To D or not to D? On estimating the microenvironment polarity of biomolecular cavities†

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Knowledge of the local polarity of specific cavities in biopolymers can facilitate the design of selective low MW ligands that impact the structure and function of macromolecules. The most common tools for interrogating local polarity are fluorescent probes that are sensitive to their microenvironment. Researchers often evaluate and express this local polarity using dielectric constants, a parameter that reflects an inherent bulk property. A more appropriate expression should take into account solvent–solute interactions at the molecular level. Reevaluation of commonly used fluorophores illustrates the improved correlation between observed Stokes shift changes and $E_T(30)$ values as compared to the corresponding dielectric constants.

Introduction

Experimentalists as well as theoreticians have struggled, for a while, with the proper description of the polarity within confined spaces inside biopolymers. The notion of microenvironment polarity is, of course, of key importance, since intra- and intermolecular forces (such as H-bonding) are critically dependent on their surroundings, and particularly, on the attenuating (or strengthening) power of solvent molecules.[1] In recent years, elegant approaches utilizing environmentally sensitive fluorescent probes, have been employed in an attempt to quantify the polarity of biological cavities, both in proteins and nucleic acids. These attempts are telling and informative, but suffer from two major predicaments: (a) any probe placed within the cavity to be assessed inherently modifies the molecular architecture of the native environment, and (b) many studies have utilized dielectric constants as their gauge, a parameter that defines bulk solvent property and not an anisotropic medium. It is the latter aspect that is discussed here.

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Expression of polarity

Medium effects on the structure, conformation and reactivity of small and large molecules have been at the foundation of physical chemistry. Early studies utilized dielectric constants, or relative permittivity, as a measure of polarity, expressed in units of Debye (D). Such values represent a molecule's ability to attenuate an electric field generated between macroscopically distant electrodes relative to vacuum ($\varepsilon = 1$ by definition). It can be viewed as a measure of bulk polarizability, the capability of molecules to respond to the applied field and reorganize to minimize the generated potential. Polar solvents, such as water and low MW alcohols, are capable of effectively attenuating the generated field, and therefore have relatively high dielectric constants, while apolar solvents, such as hydrocarbons, with little or no ability to respond to an applied field, are characterized by very low dielectric constants. It is intuitively clear that such values, representing bulk order or disorder, do not faithfully represent the first or second solvation spheres surrounding a solute molecule, or the environment within a small molecular cavity.[2]

Experimental support for such dichotomy has emerged in early physical organic studies where, for example, rates of solvolysis reactions were measured in diverse media and showed no correlation with dielectric constants. Early attempts to develop reliable microscopic solvent polarity scales evaluated the impact of solvents on the chemical reactivity of alkyl halides and fundamentally relied on linear free energy relationships (LFER).[3] The *Y* scale, developed by Grunwald and Weinstein,[3] yielded a quantitative measure for solvents' ionization power, but was limited in terms of its general applicability, range of solvents and cumbersome determination.[4] The turning point came when spectroscopic approaches were conceived. The use of chromophoric charge transfer complexes to energetically define solvent polarity was pioneered by Kosower.[5] The *Z* value solvent polarity scale relied on a simple measurement of the absorption maximum of a charge transfer complex. A related scale, that has gained popularity due to its ease of use and coverage of a wide range of solvents and solvent mixtures, was developed by Dimroth and Reichardt. An $E_T(30)$ value (given in kcal mol^{-1}) is similarly determined by measuring a charge transfer band of a pyridinium betaine dye (**1**) (Fig. 1).[6,7] Values for the dielectric constant and $E_T(30)$ values for selected solvents are depicted in Table 1.

Although a number of other ways to express polarity have been explored,[7,8] this article focuses on the comparison between the commonly used dielectric constant and $E_T(30)$ values in the expression of polarity as derived from changes in the Stokes shift of polarity sensitive probes.

Table 1 Polarity parameters of common solvents[a]

Solvents	ε/D	n	Δf	$E_T(30)$/kcal mol^{-1}
Hexane	1.889	1.375	0.000	31.0
1,4-Dioxane	2.219	1.422	0.022	36.0
2-Propanol	20.190	1.377	0.277	48.4
1-Propanol	20.800	1.384	0.275	50.7
Ethanol	25.290	1.360	0.290	51.9
Methanol	33.520	1.329	0.309	55.4
Water	80.180	1.333	0.320	63.1

[a] See ESI for references.

