Mechano-capacitive properties of polarized membranes

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Biological membranes are capacitors that can be charged by applying a field across the membrane. The charges on the capacitor exert a force on the membrane that leads to electrostriction, i.e. a thinning of the membrane. Since the force is quadratic in voltage, negative and positive voltage have an identical influence on the physics of symmetric membranes. However, this is not the case for a membrane with an asymmetry leading to a permanent electric polarization. Positive and negative voltages of identical magnitude lead to different properties. Such an asymmetry can originate from a lipid composition that is different on the two monolayers of the membrane, or from membrane curvature. The latter effect is called ‘flexoelectricity’. As a consequence of permanent polarization, the membrane capacitor is discharged at a voltage different from zero. This leads to interesting electrical phenomena such as outward or inward rectification of membrane permeability. Here, we introduce a generalized theoretical framework, that treats capacitance, polarization, flexoelectricity, piezoelectricity and thermoelectricity in the same language. We show applications to electrostriction, membrane permeability and piezoelectricity and thermoelectricity close to melting transitions, where such effects are especially pronounced.

Introduction

Many signaling processes in biology involve electrical phenomena. These processes are related to the movement of ions and the orientation of polar molecules. Biological molecules typically contain charged groups that are at the origin of electrical fields and dipole moments. Furthermore, membranes and macromolecules are surrounded by electrolytes containing charged ions. At physiological ionic strength, the Debye length of electrostatic interactions in the aqueous medium is about 1 nm. It is caused by the shielding of charges by ions. However, in the hydrophobic cores of membranes and proteins, the dielectric constant is small, and no ions that could shield electrostatic interactions are present. Thus, the length scale of electrostatic interactions is significantly larger. Generally, under physiological conditions the range of the electric fields is similar to the size of biological macromolecules. In this publication we will focus on the electrostatics of membranes that determines capacitance, polarization, piezoelectricity, flexoelectricity and thermoelectricity.

There exist large concentration differences of ions across the membranes of biological cells. For instance, the concentration of potassium is about 400 mM inside and only 20 mM outside of a squid axon. If the membrane is selective for potassium, this results in a Nernst potential across the biological membrane. The combination of the Nernst potentials of different ions yields a resting potential, which for biological cells is in the range of ±100 mV. The central core of a membrane is mostly made of hydrophobic non-conductive material. Thus, the biomembrane is considered a capacitor, e.g., in the Hodgkin–Huxley model for the nervous impulse. During the nerve pulse, currents are thought to flow across ion channel proteins that transiently charge or discharge the membrane capacitor. Within this model, the membrane is assumed to be a homogeneous planar capacitor with constant dimensions. The capacitance can be calculated from the relation

$$C_m = \varepsilon \cdot \frac{A}{d}$$

(1)

where $\varepsilon$ is the dielectric constant, $A$ is the membrane area and $d$ is the membrane thickness.

Let us assume that the membrane is surrounded by a conducting electrolyte solution. In the presence of an applied voltage, the charged capacitor consists of one plate with positive charges and one plate with negative charges at distance $d$. The field inside the capacitor can be determined using the superposition of the fields of the two plates (illustrated in Fig. 1). The field inside a charged capacitor is different from zero, while it is zero outside of the capacitor. If no field is applied, the capacitor is not charged.

The charges on a capacitor generate mechanical forces on the two membrane layers. These forces can change the dimensions of the capacitor such that both the area and the thickness
of the membrane change (see Fig. 2). As a result, the capacitance is not generally a constant. The capacitance increases upon charging the membrane by an applied field because the membrane thickness decreases and the area increases. This effect is known as electrostriction. Close to phase transitions in the membrane (in which the compressibility of the membrane is large), the membrane should be considered as a nonlinear capacitor. A small change in voltage can result in large changes in thickness and capacitance. The coupling between the membrane voltage and its dimensions renders the membrane piezoelectric, i.e., mechanical changes in the membrane can create a membrane potential and vice versa. Similarly, a small change in temperature can change the capacitance leading to thermoelectricity.

On average, about 80% of the lipids of biological membranes are zwitterionic. Zwitterionic lipids possess permanent electrical dipole moments. Examples of such lipids are phosphatidylcholines and phosphatidylethanolamines. About 10% of biological lipids carry a net negative charge, including phosphatidylinositol and phosphatidylserine. It is known that biomembranes often display asymmetric distributions of lipids such that charged lipids are mostly found in the inner leaflet of the bilayer. Biomembranes also contain integral and peripheral proteins with asymmetric distribution (or orientation) between inside and outside, which carry both positive and negative charges. Due to such compositional asymmetries, a spontaneous electrical dipole moment of the membrane can be generated in the absence of an externally applied potential.

A redistribution or reorientation of polar molecules in an external field resembles the charging of a capacitor. If the membrane possesses a spontaneous polarization, the membrane capacitor in equilibrium can be charged even in the absence of an external field (illustrated in Fig. 3). In order to discharge this capacitor, a potential of $\Psi = -\Psi_0$ has to be applied. We call $\Psi_0$ the spontaneous membrane potential, or the offset potential. In a theoretical treatment one has to be very careful to correctly account for both capacitive and polarization effects.

The polarization effects described above rely on an asymmetric distribution of charges or dipoles on the two sides of a membrane. Interestingly, even a chemically symmetric lipid membrane made of zwitterionic (uncharged) lipid may be polarized. The individual monolayers of zwitterionic lipids display trans-layer voltages on the order of 300 mV. Any geometric deformation that breaks the symmetry between the two monolayers of a membrane results in a net polarization if these distortions alter the relative dipole orientation on the two layers. In particular, curvature induces different lateral pressure on the two sides of a membrane. Thereby, curvature can induce polarization in the absence of an applied field. This consideration was introduced by Meyer in 1969 for liquid crystals. It was applied to curved lipid bilayers by Petrov in 1975 (see also ref. 9 and 10). He called this effect ‘flexoelectricity’. Upon bending (or flexing) the membrane, both area, $A$, and volume, $V$, of the opposing monolayers change in opposite directions. If the polarization is a function of area and volume, the polarization of the outer monolayer is given by $P_o = P_{o,A} A + P_{o,V} V$ and that of the inner monolayer is given by $P_i = P_{i,A} A + P_{i,V} V$, respectively. Therefore, curvature can induce a net polarization across the membrane. This is illustrated in Fig. 4. This polarization is counteracted by opposing charges adsorbing to the membranes (Fig. 4b and c). In order to discharge the membrane, a potential $\Psi = -\Psi_0$ has to be applied. As in the case of chemical asymmetry, at zero applied field, the field inside the capacitor is zero. The cases of a chemically asymmetric planar membrane and a chemically symmetric curved membrane are conceptually similar.

