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In situ electrochemical and PM-IRRAS Studies of colicin E1 ion channels in the floating bilayer lipid membrane

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Abstract

Colicin E1 is a channel-forming bacteriocin produced by certain *E. coli* cells in an effort to reduce competition from other bacterial strains. The colicin E1 channel domain was incorporated into a 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) floating bilayer situated on a 1-thio- β -D-glucose modified gold (111) surface. The electrochemical properties of the colicin E1 channel in the floating bilayer were measured by electrochemical impedance spectroscopy (EIS); the configuration and orientation of colicin E1 in the bilayer were determined by polarization-modulation-infrared-reflection absorption spectroscopy (PM-IRRAS). The EIS and IR results indicate that colicin E1 adopts a closed channel state at the positive transmembrane potential, leading to high membrane resistance and a large tilt

angle of α -helices. When the transmembrane potential becomes negative, colicin E1 begins to insert into the lipid bilayer, corresponding to low membrane resistance and a low tilt angle of α -helices. The insertion of colicin E1 into the lipid bilayer is driven by the negative transmembrane potential, and the ion channel open and closed states are potential reversible. The data in this report provide new insights into the voltage-gated mechanism of colicin E1 ion channels in phospholipid bilayers and illustrate that the floating bilayer lipid membrane at the metal electrode surface is a robust platform to study membrane-active proteins and peptides in a quasi-natural environment.

Keywords: colicin E1, ion channels, EIS, PM-IRRAS, floating bilayer lipid membrane

1. Introduction

The channel-forming colicins belong to a subset of bacteriocins, which kill *Escherichia coli* and related cells by forming voltage-gated ion channels in the bacterial inner membrane. They bind to receptors in the outer membrane of the target bacterium and then translocate across the periplasm where they insert into the cytoplasmic membrane by forming a dissipative ion channel that depolarizes the host cell. Colicin E1 consists of 522 amino acid residues, comprising three domains: the N-terminal translocation (T), receptor-binding (R) and C-terminal channel-forming (C) domains.¹ The 190-residue C-domain of colicin E1 provides an outstanding system for the investigation of the ion-channel mechanism of channel-forming proteins in the lipid bilayer. The X-ray crystallographic structure of the colicin E1 C-domain consists of 10 α -helices in which helices VIII and IX are highly

hydrophobic.² A plethora of biophysical studies have been conducted on colicin E1 including fluorescence resonance energy transfer (FRET),^{3, 4} solid-state NMR⁵ and molecular dynamics simulations.⁶ These investigations revealed key insights into the closed channel state, where the hydrophobic helices VIII and IX insert into the nonpolar core of the lipid bilayer to form a hydrophobic hairpin, while the other eight helices are embedded in the membrane interfacial layer. The colicin E1 ion channels open at the negative transmembrane potentials,⁷ in which seven helices insert into the hydrophobic region of the bilayer to form ion-selective pores.⁸ The monovalent ions, Na⁺, K⁺, Cl⁻ can pass colicin E1 channels in a pH-dependent manner with a single-channel conductance larger than 10⁶ ions per second.^{9, 10}

Recently, a floating bilayer lipid membrane (fBLM) was developed to investigate potential controlled ion-channel formation by insertion of peptides into the membrane.¹¹ The floating bilayer was built at the gold (111) surface derivatized with 1-thio- β -D-glucose (β -Tg).¹¹ The hydrophobic region of the bilayer deposited onto this substrate was separated from the gold surface by a hydrophilic β -Tg monolayer,¹² which mimics the quasi-natural environment of membranes and prevents transmembrane peptides and proteins from denaturation. This architecture allows surface-sensitive techniques, such as electrochemical-impedance spectroscopy (EIS), atomic force microscopy (AFM) and polarization-modulation-infrared-reflection absorption spectroscopy (PM-IRRAS) to measure peptide insertion into the membrane as a function of the applied surface potential. The advantage of this approach was recently illustrated by investigations of voltage-gated ion channel formation by alamethicin, a 20-residue antimicrobial peptide, in zwitterionic¹³ and negatively charged lipid bilayers corresponding to the cytoplasmic membrane of bacterial cells.^{14, 15}

In the present paper, colicin E1 was introduced into the DPhPC floating bilayer,

whose structure is displayed in Figure 1(A). The β -Tg monolayer was deposited by self-assembly at the gold surface and the DPhPC bilayer with incorporated colicin E1 was built by the Langmuir-Blodgett (LB) transfer method followed by vesicle fusion. The electrochemical properties of the colicin E1 channel were measured by EIS, and the configuration and orientation of colicin E1 were determined by PM-IRRAS. The results in this paper provide a molecular description of the voltage-assisted mechanism of colicin E1 ion channel formation in a phospholipid bilayer.