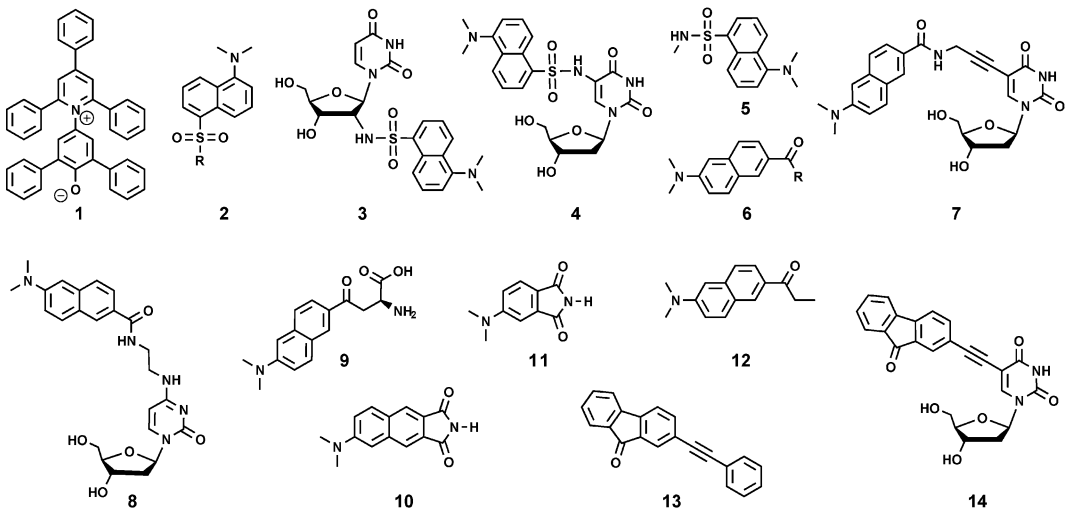

Fig. 1 Reichardt's dye (**1**), polarity sensitive fluorophores (**2**, **5**, **6**, **10**, **11**, **12**, **13**), fluorophore modified nucleosides (**3**, **4**, **7**, **8**, **14**) and fluorophore modified alanine (**9**).

How is the polarity of biomolecular cavities probed?

Inherent limitations of the natural building blocks found in nucleic acids and proteins have triggered the synthesis and implementation of designer, environmentally sensitive, fluorescent probes. Regardless of the probe's structure and its placement within a biomolecule, probing the biopolymer's polarity under native conditions is always referenced to values determined for the isolated probe in solvents or solvent mixtures of known polarity. This section outlines a typical procedure for correlating experimental observables with an unknown environmental polarity.

The solvent molecules' ability to accommodate a photoinduced dipole has a pronounced influence on the fluorescence properties of polarity sensitive fluorophores. Typically, the observed changes in emission are correlated to the orientational polarizability, Δf, of a solvent mixture. This parameter expresses the electronic and motional polarizability of solvent molecules surrounding the fluorophore in the ground and excited state, with ε and n representing the dielectric constant and the refractive index, respectively (eqn (1)).

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \qquad (1)$$

The orientational polarizability parameter is part of an equation that describes the solvent's influence on the energy difference between the ground and excited state, derived by Lippert[9] and Mataga *et al.* (eqn (2)).[10,11] In this equation, $\nu_{abs} - \nu_{em}$ is the difference in absorption maximum and the emission maximum in cm^{-1} (the Stokes shift), h represents Planck's constant and c is the speed of light. The μ_E and μ_G parameters represent the dipole moment of the excited and ground state, respectively, and a is the radius of the cavity occupied by the fluorophore that is assumed to reside in a continuum of unified dielectric constant.

$$\nu_{abs} - \nu_{em} = \frac{2}{hc}\left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right) \times \frac{(\mu_E - \mu_G)^2}{a^3} + const \qquad (2)$$

If only general solvent effects play a role, the Stokes shift is linearly dependent on the polarizability (Δf) of the solvent. Deviation from linearity implies the contribution of solvent specific effects such as hydrogen bonding, acid–base interactions or charge transfer interactions for which the approximations used in deriving the Lippert–Mataga equation cannot account.[12,13] As a result, investigators tend to utilize a limited window of linearly correlated orientational polarizability values, which typically translates into a narrow range of solvents or solvent mixtures. The final step in creating a polarity reference scale, is correlating the solvent's or solvent mixture's orientational polarizability (Δf) to its dielectric constant (ε).

