Some approuch to the fluorophore-tagging and barcoding of nano-scale objects

A.S.Lebed, S.L.Yefimova, A.V.Sorokin, I.A.Borovoy, Yu.V.Malyukin

Institute for Scintillation Materials, STC "Institute for Single Crystals", National Academy of Sciences of Ukraine, 60 Lenin Ave., 61001 Kharkiv, Ukraine

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Some approaches to the fluorophore-tagging of nano-scale objects using fluorescence resonance energy transfer (FRET) between several dyes encapsulated in nanoparticles have been reported. Sodium dodecyl sulfate micelles have been used as a model system of nanoparticles. Using tandem dyes with different FRET efficiency, fluorophore-tagging with FRET-mediated emission shifted up to 8000 cm^{-1} or tags with multiple colors under one single wavelength excitation can be created. The requirements to tandem dyes for FRET applications have been analyzed. It has been revealed that to obtain the effective FRET in nano-scale volume, such properties of the dyes as hydrophobicity, topology and electronic density distribution should also be taken into consideration.

Представлены некоторые подходы к флуоресцентному мечению наноразмерных объектов, основанные на использовании явления безызлучательного переноса энергии электронного возбуждения (FRET) между несколькими флуорофорами, помещенными в наночастицу. В качестве модельной системы наночастиц использовались мицеллы додецилсульфата натрия. Показано, что используя тандемные флуорофоры с разной эффективностью FRET можно создавать флуоресцентные метки с аномально большим сдвигом излучения по отношению к поглощению (до 8000 $см^{-1}$) или получать метки, которые будут иметь несколько полос люминесценции при одном возбуждении. Проанализированы требования к выбору тандемных флуорофоров. Показано, что при создании композиций в нанообъеме с эффективным FRET, кроме основных требований, предъявляемых к тандемным флуорофорам, необходимо учитывать такие свойства молекул, как гидрофобность, топология и распределение электронной плотности.

1. Introduction

Fluorescence techniques are widely used research tools in chemistry, physics, biology and medical diagnostics [1, 2]. The methods are very sensitive and enable studying the excited state of molecules, photochemical reactions, dynamics of fast molecular processes, structure and properties of complex chemical and biological objects [1, 2]. Fluorescent labels represent one of the most widely developing fields in biology and medicine with enormous applications [3, 4]. Traditionally, the family of fluorescent labels includes organic dyes, fluorescent proteins, and lanthanide chelates, which are still popular due to such advantages as existence of standard protocols for their usage, relative cheapness and availability [3, 4]. Recently, significant advances have led to a large variety of labeling reagents based on nanomaterials, such as quantum dots, magnetic nanoparticles, lanthanide doped-compounds, fluorophore-tagged latex/silica nanoparticles, fluorophore-labeled polymeric nanospheres etc. [3, 5, 6]. Strong interest in such objects is dictated also by the fact that nanoparticles have the potential to revolutionize the diagnosis and treatment of

