On the theory of dielectric spectroscopy of protein solutions

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Abstract
We present a theory of the dielectric response of solutions containing large solutes, of the nanometer size, in a molecular solvent. It combines the molecular dipole moment of the solute with the polarization of a large subensemble of solvent molecules at the solute–solvent interface. The goal of the theory is two-fold: (i) to formulate the problem of the dielectric response avoiding the reliance on the cavity-field susceptibility of dielectric theories and (ii) to separate the non-additive polarization of the interface, jointly produced by the external field of the laboratory experiment and the solute, from specific solute–solvent interactions contributing to the dielectric signal. The theory is applied to experimentally reported frequency-dependent dielectric spectra of lysozyme in solution. The analysis of the data in the broad range of frequencies up to 700 GHz shows that the cavity-field susceptibility, critical for the theory formulation, is consistent with the prediction of Maxwell’s electrostatics in the frequency range of 10–200 GHz, but deviates from it outside this range. In particular, it becomes much smaller than the Maxwell result, and shifts to negative values, at small frequencies. The latter observation implies a dia-electric response, or negative dielectrophoresis, of hydrated lysozyme. It also implies that the effective protein dipole recorded by dielectric spectroscopy is much smaller than the value calculated from the protein’s charge distribution. We suggest an empirical equation that describes both the increment of the static dielectric constant and the decrement of the Debye water peak with increasing protein concentration. It gives fair agreement with broad-band dispersion and loss spectra of protein solutions, but misses the δ-dispersion region.

(Some figures may appear in colour only in the online journal)

1. Introduction
Dielectric spectroscopy is a linear response technique, monitoring the dynamics of the dipole moment of a macroscopic sample of a polarizable material [1, 2]. While it is highly sensitive and provides a wealth of information about the dynamics of polarization modes active in a medium, the interpretation of spectra and the assignment of the observed relaxation to physical processes in the sample often require theoretical approaches.

The standard theoretical tool to study mixtures is the Maxwell–Wagner theory [2, 3] and its modifications, also in terms of effective-medium approaches [4]. All these theories assume that macroscopic dielectric constants can be assigned to all components of the mixture. This often becomes a significant oversimplification when highly heterogeneous solutes of nanometer dimension, such as hydrated proteins, are involved [5, 6]. The description of the polar response in terms of the molecular charge distribution is more accurate for these solutes [7]. Given the length-scale of the external field variation in a typical dielectric or light-absorption experiment, the overall charge and the dipole moment are the two main multipoles to consider [8].

Once the dipole moment has been assigned to a hydrated protein, one might assume that standard models of dipolar liquids [9, 10], involving the statistics and dynamics of molecular dipoles, could be directly extended to study protein solutions. One has, however, to recognize that proteins, and other solutes of similar dimension, possess an extended
interface with a molecular solvent, such as water, which is absent in the case of mixtures composed of molecules of comparable size. The interface of a hydrated protein involves a large number, \(\sim 300–500\), of water molecules in the first hydration layer alone. Given that the polarization of water by the protein propagates into at least the second hydration layer [11, 12], the actual size of the protein–water interface, as probed by polarization-sensitive experiments, is significantly larger.

These new physical realities require new theoretical approaches. The key question for this development is how to extend the classical theories of polar response of molecular dipoles into the realm of large solutes with extended interfaces. The key parameter for the development of dielectric theories is the Onsager cavity (or directing) field [13]. It describes the orienting torque acting on a molecular dipole when the macroscopic sample is placed in an external electric field [14]. The standard result of classical theories prescribes that the field of the external charges \(E_0\) is screened by the interfacial polarization to the cavity field [14, 15]

\[
E_c = \frac{3}{2\epsilon_s + 1} E_0,
\]

where \(\epsilon_s\) is the dielectric constant of the solvent. This cavity field then directly leads to the Onsager mean-field [16] equation for the dielectric constant of a homogeneous material and, when mutual short-range correlations of the dipoles are included, to the Onsager–Kirkwood relation [14]. The problem one faces in an attempt to describe a mixture of nanometer-size solutes with a molecular solvent is that there is no analog of either of these two equations. The fundamental line of inquiry here is whether one can extend equation (1), or its analog, to such mixtures, or a new set of rules is required. This is a nontrivial question. Some initial computer simulations have indicated that, indeed, polarized nanoscale interfaces follow rules different from those established for cavities carved in dielectrics [12, 17, 18]. The simulations are, however, limited by the nanosecond range of timescales. The question of what the polar response of a nanoscale interface is at lower frequencies remains therefore open.