To estimate the polarity of a biomolecular cavity, one then experimentally determines the Stokes shift for the biomolecule-probe conjugate. Using the reference scale generated by the Lipper–Mataga equation, one can correlate $\nu_{abs} - \nu_{em}$ to a specific Δf value (Fig. 2 path a). This Δf value is then subsequently correlated to a corresponding ε using

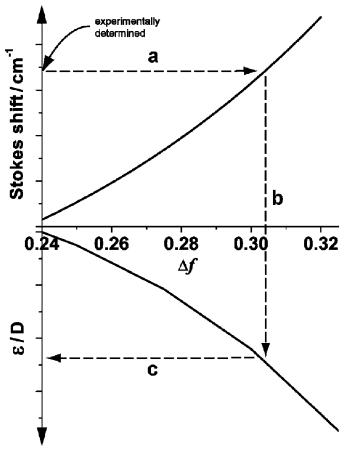

Fig. 2 A schematic illustration showing how to correlate an experimentally determined Stokes shift to the corresponding dielectric constant, ε, *via* the orientational polarizability, Δf.

a second correlation (Fig. 2 paths b and c).

Despite the fact that the Lippert–Mataga theory does not take solvent specific interactions into account, it does provide an easy tool to correlate changes in Stokes shift with polarity. To illustrate the features and caveats associated with the parameters discussed above and their applicability for estimating polarity, three different fluorescent molecules are analyzed below.

Probing polarity in biomolecules using dansyl and DAN fluorophores

The probing of DNA groove polarity was pioneered by Breslauer and Jin using non-covalently bound bisbenzimide (Hoechst 33258).[14] More recent approaches have involved the use of covalently-linked fluorophores. A popular probe, used to locate base mismatches in DNA,[15] identify adenosine–adenosine deaminase complexes,[16] and to estimate DNA groove polarity[17] and its sequence dependency,[18] is the dansyl [1-(dimethylamino)-naphthalene-5-sulfonyl] fluorophore (**2**). It has been connected to either the ribose unit (**3**)[15] or the nucleobase moiety of a nucleoside[17,18] (**4**). Despite its popularity, spectroscopic data on minimally modified sulfonamide chromophores are lacking. The *N*-methyl sulfonamide derivative **5** was therefore synthesized and its absorption and emission spectra were taken in various solvents.[19] Dansylamide **5** is characterized by minor changes in the absorption spectra but large wavelength shifts and intensity variations in the emission spectra (see ESI†).[20]

To examine the correlation between solvent polarity and the changes in absorption and emission maxima of **5**, the observed Stokes shifts are plotted against dielectric constants, the orientational polarizability and $E_T(30)$ values (Fig. 3). Plotting the Stokes shifts against dielectric constants yields a scattered relationship (Fig. 3a). A more exponential relation is observed between the Stokes shift and Δf (Fig. 3b). In contrast, a respectable linear relationship is obtained between the Stokes shift and the $E_T(30)$ indicating that this polarity scale describes the solvent-induced changes in Stokes shifts more accurately than the dielectric constant or the orientational polarizability.

The example discussed above illustrates the superior performance of microscopic solvent polarity scales, such as Reichardts's $E_T(30)$, in portraying the immediate environment of a chromophore. In studying DNA groove polarity, Majima *et al.* implicitly addressed this issue by reporting values not only in Debye, but also in the more appropriate $E_T(30)$ value.[21] Their studies were conducted with DAN (6-dimethylamino-2-acylnaphthalene) (**6**), another popular fluorophore, which is structurally related to dansyl. Both fluorophores share the naphthalene core substituted with a dimethylamine electron donating functionality in conjugation with an electron withdrawing moiety leading to strong charge transfer bands. With DAN connected to a natural nucleobase *via* either a rigid (**7**)[22] or flexible (**8**)[20,21] amide linker, the polarity of the minor and major groove of B-DNA as well as A-DNA,[21] Z-DNA[22] and the interior of a DNA-binding protein–protein complex[23] have been estimated.

