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Solvatochromic fluorescent dyes as universal tools for biological research

Andrey S. Klymchenko

Résumé

Les sondes fluorescentes solvatochromes : des outils universels en recherche biologique

Les sondes fluorescentes à même de changer de couleur et de luminosité en réponse à des modifications de polarité de leur environnement, qualifiées de solvatochromes, se sont révélées récemment être de puissants outils en recherche biologique. Parmi ces sondes, les mieux caractérisées sont celles qui présentent un transfert de charge à l'état excité amenant à un déplacement vers le rouge de leur unique bande d'émission lorsque la polarité du solvant augmente. Les sondes présentant un transfert intramoléculaire de proton à l'état excité constituent une autre classe prometteuse, montrant une émission duale sensible au solvant. Ces sondes solvatochromes ont été utilisées avec succès pour suivre l'ordre lipidique des biomembranes et étudier leurs domaines lipidiques, leur asymétrie et le phénomène d'apoptose. En outre, ces sondes greffées sur des peptides ont permis de suivre les interactions de ceux-ci avec des oligonucléotides, des protéines et des membranes lipidiques.

Mots-clés

Fluorescence, sondes solvatochromes, membranes lipidiques, peptides, ADN.

Abstract

Fluorescent dyes capable of changing their color and brightness in response to changes of their environment polarity, so-called solvatochromic dyes, have recently emerged as a powerful tool for biological research. The most well-established are dyes that undergo excited state charge transfer showing red shift of their single emission band with increase in the solvent polarity. The other promising class is dyes undergoing excited state intramolecular proton transfer and showing solvent-sensitive dual emission. Solvatochromic dyes were successfully applied for monitoring lipid order in biological membranes and to study lipid domains, transmembrane asymmetry and apoptosis. Moreover, these dyes grafted to peptides enable monitoring their interactions with oligonucleotides, proteins and lipid membranes.

Keywords

Fluorescence, solvatochromic dyes, lipid membranes, peptides, DNA.

It is difficult to imagine modern biological research without fluorescence techniques, which allow rapid and highly sensitive monitoring of biological processes at the molecular level. As biological systems are poorly fluorescent, these techniques rely on synthetic fluorescent probes or markers, commonly based on organic dyes. They could be divided into "classical" dyes (rhodamine, cyanines, bodipy, etc) and environment-sensitive dyes.

Unlike the "classical" dyes, the environment-sensitive dyes can change their fluorescence properties, fluorescence intensity or emission color, in response to changes in the physico-chemical properties of their molecular environment. While classical dyes are perfect markers of biological molecules, the environment-sensitive dyes are "smart molecules" that can be used as sensors for probing local biological environment and monitoring biomolecular interactions. Their response to the environment is driven by excited-state reactions (conformational change, charge, electron and proton transfer, etc) and their non-covalent interactions with surrounding: universal interactions (van der Waals, dipole-dipole, dipole-external electric field, etc), and specific H-bonding interactions. Here, we do not consider pH- and ion-sensitive dyes as environment-sensitive, since the response of these dyes is associated with changes in their

chemical structure: protonation/deprotonation or formation of a complex with an ion.

A widely used class of environment-sensitive fluorophores is solvatochromic dyes exhibiting shifts in their emission maxima as a function of polarity and hydration of their environment [1-2]. Typical example is 2-propionyl-6-dimethylaminonaphthalene (Prodan, figure 1) [3]. In this fluorophore, the dipole moment increases dramatically upon

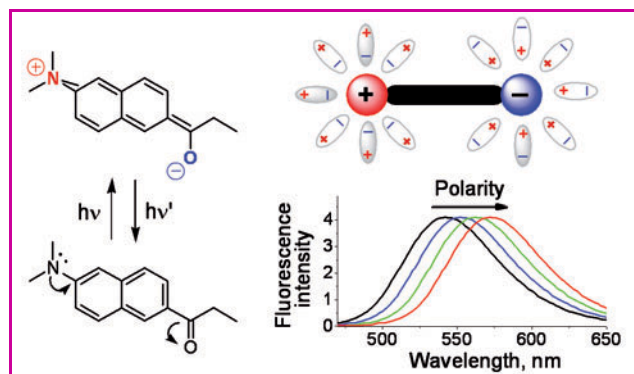


Figure 1 - Typical solvatochromic fluorescent dye (Prodan) and its excited state intramolecular charge transfer.

electronic excitation due to an intramolecular charge transfer from the electron donor, dialkylamino group, to the electron acceptor, carbonyl group (*figure 1*). The polar solvents, which present highly dipolar groups, affect the fluorophore through dipole-dipole interactions, while protic solvents, which contain hydrogen atom bound to oxygen (hydroxyl) or nitrogen (amine, amide, etc), interact with the fluorophore through H-bonding. Due to relaxation of its excited state dipole by these dipole-dipole and H-bonding interactions, this fluorophore exhibits a red-shift of its emission spectrum in response to an increase in solvent polarity. In this work, single- and two-band solvatochromic fluorophores will be presented.