This study aims to address this question by analyzing recent dielectric data obtained for solutions of lysozyme in water [19, 20]. We first develop a general formalism that does not anticipate any particular solution for the local field acting on the protein dipole. As a result of this analysis, we arrive at a surprising conclusion that the hydration layers of the protein dipole polarizes the surrounding dielectric by its own electric field such that the inhomogeneous Maxwell field \(\mathbf{E}(\mathbf{r})\) around the solute is a sum of the uniform Maxwell field of the external charges \(\epsilon_s^{-1}E_0\) and the dipolar field of the polarized interface

\[
\mathbf{E}(\mathbf{r}) = \epsilon_s^{-1}E_0 + \sum_j T(\mathbf{r} - \mathbf{r}_j) \cdot \mathbf{M}_{ij}^{\text{int}}.
\]

Here, \(T(\mathbf{r} - \mathbf{r}_j)\) is the dipolar tensor describing the electric field at point \(\mathbf{r}\) inside the solvent by a point dipole placed at \(\mathbf{r}_j\); the sum runs over \(N_0\) solutes with coordinates \(\mathbf{r}_j\).

The Maxwell field \(\mathbf{E}(\mathbf{r})\) polarizes the liquid, with the resulting local inhomogeneous polarization \(\mathbf{P}(\mathbf{r}) = (4\pi)^{-1}(\epsilon_s - \epsilon_\infty)\).

2. Dielectric response of a mixture

Dissolving a polar solute in a polar solvent leads to two distinct effects on the response of the medium to an external electric field. The first effect is the exclusion of the solvent from the volume of the solute. The second effect is the response of the charge distribution within the solute to the orienting torque of the external field. The two effects are entangled in the polarization of the interface by the solute charges and by the external field. However, their contributions to the overall dielectric response of the solution can be separated in the frequency domain. Since they also originate from distinct physical interactions, repulsive expulsion on the one hand and electrostatic interactions on the other, we start by considering the effect of the solute excluded volume and then add the contribution of the solute dipole moment to the dielectric response of the solution.

2.1. Non-polar solutes in a polar solvent

Excluding the solvent from the solute volume creates the solute–solvent interface. From the standard viewpoint of dielectric theories, any interface carries interfacial polarization when the solution is placed in a uniform field of external charges (capacitor plates of the dielectric experiment). The polarization of the interface is described in the Maxwell theory of dielectrics by the surface charge density [15]. It is given as the projection of the dipolar polarization of the dielectric \(\mathbf{P}_{\text{s}}\) at the dividing surface \(S\) on the outward normal to the surface \(\hat{n}_S\). The surface charge density then becomes \(\sigma = \hat{n}_S \cdot \mathbf{P}_{\text{s}}\). This charge density integrates to a dipole moment of the interface

\[
\mathbf{M}_{ij}^{\text{int}} = \int_S \mathbf{r}_S \sigma(r_S) \, dS,
\]

where the surface integral is taken over the closed surface \(S\) enveloping the solute.

The interface dipole polarizes the surrounding dielectric by its own electric field such that the inhomogeneous Maxwell field \(\mathbf{E}(\mathbf{r})\) around the solute is a sum of the uniform Maxwell field of the external charges \(\epsilon_s^{-1}E_0\) and the dipolar field of the polarized interface

\[
\mathbf{E}(\mathbf{r}) = \epsilon_s^{-1}E_0 + \sum_j T(\mathbf{r} - \mathbf{r}_j) \cdot \mathbf{M}_{ij}^{\text{int}}.
\]

Here, \(T(\mathbf{r} - \mathbf{r}_j)\) is the dipolar tensor describing the electric field at point \(\mathbf{r}\) inside the solvent by a point dipole placed at \(\mathbf{r}_j\); the sum runs over \(N_0\) solutes with coordinates \(\mathbf{r}_j\).