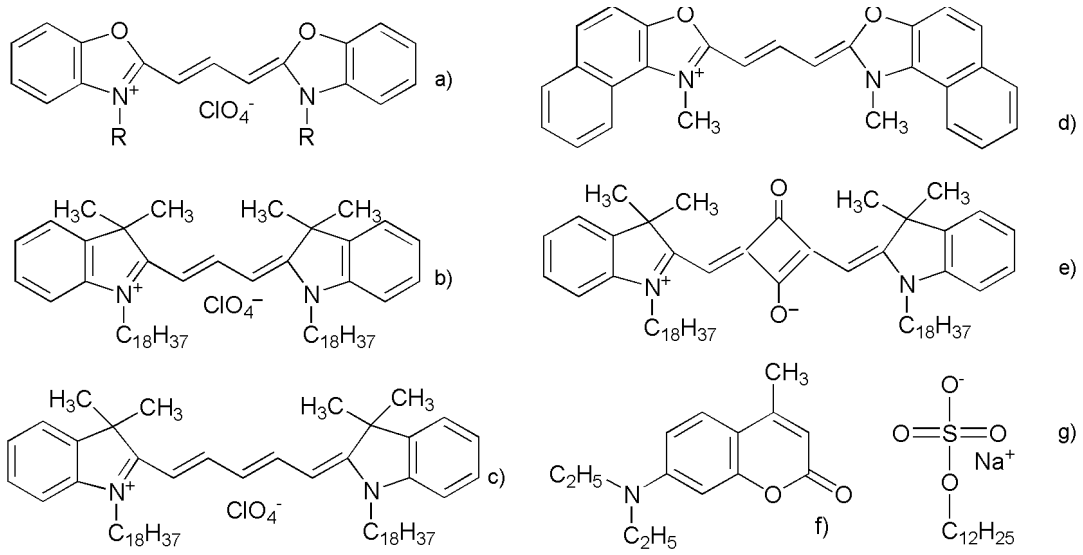

Fig. 1. Structural formulas of studied compounds: (a) $DiOC_n$ dyes. R = C_2H_5: $DiOC_2$, R = $C_{18}H_{37}$:$DiOC_{18}$; (b) DiI; (c) DiD; (d) JC-9; (e) Sq-18; (f) Coumarine; (g) SDS.

many diseases, for example cancer and infectious diseases [6]. However, nanoparticle application in biological research and medical diagnostics requires visualization of their localization in bioobjects (cells, tissue, etc.) that can be provided with fluorophore-tagging.

In this paper, we report some approach to the fluorophore-tagging of nano-scale objects. To avoid such essential limitation of conventional organic dyes as a short Stokes shift, we apply fluorescence resonance energy transfer (FRET) phenomenon between several dyes incorporated in a nano-scale volume of a surfactant micelle used as simple nanoparticles. The requirements to tandem dyes for FRET applications are analyzed.

2. Experimental section

Materials. The carbocyanine dyes 3,3′-diethyloxacarbocyanine perchlorate ($DiOC_2$), 3,3′-dioctadecyloxacarbocyanine perchlorate ($DiOC_{18}$), benzopyran derivative 1-benzopyran-2-one (Coumarine) and bis(1-octadecyl-3,3- dimethylindolin-2-ylidene) squaraine (Sq-18) were obtained from the dye collection of Dr.I.Borovoy (Institute for Scintillation Materials NAS of Ukraine) (Fig. 1). The purity of the dyes was controlled by thin layer chromatography. The carbocyanine dyes 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), 1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindodicarbocyanine perchlorate (DiD) and 3,3′-dimethyl-α-naphthoxacarbocyanine iodide (JC-9) were purchased from *Invitrogen* and used without purification. Surfactant sodium dodecyl sulfate (SDS) were purchased from *Sigma-Aldrich* and used without purification. Chloroform and ethanol (*Sigma-Aldrich*) used to prepare stock solutions of the dyes was a spectroscopic grade product. To prepare aqueous solution of the dyes with surfactant, doubly distilled water was used.

Solution Preparation. The concentration of the surfactant in the solutions was $1 \cdot 10^{-2}$ M and was kept constant for all experiments. The concentration of the dyes in the water-micellar solutions were varied within the $1 \cdot 10^{-7}$–$5 \cdot 10^{-4}$ M range. First, stock solutions of each dye in chloroform of $1 \cdot 10^{-3}$ and $1 \cdot 10^{-4}$ M concentrations were prepared (stock solutions of JC-9 were prepared in ethanol). To prepare solutions for measurements 3 mg of SDS were mixed in a flask with the required amount of the dye stock solution. After chloroform (ethanol) evaporation the required amount of doubly distilled water was added. The solutions were heated to 80°C to ensure the uniform distribution of the components and then cooled to room temperature.