Charged capacitors, polarization, flexoelectricity and piezoelectricity all involve the spatial separation of charges. Thus, they all represent aspects of the same electrostatic phenomena. However, in the literature they are often treated as different things and they are described by a different language. In this communication we formulate a general thermodynamical description of the electrostatics of lipid membranes, which represents a generalization of a study on the capacitance of membranes previously published by our group. It will be used to generalize the effect of an externally applied electric field on
the lipid melting transition. We will introduce the thermodynamics of a polarized lipid membrane in an electric field, which then results in a generalization of electrostriction effects on lipid membranes.

Theory

When the molecules of dielectric materials are placed in an external electric field, they orient themselves to minimize the free energy. In capacitors, net macroscopic dipoles are induced in the dielectric medium and tend to counteract the applied field. As a response to an applied electric field, mechanical changes can be observed, e.g., in piezoelectric crystals. To deal with these effects, authors like Frank treated the electrostatic effects within a thermodynamical framework.\textsuperscript{11} He considered the electrical work performed on a fluid during any infinitesimal and reversible change, \(dW_{el} = Ed\psi\). This type of consideration leads to expressing the electric displacement, \(D\), in a volume, \(V\), as an extensive variable with the electric field, \(E\), as its conjugated intensive variable. Vector notation has been dropped assuming planar geometry.

When we consider a membrane capacitor, its hydrophobic core separates the two capacitor plates and acts both as a compressible and dielectric material. Choosing hydrostatic pressure \((p)\), lateral pressure \((\pi)\), temperature \((T)\) and applied electric field \((E)\) as intensive variables, we can write the differential of the Gibbs free energy as

\[
dG = -SdT + \nu dp + A d\pi - (\nu D)dE + \cdots \tag{2}
\]

where the conjugated extensive variables are \(S\) (entropy), \(\nu\) (volume), \(A\) (area) and \(\nu D\) (electric displacement). The electrical contribution to the free energy due to an applied electric field comes from the final term, which we will refer to as the electrical free energy, \(G^{el}\).

The electric displacement is related to the total polarization, \(P_{tot}\) by

\[
D = \varepsilon_0 E + P_{tot}. \tag{3}
\]

where \(\varepsilon_0\) is the vacuum permittivity. Most materials have zero polarization at zero electric field, and polarization is only induced by an external field. For a linear dielectric material the induced polarization is \(P_{ind} = \varepsilon_0 \chi_0 E\), where \(\chi_0\) is the dielectric susceptibility. We are interested in extending our considerations to a dielectric material which can display spontaneous polarization, \(P_0\), in the absence of an applied field such that

\[
P_{tot} = \varepsilon_0 \chi_0 E + P_0. \tag{4}
\]

The spontaneous polarization, \(P_0\), can originate from asymmetric lipid bilayers, e.g., from curvature (flexoelectricity) or from different composition of the two monolayers. The electric displacement takes the form

\[
D = \varepsilon(E + E_0), \tag{5}
\]

where \(\varepsilon\) is the dielectric constant, \(\varepsilon = \varepsilon_0(1 + \chi_0)\) and \(E_0 \equiv P_0/\varepsilon\) is the electric field related to the spontaneous polarization, \(P_0\), at \(E = 0\).

Using eqn (5), we can determine the electrical free energy:

\[
G^{el} = - \int_0^E (\nu D) dE' = -\varepsilon_0 (\frac{E^2}{2} + E_0 E) \tag{6}
\]

where we have assumed the volume of the lipid membrane to be constant. Assuming that the dielectric properties of the medium are homogeneous across a membrane with thickness \(d\), we can define \(Ed = \Psi\) where \(\Psi\) represents the applied electric potential difference. This leads to

\[
G^{el} = -\frac{\varepsilon A}{2d} ((\Psi + \Psi_0)^2 - \Psi_0^2) \tag{7}
\]

where \(\Psi_0\) is the offset potential related to \(E_0\) \((E_0d = \Psi_0)\). The pre-factor contains the capacitance of a planar capacitor \((C_m = \varepsilon A/d)\). Thus, the electric free energy is given by

\[
G^{el} = -\frac{1}{2}C_m ((\Psi + \Psi_0)^2 - \Psi_0^2). \tag{8}
\]

At \(\Psi = 0\) the electrical contribution to the free energy is zero.

Capacitive susceptibility, piezoelectricity, flexoelectricity and thermoelectric effect

The polarization of a membrane can change by charging, compressing, stretching, bending or heating of the membrane. The corresponding electrostatic phenomena are called electrostriction, piezoelectricity, flexoelectricity and thermoelectricity. In the past, some simple relations were derived by A.G. Petrov.\textsuperscript{12} For instance, piezoelectricity was described as the area-dependence of polarization. Correspondingly, flexoelectricity was described as the curvature-dependence of the polarization assuming that polarization is zero in the planar state of the membrane. However, upon changing the membrane area, its capacitance also changes. Thus, in the presence of a field not only the
polarization but also the charge on the capacitor can change upon changing area. In the case of membrane curvature, the polarization may be different from zero in the planar state. In the following, we derive general equations for electrostriction, piezoelectricity, flexoelectricity and thermoelectricity. We will find that some relations previously derived by Petrov are special cases of our more general description.