Figure 1. (A) Schematic diagram of the DPhPC/colicin floating bilayer at β -Tg modified gold (111) surface. The red numbers in the colicin E1 structure correspond to the protein sequence for each helix. The left cartoon shows the equivalent circuit used in the EIS analysis. (B) Chemical structures of DPhPC and β -Tg.

2. Experimental Section

2.1 Materials

The wild-type C-domain of colicin E1 (P190) was prepared and purified from transformed *lex A⁻ Escherichia coli* IT3661 cells as previously described.¹⁶ Before

use, colicin E1 was stored in 10 mM sodium phosphate and 50 mM NaCl buffer (pH = 7.4). The concentration of colicin E1 in the stock solution was 20 mg mL⁻¹. Potassium phosphate monobasic (KH₂PO₄, BioUltra, ≥ 99.5%), 1-thio- β -D-glucose sodium salt (β -Tg) and deuterium oxide (D₂O, 99.9 atom % D) were purchased from Sigma-Aldrich (Oakville, ON, CA). 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was obtained from Avanti Polar Lipids (Alabaster, AL). All chemicals were used directly without further purification. All aqueous solutions were prepared using water from the Milli-Q (Millipore, Bedford, MA) ultrapure water system (resistivity > 18.2 M Ω cm).

The single crystal gold (111) electrodes, grown, oriented, and cut in house, were employed as the working electrodes in the EIS and PM-IRRAS experiments. The EIS and PM-IRRAS measurements were performed in all-glass, three-electrode cells. The Ag/AgCl (saturated KCl) electrode (Pine Research Instrumentation, NC) was used as the reference electrode. The cell was de-aerated by purging with argon (Linde Canada, Guelph, Ontario, Canada) for 30 minutes before each measurement. An argon blanket was maintained above the electrolyte throughout the experiments to prevent the influx of oxygen. All potentials reported in this paper are quoted versus the Ag/AgCl (saturated KCl) electrode and the experiments were performed at room temperature (21 $\pm$ 2°).

2.2 Assembly of the DPhPC/colicin floating bilayer at β -Tg modified gold (111) surface

The gold (111) electrode was flame-annealed, cooled in air and then immersed into a 2 mM solution of β -Tg in methanol (Fisher Scientific, HPLC grade) overnight. Afterwards, the electrode was thoroughly rinsed with methanol to remove adsorbed β -Tg molecules, and with copious amount of water to remove methanol.

The inner leaflet contained pure DPhPC. A monolayer of the lipids was spread at the surface of a Langmuir trough and compressed to the surface pressure of 30 mN m^{-1} . It was then transferred onto the β -Tg covered gold (111) electrode using the vertical withdrawal (Langmuir-Blodgett method).¹⁷ The transfer ratio is defined as the ratio between the decrease in monolayer area during deposition (A_m) and the area of the substrate (A_s), where the ratio = 1 corresponds to the transfer of a completed monolayer of lipid on the substrate surface. In these experiments, the transfer ratio was 1.0 ± 0.1 . A KSV Mini-Micro trough (KSV NIMA, Biolin Scientific, Gothenburg, SE) with two hydrophilic barriers and a Wilhelmy plate controlled by KSV LB5000 software was utilized for the inner leaflet transfer. The electrode was dried for 1 h after the transfer. The outer leaflet of the floating bilayer was deposited by vesicle fusion. Solutions of small unilamellar vesicles of DPhPC were prepared according to the previous report.¹⁸ A stock solution of DPhPC in chloroform was added to a test tube to give the total mass of DPhPC at 1 mg. The chloroform was dried by mixing on a vortex in a test tube under a flow of argon until a thin film of lipid formed on the inside wall of the test tube. Dried films were placed in a vacuum desiccator to remove residual solvent. Finally, 1 mL of 0.1 M KH_2PO_4 buffer solution (pH=4) was added into the test tube and the lipid was sonicated for 30 minutes to obtain a 1 mg mL^{-1} solution of small unilamellar vesicles. The inner leaflet-covered gold (111) electrode was immersed into the vesicle solution for 12 h to deposit the outer leaflet. After formation of the DPhPC bilayer was completed, 5 μL of colicin E1 stock solution was added into the DPhPC vesicle solution. The concentration of colicin E1 in the DPhPC vesicle solution was 0.1 mg mL^{-1} , and the molar ratio between DPhPC and colicin E1 in the vesicle solution was $\sim 250:1$. The bilayer-covered electrode was incubated in the mixed solution of DPhPC and colicin E1 for another 3 h to allow for complete incorporation of colicin E1 into the lipid bilayer.

