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Polarity of Hydrated Phosphatidylcholine Headgroups

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Abstract

The headgroup (H) stratum (sometimes called the polar region) of membrane bilayers is a relevant yet poorly understood solvation phase for small molecules and macromolecules interacting with the membranes. Solvation of compounds in bilayer strata is characterized experimentally by wide- and small angle X-ray scattering, neutron diffraction, and various NMR techniques. The quantification is tedious and only available for a limited set of small molecules. Our recently published model of liposome partitioning of small molecules shows that solvation of compounds in the H-stratum of fluid phosphatidylcholine (PC) bilayers correlates well with their solvation in hydrated diacetyl phosphatidylcholine (DAPC), and solvation in the core (C) depends in a similar way on that in *n*-hexadecane. These two correlations became a basis for a model describing the location of compounds in the H- and C-strata, and at the connecting interface, as a nonlinear function of the fragment solvation characteristics of the compounds. In this study, refractivity of hydrated DAPC phases with varying water content was measured and polarity determined using steady-state fluorescence of indole and Nile Red. The results were compared with the published data obtained by other techniques for PC bilayers in liposomes or on solid supports. The demonstrated qualitative agreement, as well as the polarity and refractivity dependencies on the DAPC concentration, support the suitability of hydrated DAPC as the H-stratum surrogate. Interestingly, depending on hydrations typical for H-strata of fluid PC bilayers, the dielectric constant could decrease significantly, from 31.0 to 7.3 for 16 and 8 water molecules per headgroup, respectively. Although additional experiments are needed for a confirmation, this observation could help set proper dielectric constant magnitudes in continuum-based computational models of accumulation and crossing of the PC bilayers with varying hydration levels thanks to the temperature or the structure of fatty acid chains.

Graphical Abstract

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Keywords

phospholipid; bilayer; phosphatidylcholine; DAcPC; DMPC; DOPC; DPPC; DSPC; intrabilayer distribution; partition coefficient; polarity; dielectric constant; Reichardt's polarity parameter; headgroups; core; interface; *n*-hexadecane

INTRODUCTION

The interactions with the headgroup (H) and core (C) strata of bilayers play important roles in absorption and distribution of drugs and other small organic molecules, peptides, and nucleic acids in organisms,¹ as well as in transporter and channel² functions of membrane embedded proteins. The equilibrium distribution of compounds in the phospholipid bilayer strata³ can be determined by several complementary experimental methods, mainly neutron diffraction,^{4–7} small^{8;9} and wide angle X-ray scattering,^{10;11} various nuclear magnetic resonance (NMR)^{12–15} and electron paramagnetic resonance¹⁶ techniques, surface plasmon resonance,¹⁷ as well as fluorescence¹⁸ and fluorescence quenching.¹⁹ Except neutron diffraction, these methods usually do not provide a complete intrabilayer distribution profile and rather pinpoint the preferred stratum. All mentioned methods are quite tedious and not amenable to a higher throughput.

To extrapolate the valuable bilayer distribution data to other compounds and quickly obtain estimates of solvation free energies of compounds in the bilayers or their strata, different surrogate phases have been examined, ideally exhibiting similar composition as the imitated bilayer strata.

In this aspect, diacetyl phosphatidylcholine (DAcPC) occupies a unique position: it contains all structural fragments and represents the closest match to the headgroup of phosphatidyl choline (PC), the most abundant mammalian phospholipid. Hydrated DAcPC is an adequate surrogate phase of the H-stratum of the PC bilayer,²⁰ albeit it is isotropic. DAcPC has a high aqueous solubility, allowing its use in the hydration states close to those of a fluid PC bilayer at room and body temperatures, which are represented by 8 – 16 water molecules per headgroup.^{21–24}

Longer hydrocarbons, e.g. *n*-hexadecane (C16), are appropriate bilayer core surrogates²⁵ as evidenced by experimental observation of similar molecular packing²⁶ and dynamics^{27;28} of the fatty acyl chains in the core of the bilayer and in bulk liquid alkanes.

In low concentrations typical for pharmacotherapy situations, interactions of drugs with hydrated DAcPC and C16 phases can be more conveniently described in terms of partitioning than as binding.²⁹ We showed that the C16/DAcPC partition coefficients of organic compounds perfectly discriminate between the lipophiles accumulating in the core and cephalophiles preferring the H-stratum, in contrast to other, more commonly used partition coefficients.³⁰ Fragment contributions to the C16/water and C16/DAcPC partition coefficients were used to construct accurate structure-based models of intrabilayer drug distribution.³¹ These facts further support the suitability of hydrated DAcPC and C16 as the surrogate phases of the H- and C- strata. Whether the lacking anisotropy of hydrated DAcPC affects its ability to emulate solvation behavior of the PC H-stratum bilayer in some situations remains to be examined by additional experiments. One of them is the comparison of polarities of the two phases, which was performed in this study.

Computational approaches for studying macromolecules and drugs in the bilayer have used different levels of approximation for the bilayer structure, ranging from implicit continuum models and coarse-grained mesoscale models³² to fully atomistic representations,^{33;34} and their combinations.³⁵ Our fragment-based approach³¹ formally represents a simple continuum model.. The continuum models often require the dielectric constant value to calculate the electrostatic interaction energies. In some cases, similar solvation properties of the H-stratum and bulk water were assumed,^{36–40} stemming from the high headgroup hydration. Plausibility of the bulk water approximation is lessened by stronger interactions of aromatic substructures of drugs with hydrated PC headgroups than with bulk water,³⁰ and by the diminished residual hydration capacity of water molecules because of the interactions with the headgroups.⁴¹ The H-stratum of a fluid PC bilayer contains 8 – 16 water molecules per headgroup at room and body temperatures.^{21–24} The PC headgroups interact with water hydrogens through their H-bond acceptor groups (phosphate, carbonyls, and glycerol oxygens), with the strength decreasing in this order. Water molecules around the trimethylammonium group are organized in a loose, clathrate-like structure, with oxygens mostly pointing towards the positively charged nitrogen.^{41–43} The clathrate is stabilized, to some extent, by unique hydrogen bonds involving the hydrogens in methyl groups^{42;43} and methylene group^{41–43} surrounding the nitrogen. The bonds were observed by small angle neutron scattering⁴¹ and IR spectroscopy,⁴² and supported by reliable computational chemistry approaches.^{41–43} Complexity and networking of H-bond interactions in the H-stratum are presumable reasons for a better description of the distribution of a limited set of small molecules in bilayer strata by our semi-empirical, continuum, fragment-based model³¹ compared to the atomistic molecular dynamics simulations utilizing current force fields.³⁴ Participation of the majority of hydrating water molecules in these interactions⁴¹ affects polarity of the H-stratum.

