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Advanced Fluorescence Reporters in Chemistry and Biology III

Applications in Sensing and Imaging

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Aims and Scope

Fluorescence spectroscopy, fluorescence imaging and fluorescent probes are indispensible tools in numerous fields of modern medicine and science, including molecular biology, biophysics, biochemistry, clinical diagnosis and analytical and environmental chemistry. Applications stretch from spectroscopy and sensor technology to microscopy and imaging, to single molecule detection, to the development of novel fluorescent probes, and to proteomics and genomics. The *Springer Series on Fluorescence* aims at publishing state-of-the-art articles that can serve as invaluable tools for both practitioners and researchers being active in this highly interdisciplinary field. The carefully edited collection of papers in each volume will give continuous inspiration for new research and will point to exciting new trends.

Preface

The key element of any fluorescence sensing or imaging technology is the fluorescence reporter. It is a molecular or nanoscale device that transforms the information on molecular interactions and dynamics into measurable signal of fluorescence emission. Due to these reporters, fluorescence technologies feature the unique combination of very attractive properties, such as extreme sensitivity up to individual molecules, ultrahigh resolution in time, potential for multiplexing applications, and remote accessibility with the possibility of obtaining the high-resolution images. These properties are in the background of innumerable applications. Within this content, the aim of three volumes under a common title: "Fluorescence Reporters in Chemistry and Biology" is to provide the comprehensive overview of fluorescence reporters, concentrating on those of them that represent organic fluorophores or exhibit similar spectroscopic behavior. Being historically the first, organic dyes maintain their leading positions both in basic studies and technology developments. In logical sequence, Part I was devoted to fundamentals and design of organic fluorophores and to optimization of their valuable properties. Part II addressed nanoscale constructions that are designed based on organic fluorophores and the systems that behave like these reporters but display strongly improved functioning – the clusters of only few noble metal ions and the conjugated polymers. The present Part III is the final part highlighting the applications of fluorescence in sensing and imaging, from the nanoscopic properties of materials to the biological whole-body imaging.

Heterogeneity in structural and dynamic properties is peculiar to any condensed state system. Special attention is required to those systems, in which such heterogeneity reveals on a very short length scale comparable with the size of molecular reporters. The introductory chapter addresses this important issue and critically analyzes different approaches based on physical theory, empirical correlations, and computer simulations. A strong attempt is made toward reducing ambiguity in interpretation of experimental data.

Improving old and designing new materials require new developments in fluorescence techniques. Static and dynamic properties of synthetic polymers and of their change during the synthesis, processing, and aging are the subject of the special chapter. The readers interested in unique properties of ionic liquids as the

media of prospective "green chemistry" will obtain the important information on them derived from molecular probing. The systems with nanoscale anisotropy in molecular interactions and dynamics include liquid crystals, molecular-scale thin films, micelles, and bilayers formed of surfactants and lipid molecules. Strong electric field gradients are the characteristic of them. Physical modeling and structural data with atomic-level resolution help in understanding the spectroscopic response in proteins and nucleic acids.

The most extensive and efficient applications of fluorescence techniques are in the studies of biological macromolecules in different aspects – for sensing of other molecules as the targets and for obtaining the distributions of sensed molecules within the living cells and tissues. Operating with organic fluorophores within living cells is a challenging task and it can be addressed by the design of biosynthetically produced protein and peptide tags. Three chapters of this book address the design of organic fluorophores and their covalent modification for fitting these tags inside the cells.

Fluorescence reporters possess extending possibilities for applications on tissue and whole-body levels. They became realized with the design of bright fluorescent dyes absorbing and emitting light in the near-IR range of spectrum. In vivo imaging of vascular targets and recognition of hematopoetic and cancer cells belong to successful applications of organic dyes as fluorescence reporters. Concluding chapters of this book demonstrate recent progress in these practically important areas.

In line with other books of these series, this volume demonstrates the advancement in a rapidly developing interdisciplinary field of research and development. Therefore, it addresses an interdisciplinary audience starting from photophysicists and organic chemists to specialists in material science, chemical technology, and also to researchers working with living objects on molecular and cellular levels. This knowledge will provide additional stimulus to continuous progress in industrial technologies and biotechnologies.

Kyiv, Ukraine
January 2011

Alexander P. Demchenko

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Part I
General Aspects

Interfacial Behavior of Fluorescent Dyes

Power and Weakness of Nanoscopic Description

Alexander P. Demchenko and Semen O. Yesylevskyy

Abstract Our macroscopic world and the world of atoms and small molecules are separated by length scales differing by seven or more orders of magnitude. Describing the latter world with fluorescence probes in terms of structure and dynamics has both merits and difficulties due to peculiarities and limitations of fluorescence method. Demonstrating unique resolution in time and very high sensitivity to interaction energies, this method generally lacks structural resolution on the level of atomic details. Therefore, presentation of fluorescent probe by its molecular structure or its derivatives (size, charge distribution, dipole moment, etc.) and of its tested molecular environment in terms of continuous medium (such as micropolarity, microfluidity, or proticity) became the common method of analysis. This description that combines molecular-level parameters and reduced to molecular-level macroscopic parameters can be termed "nanoscopic". The strong demand towards rational description of systems with molecular and nanoscale heterogeneity (surfaces of liquids and solids, liquid–liquid and liquid–solid interfaces, nanoparticles and porous nanocomposites) requires critical analysis of methodology when applied to these systems. This will be the subject of the present chapter.

Keywords Fluorescence reporters · Nanocavities and nanocomposites · Nanoscale polarity · Nanoscale viscosity · Solvatochromy · Surfaces and interfaces

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Contents

1 Introduction

Determination of composition, structural arrangement, and dynamics of molecules and their groups of atoms at liquid–liquid and liquid–solid interfaces are extremely important for understanding various phenomena related to adsorption and catalysis and for technologies of chemical synthesis and separation/purification of reaction products. The properties of nanoscale porous materials and of nanoparticles, possessing very high surface-to-volume ratios, can be to a dramatic extent determined by interactions at their solvent interface. Structure and stability of synthetic polymers and biopolymers (proteins, polysaccharides, and DNA) are governed by interactions with the solvent and with the adsorbed low-molecular ligands. Understanding the behavior of these materials requires molecular-size

tools integrated into these composite systems and serving as the reporters. Fluorescence probing methodology can provide such tools that are simple, highly sensitive, and nondestructive.

Fluorescence reporting focuses on *nanoscopic* properties of matter. It uses different organic dyes, luminescent metal complexes, labeled macromolecules, and different kinds of nanoparticles to evaluate local properties of their environment and of their intermolecular interactions. Sensing local field effects, the fluorescent reporter probes simultaneously the local polarity of the host medium, specific chemical interactions, and geometrical or morphological constraints. Meantime, the description of the probed system on the level of atomic details here is not available (exceptions are the formations of strong complexes and of covalent bonds by the probes, which is generally outside the probing methodology). This limitation is simply due to the size of reporters that is larger than atomic. Another limitation comes with the restricted geometry of probe location and orientation in structurally inhomogeneous systems that induces new difficulties in the understanding of their properties. Using fluorescence method, we possess a very limited number of parameters (they are intensity, anisotropy, lifetime, and the changes in excitation and/or emission spectra) that could provide informative reporter signal.

Because of these peculiarities, analysis of fluorescence data depends strongly on physical modeling leading to simplification of molecular systems or on empirical correlations relating spectroscopic parameters and intermolecular interactions [1]. Both approaches lead to quasi-continuous characterization of reporter surrounding. They allow exploration of such terms as micropolarity, microfluidity, or proticity as the *nanoscopic* analogs of parameters characterizing *macroscopic* condensed media. Such correlation of parameters that refer to macroscopic scale and molecular scale is not easy and requires different assumptions and approximations that are rarely discussed in original works.