The vesicle fusion technique was employed because it requires a small volume of solution ~ 1 mL. Using this technique the concentration of colicin E1 in the bilayer should be the same as in the vesicles. The procedure in which the bilayer was formed first, followed by incubation with vesicles containing colicin E1, reduced the time that colicin E1 was exposed to ambient atmosphere and reduced the probability of the protein denaturing. The molar ratio between DPhPC and colicin E1 in the vesicles solution was 250:1. After the vesicle fusion and colicin E1 incorporation, the DPhPC/colicin floating bilayer-covered electrode was carefully rinsed with water to remove extraneous molecular residue from the membrane surface.

2.3 Electrochemical instrument and measurements

The potential of zero free charge (E_{pzfc}) of the DPhPC floating bilayer at β -Tg modified gold (111) surface in 0.1 M KH_2PO_4 electrolyte was determined by the immersion method.^{19, 20} A freshly prepared bilayer was used for each immersion measurement. The procedures of the E_{pzfc} measurement were previously described.²⁰

The EIS measurements were performed using the Solartron SI 187 electrochemical interface and Solartron SI 1260 impedance/gain-phase analyzer (Ametek Scientific Instruments, Depew, NY, US). Spectra were collected in the frequency range from 10^{-2} to 10^3 Hz with the excitation amplitude of 10 mV. The ZView software (Scribner Associates Inc.) was used for the data analyses. Details of the EIS measurements were described in a previous report.¹⁷

According to an earlier report,²¹ the optimum pH for the incorporation of colicin E1 into the lipid vesicles is pH 4. Due to the inherent difficulty to prepare a low pH buffer in D_2O , 0.1 M KH_2PO_4 buffer solution (pH=4.5) was chosen as the electrolyte in the EIS and PM-IRRAS measurements to ensure incorporation of colicin E1 into the lipid bilayer during the measurements.

2.4 PM-IRRAS experiments

PM-IRRAS experiments were performed using a Thermo Nicolet Nexus 870 spectrometer (Madison, WI), equipped with an external tabletop optical mount (TOM) box. The PM-IRRAS spectra were measured with the photoelastic modulator (PEM) set for half-wave retardation at 1600 cm^{-1} and the angle of incidence was set at 60° to obtain a large enhancement of the mean square electric field strength (MSEFS) of the *p*-polarized radiation at the electrode. The calculations used to select the optimal angle of incidence were described previously²². D₂O solution was employed to avoid the IR absorption by H₂O in the 1650 cm^{-1} region. At each potential, 4000 IR scans were added and averaged with the instrumental resolution of 4 cm^{-1} . The detailed description of PM-IRRAS measurements that were used in these experiments are described in a previous report.^{22, 23}

2.5 Calculation of the tilt angle

The PM-IRRAS spectra of the DPhPC/colicin floating bilayers at β -Tg modified gold (111) surface can be simulated using a model of five homogeneous, parallel phases (Au/ β -Tg/(DPhPC/colicin)/D₂O/CaF₂) and custom written software that solves Fresnel equations using the transfer matrix method.²⁴ The theoretical background for these calculations were described earlier²². The average tilt angles of α -helices in colicin E1 (γ_{colicin}), with respect to the surface normal were calculated from the integrated intensity of the corresponding IR bands in the measured and simulated PM-IRRAS spectra. The error analysis of the tilt angle calculation was described earlier.²⁵ Detailed descriptions of spectra simulation and tilt angle calculations were previously reported¹⁷ and can also be found in the Supporting information.