Macroscopic solvent polarity is generally understood as the solvation capability of the solvent determined by its specific and non-specific interactions with the solute molecules except the interactions altering chemical structure of the solute, such as protonation/deprotonation, oxidation/reduction, complex formation, etc.^{44,45} Fluorescence is widely used to measure solvent polarity thanks to different stabilization of photo-excited states of fluorophores by solvent molecules, resulting in varying emission characteristics, including emission peak wavelengths, quantum yields, and lifetimes.⁴⁶ Macroscopic solvent polarities

are most often characterized by the continuum-based dielectric constant (equal, for the static electric field, to relative permittivity at room temperature) and empirical parameters, which take into account local interactions of the reporter molecules with the solvent, such as the Reichardt's E values.⁴⁴ The $E_T(30)$ solvent parameters represent the excitation energies, in kcal/mol, of 2,6-diphenyl-4-(2,4,6-triphenylpyridin)-1-phenolate. They can be normalized to dimensionless $E_T^N = [E_T(30) - 30.7]/32.4$, where 30.7 is $E_T(30)$ for tetramethylsilane ($E_T^N = 0$) and 32.4 is the difference between the $E_T(30)$ values of water ($E_T^N = 1$) and tetramethylsilane.⁴⁴

The dielectric constant of the PC H-stratum has been reported^{47;48} to range from 30 to 34 but the value could also be as low as 4 depending on the gel/liquid-crystal phase transition and ion binding.⁴⁸ Here, we determine the dielectric constant and the normalized Reichardt's E_T^N polarity indices for the hydrated DACPC indirectly, in the steady-state fluorescence studies of Nile Red (NR) and indole. The modeling of the fluorescence spectral shifts requires the refractivity data of DACPC solutions, which are also measured. The polarity and refractivity indices are compared with published values obtained for the PC bilayer headgroups in liposomes.

EXPERIMENTAL SECTION

Chemicals

DACPC was synthesized and purified as described previously.⁴⁹ All solvents were procured from commercial vendors through VWR International, Inc. and used as received: chlorobenzene and *n*-hexadecane (99%) from Alpha Aesar, ethanol (ACS grade) from BDH, and dry solvents acetonitrile, benzene, chloroform (ethanol stabilized), dichloromethane, 1,2-dichloroethane, 2-propanol, *N,N*-dimethylformamide, benzene, ethyl acetate, methanol, tetrahydrofuran, and dimethylsulfoxide (DMSO) from EMD Millipore. Used water was prepared in-house using a Barnstead Nanopure™ TOC Life Science ultrapure water system. The used fluorescent dyes, indole and 9-diethylamino-5-benzo[*a*]phenoxazinone (Nile Red, NR), were obtained from Acros.

Refractometry Studies

Aqueous DACPC solutions were prepared by adding the required amount of water to crystalline, lyophilized DACPC to obtain the highest used concentration (2.5 M). This solution was diluted with required amounts of water to obtain lower concentrations.

An Abbe 201 refractometer, working at sodium D-line (589 nm), was used to measure the refractive index of the aqueous DACPC solutions at 25 °C. The results in degrees Brix were converted to the refractive index using verified results of the FermCalc calculator.⁵⁰ The calculator is based on the dependence of the Brix degree on the refractive index, expressed by a fourth-degree polynomial. The corresponding refractive index values are obtained by iterative approach,⁵⁰ and were verified using the underlying equation.

Fluorescence Studies

Indole solutions in the used organic solvents and aqueous DAcPC solutions were prepared directly by dissolving the compound in the solvents.

Nile Red is sparingly soluble in water but dissolves better in aqueous DAcPC solutions and is soluble in used organic solvents. The NR solutions (2 μM) in the organic solvents were prepared directly. For the aqueous DAcPC solutions, NR was initially dissolved in the 2.5 M DAcPC solution and then diluted with appropriate DAcPC solutions to obtain required concentrations ($< 5 \mu\text{M}$).

Steady-state fluorescence absorption and fluorescence spectra were measured using a Varian Cary Eclipse fluorescence spectrophotometer in 1-cm path quartz cells, using spectral band width 0.1 nm and a scanning speed 600 nm/min at 25 °C. For the measurement of fluorescence spectra, the samples were excited at the absorption maximum (Tables 1 and 3). For flat absorption peaks consisting of several overlapping bands of similar (within 5%) intensities, the band with the longest wavelength was used. This was the situation for indole in solvents 1, 8, 9, and 15 (Table 1) and in all DAcPC solutions (Table 3), and Nile Red in solvents 8, 9, and 12–15 (Table 1).

The fluorescence spectra were fitted with the sum of Gaussian functions, with the number of used functions selected by the best fit with no negative peaks. Three Gaussians were used for aqueous solutions of indole with $c_{\text{DAcPC}} < 1.9 \text{ M}$ and for NR in most solvents and in aqueous solutions with $c_{\text{DAcPC}} > 1.9 \text{ M}$. Four Gaussians proved the best choice for more concentrated aqueous indole solutions and also for NR in less concentrated aqueous DAcPC solutions and in benzene, chlorobenzene and *n*-hexadecane. Five Gaussians were needed for indole in solvents. The fitted functions aided in identification of the peak wavelengths and corresponding intensities. The fits lacked systemic deviations in any region of the spectrum and were characterized by the squared correlation coefficients $r^2 > 0.999$.

Solvent polarity is typically evaluated using the absorption and fluorescence emission peak wavelengths, λ_a and λ_f , resp. The difference of the inverse values of these wavelengths is called the Stokes shift, $S = 1/\lambda_a - 1/\lambda_f$. The underlying theory was developed from the quantum-mechanical second-order perturbation and the Onsager's model, assuming that the solvent rearrangements are much faster than the fluorescence event.⁵¹ Applying several approximations and simplifying assumptions regarding the probe polarizability and the shape of the Onsager cavity, several models relating the wavelengths to the dielectric constant, ϵ , and the refractive index, n , were obtained in the following forms:

$$1/\lambda_a - 1/\lambda_f = m_1 f(\epsilon, n) + \text{const} \quad (1)$$

$$1/\lambda_a + 1/\lambda_f = -m_2 [f(\epsilon, n) + 2g(n)] + \text{const} \quad (2)$$