The Maxwell field \(\mathbf{E}(\mathbf{r})\) polarizes the liquid, with the resulting local inhomogeneous polarization \(\mathbf{P}(\mathbf{r}) = (4\pi)^{-1}(\epsilon_s - \epsilon_\infty)\).
\(1\) \(\mathbf{E}(\mathbf{r})\), decaying to the homogeneous polarization \(\mathbf{P} = (4\pi)^{-1}(1 - \epsilon^{-1})\mathbf{E}_0\) produced by the external charges far from the solute–solvent interface. The overall dipole created in the solution is the integral of \(\mathbf{P}(\mathbf{r})\) over the volume \(\Omega\) occupied by the solvent

\[
\mathbf{M}_{\text{mix}} = \int_{\Omega} \mathbf{P}(\mathbf{r}) \, d\mathbf{r}. \tag{4}
\]

Here, the subscript ‘mix’ identifies the solvent–solute mixture. Assuming that the interfacial dipoles of solutes are independent of each other, one obtains [12]

\[
\mathbf{M}_{\text{mix}} = \mathbf{M}_{\text{hom}} - N_0\Omega_0\mathbf{P} - (2/3)(\epsilon_s - 1)N_0\mathbf{M}_0^{\text{int}}. \tag{5}
\]

Here, \(\mathbf{M}_{\text{hom}} = V\mathbf{P}\) is the dipole moment of the corresponding homogeneous (without solutes) polarized solvent and \(\Omega_0\) is the volume of the solute. The second summand in equation (5) represents the dipole moment cut from the liquid by inserting \(N_0\) voids. Finally, the last term is an additional polarization induced in the surrounding liquid by the surface charge density \(\sigma_p\).

The value of the interface solute dipole \(\mathbf{M}_0^{\text{int}}\) will depend on the specifics of the solute–solvent interactions and the local polarization of the solvent created by these interactions. While it is a complex function of the entire mosaic of pairwise solute–solvent interactions for a realistic solute, an estimate of this parameter can be obtained from dielectric theories for a spherical void in a dielectric. The interface dipole reads in this case [15]

\[
\mathbf{M}_0^{\text{int}} = -3\Omega_0\mathbf{P}/(2\epsilon_s + 1), \tag{6}
\]

where the superscript ‘\(M\)’ specifies Maxwell’s boundary conditions at the dividing surface not affected by the local solute–solvent interactions. In order to quantify deviations from this generic result, one can introduce the ratio

\[
\alpha = \mathbf{M}_0^{\text{int}}/\mathbf{M}_0^{\text{M}}. \tag{7}
\]

The dipole moment of the mixture is related to the mixture dielectric constant \(\epsilon_{\text{mix}}\) as \(\mathbf{M}_{\text{mix}}/V = (4\pi)^{-1}(1 - \epsilon_{\text{mix}}^{-1})\mathbf{E}_0\). One then obtains for the dielectric constant of the mixture

\[
\frac{\epsilon_s}{\epsilon_{\text{mix}}} = 1 + \eta_0(\epsilon_s - 1) \left[ 1 - 2\alpha \frac{\epsilon_s - 1}{2\epsilon_s + 1} + R_1(\eta_0) \right], \tag{8}
\]

where \(\eta_0 = N_0\Omega_0/V\) is the volume fraction of the solutes in the sample with the overall volume \(V\). We have put an extra term \(R_1(\eta_0)\) in the above equation to indicate terms non-linear in the volume fraction that appear when mutual polarization of the interface dipoles is taken into account [22]. Similar non-linear terms appear in the response of a mixture of water with dipolar solutes discussed below. There is presently no consistent formalism to include these effects and we neglect them at the current stage of the theory development recognizing that the theory might run into conflict with the data collected for concentrated solutions.