3. Results and Discussion

3.1 Electrochemical data

Figure 2 compares the EIS data of the DPhPC bilayers without and with colicin E1 floating at β -Tg modified gold (111) surface in 0.1 M KH_2PO_4 (pH=4.5) at the electrode potential of 0.2 V. The total impedance (Figure 2(A)) of the electrode is slightly higher in the presence of the pure DPhPC bilayer than that of the DPhPC/colicin bilayer. The phase angle of the DPhPC bilayer displays a plateau in the low frequency region with a value $\sim 85^\circ$ indicating that the bilayer has very low density of defects and its value is dominated by the capacitive element. In contrast, the phase angle of the DPhPC/colicin bilayer decreases when the frequency is lower than 0.5 Hz. Valincius et al.²⁶ have shown that the decrease of the phase angle plot at low frequencies (minimum) indicates the formation of pores and defects in the bilayer. Therefore, the phase-angle plots indicate that incorporation of colicin E1 leads to the formation of pores or defects in the DPhPC bilayer.

Figure 2. (A) Total impedance and (B) phase-angle plots of EIS spectra of the DPhPC (solid black line) and DPhPC/colicin (dashed red line) floating bilayers at β -Tg modified gold (111) surface in 0.1 M KH_2PO_4 (pH=4.5) at $E=0.2$ V vs. Ag/AgCl.

Figures 3A and B show results of EIS measurements for the gold (111) electrode

with DPhPC/colicin fBLM at different electrode potentials. The symbols show the measured data, and the solid lines represent the fitted results. The total impedance curves (Figure 3(A)) indicate that the impedance of the DPhPC/colicin bilayer slightly decreases when the potential becomes more negative. The phase-angle plots (Figure 3(B)), show that the decrease of the phase angle at frequencies $f < 0.5$ Hz becomes more pronounced at negative potentials, indicating an increase of the density of pores or defects in the bilayer.

Figure 3. (A) Total impedance and (B) phase-angle plots generated from EIS spectra of the DPhPC/colicin floating bilayer at β -Tg modified gold (111) surface in 0.1 M KH_2PO_4 (pH=4.5) at different electrode potentials. Symbols are the measured data, and the solid lines of the same color, represent the fitted results using the equivalent circuit shown in Figure 1(A).

To get further information about the properties of the bilayer, the impedance data were fitted to the equivalent circuit shown in Figure 1(A). This model was proposed by Valincius et al.,²⁶ taking into account that the fBLM is separated from the gold surface by a spacer provided by a hydrated monolayer of β -Tg. In this circuit, membrane resistance (R_m) and constant-phase element (CPE_m) represent the resistance and capacitance of the bilayer lipid membrane, respectively. CPE_{sp}

corresponds to constant phase element of the spacer region, which describes the electrochemical properties of the β -Tg monolayer and water layer between the lipid bilayer and the gold surface. The impedance of the CPE is defined as:

$$Z_{CPE} = \frac{1}{Q(j\omega)^\alpha} \quad (1)$$

where Q is the constant phase element coefficient measured in $\mu\text{F cm}^{-2} \text{s}^{\alpha-1}$ and the exponent α is an empirical constant related to the frequency dispersion. When α is close to 1, Q is approximately equal to the capacitance and when α is close to 0, the impedance of CPE is dominated by resistance.

The numerical values of the equivalent circuit elements determined from the fit to the data in Figure 3 are listed in Table 1. Since the values of α_m are close to 1, Q_m can be regarded as the capacitance of the DPhPC/colicin floating bilayer. The value of α_m is a lower than 0.9 at $E=0.4$ V, which corresponds to the onset of adsorption of H_2PO_4^- anion.

Table 1. Numerical values of the equivalent circuit elements of the DPhPC/colicin floating bilayer in 0.1 M KH_2PO_4 (pH=4.5) at different electrode potentials.

$E / \text{V vs. Ag/AgCl}$	$Q_m / \mu\text{F cm}^{-2} \text{s}^{\alpha-1}$	α_m	$R_m / \text{M}\Omega \text{ cm}^2$	$Q_{sp} / \mu\text{F cm}^{-2} \text{s}^{\alpha-1}$	α_{sp}
0.4	17.4 ± 1.7	0.885 ± 0.008	1.72 ± 0.17	1.6 ± 0.05	0.257 ± 0.050
0.3	12.4 ± 1.2	0.924 ± 0.004	0.85 ± 0.11	2.4 ± 0.11	0.375 ± 0.052
0.2	10.8 ± 1.1	0.941 ± 0.007	0.79 ± 0.14	2.5 ± 0.35	0.440 ± 0.035
0.1	10.4 ± 1.0	0.945 ± 0.006	0.74 ± 0.07	3.3 ± 0.53	0.472 ± 0.031
0.0	10.2 ± 1.0	0.950 ± 0.006	0.60 ± 0.06	3.2 ± 0.24	0.436 ± 0.026
-0.1	10.5 ± 1.1	0.952 ± 0.006	0.56 ± 0.05	3.6 ± 0.20	0.454 ± 0.025