**The charge on a capacitor.** The charge on a capacitor is given by

$$q = A \cdot D = A(zE + P_0) = \varepsilon_0 A \left( \Psi + \Psi_0 \right) = C_m \left( \Psi + \Psi_0 \right). \quad (9)$$

The dependence of the charge on potential, $\Psi$, surface area, $A$, curvature, $c$, and temperature $T$ is given by

$$dq = \left( \frac{\partial q}{\partial \Psi} \right)_{A,c,T} d\Psi + \left( \frac{\partial q}{\partial A} \right)_{\Psi,c,T} dA + \left( \frac{\partial q}{\partial c} \right)_{A,T} dc + \left( \frac{\partial q}{\partial T} \right)_{\Psi,A,c} dT. \quad (10)$$

Here, we assume that $\Psi$, $A$, $c$ and $T$ are variables that can be independently controlled in the experiment (which is not generally the case in all experiments). Thus, the change of the charge on a capacitor as a function of potential, area, curvature and temperature is given by:

$$dq = \left[ (\Psi + \Psi_0) \left( \frac{\partial C_m}{\partial \Psi} \right)_{A,c,T} + C_m + C_m \left( \frac{\partial \Psi_0}{\partial \Psi} \right)_{A,c,T} \right] d\Psi$$
$$+ \left[ (\Psi + \Psi_0) \left( \frac{\partial C_m}{\partial A} \right)_{\Psi,c,T} + C_m \left( \frac{\partial \Psi_0}{\partial A} \right)_{\Psi,c,T} \right] dA$$
$$+ \left[ (\Psi + \Psi_0) \left( \frac{\partial C_m}{\partial c} \right)_{\Psi,A,T} + C_m \left( \frac{\partial \Psi_0}{\partial c} \right)_{\Psi,A,T} \right] dc$$
$$+ \left[ (\Psi + \Psi_0) \left( \frac{\partial C_m}{\partial T} \right)_{\Psi,A,c} + C_m \left( \frac{\partial \Psi_0}{\partial T} \right)_{\Psi,A,c} \right] dT.$$

or in abbreviated form as

$$dq = [(\Psi + \Psi_0) \frac{\partial C_m}{\partial \Psi} + C_m + C_m \beta_{A,T}] d\Psi$$
$$+ [(\Psi + \Psi_0) \frac{\partial C_m}{\partial A} + C_m \beta_{A,T}] dA$$
$$+ [(\Psi + \Psi_0) \frac{\partial C_m}{\partial c} + C_m \beta_{A,T}] dc$$
$$+ [(\Psi + \Psi_0) \frac{\partial C_m}{\partial T} + C_m \beta_{A,T}] dT. \quad (11)$$

The first term describes the change of charge on a capacitor allowing for the possibility that both capacitance and polarization can depend on voltage. The second term describes piezoelectricity, i.e., the change of charge by changing area, taking into account the area dependence of both capacitance and polarization. One could write similar equations, if the lateral pressure, $\pi$, were controlled instead of the area, $A$. The third term describes flexoelectricity, which relates to the change of charge caused by changes in curvature. Here, both dependence of capacitance and polarization on curvature are considered. The last term describes the thermoelectric (Seebeck) effect, i.e., the dependence of the charge on a capacitor on temperature. We will discuss experimental situations below where two variables, for instance temperature and area, are coupled and not independent. In such situation one has to adapt the above equations and adjust the coefficients of the parameters $\alpha$ and $\beta$.

**Capacitive susceptibility.** The capacitive susceptibility is given by

$$\hat{C}_m = \left( \frac{\partial q}{\partial \Psi} \right)_{A,c,T} = (\Psi + \Psi_0) \alpha_{c,T} + C_m + C_m \beta_{c,T}. \quad (13)$$

Here, we have omitted the index $A$ because area is not kept constant. Further, if the spontaneous polarization is constant, this reduces to

$$\hat{C}_m = C_m + (\Psi + \Psi_0) \left( \frac{\partial C_m}{\partial \Psi} \right)_{c,T}. \quad (14)$$

If the spontaneous polarization is zero at all voltages, this reduces to

$$\hat{C}_m = C_m + \Psi \left( \frac{\partial C_m}{\partial \Psi} \right)_{c,T} \quad (15)$$

which is the relation given by Heimburg (2012). If both capacitance and polarization are constant, $\hat{C}_m = C_m$. However, eqn (13) also implies that the capacitive susceptibility generally depends on the voltage-dependence of the polarization.

**Piezoelectricity.** Piezoelectricity is the area or thickness dependence of the charge on a capacitor, or more generally, the influence of geometry on the charge of a capacitor. Let us assume that in eqn (12) $\Psi$, $c$ and $T$ are constant. We then obtain

$$dq = [(\Psi + \Psi_0) \frac{\partial C_m}{\partial \Psi} + C_m \beta_{c,T}] d\Psi$$
$$+ [(\Psi + \Psi_0) \frac{\partial C_m}{\partial A} + C_m \beta_{c,T}] dA$$
$$+ [(\Psi + \Psi_0) \frac{\partial C_m}{\partial c} + C_m \beta_{c,T}] dc$$
$$+ [(\Psi + \Psi_0) \frac{\partial C_m}{\partial T} + C_m \beta_{c,T}] dT. \quad (16)$$

At $\Psi = 0$, we obtain for a small change in area, $\Delta A$,

$$\Delta q \approx (\Psi_0 \frac{\partial \Psi}{\partial c} + C_m \beta_{c,T}) \Delta A. \quad (17)$$

If $\Psi_0(\Delta A = 0)$ is zero, the capacitor is uncharged for $\Psi = 0$. Then the charge on the capacitor after a change in area of $\Delta A$ is given by

$$q(\Delta A) = C_m \beta_{c,T} \Delta A \text{ or } \Psi_0(\Delta A) = \beta_{c,T} \Delta A. \quad (18)$$

A similar relation was given by Petrov and Usherwood. As evident in eqn (16), the piezoelectric effect is a function of the externally applied potential if the capacitance is influenced by area changes (which usually is the case).

**Inverse piezoelectric effect.** The elastic free energy density of membrane compression is given by $g = \frac{1}{2} K^2_{m} (\Delta A / A_0)^2$, where $K^2_{m}$ is
the lateral compression modulus and $A_0$ is the equilibrium area prior to compression. In the presence of an applied potential, the free energy density is given by

$$g = \frac{1}{2}K^2 A \left( \frac{\Delta A}{A_0} \right)^2 - \frac{1}{2} C_m A_0 \left( \Psi + \Psi_0 \right)^2,$$

(19)

which contains an elastic and an electrostatic term. In order to obtain the free energy, $G$, this has to be integrated over the surface area of the lipid membrane. At constant compression modulus, $K^2$, and constant potential $\Psi$, the area change $\Delta A$ equilibrates such that

$$\frac{\partial g}{\partial A} = K^2 \frac{\Delta A}{A_0} - \frac{C_m}{A_0} \left( \frac{\partial \Psi_0}{\partial A} \right)_{\Psi,T} \Psi + \frac{1}{2} \frac{\partial C_m}{\partial A} \left( \Psi + \Psi_0 \right)^2 \left( \Psi + \Psi_0 \right)^2.$$

