Supplementary Material

Transport of siRNA through Lipid Membranes driven by Nanosecond Electric Pulses: an Experimental and Computational Study

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METHODS

GUVs preparation

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (DOPE-Rhodamine) were purchased from Avanti Polar Lipids (Alabaster, AL). DOPC was dissolved in chloroform at a mass concentration of 0.5 mg/mL. The dye DOPE-Rhodamine was added at a 1% molar concentration and the solution was stored at -20°C.

The vesicles were prepared at 6°C using the electroformation protocol described by Angelova.[1] 15 µL of the lipid solution was deposited on the conducting side of two glass slides coated with indium tin oxide (Sigma, Saint Louis, MO). The slides were then kept under vacuum for two hours in a dessicator to remove all traces of organic solvent. A chamber was assembled with the slides spaced by a 1.5 mm silicone isolator (Sigma, Saint Louis, MO). The chamber was filled with a sucrose solution (240 mM sucrose, 1mM NaCl, 1mM KH_2PO_4/K_2HPO_4). The slides were connected to a function/arbitrary waveform generator (HP Agilent 33120A, Santa Clara, CA) and sinusoidal voltage of 25 mV peak to peak and 8 Hz was applied. The voltage was increased by 100 mV steps every 5 minutes, up to a value of 1225 mV and maintained under these conditions overnight. Finally, square-wave Ac field of the same amplitude was applied at 4 Hz for one hour in order to detach the GUVs from the slides.

siRNA

The fluorescently labeled siRNA (sense strand 5'r(GCUACGGGCAGCAGAACCC)d(TT)-FITC-3' and antisense strand 5'-(GGGUUCUGCUGCCCGUAGC)d(TG)-FITC-3') were purchased from Eurogentec (Seraing, Belgium). Each strand is labeled by a fluorescein isothiocyanate moiety. The siRNA solution was diluted in the pulsation buffer (260 mM glucose, 1mM NaCl, 1mM KH_2PO_4/K_2HPO_4) to a concentration of 500 nM.

Vesicle exposure to electric pulses

20 µL of the GUVs solution was mixed with 20 µL of siRNA solution and 160 µL of pulsation buffer. A control solution was also prepared with 20 µL of the GUVs solution and 180 µL of pulsation buffer. These preparations were transferred in conventional electroporation cuvettes (STD, Dutcher, Issy les Moulineaux, France). The distance between the two planar electrodes of the cuvette is d =0.1 cm. A single 10 ns pulse was then applied to the cuvette/ A commercial generator purchased from FID (FID GmbH, Model FPG 10-ISM10, Burbach, Germany) with an output impedance of 50 Ω was used. It generates trapezoidal monopolar pulses with a full-width at half maximum of 10 ns. The output voltage magnitude applied were U = 2 kV, U = 3.2 kV and U = 6kV. This set-up was previously described.[2]

Confocal microscopy

After the pulse delivery, vesicles were removed from the electroporation cuvette and deposited inside a chamber consisting of a glass coverslip stuck onto a glass slide with heated parafilm. All images were obtained with an inverted confocal microscope (Zeiss LSM510 ; Carl Zeiss, Jena, Germany) equipped with an objective Plan Apochromat 63x/ ON 1.4. For the red channel (Rhodamine-labeled vesicles), the excitation was fixed at 543 nm and the emission filter was a 560 nm long pass. For the green channel (FITC-labeled siRNA), the excitation wavelength was 488 nm and the emission filter was a 500-530 band pass.

Image and data processing

The fluorescence intensity was measured on the confocal images with Image J (National Institute of Health, Bethesda, MD). For each vesicle, the mean fluorescence intensity was measured within a rectangular region covering the center of the vesicle.

