the co-assemblies. (Data shown are mean ± SD, n ≥ 3)

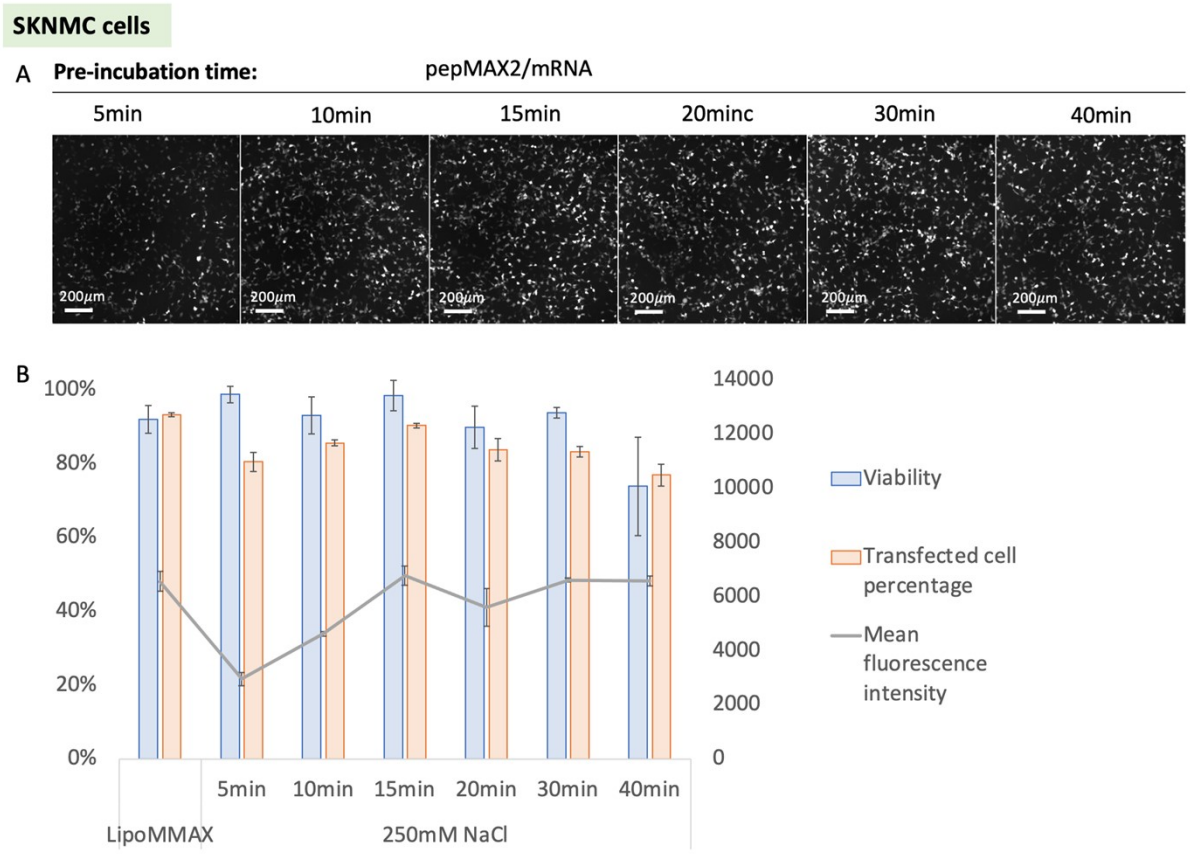
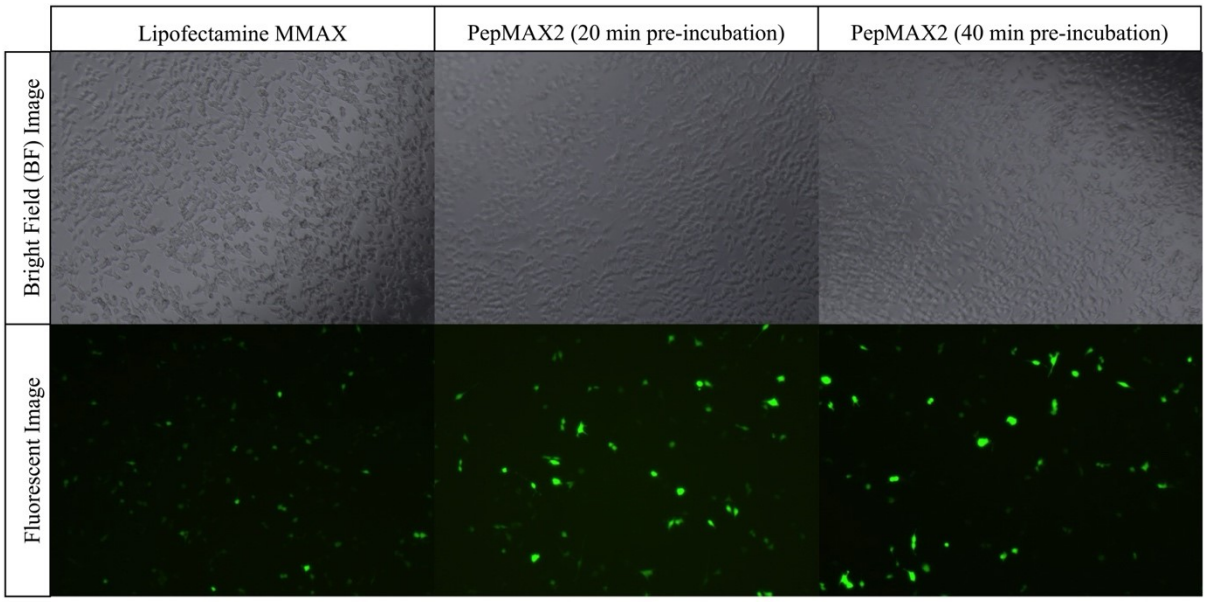


