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Measurement of Dipole Potential in Bilayer Lipid Membranes by Dielectric Spectroscopy

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Abstract Planar bilayer lipid membranes formed from egg phosphatidylcholine in aqueous media containing the lipophilic anion, dipicrylamine (DPA), were studied by dielectric spectroscopy over a frequency range of 10 Hz–10 MHz. The membranes showed dielectric relaxation due to the translocation of DPA between the membrane interfaces. Incorporating either cholesterol or 6-ketocholestanol into the membranes increased the characteristic frequency of the relaxation, which is proportional to the translocation rate constant of DPA. The results suggested that the sterol dipoles induced positive potential changes within the membrane interior. The changes of the dipole potential were 70 mV for cholesterol and 150 mV for 6-ketocholestanol when the sterol mole fraction was 0.67. The opposite effect was caused by phloretin added to the aqueous media, and the maximum dipole potential change was −90 mV at 100 μM.

Keywords Dielectric relaxation · Lipid bilayer membrane · Lipophilic anion · Dipicrylamine · Dipole potential · Dielectric spectroscopy

Introduction

It is known that there is a positive electrostatic potential within a bilayer lipid membrane (BLM) originating from alignment of dipolar residues of lipids and water molecules (Clarke 2001; Wang 2012). The potential is termed dipole potential and is thought to be important in regulating the functions of membrane proteins (Maggio 1999; Starke-Peterkovic et al. 2005) and the interactions of various bioactive compounds with the membranes (Cladera and O'Shea 1998; Alakoskela et al. 2004; Luchian and Mereuta 2006). The dipole potential has been measured using several techniques that target on lipid monolayers, planar BLMs, lipid vesicles, and cells (Clarke 2001; Wang 2012). The values estimated by different techniques, however, are no longer in good agreement with each other, and improvements in the measurement techniques are required.

The dipole potential in planar BLMs has been estimated from the ion translocation rates of lipophilic ions and ionophore-ion complexes in BLMs following the membrane potential model of Flewelling and Hubbell (1986). The ion translocation rates are obtained by the voltage jump and charge-pulse experiment (Ketterer et al. 1971; Benz and Cros 1978; Pickar and Benz 1978; Benz 1988; Peterson et al. 2002) and the steady-state conductance measurement (Efimova and Ostroumova 2012; Ostroumova et al. 2013). Ketterer et al. (1971) first proposed a kinetic model for the translocation of lipophilic ions in BLMs and analyzed time-dependent current in response to a step voltage applied to BLMs. The time-dependent current predicts frequency dependence of the membrane capacitance and conductance, i.e., dielectric relaxation of the membrane. Indeed, the relaxation of the membrane capacitance induced by lipophilic anions has been measured by dielectric (or impedance) spectroscopy in a few studies (De Levie et al. 1974; De Levie and Vukadin 1975; Pickar and Brown 1983). The dielectric relaxation, however, has not been analyzed in connection to the dipole potential. On the other hand, the dielectric relaxation of the membrane due to lipophilic anions has been studied with single cells by the electrorotation technique (Sukhorukov and Zimmerman 1996; Kürschner et al. 1998; Sukhorukov

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et al. 2001; Zimmermann et al. 2008) and with suspensions of cells and lipid vesicles by dielectric spectroscopy (DS) (Asami 2013). Sukhorukov et al. (2001) estimated the dipole potential changes in the membranes of cultured cells by addition of phloretin.

In this study, we demonstrate that DS is a useful tool for estimating the changes of the dipole potential in planar BLMs. In addition, the effects of typical dipole potential modifiers (cholesterol, 6-ketokorestanol and phloretin) are examined. In general, DS uses a small ac voltage to provide a minimal perturbation to the membrane and is relatively insensitive to external electrical noises. Since DS is also applicable to lipid vesicles, DS would enable us to directly compare the dipole potential between planar BLMs and lipid vesicles formed from the same lipids. This is another merit of DS in dipole potential measurement.