Spectroscopic Measurements. Visible absorption spectra were recorded using microspectrometer USB4000 (Ocean Optics, USA) supplied with an incandescent lamp. Solutions were placed in a quartz cuvette of 2 mm optical path length. Luminescence spectra were taken with a spectrofluorimeter on the base of two grating monochroma-

tors MDR-23 and a xenon lamp. One of the monochromators was used to select a required wavelength (FWHM ~ 0.5 nm), the other one was used for the luminescence collection.

3. Results and Discussion

All the dyes used in the experiment (Fig. 1) possess the significant hydrophobisity (except of $DiOC_2$ with short alkyl tails) and are poorly soluble in water. However, each of these dyes demonstrates a tendency to be incorporated into the SDS micelles in water-micellar solutions. In our previous works the interaction of carbocyanine dyes $DiOC_n$, DiI and DiD with the SDS micelles were studied [7–10]. The abrupt changes in both absorption and luminescent spectra of the dyes (in particular, a significant intensity growth and a maxima red shift) were observed, when the SDS micelles are formed in aqueous solutions containing the dyes [7–10]. The same results are observed for the other dyes used: Coumarine, JC-9 and Sq-18 (data not presented).

To obtain FRET compositions in a nano-scale volume of surfactant micelles, one should incorporate at least two different dye molecules (electronic excitation energy donor and acceptor, respectively) into the micelle [1]. Thus, important questions are (i) how many dye molecules could be solubilized in one SDS micelle? and (ii) what are the requirements to the dyes to be incorporated into nano-scale volume?

We can estimate the approximate number of dye molecules (n) incorporated into a single micelle using the relation [11]:

$$n = \frac{C_d}{C_{mic}}, \qquad (1)$$

where C_d is the dye concentration in the solution, C_{mic} is the SDS micelle concentration that can be calculated as [11]

$$C_{mic} = \frac{C_{SDC} - CMC}{N_{agg}}, \qquad (2)$$

were C_{SDS} is the surfactant concentration ($1 \cdot 10^{-2}$ M), CMC is the critical micelle concentration (CMC of the SDS in the presence of the dyes is $8 \cdot 10^{-3}$ M [12]), N_{agg} is the surfactant aggregation number ($N_{agg} = 64$ for SDS micelles [13]).

So, in the studied solutions, the SDS micelle concentration $C_{mic} = 3 \cdot 10^{-5}$ M.

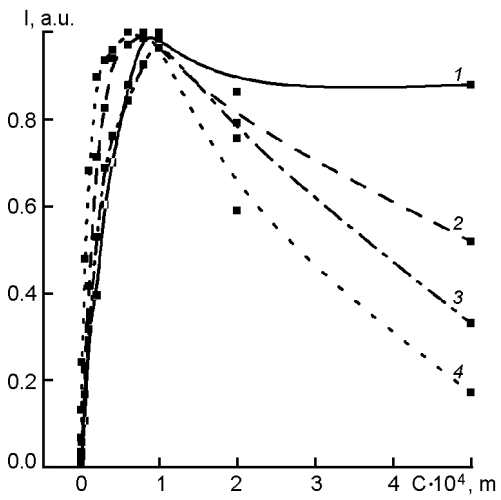

Fig. 2. $DiOC_{18}$ (*1*), $DiOC_2$ (*2*), DiI (*3*) and DiD (*4*) luminescence intensity change as a function of the dye concentration in a water-micellar solution. [SDS] = $1 \cdot 10^{-2}$ M; [Dyes] varies from $1 \cdot 10^{-7}$ M to $5 \cdot 10^{-4}$ M.