Single-band solvatochromic dyes

The most common single-band solvatochromic dyes are aromatic conjugated molecules containing electron acceptor and electron donor groups at the opposite poles (*figure 2*). One of the simplest examples is Prodan [3], which was already mentioned above. Though its sensitivity to solvent polarity is remarkable, its absorption in ultraviolet region (360 nm) limits its applications in cellular studies. In order to shift the absorbance of Prodan to the red, Lu *et al.* have synthesized its benzo-analogue, 2-propionyl-6-dimethylaminoanthracene, Anthradan [4]. This dye showed desired red-shifted absorption (around 430 nm); however, its brightness was limited due to low absorption coefficient. Recently in our group, we extended the electronic conjugation of Prodan by substituting its naphthalene core with fluorene [5]. The obtained fluorene derivative FR0 (*figure 2*) showed a red-shifted absorption (close to 400 nm), with twice as large absorption coefficient, and a manifold larger two-photon absorption cross section (400 Goeppert-Mayer) compared to Prodan. Moreover, studies in organic solvents revealed much stronger dependence of its emission maximum on solvent polarity, which is connected with its twice as large change in the dipole moment (14.0 Debye) (*figure 3*).

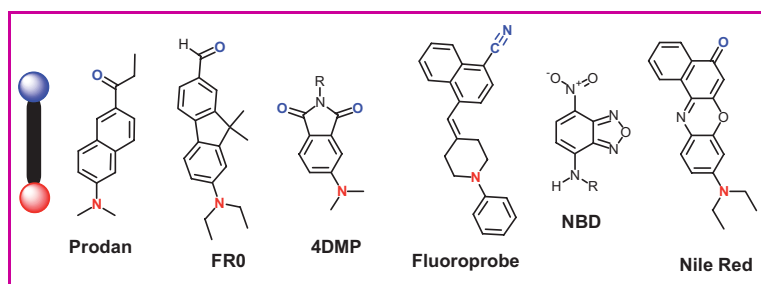


Figure 2 - Examples of fluorescent solvatochromic dyes.

4-*N,N*-dimethylaminophthalimide (4DMP) is another fluorescent solvatochromic dye, which recently attracted attention of biophysicists. This relatively small and rigid molecule presents a very strong solvent sensitivity; however, similarly to Prodan, it shows absorption in UV region (380-390 nm) and very low extinction coefficient. To improve its properties, the 4DMP fluorophore was extended, giving a new environment-sensitive dye, 4-*N,N*-dimethylamino-1,8-naphthalimide (4DMN) [6]. It should be noted that 4DMP and all its analogues are nearly non-fluorescent in water, and become highly fluorescent in aprotic media, which is important for detection of biomolecular interactions [6]. On the other hand, their poor fluorescence in water makes them

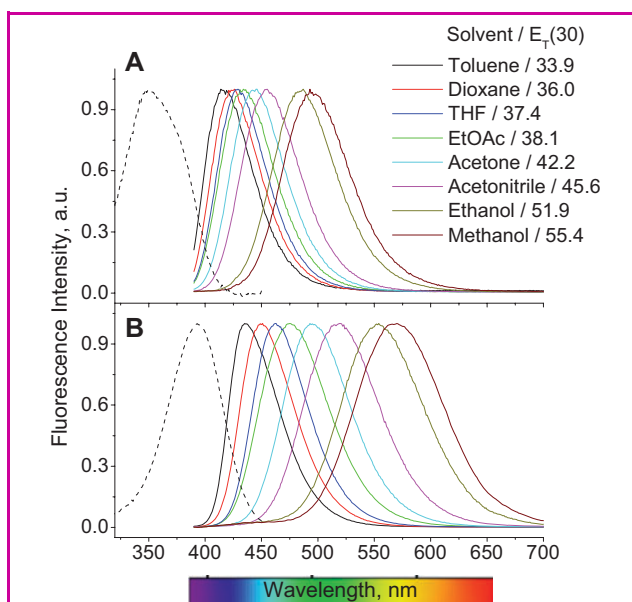


Figure 3 - Absorption (dash) and fluorescence (solid) spectra of Prodan (A) and FR0 (B) in organic solvents of different polarity. Absorption spectra were recorded in toluene. $E_T(30)$: empirical polarity index [1].

inefficient in the cases where the label shows significant water exposure at all steps of the interaction.