In the past, polarity sensitive fluorophores attached to long hydrocarbons have been used to probe biological membranes.[24] A related approach, where amino acids are substituted with chromophoric residues, is typically undertaken in exploring local polarity in proteins.[25] One of the most elegant and recent examples describes the synthesis and incorporation of Aladan, a chromophoric amino acid obtained by conjugating DAN to alanine (**9**, Fig. 1). Incorporation of this unnatural building block into a polypeptide, facilitates the estimation of local polarity by means of steady state and time-resolved fluorescence.[26,27] The useful properties of such chromophores have inspired the development of related fluorophores, including 6DMN (6-*N*,*N*-dimethylamino-2,3-naphthalimide) (**10**)[28] and the smaller 4-DMAP (4-(*N*,*N*-dimethylamino)-phthalimide) (**11**),[29] by Imperiali and coworkers (Fig. 1).

The remarkable sensitivity of DAN for its environmental polarity has been established in 1979 with Prodan (6-propionyl-2-(dimethylamino)-naphthalene, **12**), by Weber and Farris.[30] The Stokes shift data for Prodan, as reported by the authors, are plotted against the corresponding dielectric constants (Fig. 4a), the orientational polarizability (as in the original paper, Fig. 4b), and $E_T(30)$ values (Fig. 4c) of the

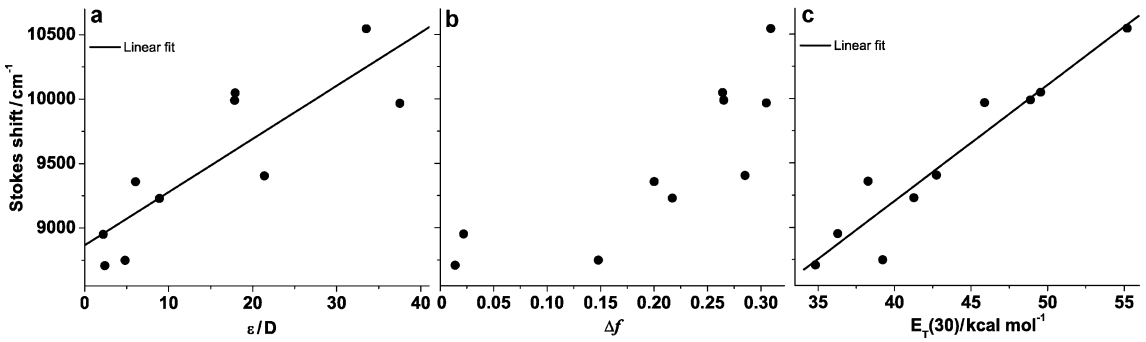

Fig. 3 Plots of the stokes shifts of dansyl **5** in various solvents against (a) ε, (b) Δf and (c) $E_T(30)$.

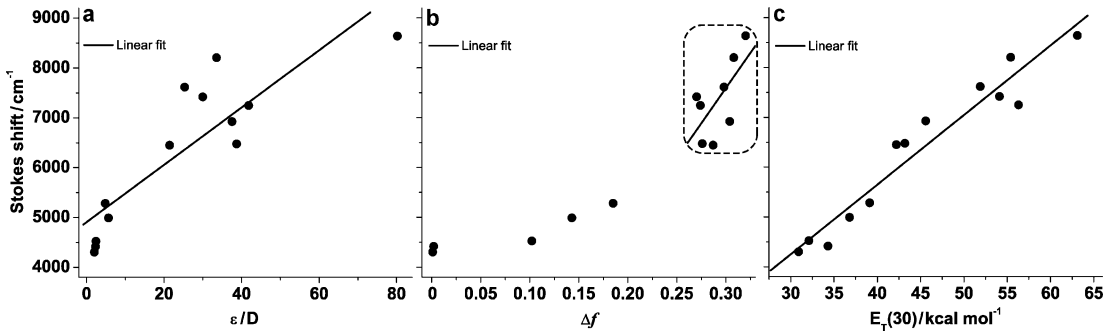

Fig. 4 Plot of the Stokes shift of Prodan (**12**)[30] against (a) ε, (b) Δf, additional frame displays the more linear part of the curve used for polarity determination in biocavities, and (c) $E_T(30)$.

solvents used.[31] The lack of correlation between the Stokes shifts and dielectric constants suggests that a solvent bulk polarity parameter does not adequately describe the immediate microenvironment of the chromophore. Although not linear, a seemingly exponential trend is revealed upon correlating the Stokes shifts with orientational polarizability similar to dansyl (**5**). This better correlation is intriguing since this polarity scale still relies on dielectric constants and refractive indexes, both bulk solvent parameters (eqn (1)). Note that to overcome this challenge, researchers often use a narrow range of this correlation where a semi-linear correlation can be deduced (see Fig. 4b).