If the Maxwell result for a void in a dielectric holds, \(\alpha = 1\) and the dielectric constant of the mixture becomes

\[
\frac{\epsilon_s}{\epsilon_{\text{mix}}} = 1 + \eta_0 \frac{3(\epsilon_s - 1)}{2\epsilon_s + 1}. \tag{9}
\]

Equation (8), with \(R_1(\eta_0)\) omitted, and equation (9) describe the dielectric constant of a mixture of non-interacting, non-polar solutes with a polar solvent. Equation (9) also reduces to the standard result of the Maxwell–Wagner theory in the limit of low volume fraction of the solutes [2, 3]. One can also account for the electronic polarizability of the protein not mentioned so far. If the refractive index \(n_p\) can be assigned to the protein, one needs only to realize that the boundary conditions of the dielectric theories are sensitive to the ratio of the two dielectric constants at the dividing surface, \(\epsilon_s/n_p^2\). Equation (9) then extends to

\[
\frac{\epsilon_s}{\epsilon_{\text{mix}}} = 1 + \eta_0 \frac{3(\epsilon_s - n_p^2)}{2\epsilon_s + n_p^2}. \tag{10}
\]

Equation (8) can be alternatively written in terms of the cavity field \(E_c\) inside a spherical void in a uniformly polarized liquid. The electric field inside the cavity is proportional to the external field, with the susceptibility \(\chi_c = E_c/E_0\). In terms of this susceptibility, (8) becomes [18]

\[
\frac{\epsilon_s}{\epsilon_{\text{mix}}} = 1 + 3\eta_0 [\chi_c(\epsilon_s - 1)]. \tag{11}
\]

The standard prescription of Maxwell’s theory of dielectrics yields (cf equation (1)) [14, 23]

\[
\chi_c^M = \frac{3}{2\epsilon_s + 1}. \tag{12}
\]

The connection between the susceptibility \(\chi_c\) and the parameter \(\alpha\) (equation (7)), which is required to obtain equation (11) from (8), is derived from the following arguments. The polarization \(\mathbf{P}(\mathbf{r})\) in the solvent, induced by the Maxwell field in equation (3), creates a non-vanishing electric field inside the solute given by the equation

\[
E_c = E_0 + \int_{\Omega} \mathbf{T}(\mathbf{r}) \cdot \mathbf{P}(\mathbf{r}) \, d\mathbf{r}. \tag{13}
\]

Upon substitution of \(\mathbf{P}(\mathbf{r}) = (4\pi)^{-1}(\epsilon_s - 1)\mathbf{E}(\mathbf{r})\) and equation (3) into this relation, one arrives at the connection between \(\chi_c\) and \(\alpha\)

\[
3\epsilon_s \chi_c = \epsilon_s + 2 - \alpha \frac{2(\epsilon_s - 1)}{2\epsilon_s + 1}. \tag{14}
\]

Combining equations (8) and (14), one arrives at equation (11).

2.2. Polar solutes in a polar solvent

When a solute carries dipole moment \(m_0\), it aligns along the external field such that the average dipole \(\langle m_0 \rangle_E\) in a weak external field is given by the linear susceptibility \(\chi_0\) [23]

\[
\langle m_0 \rangle_E = \chi_0\Omega_0E_0, \tag{15}
\]

where \(\langle \cdots \rangle_E\) denotes an ensemble average in the presence of the external field and

\[
\chi_0 = \chi_00 + \chi_{0b} = (\beta/3\Omega_0)\langle \delta m_0 \cdot \delta M_{\text{mix}} \rangle. \tag{16}
\]

In this equation, \(\delta m_0 = m_0 - \langle m_0 \rangle\) and \(\delta M_{\text{mix}} = M_{\text{mix}} - \langle M_{\text{mix}} \rangle\) are the deviations of the solute dipole and the dipole of
the sample $M_{\text{mix}}$ from their average values and $\beta = 1/(k_B T)$ is the inverse temperature.

The solute susceptibility in equation (16) is split into the self, $\chi_{00}$, and solute–solvent, $\chi_{0s}$, parts. The former is given by the variance of a single solute dipole

$$\chi_{00} = (\beta/3\Omega_0)(\langle \delta m_0 \rangle^2).$$ (17)

Correspondingly, the cross susceptibility is the correlation of a single solute dipole with the dipole moment $\delta M_s$ of the entire solvent in the sample [21]

$$\chi_{0s} = (\beta/3\Omega_0)(\delta m_0 \cdot \delta M_s).$$ (18)

Equation (17) neglects correlations between dipole moments of the solutes in the solution represented by the corresponding Kirkwood factor. Since the latter describes short-range correlations of the length-scale of the molecular diameter [9], they can be safely omitted in the type of theory developed here.