In the search for advanced environment-sensitive dyes, a bichromophoric dye Fluoroprobe was developed [7] (*figure 2*). This dye exhibits a charge transfer through space, which generates an exceptional change in the dipole moment (27 Debye) and thus solvent sensitivity. For the moment, Fluoroprobe remains the most solvatochromic fluorescent dye. However, it found no applications in biology, due to the extremely strong quenching of its fluorescence in polar media, UV absorption maximum (308 nm) and very low absorption coefficient.

The vast majority of environment-sensitive dyes show absorption in the UV or near-UV region, while few dyes present significantly red shifted absorption and emission. Remarkable exceptions are NBD and Nile Red dyes and their analogues (*figure 2*) showing absorption maxima around 460-480 nm and 530 nm, respectively. However, both dyes show limited fluorescence solvatochromism, which is much lower than that for other dyes mentioned above. Thus, the critical overview on the existing environment-sensitive dyes shows that there is a strong need for solvatochromic fluorescent dyes presenting both strong solvatochromism and good fluorescence properties.

Two-band solvatochromic dyes based on ESIPT

An alternative mechanism of solvent sensitivity can be realized by utilization of so-called excited state intramolecular proton transfer (ESIPT). Particularly interesting, ESIPT dyes are 3-hydroxychromones (3HC), presenting dual emission originated from the normal excited state (N^*) and the ESIPT tautomer (T^*) [8] (*figure 4*). The pathway for ESIPT in 3HCs is provided by the intramolecular H-bond through a 5-membered cycle, which is much weaker than the 6-membered cycle presented by other ESIPT systems. Therefore, it can be easily

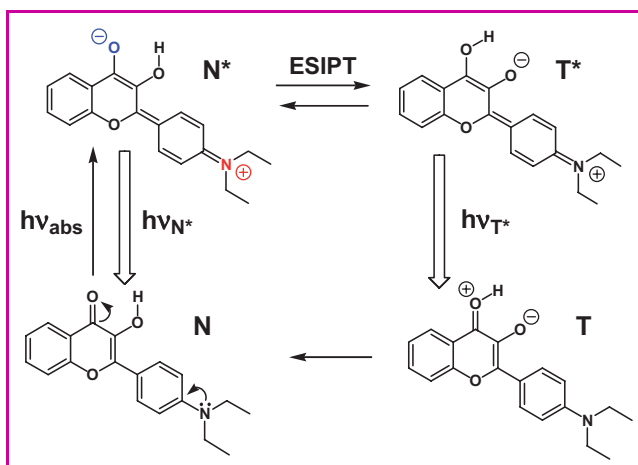


Figure 4 - Photophysical cycle of 4'-(N,N-diethylamino)-3-hydroxyflavone dye.

On electronic excitation ($N \rightarrow N^*$), a charge transfer from 4'-dialkylamino group to 4-carbonyl takes place followed by an ESIPT process. It should be noted that after $T^* \rightarrow T$ transition the proton remains at the 4-carbonyl group, producing a zwitterionic T state that rapidly converts into the stable N state.

perturbed by H-bonding interactions, thus modulating dual emission of 3HCs.

Among 3HCs developed so far, 4'-(dialkylamino)-3-hydroxyflavone (see 3HC-A in figure 5) is probably the most interesting solvatochromic dye [9-10]. Due to the 4'-dialkylamino group, the N^* excited state in this compound exhibits a large dipole moment, where the electronic charge is transferred from the dialkylamino group to the chromone moiety (figure 4) [9]. In contrast, the excited state tautomer T^* exhibits much lower charge separation and, thus, lower dipole moment. Therefore, the N^* state, unlike the T^* state, shows a significant shifts to the red on increase in solvent polarity (figure 5). This red shift is accompanied by an increase in the relative intensity of the N^* band, because this state becomes energetically more favorable than the T^* state [10]. Therefore, intensity ratio of the N^* and T^* bands, N^*/T^* , is an important indicator of solvent polarity [10]. However, 3HC-A shows dual emission only in the range between low polar and polar aprotic solvents (figure 5). In polar protic solvents, including water,

the ESIPT is efficiently inhibited, so that the T^* emission is no more observed [10]. Therefore, this dye could be applied for probing biological environments of relatively low polarity and hydration, namely biological membranes (see below).