-0.2	11.2 ± 1.1	0.953 ± 0.007	0.52 ± 0.07	3.5 ± 0.13	0.417 ± 0.030
-0.3	12.2 ± 1.2	0.954 ± 0.007	0.46 ± 0.05	3.7 ± 0.16	0.378 ± 0.038

At the two most positive potentials, the values of α_{sp} is smaller than 0.5 indicating that the impedance of the spacer layer has predominantly resistive properties. However, $\alpha_{sp} \sim 0.5$ at more negative potentials. The value of $\alpha = 0.5$ is characteristic of the Warburg impedance. Valincius et al.^{26, 27} showed that $\alpha_{sp} \sim 0.5$ is observed when the impedance is controlled by ionic transport through the pores and when the pore density is small and the pores are isolated. Therefore the impedance data indicate pore formation at $E < 0.2$ V vs SCE.

For comparison, the DPhPC floating bilayer without colicin E1 was deposited. The first leaflet was formed by the LB transfer method at the surface pressure of 30 mN m⁻¹ and the second leaflet was added by vesicle fusion. The impedance spectra of the colicin free fBLM were measured and are shown in Figure S1 and S2 of the Supporting Information (SI). These impedance spectra were fitted by the equivalent circuit displayed in Figure S1(A).¹³ The values of the equivalent circuit elements determined from the fit are listed in Table S1. They will be compared with the EIS data for the fBLM with colicin E1 below.

In addition, Figure S3 is a plot of the charge density of the gold (111) electrode with DPhPC floating bilayer at β -Tg modified gold (111) surface in 0.1 M KH₂PO₄ electrolyte (pH=4.5), determined using the immersion method.²⁰ A linear regression applied to the charge density versus potential plot allows one to determine the potential of zero free charge $E_{pzfc} = 0.38 \pm 0.03$ V vs. Ag/AgCl, of the gold (111) electrode with the DPhPC floating bilayer. Since DPhPC bilayer consists of electroneutral (zwitterionic) lipids, the gold surface is negatively charged at potentials negative to E_{pzfc} and positively charged at $E > E_{pzfc}$. Knowing E_{pzfc} allows one to plot

experimental data versus $E-E_{\text{pzfc}}$, which is a measure of the transmembrane potential.

Figure 4 (A) compares R_m of the DPhPC and DPhPC/colicin floating bilayers at different potentials. The bottom x -axis represents the applied electrode potential, and the top x -axis, $E-E_{\text{pzfc}}$. The resistance of the DPhPC bilayer without colicin E1 is large and potential independent for $E-E_{\text{pzfc}} > -0.3$ V. It decreases at more negative transmembrane potentials. The phase angle plot in Figure S1 of SI shows that concomitantly with the decrease of R_m , a decrease of the phase angle is observed at $f_m < 0.1$ Hz. This behavior indicates that the decrease is caused by electroporation of the membrane when $E-E_{\text{pzfc}} < -0.3$ V. The membrane resistance of the DPhPC/colicin bilayer is more than an order of magnitude smaller than the bilayer without colicin E1, demonstrating that in the presence of colicin E1, the bilayer has considerably more pores and membrane defects. The inset to Figure 4A shows that the resistance of this membrane continuously decreases when $E-E_{\text{pzfc}}$ becomes more negative, indicating an increase of the density of pores and defects.

The values of Q_m for the two membranes are compared in Figure 4B. Since α_m are close to unity they represent changes of the membranes capacitance. For $E-E_{\text{pzfc}} > -0.3$ V, the comparable values of $\sim 11 \mu\text{F cm}^{-2}$ are observed for the two bilayers. Such a value of the capacitance is typical when the phospholipid membrane is separated from the gold surface by a highly hydrated spacer layer.^{26, 27} The head group region of the lipids is then quite hydrated.^{28, 29} At $E-E_{\text{pzfc}} < -0.3$ V, the capacitance increases due to electroporation. Apparently, water molecules penetrate into the hydrocarbon region of the bilayer.^{28, 29} The capacitance increase is much more pronounced in the absence of colicin E1. The membrane capacitance equals ϵ/d , where ϵ is the permittivity and d is the membrane thickness. Although, colicin E1 has a strong effect on membrane resistivity, its effect on the membrane permittivity and thickness is rather small. Different dependence of the capacitance on the transmembrane potential suggests a

unique effect of $E - E_{\text{pzfc}}$ on the membrane properties. In the absence of colicin E1, $E - E_{\text{pzfc}} < -0.3$ V causes electroporation, penetration of water into the membrane and an increase of permittivity. In the presence of colicin E1, the potential-induced changes of Q_m are small, suggesting an insertion of the peptide into the bilayer. In the next section, the PM-IRRAS technique is employed to provide a molecular level interpretation to the impedance data.