To answer the first question and find n_{max}, the changes in luminescence intensities of several dyes in water-micellar solutions were analyzed depending on a dye concentration (Fig. 2). For all dyes investigated, the changes are revealed to be of the same character. Within the $1 \cdot 10^{-7}$–$3 \cdot 10^{-5}$ M concentration range, the dye luminescence intensity increases gradually (Fig. 2). In this concentration range, all dye molecules are solubilized into the SDS micelles. As follows from Eq. 1, one SDS micelle solubilizes no more than one dye molecule. In the $3 \cdot 10^{-5}$–$1 \cdot 10^{-4}$ M concentration range, the concentration growth causes only a slight increase in the luminescence intensity (Fig. 2). According to Eq.1, at these concentrations several dye molecules can be incorporated into one SDS micelle. When the dye concentration is more than $1 \cdot 10^{-4}$ M, the luminescence concentration quenching is observed that is accompanied with the red shift of the luminescence maxima [1, 14]. So, the dye concentration of $1 \cdot 10^{-4}$ M is considered to be the critical amount of the dye that can be solubilized by the micelles at a given amount of the SDS. At this concentration, according to Eq.1 $n_{max} = 4$. In other words, the "solubilizing capacity" of the SDS micelle is equal to 4 dye molecules.

To observe FRET between pair of dyes (donor and acceptor) they must meet some primary requirements [1]:

(i) donor and acceptor must be in close proximity (<10 nm);

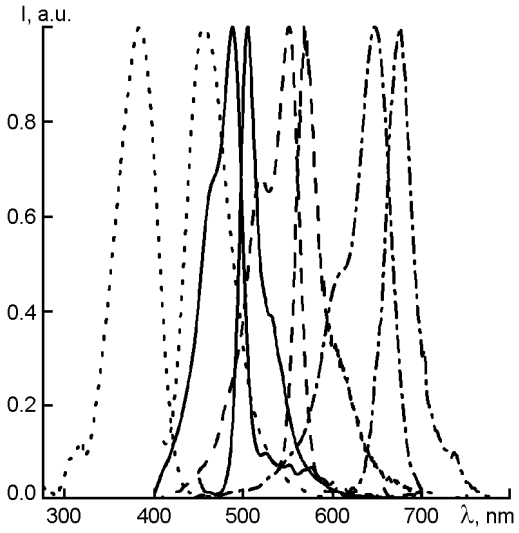

Fig. 3. Overlap of the absorption and emission spectra of the dyes used in the FRET compositions: Coumarine (dash-dotted lines), DiO (strait lines), DiI (dotted lines) and DiD (dashed lines).

(ii) absorption spectrum of the acceptor must overlap fluorescence emission spectrum of the donor;

(iii) donor and acceptor transition dipole orientations must be in a favorable mutual orientation (for optimal energy transfer);

(iv) sufficient quantum yield of both the donor and the acceptor.

Carbocyanine dyes $DiOC_{18}$ and DiI (Fig. 1a,b) seem to be a perspective tandem dyes for FRET in SDS micelles [4]. The overlap of donor ($DiOC_{18}$) luminescence spectrum and acceptor (DiI) absorption spectrum is quite effective (Fig. 3). As the SDS micelle diameter is about 5 nm [15, 16], the forced concentration of the dyes in the micelle nano-volume provides the necessary distance between the molecules that is one of the primary condition for FRET [1].

The luminescence was excited at 440 nm (short-wavelength edge of the $DiOC_{18}$ absorption band). At this wavelength DiI luminescence is practically not excited. In all the measurements donor concentration was $3 \cdot 10^{-5}$ M (equal to the micelles concentration). Concentration of the acceptor was varied in a wide range ($1 \cdot 10^{-7}$–$1 \cdot 10^{-4}$ M). The most effective energy transfer between hydrophobic dyes $DiOC_{18}$ and DiI have been observed when the acceptor and donor concentrations were equal (Fig. 4, curve *1*). In this case each SDS micelle solubilizes one donor and one acceptor molecule. This composition gives a "Stokes shift" about

