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Ion-Pairing Mechanism for the Valinomycin-Mediated Transport of Potassium Ions Across Phospholipid Bilayers

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Abstract

The role of the anion on the ionophore properties of valinomycin was studied in a model floating bilayer lipid membrane (fBLM) using supporting electrolytes containing K^+ with four different counter anions (ClO_4^- , $H_2PO_4^-$, Cl^- and F^-). The electrochemical impedance spectra indicate that the membrane resistance of the bilayer decreases with the decrease of Gibbs free energy of anion solvation. The IR spectra demonstrate that valinomycin does not readily bind to K^+ in the KH_2PO_4 , KCl and KF electrolyte solutions, but in the presence of $KClO_4$, valinomycin readily binds to K^+ forming a valinomycin- K^+ complex. The results in the present paper reveal the role of the counter anion on the transport of cations by valinomycin across the lipid bilayer. The valinomycin-cation complex creates an ion pair with the anion and this ion pair could enter the hydrophobic region of the bilayer and transport cation across the bilayer. Anions with low solvation energies facilitate the formation of the ion pair. This paper sheds new light on the transport

mechanism of valinomycin ionophores and provides new information about the bioactivity of this molecule.

Keywords: valinomycin, floating bilayer lipid membrane, anion, Gibbs free energy of hydration, EIS, PM-IRRAS

1. Introduction

Valinomycin is a cyclic neutral peptide with twelve alternating amino acids, which has ionophore properties in biological cell membranes and artificial lipid bilayers.¹⁻² Valinomycin is selective for alkali cations by forming a complex with K^+ , Rb^+ and Cs^+ , but not with Na^+ or Li^+ .³⁻⁴ XRD data indicates that the valinomycin- K^+ complex adopts a bracelet conformation with a hydrophobic exterior and a polar cavity in the center of the valinomycin peptide where K^+ is coordinated with six ester $C=O$ bonds.⁵ In contrast, free (non-complex) valinomycin assumes a propeller conformation forming hydrogen bonds with the surrounding solvent molecules.⁶

There is a renewed interest in the antibacterial and antiviral properties of valinomycin.⁷ A recent paper reported that valinomycin could potentially be used as a novel antiviral agent against the SARS-CoV-2 virus.⁸ This new application has stimulated efforts to better understand the mechanism of valinomycin bioactivity. Traditional mechanistic studies assume that the polar center of the valinomycin- K^+ complex is surrounded by the hydrophobic exterior in a bracelet conformation. This structure allows the complex to enter the hydrophobic region of a biological membrane and transport alkali cations across the membrane.⁹⁻¹¹ However, several recent reports concerning the ionophore properties of valinomycin show that the mechanism depends on the anion present in the

electrolyte. Shirai et al.¹²⁻¹³ used cyclic voltammetry to study the transmembrane current of valinomycin in the black lipid membrane (BLM) and found that the transmembrane current depends on the Gibbs free energy of hydration of anions. Wittenkeller et al.¹⁴ employed NMR and IR techniques to probe the formation of an ion pair between valinomycin and anions in the lipid vesicles. In our previous study,¹⁵ electrochemical impedance spectroscopy (EIS) and polarization-modulation infrared reflection and absorption spectroscopy (PM-IRRAS) techniques were employed to show that, in a tethered bilayer lipid membrane (tBLM), valinomycin forms the complex with K^+ in $KClO_4$ but not in KF solution. Additionally, we have confirmed that in a floating bilayer lipid membrane (fBLM) of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), valinomycin forms a complex with K^+ in $KClO_4$ but not with Na^+ in $NaClO_4$ solution.¹⁶

The objective of the present study is to investigate the mechanism of potassium transport by valinomycin across the floating bilayer of DPhPC using four different potassium salts ($KClO_4$, KH_2PO_4 , KCl and KF). The chemical structures of valinomycin, DPhPC, and β -Tg are presented in Figure S1A and 3D models of the propeller-type conformation (free valinomycin) and bracelet-type conformer (valinomycin- K^+ complex) are displayed in Figures S1B and C, respectively. The hydration energies of the anions varied between -229 and -515 kJ/mol.¹⁷⁻¹⁸ EIS was employed to measure membrane conductivity while PM-IRRAS allowed determining valinomycin- K^+ complex in the bilayer. Since the IR band intensity is proportional to the concentration of the complex in the membrane, we are able to provide information that the membrane conductivity is proportional to the concentration of the valinomycin- K^+ complex in the bilayer. We also demonstrate that the concentration of the valinomycin- K^+ complex and the membrane conductance similarly depend on the hydration energy of the anion. These results provide

proof that valinomycin is transporting K^+ in phospholipid bilayers as an ion pair with the anion in the surrounding electrolyte. These unique results provide new information concerning the ionophore properties of valinomycin, relevant for the understanding of the nature of its bioactivity.

