Probing DNA–Lipid Membrane Interactions with a Lipopeptide Nanopore

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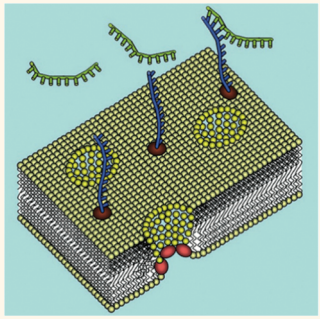
ABSTRACT Association of DNA molecules with lipid bilayer membranes is of considerable interest for a large variety of applications in biotechnology. Here we introduce syringomycin E (SRE), a small pore-forming lipopeptide produced by the bacterium *Pseudomonas syringae*, as a facile sensor for the detection of DNA interactions with lipid membranes. SRE forms highly reproducible pores in cellular and artificial membranes. The pore structure involves bilayer lipids, which have a pronounced influence on open channel conductance and gating. SRE channels act as ionic diodes that serve as current rectifiers sensitive to the charge of the bilayer. We employ this intrinsic property to electronically monitor the association of DNA molecules with the membrane in a variety of different settings. We show that SRE can be used for quantitatively probing electrostatic interactions of DNA and DNA–cholesterol conjugates with a lipid membrane. Furthermore, we demonstrate that SRE channels allow monitoring of hybridization reactions between lipid-anchored probe strands and complementary strands in solution. In the presence of double-stranded DNA, SRE channels display a particularly high degree of rectification. Finally, the formation of multilayered structures assembled from poly-(L)-lysine and DNA oligonucleotides on the membrane was precisely monitored with SRE.



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Attachment of DNA molecules to lipid bilayer membranes is of considerable interest for a large variety of applications in biotechnology. For instance, DNA–lipid conjugates can be used as sequence-addressable anchors for the controlled attachment of vesicles to surfaces.[1] It has also been shown that vesicle fusion can be controllably mediated by hybridization between complementary DNA strands attached to the vesicles,[2] and DNA hybridization has been utilized to build multilayered lipid nanocontainers.[3] Packaging of DNA into lipid complexes is a promising route toward gene or RNA delivery.[4] Finally, the attachment of DNA-based nanoconstructs to membrane structures is of considerable interest for the development of cell-like hybrid structures for bionanotechnology. Characterization of lipid–DNA hybrid structures is typically performed using fluorescence microscopy, requiring labeling of the molecules, and spectroscopic, surface plasmon resonance, or scattering techniques, often requiring large amounts of material and complicated and expensive equipment.[5–8] Here, we introduce a different method for the characterization of DNA–membrane interactions that employs the current rectification properties of nanopores formed by the lipopeptide syringomycin E (SRE). Applications of nanopore sensors cover a large variety of topics such as single-molecule sensing, monitoring of biochemical reactions, the creation of biobatteries, or an all-electronic approach toward DNA sequencing.[9–16]

Nanopore sensors are of two types. The first type uses the "resistive pulse" technique: analytes are electrophoretically driven through a nanopore—typically a biological channel—like those formed by alpha-hemolysin, aerolysin, or MspA, or a synthetic "solid-state" nanopore.[17–20] By measuring changes in the ionic current through the pore, it is possible to detect individual translocating molecules. The degree and duration of ion current reduction can provide further information about the structure or size of the analyte, for instance, the length or secondary structure of a translocating oligonucleotide.[21] To observe an effective nanochannel blockage and, as a result, a detectable electrical signal, the sizes of the analyte and nanopore must be comparable. Thus, it is necessary to employ different pores for different analytes, which is difficult when the structure of the analyte is not characterized or molecular complexes of variable sizes form over time. Moreover, for applications such as DNA sequencing, analyte translocations through nanopores occur at rates that are too fast for sensing, with resistive pulses necessitating the use of chemical tags or "molecular brakes".[22,23]