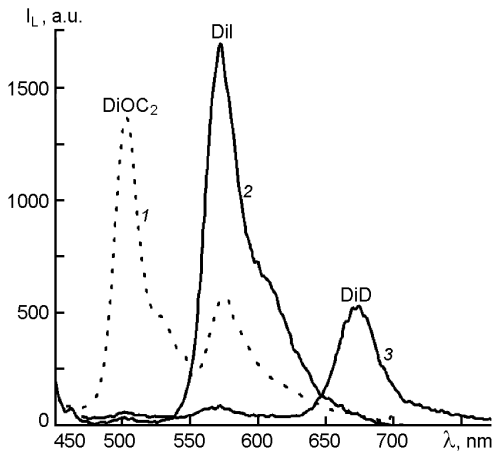

Fig. 4. Luminescence spectra of water-micellar solutions of: *1* – $DiOC_{18}$/DiI mix composition; *2* – $DiOC_2$/DiI composition; *3* – $DiOC_{18}$/DiI/DiD composition. [SDS] = $1 \cdot 10^{-2}$ M, [Dyes] = $3 \cdot 10^{-5}$ M; λ_{exc} = 400 nm.

5250 cm^{-1} (Fig. 4, curve *1*). The FRET efficiency (E) calculated as [1]:

$$E = 1 - \frac{I_{DA}}{I_D}, \qquad (3)$$

where I_{DA} and I_D are the donor luminescence intensities in the presence and the absence of the acceptor, respectively, is 93 %.

It was found that the dye hydrophobicity plays an important role in FRET efficiency within a micelle nano-scale volume. When we try to use $DiOC_2$ dye as an excitation energy donor, we did not obtain the effective energy transfer from $DiOC_2$ to DiI dye: the donor luminescence is half quenched, whereas the acceptor luminescence is not intensive, as compared to the case of the $DiOC_{18}$/DiI pair (Fig. 4, curve *2*). The FRET efficiency calculated for this pair using Eq.3 is 63 %. Let us note that $DiOC_2$ differs from $DiOC_{18}$ only in the length of the hydrocarbon radicals, i.e. hydrophobicity (Fig. 1a). The dyes have the same luminescence spectra profile (Fig. 3), so the overlap integral for $DiOC_{18}$/DiI and $DiOC_2$/DiI pairs are the same. In our previous work we analyzed the effect of dye $DiOC_n$ hydrophobicity on the dye-to-micelle binding efficiency [10]. It was revealed that hydrophobic dye $DiOC_{18}$ exhibits 12 times higher dye-to-micelle binding ability than the less hydrophobic dye $DiOC_2$ [10]. In $DiOC_n$ molecules hydrocarbon tails act as "anchors" holding the dyes in SDS micelles in such a way that the hydrocarbon tails anchor in the micelle hydrocarbon core, while the chromophore

