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Transport of siRNA through Lipid Membranes Driven by Nanosecond Electric Pulses: An Experimental and Computational Study

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Supporting Information

ABSTRACT: The use of small interfering RNA (siRNA) is a blossoming technique for gene regulation. However, its therapeutic potential is today severely hampered by the lack of an efficient means of safely delivering these nucleic acids to the intracellular medium. We report here that a single 10 ns high-voltage electric pulse can permeabilize lipid vesicles and allow the delivery of siRNA to the cytoplasm. Combining experiments and molecular dynamics simulations has allowed us to provide the detailed molecular mechanisms of such transport and to give practical guidance for the design of protocols aimed at using nanosecond-pulse siRNA electro-delivery in medical and biotechnological applications.

Small interfering RNA (siRNA) has recently drawn a lot of attention as it mediates RNA interference, one of the most promising approaches in gene regulation, with wide therapeutic applications in the prevention and treatment of diseases such as cancer that are caused by inherited or deregulated genes.[1] After crossing the plasma membrane, siRNA binds to the RNA-induced silencing complex in the cytoplasm, eventually preventing its target mRNA from being used as a translation template to produce its dedicated protein.[2] As such, siRNAs are part of a new generation of biodrugs, and their efficiency is being tested in preclinical and clinical trials.[3] One of the major challenges that hampers the use of nucleic acids as drugs relates to their efficient intracellular delivery to specific tissues and organs that express the target gene. Indeed, highly negatively charged naked nucleic acids such as siRNA can barely cross the barrier constituted by the hydrophobic lipid plasma membrane and enter the cytoplasm. Some methods to improve the uptake of naked nucleic acids by transiently permeabilizing the membrane have been proposed, among which are hydrodynamic injection[4] and application of electric pulses.[5]

The application of high electric fields to cells promotes electroporation (EP), a process in which lipid membranes are permeabilized.[6] As it enables the uptake of molecules that usually display poor transmembrane-crossing abilities, EP is widely used in biomedicine and biotechnology to enhance the transport of drugs, molecular probes, and nucleic acids.[7] Electrodelivery of nucleic acids (DNA plasmids and siRNA) to cells ensures that these highly biodegradable molecules are taken up quickly. This technique has been successful using low-magnitude microsecond pulses in vitro[8,9] and in vivo.[10−12] Recently, devices have emerged whose pulses in the kV/cm magnitude range can reach the nanosecond time scale (nanosecond electric pulses, or nanopulses).[13] In such a case, high-magnitude pulses (around a few hundred kV/cm) enable reversible EP not only of the plasma membrane but also of the membranes of internal organelles.[14] These nanopulses are particularly interesting because, contrary to other delivery techniques, deleterious thermal effects are avoided, minimizing the damage to the biological tissue.[15] The effect of nanosecond EP on cells strongly depends on the parameters of the pulses (magnitude, duration, frequency of repetition, and total number of pulses),[16] and the study of the consequences of 10 ns pulses remains at an early stage.[17]

The electrotransfection of siRNA by nanosecond pulses has not been studied to date, and the mechanisms of lipid bilayer crossing by nucleic acids, if any, remain unknown. To address these questions specifically, we set up protocols and strategies combining experimental and theoretical investigations. The nanosecond pulses were applied to giant unilamellar vesicles (GUVs), which are well-known simple models for cells. To mimic the cell membrane, the GUVs were synthesized with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a representative of phosphatidylcholine lipids, which are main constituents of the cell membrane. Using transmission and confocal microscopies, we investigated the stability of such GUVs and their uptake of siRNA when subjected to nanosecond pulses. Finally, we used molecular dynamics (MD) simulations to characterize the effect of such pulses at atomic resolution.

The vesicles were prepared using the electroformation technique.[18] The resulting solutions contained GUVs with diameters ranging from 10 to 100 μm, with a majority having diameters of 20 μm. The vesicles were loaded with sucrose at 240 mM and plunged into a 260 mM glucose solution [see the Supporting Information (SI)]. The difference between the refractive indexes of the two media ensured enough contrast to allow for the indirect observation of the vesicles by transmission microscopy (Figure S1A in the SI). This contrast was reduced when glucose and sucrose were exchanged between the internal and external media, thereby signaling a permeabilization of the vesicles. When subjected to a 10 ns, 3.2 kV/mm pulse, most of

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the vesicles remained intact, but some displayed a variable contrast loss (Figure S1B). After the vesicles were subjected to a 10 ns pulse of higher magnitude (5.8 kV/mm; Figure S1C), the contrast of most of the GUVs weakened or completely disappeared. These first results show that a single nanopulse with a magnitude of a few kV/mm can trigger the exchange of small molecules between the interior and exterior of the GUVs without any overall change in the shape of the vesicles, indicating that such nanopulses are able to permeabilize lipid vesicles of a size comparable to that of cells.