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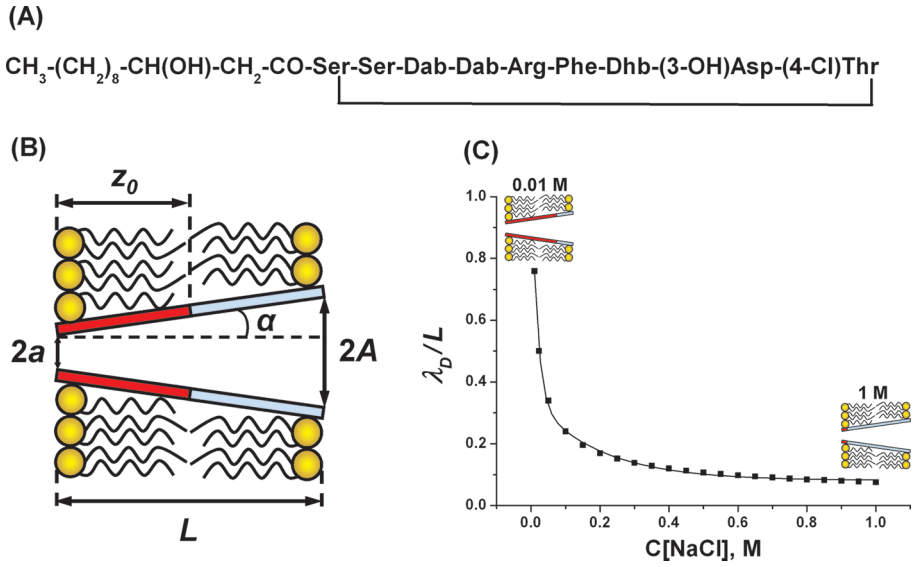

Figure 1. (A) Structure of SRE molecule in zwitterionic form. Abbreviations: Arg, arginine; (3-OH)Asp, 3-hydroxyaspartic acid; Dab, 2,4- diaminobutyric acid; Dhb, dehydro-2-aminobutyric acid; (4-Cl)Thr, 4-chlorothreonine; Ser, serine; Phe, phenylalanine. (B) Scheme of an SRE channel as a conical diode, where *A*, *a*, radii of pore openings; α, half opening angle of the cone; *L*, membrane thickness; z_0, typical length of positively charged region. (C) Dependence of λ_D/L on the concentration of the bathing solution.

The second type of nanopore sensor uses changes in current−voltage curves that are monitored before and after introduction of an analyte. The presence of an analyte molecule inside or close to a nanopore mouth can cause two different effects: occlusion of the pore (without translocation) and/or modification of the surface charge.[12,24,25] In both cases, the presence of the analyte can be detected as a change in the magnitude of an ionic current passing through the nanochannel. To exploit these effects, microfabricated conical nanochannels that are essentially nanofluidic diodes have been recently utilized as a biosensing platform.[26−28] For these nanochannels, ion current rectification and selectivity depend on sensitivities to surface charge density and effective diameter. If binding of an analyte results in a change in these parameters, a measurable change in ionic current will be induced. Ionic diodes are best characterized by the current rectification degree Q_{rec}, which compares currents recorded at voltages of one polarity with those obtained for the opposite one:

$$Q_{rec} = \frac{|I(-V)|}{|I(V)|} \quad (1)$$

Following the strategy of the second type, we employed syringomycin E, a small pore-forming lipopeptide (Figure 1A), as a tool for the detection of DNA interactions with lipid membranes. SRE belongs to a class of cyclic lipodepsipeptides produced by *Pseudomonas syringae* pv. *syringae* and plays an important role in the interactions of these bacteria with plants.[29] Lipid bilayer studies have shown that SRE acts by inserting into lipid bilayer membranes followed by the formation of ion-conducting pores and that its presence on only one side of the membrane is sufficient for pore formation.[30,31] SRE forms nanopores of "large" and "small" conductance, where the "large" pores are in fact clusters of the "small" (elementary) pores.[32] The conductances of the clusters are integer multiples of the elementary channel conductances, and the ionic selectivities of the two coincide. The elementary SRE channel is an asymmetric peptide-lipid pore with a conical shape. The wider *trans*-opening, with an estimated diameter of 1.0−1.8 nm, is formed from host lipid molecules, whereas the *cis*-opening, containing the SRE molecules, is only 0.5−0.7 nm wide.[33] SRE channels are preferentially permeable to anions, and their conductance and gating kinetics are strongly voltage-dependent.[34] At least six SRE molecules are required for channel formation.[30] When incorporated in uncharged membranes, SRE channels open at negative transmembrane voltages and close with positive voltages. Schagina and coauthors[35] previously studied SRE conductance−voltage curves for various fractions of negatively charged dioleoylglycerophosphoserine (DOPS)-containing lipid bilayers. It was found that SRE channel rectification varies from 0.7 for uncharged membranes (0% DOPS) to 1.9 for membranes consisting of 100% DOPS (in all cases membranes were bathed by 0.1 M NaCl, pH 6). Further modulation of Q_{rec} was achieved by the addition of negatively charged polyanions such as polyglutamic acid or Koenig's polyanion to the *cis*- or *trans*-side of the bilayer (between 0.35 and 7) and with ionic strength of the bathing solution.[35,36] On the basis of these findings it was suggested that the presence of charges affects the energy barriers for anions that pass through the SRE channel.[36]

Its pronounced charge sensitivity and conical shape make the SRE channel a promising natural analogue to

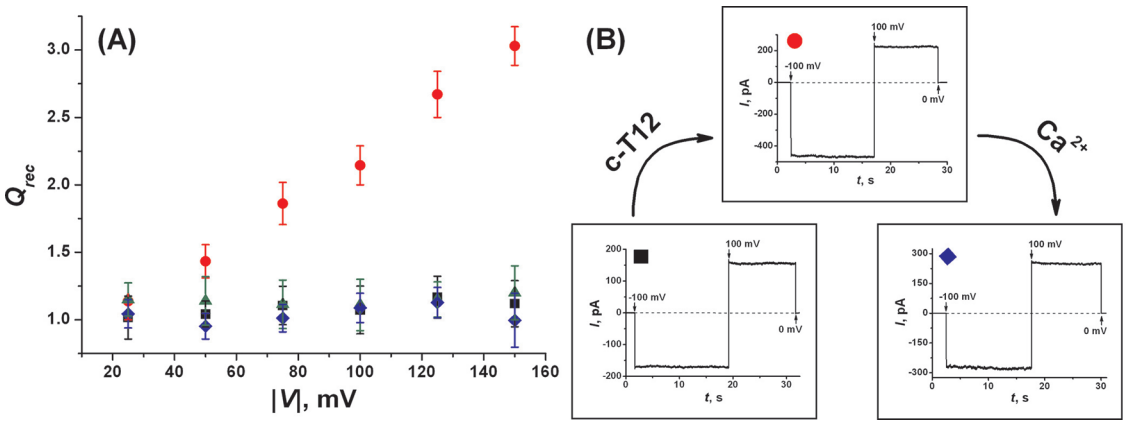

Figure 2. (A) Before addition of DNA initial (control) Q_{rec} values were measured in the voltage range of 25−150 mV (black squares) according to eq 1. Binding of T12 ssDNA with cholesterol attached to the 5′ end (c-T12) up to 1.12 μM to the *trans*-monolayer results in a local increase of negative membrane charge and, hence, leads to a shift in conductance ratios (red circles). Unmodified T12 DNA had no effect on Q_{rec} at the same concentration (green triangles). Subsequent addition of 5 mM Ca^{2+} neutralizes the DNA molecules and restores the initial situation (blue diamonds). (B) Typical current traces at −100 and 100 mV observed initially (left), in the presence of c-T12 DNA at the *trans*-side (center), and after subsequent Ca^{2+} addition (right). Membrane-bathing solution was 50 mM NaCl at pH 6. Lipid bilayer was formed from DPhPC.