2. Experiment

DPhPC was purchased from Avanti Polar Lipids (Alabaster, AL, US). Valinomycin (val, 98%), 1-thio- β -D-glucose sodium salt (β -Tg), potassium perchlorate ($KClO_4$, 99%), potassium phosphate monobasic (KH_2PO_4 , 99.0%), potassium chloride (KCl, BioXtra, 99.0%), potassium fluoride (KF, BioUltra, 99.5%), and deuterium oxide (D_2O , 99.9 atom % D) were obtained from Sigma-Aldrich (Oakville, ON, CA). All aqueous solutions were prepared using the ultrapure water from a Milli-Q UV plus (Millipore, Bedford, MA) water system (resistivity $> 18.2 \text{ M}\Omega \text{ cm}$). The single crystal gold (111) electrodes, which were grown, oriented, and cut in-house, were used as the working electrodes in the electrochemical and PM-IRRAS experiments. The reference electrode, purchased from Pine Research Instrumentation, was Ag/AgCl (sat. KCl). All experiments were performed at room temperature at $20 \pm 2 \text{ }^\circ\text{C}$ and all potentials are reported versus the Ag/AgCl (sat. KCl) electrode.

The β -Tg monolayer was deposited onto the gold (111) surface by self-assembly in a 2 mM β -Tg methanol (HPLC grade) solution for 12 hours. The DPhPC/val (9:1, mole ratio) fBLM was constructed on top of the β -Tg monolayer using a combination of Langmuir-Blodgett and Langmuir-Schaefer (LB-LS) methods performed in a KSV Mini-Micro trough (KSV NIMA, Biolin Scientific, Gothenburg, SE). The mole ratio between DPhPC and valinomycin in the bilayer was 9:1. The inner and outer leaflets of the bilayer

were transferred at a constant surface pressure of 30 mN m⁻¹. The procedure of the LB-LS transfer was described in Ref.¹⁶.

Electrochemical and PM-IRRAS measurements were performed in all-glass three-electrode cells deoxygenated with argon (Praxair, Kitchener, ON). EIS was measured using the Solartron SI 1287 electrochemical interface and Solartron SI 1260 impedance/gain-phase analyzer (Ametek Scientific Instruments, Depew, NY) with the ac voltage amplitude of 10 mV rms. PM-IRRAS measurements were carried out with a Nicolet Nexus 8700 spectrometer (Thermo Fisher Scientific, Mississauga, ON) with an external tabletop optical mount (TOM) box. D₂O was chosen as the solvent to avoid the huge IR absorption of water around the 1650 cm⁻¹ region. In each measurement, 4000 scans were collected and averaged with an instrumental resolution of 4 cm⁻¹.

After correction for PEM response functions, the PM-IRRAS signal ΔS is described by:

$$\Delta S = \frac{2(I_s - I_p)}{I_s + I_p} = 2.3A = 2.3\Gamma\varepsilon \quad (1)$$

where I_s and I_p are the intensities of the s - and p -polarized radiation; A , Γ and ε are the absorbance, surface concentration and decimal molar absorption coefficient of the surface species, respectively. The normalized PM-IRRAS spectra were defined as:¹⁶

$$\Delta S_{\text{nor}} = \frac{\Delta S}{\langle E_{p, z=0}^2 \rangle / \langle E_{p0}^2 \rangle} \quad (2)$$

where $\langle E_{p0}^2 \rangle$ is the mean square electric field strength (MSEFS) of the p -polarized incident beam and $\langle E_{p, z=0}^2 \rangle$ is the MSEFS of the p -polarized beam on the gold surface.

The ratio $\langle E_{p, z=0}^2 \rangle / \langle E_{p0}^2 \rangle$ is the surface enhancement, which can be calculated using a

model of five homogeneous, parallel layers (gold/ β -Tg/bilayer/D₂O/CaF₂).¹⁹ The normalized spectra eliminate the influence of the angle of incidence and the thickness of the solution layer on the intensities of the absorption bands and could be used to quantitatively compare the IR results from different measurements.

More details about the experimental procedures and data analysis were described in Ref. ¹⁶.