The terms m_1 and m_2 contain the values of the dipole moments of the probe in the ground and excited states. Hence, the measurements of λ_a and λ_f in several solvents are often used to determine the dipole moment in the excited state. The models differ in the form of

functions $f(\epsilon, n)$ and $g(n)$, also called 'polarity functions',⁵² obtained for different approximations and assumptions.⁵¹ Since these are difficult to assess for individual cases, the usual approach is to examine all models listed below and select the best one for the estimation of desired characteristics (ϵ in this case). The simplest Lippert-Mataga model^{53;54} uses eq 1 and

$$f(\epsilon, n) = \frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (3)$$

The Bakshiev model⁵⁵ differs only in a modified $f(\epsilon, n)$:

$$f(\epsilon, n) = \frac{n^2 + 1}{n^2 + 2} \left(\frac{\epsilon - 1}{\epsilon + 2} - \frac{n^2 - 1}{n^2 + 2} \right) \quad (4)$$

The Kawski-Chamma-Viallet (KChV) model^{51;56-60} uses eq 2, the same $f(\epsilon, n)$ as in the Bakshiev model, and

$$g(n) = \frac{3}{2} \left[\frac{n^4 - 1}{(n^2 + 2)^2} \right] \quad (5)$$

Reichardt's E_T^N indices are defined using the inverse value of the absorption peak wavelength of a pyridinium N-phenolate betaine dye.⁴⁴ For many other probes, including indole and NR, the range of the absorption peak wavelengths is insufficient. Better results are achieved using the Stokes shifts, assuming that the emission peak wavelengths are linearly related to their absorption counterparts, at least for solvents with similar interactions with the probe. The best correlation between the Stokes shift and E_T^N is then linear:⁶¹

$$1/\lambda_a - 1/\lambda_f = aE_T^N + const \quad (6)$$

The used approach is based on the calibration of models for the absorption and fluorescence peak wavelengths of indole and NR in organic solvents with known dielectric constants and refractive indexes or the E_T^N values. The dielectric constants or the E_T^N of aqueous DAcPC solutions are then estimated from the absorption and fluorescence wavelengths of indole and NR measured in these solutions using the best calibrated model.

Several datasets were fit by the Boltzmann sigmoid equation:

$$y = A + (B - A) \left[1 + e^{\left(\frac{x - C}{D} \right)} \right] \quad (7)$$

where A – D are optimized parameters. Equation 7 was used as an empirical equation, without any mechanistic background. The goal was to provide a tool for inter- and extrapolation. All fits by eq 7 and all linear fits were made using the Origin software.⁶²

RESULTS AND DISCUSSION

Polarity of DAcPC hydrated to different levels was measured using shifts in the absorption and fluorescence peak wavelengths of fluorescent probes. Selection of the used fluorescent probes was guided by the desire to avoid complications with ionization, and to examine potential interactions with DAcPC molecules. The chosen probes, Nile Red as an H-bond acceptor and indole as an H-bond donor, have been used for polarity estimation before.^{63;64} Nile Red is a hydrophobic dye, mainly used to visualize lipid components of cells.⁶⁵ Indole represents the fluorophoric portion of the amino acid tryptophan, fluorescence of which has been used to probe polarity in the protein interior.⁶⁶

The wide range of DAcPC concentrations (0.052 M – 2.5 M) for polarity examination was chosen for two reasons: (i) the higher concentrations imitate the hydration levels that are normally seen in fluid PC bilayers at room and body temperatures (8–16 water molecules per headgroup)^{21–24} and (ii) the lower concentrations allow examination of possible aggregation of DAcPC molecules.

In this study, the refractive index, n , was determined in all DAcPC solutions. We measured the absorption and fluorescence spectra, and correlated the peak wavelengths with the dielectric constant, ϵ , and the refractive index, n . The models, calibrated using the solvents with known ϵ and n values, allow for the estimation of ϵ of hydrated DAcPC.

Calibration of the Dependencies of Stokes Shifts on Solvent Properties

Fifteen organic solvents, covering (1) the polarity range between alkanes and water, and (2) the refractive index range of the DAcPC solutions were used. The spectroscopic results are summarized in Table 1, with the solvents listed in the order of the decreasing magnitude of the dielectric constant. In this order, the absorption and fluorescence peak wavelengths of both indole and NR exhibit an approximate blue shift trend that is more pronounced for fluorescence.

Indole Spectra are more diverse thanks to the shift of the emitting excited state from the 1L_b state in solvents devoid of H-bonding capability (e.g., *n*-hexadecane) to the 1L_a state in solvents capable of H-bonding (e.g., water).⁴⁶ The mechanism of the phenomenon is still a matter of discussion.^{52;67–70} The peak wavelengths are in reasonable agreement (within a few nanometers) with the previously published data,⁵² given the fact that the absorption peaks have a complex structure and contain multiple peaks. The peaks were separated in benzene and *n*-hexadecane, while in other solvents they were lumped together, sometimes forming a plateau-like shape (solvents 1, 8, 9 and 15 in Table 1). In our data, the emission from the 1L_b state was clearly manifested only for benzene and *n*-hexadecane by the split of otherwise compact absorption and fluorescence peaks, and by a deviation from the line in polarity functions (Figure 1A).

Nile Red Spectra are more uniform, although the absorption spectra are also composed of multiple, overlapping peaks, which were quite flat in solvents 8, 9, and 12–15 (Table 1). There are no marked differences in the spectra for solvents of varying polarity, except for *n*-hexadecane, where the fluorescence spectrum shows two peaks. However, the *n*-hexadecane data adhere to the fit of other solvent data to polarity functions (Figure 1B).

Fits of Model Polarity Functions to the Data

The results of the calibration of the dependencies of indole and Nile Red absorption and fluorescence peak wavelengths on the dielectric constants^{44,71,72} and the refractive indices (eqs 1 – 5) are shown in Figures 1A and 1B for indole and Nile Red, respectively, and summarized in Table 2. The error bars of the wavelength terms in Figures 1A and 1B are not shown because the standard errors of the means (SEM) of the peak wavelengths (Table 1) lead to the errors of the wavelength terms, which are smaller than the diameter of the shown points. The errors range from $2.3 \times 10^{-6} \text{ nm}^{-1}$ to $1.3 \times 10^{-5} \text{ nm}^{-1}$ for indole (Figure 1A), and from $4.2 \times 10^{-7} \text{ nm}^{-1}$ to $3.6 \times 10^{-6} \text{ nm}^{-1}$ for Nile Red (Figure 1B).

For indole, the spectra in benzene (Table 1, no. 14) and *n*-hexadecane (no. 15) indicated the change in the emission state to the 1L_b state, in contrast to other solvents stabilizing the 1L_a state,^{52;67–70} and the corresponding data were omitted in all fits shown in Figure 1A.