Materials and Methods

Planar BLMs were formed on a Teflon support with a circular hole (1 mm in diameter) by a painting method. The membrane forming solution contained egg phosphatidylcholine and sterol in n-decane at different mole fractions. The total lipid concentration was 0.033 M, and either cholesterol or 6-ketocholestanol was used as the sterol. The aqueous phases of the membrane were 0.1 M KCl with and without 2 μM DPA. Effects of phloretin were examined by adding phloretin to the aqueous phases at 10–100 μM. Platinized platinum electrodes were placed in the aqueous phases for dielectric measurement.

Admittance was measured across the membrane between the electrodes with a 4192A Impedance Analyzer (Hewlett-Packard) at an applied voltage of 10 mV_{rms}. Measurement temperature was 24–26 °C.

The formation of the BLMs was monitored with a VH-5500 digital microscope (Keyence, Osaka, Japan), and the digital images were analyzed by ImageJ (obtained from http://rsbweb.nih.gov/ij/) to determine the membrane area.

Results and Discussion

Dielectric Spectra of BLMs in the Absence and Presence of DPA

Planar BLMs were formed from a mixture of egg phosphatidylcholine (PC) and cholesterol (CL) (CL mole fraction: 0.67) in 0.1 M KCl in the absence and presence of 2 μM DPA. The admittance Y of the system including the membrane and the aqueous media was measured as a function of the frequency f of the applied ac field and was converted into the equivalent capacitance C and conductance G using the relation of $Y = G + j\omega C$ with $\omega = 2\pi f$ and the imaginary unit j. The frequency dependence of the C and G is shown in Fig. 1. In the absence of DPA, the C and G showed dielectric relaxation that was represented by a Debye type function and is due to charging of the membrane through the aqueous media. When DPA was added to both of the aqueous media symmetrically, another dielectric relaxation appeared at low frequencies.

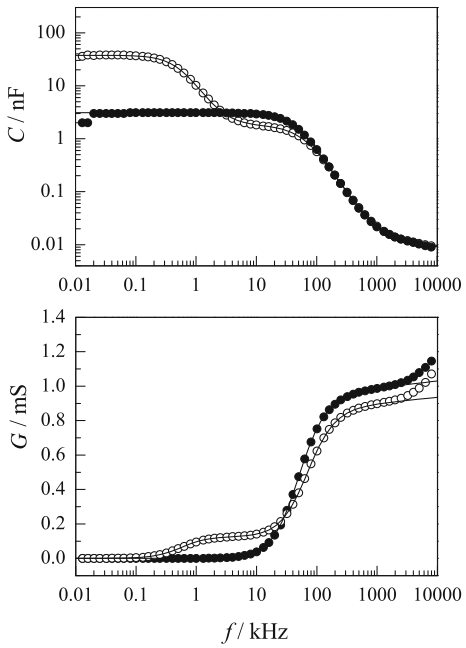

Fig. 1 The capacitance C and conductance G of egg PC-CL membranes (CL mole fraction: 0.67) in 0.1 M KCl in the absence (*black circle*) and presence (*white circle*) of 2 μM DPA. The surface area of the membranes was 0.77 mm^2 (*black circle*) and 0.58 mm^2 (*white circle*). The *solid lines* were the best-fit curves calculated assuming one (*black circle*) and two (*white circle*) Debye type functions

The membrane-medium system can be modeled by a combination of the impedances of the membrane and the aqueous medium in series. The admittance of the system Y is, therefore, related to the admittances of the membrane Y_m and the aqueous medium Y_a as

$$\frac{1}{Y} = \frac{1}{Y_m} + \frac{1}{Y_a} \tag{1}$$

where $Y_m = (G_m + j\omega C_m)S$ with the specific membrane conductance G_m and capacitance C_m and the membrane area S, and $Y_a = G_a + j\omega C_a$ with the medium conductance G_a and capacitance C_a.