3. Results and Discussion

3.1 Electrochemical characterization

Initially, the effect of anions on the properties of valinomycin-incorporated fBLMs was investigated by measuring the differential capacitance (DC) of the gold electrode with assembled membranes. Figure 1 compares the DC curves recorded in four different potassium salts. The curves display a minimum within a potential window of ~ 0.2 to -0.3 V vs Ag/AgCl in KClO₄, KH₂PO₄ and KF solutions independent of the nature of the anion. The capacitance increases at more positive or more negative potentials due to electroporation and onset of electrodewetting of the membrane from the gold electrode surface. The capacitance measured in 0.1 M NaClO₄ solution has a minimum at more negative potentials than capacitance curves recorded in KClO₄, KH₂PO₄ and KF solutions. We will show later that this difference is due to the formation of ion pairs between the anion and valinomycin-K⁺ complex and the lack of complexation between valinomycin and Na⁺. The capacitance curve has a significantly different shape in the 0.1 M KCl solution. Figures S2 and S3 of the Supporting Information show that this behavior is caused by the penetration of chloride anions through the membrane to the gold surface and specific adsorption of these anions at potentials higher than -0.1 V vs Ag/AgCl. A monolayer of β -

Tg is unable to suppress adsorption of chloride anions and chloride is partially displacing β -Tg from the electrode surface. However, the capacitance of the gold electrode modified with a monolayer of thioglucose is reduced when a nonpolar phospholipid bilayer (~ 5 nm thick) is deposited on top of thioglucose spacer layer.

Figure 1. Differential capacitance curves of the DPhPC/val fBLM in 0.1 M KClO_4 (1, red), 0.1 M KH_2PO_4 (2, magenta), 0.1 M KCl (3, dark cyan), 0.1 M KF (4, blue) and 0.1 M NaClO_4 (5, black) as a function of the applied potential. The DC curves in KClO_4 and NaClO_4 electrolytes were taken from Ref. ¹⁶.

The differential capacitance plots were determined using a single ac voltage frequency and simplified series RC circuit. Therefore, EIS spectroscopy was employed to more accurately quantify the electrochemical properties of the membrane. Figures 2A and B plot the impedance spectra of the DPhPC/val fBLM in electrolyte solutions consisting of four different anions at $E = 0.0\text{V}$ vs Ag/AgCl . The absolute magnitude of the impedance

is plotted in Figure 2A and the phase angle data is presented in Figure 2B. In addition, Figure S4 to S7 of the Supporting Information shows the EIS data for several selected potentials within the minimum region of the DC curves for all four electrolytes. The points represent experimental data and the solid lines represent the corresponding fits using the equivalent circuit shown in Figure 2A. This circuit was proposed by Valincius et al.,²⁰⁻²¹ based on a physical model describing properties of a phospholipid bilayer separated from the gold electrode surface by a water-rich spacer layer. In this model, R_{sol} is the resistance of the supporting electrolyte and R_m and CPE_m represent the resistance and constant phase element (CPE) of the lipid bilayer, respectively. The spacer region between the bilayer and gold surface is expressed as a constant phase element (CPE_{sp}). The impedance of CPE is defined as:

$$Z_{CPE} = \frac{1}{Q(j\omega)^\alpha} \quad (3)$$

where ω is the angular frequency, Q is the constant phase element coefficient and α is an empirical constant related to the frequency dispersion. The value of α may vary between 0 and 1. CPE behaves as a capacitor when α is close to 1 and as a resistor when α is close to 0. The numerical values of the elements of the equivalent circuits are listed in Tables S1 to S4 of the Supporting Information. For $E = 0.0$ V vs Ag/AgCl, the numerical values of the equivalent circuit are listed in Table 1.

Figure 2 Electrochemical impedance spectra of the DPhPC/val fBLM in different supporting electrolytes at $E = 0.0$ V. The KClO_4 and NaClO_4 data sets were obtained with permission from Ref. ¹⁶.

