Impedance Analysis of Ion Transport Through Supported Lipid Membranes Doped with Ionophores: A New Kinetic Approach

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Abstract Kinetics of facilitated ion transport through planar bilayer membranes are normally analyzed by electrical conductance methods. The additional use of electrical relaxation techniques, such as voltage jump, is necessary to evaluate individual rate constants. Although electrochemical impedance spectroscopy is recognized as the most powerful of the available electric relaxation techniques, it has rarely been used in connection with these kinetic studies. According to the new approach presented in this work, three steps were followed. First, a kinetic model was proposed that has the distinct quality of being general, i.e., it properly describes both carrier and channel mechanisms of ion transport. Second, the state equations for steady-state and for impedance experiments were derived, exhibiting the input–output representation pertaining to the model's structure. With the application of a method based on the similarity transformation approach, it was possible to check that the proposed mechanism is distinguishable, i.e., no other model with a different structure exhibits the same input–output behavior for any input as the original. Additionally, the method allowed us to check whether the proposed model is globally identifiable (i.e., whether there is a single set of fit parameters for the model) when analyzed in terms of its impedance response. Thus, our model does not represent a theoretical interpretation of the experimental impedance but rather constitutes the prerequisite to select this type of experiment in order to obtain optimal kinetic identification of the system. Finally, impedance measurements were performed and the results were fitted to the proposed theoretical model in order to obtain the kinetic parameters of the system. The successful application of this approach is exemplified with results obtained for

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valinomycin- K^+ in lipid bilayers supported onto gold substrates, i.e., an arrangement capable of emulating biological membranes.

Keywords Valinomycin · Impedance · Supported membranes · Ionophores

1 Introduction

Ion transport across biomimetic membranes has attracted great attention with the objective of gaining fundamental understanding of this vital process as it takes place in the biological membrane [1, 2] and for the development of potentiometric ionophore-based membrane sensors [3]. The membrane itself represents a high-energy barrier for hydrophilic ions such as K^+ , Na^+ , etc.; however, transport results are facilitated by transporters incorporated into the membrane, in the form of channel-forming peptides [4] or mobile carriers [5] that selectively respond to these alkali cations.

As a cation carrier for potassium transport across cell membranes, valinomycin is of considerable biological importance [6]. It is a cyclodepsipeptide, cyclo(L-Val-D-Hyl-D-Val-L-Lac)₃ that contains a single 36-member ring. This structure confers on the carrier the ability to form lipid-soluble ionic complexes [7]. The general features of the response mechanism of valinomycin-mediated K^+ transport in phospholipid membranes are well understood. However, certain aspects still need to be elucidated, namely, whether there is a rate-limiting step or the location for the site of ionophore-ion complex formation.

Läuger and coworkers [8] have given preliminary evidence, from valinomycin- K^+ and monactin- K^+ complexes in lipid bilayer experiments, that the rate constants of the process can only be individually obtained with the additional use of electrical relaxation techniques. Using voltage-jump experiments, they recognized that experimental data may not have sufficient resolution to identify all of the characteristic time constants of a mechanism [9]. This and other limitations can be overcome by the use of electrochemical impedance spectroscopy (EIS). The advantages of EIS for kinetic and mechanism analysis include the fact that it employs small signal analysis and that it is capable of probing relaxations over a wide frequency range using readily available instrumentation. However, the use of EIS in preference to other relaxation techniques is the result of an identifiability and distinguishability analysis previously performed in order to ensure optimal kinetic identification of the system [10].

In this work, we adopted a new approach to derive kinetic information relevant to mediated transport in supported lipid membranes. The successful application of this approach is exemplified with results obtained for valinomycin- K^+ in dimethyldioctadecylammonium chloride (DODAC)-supported membranes. Appropriate simplifications of the theory allowed us not only to corroborate the experimental findings theoretically but also to shed light on several open questions.