Another interesting 3HC derivative is 2-(2-furyl)-3-hydroxychromone (3HC-B). Due to much weaker electron donor 2-aryl group (2-furyl vs. 4-dialkylaminophenyl in 3HC-A), the dipole moment of its N^* state is relatively low. Therefore, this relatively apolar state cannot be stabilized in polar aprotic solvents, so that in acetonitrile the N^* band emission is almost negligible (figure 5). Moreover, in contrast to 3HC-A, the ESIPT inhibition by protic solvents is not complete, so that a clear dual emission, which depends on polarity of protic solvent, is observed [11]. Thus, the 3HC-B dye is suitable for probing polar protic environments characterized by high hydration, which corresponds well to peptides and nucleic acids (see below).

We should note the key differences between single-band solvatochromic fluorescence dyes and 3HC dyes. While the former shift their emission maximum in response to solvent polarity, 3HC dyes change also the intensity ratio of their two emission bands. This ratio is an additional channel of spectroscopic information, which allows more detailed (multiparametric) characterization of the probe environment [10]. Moreover, due to ESIPT, 3HC dyes are focused in a more narrow polarity range, where they can show higher sensitivity to properties of environment compared to single-band solvatochromic dyes.

Applications of solvatochromic dyes for biomembrane research

Biological membranes are an interesting object of study with fluorescent solvatochromic probes because the key biological functions of biomembranes are strongly dependent on their physical properties. Here, the importance of membrane probes for monitoring membrane composition and lipid order will be shown.

Monitoring lipid order

Lipid order and membrane phase state define the membrane mechanical properties, permeability and insertion and activity of membrane proteins. Two phases could be found in biological membranes. Liquid disordered (Ld) phase, mainly composed of unsaturated phospholipids and cholesterol, is characterized by relatively poor ordering of the fatty acid chains. In contrast, liquid ordered (Lo) phase, presented by saturated lipids and cholesterol, is characterized by high level of lipid order. The latter phase is believed to be responsible for the formation of so-called lipid rafts, "swimming in the sea" of disordered phase in cell membranes, and plays a key role in the function of certain membrane proteins [12]. Therefore, visualization of these lipid membrane structures is of particular importance.

Solvatochromic dyes are powerful tools for distinguishing Lo and Ld phases, as these two phases present very different polarity and hydration of their interior. The solution is to place a solvatochromic dye into the lipid membrane region at the level between alkyl chains and polar head groups, where the level of water should be strongly affected by the lipid order. One of the most known examples is a derivative of Prodan, Laurdan. Due to its apolar fluorophore and a long alkyl chain, it incorporates strongly inside lipid bilayers. Being bound to Lo phase, it shows significantly blue shifted emission

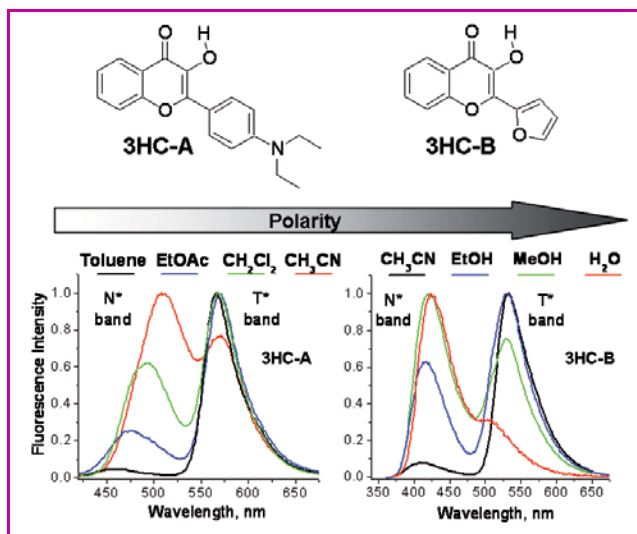


Figure 5 - Chemical structure of 3HC dyes and their solvent-dependent dual emission.

compared to the Ld phase, because the former is characterized by much lower hydration and polarity. These changes in the color were used for color imaging of Lo and Ld phases in giant lipid vesicles [13]. However, this dye has limited applications for cellular research because it absorbs in UV region and it internalizes rapidly inside the cells.