The entrance of siRNA into giant unilamellar vesicles subjected to a single electric nanopulse was monitored by confocal microscopy. Rhodamine-labeled vesicles of DOPC (red fluorescence) were submitted to 10 ns electric pulses of magnitude from 2 to 5.8 kV/mm in the presence of 500 nM fluorescein isothiocyanate (FITC)-labeled siRNA (green fluorescence). Confocal microscopy images were taken a few minutes after the application of the pulse, and fluorescence intensity profiles were estimated (see the SI) to allow for a better characterization of the system. In the absence of siRNA, no green fluorescence was detected (Figure 1A), whereas in the presence

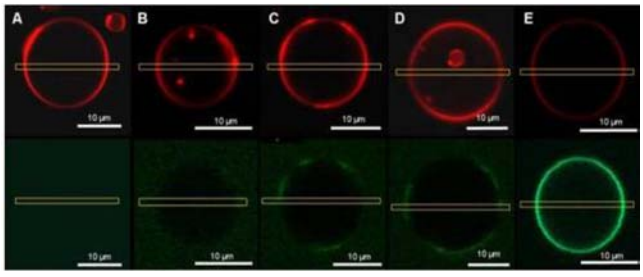

Figure 1. Confocal microscopy images highlighting the effect of a 10 ns electric pulse on representative DOPC GUVs labeled with 1% DOPE–rhodamine lipids (red fluorescence; upper row) in the presence of FITC-labeled siRNA (green fluorescence; bottom row). (A) DOPC vesicle in glucose buffer. (B) DOPC vesicle in the glucose buffer with 500 nM siRNA. (C–E) same as (B) after a 10 ns pulse with a magnitude of 2, 3.2, or 5.8 kV/mm, respectively.

of 500 nM siRNA (no pulse applied), the green fluorescence was homogeneous and visible only outside of the GUVs (Figures 1B and 2B). In the absence of any electric field, the siRNA molecules did not undergo passive diffusion through the membrane of the GUVs for at least a few minutes.

After the application of a 10 ns pulse at 2 kV/mm (Figures 1C and 2C) or 3.2 kV/mm (Figures 1D and 2D), no green fluorescence could be detected inside the vesicles. However, a substantial increase in the green fluorescence at the membrane of the vesicles was detected. While one would expect the lipid membrane to repel siRNA, a hydrophilic molecule, the data suggest that, to a certain extent, application of the pulse causes some siRNA molecules to become associated with the lipid. The increase in green fluorescence at the GUV membrane may also result from the fact that as the siRNA approaches the membrane, the FITC fluorescent tag penetrates the lipid hydrophobic environment, resulting in a decreased quenching of the probe fluorescence with respect to the aqueous environment.[19,20] At any rate, and quite surprisingly, a single 10 ns pulse appeared to be sufficient to drag siRNA molecules toward the lipid membrane electrophoretically and to trigger their association with the latter. The green fluorescence was not homogeneously distributed throughout the membrane, nor was it present only at the two

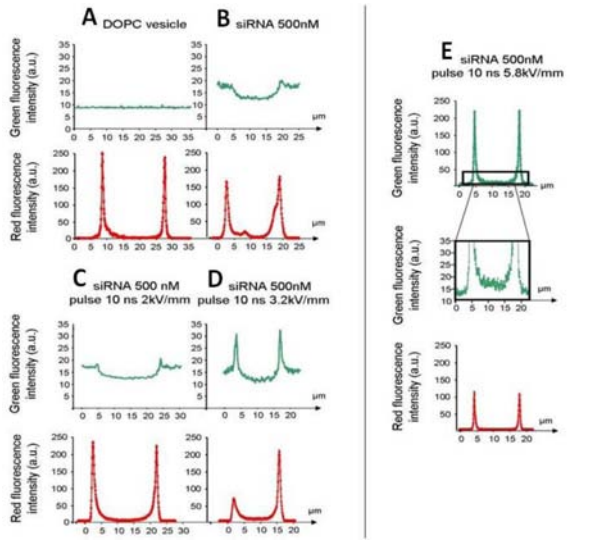

Figure 2. Fluorescence intensity profiles of FITC-labeled siRNA (green) and rhodamine-labeled lipids (red) estimated across a GUV section. The mean values were estimated from the orange rectangular sections in the confocal microscopy images in Figure 1.

poles of the vesicles. This was probably due to the high mobility of the phospholipids:[21] the siRNA molecules involved in strong interactions with the membrane phospholipids follow the movement of these lipids to a certain extent. For a field of 5.8 kV/mm, siRNA molecules were still found at the membrane, as evidenced by the strong and homogeneous green fluorescence at the boundaries of the GUVs (Figures 1E and 2E). At such a high voltage pulse, the red fluorescence decreased, indicating a degradation of the fluorescent rhodamine moiety, as previously described under different reactive conditions.[22] However, the most striking feature is that the green fluorescence inside the GUVs was stronger than the outside fluorescence, indicating that siRNA molecules had been taken up by the vesicles (Figure 2E) and highlighting moreover that this uptake was driven by electrophoresis as opposed to a pure diffusive mode due to a concentration gradient (see the discussion in the SI).