Both standard arguments of the dielectric theories [14] and microscopic derivation [12] suggest a simple connection between the solute dipolar susceptibility $\chi_0$ and the self-susceptibility $\chi_{00}$

$$\chi_0 = \chi_c \chi_{00}.$$ (19)

This relation implies that the account of the solute–solvent cross-correlations entering the susceptibility $\chi_{0s}$ amounts to introducing the cavity field acting on the average solute dipole. The same cavity field is also built into the Onsager theory of dipolar liquids and defines the torque acting on a selected dipole in the liquid (directing field) [13].

Adding the dipolar polarization of the solutes to equation (11) for the dielectric constant of the liquid with spherical voids, one arrives at the dielectric constant of the solution

$$\frac{\varepsilon_s}{\varepsilon_{\text{mix}}}(\omega) = 1 - 3\eta_0 + 3\eta_0\varepsilon_s(1 - y_0),$$ (20)

where $y_0 = (4\pi/3)\chi_{00}$. This equation clearly reduces to (11) in the limit of non-polar solutes when $y_0 \rightarrow 0$.

2.3. Frequency-dependent response

The static arguments presented in the previous sections can be extended to the frequency domain of main interest to broad-band dielectric spectroscopy. The dielectric constants of both the solvent and the mixture become frequency-dependent functions, $\varepsilon_s(\omega)$ and $\varepsilon_{\text{mix}}(\omega)$. The dipolar susceptibility of an isolated solute transforms into a linear response function, instead of a static correlator of equation (17). The relevant formalism is well developed and the result is the following response function of the solute dipolar fluctuations [24, 25]:

$$\chi_{00}(\omega) = \chi_{00}(1 + i\omega S_{00}(\omega)).$$ (21)

Here, $S_{00}(\omega)$ is the Laplace–Fourier transform of the normalized time correlation function of the solute dipole $m_0(t)$

$$S_{00}(t) = [(\delta m_0(t)^2)]^{-1}(\delta m_0(t) \cdot \delta m_0(0)).$$ (22)

This function was fitted to multi-exponential decay when applied to the analysis of the molecular dynamics (MD) simulation data presented below

$$S_{00}(t) = \sum_i A_i e^{-t/\tau_i}, \quad \sum_i A_i = 1,$$ (23)

where $\tau_i$ are the relaxation times and $A_i$ are the relative weights of the relaxation components. From this equation, one gets the frequency-dependent function $y_0(\omega)$

$$y_0(\omega) = y_0 \sum_i \frac{A_i}{1 - i\omega \tau_i}.$$ (24)

The frequency-dependent dielectric constant of the solution becomes

$$\frac{\varepsilon_s(\omega)}{\varepsilon_{\text{mix}}(\omega)} = 1 - 3\eta_0 + 3\eta_0\varepsilon_s(1 - y_0(\omega)).$$ (25)

Our arguments so far have not introduced any approximations except for neglecting the mutual polarization of the solute cavities at their high concentration and short-range correlations of the solute dipoles entering the Kirkwood factor of the solutes. However, equations (20) and (25) contain an unknown cavity-field susceptibility $\chi_c(\omega)$. The Maxwell result for this function refers to a free surface separating a dielectric from a void. It is a priori not obvious that this function can describe the complex and heterogeneous protein–water interface involving both weak protein–water interactions at hydrophobic patches and strong binding to charged surface residues. However, one can use the experimental input for the dielectric constants of the mixture and pure water in equation (25) to extract the cavity-field susceptibility $\chi_c(\omega)$.

Figure 1 shows the real part $\chi_c(\omega)$ extracted from equation (25) using frequency-dependent dielectric constants of lysozyme solutions from broad-band dielectric spectroscopy below 50 GHz [19] and from separate measurements in the frequency range 70–700 GHz [20]. The dotted line shows $\text{Re}[\chi_c^M]$ from equation (12); the break in the curve signals the transition from water to buffer used at higher frequencies in [20]. The cavity-field susceptibility follows very closely the Maxwell prediction in the range of frequencies 10–200 GHz, but then deviates downward outside this range. The behavior at low frequencies is particularly noteworthy.