(20)

Therefore,

$$\Delta A(\Psi) = A_0 \left[ \frac{C_m \beta_{\Psi,T}}{K^2} + \frac{3 \Psi_0}{K^2} \left( \Psi + \Psi_0 \right)^2 \left( \Psi + \Psi_0 \right)^2 \right].$$

(21)

Here, the first linear term is due to the area dependence of the membrane polarization, while the second quadratic term originates from the area dependence of the capacitance. It is evident that the area change induced by an applied voltage will be much larger with the transition regime of a lipid membrane than in the pure phases. 3

**Flexoelectricity.** Flexoelectricity describes the dependence of the charge on the membrane capacitor induced by membrane curvature. Let us assume that in eqn (11) $\Psi$, and $T$ are constant. It is questionable whether one can define experimental conditions where the membrane area is independent of curvature. Therefore, we consider area a variable depending on curvature, which is not independently controlled. Then we find

$$dq = \left[ (\Psi + \Psi_0) x_{\Psi,T} + C_m \beta_{\Psi,T} \right] dc.$$

(22)

This is the (direct) ‘flexoelectric effect’. If we make the simplifying assumption that the capacitance $C_m$ does not depend on curvature and that the coefficient $\beta_{\Psi,T}$ is constant, we obtain

$$q(c) = C_m (\Psi + \Psi_0) + C_m \beta_{\Psi,T} c,$$

(23)

where $C_m (\Psi + \Psi_0)$ is the membrane charge at $c = 0$. If further the applied potential, $\Psi$, is zero and the polarization in the absence of curvature is also assumed being zero, we obtain

$$q(c) = C_m \beta_{\Psi,T} c \quad \text{or} \quad \Psi_0(c) = \beta_{\Psi,T} c.$$

(24)

Thus, the offset potential $\Psi_0$ is proportional to the curvature. This relation is a special case of the flexoelectric effect described in eqn (22). It was previously discussed by Petrov. 12

He introduced a flexoelectric coefficient, $f$, which is given by $f \equiv \varepsilon \beta_{\Psi,T}$. Petrov found experimentally that $f = 10^{-18}$ [C], or $\beta_{\Psi,T} = 2.82 \times 10^{-8}$ [m] for $\varepsilon = 4 \varepsilon_0$, respectively.

**Inverse flexoelectric effect.** In the absence of a spontaneous curvature, the elastic free energy density of bending is given by $g = \frac{1}{2} K_B c^2$, where $K_B$ is the bending modulus. In the presence of an applied potential and assuming that $C_m$ does not depend on curvature, the free energy density is given by

$$g = \frac{1}{2} K_B c^2 - \frac{1}{2} C_m \left( \Psi + \Psi_0 \right)^2 .$$

(25)

which contains an elastic and an electrostatic term as in the expression for piezoelectricity. In order to obtain the free energy, $G$, this has to be integrated over the surface area of the lipid membrane. At constant potential $\Psi$, the curvature $c$ equilibrates such that

$$\frac{\partial g}{\partial c} = K_B c - \frac{C_m}{A} \left( \frac{\partial \Psi}{\partial c} \right)_{\Psi,T} \Psi = K_B c - \frac{C_m}{A} \beta_{\Psi,T} \Psi = 0.$$

(26)

Therefore,

$$c(\Psi) = \frac{C_m}{A} \frac{\beta_{\Psi,T}}{K_B} \Psi = \frac{\varepsilon \beta_{\Psi,T}}{\varepsilon_0} \Psi.$$

(27)

This effect is called the ‘inverse flexoelectric effect’. It describes how curvature is induced by an applied potential. It depends on the bending modulus and the dependence of polarization on curvature, $\beta_{\Psi,T}$. In melting transitions, the curvature-induction by voltage is enhanced because $K_B$ approaches a minimum. 4 This implies that in the presence of an applied field, the curvature of a membrane changes upon changing the temperature – in particular close to transitions.

Both, the investigation of flexoelectric and inverse flexoelectric effects have been pioneered by Petrov. 12 In Petrov’s nomenclature, eqn (27) assumes the form $c(\Psi) = \int f dK_B \Psi$.

**Thermoelectricity.** Let us assume that in eqn (11) $\Psi$, and $c$ are constant, and $A$ is a function depending on temperature. Then we find

$$dq = \left[ (\Psi + \Psi_0) x_{\Psi,T} + C_m \beta_{\Psi,T} \right] dT.$$

(28)

This is the Seebeck effect. It describes the charging of a capacitor by changing temperature. Since the changes in capacitance are especially strong in the melting transition of membranes, the coefficient $x_{\Psi,T}$ (which is positive) typically displays a maximum at $T_m$. 2 The coefficient $\beta_{\Psi,T}$ that describes the change in spontaneous polarization with temperature will also display an extremum. In the absence of spontaneous polarization, eqn (28) reduces to

$$\frac{dq}{dT} = \Psi \left( \frac{\partial C_m}{\partial T} \right)_{\Psi,T}.$$

(29)

Due to changes in area and thickness, the dependence of the capacitance on temperature is especially large in the melting transition of a membrane. This effect was described in Heimburg (2012). 2 A related effect, the induction of temperature changes by charging a membrane is the Peltier effect. It can occur in membranes due to charge-induced transitions that lead to an absorption or release of latent heat from the membrane.
Applications

Electrostriction

The charges on a capacitor attract each other. These attractive forces can change the dimensions of the membrane and thereby change the capacitance. If \( \Psi_0 = 0 \), the electric contribution to the free energy according to eqn (8) is \( G^e = \frac{1}{2} C_m \Psi^2 \). For \( A \approx \text{const.} \) and \( \Psi = \text{const.} \), the force \( \mathcal{F} \) acting on the layers is

\[
\mathcal{F} = \frac{\partial G^e}{\partial d} = -\frac{1}{2} \left( \frac{\partial C_m}{\partial d} \right) \Psi^2 = \frac{1}{2} \frac{C_m}{d} \Psi^2.
\]

This is the force acting on a planar capacitor given in the literature (e.g., ref. 2). If there exists a constant offset potential \( \Psi_0 \), we find instead (eqn (8))

\[
\mathcal{F} = \frac{1}{2} \frac{C_m}{d} ((\Psi + \Psi_0)^2 - \Psi_0^2).
\]

Thus, one expects that the force on a membrane is a quadratic function of voltage which displays an offset voltage when the membrane is polarized. This force can reduce the membrane thickness and thereby increase the capacitance of a membrane. Note, however, that for \( (\Psi + \Psi_0)^2 - \Psi_0^2 < 0 \), the force \( \mathcal{F} \) is negative. As a consequence, capacitance will be decreased.