The values of C_a and G_a can be obtained by extrapolating those of C and G to high frequencies, where the membrane is "short-circuited" due to displacement current. Hence, Y_m is calculated from Eq. (1) and is converted into the complex membrane capacitance C_m^* as:

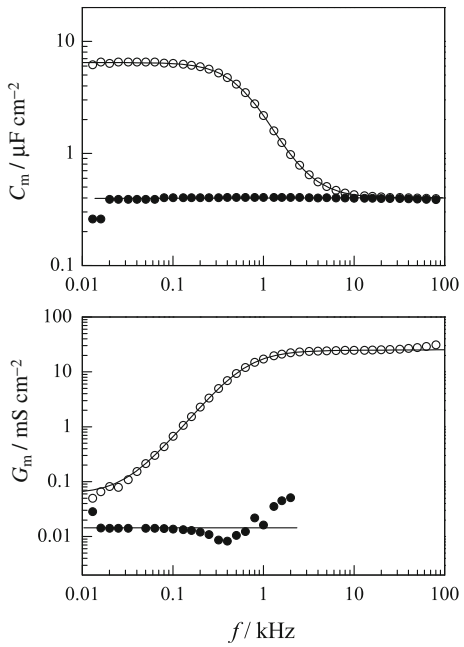

Fig. 2 The capacitance C_m and conductance G_m of the membranes as a function of frequency f in the absence (*black circle*) and presence (*white circle*) of 2 μM DPA. The C_m and G_m were calculated from the data shown in Fig. 1. The *solid lines* through data points (*white circle*) are the best-fit curves calculated from Eqs. (4) and (5) with $C_{mh} = 0.40$ μF cm^{-2}, $\Delta C_m = 6.07$ μF cm^{-2}, $\tau_m = 0.250$ ms, $G_{ml} = 0.06$ mS cm^{-2}, and $\Delta G_m = 24.3$ mS cm^{-2}

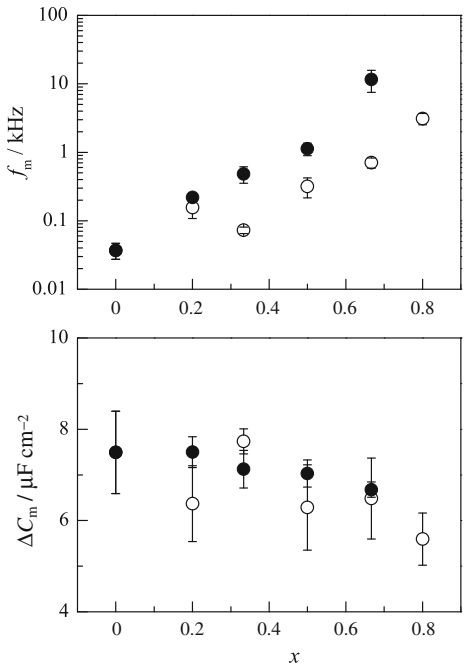

Fig. 3 The relaxation parameters f_m and ΔC_m of egg PC-sterol membranes as a function of the mole fraction x of sterol. Each *symbol* represents the mean ± SD: $n = 6$–22 for cholesterol (*white circle*) and $n = 12$–14 for 6-ketocholestanol (*black circle*)

$$C_m^* \equiv C_m + \frac{G_m}{j\omega} = \frac{Y_m}{j\omega S}. \tag{2}$$

Figure 2 shows the frequency dependence of the C_m and G_m calculated from the data in Fig. 1. In the absence of DPA, the C_m was independent of frequency up to 100 kHz, and the G_m was flat at least below 1 kHz. The data of G_m for the bare BLM are not shown at frequencies above a few kHz, where the calculation of G_m includes large errors in the case of $G_m \ll G_a$. In the presence of DPA, however, marked dielectric relaxation was found around 1 kHz, which was represented by a Debye type function.