Hence, these experiments were the first to demonstrate that siRNA uptake can be induced by a single nanosecond pulse. However, the mechanism by which siRNA crosses the lipid bilayer remained unclear at this point, requiring us to use a complementary technique to investigate such a process at a molecular level. Consequently, we chose to use atomistic MD simulations to study the effect of high-magnitude nanosecond EP on the electrotransfer of siRNA. Because of obvious computational limitations, we modeled here only a fraction of the vesicle surface, namely, the portion that is perpendicular to the applied field. We considered planar POPC bilayer patches in which a 22 base pair double-stranded siRNA molecule was placed ~1 nm away from the membrane–solution interface (see the SI for further simulation details). In simulations, it is possible to mimic the experimental conditions of nanosecond EP by modeling the application of an external electric field $\mathbf{E}$ (of the same duration) in the form of an external force $\mathbf{F} = q_i\mathbf{E}$ acting on each particle i carrying a charge q_i.[23] Because of the use of periodic boundary conditions, the effect of an electric field on a bilayer, in particular the induced transmembrane voltage (and therefore the propensity to electroporate the membrane), depends not only on the field strength but also on the size of the system (see the SI for details).[24] In our system, the field magnitude needed to

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generate a transmembrane voltage of ~1.6 V and therefore yield the membrane electroporation was found to be 140 kV/mm; we considered this as a threshold value (E_{thr}). Because of the setup, simulations at a field in the same magnitude range as the experiments (7 kV/mm) yielded a voltage too low to electroporate the membrane or even to stabilize a preformed pore (Table S1 in the SI). Therefore, in the following simulations, we used electric fields below and at E_{thr}. Several MD simulations of an siRNA strand placed near an intact lipid bilayer headgroup interface and subjected to a constant electric field were conducted. For fields well below E_{thr}, we witnessed a condensation of the double strand at the interface but no further translocation across the bilayer, despite the electrophoretic force acting on the negatively charged siRNA. Under these conditions, the siRNA double strand came close enough to the positively charged choline moieties of the lipid headgroups to engage in strong electrostatic interactions (Figure S2, and Movie S1). Such a finding is similar to those reported in early investigations of the condensation of DNA double strands used as gene carriers on the choline groups of zwitterionic lipid multilamellar stacks.[25] The present MD simulations showed that such interactions are very stable, as manifested by the fact that the siRNA remained anchored to the lipid head groups even long after the field was switched off.

Above E_{thr}, in agreement with other simulations,[26] hydrophilic pores formed in the bilayer within a couple of nanoseconds (Figure 3 and Movie S2). From this point on, prolonged

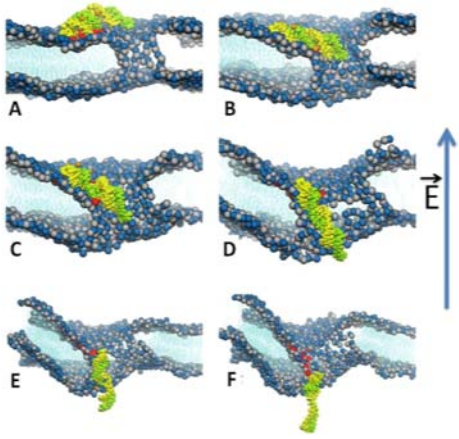

Figure 3. Molecular snapshots of the mechanism of electrotransfer of a double-stranded siRNA (shown as yellow and green spheres) through an aqueous pore (water omitted for clarity) within a POPC membrane (headgroups shown as blue and gray spheres and hydrophobic tails as cyan lines). Snapshots were taken at ~0 (A), 2 (B), 4 (C), 6 (D), 8 (E), and 10 ns (F) of an MD trajectory. The lipid headgroups initially in interaction with the siRNA strand are depicted in red to highlight their lateral diffusion.