When the averaged properties of the solvent as the "bulk medium" are of primary importance, then the quasi-continuous models (such "*continuum solvation models*") that ignore the solvent molecular structure are effective in description. Meantime, local field effects that deviate from that described in these models and a restricted geometry of probe location and orientation can be important parameters for the full understanding of spectroscopic behavior in such complex systems. Orientation of amphiphilic molecules at the polar–nonpolar interface and formation of specific interactions (such as charge-transfer complexes or H-bonds) may result in additional geometrical or morphological constraints. Then the models based on exact molecular structures (such as "*explicit solvation models*") should be applied.

The sensitivity of fluorescence emission from dye molecules to weak intermolecular interactions and their dynamics has been recognized as an important means to probe local field effects not only in homogeneous systems but also in the systems with molecular and nanoscale heterogeneity. This chapter is focused on mesoscopic description of the systems with this type of heterogeneity based on the data obtained in fluorescence probing. We analyze different methodologies in this description.

2 Essentials of Mesoscopic (Nanoscopic) Analysis Based on Computational Approach

Mesoscopic objects are the objects dealt in physics of condensed systems that are larger than atoms but small enough to observe fluctuations of statistically averaged variables. Fluorescent dyes always probe their *local* molecular environment, whatever is the dimension of studied object, in which they are incorporated. The system composed of the dye and of its local environment is always mesoscopic. Because of nanometer size of such systems they are often called *nanoscopic*. The description of nanoscopic systems and, particularly, those properties that give rise to spectroscopic changes should require some combination of classical and quantum-mechanical variables. Electronic excitations and redistributions of electronic density that accompany them are described by the laws of quantum mechanics. Meantime, there is a possibility to use intermolecular potentials derived from classical mechanics to describe intermolecular interactions in the ground and excited states. This allows considering the change of energies of electronic transitions as information on these interactions.

Why we are not satisfied by just empirical correlations between spectroscopic and macroscopic-like properties and need going deeper in the analysis of molecular and electronic structures? Our first aim is to reduce ambiguity in interpretation that commonly exists even in neat solvents. For instance, the strong shifts in fluorescence spectra could be due to the change of polarity or of H-bonding potential in the dye environment. But it can be also due to some photophysical reaction in the dye coupled with the dynamics in this environment. Even more difficult is the analysis of structure and dynamics in heterogeneous systems. Imagine that we study the properties of two interacting media of macroscopic dimensions. We can describe them in *macroscopic* terms, such as polarity and viscosity. However, this does not allow for the understanding of the properties of interface, such as the sorption of amphiphilic molecules (e.g., detergents), aggregation of nanoparticles, and the interfacial catalysis. On the other hand, *atomistic* description of these systems is very hard to achieve experimentally due to limitations of the methods that are commonly used for structural analysis, such as X-ray diffraction and NMR. But even if we do so by overcoming the problems of sample crystallization or isotope enrichment, we get a huge amount of extra structural information that is hard to analyze. We will then search the possibilities for *nanoscopic* description.

An "in silico" experiment with molecular dynamic (MD) simulations [2, 3] has a much broader applicability. This approach is based on application of classical mechanics and allows computing the forces between all atoms in the system and equilibrating the structure in chosen thermodynamic ensemble. This provides the atomistic detail in structure together with realistic dynamics of individual atoms. Meantime, it is often hard to operate with such large massive of information. Numerous atomic details can mask the general picture of physical and chemical processes that depend on statistical behavior of molecular ensembles rather than on detailed atom–atom interactions. Therefore, the technique of MD simulations is

moving toward the *coarse-grained models*. In these models, the groups of adjacent atoms are combined into the "beads", which interact with each other by means of empirical potentials. Since the number of beads is much smaller than the number of individual atoms, significant speed up of computations could be achieved. The coarse-grained models could describe slow collective motions of complex molecules (such as large proteins) or macromolecular assemblies like the membranes of liposomes [4]. The coarse graining provides the *nanoscopic* description of the studied systems instead of purely atomistic treatment.

The difference in the characteristic times for different components of the studied system is crucial for adequate interpretation of MD results. Very slow rotational degrees of freedom of large solutes, such as proteins, DNA, and membranes, are never sampled adequately in MD simulations. However, the dynamics of solvent molecules is usually so fast that the solvent could be considered in local thermodynamic equilibrium for each given position and orientation of the solute. This allows averaging the solvent properties effectively and obtaining integral characteristics, such as local effective dielectric constant, local charge density, local hydrogen bonding propensity, local ionic concentrations, local electric field, etc. Such local properties could be computed around active sites of the protein and even in the vicinity of individual amino acids exposed to the solvent. As a result, unnecessary details of fast solvent motion are averaged out, while the solute is still described with atomic resolution. This represents another facet of the nanoscopic description of the system.

One of the most significant drawbacks of classical MD simulations that deal with atoms as classical bodies but not the electrons is the inability to handle the excited states, redistributions of the electronic density inside the molecules, and chemical reactions. Thus, the development of hybrid simulation techniques, which combine MD with the quantum mechanics [5, 6], has boosted in recent years. Such combined techniques allow computing electronic properties of small critical subsystems (such as organic dye molecules together with their binding sites) in realistic dynamic environment, which is described in the terms of classical mechanics. The interactions of fluorophores with their environment become dependent on their electronic state in this approach.

All MD or hybrid simulations provide the trajectories of individual atoms and, in the case of the hybrid simulations, the electronic densities of the quantum subsystems. These quantities should be integrated over the statistical ensemble to obtain useful characteristics of the system, such as viscosity, polarity, diffusion coefficient, interaction or solvation free energies, etc.

In contrast to the methods that provide atomic structural resolution, fluorescence spectroscopy allows achieving response to intermolecular interactions already in integrated manner. A *nanoscopic* level of details can be addressed here by describing the studied object as an integral but nonhomogeneous system with some of its essential properties presented on molecular level. The other properties appear as integrated over elementary interactions and their dynamics, which requires introduction of quasi-continuous description in such terms as "polarity" or "viscosity". Essentials in spatial resolution here are not lost if they are determined by the

structural location and orientation of reporting fluorophore [7]. The nanoscopic view could also be achieved in MD simulations by performing limited statistical averaging, which preserves the intrinsic heterogeneity of the system. For example, the averaging in the plane of the lipid bilayer provides nanoscopic quantities, such as the profiles of polarity, electric field, dipole momentum, etc., across the bilayer [7].

Atomistic simulations of the compounds in the excited electronic states are rather challenging. Excitation is an electronic process that can only be described adequately by the methods of quantum chemistry, which are extremely intensive computationally. Therefore, the reasonable compromise is needed between the accuracy of the quantum description and the computational speed of the classical one. The rational choice of different computational schemes can be made in several distinct cases:

- *An excited state is long living compared to the characteristic times of rotational and translational diffusion. There is no significant time-dependent electronic charge transfer inside the excited molecule and between this molecule and its environment*. In this case, the classical MD could be used because the charge distribution in the excited molecule could be approximated adequately by the point charges on individual atoms. This distribution is computed on the quantum level when the empirical parameters of the force filed are determined (the parameterization stage). Only one such computation is needed, which makes it realistic even for relatively large molecules. As a result, the molecules in the ground and excited states differ only by their point charges. However, there are a number of complications in this approach. For example, the energies of bond stretching and angle bending in the excited state may be very different from the ground state, while empirical force field parameters for these energy terms are usually determined in the ground state. This can lead to systematic errors in the local dynamics and mobility of the studied compounds, although no systematic studies of these issues seem to be performed.
- *An excited state may be short living, but the perturbations of charges of surrounding molecules, caused by the excitation, are localized in the vicinity of the chromophore*. In this case, the hybrid quantum mechanics/molecular mechanics (QM/MM) methods could be used to reduce the computational burden of the quantum dynamics while keeping the accurate quantum description of the excited states. In the hybrid techniques, a small part of the system in the vicinity of the excited molecule is described on the quantum level, while the rest of the system is purely classical (Fig. 1). The quantum part usually includes the chromophore itself and the groups, which interact with it directly. Time-dependent intra- and intermolecular charge transfer could occur in the quantum subsystem as well as any chemical reactions. On each simulation step, the forces from the classical and quantum subsystem are updated in a consistent manner. This requires one quantum calculation per time step, which is feasible only for relatively small quantum subsystems.