artificial microfabricated diode sensors.[26,27] Besides naturally rectifying channels (like the IRK^+ channel[37,38]) only a few biological ion channels have been employed as ionic diodes, *e.g.*, a mutated form of OmpF porin containing a cluster of positively charged arginines,[39] and charged derivatives of gramicidin A (gA).[9] Maximum rectification degrees observed are 3.14 ± 0.28 for OmpF and 4.8 ± 0.1 for gA mutants, respectively. Similar to microfabricated nanochannels, biological diodes have also been employed as sensors for processes that affect membrane charge. For instance, gA was successfully used to monitor the action of phospholipase enzymes on lipid membranes.[10]

In the present work, we study modulations of the SRE channel rectification degree induced by DNA molecules interacting with lipid bilayer membranes in a variety of different situations. We first show that SRE channels can be used to characterize binding of cholesterol-labeled DNA oligonucleotides to the membrane. This process leads to a strong change in Q_{rec}, which is attributed to an increase in negative charge close to the pore mouth that is conferred by the DNA. The effect can be reversed by neutralization of the DNA charge with appropriate counterions such as Ca^{2+}. It is found that the DNA concentration-dependent shift of Q_{rec} is well described by the Langmuir adsorption model. We also demonstrate that SRE nanochannels can be used to monitor DNA hybridization reactions between lipid-anchored DNA probes and complementary DNA strands in solution. For dsDNA-modified lipid membranes, very high Q_{rec} values, up to 8.5 ± 0.9, were observed, comparable to the rectification of the OmpF channel under strongly asymmetric pH conditions.[40] Finally, formation of membrane-supported multilayered charged complexes between negatively charged DNA and positively charged poly-(L)-lysine can be efficiently monitored by this technique.

RESULTS AND DISCUSSION

After adsorption on uncharged membranes, SRE molecules together with lipid molecules (red and light blue bars in Figure 1B, respectively) form channels resembling unipolar diodes with positively charged and neutral regions. Parameters used to describe microfabricated nanodiodes are employed here for the SRE channel.[26,41,42] The small (*cis*-) opening of the SRE channel has a mean width of roughly $a \approx 0.3$ nm, the *trans*-opening is approximately $A \approx 0.7$ nm wide, and the pore length is virtually equal to the solvent-free 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) membrane thickness of 4 nm; hence the cone angle is $\alpha \approx 3°$. For nanofluidic diodes, the extension of the charged regions within the channels is an important parameter for their rectification properties. In contrast to microfabricated pores, however, SRE pore walls are not uniformly charged, with its few positive charges preferentially residing at the pore entrance. As a rough estimate for the "polarity" of the diode, one can compare the interaction range of these charges, *i.e.*, the Debye length λ_D, with the pore length L. At high salt concentrations, the positively charged region is predicted to be strongly localized to the pore entrance, while at low salt concentration the charge is distributed over a larger portion of the entire channel (Figure 1C), resulting in more pronounced rectification properties. Here all measurements were performed at 50 mM NaCl, for which $\lambda_D/L \approx 1/3$.

Detection of DNA−lipid interactions was based on the measurement of changes in the channel rectification degree Q_{rec}. After the initial formation of a lipid bilayer membrane, SRE was added to the *cis*-compartment of the chamber at a sufficiently high concentration to ensure the formation of multiple pores. After the number of open SRE channels reached steady state, initial (control) Q_{rec} values were measured in the voltage range of 25−150 mV (Figure 2A, black squares) according to eq 1.

Following the addition of c-T12 ssDNA (consisting of 12 thymine nucleotides with a cholesterol anchor attached to the 5′ end) to the *trans*-compartment, the bathing solution was stirred and the transmembrane voltage was switched to +50 mV to stimulate the attraction of DNA to the membrane. In order to monitor the adsorption process, Q_{rec} was measured every 2 min until a new steady value was reached (Figure 2A, red circles). Rectification increased with the addition of negative charges to the *trans*-side, consistent with previous findings, and was qualitatively similar to the substitution of neutral with negatively charged lipids as previously described,[35] and the addition of DNA to the *trans*-side is viewed as switching from unipolar to bipolar diode behavior. The results are consistent with the fact that bipolar diodes rectify better than unipolar ones.[42] Also consistent with these interpretations, the addition of divalent Ca^{2+} cations to the solution after adsorption of DNA on the membrane neutralized the negative DNA charge, thus resulting in a decrease of rectification toward the initial state (Figure 2A, blue diamonds). It is important to note that no shift of Q_{rec} was observed for unmodified T12 ssDNA at the same concentration (Figure 2A, green triangles), in agreement with results of Tsuji and Yoshikawa,[43] who also found that unmodified DNA did not adsorb onto membranes composed of lipids with phosphocholine headgroups.