Table 2 shows that the Lippert-Mataga model is clearly better than the other two models, in terms of the both the correlation coefficient (r) and the standard deviation (SD). For Nile Red, the data from all fifteen solvents could be used (Figure 1B). The Lippert-Mataga and Bakshiev model fits exhibit almost identical statistical indices r and s , and provide better fits than the Kawski-Viallet-Chama model. Using the principle of parsimony, the simpler Lippert-Mataga model was used in determining the dielectric constant for the DAcPC solutions.

The Reichardt's E_T^N indices are defined using the inverse value of the absorption peak wavelength of a pyridinium N-phenolate betaine dye.⁴⁴ To expand the wavelengths' range, absorption and fluorescence data can be combined for other probes. The best correlation between the Stokes shift and E_T^N is linear (eq 6) for solvents with similar binding characteristics. The results for our solvent set (Table 1) are plotted in Figure 2, the top set (black symbols) for indole and the bottom set (red symbols) for Nile Red. Separate linear correlations were observed for nonpolar solvents containing only carbons, hydrogens, and halogens and for other solvents. The line characteristics are summarized in Table 2.

Refractivity of DAcPC Solutions

Table 3 below lists the refractive index, n , measured for the 589 nm wavelength (sodium D-line) at 25 °C. The n values increase with the DAcPC concentration, from $n = 1.3324$ for water to $n = 1.4286$ for a 2.5 M DAcPC solution. The dependence of n on DAcPC molarity, with the slope 0.03848 ± 0.00001 and the intercept 1.33241 ± 0.00001 , is perfectly linear in the given range ($r = 1.000$ and $SD = 2.349 \times 10^{-5}$). The differences of n between water and more concentrated DAcPC solutions are significant and need to be considered in the correlation between the peak shifts and solvent properties.

Spectroscopy of DAcPC Solutions

The results for indole and Nile Red are summarized in Figure 3 and Table 3. The wavelengths of the fluorescence peaks exhibit a significant blue shift with the increasing DAcPC concentration, in contrast to those of the absorption peaks, which do not vary much. The blue shift indicates a decrease in polarity. For indole (Figure 3A), the fluorescence intensity is at the highest point for water (Figure 3A, dotted line) and decreases with the increasing DAcPC concentrations. Nile Red (Figure 3B) has the highest fluorescence intensity for the most concentrated DAcPC solution, and exhibits progressively lower intensities with increasing water content. The intensities and shapes of the peaks determine the standard error of the mean (SEM) of the λ_{max} values (Table 3). For indole, the increase in DAcPC concentrations leads to lower intensities and flatter peaks, causing the increase in the SEM values. The Nile Red data show the opposite trend, which is making them more suitable for estimation of polarity indices of the concentrated DAcPC solutions, imitating the H-stratum.

The dielectric constants, ϵ , of the DAcPC solutions were extracted from the solution-calibrated Lippert-Mataga model (Table 2) for indole and NR. They are summarized in Table 3 and Figure 4A. The errors calculated using the SEM values of the absorption and fluorescence peak wavelengths are high for solutions with low DAcPC concentrations. The magnitude of the errors is caused mainly by the shape of the functional dependence of ϵ on the peak wavelengths, λ , and the refractivity indices, n , obtained from eqs 1 and 3. This influence is becoming less significant for the more concentrated DAcPC solutions. The errors for the NR data are small thanks to the small SEM errors of the fluorescence peak wavelengths (Table 3) and render the data suitable for determining the ϵ values. In contrast, the errors for the indole data are magnified by the large SEM values of the fluorescence peak wavelengths (Table 3) and the reliability of the results is lower.

Hydration levels of fluid PC bilayer at room and body temperatures^{21–24} are best represented by 1.832 M - 2.5 M DAcPC/water systems (Table 3), which will be referred to as headgroups-mimetic systems. For these systems, the more reliable Nile Red data provide the dielectric constant, ϵ , ranging from 7.3 to 31.0. The indole data lead to similar albeit a bit higher indices: the ϵ value ranges from 14.1 to 43.8. For lower DAcPC concentrations, the ϵ values asymptotically approach that of water ($\epsilon = 78.36$). The entire dependence (Figure 4A) can be described by the empirical eq 7, with the dependent variable being the dielectric constant and the independent variable represented by the molarity of the DAcPC solutions. The optimized regression coefficients and the fit characteristics are given in the first double row of Table 4.

Reichardt's E_{N}^{T} parameters were calculated from the solvent-calibrated eq 6 (Table 2), and are summarized in Table 3 and Figure 4B. For the indole data, the equation for other solvents was used for all DAcPC solutions. For Nile Red, this approach would lead to unrealistic negative or very low values for the most concentrated DAcPC solutions. Therefore, for the headgroups-mimetic concentrations (1.832 M - 2.5 M), the equation for the nonpolar solutions was used, and the equation for other solutions provided the estimates for the remaining concentrations. This choice was guided by the smoothness of the curve in Figure 4B.

The errors are acceptable, except for the indole values in more concentrated DAcPC solutions (Figure 4B). For these larger errors, the large SEM values of the fluorescence peak wavelengths are responsible, as seen for the dielectric constants (Figure 4A). For the headgroup-mimetic DAcPC systems, the E_N^T parameters range from 0.323 to 0.437 for the more reliable NR data, and from 0.325 to 0.628 for the indole data. For lower DAcPC concentrations, the E_N^T values increase but do not reach that of water ($E_N^T = 1$). This fact points to poor coverage of eq 6 of the entire range of studied polarities. The entire dependence can be described by the empirical eq 7, having the E_N^T parameter as the dependent variable and the DAcPC molarity as the independent variable. The optimized coefficients and the fit characteristics are given in the second double row of Table 4.

To relate the DAcPC-based results to the situation in PC bilayers, the number of water molecules per headgroup is shown in the second column of Table 3. Naturally, higher water content leads to higher polarity. The values of the refractivity index and both polarity indices for the realistic water/PC ratios, 8 to 16 water molecules per headgroup, are plotted in Figure 5. The lines correspond to eq 7 with the dependent variable being the refractive index or one of the polarity indices, and the independent variable representing the number of water molecules per headgroup. The optimized values of the regression coefficients are listed in the rows 3 – 5 of Table 4. The fits of eq 7 provide for easy intrapolation (e.g., for different temperatures) and extrapolation (e.g., for PCs with shorter fatty acid chains)²² of the indices for other water/PC ratios and for the comparison with the real situation in PC bilayers. The measurement temperature of the PC bilayer determines the surface area per lipid and the average number of water molecules hydrating a headgroup (see below).