To overcome these problems, our group selected Nile Red, which is a solvatochromic dye presenting strongly red shifted absorption (~ 530 nm) and emission (~ 600 nm). Moreover, we found that Nile Red can undergo reversible redox cycle by action of sodium dithionite and air, allowing a controlled “on-off” switching of the dye. To localize the fluorophore at the lipid bilayers interface and avoid probe internalization inside the cells, we conjugated Nile Red fluorophore with an “anchor” containing zwitterionic group and long hydrophobic chain (probe NR12S, *figure 6*) [14]. By using the “on-off” switching capability of Nile Red fluorophore, we could evidence the selective binding of NR12S to the outer membrane leaflet with almost negligible flip-flop. Moreover, the emission maximum of NR12S in model vesicles exhibited a significant blue shift in Lo phase as compared to Ld phase. As a consequence, these two phases could be clearly distinguished in NR12S-stained giant vesicles by fluorescence microscopy based on two-color ratiometric imaging (*figure 7*). Being added to living

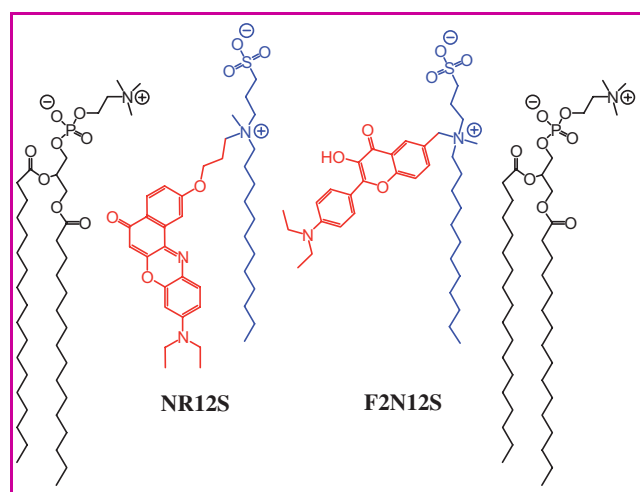


Figure 6 - Chemical structure of membrane probes NR12S and F2N12S and their approximate localization with respect to lipids (in black) in lipid bilayers. Anchor group is shown in blue, while the fluorophore is shown in red.

cells, NR12S binds selectively cell plasma membranes and its emission color correlates well with the cholesterol content (*figure 7*), which defines the content of Lo phase in the biomembranes. Remarkably, after cholesterol extraction with methyl- β -cyclodextrin, the cells appeared in different colors, which could probably reflect the different efficiency of the extraction process from cell to cell. However, we could not detect the presence of lipid rafts in intact live cells, as the color of the membranes in our ratiometric images remained rather homogenous. It was already proposed that lipid rafts are highly dynamic nanoscopic objects; therefore their visualization will probably require utilization of recently introduced fast super-resolution fluorescence techniques in combination with our probe. The attractive photophysical and switching properties of NR12S, together with its selective outer leaflet staining and sensitivity to cholesterol and lipid order make it a new powerful tool for studying model and cell membranes.

Monitoring membrane asymmetry and apoptosis

Normal cells exhibit a remarkable asymmetry of their lipid distribution between inner and outer leaflets of cell membranes, which is lost during the early steps of apoptosis (programmed cell death) [15] (*figure 8*). These changes in the plasma membrane can be used to detect apoptosis. In this respect, the most common detection method relies on the use of fluorescently labeled annexin V, which interacts with phosphatidylserine (PS) exposed on the membrane surface of apoptotic cells [16].