Let us assume a membrane with constant area and small thickness change, \( \Delta d \ll d \). Then the change in capacitance, \( \Delta C_m \), caused by a change of thickness, \( \Delta d \), is given by

\[
\Delta C_m = -\frac{C_m}{d^2} \Delta d.
\]

Thus, the change in capacitance is proportional to the change in thickness. If the thickness is a linear function of the force \( \mathcal{F} \propto \Delta d \), one finds that the capacitance is proportional to the force \( \mathcal{F} \). Therefore, it is a quadratic function of voltage with an offset of \( \Psi_0 \).

\[
\Delta C_m \propto ((\Psi + \Psi_0)^2 - \Psi_0^2).
\]

The magnitude of the change in capacitance depends on the elastic constants of the membrane.

Relation (33) was studied by various authors. Using black lipid membranes, Alvarez and Latore\(^{14}\) found a quadratic dependence of the capacitance on voltage (Fig. 5). In a symmetric membrane made of the zwitterionic (uncharged) lipid phosphatidylethanolamine (PE), the offset potential \( \Psi_0 \) in a 1 M KCl buffer was found to be zero. In an asymmetric membrane with one monolayer made of PE and the other made of the charged lipid phosphatidylserine (PS), a polarization is induced. In a 1 M KCl buffer, the offset potential was \( \Psi_0 = 47 \text{ mV} \), while it was \( \Psi_0 = 116 \text{ mV} \) in a 0.1 M KCl buffer. It is obvious from Fig. 5 that within experimental error the shape of the capacitance profile is unaffected by the nature of the membrane. Only the offset potential is influenced by composition and ionic strength. This suggests that the offset potential has an ionic strength dependence. In this publication, we do not explore the theoretical background of this experimental fact.

In a range of \( \pm 300 \text{ mV} \) around the minimum capacitance, the change in capacitance, \( \Delta C_m \), is of the order of \( <1.5 \text{ pF} \), while the absolute capacitance, \( C_{m,0} \), at \( \Psi = 0 \) is approximately 300 pF.\(^{14}\) Thus, the change in capacitance caused by voltage is very small compared to the absolute magnitude of the capacitance.

Membrane conductance and rectification

A very interesting application of electrostriction is the influence of voltage on membrane permeability. It has been found that membranes can form pores that appear as quantized conduction events upon the application of potential difference across the membrane\(^{15-19}\) (see right insert in Fig. 6). The likelihood to form a pore is thought to be proportional to the square of the applied electric potential.\(^{20,21}\) This assumption is based on the hypothesis that an increase in voltage thins the membrane (as described in the previous section) and eventually leads to an electric breakdown linked to pore formation. Laub et al.\(^{22}\) found that the current–voltage (I–V) relation for a chemically symmetric phosphatidylethanolamine membrane patch formed on the tip of a glass pipette was a non-linear function of voltage which was not symmetric around \( \Psi = 0 \), but rather outward rectified (Fig. 6).

Blicher et al.\(^{23}\) proposed that an offset potential can explain the outward-rectification. The free energy difference between an open and a closed pore, \( \Delta G_p \), can be expressed by

\[
\Delta G_p = \Delta G_{p,0} + z ((\Psi + \Psi_0)^2 - \Psi_0^2),
\]

where \( \Delta G_{p,0} \) and \( z \) are coefficients describing the difference in free energy between open and a closed pore in the absence and the presence of externally applied voltage. The equilibrium
constant between open and closed pores is given by \( K_p = \exp(-\Delta G_p/kT) \), and the likelihood of finding an open pore is given by \( P_{\text{open}} = K_p/(1 + K_p) \), shown in the left inset of Fig. 6. The \( I-V \) relation can be expressed as

\[
I = \gamma_p P_{\text{open}} \Psi.
\]

This relation perfectly describes the experimental current–voltage data if a offset potential of \( \Psi_0 = 110 \text{ mV} \) was assumed (solid line in Fig. 6). Thus, inward and outward rectified \( I-V \) profiles can be found in pure lipid membranes in the complete absence of proteins. They find their origin in the polarization of the membrane and the effect of electrostriction.

The most likely origin of the offset potential is polarization of the membrane in the patch pipette due to curvature (flexoelectricity\(^2\)). The above analysis implies that many asymmetric phenomena of biomembranes could have their origin in a spontaneous polarization of the membrane as a whole.

### Piezoelectricity and capacitive susceptibility: influence of the potential on the membrane dimensions and capacitance close to a melting transition

As discussed above, the influence of voltage on the capacitance is small in the gel and in the fluid phase because membranes are not very compressible in their pure phases. However, close to the phase transition between gel and fluid, membranes become very compressible.\(^3\) In this transition, the thickness of the membrane, \( d \), decreases by about 16% and the area, \( A \), increases by about 24%\(^4\) for the lipid dipalmitoyl phosphatidylcholine (DPPC), with a melting temperature \( T_m = 314.15 \text{ K} \). Therefore, the capacitance of the fluid membrane is about 1.5 time higher than the capacitance of the gel phase.\(^5\)

In the following we wish to describe the influence of voltage on membrane area, capacitance and charge. According to eqn (8), the Gibbs free energy difference caused by an external electric field can be written as

\[
\Delta G_e = G_{\text{fluid}}^{\text{el}} - G_{\text{gel}}^{\text{el}} = -\frac{\Delta C_{\text{m}}}{2} \left( (\Psi + \Psi_0)^2 - \Psi_0^2 \right).
\]

where \( \Delta C_{\text{m}} \) is the difference between the capacitance of gel and fluid phase. Here, we assumed that both the offset potential \( \Psi_0 \) and the dielectric constant \( \varepsilon \) do not change with the state. We have confirmed the latter in experiments on the dielectric constant in the melting transition of oleic acid using a parallel plate capacitor (data not shown). We found that the changes of the dielectric constant caused by the melting of oleic acid (\( T_m \approx 17 \text{ °C} \)) are very small.