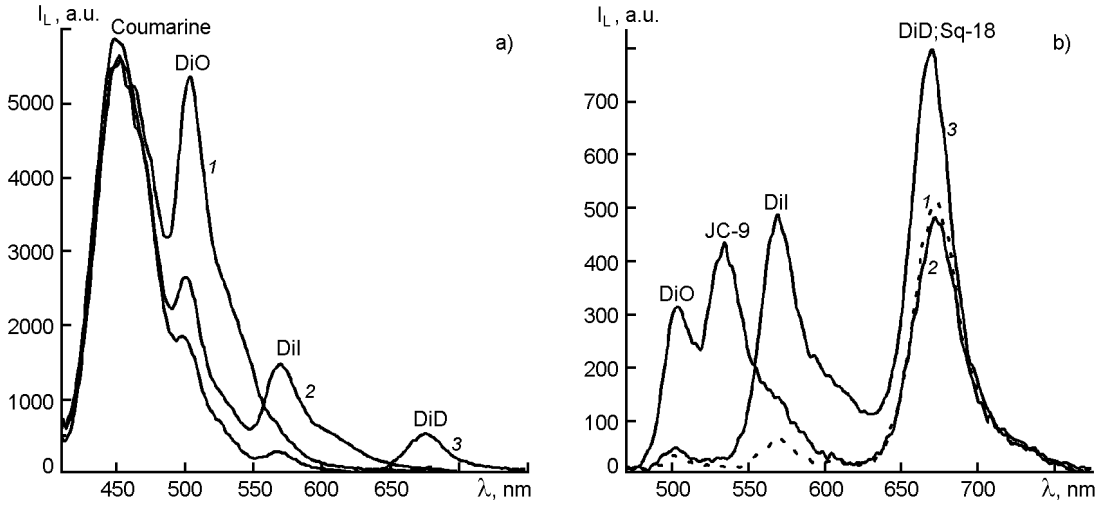

Fig. 5. Influence of the dye topology and electronic density distribution on the FRET efficiency of FRET compositions in SDS micelles. [SDS] = $1 \cdot 10^{-2}$ M, [Dyes] = $3 \cdot 10^{-5}$ M. a) Composition with Coumarine: *1* — Coumarine/$DiOC_{18}$; *2* — Coumarine/$DiOC_{18}$/DiI; *3* — Coumarine/$DiOC_{18}$/DiI/DiD. λ_{exc} = 400 nm. b) Compositions with JC-9 and Sq-18: *1* — $DiOC_{18}$/DiI/DiD; *2* — $DiOC_{18}$/JC-9/DiD; *3* — $DiOC_{18}$/DiI/Sq-18. λ_{exc} = 440 nm.

group is localized in the micelle headgroup region [4]. The shorter the tail, the weaker the hydrophobic forces holding the dye within the micelles, and the electrostatic repulsion between the positively charged donor and acceptor dye molecules can result in increasing the distance between the dyes that is principal for FRET [1]. However, the absence of a shift of the absorption and luminescence maxima indicates that the dye molecule is localized in the micellar region (Fig. 4, curve *2*). Thus, dye hydrophobicity plays important role in the effectiveness of the FRET in surfactant micelles and should be taken into consideration.

To increase a "Stokes shift", we have created two-stage cascade FRET composition between three dyes solubilized within the SDS micelle. For this purpose, the second excitation energy acceptor DiD has been selected (Fig. 1c). This dye also belongs to the carbocyanine family and possesses the same hydrophobicity as $DiOC_{18}$ and DiI dyes [4]. Fig. 4, curve *3* represents the luminescence spectrum of the $DiOC_{18}$/DiI/DiD composition. In this composition, at the fist stage the excitation energy (440 nm) is transferred from the donor $DiOC_{18}$ to the acceptor/donor DiI and then to the second acceptor DiD. As a result, almost complete quenching of the $DiOC_{18}$ and DiI luminescence and sensitizing of DiD luminescence are observed (Fig. 4, curve *3*). This composition gives the "Stokes shift" about 8000 cm^{-1} with the FRET efficiency of 98 % calculated for the first donor $DiOC_{18}$.

We tried to create three-stage cascade FRET composition using four dyes molecules solubilized in nano-scale volume. The dye from the coumarine family (Fig. 1f) was used as an energy donor for $DiOC_{18}$ in the FRET composition: Coumarine/$DiOC_{18}$/DiI/DiD. The coumarine dye meets all the requirements for FRET (high quantum yields, effective spectral overlap with acceptor $DiOC_{18}$ (Fig. 3) and high hydrophobicity). The luminescence of this solution was excited at λ_{exc} = 400 nm. At this excitation, the luminescence of three other dyes ($DiOC_{18}$, DiI and DiD) is negligible. In this composition, fluorescence resonance energy transfer and, as a result, sensitized DiD luminescence are observed (Fig. 5a, curve *3*). However, we did not achieve the complete quenching of the donor (coumarine) luminescence. In this composition the FRET efficiency in the first stage is 48 %. Such feature is also observed in Coumarine/$DiOC_{18}$ and Coumarine/$DiOC_{18}$/DiI compositions (Fig. 5a) and therefore can not be associated with the dye steric effects within the micelle nano-volume. The reasonable explanation of this observation seems to be the difference in molecular topology of coumarine and other dyes. Such a difference can lead to the different relative orientation of the dye chromophores in the micelles. According to the Ferster theory of resonance energy transfer, the relative orientation of interacting dipoles has a consid-

erable influence on the FRET efficiency [1]. Moreover, the difference in topology can cause a difference in the electronic density distribution within a molecule that also affects the FRET efficiency.