Examination of Aggregation

Nile Red has been used to detect the presence of micelles in solution and to determine the critical micelle concentration (CMC) thanks to the enhancement of the emission fluorescence intensity in hydrophobic intra-micellar environments.⁷³ A sharp break point between two lines in the concentration dependence of the intensity is used to determine the CMC. The fluorescence spectra of Nile Red in DAcPC solutions are shown in Figure 3B. The emission intensity at the wavelength of the peak in the most concentrated DAcPC solution ($\lambda_{\text{max}}=642.4$ nm, the last row of Table 3) is plotted against the DAcPC concentration in Figure 6.

The dependence is described closely by an increasing sigmoidal function, represented by eq 7. The fit characteristics are summarized in the last row of Table 4. The data are conforming to a smooth sigmoidal function, indicating the absence of a distinct break point, which would be a hallmark of micelle formation. The observed continual fluorescence intensity enhancement with an inflection point is caused by fact that polarity of DAcPC solutions falls with increasing concentrations. This observation shows that DAcPC does not form molecular aggregates in the studied concentration range, as could be perhaps expected given the small size of the acetyl chains compared to the rest of the DAcPC molecule. No light scatter was observed in the UV Vis spectra of DAcPC solutions even at the highest used concentration that would have indicated aggregate formation. This behavior is in contrast to

dipropanoyl, dibutanoyl, and dihexanoyl PCs, for which the CMCs of 405, 180, and 8.1 mM, respectively, were reported.⁷⁴

Comparison with Published Data

Hydration of the H-stratum at the measurement conditions is a key factor for a comparison of our results with published data on refractivity and polarity. The hydration varies with temperature, and is associated with the area per lipid, which depends on the length and, more significantly, on the saturation of PC fatty acid chains.²³ The differences in composition of the aqueous phase, especially the content of salts⁷⁵ (the DAcPC was dissolved in water, PC liposome preparation may use salt solutions) or co-solvents,^{75,76} decreases reliability of the estimations.⁷⁵

The area per lipid (A) can be most precisely determined by combining the x-ray and small-angle neutron diffraction data.⁷⁷ The increase in the lipid area is characterized by thermal area expansivity, $\alpha_A = (1/A)(dA/dT)$, at a constant pressure. For a series of saturated diacyl PCs (1,2-di-myristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-di-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-di-stearoyl-*sn*-glycero-3-phosphocholine (DSPC)), the α_A values range between 0.0029 and 0.0032 deg⁻¹, for the temperatures 30 – 60 °C and above the transition temperatures.⁷⁸ The lipid areas, which are needed for calculation of the changes for different temperatures below were $A = 59.9 \text{ \AA}^2$ for DMPC at 30 °C, $A = 63.3 \text{ \AA}^2$ for DPPC at 50 °C, and $A = 63.8 \text{ \AA}^2$ for DSPC at 50 °C.⁷⁸

Lipid area (A) is connected with hydration, which can be characterized as the number of water molecules per headgroup (N). The dependence of A on N is almost linear and only slightly curved, and was described by the increasing part of the parabolic equation²³ (see below). The hydration for the given lipid area A is calculated as the positive root of the quadratic equation. This is the last step that is needed to estimate hydration for a comparison of the DAcPC polarities with published data.

The polarity of the H-stratum is expected to decrease from the magnitude typical for bulk water to a lower value at the headgroup/core interface. The polarity indices for different DAcPC hydration levels (Table 3), obtained for isotropic DAcPC solutions, should correspond to the average polarity of the PC H-stratum, which has much more isotropic molecular polarizability tensor than the lipid chains.⁷⁹ Most H-stratum polarity data come from fluorescence or electron spin resonance studies of amphiphilic compounds embedded in a PC bilayer or from NMR studies of ¹³C-labelled PCs in a PC bilayer. While fluorescence results depend on the (often unknown) insertion depth of the fluorophores as well as the disturbance of the probe environment, the NMR results are largely free from these two factors

Polarity of DAcPC solutions gradually reached quite low values ($\epsilon = 7.3 - 14.7$, $E_N^T = 0.323 - 0.399$, Table 3, NR data) with decreasing hydration ($N = 8 - 14$). Low polarity ($\epsilon = 4$) was measured previously in the DPPC and DSPC liposomes using the fluorescence changes of incorporated L- α -dansyl-phosphatidyl ethanolamine but only in the gel phase, which has low hydration. With increasing temperature, the polarity was increasing and leveled off at $\epsilon = 34$ at least 10° above the transition temperatures (41 and 52 °C,

respectively).⁴⁸ A quantitative comparison cannot be made because the liposomes were prepared in the Tris-HCl buffer.

The polarity of the H-stratum ranges from that of water ($E_N^T = 1.00$ by definition) to a lower value at the headgroup/core interface. A comparison of ^{13}C NMR shifts of DMPC molecules intercalated in the minimal-size unilamellar liposomes at 45 °C with those in solvents showed that the lowest H-stratum polarity, at the level of fatty acid carbonyls, is characterized by $E_T(30) = 50$ kcal/mol,⁸⁰ which corresponds to $E_N^T = 0.60$. Hydration of the measured samples needs to be estimated. For DMPC in unilamellar liposomes, lipid area $A = 59.9 \pm 1.2 \text{ \AA}^2$ at 30 °C⁷⁸ is associated with hydration $N = 7.4 \pm 1.9$ water molecules per headgroup.²³ Lipid area at 45 °C can be estimated as $A = 59.9 \text{ \AA}^2 \times (1 + 0.0032 \text{ deg}^{-1} \times 15 \text{ deg}) = 62.8 \text{ \AA}^2$. As outlined above, lipid area is related to hydration as $A = a + bN + cN^2$. The values of the optimized coefficients for DMPC unilamellar vesicles were $a = 57.246$, $b = 0.402$, and $c = -0.00197$.²³ The hydration for the given lipid area A is calculated as the positive root of the quadratic equation: $N = [-b + (b^2 - 4c(a - A))^{0.5}] / 2c$. For $A = 62.8$, hydration is $N = 14.9$ water molecules per headgroup. The E_N^T value of the DAcPC solution, calculated from eq 7 using the optimized coefficients listed in row 4 of Table 4, is $E_N^T = 0.42$, which is substantially lower than the DMPC published $E_N^T = 0.60$. A possible explanation is the difference in composition of used aqueous media: water for DAcPC hydration and the 0.1 M phosphate buffer for the liposome preparation.⁸⁰

The measured refractive index for the most concentrated DAcPC solution (2.5 M; 8 waters per headgroup), $n = 1.4286$ at 589 nm, should be quite comparable to the n of the H-stratum of the fluid PC bilayer because of a similar hydration and the fact that the headgroups have much more isotropic molecular polarizability tensor than the lipid chains.⁷⁹ Unfortunately, a direct comparison is hampered by the lack of proper data. The closest systems with measured n values are complete PC bilayers at solid supports. In such systems, the refractive index does not consist only of that of the H-stratum, but also includes the contributions of the lipid core, increasing with the chain order, and of the hydration layer on the solid surface. Therefore, the n values of solid-supported bilayers represent the upper limits for the DAcPC n values, and no direct equality can be expected. The PC bilayers are immobilized on a solid support by several approaches, each leading to a different quality of the bilayer.