$$C_m^* = C_{mh} + \frac{\Delta C_m}{1 + j\omega\tau_m} + \frac{G_{ml}}{j\omega} \tag{3}$$

where C_{mh} is the high-frequency limit of the membrane capacitance, ΔC_m is the intensity of the relaxation, τ_m is the relaxation time, and G_{ml} is the low-frequency limit of the membrane conductance. Separating the real and imaginary parts of C_m^* in Eq. (3), C_m and G_m are represented by

$$C_m = C_{mh} + \frac{\Delta C_m}{1 + (\omega\tau_m)^2} \tag{4}$$

$$G_m = G_{ml} + \frac{\Delta G_m (\omega\tau_m)^2}{1 + (\omega\tau_m)^2} \tag{5}$$

where $\Delta G_m = \Delta C_m/\tau_m$. The values of C_{mh}, ΔC_m, G_{ml}, and ΔG_m were obtained by fitting Eqs. (4) and (5) to the spectra of C_m and G_m.

Effects of Cholesterol, 6-Ketocholestanol, and Phloretin

Cholesterol (CL), 6-ketocholestanol (KC), and phloretin (PL) are known as dipole potential modifiers (Franklin and Cafiso 1993; Starke-Peterkovic et al. 2006; Efimova and Ostroumova 2012). CL and KC provide positive changes in the membrane dipole potential, whereas PL does negative ones. The effects of the modifiers on the dielectric relaxation of the membrane have been studied.

The dielectric relaxation parameters $f_m = 1/(2\pi\tau_m)$ and ΔC_m were determined when the mole fraction x of the sterols in egg PC membranes was varied, being shown in Fig. 3. The value of f_m increased markedly with x, and the increment of f_m for KC was about ten times larger than that for CL, comparing at the same x. On the other hand, the value of ΔC_m decreased slightly with x and there was a little difference between the sterols.

Effects of PL have been studied with egg PC-CL membranes of $x = 0.67$ by varying the PL concentration c_p in both of the aqueous media from 10 to 100 μM (Fig. 4). The f_m decreased with c_p and level off above 50 μM. PL reduced the ΔC_m largely compared with the sterols.

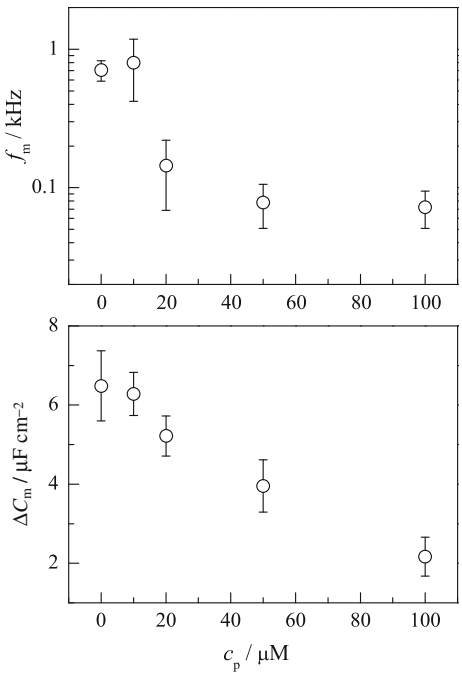

Fig. 4 The relaxation parameters f_m and ΔC_m of egg PC-CL membranes (CL mole fraction: 0.67) as a function of the phloretin concentration c_p in the aqueous medium. Each *symbol* represents the mean ± SD (n = 11–17)

Translocation of Mobile Ions in the Membrane and Dipole Potential

The dielectric relaxation of the membrane is due to the translocation of DPA (monovalent anion) across the membrane in response to the applied ac field. DPA ions distribute between the membrane interface and the aqueous medium; c is the DPA concentration in the aqueous medium, and β and k are the partition coefficient and the rate constant of the ions, respectively. The ions adsorbed at the membrane interfaces move across the membrane at rate constant k_i. According to the kinetic model of Ketterer et al. (1971), the dielectric relaxation parameters in Eqs. (3)–(5) are related to the kinetic parameters as:

$$\Delta C_m = \frac{F^2}{RT}\frac{k_i}{(k+2k_i)}\beta c \tag{6}$$

$$\tau_m = \frac{1}{2\pi f_m} = \frac{1}{k+2k_i} \tag{7}$$

$$G_{ml} = \frac{F^2}{RT}\frac{kk_i}{k+2k_i}\beta c \tag{8}$$

$$\Delta G_m = \frac{F^2}{RT}\beta c k_i \tag{9}$$

where F, R, and T have their usual meanings. The derivation of Eqs. (6)–(9) are described in Appendix.