  The consistent coupling between the quantum and classical parts is one of the most challenging problems in QM/MM calculations, especially if these regions

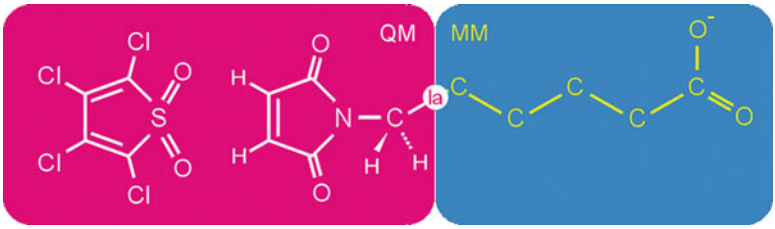

Fig. 1 The scheme of QM/MM simulation. Simulation of the Diels–Alder cyclo-addition reaction is shown as an example. The reacting groups are treated at quantum level, while the rest of the system is purely classical. The *white circle* corresponds to so-called linking atom, which is an auxiliary particle used to connect quantum and classical subsystems seamlessly. Picture from http://www.dddc.ac.cn/embo04/practicals/qmmm/qmmmvacuum.html

are covalently linked. Despite many technical and theoretical difficulties, the QM/MM functionality is now present in the widely used MD packages such as Gromacs [8] and Amber [9]. The questions, which can be addressed with the QM/MM technique, range from the optical properties of small molecules in different solvents [10] to the charge-transfer phenomena and the mechanisms of enzymatic reactions in proteins [11, 12].

- *Electronic excitation causes significant change of the electronic structure and the charge distribution, which is not localized.* For example, the long-distance charge transfer or fast diffusion of the excited molecules. In this case, the methods of the so-called quantum dynamics allow studying dynamic behavior of the system and its evolution in time on a quantum level. The most widely used variant of the quantum dynamics is the Car–Parrinello method [13], which incorporates electronic degrees of freedom directly into the equations of motions of atomic nuclei. The quantum dynamics is extremely intensive computationally because the quantum calculations of the whole system should be performed on each step. This method is usually limited to isolated molecules or small clusters with at most few dozens of atoms. Recent studies of such relatively complex heterocyclic molecules as coumarin dye show that purely quantum dynamics could be used to study their excited state in the gas phase and in the solution [14].

The treatment of *molecular environment* is one of the most important issues in the simulations of the fluorescent probes and other compounds in the excited state. The influence of the solvent on the spectral properties is often the major point of interest in such studies. As it was already stated before, an explicit solvation model includes all solvent molecules surrounding the solute (see Fig. 2a). The dynamics of the solvent should be modeled for rather long period of time, which should be sufficient for obtaining reliable statistical averages. This is usually prohibitively time consuming for quantum dynamics simulations. Alternative approach is to account for the solvent implicitly. In this case, the molecule of interest is placed into the cavity formed in the surrounding continuous dielectric medium (Fig. 2c).

In the MD simulations, the variety of methods based on the Generalized Born concept is used [16]. In these methods, each atom is represented by the sphere,

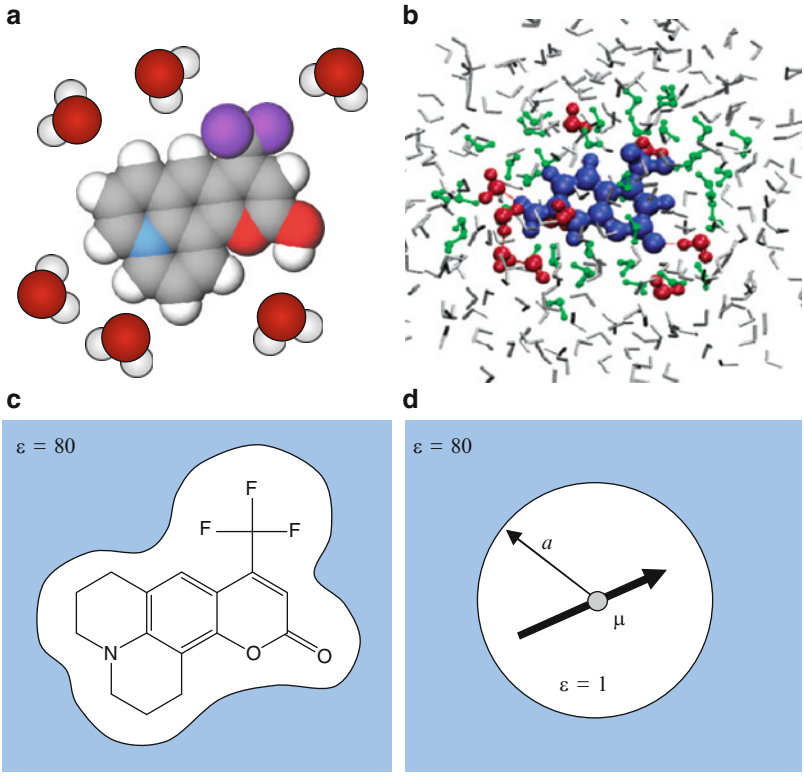

Fig. 2 Schematic view of various solvation models. (**a**) Explicit solvation. (**b**) Hierarchical solvation in quantum dynamics [15], different solvation shells are shown in *different color*. (**c**) Implicit solvation. (**d**) Simple Debye–Onsager model. The coumarin 153 probe in water is used as an example

which is cut out of the continuous dielectric medium and the electrostatic energy of such exclusion is computed. The radii of the spheres, called the Born radii, are either assigned based on the chemical identity of the atoms or updated during the simulation using various complicated algorithms. In quantum simulations, the COSMO implicit solvation model is widely used [17]. In this model, the cavity with the shape of the studied molecule is cut out in the continuous dielectric medium (Fig. 3). It screens the electronic charge density according to the dielectric constant of the medium and provides quite accurate approximation of the solvation effects. Particularly, it was shown that COSMO solvation model allows computing fluorescence spectra of 3-hydroxychromone fluorescent dyes in different solvents quite accurately [18].

Recently, improved implicit solvent models were used to study the dynamics of electronic excitations. Particularly, the effects of volume polarization and the penetration of the electronic density through the walls of dielectric cavity [19] were taken into account. It was shown that these effects play an important role in the excitations of small test molecules [20].

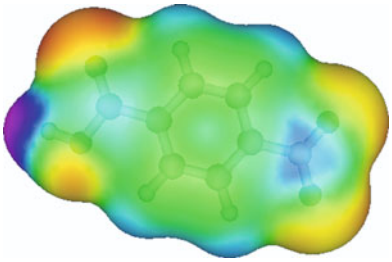

Fig. 3 COSMO surface of 4-nitrobenzoic acid colored according to the screened surface charges induced by the enclosed molecule. *Red* corresponds to the positive charge, *blue* to the negative. Picture from http://commons.wikimedia.org/wiki/File:Nitrobenzoic_acid.png

Another direction of improvement of implicit solvent models is the accounting for time-dependent solvation effects. It was shown that introduction of the time-dependent continuous polarization into the quantum dynamics allows reproducing the experimental time-dependent Stokes shift in coumarin 153 and the charge transfer in *N*,*N*-dimethylaniline extremely well [21].

There are attempts to develop the methodology, which keeps an atomic representation of the solvent, but improves the computational efficiency significantly in comparison to purely explicit solvent model, which is especially important for quantum dynamics simulations. The hierarchical approach is utilized in such methods (Fig. 2b). Solvent molecules from the first solvation shell, which contact directly with the solute, are modeled explicitly at the quantum level with full precision. Few more distant solvent shells are still treated at the quantum level but the approximation of frozen charge density is used, which speeds up the simulations. Even more distant solvent molecules are considered flexible, but their geometries are taken from the precomputed snapshots. Finally, the outer solvent shell is represented by the rigidly fixed solvent molecules [15].