It has been shown experimentally that in the vicinity of the lipid melting transition changes of various extensive variables are proportionally related.\(^2\) For instance, changes in enthalpy are proportional to changes in area, in volume and we assume that a similar relation holds for changes in thickness. Further, close to transitions the elastic constants are closely related to the heat capacity. For instance, the temperature-dependent change of the isothermal compressibility is proportional to heat capacity changes. Thus, membranes are more compressible close to transitions, and it is to be expected that the effect of potential changes on membrane capacitance is enhanced. This will be calculated in the following.

We assume that the lipid melting transition is described by a two-state transition governed by a van’t Hoff law, so that the equilibrium constant between the gel and the fluid state of the membrane can be written as\(^2\):\(^27\)

\[
K(T, \Psi) = \exp \left( \frac{-n \Delta G}{RT} \right)
\]

where \( n \) is the cooperative unit size which describes the number of lipids that change state cooperatively (for LUVs of DPPC we used \( n = 170\))\(^3\). The free energy difference between gel and fluid membranes is given by

\[
\Delta G = (\Delta H_0 - T \Delta S_0) + \Delta G_e
\]

where \( \Delta H_0 = 35 \text{ kJ mol}^{-1} \) and \( \Delta S_0 = 111.4 \text{ J mol}^{-1} \text{ K}^{-1} \) (for DPPC). From the equilibrium constant we can calculate the fluid fraction, the average fraction of the lipids that are in the fluid state,

\[
f_i(T, \Psi) = \frac{K(T, \Psi)}{1 + K(T, \Psi)}
\]

For DPPC LUV, the thickness in the gel and fluid state is given by \( d_g = 4.79 \text{ nm} \) and \( d_f = 3.92 \text{ nm} \), respectively. The area per lipid is \( A_g = 0.474 \text{ nm}^2 \) and \( A_f = 0.629 \text{ nm}^2 \).\(^3\) We assume a dielectric constant of \( \varepsilon = 3 - \varepsilon_0 \) independent of the state of the membrane, and that the offset potential \( \Psi_0 = 70 \text{ mV} \) is also a constant. The area is described by \( A(T, \Psi) = A_g + f_i \Delta A \), and the membrane thickness by \( d(T, \Psi) = d_g - f_i \Delta d \), respectively.

---

**Fig. 6** Current through a lipid membrane (10:1 DMPC : DLPC, \( T = 30 \text{ °C}, 150 \text{ mM} \)) on a patch pipette as a function of voltage. The current–voltage relation can nicely be fitted by the simple model described in the text. The offset potential is \( \Psi_0 = 110 \text{ mV} \). Left inset: Open probability of lipid membrane channels. Right inset: Opening and closing of individual lipid channels as a function of time at a voltage of 50 mV. Data adapted from ref. 24 and 23.
Both, temperature and voltage-dependent area, \( A(T, \Psi) \), and capacitance, \( C_m = \frac{\partial A(T, \Psi)}{\partial (T, \Psi)} \), are shown in Fig. 7. For small variations of the potential around \( \Psi = -\Psi_0 \), the change in capacitance is well approximated by a quadratic function (insert in Fig. 7). The gel phase capacitance is smaller than the fluid phase capacitance. Due to the effect of electrostriction, an increase in voltage can induce a melting transition. In this transition, the capacitance increase until the value for the fluid phase is reached. By necessity, the charge on the membrane also undergoes a stepwise change (Fig. 7, top right). For this reason, the capacitive susceptibility \( \tilde{C}_m = \frac{\partial \tilde{q}}{\partial \tilde{C}_m} \), given by eqn (14) displays maxima at the transition voltage if the temperature is within or below the melting regime. Due to the presence of an offset potential, the transition profiles are not symmetric around \( \Psi = 0 \). One can recognize that the sensitivity of the capacitance to voltage changes close to the transition is much larger than that of the pure phases (Fig. 5). It is also a sensitive function of the temperature. Fig. 7 shows \( C_m(\Psi) \) for five different temperatures close to the melting temperature of DPPC at 314.15 °C. At \( T = 314.5 \) K, the change in capacitance at \( \Psi = -\Psi_0 = 300 \) mV is approximately 3% compared to the about 0.5% experimentally measured in the absence of a transition (Fig. 5). Due to the presence of a melting transition, the curve profile in Fig. 7 is only a quadratic function of potential close to \( \Psi = -\Psi_0 \). If the temperature is lower than \( T_m \), one finds voltage-induced transitions with a temperature-dependent transition voltage. Above this critical voltage the change in capacitance can be as high as 50%. The coefficient \( \alpha_{c,T} \) in eqn (14) is given by

\[
\alpha_{c,T} = \frac{1}{(\Psi + \Psi_0)} (\tilde{C}_m - C_m).
\]  

(40)

Close to the transition, it is a non-linear function of the thermodynamic variables (Fig. 8). In the absence of a transition, \( \alpha_{c,T} \) is zero because \( (\tilde{C}_m - C_m) \approx 0 \). If the spontaneous polarization \( \Psi_0 \) is independent of the applied potential, as assumed here, the coefficient \( \beta_{c,T} \) is zero. However, generally the coefficients \( \alpha_{i,k} \) and \( \beta_{i,k} \) are nonlinear functions of the variables close to transitions. Under these conditions, the electrostatics of membranes is especially interesting.

The dependence of the melting temperature on the applied potential. The total free energy difference between gel and fluid phase, \( \Delta G \), given by eqn (38) consists of an enthalpic and an
entropic contribution. At the melting temperature, \( T_m \), the Gibbs free energy difference \( \Delta G \) is zero, so that

\[
T_m = T_{m,0} \left( 1 + \frac{\Delta G^{el}}{\Delta S_0} \right)
\]

\[
= T_{m,0} \left( 1 - \frac{1}{2} \frac{\Delta C_a}{\Delta S_0} \left( (\Psi + \Psi_0)^2 - (\Psi_0)^2 \right) \right) .
\]

where \( T_{m,0} = \Delta H_m / \Delta S_0 \) is the melting temperature in the absence of an external field (for DPPC: \( \Delta H_m = 35 \text{ kJ mol}^{-1} \), \( T_{m,0} = 314.15 \text{ K} \) and \( \Delta S_0 = 111.4 \text{ J mol}^{-1} \text{ K}^{-1} \) (ref. 3)). This result describes the effect of electrostriction on the lipid melting transition in the presence of spontaneous polarization. It is a generalization of the electrostriction effect described by Heimburg\(^2\) who treated this phenomenon in the absence of polarization effects. Fig. 9 shows the dependence of \( T_m \) on an applied voltage for three different offset potentials, \( \Psi_0 \). It can be seen that in the presence of an applied potential, the spontaneous polarization and its sign influences the melting temperature.