In contrast to the questionable correlations illustrated above, a very good linear relationship between the Stokes shifts and $E_T(30)$ values is observed (Fig. 4c). It is reassuring that, in spite of the large variation of the solvents used, the best correlation is indeed observed when the Stokes shifts are correlated to a parameter that inherently reflects specific solvent–solute interactions. This observation underlines the wide applicability of Reichardt's dye (**1**) and the resulting polarity scale that allows for parameterization of virtually all solvents and solvent mixtures, regardless of their solvent specific interactions. For this reason, cyclohexane and water, two solvents with obviously very different characteristics, are found on the same linear fit (Fig. 4c and ESI). Thus, where the dansyl probe only indicates the more appropriate use of the $E_T(30)$ value, the results obtained with the DAN fluorophore strongly support the use of the $E_T(30)$ scale over the use of dielectric constants.

An extremely water sensitive fluorophore

Certain fluorophores display considerable sensitivity to the presence of H-bonding solvents. In certain cases, such solvents are excluded from the evaluation due to their "specific solvent–solute interactions", as the approximated Lippert–Mataga correlation does not handle such interactions properly.[32] To illustrate an extreme case of such a sensitive fluorophore, we describe the photophysical features of 2-phenylethynylfluorenone (**13**).[33] This emissive fluorenone derivative represents the chromophoric portion of a modified nucleoside (**14**) we have recently synthesized and examined.

Spectroscopic characterization of **13** in common solvents reveals almost identical absorption spectra, but displays a remarkable drop in fluorescence intensity with concomitant red shift of the emission maximum (see ESI†).[20] This finding prompted us to study **13** in a binary mixture of dioxane–water (Fig. 5b). With this binary system, in which the two components are miscible in all ratios, a wide polarity range from 2.2 D ($E_T(30) = 36.0$ kcal mol^{-1}) to 80.2 D ($E_T(30) = 63.1$ kcal mol^{-1}) was obtained. The stark decrease in emission with concomitant red-shift upon increasing water content established the acute sensitivity of the probe for water or better, hydrogen bond donating solvents.

To unveil the apparent relationship between solvent polarity and the minimal changes in the absorption characteristics but large shifts in the emission maximum, the Stokes shifts are plotted against three solvent polarity parameters: ε, Δf and $E_T(30)$, for both pure solvents (filled circles) and water–dioxane mixtures (open circles) (Fig. 5). Notably, the relation between the ε and the Stokes shift seems to be of a different nature for the pure solvents than the water–dioxane mixtures. In the case of the pure solvents, there

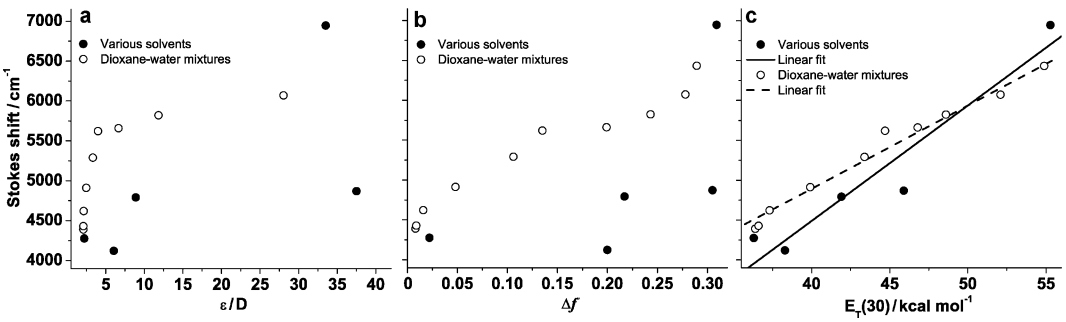

Fig. 5 Plots of the Stokes shifts of 2-phenylethynylfluorenone (**13**) in pure solvents (filled circles) and water–dioxane mixtures (open circles) against: (a) dielectric constant, (b) orientational polarizability, and (c) $E_T(30)$ value.

is no clear correlation, while the solvent mixture displays an exponential correlation. A similar behavior is seen for the correlation of the Stokes shifts with Δf.[34] By using $E_T(30)$ values, however, both pure solvents and solvent mixtures show a similar linear trend, illustrating again the benefit of using a microscopic solvent polarity scale that inherently accounts for solvent–solutes interactions.