Figure 4. (A) Membrane resistance (R_m) and (B) constant phase element coefficient (Q_m) of the DPhPC (black rectangles) and the DPhPC/colicin (red circles) floating bilayers at β -Tg modified gold (111) surface in 0.1 M KH_2PO_4 (pH=4.5) in D_2O at different potentials. The insert of Figure 4(A) displays R_m of the DPhPC/colicin floating bilayer between 0.3 V and -0.3 V.

3.2 PM-IRRAS measurements

Figure 5(A) displays the PM-IRRAS spectra in the amide I region of the DPhPC bilayer with colicin E1 at three selected potentials. The IR band between 1760 and 1700 cm^{-1} corresponds to the carbonyl absorption of DPhPC molecules,³⁰ and the band between 1700 and 1600 cm^{-1} comes from the amide I absorption of colicin E1.^{31, 32} In the PM-IRRAS spectra, the peak center and shape of the amide I band depends on different types of the secondary structure (α -helix, β -sheet, β -turn, and random) in the protein;³³ while the intensity of the amide I band is related to the orientation of the protein in the bilayer.³⁴ It can be observed that the shapes of the amide I band at different potentials are similar, indicating that the secondary structure of colicin E1 does not change with potential. The intensity of the amide I band increases when the electrode potential becomes more negative, demonstrating that the orientation of colicin E1 changes as a function of the potential. Since the C-domain of colicin E1 consists of 190-amino acid residues with predominantly helical secondary structure connected with short loop regions, the envelope of amide I band of colicin E1 contains several sub-bands. Second derivative (SD) and Fourier self-deconvolution (FSD) techniques were used to assist the amide I band deconvolution. The Fourier self-deconvolution is a procedure that allows one to narrow individual absorption bands hidden in a broad spectral envelope resulting from bands overlap as previously described^{23, 35}. The SD and FSD analysis of the amide I band is shown in Figure S4. The deconvolution of the PM-IRRAS spectrum of the DPhPC/colicin bilayer at $E=0.0$ V vs Ag/AgCl is plotted in Figure 5(B). The amide I band of colicin E1 contains 6 sub-bands. The strongest band at 1656 cm^{-1} corresponds to the α -helices, which constitute the predominant secondary structure of colicin E1.^{2, 32} The band at 1647 cm^{-1} comes from the protein random structure elements, two bands in 1633 and 1614 cm^{-1} are related to some minor β -sheet structure, and two bands in 1668 and 1678 cm^{-1} originate from β -turn structure.^{2, 33}

The average tilt angle of the main axis of α -helices with respect to the gold surface normal (γ_{colicin}) can be determined from the integrated intensity of the corresponding band in the PM-IRRAS spectra and the theoretical simulated IR spectrum of random-oriented colicin E1 molecules. The tilt-angle calculations assume that the lipid to peptide molar ratio in the bilayer was the same as in the vesicles (250:1). Details of the tilt-angle calculations are described in the Supporting Information. The PM-IRRAS spectra of the DPhPC/colicin bilayer at different potentials and the simulated spectrum are shown in Figure S5 of SI. The optical constants of the DPhPC/colicin mixture in the 1800-1550 cm^{-1} are presented in Figure S6 of SI.

Figure 5. (A) PM-IRRAS spectra of the DPhPC/colicin floating bilayer at β -Tg modified gold (111) surface in 0.1 M $\text{KH}_2\text{PO}_4/\text{D}_2\text{O}$ (pD = 4.5) in the amide I region at the potential of 0.4 V (black), 0.0 V (blue), and -0.4 V (red). (B) Deconvolution results of the PM-IRRAS spectrum of the DPhPC/colicin floating bilayer at $E=0.0$ V.