2 Experimental

Lipid vesicles were prepared by mechanical dispersion using a chloroform solution of a mixture containing 80% and 20% w/w egg yolk phosphatidylcholine (from Sigma) and positively charged dimethyldioctadecylammonium chloride (from Sigma), respectively. The solvent was removed by evaporation with nitrogen stream. The dry film was shaken in buffer solution 10 mM Tris (tris-(hydroxymethyl)aminomethane), pH 7.4. The final

concentration of the dispersion was 3% w/v. Typically, this method yields large multilamellar vesicles with varying sizes over 1 μm in diameter. Disruption of vesicles in the suspension using sonic energy (sonication) typically produces small, unilamellar vesicles with diameters in the range of 15–50 nm. Previously, ionophores were added to buffer solutions during sample preparation for the two different systems. Gramicidin D (purchased from Sigma) dissolved in methanol was added to a final concentration of 100 μM , while valinomycin (purchased from Fluka) dissolved in dimethyl sulfoxide was added to a final concentration of 6 μM .

A gold electrode was modified by immersion in 10-mM 3-mercaptopropionic acid (from Sigma) ethanolic solution for 10 min. In this way, a self-assembled mercaptocarboxylic acid monolayer forms directly on gold surfaces. This method provides an adequate substrate for the deposition, on top of this monolayer, of stable self-assembled lipid bilayers by vesicle fusion. The bilayer was formed by dipping the electrode in the vesicle dispersion for 1 h. For the experiments, the buffer solution was replaced by 0.1 M KCl solutions in Tris 10-mM buffer at pH 7.4. Mercaptopropionic acid was employed in order to obtain a negatively charged surface. At the pH of the experiments, the carboxylic group is dissociated, having a negative charge. Subsequent addition and fusion of lipid vesicles generates electrostatic fixation of the bilayer, exhibiting a positive surface charge as a result of using positively charged DODAC in the lipid mixture formulation.

During the surface modification process, capacitance values were obtained from impedance measurements in order to check proper completion of each step.

EIS data were measured with a Solartron SI 1254 device; a 10-mV amplitude sine-wave signal perturbation was applied in the 10 mHz–65 KHz frequency range. The electrochemical cell in a three-electrode configuration contains a saturated calomel reference electrode, a Pt counter electrode, and the modified gold surface as the working electrode. Data were analyzed by using complex nonlinear least-squares fitting based on the Levenberg–Marquard algorithm.

3 Theory

3.1 Background

Bellow, we initially illustrate the experimental system and subsequently outline the corresponding mathematical formulations for the proposed theoretical mechanism.

A system emulating a biological membrane was prepared. It consists of a supported lipid bilayer on gold electrodes fixed by vesicle fusion. At this point, it is perhaps worth mentioning that fusion has been shown to be a charge-induced effect and thus vesicle fusion with bilayer formation rather than vesicle adsorption takes place in this and analogous experimental arrays [11, 12]. In order to analyze K^+ transport through the lipid bilayers, they were conveniently doped with valinomycin as shown in Fig. 1.

In order to obtain one general expression that involves different processes of ion translocation through membranes facilitated by ionophores, we will consider both basic mechanisms for facilitated ion transport, namely channels and carriers.

In the presence of an ion-channel-forming peptide as gramicidin, ionic species M^+ arrives at the aqueous–membrane interface, adsorbs at the mouth of an active channel, and incorporates into it. This process can be described by first-order interfacial transfer kinetics [13–15]. Subsequently, the cation permeates the bilayer through the channel by a second

Fig. 1 Schematic representation of the experimental system showing a supported bilayer doped with valinomycin

electrochemical step, which is characterized by a single activation energy barrier, according to de Levie's [13] single jump model.

The process of ion transport mediated by the neutral carrier valinomycin is initiated by the interfacial reaction of a 1:1 valinomycin- K^+ complex formation. According to the single jump approach adopted here, the height of the barrier determines that, at any time, most of the ions and molecules in the membrane will reside in energy wells on both sides of the barrier. Consequently, concentrations of the neutral carrier and the K^+ -complex are expressed in terms of moles per unit membrane area rather than volume concentrations. Indirect evidence of the validity of this assumption originates in studies with different systems that exhibit similar interfacial kinetics, like two immiscible electrolyte solutions. Thus, these studies suggest that the alkali cation is transferred from the aqueous phase to the organic phase at an active interfacial site, in a single step [16, 17]. Moreover, studies by molecular dynamics simulations hint at conformational changes in the carrier molecule in the nonhomogeneous environment of the interface between the aqueous solution and the membrane. A corollary to this is that the simulations demonstrate that the neutral carrier concentrates at the interface and does not primarily reside in the membrane interior [18].