It turns out that the dipole moment induced at the protein by an external field is over-screened [26] by the hydration layers, and perhaps the ionic atmosphere, to nearly zero. In fact, $\chi_c$ is below zero at $\nu < 1$ GHz, implying a dia-electric response, i.e. repulsion of the protein dipole from a stronger electric field. This phenomenon, known as negative dielectrophoresis, is well documented for hydrated nanoparticles [27], but has not been broadly observed for proteins. Our recent extensive simulations of ubiquitin [12], which is neutral at pH 7.0, have pointed to exactly this scenario: a negative $\chi_{0s}$, larger in magnitude than the positive $\chi_{00}$, thus resulting in a slightly negative $\chi_0$ in equation (16). However, this result has not been detected from simulations of charged proteins, including lysozyme, probably due to the neglect of the ionic atmosphere in the analysis.
Figure 1. Real part of the cavity-field susceptibility $\chi_c(\omega)$ extracted from experimental dielectric measurements according to (25). The results combine the broad-band dielectric measurements from [19] (solid and dashed lines) with high-frequency data from [20] (dash-dotted lines). The dotted line (labeled w/b) indicates $\chi^M_0$ from equation (12) for pure water (at lower frequencies) and for the buffer (at higher frequencies). The gap between the two sets of curves represents the frequency window between the measurements; the two high-frequency lines referring to different concentrations almost coincide on the scale of the plot. Partial protein association in no-buffer solution [19] may account for the differences between the two low-frequency lines. The inset shows the magnified low-frequency portion of the plot.

Figure 1 suggests that dielectric models of the cavity-field susceptibility do not provide an adequate description in the entire range of frequencies of interest to broad-band spectroscopy. However, the modeling can proceed along separate routes since the expulsion of polar water from the solute core is significant only at high frequencies, while the polar response of the protein dipole, described by $y_0(\omega)$, dominates at low frequencies. One therefore can keep the Maxwell result for $\chi_c(\omega)$ for the former component, as realized in equation (9). Since there is currently no model allowing the description of the overscreening observed at low frequencies, we have resorted to an empirical approximation. Replacing $\chi_c y_0$ in equations (20) and (25) with $\chi^M_0 y_0$ accomplishes most of what is seen to occur in figure 1 and allows us to arrive at a compact relation for the dielectric constant of the solution

$$\frac{\varepsilon_s(\omega)}{\varepsilon_{\text{mix}}(\omega)} = 1 + \frac{3\eta_0}{2\chi_c(\omega) + 1} \left[\varepsilon_s(\omega) - 1 - 3y_0(\omega)\right]. \quad (26)$$

2.4. Dielectric instability

Equation (20) predicts a point of dielectric instability at which the assumption of a uniform solution of weakly interacting protein dipoles breaks down. The instability is toward clustering of dipoles and is associated with the divergence of the dielectric constant $\varepsilon_{\text{mix}}$. It is reached at the critical volume fraction

$$3\eta_c = [1 + \varepsilon_s \chi_c(\gamma_0 - 1)]^{-1}. \quad (27)$$

If the Maxwell form of the cavity-field susceptibility is used in this equation, the critical point $\eta_c = 0.01 (\gamma_0 \simeq 16)$ corresponds to a concentration of 8 mg mL$^{-1}$ for lysozyme in solution. Lysozyme solutions are stable in this range of concentrations and this estimate is clearly too low. On the contrary, the overscreening scenario shown in figure 1 makes $\eta_c$ negative, thus removing the instability altogether. While other forms of aggregation are still possible [28, 29], it might be quite possible that overscreening of the protein dipole eliminates instability toward dipolar clustering (such as formation of dipolar chains) and lowers the sensitivity of proteins in solutions to inhomogeneous electric fields always present in vivo.