MD Simulations procedures

System: The complete simulation system consists of a solvated membrane together with a 22 base pair siRNA molecule. The membrane model used for this study is an equilibrated fully hydrated palmitoyl-oleoyl-phosphocholine (POPC) bilayer. In some occurrences, it contains a hydrophilic pore of initial diameter ~25 Å. It consists of 1152 lipids units and 84,014 water molecules organized in two lamellae above and below the lipids. At the temperature set for the study, *i.e.* 300 K, the bilayer is in the biologically relevant liquid crystal Lα phase. The siRNA coordinates were extracted from the 2F8S PDB structure and the molecule was set just above the top membrane/solution interface.[3] To counterbalance the negative charge on the siRNA, 42 sodium ions were added randomly to the solution. The final dimensions of the system before extending it in the z direction were 168 x 183 x 130 $Å^3$ and the total number of atoms was 338,730.

Simulations parameters: The MD simulation presented was carried out using the program NAMD2 targeted for massively parallel architectures.[4,5] The systems were examined at constant pressure and constant temperature (1 atm and 300 K) or at constant volume and constant temperature (300 K) employing the Langevin dynamics and Langevin piston method. The equations of motion were integrated using the multiple time-step algorithm. A time-step of 2.0 fs was employed. Short- and long-range forces were calculated every 2 and 4 time-steps, respectively. Chemical bonds between hydrogen and heavy atoms were constrained to their equilibrium value. Long-range, electrostatics forces were taken into account using a fast implementation of the particle mesh Ewald (PME) method,[6,7] with a direct space sum tolerance of 10^{-6} and a spherical truncation of 11 Å. The water molecules were described using the TIP3P model[8] and a united-atom representation was adopted for the acyl chains of the POPC lipid molecules.[9]

Simulations protocols: The system was first equilibrated at constant temperature (300 K) and constant pressure (1 atm), using full 3D periodic boundary conditions as in the simulations of multi-lamellar stacks of lipids. Further simulations were performed using the standard "electric field method" for which a constant electric field $\vec{E}$, normal to the bilayer, was applied to each particle of the system bearing a charge q_i of the system in the form of a force $F=q_i.E$, using 3D periodic boundary conditions. Using MD simulations, the value of the electric field that enables to porate the membrane is around 140 kV/mm. Due to the length of the simulation box (125 Å), the transmembrane voltage amounts then to ~1.6 V. Compared to the field used in the experiments, it is one order of magnitude higher. However, due to the limitations of the simulation setup, fields of this magnitude (simulations #4-7) are not even able to maintain open an already formed pore, let alone create a transmembrane voltage large enough to porate the membrane.

SUPPLEMENTARY DISCUSSION

Due to its instability in the blood flow where it can be degraded or opsonized, siRNA has either to be protected or the lapse time of its circulation in the blood needs to be reduced. Practically, the use of naked siRNA requires an injection at the site of the disease that has to be treated (for example the tumor) followed rather quickly by the electric treatment so as to facilitate the rapid uptake by the cells and to avoid the degradation of the nucleic acids.

SiRNA fluorescence is larger inside the vesicles compared to outside mainly as a consequence of the fact that uptake of these nucleic acids by the GUVs is a phenomenon driven by electrophoresis and not by the concentration gradient. Indeed, in the later case, the result should be a lower internal concentration or, at best, an equilibrium between internal and external concentrations. One could argue that an equal translocation at both poles of the vesicle facing the electric field is to be expected. However, before the pulse is applied, the vesicle is empty i.e. the accumulation (and potential exit) of the siRNA molecules on the other side of the vesicle is initially null or almost null. This provides for a net accumulation of siRNA molecules inside the cell.

We have concluded in this work that the translocation of the siRNA takes place on a 10 nanosecond time scale based solely on the experimental results. Indeed, as the post pulse images are recorded a couple of minutes after the 10 ns pulse, one may raise the possibility of siRNA uptake (or translocation) after the ns pulse by passive diffusion of the molecules that were being trapped in the membrane. If so, one would have detected SiRNA inside the vesicle also for the low voltage experiments (3.2kV/mm pulse) that were shown by bright field microscopy experiments to lead to electroporation of the membrane. Si RNA would have been detected in all cases above the vesicle electroporation threshold. We conclude, following the scenario sampled in the atomistic simulations (see figure S4) that the uptake of siRNA molecules is driven by the electrophoretic force through an open pore in the membrane, and that translocation of the siRNA must take place mostly (or even exclusively) within 10 nanoseconds.