It is possible to conclude that the computational approaches to describe the interactions of fluorescent dyes with their environments become more and more popular in recent years. Rapid advances in computer hardware and computational software allow in silico simulations of either very large systems or intricate time-dependent quantum phenomena, such as electronic charge transfers. The general trend of modern computational methods is the combination of different techniques, which describes the system on different hierarchical levels in order to obtain the general mesoscopic picture with an emphasis on quantum behavior of critical sites of interest. Such decomposition keeps the balance between the accuracy of quantum chemistry and the speed of classical empirical MD simulations and thus is very promising for large heterogeneous systems. Despite the exceptional spatial and time resolution of computational techniques, they depend strongly on experimental reference information, which is used to develop the force fields and to prepare the realistic starting structure for simulations. Thus, the “golden standard” of modern mesoscopic studies includes both experimental and computational approaches to the same system, which complement each other.

3 Analytical Descriptions and Empirical Correlations Exploring the Term "Polarity"

The term "polarity" is often used in a very general sense describing solvating capability of the medium. Whereas chemical definition of polarity is based on distribution of compounds between aqueous and highly hydrophobic phases (e.g., using the index log P, which is the logarithm of the octanol–water partition coefficient), physicists and physical chemists addressing the properties of materials use two different concepts based on *physical modeling* or on *empirical correlations* that will be discussed below.

3.1 Physical Modeling of Polarity Effects

Physical definition of polarity is quite different from that used by chemists and physical chemists. Here, polarity is considered as the composition of two basic effects in accordance to two general ways to stabilize a given molecule in a particular environment. One is the *electronic polarizability* of the medium that provides ultrafast response that can be described as the function of the square of refraction index, n^2, and is frequently presented as $f(n^2) = (n^2 - 1)/(2\ n^2 + 1)$. The other is the *nuclear polarizability* that describes the presence of molecular dipoles interacting with the probing dye and their much slower motion in the electric field created by this dye. This effect is expressed as a function of dielectric constant, ε. When the effects of electronic polarizability are small (e.g., in fluorescence spectra in highly polar liquids), the Onsager polarity function $f(\varepsilon) = 2(\varepsilon - 1)/(2\ \varepsilon + 1)$ can be used as a simplified estimate of *polarity*. In addition, there are the effects of generation of induced dipoles that need a more complicated description in both ε and n^2 terms. The interactions giving rise to electronic and nuclear polarizability in the fluorophore environment are considered "universal" in contrast to "specific" interactions that could be the charge-transfer (CT) complexes and H-bonds [22].

All mesoscopic models used for describing the solvation effects operate with the concept of Onsager *reactive field*. Here, the multitudes of real weak (Van der Waals) intermolecular interactions are represented in an integrated manner as the macroscopic electric field acting on a dye molecule at the site of its location. It may be considered as the field created and sensed by the solute dipole in its dielectric environment. Its electric dipole moment polarizes the solvent so that the solute itself experiences an electric field, the reaction field. The reaction field strength is proportional to the solute dipole moment in the ground and excited states. The energy shift on transition from vacuum ($h\nu_0$) to dielectric environment ($h\nu$) is proportional to the product of reactive field vector R and $\Delta\mu = \mu_e - \mu_g$, the change of dipole moment on electronic excitation:

$$h\Delta\nu = h\nu_0 - h\nu = \Delta\mu R/h.$$

The simplest models consider the dye as a point dipole located in the center of spherical cavity of radius corresponding to the dye dimension and dielectric constant equal to 1 (Onsager sphere radius a) (see Fig. 1d). The frequently used Lippert–Mataga equation [23] is based on approximation, in which all polarization effects except the generation of reactive field are neglected and the dipoles of the ground and the excited states (μ_g and μ_e) are oriented in the same direction. As the spectroscopic parameter, the model uses the Stokes shift, which is the difference between the positions of dye absorption and emission maxima on the wavenumber scale (in cm^{-1}). It describes how the general solvent effects expressed as a function of n^2 and ε can produce the relative shifts between absorption and fluorescence emission spectra:

$$\bar{\nu}_A - \bar{\nu}_F{}^- = \frac{2}{hc}\left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right)\frac{\left(\mu_e - \mu_g\right)^2}{a^3} + \text{const.} \tag{1}$$

Here, c is the speed of light and h is Planck's constant. The function $\Delta f(\varepsilon,n)$

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1},$$

that is called *orientational polarizability* is the difference between two terms inside large parentheses of (1). The refractive index contribution accounts for the ability of the electrons belonging to polarizable groups in the environment to polarize in order to stabilize the dipole moment of the fluorophore in the excited state, and this factor decreases the Stokes shift. Such polarization is instantaneous and occurs during the absorption process. It shifts the absorption spectrum to lower energies. In contrast, the dielectric constant term accounts for the relaxation process that involves the rotational or translational motions of groups of atoms or whole molecules. It develops in time and results in shifts of fluorescence spectrum in the direction of decrease of the energy difference between the ground and excited states. The constant term in (1) accounts for small additional spectral shifts due to excitation and emission to higher vibrational levels. The Δf values calibrated in different aprotic solvents are commonly used as the measures of polarity [24, 25].

There were a number of attempts to improve the Lippert–Mataga equation (see [26]). Thus, Bakhshiev considered the induced polarization terms and the nonparallel orientation of ground-state μ_g and excited-state μ_e dipole moments [27]. Somewhat different Δf values are used for calibration when the generation of induced dipoles resulting in more complex functions of ε and n are accounted.

Specific interactions, such as CT complexes and H-bonds, cannot be integrated easily into this treatment. But there is a frequently used possibility to consider the complexes formed by these interactions as discrete molecular entities that do not reorganize during electronic excitation. Such approach is not possible with the structures reorganized in the excited state (e.g., excimers and exciplexes), which

require different description. In all these treatments, the involvement of specific interactions can be detected as the deviations from linearity in Stokes shift versus Δf plots (the Lippert plots).

The experimental data show that polarity is an efficient variable describing the interaction forces of the probe with the solvent and its components. Their description must involve several molecular properties (electronic and nuclear polarizabilities, abilities to form specific bonding), and therefore it is difficult to provide a strict definition of polarity and provide its measurement in a straightforward manner.

Accounting for polarity in quantum and QM/MM simulations is also nontrivial. The polarity is an integral property averaged over many solvent molecules during sufficiently large period of time, which should be larger than the characteristic time of rotational diffusion of the solvent molecules. Such averaging is problematic in quantum simulations due to huge amount of computational time required. However there are several approximations, which allow effective accounting for polarity effects. The method of effective fragment potentials (EFP) subdivides the system into the solute, which is treated at full precision according to selected basis set, and the solvent, which is treated at the simplified semiclassical level (Fig. 4). Such semiclassical description is, nevertheless, more precise than purely classical treatment in traditional QM/MM but is also more expensive computationally. Recently, the influence of the polar water environment on the excited state of Coumarin 151 dye was successfully modeled using this technique [14]. Another approach to the polarity effects in quantum calculations is based on the continuous implicit solvent model with volume polarization effects [19, 20].