Figure 2B presents typical current traces at −100 and 100 mV observed initially without DNA (left), in the presence of c-T12 DNA at the *trans*-side (center), and after subsequent Ca^{2+} addition (right). Shift of Q_{rec} can originate from change of pore conductance and/or probability of open state (P_{on}) at opposite voltages after adsorption of DNA. Previously it was shown that for the bipolar state of the SRE channel (membrane from equimolar mixture of DOPS and DOPE bathed by 0.1 M NaCl, pH 6), which is qualitatively similar to the case of c-T12 DNA adsorption on the *trans*-side of the bilayer, P_{on} does not depend on transmembrane voltage.[44] Hence here we attribute observed Q_{rec} alteration to change of pore conductance rather than shift of probability of pore open state, although for the exact answer, measurements of P_{on} of the SRE channel in the presence of c-T12 DNA are necessary.

The addition of c-T12 caused Q_{rec} to reach a value of ~3 at $V = |150\ mV|$ (Figure 2A, red circles), which is considerably higher than the Q_{rec} = 1.9 previously observed for 100% negatively charged membranes at the same voltage.[35] In order to find the maximum Q_{rec} inducible under these conditions, we measured the dependence of Q_{rec} on the concentration c of DNA in solution (Figure 3).

As expected for an adsorption process, Q_{rec} first increased linearly with c and then saturated at high concentrations. As indicated by the fit in Figure 3, this behavior is well described by the Langmuir adsorption

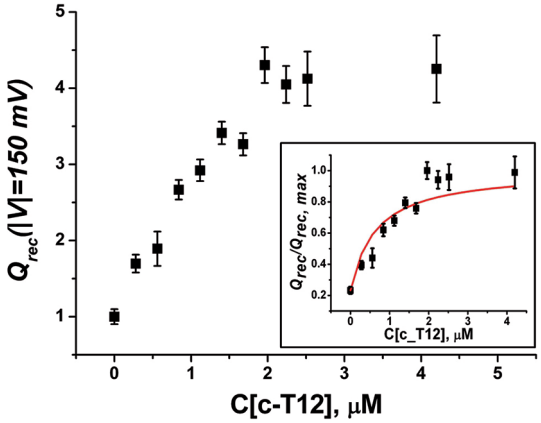

Figure 3. Dependence of rectification degree, measured at $|V|$ = 150 mV, on c-T12 concentration. Inset: c-T12 adsorption process obeys Langmuir adsorption isotherm. Red curve presents the fitting according to eq 3. Data were obtained for DPhPC bilayers in 50 mM NaCl, pH 6 solutions.

isotherm,[45,46] which can be written in the following form:

$$\theta(c) = \frac{Bc}{1 + Bc} \qquad (2)$$

Here, θ is the fractional surface coverage, c is concentration of the adsorbing species, and B is the adsorption constant. For the fit, the Langmuir isotherm was rewritten in terms of the rectification degree as follows:

$$\frac{Q_{rec}(c)}{Q_{rec,\ max}} = \frac{Q_{rec}(0)/Q_{rec,\ max} + Bc}{1 + Bc} \qquad (3)$$

where $Q_{rec}(c)$ is the rectification degree at the current concentration of c-T12 and $Q_{rec}(0)$ and $Q_{rec,max}$ are rectification degrees in the absence of DNA and at its maximum value, respectively. The fit yielded a binding constant of $B = 1.55\ \mu M^{-1}$. The binding of DNA to the lipid membrane bears some similarities to the fabrication of DNA microarrays and also probe−target hybridization in such arrays.[47] For microarrays, the saturation of the binding curve is caused by steric hindrance on the surface and mutual electrostatic repulsion of the molecules, which in turn is influenced by the salt concentration.[48]

A fully quantitative treatment of the rectification properties of this system requires a description within a Nernst−Planck model, for which an accurate potential landscape would need to be determined. Such an analysis is beyond the scope of the current work, and instead, an empirical explanation is provided here of the working principle of the SRE diode. The observed changes in rectification degree can be understood in terms of the electric field distribution and the corresponding energy profiles for anion transport through the SRE channel, as schematically shown in Figure 4.