SUPPLEMENTARY FIGURES

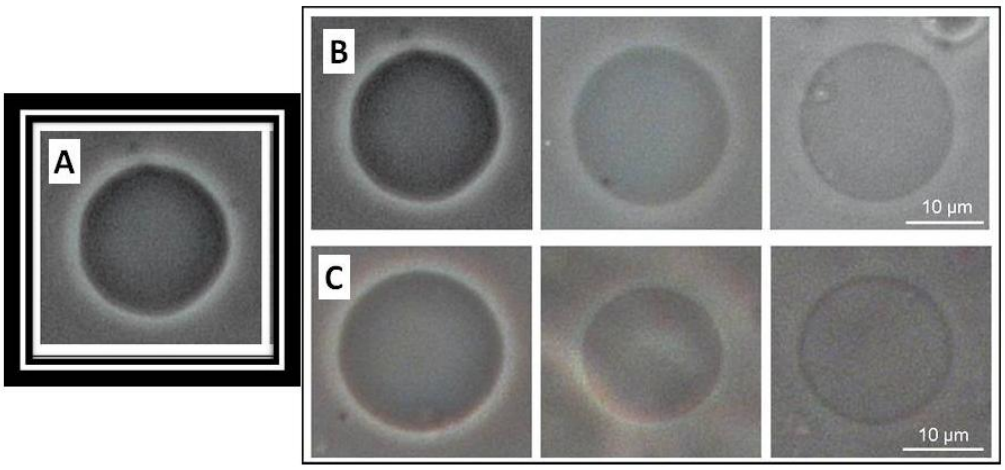

Figure S1. Representative transmission microscopy images of different DOPC giant unilamellar vesicles (GUVs) treated with a single nanopulse. The images were collected before (A) or a couple of minutes after the application of a 10 ns pulse of 3.2kV/mm (B) or of 5.8kV/mm (C). Note the fading contrast between the internal media (sucrose 240 mM) and external media (glucose 260 mM), indicative of an exchange of sugars across the lipid membranes and therefore an electroporation of the vesicles.

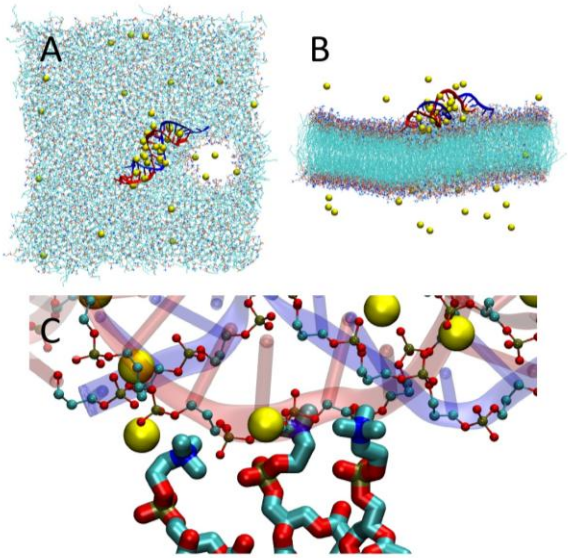

Figure S2. Simulation system. (A-B) Top and side view of the simulation system, consisting of a 22 bp ds siRNA deposited on a readily porated POPC bilayer solvated in water. The two strands of siRNA are represented as blue and red ribbons. Lipids are represented as coloured sticks and sodium ions as yellow spheres. Water is not shown for clarity. (C) Characteristic snapshot of the interaction of siRNA backbone (as small spheres, phosphate in brown, oxygen in red and carbons in cyan) with the choline moieties of the lipid headgroups (licorice representation) and sodium ions (yellow spheres).