For a fluid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayer prepared by the Langmuir-Blodgett (the first monolayer) and Langmuir-Schaefer (the second monolayer) techniques, $n \sim 1.376$ was obtained by optical light-mode waveguide spectroscopy (632.8 nm, 24 °C).^{81;82} The measured value is lower than the n of the most concentrated (2.5 M) DAcPC solution ($n = 1.4286$), and would be further reduced if corrected for the used wavelength (see below). This fact, combined with the higher n of other bilayer-based systems below, can be explained by the incomplete bilayer with a lower lipid chain alignment and mass density, which the described deposition approach may produce.^{81;82}

The fluid bilayers prepared by the solvent spreading and analyzed by the plasmon-coupled waveguide resonance provided $n = 1.435$ for 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and $n = 1.445$ for POPC (averages for 543.5 and 632.8 nm).⁸³ A higher n than that for the Langmuir-Blodgett/Langmuir-Schaefer bilayers seems to confirm that the solvent

spreading techniques may lead to a better surface coverage by phospholipids. However, the measured n is lower than for the spontaneously adsorbed phospholipids (see below), which does not support the expected increased lipid chain ordering by residual solvent.^{81;82}

The self-assembly from a liposome suspension seems to produce the deposits closest to the biological bilayers. Dual-polarization interferometry at 632.8 nm provided the following results for fluid bilayers at 20 °C: $n = 1.4778$ for POPC, $n = 1.4560$ for DOPC, and $n = 1.4736$ for DMPC. For the gel phase DMPC bilayer, $n = 1.4810$ was measured.⁸⁴ Dual-wavelength (670 and 785 nm) surface plasmon resonance measured $n = 1.4728$ – 1.4845 for fluid DOPC bilayer and $n = 1.4725$ – 1.4775 for the fluid POPC bilayer, depending on the wavelength and the modeling approach.⁸⁵ Similar n values were also obtained from calculations utilizing bond polarizabilities and simplified bilayer structures for the field perpendicular to the bilayer plane.⁸⁶

The experimental and calculated values would be a bit lower for the 589 nm beam (the dispersion index, $dn/d\lambda \sim -4 \times 10^{-5} \text{ nm}^{-1}$)⁸⁵ but still higher than the measured n value for the isotropic 2.5 M DAcPC solution. As mentioned above, a positive discrepancy is expected because of the refractivity contributions from the lipid core and the hydration layer.

The measured $n = 1.4286$ of the 2.5 M DAcPC solution agrees better with $n = 1.42$ of a 50% aqueous solution of sucrose,⁵⁰ which is used to match the refractive index of liposomes, suppress light scattering and enable linear dichroism studies.⁸⁷

CONCLUSION

In this study, for DAcPC hydrated to different levels, refractivity was measured and polarity was characterized as the macroscopic dielectric constants and the microscopic normalized Reichardt's parameters using the Stokes shifts of indole and NR. The NR data are more reliable because the intensity of the fluorescence peak is increasing with the decreasing water content, in contrast to the indole spectra. For hydrated DAcPC, both refractivity and polarity exhibit characteristic concentration dependencies: refractivity is increasing in a linear way and polarity exhibits a sigmoidal decrease with the loss of water. The dielectric constant for low but biologically relevant hydration ($N = 8 - 14$) reached quite low values ($\epsilon = 7.3 - 14.7$) thanks to the prevalence of bound water. Agreement of refractivity and polarity with published data was only qualitative because of the differences in the used aqueous phases, the preparation techniques, and the lack of suitable systems. The facts that (1) polarity of DAcPC solutions decreases with decreasing water content in a similar way as in headgroups of PC bilayers and (2) DAcPC does not form micelles even at higher concentrations support further use of the DAcPC solutions as a bilayer H-stratum surrogate for a fast prediction of the molecular propensities to accumulate in this region during transbilayer transport and accumulation.³⁰ The drug fragment contributions to C16/water and C16/DAcPC partition coefficients provided a fast and reliable model for prediction of drug accumulations in individual bilayer strata from drug structure.³¹

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Figure 1.

Fluorescence and absorbance maxima of indole (A) and Nile Red (B) as functions of the dielectric constant, ϵ , and the refractivity index, n (data in Table 1), according to the models of Lippert-Mataga (eqs 1 and 3, black points, the left axes), Bakshiev (eqs 1 and 4, red squares, the left axes), and Kawski-Viallet-Chama (eqs 2 and 5, blue triangles, the right axes). The fits are summarized in Table 2. The crossed-out points for solvents 14 and 15 in part A were omitted from the correlations (see text). The number (2) indicates two black and red overlapping points.

Figure 2.

Fluorescence and absorbance maxima of indole (black) Nile Red (red) as functions of the E_T^N index for nonpolar (8, 9, 12–15) and other solvents (1–7, 10, 11). The crossed-out data for benzene (14) and *n*hexadecane (15) were omitted for indole (as in Figure 1A) because of possible different excitation mechanism. Data are summarized in Table 1, line characteristics in Table 2.

Figure 3.

Fluorescence emission spectra of indole (A) and Nile Red (B) in aqueous DAcPC solutions with concentrations (M): 0.052 (black), 0.112 (red), 0.205 (green), 0.384 (blue), 0.719 (magenta), 1.210 (cyan), 1.832 (dark yellow), 1.959 (orange), 2.119 (wine), 2.251 (pink), and 2.500 (violet). In A, the indole spectrum in water is shown as a dotted line, and the spectra for DAcPC concentrations 1.959 – 2.500 M are not shown because they significantly overlap with that for 1.832 M DAcPC. The Nile Red spectrum in water is missing in B as it could not be measured because of insufficient solubility. The arrows indicate the DAcPC hydration (Table 3) for individual peaks from high to low intensities.

Figure 4.