In the absence of DNA, an anion (which is the dominating translocating species in SRE channels in

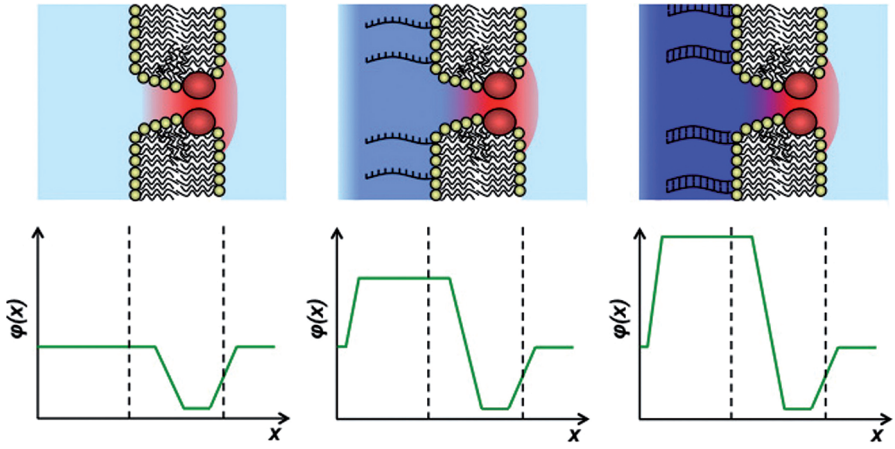

Figure 4. Sketch of electric field distribution in the absence (left) and presence of ssDNA (center) or dsDNA (right) on the *trans*-side. All configurations are in the absence of transmembrane voltage. (Bottom) Energy profiles of anion transport through SRE channel for the corresponding situation.

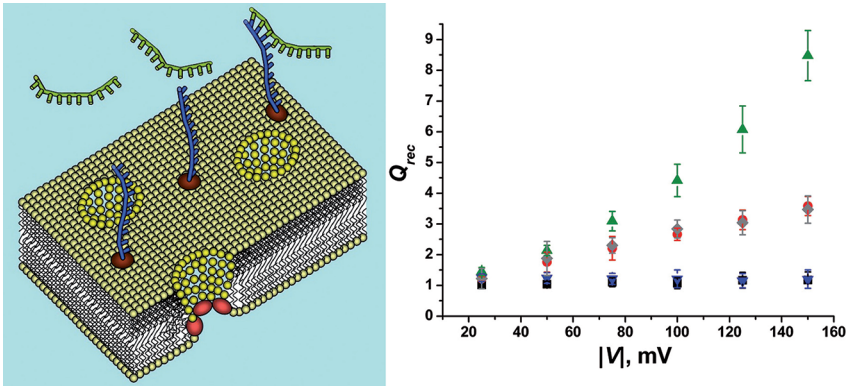

Figure 5. Hybridization of complementary strands on the membrane. After formation of SRE channels (black squares) single-stranded DNA with cholesterol anchor (c-DmiR21comp; depicted in the sketch as a blue strand with dark red "anchor", $c = 0.56\ \mu$M) is adsorbed on the *trans*-monolayer and the first shift of rectification degree occurs (red circles). Addition of the complementary strand (DmiR21; depicted in green, $c = 1.12\ \mu$M) leads to DNA hybridization on the membrane surface and a change of Q_{rec} (green triangles). Addition of DmiR21 in the absence of c-DmiR21comp or addition of noncomplementary T12 after adsorption of c-DmiR21comp did not change Q_{rec} (blue and gray triangles, respectively; in both cases concentration of added DNA was 1.12 μM). Lipid bilayer was formed from DPhPC and bathed in 50 mM NaCl, pH 6 solution.