**Generalization for \( \Psi_0 \neq \text{const} \).** The orientation of lipid dipoles can change upon lipid melting. It seems to be obvious from lipid monolayer experiments that the polarization of liquid expanded and solid condensed layers is different.\(^2\) We assume the same to be true for bilayers. Let us assume that the net offset potentials originating from membrane polarization in the gel and the fluid phase are given by \( \Psi_{0g} \) and \( \Psi_{0f} \), respectively. The free energy is now given by

\[
\Delta G^{el} = -\frac{\Delta C}{2} \left( (\Psi + \Psi_{0g})^2 - (\Psi_{0g})^2 \right) - C_v \Psi (\Psi_{0f} - \Psi_{0g})
\]

This can be inserted in eqn (38) and (41) to obtain the change in melting temperature due to an applied field.

**The thermolectric effect**

The charge on a capacitor is given by \( q = C \Delta \Psi \), where the capacitance \( C \) can be a function of temperature. If the spontaneous polarization \( \Psi_0 \) is independent of temperature, the dependence of the charge on the temperature changes is given by (see eqn (28))

\[
dq = (\Psi + \Psi_0) c \Psi \, dT .
\]

This is shown in Fig. 10 for two different fixed voltages, \( \Psi = 100 \text{ mV} \) and \( \Psi = -100 \text{ mV} \), and an offset potential of \( \Psi_0 = 70 \text{ mV} \). It can be seen that close to a transition the charge changes in a stepwise manner. Heimburg\(^2\) called this change an ‘excess charge’. This absorption or release of charge upon temperature change could be called a thermolectric effect. Close to transitions, the sudden change in temperature can lead to a capacitive current.

One can also alter the membrane charge in an adiabatically shielded system. The induction of a phase transition by changing the membrane leads to a release or an absorption of the latent heat which would alter the temperature in the aqueous reservoir. This is the Peltier effect.

**The dielectric susceptibility**

In ref. 2 we defined a capacitive susceptibility, \( \hat{C}_m = \langle \partial \epsilon / \partial \Psi \rangle \), which is given by:

\[
\hat{C}_m = \epsilon_0 \hat{C}_m = \epsilon_0 \langle \partial \epsilon / \partial \Psi \rangle = \epsilon_0 \hat{C}_m + \epsilon_0 \langle \partial \epsilon / \partial \Psi \rangle .
\]

This susceptibility has a maximum at the melting temperature, which is a consequence of the fact that the capacitances of gel and fluid lipid phases differ. By analogy, we now introduce a dielectric susceptibility, \( \hat{\epsilon} = \langle \partial \epsilon / \partial \Psi \rangle \), which is given by:

\[
\hat{\epsilon} = \epsilon + E \left( \frac{\partial \epsilon}{\partial E} \right) + \left( \frac{\partial \Psi_0}{\partial E} \right).
\]
Thermodynamic susceptibilities are linked to fluctuation relations. For instance, in ref. 2 we showed that the capacitive susceptibility is given by \( \hat{C} = \frac{\langle q^2 \rangle - \langle q \rangle^2}{kT} \), i.e., it is proportional to the fluctuations in charge. This fluctuation relation is valid as long as the distribution of states is described by Boltzmann statistics and the area and thickness are kept constant. Analogously, for constant volume, \( \nu \), the dielectric susceptibility, \( \hat{\varepsilon} \), is given by

\[
\hat{\varepsilon} = \frac{\nu \langle D^2 \rangle - \langle D \rangle^2}{kT}.
\]

Since this is a positive definite form, \( \hat{\varepsilon} \) is always larger than zero. The mean displacement, \( \langle D \rangle \), always increases with an increase in the electric field, \( E \). If either \( \varepsilon \) or the permanent polarization \( P_0 \) are different in the gel and the fluid state of a membrane, one can induce a transition. In this transition, the dielectric susceptibility displays an extremum.

**Summary and discussion**

In this publication, we provided a general thermodynamic treatment of polarization effects on the properties of lipid membranes. When applied to a membrane in an electrolyte, these electric effects can all be related to the charging (or discharging) of capacitors by either potential, curvature, area (or lateral pressure) or temperature changes. Curvature and area changes can lead to an offset potential or a spontaneous polarization. This is important because biological membranes are known to be polar and changes in voltage are generally considered to be central to the understanding of the functioning of cells. We show that a permanent or spontaneous polarization of a membrane influences the properties of a membrane capacitor such that it is discharged at a voltage different from zero. We relate this voltage to an “offset potential” \( \Psi \). The existence of this potential has the consequence that membrane properties even of chemically symmetric membranes are controlled differently for positive and negative voltages. We derived equations for the piezoelectric and inverse piezoelectric effect. The first considers the change in the offset potential when changing the membrane area. The second considers the change in membrane area by an applied field, which depends on the elastic modulus of the membrane. Finally, we derived general relations for the flexoelectric and the inverse flexoelectric effect. We showed that in some simple limiting cases, our derivations lead to relations identical to those of Petrov\(^1\) who pioneered the field of membrane flexoelectricity (e.g., ref. 8, 12, 13, 29–33).

An electric field applied across a lipid membrane generates a force normal to the membrane surface due to the charging of the membrane capacitor. The resulting reduction in membrane thickness is called electrostriction.\(^2\) For fixed membrane dimensions, the electrostrictive force is a quadratic function of voltage. Due to membrane thinning induced by the forces, one finds an increase in membrane capacitance. This has been demonstrated for symmetric black lipid membranes made from phosphatidylethanolamines (Fig. 5, Alvarez \( et \ al. , 1978 \)\(^1\)). However, for an asymmetric membrane made of charged lipids on one side and zwitterionic lipids on the other side (thus displaying polarity) the minimum capacitance is found at a voltage different from zero (Fig. 5, ref. 14). This indicates that a spontaneous electric polarization of the membrane influences the capacitive properties of a membrane. This has also been found in biological preparations. Human embryonic kidney cells display an offset potential of \(-51 \text{ mV} \)\(^,3\) This indicates that the capacitance in electrophysiological models such as the Hodgkin–Huxley model\(^1\) is incorrectly used because offset potentials are not considered. However, it is very likely that the offset potentials are closely related to the resting potentials of membranes. It should also be noted that the capacitance is typically dependent on the voltage. This effect has also not been considered in classical electrophysiology models. We treat that here in terms of a ‘capacitive susceptibility’ (eqn (13), cf. ref. 2).