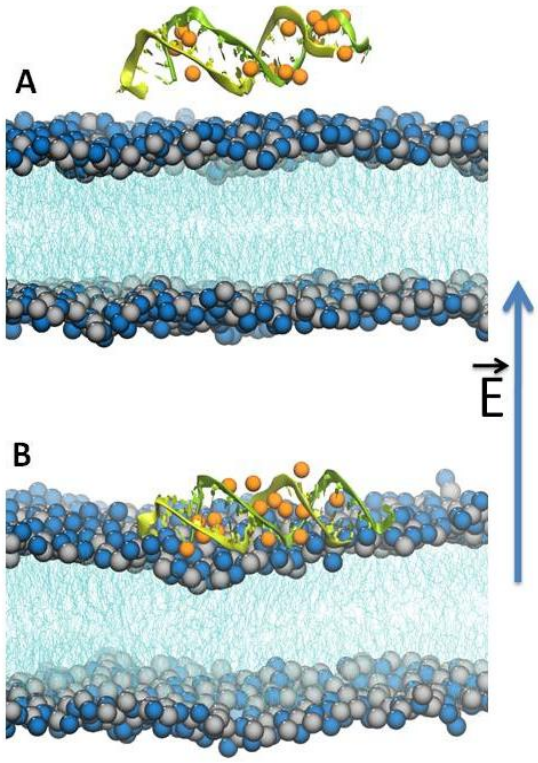

Figure S3. Location of siRNA (yellow ribbons) with respect to the membrane (polar headgroups represented as blue and grey spheres and hydrophobic tails as cyan lines) before (A) and after (B) the application of an electric field E normal to the bilayer. The field drags the negatively charged siRNA towards the membrane surface. Note the presence of counter ions (Na^+, orange spheres) that remain in interaction with the nucleic acid throughout the simulation.

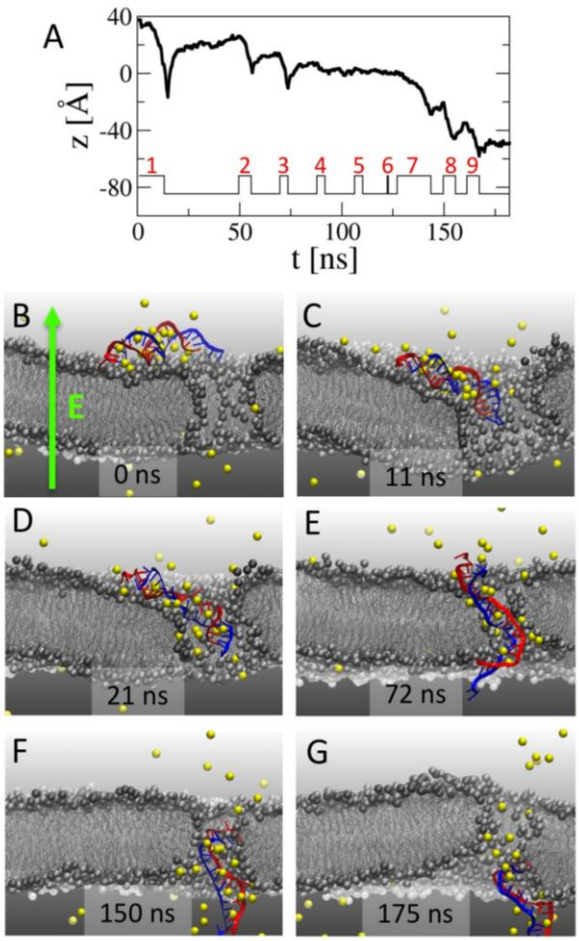

Figure S4. SiRNA transfer mechanism (Sim #11, see table S1) . (A) Position of the siRNA center of mass along the membrane normal (0 stands for the center of the bilayer) as a function of the simulation time (thick line). The electric field pulse sequence is shown as a thin line at the bottom of the plot. Pulses are numbered 1 through 9. (B-G) Snapshots of the siRNA transfer through the aqueous pore within the membrane. The two strands of siRNA are represented as blue and red ribbons. Lipids are represented in grey (spheres for the headgroups and lines for the lipid tails) and sodium ions as yellow spheres. Water is not shown for clarity. The field direction is indicated with the green arrow. (B) The siRNA is located at the top membrane/solution interface, in the vicinity of a preformed hydrophilic pore. (C-D) The electric field pulse drags the negatively charged siRNA downwards while the hydrophilic pore grows in diameter. (E) After switching off the field, the pore size reduces, trapping thereby the siRNA in a transmembrane location. (F-G). Only switching on the field again, enlarges the pores and drags the siRNA through it.