Since the experimental results showed that the value of ΔG_m was much larger than that of G_{ml} (see Fig. 2), the relation of $k_i \gg k$ holds for DPA-doped BLMs, and therefore ΔC_m, f_m, and G_{ml} are approximately written as $\Delta C_m = \left(F^2/RT\right)(\beta c/2)$, $f_m = k_i/\pi$ and $G_{ml} = \left(F^2/RT\right)(k\beta c/2)$. Since ΔC_m is directly proportional to βc (the concentration of ions adsorbed at the interface), the experimental results on ΔC indicate that the adsorption of DPA is not influenced by CL and KC but is inhibited by PL as described in previous papers (Franklin and Cafiso 1993; Sukhorukov et al. 2001). The k_i that is equal to πf_m is seriously affected with the dipole potential modifiers.

The change of the dipole potential $\Delta\psi_d$ induced by a modifier is related to the transport kinetics of lipophilic ions (Benz and Cros 1978; Pickar and Benz 1978):

$$\Delta\psi_d = \frac{RT}{F}\ln\frac{\beta k_i}{\beta^0 k_i^0} \tag{10}$$

where k_i^0 and β^0 are, respectively, the translocation rate constant and the partition coefficient in the absence of the modifier. Equation (10) is rewritten using the dielectric parameters f_m, ΔC_m, and ΔG_m given by Eqs. (6), (7) and (9):

$$\Delta\psi_d = \frac{RT}{F}\ln\frac{f_m\Delta C_m}{f_m^0\Delta C_m^0} = \frac{RT}{F}\ln\frac{\Delta G_m}{\Delta G_m^0} \tag{11}$$

where superscript 0 refers to the membrane without the modifier, which is either the egg PC membrane without sterol or the egg PC–CL membrane (x = 0.67) in the absence of PL.

Figure 5a shows the change of dipole potential as a function of the mole fraction x of sterol. The value of $\Delta\psi_d$ increases with x and the increment for KC is about twice that for CL ($\Delta\psi_d$ = 70 mV for CL and $\Delta\psi_d$ = 150 mV for KC at x = 0.67). This is consistent with the fact that the dipole moment of KC perpendicular to the membrane surface is twice compared with that of CL (Starke-Peterkovic et al. 2006). There are a few reports on the values of $\Delta\psi_d$ in egg PC membranes induced with sterols, and those vary widely among different measurement techniques. Benz and Cros (1978) studied the influence of sterols on transport of lipophilic ions through planar BLMs, and reported that CL little influenced the dipole potential in dioleoyl PC membranes but induced about 40 mV for monoolein membranes, which was calculated at x = 0.67 from their data on the ion transport. The $\Delta\psi_d$ of about 50 mV was obtained with vesicles of egg PC–KC (x = 0.15) by EPR measurement with spin-labeled lipophilic ions (Franklin and Cafiso 1993), corresponding to 220 mV at x = 0.67. Starke-Peterkovic et al. (2006) used voltage-sensitive dyes to probe electrostatic potential in the