We made an attempt to monitor apoptosis using probe based on 3HC-A fluorophore, which is highly sensitive to membrane surface charge and phase state [17]. The sensitivity to the surface charge is important to detect exposure of the negatively charged PS during apoptosis. Moreover, the sensitivity to the lipid phase could also be important for detection of apoptosis, because the phase state can be modified by the apoptotic loss of the transmembrane asymmetry. To detect the loss of transmembrane asymmetry, we needed a probe that binds selectively the outer leaflet of the cell plasma membrane. Therefore, we have developed a fluorescent probe F2N12S bearing anchor group, similar to that of NR12S (*figure 6*). Fluorescence spectroscopy, flow cytometry and microscopy measurements showed that the ratio of the two emission bands of the probe changes dramatically in response to apoptosis (*figure 8*) [18]. This response reflects the exposure of PS lipid together with the decrease in the lipid order at the

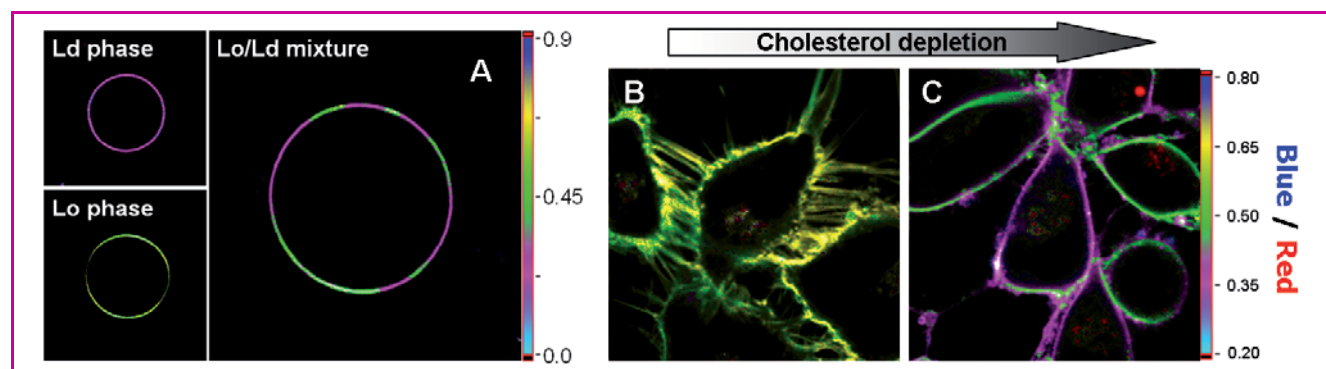


Figure 7 - Application of NR12S for fluorescence imaging of separate Lo and Ld phase domains in giant vesicles (A) and changes in the lipid order in cell membranes on cholesterol depletion by methyl- β -cyclodextrin (B and C).

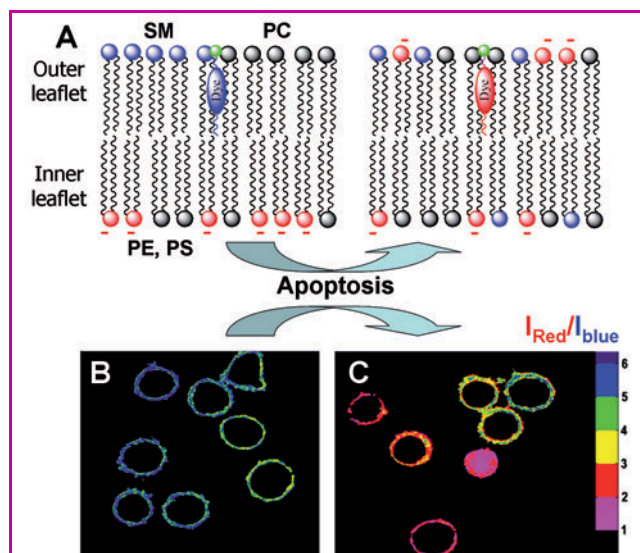


Figure 8 - Apoptosis detection using probe F2N12S: changes in membrane asymmetry on apoptosis (A) and ratiometric images of normal (B) and actinomycin D-treated (C) CEM cells stained with F2N12S. The size of the images is 60×73.1 μm . SM: sphingomyelin; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine.

outer leaflet due to apoptotic loss of the transmembrane asymmetry. Being ratiometric, the response of F2N12S can be easily quantified on an absolute scale. This allows monitoring by laser scanning confocal microscopy the degree and spatial distribution of the apoptotic changes at the cell plasma membranes [18], a feature that can be hardly achieved with the commonly used fluorescently labeled annexin V assay.