3. Application to experiment: lysozyme solution

Dielectric measurements of solutions typically provide the real and imaginary parts of the dielectric constant as functions of frequency and solution composition [19, 30–33]. The existence of these two coordinates, frequency and solute concentration, allows one to learn about the specific pattern of interfacial polarization realized for a given solute and the dynamics of processes contributing to the relaxation of the sample dipole moment. We start with the analysis of the concentration dependence at a given frequency, followed with the analysis of the frequency dependence at a fixed concentration.

3.1. Decrement of the water Debye peak

Independently of the details of the dynamics of the protein itself and its coupling to the interfacial waters, the timescales of these motions are significantly lower than the characteristic time of dielectric relaxation of water. The global motions of the solute are dynamically frozen at the frequency of the water Debye peak ($\nu_D \sim 18$ GHz). This implies that $y_0(\omega_D)$ can be dropped from equation (26). One then arrives at the dielectric constant of the mixture of polar water and effectively non-polar solutes (equations (8) and (9)). Any sufficiently high frequency can in principle be taken for this analysis.

The decrement of the Debye peak of water in the solution versus the solute concentration is often reported [8] and can be used, in the framework of the present theory, as a convenient source of data to extract the information about the parameters $\alpha$ and $\chi_c$.

Our formalism is applied to recent measurements of dielectric spectra of lysozyme solutions [19, 20]. Figure 2 shows the dependence of the decrement in the amplitude of the water Debye peak $\Delta\varepsilon(\omega_D)$ in the solution, $\Delta\varepsilon(\omega_D) = \varepsilon_{\text{mix}}(\omega_D) - \varepsilon_s(\omega_D)$, versus the protein concentration. The circles show the experimental data from [19], while the solid and dashed lines refer to equations (9) and (26), respectively. For the latter, $y_0(\omega_D)$ calculated from MD simulations [12], and discussed below for the analysis at lower frequencies, was used. Clearly, the protein permanent dipole can be safely neglected. The transformation from the solution concentration reported experimentally to the volume fraction required by (9) and (26) was performed by using the volume of lysozyme $\Omega_0 = 29.8$ nm$^3$. The latter was calculated from the crystallographic structure of the protein (3FE0, PBD database) by using the algorithm developed by Till and Ullmann [34].
Decrement of the water dielectric constant in the solution of lysozyme in water, $\Delta \varepsilon(\omega)/\varepsilon(\omega) = \varepsilon_{\text{mix}}(\omega)/\varepsilon(\omega) - 1$, as a function of the protein concentration $C$. The points are the experimental data at the frequency of the water Debye peak $\nu_D \approx 18$ GHz (circles) [19], and at $\nu = 72$ GHz (diamonds) [20]. The solid and dash-dotted lines refer to the calculations using equations (9) and (10) in the order of increasing frequency. The dashed line, nearly coinciding with the solid line on the scale of the plot, is the calculation incorporating the dynamics of the protein dipole according to equation (26), with $\gamma_0(\omega)$ calculated from MD simulations [12]. The lysozyme molecular volume of $\Omega_0 = 29.8$ nm$^3$ is used to convert from the volume fraction to the solution concentration. The dotted line connects the experimental points.

In accord with the results shown in figure 1, the cavity-field susceptibility is well described by the Maxwell form (equation (12)) at the frequency of the water Debye peak, and the agreement between theory and experiment is excellent. It becomes less satisfactory at a higher frequency of 72 GHz [20], also shown in figure 2. The refractive index of the protein starts to affect the result at this high frequency and $n_p = 1.7$ from [35] was adopted in the calculations using equation (10). The theoretical slope with increasing protein concentration is higher than experimentally reported and is likely related to deviations of the cavity-field susceptibility from the Maxwell form, as is seen in figure 1.

### 3.2. Dielectric spectra of solutions

The results for the dispersion and loss spectra of lysozyme solutions are shown in figure 3. Experimental data from [36] were used for $\varepsilon_{\text{s}}(\omega)$ and MD simulations of a single lysozyme protein hydrated in a simulation box of TIP3P water [12] were used to produce $\gamma_0(\omega)$ in (24). The relaxation parameters in (24) are $A_i = [0.13, 0.06, 0.81]$, $\tau_i = [0.037, 0.295, 14.6]$ ns, $\gamma_0 = 16.3$. The dominant relaxation component of the solute dipole, with a relaxation time of 14.6 ns, can be assigned to protein tumbling. A relaxation time of 9.1 ns was reported for this relaxation component from the analysis of proton NMR at low resonance frequencies [37].