Figure 6 is a plot of changes of the average tilt of the main axes of α -helices of the colicin E1 molecule (γ_{colicin}) and the resistance of the bilayer in the presence of colicin E1 as a function of the applied potential. The bottom axis plots potential measured versus the Ag/AgCl reference electrode. The top axis plots the transmembrane potential $E-E_{\text{pzfc}}$. Due to a positive value of E_{pzfc} , the tilt angles were determined for predominantly negative values of $E-E_{\text{pzfc}}$. An onset of the membrane detachment and phosphate ion adsorption prevented measurements at more positive potentials. At the most positive transmembrane potential, $E-E_{\text{pzfc}} = 0.02$ V, the value of γ_{colicin} is $\sim 48 \pm 2^\circ$. This value is close to the average tilt angle of α -helices in colicin E1 reconstituted into a stack of multiple DMPC bilayers supported by the AgCl window, determined to be between 45° and 50° using the ATR technique.³² Rath et al.³² estimated that the average tilt angles between 45 and 50° correspond to two helical hairpins of colicin E1 with ~ 70 residues inserted into the membrane and the remaining ~ 100 residues assuming a predominantly parallel orientation with respect to the membrane surface. The C-forming domain of colicin E1 has ten α -helices. In the closed channel state, colicin E1 adopts an extended two-dimensional helical array at the membrane surface with two helices inserting into the hydrophobic region of the bilayer.^{3, 5, 6} In the open channel state, seven helices insert into the bilayer to form a toroidal pore.⁸ Therefore, the value of γ_{colicin} is $\sim 48 \pm 2^\circ$ at $E-E_{\text{pzfc}} = 0.02$ V suggests the existence of the closed state of the channel. However, this conclusion is not consistent with the observed significant decrease of membrane resistivity in the presence of colicin E1 (see Figure 4A). The fact that colicin

decreases the membrane resistance without affecting its capacitance suggests that colicin E1 forms channels rather than defects in the bilayer. To reconcile IR and EIS data, it is reasonable to assume that $\gamma_{\text{colicin}} \sim 48 \pm 2^\circ$ corresponds to a fraction of colicin E1 molecules forming channels with all seven helices inserted into the bilayer and the remaining molecules residing in the closed state with helices parallel to the surface.

As the transmembrane potential decreases, so does the value of γ_{colicin} , demonstrating that more α -helices insert into the bilayer as the potential becomes more negative. The decrease of γ_{colicin} is concomitant with a decrease of the membrane resistivity indicating that insertion of α -helices into the membrane leads to ion channel formation. When the transmembrane potential reaches -0.98 V, the tilt angle γ_{colicin} becomes equal to $37 \pm 2^\circ$. This value implies that a significant fraction of colicin E1 molecules forms open channels at this negative transmembrane potential. The electrostatic field of the metal is acting on the membrane and its strength is quite weak outside the membrane, where it is screened by ions in the diffuse layer. Consequently, the change in the tilt of the helices must correspond to insertion into the membrane. Since helices VIII and IX are hydrophobic and the remaining helices are amphipathic,² the insertion must lead to aggregation of helices and to channel formation. The results of PM-IRRAS measurements correlate nicely with the EIS data.

Figure 6. Variation of the average tilt angles of the α -helices of colicin E1 (red circles) and membrane resistance (black triangles) of the DPhPC/colicin floating bilayer as a function of the potential. The error bars for the tilt angle of α -helices were calculated using the procedure described earlier²⁵. The error bars for the membrane resistance correspond to uncertainties of fitting the experimental data to the equivalent circuit.

The reversibility of the colicin E1 channel formation was also tested by the PM-IRRAS technique. Figure 7 is a plot of the value of γ_{colicin} determined from the IR spectra during the potential scanned from positive to negative, and then back to positive values. When the potential was scanned from $E - E_{\text{pzfc}} = 0.02 \text{ V}$ to $-E - E_{\text{pzfc}} = -0.8 \text{ V}$, the value of γ_{colicin} decreases from $48 \pm 2^\circ$ to $39 \pm 2^\circ$. When the $E - E_{\text{pzfc}}$ was scanned back from -0.8 V to 0.02 V , the value of γ_{colicin} increased to reach $46 \pm 2^\circ$ at $E - E_{\text{pzfc}} = 0.02 \text{ V}$, which is almost equal to the initial value. The IR results verify that the potential controlled ion-channel state of colicin E1 in the DPhPC floating bilayer is reversible.

Figure 7. Average tilt angles of the α -helices of colicin E1 as a function of the potential.