Applications for monitoring biomolecular interactions

Monitoring biomolecular interactions is a fundamental problem in biosensing with numerous applications ranging from biological research up to clinical diagnostics. The fluorescence techniques suggest several approaches. The most established one is Förster resonance energy transfer (FRET) based assay, where interacting partners are labeled with donor and acceptor molecules. Then, the interaction event results in the efficient energy transfer between the proximal donor and acceptor, providing the analytical signal. Though the approach is robust, it requires double labeling, which is complicated and cannot be realized in many screening assays. Therefore, the single fluorescence labeling techniques, where only one of the partners is labeled, are of high interest. The most well-established single-labeling approach is based on monitoring by fluorescence anisotropy changes in the mobility of the fluorescent label, grafted to one of the interacting partners. The other, which emerged recently, is the utilization of solvatochromic dyes. In this approach, the interaction between the molecules decreases the polarity of the local site of interaction, which can affect fluorescence properties (emission maximum or intensity) of the solvatochromic labels (figure 9). The representative examples are works of Imperiali *et al*, presenting applications of environment-sensitive dyes based on Prodan, 4DMP and its analogues for monitoring protein-protein interactions [6]. Below, some recent developments of our laboratory will be presented.

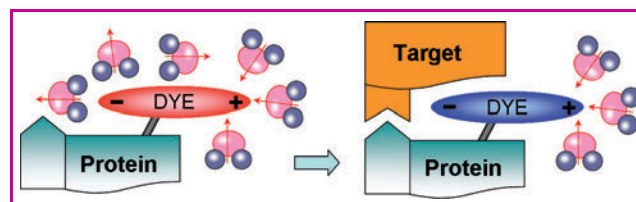


Figure 9 - Monitoring biomolecular interactions by a solvatochromic fluorescent dye.

Polycation-DNA interactions

A simple example of interaction of two (bio)molecules is binding of oligocation spermine to DNA. In this study we labeled spermine with a reactive derivative of 3HC-B, because the latter is optimal for working in highly polar environments. On binding to a double-stranded DNA (dsDNA), the ratio of the two emission bands of the 3HC conjugate changed about 16-fold, while only moderate changes were observed on binding to a single-stranded DNA (ssDNA) (figure 10) [19]. The results suggested that in the dsDNA complex, 3HC fluorophore is efficiently screened from the bulk water due to its intercalation between the bases, while in the ssDNA the screening is much less efficient. Thus, the 3HC fluorophore being conjugated to spermine discriminates the binding of the polycation to dsDNA from that to ssDNA.

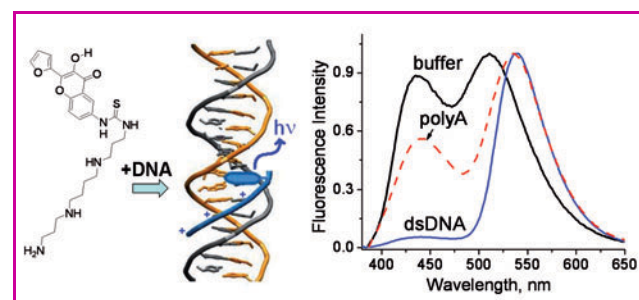


Figure 10 - Conjugate of spermine with 3HC label and its fluorescence response to binding to dsDNA (double-stranded DNA from calf thymus) and ssDNA (single-stranded DNA, poly A).

Peptide-DNA interactions

To extend the application of 3HC-B fluorophore to peptides, it was attached to the N-terminus of a nucleocapsid peptide (NCp7) from human immunodeficiency virus (HIV1) using solid state peptide synthesis (figure 11). This peptide plays an important role in HIV-1 and shows high affinity to a viral oligonucleotide (ODN) cTAR, which is a complementary sequence of the transactivation response element from HIV-1 genome. The interaction of the labeled NCp7 fragment with cTAR changed dramatically the emission color of the 3HC probe [11]. Different ODN sequences have been tested and the obtained spectroscopic data were correlated with the known NMR structure of the peptide-ODN complexes (figure 11). The results suggested that the 3HC label senses proximity of the peptide labeling site (N-terminus) to the ODN bases. This approach allowed us to determine the peptide-ODN binding parameters and distinguish multiple binding sites in ODNs, which is rather difficult using other fluorescence methods. Moreover, this method was found to be more sensitive than