DPhPC membranes[31]) entering the channel from the *cis*-side initially experiences an attractive potential caused by positively charged SRE molecules. As discussed above, the range of this potential is approximately given by the Debye length (1.3 nm with 50 mM NaCl). In addition, the potential landscape is relatively flat, which indicates a unipolar diode and the "low" rectification state of the SRE channel (Figure 4, left). The presence of negatively charged DNA molecules on the membrane creates an energy barrier for anions, and hence the SRE channels shift to bipolar diode characteristics and a "high" rectification state (Figure 4, center).

The charge sensitivity of the SRE pore conductance was exploited to measure membrane-localized DNA hybridization events (Figure 5).

The membranes were initially loaded with cholesterol-modified probe strands (c-DmiR21comp), and the further addition of (unmodified) complementary DNA strands (DmiR21) gave an additionally enhanced change in Q_{rec}. In control experiments without probe strands, no change was observed. Furthermore, the addition of non-complementary DNA strands also failed to result in an appreciable change in Q_{rec}. The results are therefore consistent with the formation of DNA duplexes on the membrane. The high negative charge density of DNA duplexes leads to a much stronger accumulation of counterions than for single-stranded DNA, which in turn confers a higher rectification degree (Figure 4, right). Our observations are in agreement with previous work where hybridization was observed at similar micromolar DNA concentrations using synthetic diodes.[27] The sensitivities of the two methods are comparable.

In addition to ordinary DNA hybridization events, SRE channels can be used to study the formation of complexes between DNA and other charged molecules such as DNA-binding proteins or polymers. Such interactions play an important role in many biological processes such as gene transcription and regulation, DNA recombination, or compaction of DNA in

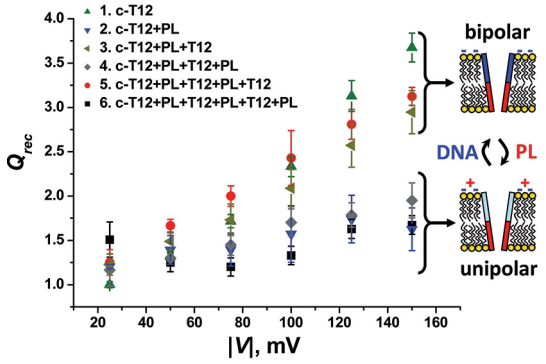

Figure 6. Formation of ssT12 DNA–PL complexes on the membrane is accompanied by switching between bipolar and unipolar forms of diode. Step 1 is the adsorption of 1.4 μM c-T12 on a membrane (only here T12 DNA with a cholesterol anchor is required). In a second step positively charged PL up to a concentration of 14 μg/mL is added (step 2). After rectification degree measurements we add 1.4 μM T12 DNA (steps 3 and 5). Steps 4 and 6 are equal to step 2. Data were obtained for DPhPC bilayers in 50 mM NaCl, pH 6 solutions.

chromosomes. Furthermore, the formation of lipid–DNA or polymer–DNA complexes is of considerable interest for biomedical applications in gene delivery.[49] Therefore, we examined if SRE channel conductance can be used to monitor the formation of a membrane-supported, multilayered structure composed of positively charged poly-(L)-lysine (PL) and DNA.

As before, cholesterol-modified c-T12 DNA was initially used to create a DNA–lipid "basement", setting the pore into a bipolar high-rectification state (Figure 6, green triangles). The addition of PL to the membrane caused a reversal to the unipolar "low" state due to the neutralization of the negative charge on the DNA (Figure 6, blue triangles). Subsequent addition of T12 DNA *without* cholesterol anchor (step 3, dark yellow triangles on Figure 6) to the membrane led to a subsequent increase of Q_{rec}. Since unmodified DNA does not bind to the naked membrane, the observed binding must be mediated by the presence of the PL. Similar to the well-known layer-by-layer deposition technique,[50] alternate addition of DNA and PL can be repeated several times (steps 3–6). Detection of this repetitive adsorption process will be constrained at a point when the top layers are too distant from the membrane surface to be registered by the SRE channel. In our experiments, a maximum of three subsequent DNA–PL layers was achieved, corresponding to an estimated maximum thickness of ~15 nm.[51]