In the valinomycin- K^+ complex the alkali ion is situated inside the ring-shaped valinomycin molecule, forming an electrically charged complex with compact and globular conformation. In a second step, this species surmounts a potential barrier in order to cross the membrane. The reverse process occurs at the opposite membrane interface, i.e., the ion is transferred to the aqueous phase [19].

Consequently, the kinetic mechanism can be described by a first step corresponding to a surface reaction involving an ion M^+ from the aqueous phase and a carrier S from the membrane (1). The form of (1) is the result of a location at the interface for the ionophore-ion complex formation as indicated by Senda et. al. [20]. This reaction step is characterized by the formation and dissociation interfacial rate constants k_r and k_d for MS^+ . The second step corresponds to the translocation process characterized by the rate constant k_{MS} for the charged complex MS^+ (2).

For the sake of simplicity, we assume as previously suggested by de Levie [13] that all membrane properties are symmetrical with respect to the plane equidistant from each side of the membrane ($x=0$ or $x=d$).

where $MS_{x=0}^+$ and $MS_{x=d}^+$ correspond to the complex at each side of the membrane.

The potential (E) dependence of the rate constants can be expressed by an exponential law:

$$k_i = k_i^o \exp(b_i E) \quad (3)$$

$$k_{-i} = k_{-i}^o \exp(b_{-i} E) \quad (4)$$

where $b_i = -\alpha F/RT$ and $b_{-i} = (1 - \alpha)F/RT$, k_i^o and k_{-i}^o denote standard rate constants, α is charge transfer coefficient ($0 < \alpha < 1$), F is the Faraday constant ($\approx 96,500$ C/mol), R is the gas constant and T is the absolute temperature.

Finally, the following two assumptions were also made in writing the formalism:

1. Because the steady-state condition selected for the experiments entails a cathodic polarization, the backward anodic reaction is considered negligible.
2. No reaction step is considered to be rate determining. This implies a relaxation of the usual condition of a fast reaction under equilibrium for step 1 [8].

3.2 Derivation of the Fundamental Equations

The maximum number of sites per unit surface of the membrane interface consisting of neutral membrane-confined carriers which can be reached by the species M^+ is characterized by the coefficient β and the fraction of these sites actually occupied forming complex molecules by X ($0 < X < 1$). As a consequence, the surface concentration of free valinomycin molecules associated with electroactive sites is given by $\beta(1-X)$.

Mass balance for the transport process can be calculated by the expression:

$$\beta \frac{dX}{dt} = k_R(1-X)c - k_D X - k_{MS} X \equiv g(E, c, X) \quad (5)$$

while the overall faradaic current, I , according to the proposed reaction scheme is given by

$$I = FA[k_R(1-X)c - k_D X + k_{MS} X] \equiv I(E, c, X). \quad (6)$$

Two experimental procedures will be considered that differ according to the type of input (perturbing potential signal): steady-state experiments, where a constant input $E(t)$ takes various constant values, and impedance experiments, where $E(t)$ takes the form of a sinusoidal wave of small amplitude.

3.2.1 Steady-State Analysis

Under steady-state conditions, $dX/dt=0$ and the fractional coverage of the surface can be expressed as

$$X^{ss} = \frac{k_R c}{k_R c + k_D + k_{MS}} \quad (7)$$

and the steady-state forward faradaic current as

$$I^{ss} = 2FA \frac{k_R c k_{MS}}{k_R c + k_D + k_{MS}} \quad (8)$$

where A represents the electrode area and c the aqueous concentration of M^+ at the membrane surface. It is clear that $k_R c$ and k_{MS} in (8) are interchangeable and that there are two sets of kinetic parameters that represent two possible solutions. Hence, the parameters are not identifiable from this steady-state study. This ambiguity may be removed by carrying out several experiments, using different values for c , although impedance spectroscopy can be expected to improve identifiability compared with steady-state experiments [21]. Analogously, Stark et al. [8] showed that from steady-state conductance measurements only certain combinations of the constants can be obtained. In this case, these authors were able to determine individual values for each parameter by using additional electrical relaxation measurements, namely, by recording the membrane current transient after the application of a voltage jump.