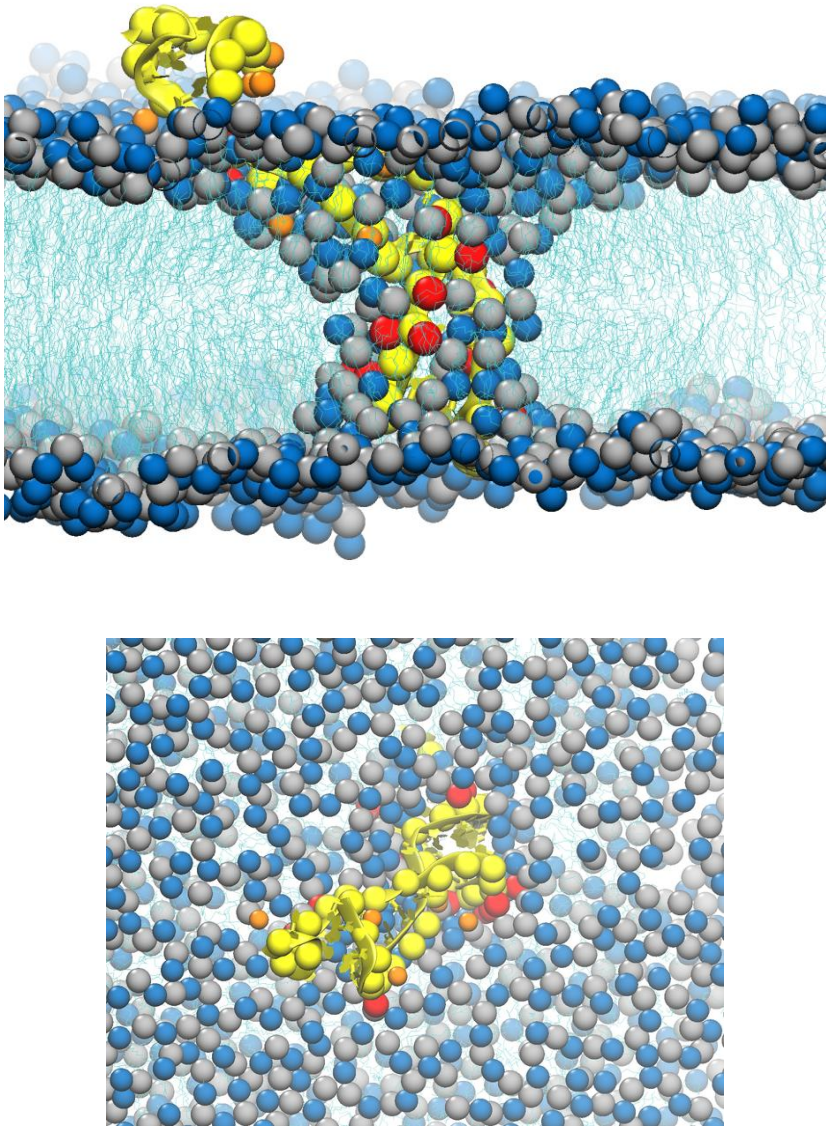

Figure S5. Side and top views of a configuration of the siRNA (yellow ribbons) entrapped in the bilayer after pore collapse (following switching off the electric field) from MD simulations. Color code: The lipid polar headgroups are represented as blue and grey spheres and the hydrophobic tails as cyan lines. Are drawn as yellow spheres the phosphates of the siRNA, and as red spheres, the choline $(NCH_3)_3$ lipid groups located within 5 Å of the siRNA phosphates. The Na^+ counter ions still solvating the siRNA are drawn as orange spheres.