Figure S7. Fluorescent microscope images of SKNMC cells transfected with co-assemblies initially formed in 250mM NaCl solution for different times. Transfection efficiency of co-assemblies quantified by flow cytometry, with cell viability, percentage of cells transfected and mean fluorescence intensity of the positive cells. LipoMMAX served as a control with the same mRNA loading as 80ng/100μl in co-assemblies. (Data shown are mean ± SD, n ≥ 3)



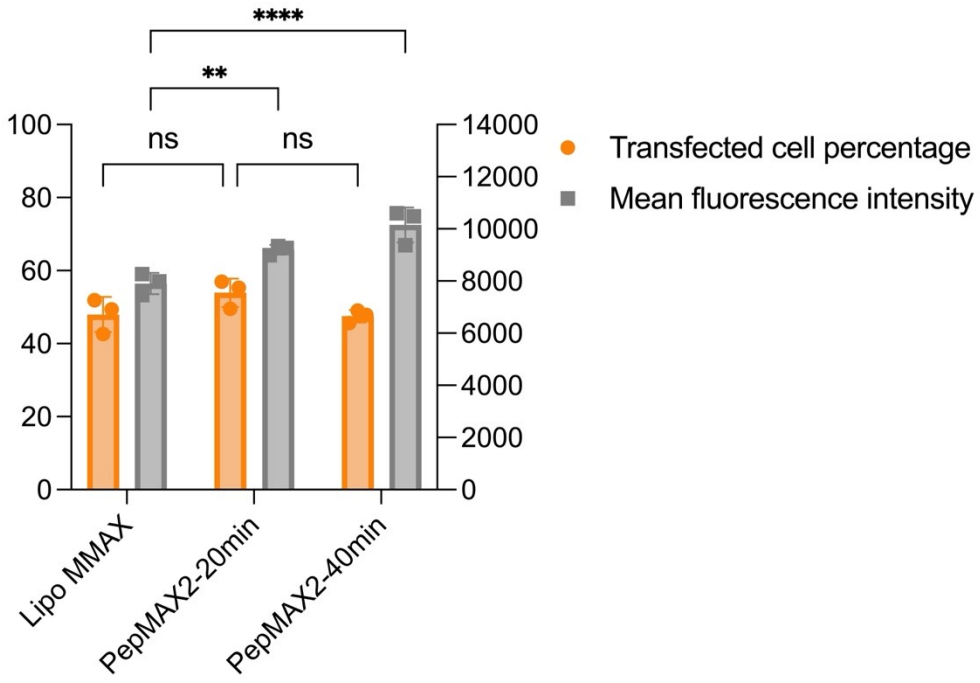


Figure S8. Bright-field and fluorescent microscope images of PC12 cells transfected with PepMAX2-mRNA assemblies formed in a 250 mM NaCl solution are presented in the upper figure, with pre-incubation times of 20 minutes and 40 minutes. LipoMMAX was used as a control, maintaining the same mRNA loading of 80 ng/100 µl in the co-assemblies. The bar plot below quantifies the data obtained from fluorescent imaging, analyzed using ImageJ software. A blank cell was used as a negative control to eliminate background interference, allowing for the overlay of green positive cells and measurement of fluorescent intensity by setting a threshold for each region of interest (ROI). A minimum of three frames were analyzed

for each image (Created with BioRender.com). P-values are indicated as follows: **** <0.0001, ** <0.0021, ns = not significant.

Table S1: Binding Energy estimations calculated using the MMPBSA method between PepMAX2-PepMAX2 and PepMAX2- model mRNA by the trajectories of 400 ns of all atomistic MD simulations. All energy values are in kJ/mol. Binding Energy is represented as a summation of four individual energy components (Van der Waal Energy, Electrostatic Energy, Polar solvation Energy, and Non-polar Energy)

Interaction Pair	PepMAX2-PepMAX2			PepMAX2- mRNA		
Salt Concentration (NaCl)	0 mM	250 mM	2500 mM	0 mM	250 mM	2500 mM
Van der Waal Energy	-614.32±121.84	-671.27±49.05	-610.94±75.15	-393.95±49.46	-408.75±68.14	-328.90±70.50
Electrostatic Energy	1626.60±285.17	1543.29±663.04	2548.78±354.61	-10041.20±770.52	-11918.55±665.20	-7928.09±580.74