3.2.2 Impedance Spectroscopy Analysis

In order to derive an expression for the reaction impedance, mass and charge balances $g(E, c, X)$ and $I(E, c, X)$ ((5) and (6), respectively) should be linearized for small amplitudes of the perturbing signal, according to a Taylor series expansion, retaining only terms with first-order derivatives:

$$\beta \frac{d\Delta X}{dt} = \left(\frac{\partial g}{\partial E} \right) \Delta E + \left(\frac{\partial g}{\partial X} \right) \Delta X + \left(\frac{\partial g}{\partial c} \right) \Delta c \quad (9)$$

$$\Delta I = \left(\frac{\partial I}{\partial E} \right) \Delta E + \left(\frac{\partial I}{\partial X} \right) \Delta X + \left(\frac{\partial I}{\partial c} \right) \Delta c. \quad (10)$$

Partial derivatives in (9) and (10) correspond to steady-state conditions. Considering the application of a small ac perturbation signal $\Delta E = \tilde{E} \exp(j\omega t)$ and the resulting sinusoidal responses ΔI for the faradaic current, Δc for the ion concentration, and ΔX for the fraction of occupied sites, (9) and (10) can be rewritten as

$$\beta j\omega \frac{\Delta X}{\Delta E} = \left(\frac{\partial g}{\partial E} \right) + \left(\frac{\partial g}{\partial X} \right) \frac{\Delta X}{\Delta E} + \left(\frac{\partial g}{\partial c} \right) \frac{\Delta c}{\Delta I} \left(-\frac{1}{Z} \right), \quad (11)$$

$$-\frac{1}{Z} = \frac{\Delta I}{\Delta E} = \left(\frac{\partial I}{\partial E} \right) + \left(\frac{\partial I}{\partial X} \right) \frac{\Delta X}{\Delta E} + \left(\frac{\partial I}{\partial c} \right) \frac{\Delta c}{\Delta I} \left(-\frac{1}{Z} \right) \quad (12)$$

where $Z = -\Delta E / \Delta I$ is the faradaic impedance (the negative sign arises from the assumed convention in which the cathodic current is positive).

In order to evaluate $\frac{\Delta c}{\Delta I}$, we must remember that the change in the concentration c of species M^+ , which diffuses from the solution bulk, is ruled by Fick's second law. Considering linear diffusion in the direction x perpendicular to the electrode surface with diffusion coefficient D and c varying with time according to a sinusoidal wave, Fick's second law becomes:

$$j\omega\Delta c = D \frac{\partial^2 \Delta c}{\partial x^2}. \quad (13)$$

The resulting Δc is obtained from the general solution for (13):

$$\Delta c = L \exp\left(\sqrt{j\omega/D}\right) + M \exp\left(-\sqrt{j\omega/D}\right). \quad (14)$$

The integration constants L and M can be calculated from the boundary conditions that can be expressed (following the semi-infinite linear diffusion hypotheses on the thickness of the diffusion layer) as [22]:

- for $x \rightarrow \infty$, $\Delta c = 0$, i.e., the condition of a concentration independent of time and
- for $x = 0$, $F\Delta J = -\Delta I$, expressing the continuity of the flux J .

In this case, $L = 0$ and hence:

$$\Delta c = M \exp\left(-x\sqrt{j\omega/D}\right) \quad (15)$$

according to Fick's first law:

$$\Delta J(x, \omega) = -D \frac{\partial \Delta c}{\partial x} = -\frac{\Delta I}{FA} \quad (16)$$

so that

$$\Delta I = -MFAD \cdot \exp\left((-x)\sqrt{j\omega/D}\right) \cdot \sqrt{j\omega/D} \quad (17)$$

hence, taking into account (15) results in

$$\frac{\Delta c}{\Delta I} = -\frac{N(\omega)}{FA} \quad (18)$$

where

$$N(\omega) = +\frac{1}{\sqrt{j\omega D}}. \quad (19)$$

From (11) and (12), applying Cramer's rule, Z can be calculated. After evaluating the derivatives in these equations with $\alpha = 0.5$ and replacing X by its steady-state value, the final expression for the impedance can be obtained:

$$Z = \frac{j\omega + \frac{Q}{\beta} + PNj\omega + 2PN \frac{k_{MS}}{\beta}}{R\left(j\omega cP + 2cP \frac{k_{MS}}{\beta}\right)} \quad (20)$$

where $P = \frac{k_R(k_D + k_{MS})}{k_R C + k_D + k_{MS}}$ $Q = k_R C + k_D + k_{MS}$ $R = \frac{F^2 A}{RT}$ and $N = + \frac{1}{\sqrt{j\omega D}}$.