Theoretical description of the polarity effects on the nanoscopic length scale also progressed in recent years. Particularly, the theory of the electronic polarization of

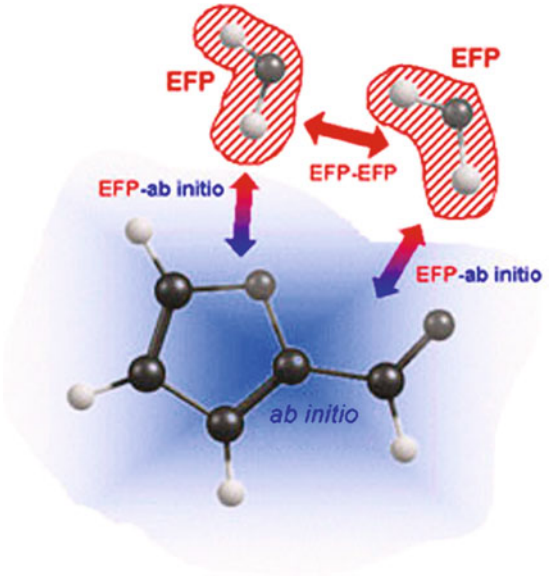

Fig. 4 The scheme of the EFP method. The solute (*blue*) is treated at the quantum ab initio level, while the solvent (*red*) is treated at the simplified semiclassical level

the molecules based on the concept of nonlocal dielectric function was built [28]. This theory also includes the contribution from spontaneous quantum fluctuations in polarization, which are rarely taken into account in traditional approaches. Although such theoretical works are of little practical importance now, they may lead to the development of innovative computational techniques in the future.

Extended versions of physical theory allow closer approximations of real systems accounting real shape of the dye molecule and its charge distribution including not only dipolar but also octupolar and higher order moments and also anisotropy in solvent polarization. Meantime, the basic theory is sufficient for many applications. The most important advantages in this analysis are the following:

- The shifts in both absorption and fluorescence spectra are described in a consistent way as the differences in energy of the ground and excited states.
- The time-dependent shifts of fluorescence spectra can be described within the same formalism in terms of relaxation of surrounding dipoles leading to changes in energies of these interactions. Thus, dynamics in the medium can be characterized.
- The physical parameters of reporting dyes are included into the analysis so that they can nicely reproduce the most important spectral features, including frequencies, intensities, and band shapes, based on a few molecular parameters that are kept fixed in all solvents. Therefore, this analysis can serve as a guideline for developing new probing dyes. The responses of dyes exhibiting specific interactions can be recognized as deviations from "universal" behavior.
- The models are extendable for describing the mechanisms of excited-state reactions, such as intramolecular charge transfer (ICT) and excited-state intramolecular proton transfer (ESIPT), in which the motions along "the solvation coordinate" play an important role. These effects can provide additional tools for characterizing the dye environment.

3.2 Effects of Local Electric Fields

Electric fields, either external or that produced by nearby atomic charges, influence the energies of electronic transitions resulting in spectral shifts [29] providing the tools for characterizing electrostatic interactions on a molecular scale. Local electrostatic fields generated by closely located charges are sensed in the same way as the effects of polarity being an additional component to the reaction field [30]. In these cases and, particularly, when the densely packed molecules form charged interfaces, such as in Langmuir–Blodgett films [31] and biomembranes [7], reorientation of the dye with respect to the field can distinguish the electric field effects. A group of voltage-sensitive styryl or naphthyl styryl dyes has been successfully applied for the visualization of the changes of biomembrane potentials [32].

The electrochromic dye senses the integrated electric field at the site of its location whenever this field is applied externally in a macroscopic device or internally, on a molecular level, produced by nearby charges. The "mesoscopic"

approach considering the dye π-electronic system as a point dipole, electric field as a vector $\vec{F}$ that averages all the fields influencing this system and its surrounding as the medium with effective dielectric constant ε_{ef} can be used for the description of electrochromism in the simplest dipole approximation. The direction and magnitude of the shift, $\Delta\nu_{obs}$, is proportional (in the first approximation) to the electric field vector $\vec{F}$and the change of dipole moment associated with the spectroscopic transition $\Delta\vec{\mu}$:

$$h\Delta\nu_{obs} = -(1/\varepsilon_{ef})|\Delta\vec{\mu}|\left|\vec{F}\right|\cos(\theta),$$

where θ is the angle between $\Delta\vec{\mu}$ and $\vec{F}$ vectors. It follows that in order to show maximal sensitivity to electrostatic potential, the probe dye should be located in low-polar environment (low ε_{ef}) and oriented parallel ($\cos\theta = 1$) or antiparallel ($\cos\theta = -1$) to the electric field. It is also important that the correlation between the electric field strength and the spectroscopic effect within the applied approximation is linear, which allows, in principle, easy calibration of this effect in absolute values.

It is essential to note that electrochromism (the response to external electric field) and solvatochromism (the response to dielectric interactions with molecular environment) are based on the same physical mechanism [30]. Practically, this means that the effects of dipoles chaotically surrounding the dye in a polar liquid (effects of polarity) are basically not distinguishable from that of dipoles arranged in organized ensemble and generating the electrostatic potential. In experiments with the charged interfaces, the distinguishing feature can only be based on the fact of high structural anisotropy of this interface and the possibility of locating the probe also anisotropically, orienting it along, reverse or perpendicular to the interface, as it was shown for lipid bilayers [33]. Therefore, the effects that do not depend on dye orientation should be attributed to the effects of polarity and those that show such dependence should be to electrostatic potentials.

Because of stringent demands on the dye location and orientation, the correlation of spectroscopic effects with the applied field strength is possible only if its location and orientation relative to the field are fairly known.

It is important to note that the concept of *dielectric constant*, which is used in nearly all continuous models of environment, becomes invalid for atomistic simulations, such as MD or QM/MM techniques. In these simulations, the Coulomb forces are treated explicitly without the need for involving continuous medium with certain dielectric constant. In the classical subsystem, all pair-wise interactions of the point charges are computed either explicitly or by means of Particle-Mesh Evald summation [9]. In the quantum subsystem, the electron density and the nuclei are treated explicitly. As a result the dielectric effects appear naturally from the motions of individual atoms and the redistributions of their electronic density.

The *electronic polarization* is described directly as a redistribution of the electronic density in the quantum subsystem. This term of polarization is naturally missed in the classical part of the system. The *orientational polarization* comes from the translation of charges and rotations of dipoles in the system. If the

simulation time is large enough to average out all rotational and translational motions, then the "global" effective dielectric constant of the whole system could be computed as a macroscopic statistical average. However, this is possible only for relatively simple systems. The dynamics of such complex systems as proteins embedded into the lipid membrane is so slow that true thermodynamic equilibrium is never reached in simulations (for example, the rotational degrees of freedom of a protein are almost never sampled in MD simulations). Despite this undersampling, the orientational polarization of the solvent in the vicinity of the active sites of proteins or other molecules of interest is still taken into account accurately. This can be done because the dynamics of the solvent is considered to be fast enough to produce reliable statistical averages.

3.3 Hydrogen Bonding Effects

Formation of intermolecular H-bonds by the probing dyes can be used for characterizing the H-bond donor and acceptor potential of the medium. These bonds can be typically formed by carbonyl groups as proton acceptors and hydroxyl groups as proton donors. When these bonds are formed, different changes of fluorescence emission can be observed: the changes in intensity (enhancement or quenching) and wavelength shifts. The general rule is that the formation of H-bonds with proton donors produces much stronger effects than with proton acceptors, which allows observing strong effects on the changes of solvent *proticity*. The other rule is that the H-bonding in the excited state is much stronger than in the ground state (due to increase of partial charges on correspondent groups), which, in some cases, may allow following the dynamics of formation of new bonds.

Hydrogen bonds of the dye carbonyl groups with protic partners can be recognized by strong spectral shifts and the changes in shape of fluorescence spectra at low protic component concentrations, at which there should be no significant polarity effects. There are interesting reported cases when in contrast to polarity effects that display the spectral shifts only, the essential transformations of spectra are observed and in this case they are so specific that can distinguish primarily formed strong H-bonds and weaker bonds formed at high protic cosolvent content [34]. In common solvatochromic dyes, such as Prodan, these effects occur in the same direction as the increase of polarity and, therefore, it is hard to distinguish them. They can be of the same magnitude as the spectral shifts over the full scale of polarity [35].