Polar solvation Energy	473.62±156.96	373.31±208.39	301.86±128.16	1876.43± 301.11	1843.09±363.82	1233.50±198.43
Non-polar Energy	-55.83±10.17	-55.95±9.19	-58.12±6.70	-44.64±4.06	-36.84±5.70	-44.36±5.42
Binding Energy	1430.07±235.31	1189.38±221.03	2181.57±265.68	-8603.36±438.02	-10521.05±433.72	-7167.85±450.43

Table S2: Aggregation propensity (AP) parameter, estimated for pepMAX2-model mRNA assembly using 400ns all atomistic MD simulation trajectories at three different salt concentrations.

The concentration of NaCl (mM)	AP
0	1.63
250	1.94
2500	1.64

Supporting video 1: **Dynamics of pepMAX2 molecules in the absence of salt**. The all-atomistic MD simulation of 400 ns NPT production run of two pepMAX2 molecules in the presence of water. (Water molecules were removed for visualization). Two pepMAX2 molecules are colored red and blue.

Supporting video 2: **Co-assembly of pepMAX2 and model mRNA at 250 mM salt condition.** The all-atomistic MD simulation of 400 ns NPT production run of pepMAX2 and model mRNA at 250 mM NaCl concentration. (Water molecules were removed for visualization). Color code: dark blue for Na^+, pale blue for Cl^-, red for pepMAX2, and yellow for mRNA.

Supporting video 3: **Salt ions on pepMAX2 molecules at 2500 mM salt condition**. The all-atomistic MD simulation of 400 ns NPT production run of pepMAX2 molecules at 2500 mM NaCl concentration. (Water molecules were removed for visualization). Color code: dark blue for Na^+, and pale blue for Cl^-, Two pepMAX2 molecules are colored red and yellow.

Supporting video 4: **Real-time tracking of sustained cellular uptake from 20-120min post-transfection.** We tracked the Cy5-labeled mRNA in HeLa cells and labelled the late endosome/lysosome with Lysotracker Red. Color code: red for Lysotracker Red, green for EGPF, and blue for Cy5 labelled mRNA. Scale bar: 30μm.

Supporting video 5: **Real-time tracking of mRNA release 120min post-transfection.** As shown from 120min to 160min, direct cytosol delivery of mRNA without endosomal entrapment was observed. Color code: red for Lysotracker Red, green for EGPF, and blue for Cy5 labeled mRNA. Scale bar: 30μm.