The electrochemical properties of the water-rich spacer are presented in Figure 3. Figures 3A and 3B plot Q_{sp} and α_{sp} measured for the four electrolytes with respect to the applied electrode potential. For all membrane systems, the values of Q_{sp} decrease, and consequently the impedance values of the spacer layer increase (see Eq 3) when the

Figure 3. Variation of (A) Q_{sp} and (B) α_{sp} of the DPhPC/val fBLM in 0.1 M $KClO_4$ (red circles), 0.1 M KH_2PO_4 (magenta rectangles), 0.1 M KCl (dark cyan diamonds) and 0.1 M KF (blue triangles) as a function of potential. The $KClO_4$ data was taken with permission from Ref. ¹⁶.

potential becomes more positive. The highest values of Q_{sp} are observed in $KClO_4$ indicating the lowest impedance of the spacer layer in this electrolyte. The values of α_{sp} are close to unity for $E \geq 0.0$ V vs Ag/AgCl for all electrolytes. Consequently, the spacer layer has predominantly capacitive properties in the $KClO_4$ and in the KF , KCl , and KH_2PO_4 solutions at $E \geq 0.0$ V vs Ag/AgCl. As the potential is decreased below 0.0 V in KF , KH_2PO_4

and KCl solutions, the values of α_{sp} rapidly decrease achieving a value close to 0.5. Dispersion coefficient values of 0.5 are characteristic of Warburg impedance and suggest that the impedance of the spacer layer becomes controlled by the diffusion of ions through defects or pores formed at negative potentials.

Figure 4. Variation of (A) membrane capacitance (Q_m) and (B) membrane resistance (R_m) of the DPhPC/valinomycin fBLM in 0.1 M KClO_4 (red circles), 0.1 M KH_2PO_4 (magenta rectangles), 0.1 M KCl (dark cyan diamonds) and 0.1 M KF (blue triangles) as a function of potential. The KClO_4 data was taken with permission from Ref. ¹⁶.

The membrane capacitance, Q_m , and resistance, R_m , for the four different potassium electrolytes are illustrated in Figure 4A and 4B, respectively, as a function of the applied potential. The values obtained for the coefficient α_m , in all four systems, are close to unity for all studied potentials implying that Q_m represents the capacitance of the fBLM coated-

gold electrode interface. The values of Q_m are in good agreement with DC plots shown in Figure 1 indicating that the simplified RC circuit used to calculate differential capacitance was a good approximation. The Q_m curves, shown in Figure 2A, of the KClO_4 , KH_2PO_4 and KF electrolytes display a minimum at ~ 0.0 V vs Ag/AgCl and have similar minimum values of $8 \mu\text{F cm}^{-2}$. The equivalent circuit in Figure 2A accounts for both the capacitance of the membrane and spacer layer. These values of Q_m are typical for fBLMs made of DPhPC floating on a β -Tg modified gold electrode surface.²² In the experiments involving the KCl electrolyte, EIS measurements were only performed at potentials where $E \leq 0.0$ V vs Ag/AgCl since Cl^- adsorption occurs at more positive voltages. The membrane resistance values, plotted in Figure 4B, display a maximum at $E \sim 0.0$ V vs Ag/AgCl ($E_{R_{\text{max}}}$) in KF and KH_2PO_4 electrolytes. Table 1 shows that the resistance of the fBLM without valinomycin is equal to $5.9 \text{ M}\Omega \text{ cm}^2$,¹⁶ while the values of R_m are much lower in the presence of valinomycin.

Table 1 Numerical values of the elements in the equivalent circuit for the DPhPC/val fBLM in different supporting electrolytes at $E = 0.0\text{V}$. The ΔG_{hyd} values were taken from Ref. ¹⁷⁻¹⁸. The first column lists the data of the pure DPhPC fBLM (without valinomycin) in KClO_4 solution.¹⁶

Bilayer	DPhPC	DPhPC/val			
Electrolyte	KClO_4	KClO_4	KH_2PO_4	KCl	KF
$-\Delta G_{\text{hyd}} / \text{kJ mol}^{-1}$	229	229	330	381	515
$Q_m / \mu\text{F cm}^{-2} \text{ s}^{\alpha-1}$	9.4 ± 1.0	7.9 ± 0.8	7.8 ± 0.8	17.4 ± 1.7	7.7 ± 0.8
α_m	0.945 ± 0.004	0.924 ± 0.009	0.936 ± 0.005	0.905 ± 0.007	0.952 ± 0.004

$R_m / \text{k}\Omega \text{ cm}^2$	$5.9 \pm 0.4 \times 10^3$	36.3 ± 3.8	143 ± 16	84.7 ± 10	308 ± 30
$Q_{\text{sp}} / \mu\text{F cm}^{-2} \text{ s}^{\alpha-1}$	6.2 ± 0.5	3.4 ± 0.2	1.33 ± 0.06	0.97 ± 0.15	1.13 ± 0.04
α_{sp}	0.500 ± 0.032	0.948 ± 0.028	0.795 ± 0.015	0.992 ± 0.070	0.777 ± 0.015

The presence of valinomycin reduces the barrier properties of the membrane and the membrane resistance depends on the nature of the anion. This dependence is most prevalent around $E = 0.0 \text{ V vs Ag/AgCl}$ and diminishes at more positive or negative potentials. This behavior indicates that at E_{Rmax} , the ionic transport is controlled by the presence of the ionophore, while defects and/or pores may contribute to the non-discriminating transport through the membrane when potentials deviate from E_{Rmax} .