Polarity indices of DAcPC solutions plotted against DAcPC concentration. The dielectric constants (A) were determined from the solvent-calibrated Lippert-Mataga model (eqs 1 and 3). Reichardt's E_T^N polarity indices (B) were determined from the solvent-calibrated eq 6. The Nile Red data are indicated by red squares and indole data by black points. For many red points, the error bars are hidden inside the squares. The dependencies were fit by the eq 7, as summarized in Table 4 (in the first and second double rows, for A and B, respectively).

Figure 5. Dependence of the refractive index (A), the normalized Reichardt's polarity parameter (B), and the dielectric constant (C) on hydration (8–16 water molecules per DAcPC). The values of the polarity characteristics were determined from the Nile Red Stokes shifts. The data are in the last five rows of Table 3. The dependencies were fit by eq 7, as summarized in the rows 3 – 5 of Table 4.

Figure 6. Relative fluorescence intensity (relative units) plotted against the DAcPC concentration. The curve corresponds to eq 7 with optimized coefficients listed in Table 4, line 6. The wavelength of the peak in the most concentrated DAcPC solution (642.4 nm) was used.

Table 1.

INDOLE and NILE RED ABSORPTION and FLUORESCENCE PEAK WAVELENGTHS in ORGANIC SOLVENTS with LISTED POLARITY INDICES^a and the REFRACTIVE INDEX VALUES.

No.	Solvent	ϵ	E_T^N	n	indole $\lambda_{\max}$ (nm)		Nile Red $\lambda_{\max}$ (nm)	
					absorption	fluorescence	absorption	fluorescence
1	water	78.36	1.000	1.3324	283.7±0.1	347.4±0.4	570.9±0.2 ^b	657.8±0.2 ^b
2	dimethylsulfoxide	46.45	0.444	1.4771	279.7±0.6	331.1±0.1	549.6±0.7	628.0±0.1
3	<i>N,N</i> -dimethylformamide	36.71	0.386	1.4270	277.2±0.8	324.9±0.5	548.7±1.0	619.7±0.2
4	acetonitrile	35.94	0.460	1.3403	267.0±0.8	320.7±0.1	544.5±0.1	614.0±0.5
5	methanol	32.66	0.762	1.3270	274.8±0.3	330.8±0.4	556.3±0.4	635.2±0.8
6	ethanol	24.55	0.654	1.3611	274.3±0.6	327.6±1.0	549.7±0.1	629.5±0.1
7	2-propanol	19.92	0.546	1.3761	274.7±0.1	325.4±0.2	549.7±0.1	622.6±0.1
8	1,2-dichloroethane	10.36	0.327	1.4440	273.0±0.1	315.1±0.9	544.4±0.1	601.6±0.5
9	dichloromethane	8.93	0.309	1.4211	272.8±0.6	310.5±0.7	545.1±0.2	598.4±0.2
10	tetrahydrofuran	7.58	0.207	1.4040	271.4±0.8	311.7±0.7	536.4±0.8	593.3±0.8
11	ethyl acetate	6.02	0.228	1.3758	274.8±0.5	310.7±0.7	531.6±0.1	588.8±0.4
12	chlorobenzene	5.62	0.194	1.5230	270.4±0.2	298.3±0.1	544.4±0.6	588.5±0.1
13	chloroform	4.89	0.259	1.4440	254.4±0.5	278.6±0.3	547.5±0.2	597.6±0.1
14	benzene	2.27	0.071	1.4956	282.0±0.7	304.5±0.3	542.0±0.1	571.0±0.1
15	<i>n</i> -hexadecane	2.05 ^c	0.012 ^d	1.4325	275.0±0.8	298.7±0.1	513.7±0.4	531.2±0.1

^aAll data for 25 °C, unmarked polarity indices from ref.⁷¹

^bThe values extrapolated from methanol/water data because of low solubility.

^cFrom ref.⁷²

^dThe value was not available, so the value for *n*-dodecane was used, which was identical to, or only slightly different from, the values for alkanes from *n*-pentane to *n*-decane.⁴⁴

Table 2.

FITS of the RELATIONSHIPS between the ABSORPTION/FLUORESCENCE MAXIMA and SOLVENT CHARACTERISTICS ACCORDING to INDIVIDUAL MODELS for INDOLE and NILE RED.

Probe ^a	Model ^b	Slope	Intercept	N	r ^c	SD ^d
Indole	LM	$(1.720 \pm 0.083) \times 10^{-3}$	$(9.175 \pm 2.085) \times 10^{-5}$	13 ^e	0.987	1.690×10^{-5}
	B	$(5.189 \pm 0.424) \times 10^{-4}$	$(1.557 \pm 0.301) \times 10^{-4}$	13 ^e	0.965	2.791×10^{-5}
	KChV	$-(1.320 \pm 0.321) \times 10^{-3}$	$(8.450 \pm 0.400) \times 10^{-3}$	13 ^e	-0.777	1.750×10^{-4}
	E _T ^N nonpolar	$(1.030 \pm 0.417) \times 10^{-3}$	$(1.276 \pm 1.150) \times 10^{-4}$	4 ^e	0.867	4.489×10^{-5}
	E _T ^N others	$(2.450 \pm 0.588) \times 10^{-4}$	$(4.314 \pm 0.337) \times 10^{-4}$	9	0.844	4.232×10^{-5}
Nile Red	LM	$(4.833 \pm 0.330) \times 10^{-4}$	$(7.671 \pm 0.771) \times 10^{-5}$	15	0.971	1.252×10^{-5}
	B	$(1.647 \pm 0.112) \times 10^{-4}$	$(8.136 \pm 0.741) \times 10^{-5}$	15	0.971	1.250×10^{-5}
	KChV	$-(4.220 \pm 0.684) \times 10^{-4}$	$(3.980 \pm 0.081) \times 10^{-3}$	15	-0.863	6.601×10^{-5}
	E _T ^N nonpolar	$(3.497 \pm 0.253) \times 10^{-4}$	$(6.082 \pm 0.582) \times 10^{-5}$	6	0.990	6.930×10^{-6}
	E _T ^N others	$(6.374 \pm 1.626) \times 10^{-5}$	$(1.783 \pm 0.093) \times 10^{-4}$	9	0.828	1.170×10^{-5}

^aThe data (Table 1) and lines are shown in Figures 1 – 3.

^bLM, Lippert-Mataga (eqs 1 and 3), B, Bakshiev (eqs 1 and 4), KChV, Kawski-Chamma-Viallet (eqs 2 and 5), E_T^N nonpolar and others (eq 6) for solvents (Table 1) classified as nonpolar (8, 9, 12–15) and other (1–7, 10, 11).

^cThe correlation coefficient.

^dStandard deviation.

^eSolvents 14 and 15 are excluded because of a different excitation mechanism.