3.4 Empirical Correlations

Both scientists and users of scientific results like simplicity. The empirical methods offer such simplicity by describing the polarity with a single parameter. Providing often satisfactory description of polarity-related properties in many systems, they

are very popular. They use the calibration on the basis of the correlation of spectroscopic changes with the indices characterizing solvent polarity. Several empirical solvent polarity scales were suggested [1, 35]. They are not totally equivalent and the scatter of experimental data against these scales is significant, even within the families of structurally related solvents. One of these scales is based on the wavelength shifting of the long-wavelength CT absorption band for *nonfluorescent betaine* dye 2,6-diphenyl-4-(2,4,6-triphenylpyridinium-1-yl)phenolate. The polarity index $E_T(30)$ is the spectral shift translated to kcal/mol, and its normalized version, E_T^N, describes relative variation of polarity between two extremes: highly polar water ($E_T^N = 1.0$) and low polar tetramethylsilane ($E_T^N = 0$) [1].

This scale is considered classical as the probing dye is soluble in a vast majority of solvents and solvent mixtures and its single parameter reflects both polarity and, to some extent, H-bonding interactions. In many investigations, the parameter E_T^N is used to check the response to polarity of different *fluorescent* dyes, which extends the range of application of this approach. Meantime, the more recently suggested dyes as polarity calibrators based on absorption or fluorescence are neither universal enough to cover the broadest range of solvents nor specific enough to investigate the mechanisms of deviations from simple regularities provided by specific features of every solvent. They allow, however, in a semiquantitative manner to characterize both polarity and H-bonding properties of the environment [36].

There were attempts to extend the single-parameter approach to distinguish several types of intermolecular interactions by using the comparison between the effects of similar dyes with different response to H-bonding as donors and acceptors [1]. For characterizing heterogeneous systems, this approach is of questionable value as the relative distribution of these dyes in studied systems can vary in an unpredictable manner.

Other empirical polarity scales are also known. The relative intensities of two vibronic bands of fluorescence spectrum of *pyrene* exhibit the polarity-dependent change, and this effect was also suggested for polarity probing under the name of Py scale [37]. Computational tools allow providing interpretation of this effect [38]. Development of the methods of covalent immobilization of pyrene on solid surfaces [39] opened many possibilities in the studies of interfaces.

The empirical correlations between fluorescence lifetime and polarity in some *polymethine dyes* were also suggested for determining this solvent parameter [40].

Concluding this section we can state that whereas polarity of a molecule can be easily calculated from distribution of its charge density, regarding the polarity in molecular ensembles and in condensed media we have to consider a composite function that involves different types of intermolecular interactions. These noncovalent interactions provide significant contribution to the energy of solute solvation (stabilization) in condensed medium. If the solute is a chromophore, then these interactions influence the energies of electronic transitions that are seen as the shifts in light absorption and fluorescence spectra. Physical modeling that does not consider specific interactions demonstrate that the interactions of dipoles and localized charges play the most important contribution to composite polarity function. This contribution can be most closely associated with the dielectric

constant ε. Specific interactions such as the H-bonding can play an equally important role that makes the polarity function essentially multidimensional. In using empirical polarity scales, one strongly relies on the selection of reference solvents, and the derived polarity is "an equivalent polarity" based on comparison with these references.

4 Dynamics of Molecules and "Microfluidity"

Characterizing dynamics in condensed systems on the level of molecules and their groups of atoms is the great task. Here also, the mesoscopic approach is inevitable as any macroscopic properties such as diffusion rate and viscosity are not always descriptive and the observation of dynamics of selected atoms and of their groups does not allow characterizing the whole system. Macroscopic measurements of viscosity (as of the resistance to liquid flow) hide special features of dynamics on molecular level, which cannot be tolerated in the studies of nano-size objects. This required introduction of "microviscosity" or "*nanoviscosity*" as molecular-scale analogs of macroscopic property that are based on molecular properties of the probe and on the approximation of its environment as a continuous medium. Here again, we observe the division into empirical and model-based approaches.

4.1 Empirical and "Rotating Sphere" Methods

Empirical methods use various types of fluorescence response calibrated in standard solvents with the known "macroscopic" viscosity. This allows using the same *poise* units as for macroscopic viscosity. Selecting the dyes as viscosity sensors is also simple: the strong temperature dependence of response in high-viscosity liquids, such as glycerol and triacetin, can be used for that. Then, based on these data on dynamics in different liquid compositions, nanostructured systems and even the condensed media with poorly understood molecular properties, such as ionic liquids and supercritical liquids, can be characterized.

For sensing MD in condensed-phase systems such as liquids, polymers, and glasses, a fluorescent dye should respond to the change of its *translational or rotational diffusion* in this system by the change of any of its spectroscopic parameters. The oldest and simplest method introduced by Perrin to measure solvent viscosity is to apply the dye exhibiting molecular rotation during fluorescence lifetime with the detection of anisotropy [41]. The *rotational correlation time* φ in isotropic liquid medium can be determined from the measurement of anisotropy, r, and lifetime, τ_F, based on Perrin equation:

$$r = \frac{r_0}{1 + \tau_F/\varphi}.$$

This method in its present version needs the measurement of time-resolved anisotropy. Often these studies show deviations from the behavior of isotropic rotor due to nonspherical shapes of probing molecules, their specific interactions with the environment, and nonexponential decay of intensity. Structural anisotropy of the medium is also of great concern, but the dynamic component can be recognized in time-resolved experiment, so that:

$$r(t) = [(r_0 - r_\infty)\exp(-t/\varphi)] + r_\infty,$$

where φ is the apparent orientational relaxation time, r_0 is the fundamental anisotropy, and r_∞ is residual anisotropy.

The rotational correlation time can be related to viscosity η assuming that the medium is isotropic and that the rotating unit of volume V has a spherical shape (Debye–Stokes–Einstein model):

$$\varphi = \frac{\eta V}{kT}. \tag{2}$$

In order to account the deviation of probing molecule from spherical shape and dielectric friction during probe rotation, (2) is decorated with additional factors. By assuming a continuous solvent model, it can provide quantitative results only on the condition that the individual solvent molecules are smaller than the solute. Specific solvent–solute interactions produce additional problems [42]. Therefore, scientists often prefer to use empirical correlations between φ and macroscopic viscosity η that depend upon the probing dye and, of course, are different for H-bonding/polar and bond-free/nonpolar environments [43].

The time-resolved anisotropy can be combined with the observations of wavelength shifts. The benefit of that is the possibility of more detailed characterization of dye location sites in terms of polarity and rotational freedom. This is especially important in the studies of systems that cannot be easily characterized by other methods, such as silica gel-glass nanoporous materials [44].

Fluorescence quenching is another approach that can be used for evaluation of nanoscopic fluidity. There are a number of dyes with segmental mobility that provides channels for relaxation to the ground state without emission. In low-viscosity medium their segments are free to rotate, and such rotation induces the quenching [45]. In rigid media, such dynamics is frozen and a bright emission appears. Typical in this respect is the behavior of triphenylmethane dyes, such as Crystal Violet and Malachite Green. They possess the three-blade propeller-like phenyl rings joined by the central carbon atom. Their lifetimes τ_F depend strongly on the solvent viscosity and, due to the absence of groups forming specific non-covalent bonds, they practically do not depend on other solvent properties. Depending on viscosity, τ_F may change by as much as four orders of magnitude.

A class of organic dyes called *molecular rotors* responds to viscosity due to control by solvent dynamics of excited-state twisting [46]. In addition to segmental diffusion, their response can be due to formation of twisted intramolecular charge

transfer (TICT) states. An example of such molecules is 4-tricyanovinyl-[*N*-(2-hydroxyethyl)-*N*-ethyl]aniline (TC1). Such molecules can be incorporated into nanoscale system without loss of sensitivity to fluidity [47]. Time-resolved microscopy allows applying molecular rotors for measuring viscosity inside the living cells [48].