Figure 5 plots the changes in membrane resistance of valinomycin-incorporated fBLMs as a function of the Gibbs free energy of anion hydration in the potassium salts. The results show that R_m increases almost linearly with the absolute value of the hydration energy with the exception of the point corresponding to chloride ions at 0.0 V vs Ag/AgCl , which, as discussed earlier, affects the barrier properties of the fBLM because chloride ions are able to penetrate into the β -Tg monolayer and specifically adsorb onto the gold surface. The quasilinear dependence of R_m on the Gibbs energy of anion hydration indicates that anions with low solvation energies facilitate the transport of K^+ across the bilayer. Consequently, it indicates that anions become incorporated into the membrane when potassium ions are transported across the bilayer. This result is consistent with measurements of conductivity of a lipid membrane with valinomycin (black lipid membrane) separating two solutions of potassium salts reported by Shirai et al.¹²⁻¹³ These authors concluded that in the case of black lipid membranes potassium ions and the counter

anion are moving across the membrane in opposite directions. In the present case using the fBLM, the very high impedance of the spacer layer shows that there is no or very little electrolyte in the spacer layer. Therefore, the potassium ion and the counter anion must move across the fBLM in the same direction. Below, the PM-IRRAS technique will be employed to provide the molecular level interpretation of these results.

Figure 5 Variation of the membrane resistance at $E = 0.0 \text{ V}$ (red circles) and -0.1 V (black rectangles) with respect to the standard Gibbs free energy of hydration of the counter anions in the supporting electrolyte.

3.2 Infrared studies

3.2.1 C-H stretching region

The PM-IRRAS spectra of the acyl chain region of DPhPC and DPhPC/val fBLMs in different supporting electrolytes were similar to those published in Ref. ¹⁶. The representative spectrum is shown in Figure S8A of the Supporting Information. The IR spectrum in the C-H stretching region consists of broad bands containing several

overlapping C-H vibrations under its envelope. The procedures developed by Leitch et al.²³ were employed for the band deconvolution. The deconvolution of the IR spectrum is plotted in Figure S8B. Table 2 lists positions of the symmetric stretching of CH₂ group ($\nu_s(\text{CH}_2)$) and asymmetric stretching of CH₂ group ($\nu_{as}(\text{CH}_2)$) bands for all studied electrolytes. Within experimental uncertainty, the peak positions for these spectra are independent of the nature of the electrolyte and located at 2855 ± 1 and $2927 \pm 1 \text{ cm}^{-1}$, respectively. These values are higher than typical numbers for a gel state bilayer (2850 cm^{-1} and 2920 cm^{-1} , respectively) indicating that the DPhPC bilayers are in a liquid crystalline state. Figure S8C plots the transmission spectrum of valinomycin in d4-MeOH. It illustrates that presence of C-H stretching bands of valinomycin account for small differences between the spectra of fBLM in the absence and presence of valinomycin.

Table 2 Peak centers of the $\nu_s(\text{CH}_2)$ and $\nu_{as}(\text{CH}_2)$ stretching vibrations and tilt angle of acyl chains (θ_{chain}) of the DPhPC and DPhPC/val fBLMs in various 0.1 M supporting electrolyte solutions. The uncertainty of bands positions is $\pm 1 \text{ cm}^{-1}$.