THE WAVELENGTHS of ABSORBANCE and FLUORESCENCE PEAKS of INDOLE and NILE RED in AQUEOUS DACPC SOLUTIONS and THEIR ESTIMATED POLARITY INDICES.

Table 3.

DACPC concentration (M)	DACPC/water molar ratio	refractive index n ($\pm$ SEM)	wavelength $\pm$ SEM (nm)				estimated polarity indices $\pm$ SD			
			absorbance peak		fluorescence peak		dielectric constant using		Reichardt's E^T_N using	
			indole	Nile Red	indole	Nile Red	indole	Nile Red	indole	Nile Red
0	-	1.3324 $\pm$ 0.00003	284.0 $\pm$ 0.4	570.2 $\pm$ 0.1 ^a	347.4 $\pm$ 0.2	657.8 $\pm$ 0.2 ^a	79.7 $\pm$ 26.1	80.6 $\pm$ 33.1	0.862 $\pm$ 0.021	0.867 $\pm$ 0.006
0.052	1:1024	1.3344 $\pm$ 0.00003	284.0 $\pm$ 0.3	570.2 $\pm$ 0.1	347.4 $\pm$ 0.1	657.5 $\pm$ 0.1	80.1 $\pm$ 19.2	75.4 $\pm$ 35.4	0.857 $\pm$ 0.016	0.856 $\pm$ 0.012
0.112	1:512	1.3367 $\pm$ 0.00004	284.2 $\pm$ 0.3	570.3 $\pm$ 0.1	347.3 $\pm$ 0.2	657.5 $\pm$ 0.1	77.5 $\pm$ 19.2	77.4 $\pm$ 19.6	0.849 $\pm$ 0.017	0.851 $\pm$ 0.006
0.205	1:256	1.3403 $\pm$ 0.00002	284.3 $\pm$ 0.4	570.3 $\pm$ 0.1	347.2 $\pm$ 0.4	657.3 $\pm$ 0.2	78.8 $\pm$ 29.1	81.1 $\pm$ 33.1	0.840 $\pm$ 0.024	0.844 $\pm$ 0.009
0.384	1:128	1.3472 $\pm$ 0.00004	284.6 $\pm$ 0.5	570.5 $\pm$ 0.1	347.1 $\pm$ 0.1	656.9 $\pm$ 0.2	78.6 $\pm$ 30.2	76.8 $\pm$ 26.7	0.822 $\pm$ 0.025	0.820 $\pm$ 0.009
0.719	1:64	1.3601 $\pm$ 0.00006	284.7 $\pm$ 0.5	570.9 $\pm$ 0.1	346.1 $\pm$ 0.3	656.4 $\pm$ 0.1	72.9 $\pm$ 27.8	76.6 $\pm$ 17.7	0.783 $\pm$ 0.027	0.782 $\pm$ 0.006
1.210	1:32	1.3790 $\pm$ 0.00016	284.9 $\pm$ 0.4	571.8 $\pm$ 0.2	344.4 $\pm$ 0.6	655.5 $\pm$ 0.3	56.9 $\pm$ 18.0	58.7 $\pm$ 17.9	0.714 $\pm$ 0.029	0.706 $\pm$ 0.015
1.832	1:16	1.4029 $\pm$ 0.00007	285.1 $\pm$ 0.6	572.9 $\pm$ 0.4	342.2 $\pm$ 0.5	652.8 $\pm$ 0.4	43.8 $\pm$ 13.0	31.0 $\pm$ 5.7	0.628 $\pm$ 0.035	0.437 $\pm$ 0.004
1.959	1:14	1.4078 $\pm$ 0.00008	285.6 $\pm$ 0.4	575.6 $\pm$ 0.3	340.9 $\pm$ 3.1	650.6 $\pm$ 0.4	29.1 $\pm$ 18.5	14.7 $\pm$ 0.9	0.558 $\pm$ 0.111	0.399 $\pm$ 0.004
2.119	1:12	1.4140 $\pm$ 0.00003	286.5 $\pm$ 0.6	576.1 $\pm$ 0.4	340.8 $\pm$ 4.3	647.4 $\pm$ 0.2	24.4 $\pm$ 18.2	10.9 $\pm$ 0.5	0.509 $\pm$ 0.154	0.373 $\pm$ 0.004
2.251	1:10	1.4190 $\pm$ 0.00005	286.8 $\pm$ 0.6	578.0 $\pm$ 0.4	339.2 $\pm$ 5.1	645.1 $\pm$ 0.5	19.0 $\pm$ 13.2	8.2 $\pm$ 0.4	0.438 $\pm$ 0.183	0.341 $\pm$ 0.005
2.500	1:8	1.4286 $\pm$ 0.00017	288.3 $\pm$ 0.5	577.9 $\pm$ 0.5	338.1 $\pm$ 6.9	642.4 $\pm$ 0.3	14.1 $\pm$ 10.0	7.3 $\pm$ 0.3	0.325 $\pm$ 0.248	0.323 $\pm$ 0.005

^a Extrapolated from methanol/water solutions because of low solubility.

Table 4. The OPTIMIZED REGRESSION COEFFICIENTS of the BOLTZMANN SIGMOID EQUATION 7 for the SHOWN VARIABLES.

Dependent variable	Independent variable	Probe	Fig.	A	B	C	D	N	r	SD
dielectric constant	DAcPC molarity	Indole	4	1^b	78.36±5.432	1.784±0.038	0.4238±0.0390	12	0.993	11.16
		Nile Red		1^b	79.32±1.644	1.578±0.047	0.3137±0.0325	12	0.995	10.45
E_T^N	DAcPC molarity	Indole	4	-2.402±0.318	0.8918±0.0165	3.925±0.130	0.9010±0.0837	12	0.997	2.498×10 ⁻⁴
		Nile Red		0.2891±0.0218	0.8566±0.0090	1.499±0.045	0.3381±0.0368	12	0.999	2.097×10 ⁻⁴
refractivity index	#waters/DAcPC	Nile Red	5	1.385±0.010	1.789±0.144	-10^b	8.462±2.423	5	0.998	8.519×10 ⁻⁷
E_T^N	#waters/DAcPC	Nile Red		0.7816±0.0165	0.2540±0.0310	20^b	6.286±1.327	5	0.999	1.008×10 ⁻⁵
dielectric constant	#waters/DAcPC	Nile Red		271.4±59.58	7.490±0.717	20^b	1.716±0.188	5	0.998	0.7075
fluorescence intensity	DAcPC molarity	Nile Red	6	122.1±10.5	0^b	2.036±0.087	0.4464±0.0385	11	0.998	4.412

^b Coefficient was not optimized.