One more approach is based on the possibility of two pyrene molecules to form intramolecular excimers. The excimer emission differs from that of pyrene monomer by strong wavelength shift, loss of vibrational structure, and decreased lifetime. The excimers are formed in a diffusion-controlled manner and when they are connected by flexible chain their response becomes concentration independent. Typically, 1,3-dipyrenylpropane is used for this purpose. This approach has found some application in the studies of solvent mixtures [49] and detergent micelles [50]. There were many attempts to apply this method to the studies of biological membranes, but this method is currently not in frequent use. The problem is in indefinite location of the probe in such media.

Isomerization dynamics can be coupled with the dynamics of formation/breaking of specific complexes with solvent molecules. Such effects were observed in asymmetric polymethine dyes [51]. Interplay between two forms, one emitting in solid (emitting at 700–710 nm) and the other in liquid environments (emitting at 760–770 nm), allows simple wavelength-ratiometric detection. Amazingly, neither the position of these bands nor the intensity ratio shows notable dependence on any solvent parameter except the viscosity [51]. Also, the styryl pyridinium dye showing the viscosity-dependent dissociation of ground-state complexes with water displays dual excitation peaks at 469 and 360 nm detected at emission wavelengths 500–650 nm. This property was useful for intracellular studies [52].

In the study of rotations of small molecules used as probes, it is the *dielectric friction* in addition to common viscous friction that determines the rotation rate [53]. The difference is that whereas viscous friction is determined by solute size, the dielectric friction is determined by solute dipole moment and senses the dynamics of its interaction with polar medium. It arises from longer range charge–dipole or dipole–dipole electrostatic interactions. When an ion or a dipolar solute moves in a polar solvent, there is an extra friction that arises from the fact that its polarization field induced in the surrounding solvent must readjust to its motion. Because this readjustment cannot occur infinitely rapidly, the solvent polarization will lag behind the solute and cause a systematic retarding force or friction on its motion [54]. In these dynamics, the rearrangements of intermolecular H-bonds can play an additional role.

The interaction of excited molecule with its dielectric medium can produce not only retardation but also acceleration effect. If the probe dye molecule is smaller than surrounding molecules, then its own rotation can be “induced” by electric field created in the medium of less mobile solvent molecules [55]. This *induced rotation* occurs with the rate of dielectric relaxations and can be recognized as an additional fast component in anisotropy decays that disappears at excitation red edge.

4.2 Spectroscopy of Molecular Relaxations

Solvation dynamics is the process of rearrangement of solvent dipoles around an instantaneously created or reorganized solute charge or dipole in the excited state. Electronic excitation results in instantaneous redistribution of electronic density generating the change in the dipole moment of the probe. Equilibrium in interaction with surrounding dipoles is disrupted and their relaxation to new equilibrium can be observed as the shift of fluorescence spectra in time. The ability to reorient under initial stress is an important characteristic of MD. The solvent relaxation is often approximated by the very simple Debye model that considers this relaxation mono-exponential. The *dielectric relaxation time* τ_R corresponds to that time obtained in dielectric measurements of bulk solvents, but it has the feature of molecular-scale resolution being used as the direct measure of MD in the dye environment. When we talk about "environment", we mean that the majority (about 85%) of response arises from the first solvation shell of a fluorescent probe [56]. This resolution is sufficient for studies of dynamics in first hydration layer in biological macromolecules [57].

The relaxation time is assumed to be equal to the rotational relaxation time of the single molecule τ_D. This time can be computed from the Stokes law in the hydrodynamic limit using well-known formulae for the coefficient of rotational diffusion

$$D_R = k_B T/(8\pi\eta R^3),$$

where η is the macroscopic solvent viscosity and R is the effective radius of the solute molecule. Using the Einstein relation for the reorientation time $\tau_D = 1/2D_R$, the Debye relaxation time is given by

$$\tau_D = 4\pi\eta R^3/k_B T.$$

The Debye treatment is very simplified. Particularly it does not account to the fact, that the local friction around the solute molecule is different from that in the bulk liquid. This means that the local viscosity is also different from the macroscopic bulk viscosity. Introducing such local viscosity at the phenomenological level leads to the so-called extended Debye treatment [58]. The relaxation time used in fluorescence measurements, τ_R, is in fact the reflection of relaxation of reactive field acting on fluorophore and the connection is:

$$\tau_R \approx [(n^2+2)/(\varepsilon+1)]\tau_D.$$

In the case of highly polar media, $\tau_R \approx 0.1\ \tau_D$ [59]. Its determination in fluorescence experiments is based on observation of time-dependent spectral shifts in picosecond–nanosecond time range [60]. For describing these shifts, the correlation function is used:

$$C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)}.$$

Here, $v(t)$ is the position of spectrum in cm^{-1} as a function of time, and $v(0)$ and $v(\infty)$ are values extrapolated to time zero and infinity, respectively. A simplified method for determination of $C(t)$ based on single decay curve and steady-state spectrum was also described [61]. The application of $C(t)$ allows connecting the motion of spectrum to a full scale of this event and making comparisons of relaxation rates obtained with different dyes. Mazurenko and Bakhshiev [59] using a continuum model with a single Debye relaxation time have shown that $C(t)$ decays exponentially with time constant τ_R. In reality its change as a function of time is often nonexponential, and then the averaged $\langle\tau_R\rangle$ value is taken as dynamics variable [56]. This approach allowed establishing very interesting phenomenon – a decrease in mobility of water molecules by as much as 2–4 orders of magnitude in nano-size molecular ensembles, such as biopolymers and biomembranes [62]. Slow structural relaxation allows keeping preorganized electrostatic fields in reactive sites of enzymes [63], which is a strong contribution to their catalytic action.

Dielectric relaxation time and, therefore, τ_R can be connected directly, though nonlinearly, with solvent viscosity [58].

The prominent example of MD simulations, which complement experimental findings, comes from the studies of slow solvent dynamics of DNA molecules [64]. In this work, the calculated solvation responses for the dye molecule Hoechst 33258 bound to DNA were decomposed in terms of the components present in the system: water, DNA, and the ions. It was shown that the longest time scale of the solvent response observed experimentally (19 ns) is caused by the DNA itself. An ability to decompose the response into the components coming from different parts of the system is unique for MD simulations and allows interpreting experimental results properly. It is expectable that such combined studies will become increasingly popular.

Many results obtained either by "fluidity-based" or "relaxation-based" approaches demonstrate that the dynamics on nanoscopic scale can be very different from that obtained by bulk viscosity measurements. The experiments show a dramatically decreased fluidity that cannot be understood simply by intuitive extrapolation of bulk properties [65]. Friction and lubrication between nanoscale materials that determine their macroscopic properties should be understood on atomic and molecular level [66].

5 Inhomogeneous Broadening and Red-Edge Effects

In order to increase the information content of fluorescence method, scientists are trying to study the complex functions of fluorescence parameters, such as the time-resolved anisotropy and the spectral dependence of lifetime and anisotropy over excitation and emission bands. In this content, the shapes of absorption and emission bands and wavelength-selective excitation with recording of spectral dependence of parameters of emission may contain both static and dynamic information about the fluorophore environment.

5.1 Static Red-Edge Effects

Usually the electronic transitions generating absorption and emission spectra are presented as two-dimensional functions of vibrational and solvation coordinates (see [9]). The main difference between these coordinates is the intrinsically overdamped nature of solvation modes and their very low frequencies. This behavior is in contrast to vibrational modes. The latter are quantized, achieved in a very fast Franck–Condon process and, according to Kasha rule, relax rapidly to a lowest energy level. This allows treating solvation coordinate as a classical coordinate with continuous availability of electronic states. Thus, at any finite temperature a Boltzmann distribution in the population of different solvent configurations is responsible for the inhomogeneous broadening in the steady-state spectra [26]. Such broadening arises from the solute–solvent distribution in excitation energy that reflects the distribution in energy of dye interactions with its dielectric environment. The stronger will be these interactions, the broader the distribution. Laser hole-burning experiments in cryogenic media allow obtaining the inhomogeneous site energy distribution function [67], which provides important insight into the systems with low level of molecular order and can be extremely important for characterizing the environments of interfacially located dyes. But because of frozen mobility in the system, it brings only static information.