The highest-frequency relaxation of the protein dipole reported by simulations [12] is $\approx 4.3$ GHz, accounting for about 13% of the overall correlation function and below the water Debye peak at 18 GHz. Higher vibrational frequencies clearly exist in the protein and contribute to the overall vibrational density of states, but they do not significantly affect the fluctuations of the protein dipole.

The usefulness of MD simulations for comparison with experiment is somewhat limited since the charge distribution in the protein studied by simulations might not entirely fit the experimental conditions. The standard force-field prescriptions for protonating/deprotonating the surface residues of lysozyme at $pH = 7.0$ produce an overall protein charge of $+7$, while a charge of $+10$ is reported at $pH = 5.5$ in the experimental study [19]. Overall, the permanent dipole moments of the proteins arise from slight deviations from a highly symmetric distribution of charge minimizing the total dipole moment [38, 39]. Shifts of the $pK_a$ values of surface residues due to the local electrostatic environment [40], ion association, and pH can therefore alter the dipole moment.

Despite remaining uncertainties regarding the magnitude of the protein dipole when experimental conditions are considered, the dipole moment $\langle m_0 \rangle = 223$ D from MD results in a fair agreement between theoretical and experimental dispersion curves $\varepsilon'_{\text{mix}}(\omega)$ at the lower concentration of the protein, 28 mg ml$^{-1}$ (figure 3(a)). The theory misses some of the static dielectric constant at the higher concentration, $\varepsilon = 110$ mg ml$^{-1}$, but the difference actually comes from the missing increment at intermediate frequencies associated with $\delta$-dispersion. This part of the spectrum is also missing in the loss spectrum (figure 3(b)). This outcome is expected since specific protein–water binding contributing to this signal [21, 41, 30] has not been incorporated into the model.

### 4. Summary

Broad-band dielectric spectroscopy is a widely used tool to interrogate the dynamics of complex systems, including
The remarkable result of this analysis is that at dielectric constants of the protein solution and pure water, susceptibility $\chi$ to this analysis since it allows us to extract the cavity-field can be applied to hydrated proteins. Equation (25) is central established for cavities carved in dielectrics, and also applied and a quasi-macroscopic subensemble of interfacial water by recognizing both the molecular nature of the protein dipole and a quasi-macroscopic subensemble of interfacial water producing interfacial polarization.

The theory thus aims to study whether the standard rules established for cavities carved in dielectrics, and also applied to calculate the local field acting on molecular dipoles [13], can be applied to hydrated proteins. Equation (25) is central to this analysis since it allows us to extract the cavity-field susceptibility $\chi_c(\omega)$ directly from the frequency-dependent dielectric constants of the protein solution and pure water. The remarkable result of this analysis is that at $\omega < 1$ GHz the susceptibility $\chi_c(\omega)$ is below $\approx 0.02$ predicted by the Maxwell equation (12) and is in the negative territory, down to $\approx -10^{-3}$. Therefore, the standard prescription derived for dielectric cavities (equations (1) and (12)) cannot be used in successful theories of the dielectric response of protein solutions.

Granted, the cavity susceptibility extracted from experimental measurements might reflect the combined response of the dielectric interface and the ionosphere. However, as a cumulative signature of the protein–water interface, it dramatically downscales the permanent dipole sensed by the dielectric experiment compared to its value calculated from atomic charges. Its low value can also help to explain the puzzling ability of proteins to stay in solution in vivo, despite significant electric-field gradients that should pull a paraelectric particle to stick to, for instance, the bilipid membrane. The dia-electric response suggested by the present analysis of experimental data, and our previous simulations [12], might be an answer to this puzzle since a dia-electric solute repels from a charged interface creating the field gradient. It also eliminates the dielectric instability toward clustering of the solute dipoles predicted by (20) and (27) when the Maxwell form of the cavity-field susceptibility is used there.

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