Bilayer	DPhPC	DPhPC/val				
Electrolyte	KClO ₄	NaClO ₄	KF	KCl	KH ₂ PO ₄	KClO ₄
$\nu_s(\text{CH}_2) / \text{cm}^{-1}$	2855	2857	2857	2857	2857	2856
$\nu_{as}(\text{CH}_2) / \text{cm}^{-1}$	2927	2927	2927	2927	2927	2927
$\theta_{\text{chain}} / \text{degree}$	31 ± 3	32 ± 3	34 ± 3	34 ± 3	34 ± 3	37 ± 3

The integrated intensities of the $\nu_s(\text{CH}_2)$ and $\nu_{as}(\text{CH}_2)$ are used to calculate the average tilt angle of trans fragments of the acyl chains, which is used as an approximation for the tilt of the acyl chains (θ_{chain}). The procedure used to calculate θ_{chain} was developed

in Ref. ²³, which is described in detail in the Supporting Information. The calculated values are listed in Table 2. Except for the bilayer in 0.1 M KClO₄, valinomycin has a small effect on the average tilt angle of acyl chains where θ_{chain} varies between $31 \pm 3^\circ$ and $34 \pm 3^\circ$. Conversely, the chains are $\sim 6^\circ$ more tilted in DPhPC/val bilayer than in the pure DPhPC bilayer in 0.1 M KClO₄. The higher tilt angle of the bilayer is caused by the hydrophobic matching of the lipid tails to the length of the inserted valinomycin-K⁺ complex.²⁴ The diameter of the valinomycin-K⁺ complex is ~ 1.3 nm, while the length of the DPhPC chain is ~ 1.5 nm. The tilt of the complex is $26 \pm 3^\circ$.¹⁶ Therefore, the acyl chains of DPhPC must tilt to a larger degree to ensure hydrophobic matching of the lipid interior with the inserted complex.

3.2.2 C=O stretching region

Figure 6A displays the normalized PM-IRRAS spectra of the DPhPC and DPhPC/val fBLMs in the C=O stretching region measured in the various potassium supporting electrolytes. The bottom curve shows the spectrum of the DPhPC bilayer in the absence of valinomycin. This spectrum consists of a single band between 1700 and 1760 cm⁻¹ corresponding to the ester C=O stretching of DPhPC lipid,²⁵ while the spectra of the DPhPC/val bilayers show two distinct regions. The bands between 1600 and 1700 cm⁻¹ refer to the amide I absorption of valinomycin and bands between 1700 and 1780 cm⁻¹ are assigned to the overlapping ester C=O bands from valinomycin and lipids.^{15-16, 26-29} The shape and position of the IR bands depend on the counter anions in the supporting electrolytes. The spectrum in KClO₄ solution is notably different from spectra recorded in the other electrolytes. The PM-IRRAS spectra of the DPhPC/val fBLMs in different electrolytes at different potentials are presented in Figure S9 to Figure S13.

Figure 6 (A) PM-IRRAS spectra and (B) FSD results of the DPhPC/val fBLM in the C=O stretching region in different supporting electrolytes (indicated in the figure) at $E=0.0$ V. The bottom purple curve is the spectrum of the valinomycin-free DPhPC fBLM in KClO_4 .

The Fourier self-deconvolution (FSD) technique, which is a bandwidth sharpening method,³⁰ was used to assist the band deconvolution. The FSD of the PM-IRRAS spectra are plotted in Figure 6B, and the band assignment and positions of the vibration modes are listed in Table 3. In the absence of valinomycin, the DPhPC bilayer has only two peaks located at 1727 and 1742 cm^{-1} corresponding to the hydrated and non-hydrated ester C=O stretching of the lipid.²⁵ The DPhPC/val bilayer in NaClO_4 displays two bands in the amide I region. The band at 1661 cm^{-1} is assigned to free valinomycin in the hydrophobic region of the bilayer while the band at 1680 cm^{-1} is attributed to free valinomycin in the hydrophilic region of the bilayer.^{15-16, 26-29} The ester C=O stretching region of DPhPC/val bilayer in NaClO_4 contains four bands where two bands at 1729 and 1741 cm^{-1} correspond to the hydrated and non-hydrated ester C=O stretching of DPhPC,²⁵ and two bands at 1748

and 1767 cm^{-1} are linked to the ester C=O stretching from free valinomycin in the hydrophilic and hydrophobic regions of the bilayer, respectively.^{15-16, 27-28} In NaClO_4 solutions, valinomycin does not complex with Na^+ , and thus all IR bands correspond to free valinomycin peptides. Consequently, no ion pair is present in sodium salt solutions.