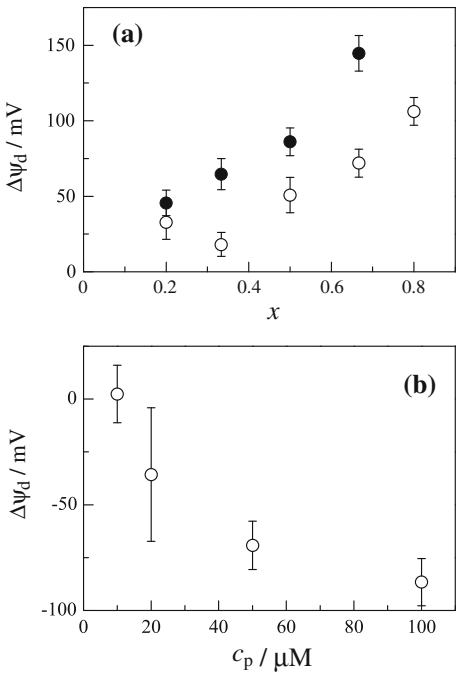

Fig. 5 The change in dipole potential $\Delta\psi_d$ as a function of **a** the mole fraction x of sterol and **b** phloretin concentration c_p. In **a**, cholesterol (*white circle*) and 6-ketocholestanol (*black circle*). The values of $\Delta\psi_d$ were calculated from the data in Figs. 3 and 4. Each *symbol* represents the mean ± SD

membranes, and the value of $\Delta\psi_d$ was about 210 mV for egg PC–CL vesicles ($x = 0.67$).

The value of $\Delta\psi_d$ decreases with increasing PL concentration c_p and tends toward saturation at 100 μM, where the value of $\Delta\psi_d$ was −90 mV (Fig. 5b). Efimova and Ostroumova (2012) estimated $\Delta\psi_d$ from changes of the steady-state conductance of planar BLMs induced by a complex of a cation with ionophore. They obtained similar saturation curves of $\Delta\psi_d$ and the maximum reductions between −90 and −150 mV depending on the lipid used. Cseh and Benz (1998) measured $\Delta\psi_d$ in lipid monolayers and bilayers as a function of c_p. The values were −150 mV for egg PC bilayers and −100 mV for egg PE bilayers at 100 μM PL, obtained by charge-pulse measurement. The dipole potential change in lipid monolayers measured by the vibrating plate method was about twice as high as that in corresponding lipid bilayers. Franklin and Cafiso (1993) obtained the $\Delta\psi_d$ of about −170 mV for egg PC vesicles containing 15 mol % PL by EPR measurement with spin-labeled lipophilic ions. The BLMs formed from different lipids showed the values of $\Delta\psi_d$ ranging between −90 and −170 mV at the maximum adsorption of PL. Nevertheless, small values of $\Delta\psi_d$ (−10 to −15 mV) were found for Human lymphoid Jurkat cells at $c_p \geq 25$ μM by the electrorotation method (Sukhorukov et al. 2001). It is uncertain whether the large difference between artificial lipid membranes and biological membranes comes from differences in their compositions or in the measurement techniques including probe molecules.

Concluding Remarks

This study has demonstrated that the dipole potential changes in planar BLMs induced by modifiers are determined by DS. Since DS is also applicable to lipid vesicles (Asami 2013), it would be possible to compare the dipole potential changes between planar BLMs and lipid vesicles formed from the same lipid using the same DS technique. This is a great advantage over the other methods whose applications are restricted to either planar BLMs or lipid vesicles.

On the other hand, there are some limitations. Although the frequency range of DS is extended by technical advances, the lower limit may be at present around 1 Hz, which corresponds to $k_i = 3\ s^{-1}$. In the case of lipophilic cations, whose k_i values are much smaller than that of lipophilic anions, dielectric relaxation of the membrane is difficult to measure. When k_i becomes more than $10^5\ s^{-1}$, the f_m is close to the characteristic frequency of the dielectric relaxation due to charging of the "geometric" membrane capacitance, and therefore the determination of f_m includes large errors. Hence, DS is applicable for lipophilic anions of k_i values ranging from 10 to $10^5\ s^{-1}$ and is not able to estimate the absolute value of the dipole potential, which is measured using both lipophilic anion and cation.