An improved Stokes shift–polarity correlation

The preferred use of $E_T(30)$ values over dielectric constants to express changes in polarity has been demonstrated by Radhakrishnan and Samanta *et al.* in determining excited state dipole moments of coumarine dyes.[35] In a modified Lippert equation (eqn (3)), the orientational polarizability term (eqn (1)) has been substituted by the unitless E_T^N scale,[7] a normalized $E_T(30)$ scale.[36]

$$\nu_{abs} - \nu_{em} = 11307.6\left[\left(\frac{\Delta\mu}{\Delta\mu_D}\right)^2\left(\frac{a_D}{a}\right)^3\right]E_T^N + C \quad (3)$$

Eqn (3) illustrates the linearity between changes in the Stokes shift ($\nu_{abs} - \nu_{em}$) and the solvent polarity parameter E_T^N. The $\Delta\mu_D$ and a_D represent the changes of the dipole moment and Onsager radius of Reichardt's dye (**1**), 9 D and 6.2 Å, respectively.[37] The $\Delta\mu$ and a are the corresponding quantities of the molecule under study and c represents a constant. The E_T^N value for any given solvent or solvent combination can be calculated based on its $E_T(30)$ value according eqn (4).[7]

$$E_T^N = \frac{E_T(30)_{solvent} - E_T(30)_{TMS}}{E_T(30)_{water} - E_T(30)_{TMS}} \quad (4)$$

The better linear correlation to the Stokes shift can be attributed to the substitution of bulk solvent parameters ε and n by E_T^N, a microscopic solvent polarity parameter. Moreover, this approach allows one to use virtually any solvent, including solvents for which the ε and n parameter are outdated, unreliable or simply unknown, since the needed $E_T(30)$ value can be easily determined experimentally. This advantage is of particular interest if solvent mixtures are used. It is somewhat surprising that this improved correlation, introduced more than a decade ago, has not been more widely adopted.

Conclusions, contemplations and prospects

Fluorescence spectroscopy with customized polarity sensitive probes could be a powerful technique in estimating the local polarity of biomolecular cavities. The three fluorophores surveyed here demonstrate that the use of dielectric constants, a bulk solvent property, while not necessarily unacceptable, is significantly inferior to employing microscopic, spectroscopy-based, solvent parameters, such as $E_T(30)$. The experimentally determined Stokes shifts are likely to continue and serve as the observable of choice in probing biomolecular cavities, since this quantity represents a simple measurable entity that probes both the ground and excited states. Correlating Stokes shifts to Δf, as shown, suffers from some serious deficiencies, particularly due to the limited linearity of this relationship, inherently resulting from the use of bulk solvent parameters in calculating Δf. The community is encouraged to consider using the modified Lippert–Mataga equation that incorporates microscopic solvent polarity parameters. In addition, probing the polarity of a biomolecular cavity by referencing it to a binary solvent mixture remains questionable. Is such a limited reference scale capable of mimicking the plethora of interactions a probe experiences inside a confined cavity?

In light of the predicaments described in the introduction, as well as the probe-dependent correlations and the ambiguity introduced when pure solvents are compared to solvent mixtures of "identical" polarity illustrated above, a critical question surfaces: do the polarity values previously estimated faithfully reflect the microenvironments probed? This is, of course, impossible to universally answer. It is intriguing to mention, however, that values determined for the polarity of the major groove of B-DNA using the general techniques described here range from 55 D to 70 D.[17,23]

Although seemingly straightforward, the experimentally measured polarity, regardless of the type of biomolecular cavity probed, is a reflection of the inherent alterations of the environment under study, and probably, to a lesser extent, the polarity of the immaculate biomolecular cavity. Infinitesimally small probes are obviously non-existent. The probe's size, shape and its intrinsic polarizability profile will inevitably evoke changes within the biomolecular cavity and its molecular constitution (*e.g.*, water and ions) that will taint the readout. The significance of the problem, coupled to the intriguing multi-faceted challenges exemplified here, are likely to accelerate the design and implementation of smaller and possibly less intrusive fluorescent probes.

Acknowledgements

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