SUPPLEMENTARY TABLE

Table S1: List of the MD simulations performed

Sim #	Electric field [kV/mm]	Length [ns]	Starting bilayer configuration	Description of the phenomena
1	140	46	Intact bilayer	RNA adsorbs to the bilayer surface, no transport
2	175	18	Intact bilayer	RNA adsorbs to the bilayer surface, no transport
3	210	18	Intact bilayer	Electroporation starts at 8 ns, and transport of siRNA follows shortly. Within 3 ns, the pore diameter has increased dramatically and full translocation of the siRNA has occurred. At 18 ns, the bilayer is completely distorted.
4	7	8	15Å diameter pore close to siRNA	Immediately after the beginning of the simulation, the pore size reduces until reaching ~3 Å diameter
5	7 + 10 dyn/cm surface tension	18	15Å diameter pore close to siRNA	Immediately after the beginning of the simulation, the pore size reduces until reaching ~3 Å diameter
6	14	8	15Å diameter pore close to siRNA	Immediately after the beginning of the simulation, the pore size reduces until reaching ~3 Å diameter
7	21	8	15Å diameter pore close to siRNA	Immediately after the beginning of the simulation, the pore size reduces until reaching ~3 Å diameter
8	70 alternative (2ns ON – 2ns OFF)	30	15Å diameter pore close to siRNA	The pore breathes, i.e. swells under the influence of the field while reduces under no field. No conduction of siRNA. The end of the trajectory is similar to its beginning.
9	70	24	15Å diameter pore close to siRNA	siRNA translocation starts after 14 ns. Full translocation occurs over the 10 remaining nanoseconds of the simulation.
10	NO continued from 20 ns into run 9	56	siRNA in transmembrane position, within a ~40 Å hydrated pore	The pore diameter reduces and the pore dehydrates within the initail few ns. The siRNA then remains trapped in a transmembrane position for the remaining 50 ns.
11	70 continued from the end of run 10 – pulses (#2-#9) of variable length (2-12 ns) interrupted by resting periods (5-10 ns).	130	siRNA trapped in a TM position in a non-hydrated pore	Application of subsequent electric pulses leads to successive reopening and reduction of the pore and of progressive drag of the siRNA to the other side of the membrane, resulting finally in a complete transfer of the latter from one side of the membrane to the other. Longer pulses (such as pulse #7 of ~16 ns or #8 and #9 of ~6 ns each) spaced out by short resting periods (~5.5 ns) seem to be the most efficient as they result in effective transport of siRNA along the membrane normal. Very short pulses, such as pulses #4, #5 (4 ns each) and #6 (0.5 ns), spaced out by longer resting periods (~12 ns) results in no effective transport at all. (Fig. S3)

SUPPLEMENTARY REFERENCES

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SUPPLEMENTARY MOVIES

Movie S1: The application of an electric field of magnitude lower than E_{thr} normal to the membrane during ~10 ns drives the negatively charged double-stranded siRNA (yellow and green ribbons) initially located ~10 Å above the membrane surface towards the latter (lipid headgroups as purple and grey

spheres and hydrocarbon tails as cyan lines). The hydrophobic membrane prevents further downward diffusion. Counter ions are represented as yellow spheres while water is not shown for clarity.

Movie S2: In the absence of siRNA, the application of an electric field of magnitude higher that E_{thr} (during ~10 ns) gives rise to the appearance of a hydrophilic pore. Water molecules (represented as transparent spheres) protrude from both sides of the membrane before eventually joining in the center. The lipid headgroups (same colors than above) then come down to line the pore and stabilize it.

Movie S3 and S4: Side and top view, respectively, of the conduction of a siRNA molecule through a membrane during a 10 ns electric pulse. The application of an electric field of magnitude higher than E_{thr} normal to the membrane gives rise to the appearance of a hydrophilic pore and to the subsequent electrophoretic drag of the siRNA across the membrane. The color scheme is the same than above.

Movie S5: Side view of the entrapment of a siRNA molecule after termination of the electric pulse. When the electric field is switched off while the siRNA double strand is still in transmembrane position, the pore collapses and the siRNA remains entrapped (stable over 100 ns). The color scheme is the same than above.