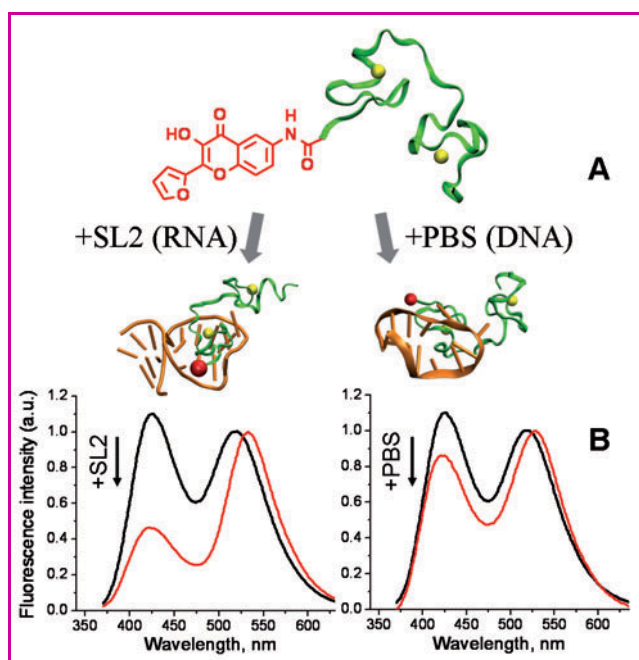


Figure 11 - A) Schematic structure of a conjugate of 3HC label with NCp7 peptide fragment and its complexes with different oligonucleotides, SL2 and PBS. B) Fluorescence spectra of the fluorescently labeled conjugate (NC-3HC) on addition of SL2 and PBS oligonucleotides.

the commonly used steady-state fluorescence anisotropy, especially in the case of small ODNs. Currently, we develop *L*-amino acids bearing 3HC fluorophore. This amino acid will be an important milestone in the probing of peptide-DNA interaction at any desired peptide site, which can be done by substituting the corresponding amino acid with the fluorescent analogue.

Protein-protein interactions

Monitoring protein-protein interaction is of key importance in the development of peptide based biosensors. To this end, an interaction between peptide labeled with SH-reactive 3HC-B dye and a target recombinant antibody fragment was studied in collaboration with Enander *et al* [20]. This protein-protein interaction changed significantly the intensity ratio of the two emission bands of the 3HC label, indicating that in the obtained complex the label is efficiently shielded from bulk water. The obtained results suggested a new methodology for development of ratiometric biosensors based on a single solvatochromic label.

Peptide-membrane interactions

Finally, we developed an approach for monitoring membrane binding and insertion of peptides using solvatochromic 3HC dye. As membrane is rather apolar medium, a reactive 3HC-A derivative was selected. It was attached to N-terminus of melittin and poly-*L*-lysine peptides, which interact with lipid membranes in a very different fashion. Binding of these peptides to lipid vesicles induced a strong fluorescence increase, which enabled to quantify the peptide-membrane interaction [21]. Moreover, the dual emission of the label in these peptides correlated well with the depth of its insertion measured by the parallax quenching method. Thus, in melittin, which shows deep

insertion of its N-terminus, the label presented a dual emission corresponding to a low polar environment, while the environment of the poly-*L*-lysine N-terminus was rather polar, in line with its surface binding. Imaging of labeled peptides bound to giant vesicles gave us some clues to orientation of the label within the membrane, which could help to estimate the peptide orientation. Thus, this label constitutes an interesting new tool for monitoring membrane binding and insertion of peptides.

Conclusions

Solvatochromic dyes, due to their ability to undergo excited-state reactions (charge and proton transfer), can change their emission color in response to variation of solvent polarity. Though a number of solvatochromic dyes has already been developed, there is a clear need in the new dyes presenting high solvatochromism together with high brightness and photostability. 3-hydroxychromones are particularly interesting example of the solvatochromic dyes due to the strong solvent-sensitivity of their dual emission generated by excited state intramolecular proton transfer. Sensitivity of the dyes to the environment polarity can be applied for probing local molecular environment in biological systems. Thus, solvatochromic molecules modified with amphiphilic anchor group can bind specifically lipid biomembranes and can monitor their lipid order and transmembrane asymmetry, which enables imaging of lipid domains and detection of apoptosis. On the other hand, solvatochromic dyes, covalently attached to peptides enable monitoring their interactions with nucleic acids, proteins and lipid membranes. Therefore, solvatochromic dyes could become a universal tool for detecting almost any kind of biomolecular interactions. However, the further success of the approach will strongly rely on the development of new improved solvatochromic fluorescent dyes.

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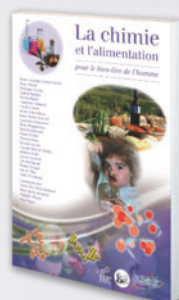
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