3.3 Identifiability and Distinguishability

The goal of this approach is to obtain optimal identification of the system in the form of a set of kinetic parameters that accurately reproduce experimental results when they are used in connection with a given theoretical model structure. If a finite number of parameters sets can be determined for a given input and for a given mechanism, then the mechanism is nonidentifiable. A mechanism is globally identifiable if and only if a unique set of parameters values can be found. Another question is whether several mechanisms can reproduce the experimental results with the same accuracy. If two different mechanisms have the same parameter set values giving the same output for a given input, then the mechanisms are nondistinguishable [10].

Fig. 2 **a** Nyquist and **b** Bode plots for a phospholipid bilayer with added valinomycin in 0.1 M KCl. Experimental data (circles) and simulated curves (line)

In this case, it can be shown that the proposed model has a single solution and thus it is globally identifiable by impedance spectroscopy. A detailed treatment for our model applied to ion transport facilitated by gramicidin channels can be found in [23]. Moreover, the proposed mechanism is distinguishable from all other possible mechanisms. Several authors described procedures that allow to check whether a given mechanism is globally or locally identifiable, as well as to find all the possible mechanisms having the same input–output model representation [24]. In this case, applying a method developed by Milocco [25] and based on the similarity transformation approach [26], it was possible to confirm that the proposed mechanism is distinguishable, i.e., no other model with a different structure exhibits the same input–output behavior, for any input, as the original. Thus, our model does not represent a theoretical interpretation of the experimental impedance but rather constitutes the prerequisite to select this type of experiment in order to obtain optimal kinetic identification of the system.

4 Results and Discussion

Impedance spectroscopy and cyclic voltammetry were used in previous works to confirm membrane integrity in this experimental array. However, the stringent specification of a very high coverage required to use this membrane in a sensor was checked in this work through the significant change in the net ion flux across the membrane due to the incorporation of the ionophore and not due to “leaky” flux through local disruptions in the membrane. Moreover, the measured potentiometric signal dependent on the aqueous ion concentration in a Nernstian manner across two-decade change in concentration, as reported previously [27], is a clear indication that the system response is due to the ion flux through the membrane.

Figure 2 displays experimental and fitted impedance spectra for a valinomycin-doped bilayer in 0.1 M KCl. A complex-plane representation (Fig. 2a) exhibits capacitive response with little structure, which contains at least two overlapped time constants. Hence, in a Bode representation (Fig. 2b), two overlapped phase maxima are present. A good agreement between experimental and simulated data was found, even though theoretical

Fig. 3 Steady-state polarization curve for a phospholipid bilayer with added valinomycin in 0.1 M KCl. Experimental data (*circles*) and simulated curve (*line*) according to the proposed model

propositions contain a number of simplifications. In turn, these simplifications could account for the deviations in Bode plots in the low-frequency range. As a result of the fitting procedure, the following individual values for the rate constants were obtained: $k_R = 4.33 \times 10^4$, $k_D = 1.46 \times 10^4$, and $k_{MS} = 5.27 \times 10^3$.

Figure 3 shows an experimental I^{ss} vs E polarization curve. The theoretical current trace is also shown in this figure. It was calculated with the theoretical expression for the dc current and the set of parameters estimated in the fitting procedure of the impedance data. It is here perhaps worth emphasizing that a single set of parameters shows good agreement between the model and experimental data obtained with both steady-state and impedance measurements. Individual values for the rate constants obtained in this work exhibit an excellent agreement with those available from the literature for a comparable experimental system containing a black lipid membrane (BLM) [28].

These results not only validate the model but also suggest that the assumptions made in writing the formalism are correct. Thus, it is possible to state that no reaction step is rate determining and that most every ion and molecule in the membrane is located at a fixed position on either side of the barrier. Finally, the results also indicate that lipid membranes supported on metallic electrodes exhibit analogous behavior to that of BLMs but with superior mechanical properties.

5 Conclusion

A new kinetic approach was presented that expands the established ways to apply impedance spectroscopy in structural characterization studies of supported lipid bilayers. A kinetic mechanism was introduced exhibiting the distinct quality of being general, i.e., it properly describes mechanisms of ion transport assisted by ionophores of different nature (mobile carriers like valinomycin and channels like those of gramicidin). The simplifications of the theory presented here both confirm the experimental findings and illuminate several open questions.

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