application of the electric pulse resulted in two main processes: (*i*) the pore diameter expanded, and (*ii*) for cases where the negatively charged siRNA was located close enough to the pore mouth, it was dragged electrophoretically against the field direction through the pore. During this electrophoretic drag, the specific interactions between the siRNA phosphates and the choline groups of the lipid molecules lining the pore seemed to be strong enough to remain fulfilled during the siRNA translocation (see the movies in the SI and Figure 3). This resulted in a sliding of these lipids along the pore from the upper to the lower leaflet. During this translocation, a few Na^+ counterions also remained bound to the siRNA. Overall, a complete translocation could be achieved under fields above E_{thr} in less than 10 ns (cf. Movies S3 and S4). Finally, for instances where the applied field was switched off before the siRNA had completed the membrane crossing, the hydrophobic pore collapsed on the strand within a fraction of a nanosecond, resulting in trapping of the siRNA within the membrane thanks to the strong association between the phospholipids and the nucleic acids (Figure 4 and Movie S5).

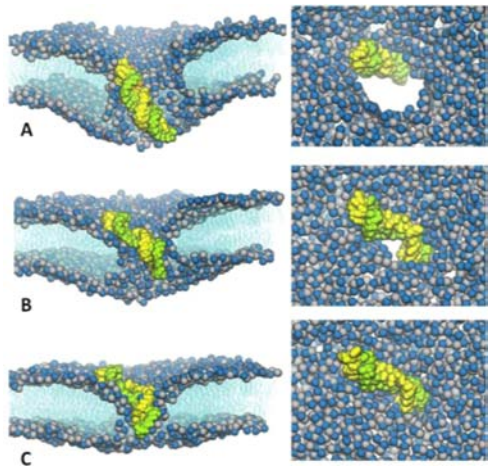

Figure 4. (left) Side and (right) top molecular views of the mechanism for trapping of the siRNA double strand after the electric field was switched off. The color scheme is the same as in Figure 3. The snapshots were taken along a 10 ns MD simulation.

The penetration of large nucleic acids (plasmid or DNA molecules) into GUVs has been observed after the application of a series of electric pulses ranging from 0.5 to 12 ms,[27] proving that the loading of vesicles with negatively charged molecules is possible thanks to membrane electropermeabilization. Here, combining theoretical and experimental studies, we have shown for the first time that siRNA can be electrotransferred into lipidic vesicles using a single 10 ns pulse. Indeed, we have shown that when siRNA molecules are submitted to low-magnitude nanopulses, they are dragged toward the membrane and strongly associate with its polar headgroups. When the field strength is increased, small nanopores through the lipid membrane can form, and siRNA can slide along these pores by electrophoresis within nanoseconds. Passive diffusion after the field is switching off can be ruled out, since pulses with magnitudes as low as 3.2 kV/mm gave rise to electroporation but did not enable measurable siRNA uptake (see the SI). Such a nanoscale process corroborates the results of experiments involving low-magnitude millisecond pulses, which suggested that the uptake mechanism of siRNA through cell plasma membranes, which takes place only through the side of the cell facing the negative electrode,[28] involves both poration of the membrane and electrophoretic dragging of the molecule. Though it was not captured in our MD simulations, we cannot rule out the possibility that large nanopores may form in the membrane under a higher electric field, allowing for direct funneling of siRNA without any specific anchoring to the lipid membrane. This mechanism could contribute to the increase in the siRNA uptake under the highest electric fields.

The MD simulations have also shown that siRNA can be trapped in the membrane thanks to electrostatic interactions

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between its negatively charged phosphates and the cholines of the lipid headgroups lining the collapsed pore (Figure S5). Although the stable nature of this state cannot be proved by this technique, it seems reasonable to infer that this mechanism, which has never been previously proposed, could also contribute to the measured increase in the green membrane-associated fluorescence after the application of a single nanopulse. The unexpected trapping of the siRNA in the membrane is an important finding. Indeed, large nucleic acids such as DNA or at least a fragment of these molecules could be more frequently trapped in the membrane during such a nanopulse procedure. This would be even more likely to occur with longer (millisecond or microsecond) pulses of low intensity, for which the electrophoretic drag would be lower. In such cases, the trapping of DNA would entail a long-term presence of DNA at the cell surface that could thus contribute to the previously described plasmid toxicity in addition to other mechanisms linked to innate immune responses inside the cell cytosol.[29]

To conclude, this study has shown that a single nanosecond pulse can induce electropermeabilization of GUVs. Combining experiments and simulations, we have demonstrated for the first time that siRNA can be electrotransferred into lipid vesicles by applying a single 10 ns pulse with a large enough magnitude. Although the electric parameters will be different under modified ionic conditions or for more complex bilayers, in particular for cells, this study gives practical guidance for the design of protocols to use nanosecond siRNA electrodelivery in biotechnological and medical applications.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, simulation protocols, and movies extracted from the MD simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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