Taking into account the k_i values so far reported for lipophilic anions, tetraphenylborate and its analog (Pickar and Benz 1978; Benz and Cros 1978; Benz 1988) and tungsten pentacarbonyl anions and their analog (Kürschner et al. 1998; Zimmermann et al. 2008; Sukhorukov et al. 2001) are probably available for the DS technique. Use of structurally dissimilar lipophilic anions is particularly interesting, for those have different depths of the adsorption plane within the membrane (Zimmermann et al. 2008), which might be one of the critical parameters in sensing changes in the dipole potential.

Appendix

Dielectric Relaxation of the Membrane Containing Lipophilic Ions

Although Ketterer et al. (1971) provided the relationships between the membrane impedance and the kinetic parameters of the ion translocation in the membrane, it is worthwhile to provide an alternative simple derivation for the dielectric relaxation of the membrane. We consider a

membrane between aqueous phases containing a kind of lipophilic ion of valency z at concentration c. The distribution of the ions between the membrane interface and the bulk aqueous phase is represented using the partition coefficient β and the rate constant k. The concentrations of the ions at the left-hand and the right-hand interfaces of the membrane are denoted by N' and N'', and $N' + N'' = 2\beta c$ at equilibrium. When a voltage U is applied to the membrane, the ions adsorbed at the interfaces move across the membrane: $k_i'$and k_i'' are the rate constants toward the right-hand interface and toward the left-hand one, respectively. Assuming the first order kinetics for the translocation of the ions, the difference between N' and N'' changes as

$$\frac{d(N' - N'')}{dt} = -k(N' - N'') - 2k_i'N' + 2k_i''N'' \tag{A1}$$

If there is a symmetrical potential energy barrier for the ion translocation in the membrane, its height at the center of the membrane changes by zFU/2. For the case of $u = FU/RT \ll 1$, $k_i' = k_i(1 + zu/2)$ and $k_i'' = k_i(1 - zu/2)$, and thus Eq. (A1) becomes

$$\frac{d(N' - N'')}{dt} = -(k + 2k_i)(N' - N'') - k_i zu\beta c \tag{A2}$$

Equation (A2) is rewritten to express the response to ac voltage $U^*(U^* = U_0 e^{j\omega t}$ with amplitude U_0, angular frequency ω, imaginary unit j and time t). When the membrane is subjected to the ac voltage, the quantity of N'-N'' oscillates at ω with amplitude A and phase angle θ.

$$N' - N'' = Ae^{j(\omega t + \theta)} \tag{A3}$$

Substituting Eq. (A3) into Eq. (A2) leads

$$N' - N'' = \frac{-k_i zu^*\beta c}{(k + 2k_i)(1 + j\omega/(k + 2k_i))} \tag{A4}$$

with $u^* = FU^*/(RT)$. Considering the membrane as a capacitor, the difference in charge density $zF(N'$-$N'')$ between the membrane interfaces corresponds to the polarization charge, and the additional charges are stored in the capacitor to compensate the polarization charge. The increment of the membrane capacitance C_i^* becomes

$$C_i^* = \frac{-zF(N' - N'')}{U^*} = \frac{\Delta C_m}{1 + j\omega\tau_m} \tag{A5}$$

where

$$\Delta C_m = \frac{z^2F^2}{RT}\frac{k_i}{(k + 2k_i)}\beta c \tag{A6}$$

$$\tau_m = \frac{1}{k + 2k_i} \tag{A7}$$

The complex capacitance of the membrane C_m^* is, therefore, represented by

$$C_m^* = C_{mh} + \frac{\Delta C_m}{1 + j\omega\tau_m} + \frac{G_{ml}}{j\omega} \tag{A8}$$

where C_{mh} is the high-frequency limit of the membrane capacitance that corresponds to the "geometric" capacitance and G_{ml} is the low-frequency limit of the membrane conductance to be identical to the steady-state membrane conductance given by

$$G_{ml} = \frac{z^2F^2}{RT}\frac{kk_i}{2k_i + k}\beta c \tag{A9}$$

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