The spectrum of the DPhPC/val bilayer in KClO_4 solution is significantly different from the spectra of NaClO_4 and the other three potassium electrolytes. Two small bands at 1661 and 1680 cm^{-1} are present, which are like those observed for valinomycin peptides in the NaClO_4 spectrum. One key difference is that the KClO_4 amide I spectral region shows an additional strong band at 1649 cm^{-1} , which is attributed to the valinomycin- K^+ complex. Furthermore, the ester C=O stretching region of the KClO_4 spectrum displays a very weak peak at 1767 cm^{-1} representing the free valinomycin peptide in the hydrophobic region, however, the C=O peak at 1748 cm^{-1} for the free valinomycin peptides in the hydrophilic region is completely absent in the KClO_4 spectrum and has been replaced by a strong band at 1757 cm^{-1} . The appearance of the 1649 and 1757 cm^{-1} bands are clear evidence that K^+ is coordinating with valinomycin to form the bracelet-like valinomycin- K^+ complex since these bands are not present in the NaClO_4 electrolyte where valinomycin is unable to form a complex with Na^+ . The IR spectra in KF , KCl and KH_2PO_4 solutions are similar. All three spectra contain strong bands associated with free valinomycin and small bands that are characteristic of the valinomycin- K^+ complex.

Table 3 Band positions and assignment of the DPhPC and DPhPC/val fBLMs in different supporting electrolytes. The data in NaClO₄ solution were obtained from Ref. ¹⁶. The uncertainty of bands positions is $\pm 1 \text{ cm}^{-1}$.

Bilayer	DPhPC	DPhPC/val				
supporting electrolyte	KClO ₄	NaClO ₄	KF	KCl	KH ₂ PO ₄	KClO ₄
valinomycin-K ⁺ complex			1649	1649	1649	1649 (s)
free valinomycin		1661 (s)	1661 (s)	1661 (s)	1661(s)	1661
free valinomycin		1681 (s)	1681 (s)	1681 (s)	1681(s)	1681
hydrated C=O in DPhPC	1727	1729	1726	1728	1727	1727
non-hydrated C=O in DPhPC	1742	1741	1741	1742	1742	1742
free valinomycin		1748	1750	1750	1751	
valinomycin-K ⁺ complex						1757 (s)
free valinomycin		1767 (s)	1767 (s)	1767 (s)	1767 (s)	1767

*(s) indicates the band is strong

To obtain additional quantitative information about the valinomycin-K⁺ complex, the IR spectra were deconvoluted. Figure 7 shows the deconvolution of the DPhPC/val PM-IRRAS spectra recorded in each of the five different electrolytes. The FSD procedure (Figure 6B) was used to identify the specific band positions. Figure 7E shows the deconvoluted PM-IRRAS spectrum of the DPhPC/val fBLM in KClO₄. The peaks

Figure 7 Deconvolution results of the DPhPC/val fBLM in (A) 0.1 M NaClO₄, (B) 0.1 M KF, (C) 0.1 M KCl, (D) 0.1 M KH₂PO₄ and (E) 0.1 M KClO₄ at $E = 0.0V$. The red curves represent the experimentally measured spectra, the blue curves corresponds to the amide I absorption of valinomycin, the dark green curves present the ester C=O stretching modes of DPhPC and the black curves relate to the ester C=O stretching modes of valinomycin.

attributed to the valinomycin- K^+ complex at 1649 and 1757 cm^{-1} are predominant in this spectrum, while the other bands corresponding to free valinomycin are small. In addition, the other three spectra for the fBLMs in potassium electrolytes show strong free valinomycin bands and weak valinomycin- K^+ complex bands indicating that only a small number of valinomycin peptides are bound to K^+ . In contrast, the 1649 cm^{-1} band is completely absent in the NaClO_4 solution since Na^+ ions cannot complex with valinomycin. Therefore, 1649 cm^{-1} band of the complexed valinomycin was integrated and its integrated intensity was plotted in Figure 8 versus the free energy of anion hydration. For comparison, the conductivity of the fBLM ($1/R_m$) is also plotted in this figure.

Figure 8. Variation of the membrane conductance ($G_m=1/R_m$, left y-axis, red circles) and normalized area of the 1649 cm^{-1} band (A_{nor} , right y-axis, black rectangles) as a function of the standard Gibbs free energy of hydration of different anions. Inset shows the variation of the membrane conductance as a function of the normalized area of the 1649 cm^{-1} band.

Figure 8 clearly shows that the intensity of the IR band for the valinomycin complex and the membrane conductivity show similar behaviors with respect to the anion hydration

energy. The inset to Figure 8 shows that the conductivity depends linearly on the absorbance of the 1649 cm^{-1} band of the valinomycin- K^+ complex (as stressed earlier, KCl is outlier due to adsorption of Cl^{-1} ions). The absorbance is proportional to the complex concentration in the bilayer. Therefore, this relation demonstrates that the membrane conductivity is proportional to the concentration of the valinomycin- K^+ complex. Figure 8 also shows that the complex concentration in the membrane depends on the hydration energy of the anion of the bathing electrolyte. These are strong arguments to support the mechanism that the valinomycin- K^+ complex enters the bilayer as an ion pair with the counter anion of the electrolyte.