The principle of sensing with the SRE channel is based on the lipopeptide's intrinsic properties and structure and does not require modification as in the case of OmpF.[39] The SRE sensor allows studies of DNA–lipid interactions without attachment of reporter tags such as dyes. Substitution of PC used in the present study with PE, for which better DNA binding is reported,[43] can potentially lead to absolutely label-free DNA detection. The range of rectification degrees available for the SRE channel is at least twice as large as for gramicidin,[9] the second smallest nanofluidic diode, and this offers higher sensitivity for the SRE-based system. The position of the SRE diode junction can be easily shifted during the experiment, and the length of the diode can be modified using lipids with hydrophobic tails of different lengths. The conical diode formed by SRE is orders of magnitude smaller than their synthetic counterparts, which also makes it attractive for screening applications using miniaturized chip devices such as the recently presented high-resolution electrical single-molecule nanopore microarray.[52]

CONCLUSION

In conclusion, we have introduced a novel all-electronic approach for the detection of DNA–lipid interactions and membrane-bound DNA hybridization events based on the rectification properties of peptide lipid membrane pores formed by SRE. The rectification degree is modified by changes in the membrane charge, which is influenced by the adsorption of DNA molecules, their counterions, and the presence of charged DNA-binding species. Cholesterol modification of the DNA molecules was observed to be critical for the efficiency of DNA binding. The SRE channel rectification degree characteristically shifts with the DNA concentration and is well described by the Langmuir adsorption model. In addition to simple DNA adsorption, the SRE method can also be used for the detection of membrane-localized hybridization events, and the adsorption of DNA-binding agents on DNA-modified membranes.

METHODS

Synthetic 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Pelham, AL, USA) and used for membrane formation. Electrolytes were of reagent grade (Sigma, Taufkirchen, Germany). Water was deionized and double distilled, and all measurements were performed with 50 mM NaCl bathing solution, pH 6 (5 mM HEPES). SRE was produced and purified as described previously.[53] Poly-(L)-lysine (molecular weight 15–30 kDa) was purchased from Sigma (Taufkirchen, Germany), and oligonucleotides were purchased from biomers.net (Ulm, Germany).

Oligonucleotides used in this study were
T12: 5′-TTTTTTTTTTT-3′
c-T12: 5′-cholesterol-TTTTTTTTTTT-3′
c-DmiR21comp: 5′-cholesterol-TCAACATCAGTCTG-3′
DmiR21: 5′-TAGCTTATCAGACTGATGTTGA-3′

DmiR21 has the same sequence as human microRNA 21 (miR21), which is associated with various types of cancers.[54] Virtually solvent-free membranes were formed as described by Montal and Mueller.[55] Two symmetrical compartments of a

Teflon chamber each with a solution volume of 0.8 mL were separated by a 25 μm thick Teflon partition containing a round aperture of 50−60 μm diameter. Squalene was used for aperture pretreatment, and a pair of Ag/AgCl electrodes was used to detect ion currents. All experiments were performed at 22 °C, and the temperature was kept constant using a BLM-TC thermocycler (Warner Instruments, Hamden, CA, USA). "Positive voltage" is defined such that the *cis*-side compartment (the side of SRE addition) is positive with respect to the *trans*-side. SRE was added to the aqueous phase from water stock solutions (1 mg/mL, pH 3) after bilayer formation. Spontaneous channel insertion was usually obtained while stirring under applied voltage. Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) in the voltage clamp mode. Signals were analyzed using built-in channel analysis software and further processed with Origin (Microcal Software, Inc., Northampton, MA, USA). PL or oligonucleotides alone had no pore-forming activity at concentrations used in experiments with SRE.

Conflict of Interest: The authors declare no competing financial interest.

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