To confirm these assumptions, the following compositions were created and compared with the $DiOC_{18}$/DiI/DiD one:

1) the $DiOC_{18}$/JC-9/DiD composition. The JC-9 molecular structure is similar to the $DiOC_n$ dyes, but JC-9 is characterized by more extended π-electron system at the expense of two additional benzene rings (Fig. 1a, d). Absorption and emission spectra of JC-9 reveal an effective overlap with luminescence spectrum of $DiOC_{18}$ and absorption spectrum of DiD, respectively (data not presented). So, JC-1 can be used as energy acceptor/donor in two-stage cascade FRET composition.

2) The $DiOC_{18}$/DiI/Sq-18 composition. Squaraine dye Sq-18 (Fig. 1e) is a π-electron analog of DiD with a central aromatic four membered ring system derived from squaric acid. The central aromatic ring imparts additional rigidity to the molecule. This fragment is also highly electron deficient that is why squaraine molecules are characterized by a donor-acceptor-donor structure and high hydrophobicity [17–19]. Sq-18 reveals the good spectral overlap with DiI and is suitable for FRET composition creation.

Luminescence spectra of the two compositions are presented in Fig. 5b (λ_{exc} = 440 nm). In both cases, two-stage cascade FRET and sensitized DiD luminescence are observed. However, in both cases, FRET is not such effective as compared with the $DiOC_{18}$/DiI/DiD composition (Fig. 4, curve *4*). In the $DiOC_{18}$/DiI/Sq-18 composition the donor luminescence is completely quenched, whereas the first acceptor luminescence is rather intensive. In the $DiOC_{18}$/JC-9/DiD composition, there is no complete quenching neither donor nor first acceptor luminescence (Fig. 5b). Thus, we can conclude that the effectiveness of the FRET in cascade compositions depends also on tandem dye topology and electronic density distribution. Using different combination of dyes with different FRET efficiency, barcode tags can be produced for multiplexed targeted FRET under single wavelength excitation.

Conclusions

FRET has been demonstrated to be the tool for fluorophore-tagging of nano-scale particles. Sodium dodecyl sulfate (SDS) micelles have been used as a model system of nanoparticles. It has been shown that to create FRET composition in nano-scale volume, tandem dyes must meet not only primary FRET requirements but should have similar molecular topology and electronic density distribution. Using carefully selected dye molecules with extra-high FRET efficiency, the tags emission spectrum can be turned so that only the longest-wavelength dye will exhibit significant fluorescence at a short-wavelength excitation. This feature will overcome the challenge of small Stokes shift of many organic dyes. In our case, the "Stokes shift" of about 8000 cm^{-1} has been achieved. On the other hand, using dyes with different FRET efficiency, barcode tags can be produced for multiplexed targeted FRET under single wavelength excitation.

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Деякі підходи до флуоресцентного мічення та кодування нанорозмірних об'єктів

А.С.Лєбєдь, С.Л.Єфімова, О.В.Сорокін, І.А.Боровой, Ю.В.Малюкін

Наведено деякі підходи до флуоресцентного мічення нанорозмірних об'єктів, що засновані на застосуванні явища безвипромінювального перенесення енергії електронного збудження (FRET) між декількома флуорофорами, інкорпорованими у наночастинку. У якості моделі наночастинок застосовано міцели додецилсульфату натрію. Показано, що, застосовуючи тандемні флуорофори з різною ефективністю FRET, можна створювати флуоресцентні мітки з аномально великим зсувом люмінесценції відносно до поглинання (8000 $см^{-1}$), або отримувати мітки, що містять декілька смуг люмінесценції при одному збудженні. Проаналізовано вимоги до вибору тандемних флуорофорів. Показано, що при створенні композицій у нанооб'ємі з ефективним FRET, крім головних вимог до тандемних флуорофорів, слід враховувати також такі властивості молекул, як гідрофобність, топологія та розподіл електронної густини.