For obtaining both site photoselection and dynamic information, a different approach can be applied that allows operating in a broad variety of experimental conditions, including room and elevated temperatures. Within the inhomogeneously broadened spectrum, one may select the species interacting stronger than the mean of this distribution with the environment by selective excitation at the long-wavelength edge of the spectrum. The positions of their fluorescence spectra, their lifetimes, and anisotropies will then be different from the mean. This is the essence of *Red-Edge effect* [68]. In structurally disordered or low-ordered systems, the strongest are the dipole–dipole interactions with polar environments. Therefore, the width of distribution and the extent of Red-Edge effects should correlate with solvent polarity.

The Red-Edge effects depend strongly on the properties of a fluorophore. The dyes with a strong increase of the dipole moment in the excited state will exhibit a broad excited-state distribution on interaction energies, and the dyes interacting stronger in the ground state – the broader ground state distributions. These features will determine their properties in spectral, anisotropy, and time domains. Meantime, if the dyes in heterogeneous system are distributed between the sites of different polarity, the spectra may also exhibit variation in these properties. In our work [69], we tried to introduce the criteria that distinguish the effects of inhomogeneous broadening from those that originate from positional (ground-state) heterogeneity. In the case of dye location at the surface or interface, the positional heterogeneity can arise from imprecise location and orientation of the dye, even on atomic scale of distances. The increased distribution on interaction

energies brings new site-selection effects that overlap those resulting from common inhomogeneous broadening.

The distinction between these cases can be observed when the excitation wavelength dependencies of fluorescence spectra are analyzed (Fig. 5). The Red-Edge effects demonstrate a very characteristic shape with the absence of the shifts of fluorescence spectra as a function of excitation wavelength at the maxima and short-wavelength shoulders of excitation bands and unlimited increase of effect at the red excitation edge [70, 71]. In contrast, the ground-state heterogeneity comes from superposition of absorption (excitation) spectra of the dyes residing

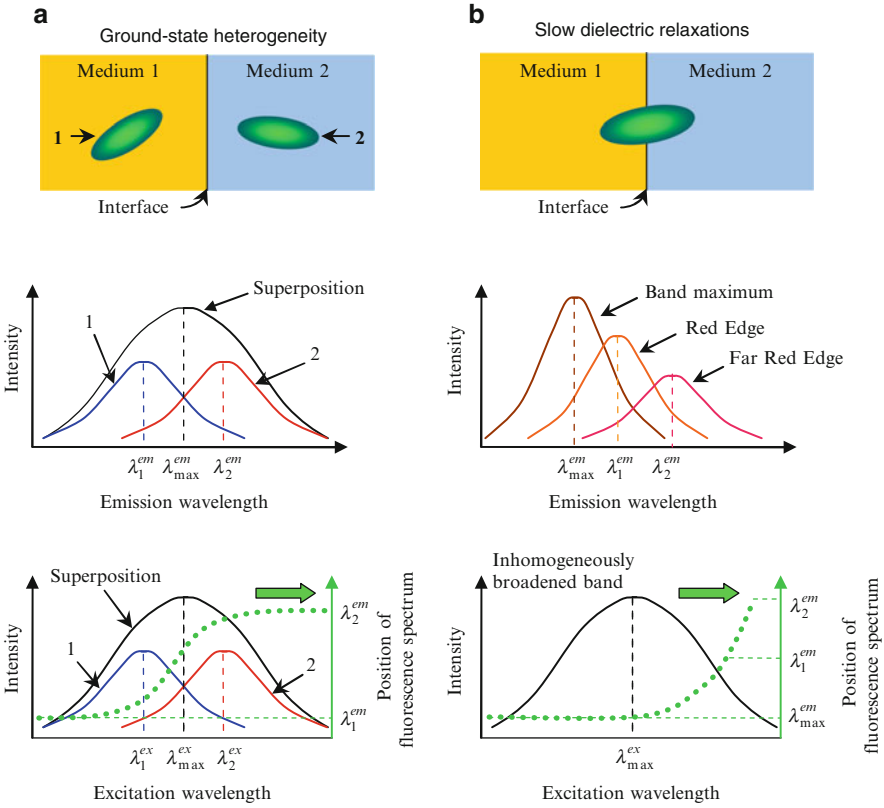

Fig. 5 Schematic representation of spectroscopic effects of (**a**) ground-state heterogeneity (e.g., the dye is distributed between two contacting media) and (**b**) slow dielectric relaxations (e.g., a single dye located at the interface and exhibiting inhomogeneous broadening) leading to Red-Edge effects. In the case (**a**) variation of excitation wavelength leads to photoselection between the species possessing difference in excitation spectra, so that the dye excited at shorter wavelengths (λ_1^{ex}) exhibits blue emission at (λ_1^{em}) and, correspondingly, excited at (λ_2^{ex}) exhibits red-shifted emission at (λ_2^{em}). The shifting of excitation wavelength leads to change of relative contributions of two emissions. In the case (**b**) there is a single ground-state form but the shift of excitation wavelength from band maximum, λ_{max}^{ex}, to the red edge of excitation leads to progressive shift of emission spectra (two of them, λ_1^{em} and λ_2^{em} are shown) to longer wavelengths

in different locations. Because the quantum yield, anisotropy, and lifetime of a dye in these locations can differ, the shapes of measured excitation-wavelength dependences can be variable, even sigmoid [24]. The dyes with low dependence for absorption spectra on solvent polarity and a very strong dependence for emission spectra could be ideal for this application. Good in this respect is tryptophan, which is the constituent of many proteins, and therefore we observe many applications of this method in protein research [68, 70]. Regarding different fluorescence dyes used for probing, the situation is variable. For instance, Nile Red with its strong depending on the environment polarity variation of both excitation and emission spectra can display both the Red-Edge effects and the ground-state heterogeneity [72], and the analysis of these effects is complicated by its strong variation of lifetime.

5.2 *Dynamic Red-Edge Effects*

When the dye molecules in the studied system are distributed between different sites (the ground-state heterogeneity), this distribution may not exhibit strong sensitivity to external conditions, such as temperature and pressure. In contrast, the distribution and therefore the positions of fluorescence bands depend strongly on the rates of dielectric relaxations in the cases when the environment dipoles move (relax) during the lifetime of emission. The relaxation mixes the environments with different excitation energies, and the differences in emission parameters between mean and site-selected dyes vanish. This is the essence of dynamic Red-Edge effects [69, 73].

The full range of these effects can be observed when the environment is rigid ($\tau_R > \tau_F$). When ($\tau_R < \tau_F$), which is the condition realized in low-viscous liquid solvents, this effect is hard to observe because of rapid averaging of species within the distribution. When $\tau_R \approx \tau_F$, with the knowledge of τ_F we get a tool for estimating τ_R from the shifts of steady-state spectra by using external perturbations that modulate τ_R (temperature or pressure) or τ_F (dynamic quenchers).

The method for obtaining the relaxation rates from the steady-state data using τ_F as the time marker is described in detail elsewhere [70, 71]. Essentially, when the Red-Edge effect in the limit of slow relaxations $\left(\nu_{t=0} - \nu_{t=0}^{\text{edge}}\right)$ is known, then τ_R can be estimated from this effect in relaxation zone:

$$\nu - \nu^{\text{edge}} = (\nu_{t=0} - \nu_{t=0}^{\text{edge}})\tau_R/(\tau_R + \tau_F).$$

Time-resolved spectroscopy allows observing the dynamics of molecular relaxations of Red-Edge selected species and comparing them with the relaxations of mean in ensemble [74, 75].

These experiments showed the appearance of the Red-Edge effect in polar solvent with the increase of solvent viscosity by lowering the temperature. The problem here is that the high and wavelength-selective temporal resolution is