Table S5 and Figure S14 of the Supporting Information show the ratio of the integrated intensity of the 1649 cm^{-1} band (of the complex) to the sum of integrated intensities of 1649 , 1661 , and 1681 cm^{-1} bands which represents the sum of intensities corresponding bands of the complex and the free valinomycin. This data shows that in 0.1 M KClO_4 solution, the fraction of the valinomycin- K^+ complex is ~ 0.8 , while in the presence of other anions, it is below 0.2 and decreases with the increasing energy of the anion hydration. This data shows that a higher membrane conductivity in the KClO_4 solution is not due to the presence of defects, which are random membrane imperfections. The IR data clearly shows that the conductivity is due to the increased concentration of the complex in the hydrophobic fragment of the membrane.

The interactions of anions with organic molecules or biological membranes are frequently discussed in terms of their structure-breaking (chaotropic) and structure-making (kosmotropic) properties.³¹ The Q_m data in Figure 4A shows that anions have small effect on the membrane capacitance indicating that the differences in their chaotropic and kosmotropic properties have small effect on the membrane structure. However, our data

also shows that the formation of the valinomycin K^+ complex in the bilayer is significantly different in the various electrolytes. These differences correlate well with the hydration energies of anion indicating that the hydration energy is the main factor affecting the ion pair formation between the valinomycin K^+ complex and anion in the fBLM.

The PM-IRRAS experiments were performed at a constant potential where the membrane is exposed to the static electric field. In contrast, the conductivity of the membrane was determined from EIS, which is a dynamic measurement where the data is collected using a 10 mV ac modulation at frequencies ranging from 0.01 Hz up to 1 kHz. The good correlation between absorbance and conductivity (acquired using static and dynamic electric fields) demonstrates that the movements of ions within the membrane assembly were fast at time scales down to 1 ms.

4. Conclusions

EIS and PM-IRRAS techniques were employed to characterize the ionophore properties of valinomycin in the DPhPC fBLM bathed in potassium electrolyte solutions with four different counter anions. The EIS results demonstrate that the conductivity of the bilayer increases with the decrease of the Gibbs free energy of hydration of the anion. The PM-IRRAS spectra demonstrated that valinomycin- K^+ complex is present in the bilayer in all potassium salts solutions. However, its concentration is very low when the Gibbs energy of anion hydration is high. In contrast, the complex concentration is high in the $KClO_4$ solution since the hydration energy of perchlorate is low. The dependence of membrane conductivity and concentration of the valinomycin- K^+ complex in the membrane on the hydration energy of anion are similar indicating that conductivity is proportional to the

concentration of the complex in the membrane. These results provide strong evidence that the transport of ions across the membrane is provided by the valinomycin- K^+ complex, which forms an ion pair with the counter anion. Recent studies of the transport of rhodamine cation through a phospholipid bilayer demonstrated that transport of this cation also involves formation of an ion pair with hydrophobic anion.³²⁻³³

Therefore ion pair formation seems to be a common rule explaining the ion transport by ionophores. This study calls for revision of the old mechanism of ionophore properties of valinomycin that assumes the complex has sufficient hydrophobic properties to enter the hydrophobic region of the bilayer. The present results strongly indicate that it is the ion pair between the complex and the counter anion of the electrolyte that is involved in the charge transfer across the bilayer as depicted in Scheme 1. The present paper sheds new light on the mechanism of valinomycin ionophore properties in lipid bilayers and provides valuable information for understanding the mechanism behind its bioactivity.

Scheme 1 Schematic diagram showing the valinomycin-assisted transport of K^+ cations across the phospholipid bilayer.

Supporting Information Available

Chemical structures of valinomycin, DPhPC and β -Tg; DC curves of the DPhPC/val fBLM and β -Tg SAM; EIS spectra of the DPhPC/val fBLM in different electrolytes; numerical values of the elements in the equivalent circuit of the DPhPC/val fBLM in different electrolytes; PM-IRRAS spectra in C-H region and calculation of the acyl chains tilt angle; PM-IRRAS spectra of the DPhPC/val fBLM in the amide I region in different electrolytes.

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