The ion-channel properties of alamethicin (Alm) in the DPhPC floating bilayer were recently studied by EIS and PM-IRRAS.¹³ Alm adopts a surface state at positive transmembrane potential, and inserts into the bilayer at negative transmembrane potential. A comparison of the electrochemical behavior of Alm and colicin E1 in the floating bilayer leads to the conclusion that Alm begins to insert into the bilayer at sufficiently negative transmembrane potentials $(E - E_{\text{pzfc}}) \leq -0.4\text{V}$, where electroporation takes place; while colicin E1 starts to insert into the bilayer when the transmembrane potential is negative. The insertion of Alm into the hydrophobic region of the bilayer is driven by the defects in the bilayer, which is caused by the electroporation of the bilayer, occurring at sufficiently negative transmembrane potentials.¹³ The insertion of colicin E1 takes place when the transmembrane potential turns to negative, which is not driven by the electroporation of the bilayer. The mechanism of Alm ion channels and colicin E1 ion channels in the lipid bilayer is quite different.

Interestingly, according to earlier reports, Alm forms barrel-stave pores in the bilayer,³⁶ while colicin E1 forms toroidal pores.³⁷

Current evidence suggests that the closed-state membrane model for the channel-forming domain of colicin E1 involves 8 amphipathic α -helices (helices I-VII and X) that adopt a two-dimensional arrangement on the membrane surface³¹ Two central hydrophobic α -helices (VIII and IX) assume a transmembrane location based on the umbrella model. Helices I and II have been shown to participate in the channel by forming a transmembrane segment in the voltage-induced open channel state. Opening colicin E1 channels with voltage increments in planar lipid bilayers or in proteoliposomes protects the protein from proteolysis by added proteases,³⁸ suggesting that the open channel embeds more deeply within the lipid bilayer in the presence of a membrane potential. It was demonstrated in planar bilayers that His-440 in a loop region within colicin E1 carries a positive charge as it moves across the membrane upon channel opening, implying that its movement is completely across the bilayer span.³⁹ It is well established that voltage-gating in the channel-forming colicins such as colicin E1 involves import of a large part of the protein from the membrane surface into the bilayer and across the bilayer core.⁴⁰ The channel opening event is a reversible, thermally induced process that is inhibited at potentials more negative than -60 mV in planar bilayers or at anionic lipid concentrations > 30 mol% at 0.1 M ionic strength.⁸ Remarkably, the potential response of the colicin E1 channel opening in planar membranes is exquisitely sensitive to the lipid concentration, ionic strength, temperature and pH of the solution. Notably, in the supported bilayer system in the present study, we observed channel formation for potentials up to -800 mV (Figure 6). There are at least two reasons for such differences. Firstly, the supported bilayer is much more stable than the planar bilayer system and the former supports

significantly larger transmembrane voltages without rupture or breakdown of the membrane bilayer. Secondly, in the planar bilayer experiment, colicin is added to the solution and then its membrane insertion is measured by following the current flowing through the planar bilayer. In our supported bilayer experiments, colicin is first inserted into vesicle membranes followed by reconstitution into the supported bilayer membrane. Hence, in the supported bilayer system, we have a partially inserted channel state at the outset of the conductivity measurements.

4. Conclusions

In situ EIS and PM-IRRAS techniques were used to study the ion channel properties of colicin E1, a channel-forming protein, incorporated into the DPhPC floating bilayer at β -Tg modified gold (111) surface as a function of the transmembrane potential. The EIS results demonstrate that the resistance of the bilayer decreases with the negative scan of the potential, and PM-IRRAS data indicate that the average tilt angle of α -helices of colicin E1 decreases at negative transmembrane potentials. The decrease of the tilt angle of α -helices indicates that they are inserted into the membrane. The concomitant decrease of the membrane resistance indicates that the inserted helices form ion channels in the membrane. Importantly, the potential-controlled formation of the colicin E1 ion channel is reversible. The reversibility of the potential-controlled insertion of α -helices correlates with the concomitant changes of the membrane resistivity and provides support to the voltage-gated mechanism of colicin E1 ion channels in the membrane bilayer.

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Supplementary Materials Available:

the EIS data of the DPhPC floating bilayer at different potentials; Secondary derivative (SD) and Fourier self-deconvolution (FSD) results of the PM-IRRAS spectrum of the DPhPC/colicin floating bilayer; PM-IRRAS spectra of the DPhPC/colicin floating bilayer at different potentials; optical constants of DPhPC/colicin (250:1) vesicles; numerical values of the equivalent circuit elements of the DPhPC floating bilayer; procedures for the calculation of the tilt angle of colicin.

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