Electrostrictive forces also influence melting transitions of lipid membranes. Since the fluid state of the membrane displays a smaller thickness than the gel phase, an electrostrictive force will shift the state of the membrane towards the fluid state. Heimburg\(^2\) calculated a decrease of the melting temperature, \( T_m \), which is a quadratic function of voltage. Since the membrane was considered being symmetric, the largest \( T_m \) is found at \( \Psi = 0 \). Here, we showed that a membrane which displays a spontaneous polarization in the absence of an applied electric field possesses an offset potential, \( \Psi_o \), in the free energy (eqn (8)). The respective equation contains the term \((\Psi + \Psi_o)^2 - \Psi_o^2 = \Psi^2 + 2\Psi_0 \Psi, \) which is approximately linear for \( \Psi \ll \Psi_o \) (eqn (8)). In fact, Antonov and collaborators found a linear dependence of the melting temperature on voltage.\(^5\)

This indicates that the membranes studied by Antonov and collaborators\(^3\) were polar. Antonov’s experiment determined the voltage-dependence of the melting temperature by measuring the permeability changes in the transition. The authors made use of the fact that membranes display maximum conductance in lipid phase transitions.\(^1\) In the ‘Applications’ section we showed how voltage can influence membrane permeability in the presence of spontaneous polarization. Surprisingly, the corresponding current–voltage \( (I-V) \) relations of membrane conductance can be inward or outward rectified, and resemble the \( I-V \) relations of many proteins.\(^19\)

Here, we investigated two possible mechanisms that can give rise to spontaneous polarization in the absence of an applied field, which both break the symmetry of the membrane. The first (flexoelectricity) acts by allowing the membrane to be curved (thus introducing a curvature, \( c \)) and a difference of the lateral tension within the two monolayers. The second mechanism acts by assuming a chemically or physically asymmetric lipid composition on the two leaflets. An example for a physically asymmetric membrane is a situation where one monolayer is in a fluid state while the other monolayer is in a gel state. Chemical asymmetry assumes a different lipid composition on the two sides of the membrane. The magnitude of the resulting offset, \( \Psi_o \), is strongly influenced by experimental conditions such as the lipid composition, salt concentration, pH, or the presence of divalent ions. Permanent polarization of the lipids can not only lead to an electrical offset but also to an enhanced dielectric constant. For biological membranes, polarization asymmetries
can originate from any constituting element of the membrane including integral membrane proteins (see Fig. 11 for a schematic description). We can also speculate that other membrane adhesive molecules with large dipoles can be used to create an asymmetric membrane, e.g., soluble proteins or lipid-associated molecules such as long-chain sugars. Depending on the nature of the asymmetry, the system can display piezoelectric properties.

The offset potential can have interesting consequences for capacitive currents. The charge on a capacitor is given by \( q = C_m(\psi + \psi_0) \). Therefore, for constant \( \psi_0 \) the capacitive current is given by

\[
I_c(t) = \frac{dq}{dt} = C_m \frac{d\psi}{dt} + (\psi + \psi_0) \frac{dC_m}{dt}
\]

(45)

For a positive change in potential, the first term in eqn (45) is positive and leads to a positive current. If the change in voltage happens instantaneously, the corresponding peak is very short. The second term describes the temporal change in the capacitance induced by the voltage change. It depends on the relaxation time of the capacitor dimensions, which close to transitions can range from milliseconds to seconds. Thus, it can be distinguished from the first term. Let us consider the situation shown in the insert of Fig. 7 (\( \psi_0 = 70 \text{ mV}, T = 314.5 \text{ K} \)) with a membrane capacitance of \( \approx 1 \mu \text{F cm}^{-2} \). Here, a jump from \( \psi = -70 \text{ mV} \) to \( \psi = -10 \text{ mV} \) yields a positive change in capacitance of \( \Delta C_m = 2.6 \text{ nF cm}^{-2} \). If the offset potential were \( \psi_0 = -70 \text{ mV} \) instead, the same jump would change the capacitance by \( \Delta C_m = -7.8 \text{ nF cm}^{-2} \). Therefore, the second term in eqn (45) is positive in the first situation but negative in the second situation. For this reason, depending on the offset potential and holding potential, the capacitive current associated to the second term in eqn (45) can go along the applied field or against the applied field. Similarly, for a jump in potential of +60 mV, the capacitive current would depend on the holding potential before the jump. For \( \psi_0 = 70 \text{ mV} \), the change in capacitance is \( \Delta C_m = -2.6 \text{ nF cm}^{-2} \) for a jump from \(-130 \text{ mV} \) to \(-70 \text{ mV} \). It is \( \Delta C_m = +8.9 \text{ nF cm}^{-2} \) for a jump from \(+70 \text{ mV} \) to \(+130 \text{ mV} \).

The typical time-scale of processes in biomembranes is a few milliseconds to a few ten milliseconds. It can be different for different voltages. Thus, slow currents on this time-scale against an applied field can originate from voltage-induced changes in lipid membrane capacitance. If the offset-potential also depends on voltage, this situation is more complicated.

Flexoelectric and piezoelectric phenomena have also be considered to be at the origin of an electromechanical mechanism for nerve pulse propagation.\(^6\) In 2005, Heimburg and Jackson proposed that the action potential in nerves consists of an electromechanical soliton. The nerve pulse is considered as a propagating local compression of the membrane with a larger area density. According to the piezoelectric effect treated here (eqn (16)), a change in membrane area can lead to the charging of the membrane capacitor. Alternatively, due to the inverse piezoelectric effect a change in the applied membrane potential can induce area changes (eqn (21)) and thus induce a density pulse. The inverse piezoelectric effect is very dependent on the lateral compressibility of a membrane. Thus, is largely enhanced in the melting transition where the compressibility is high. Further, these effects will largely depend on membrane polarization.

Finally, it should be mentioned that some of the polarization effects on artificial membranes are not very pronounced because changes in polarization due to changes in area are not very large. For instance, a voltage change of 200 mV changes the transition temperature by only 0.12 K. However, the absolute magnitude of the effect largely depends on offset polarizations. These could be influenced by lipid-membrane-associated molecules (such as proteins) with large dipole moments.

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### References