Fluorescent properties of cyanine dyes as a matter of the environment

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Abstract

In non-viscous aqueous solutions, the cyanine fluorescent dyes Cy3 and Cy5 have rather low fluorescence efficiency and short excited state lifetimes due to their structural features. In this work, we investigated the effect of solubility and rotational degrees of freedom on the fluorescence efficiency of Cy3 and Cy5 in several ways. We compared the fluorescence efficiencies of two cyanine dyes sCy3 and sCy5 with the introduction of a sulfonyl substituent in the aromatic ring as well as covalently bound to T10 oligonucleotides. The results show that because of the different lengths of the polymethine chains between the aromatic rings of the dyes, *cis-trans*-isomerization has a much greater effect on the Cy3 molecule than on the Cy5 molecule, while the effect of aggregation is also significant.

Introduction

For many years, cyanine dyes have been used as fluorescent tags for biomolecules in scientific studies such as studies of protein-nucleic acid interactions [1, 2] and nucleic acid structure studies [3, 4]. Förster resonance energy transfer (FRET) on cyanine dyes is deeply studied and widely used [5, 6] as one of a few available tools to measure distances at the nanoscale, including studying the structure and dynamics of biopolymers [7]. The application of FRET is based on the assumption that the fluorescence properties of the probe do not depend on the properties of the medium, but in reality photophysical properties of the most fluorophores depend to some extent on the environment. This can lead to incorrect results if the medium in which the fluorophore is located changes significantly during the experiment.

In this work, we investigated photophysical properties of indocyanine dyes and their derivatives known under the trade names Cy3 and Cy5. Their structures are shown in Fig. 1. Cyanine dyes are characterized by not high quantum yield (0.06–0.4) and a wide range of excited state lifetimes (from several ps to tens of ns) [8]. The main reason for the low quantum yield is the ultrafast radiation-free deactivation of the excited state of the dye molecule, which is explained by the photoinduced *cis-trans*-isomerization of the C-C bond in the polymethine chain of the cyanine dye [9, 10], with the *cis*-isomers giving negligible fluorescence at room temperature (Fig. 2) [11, 12]. Thus, the decrease in the total quantum yield of cyanine dye is due to *cis-trans*-isomerization, which increases as temperature rises, and decreasing medium viscosity [13]. An increase in the quantum yield of fluorescence can be provided by an increase in the conjugated bond stiffness and an increase in the solvent viscosity [14].

In past studies of cyanine dyes, Sanborn et al. showed that the fluorescence efficiency of the sulfated cyanine sCy3 after attachment to the DNA chain enhances significantly due to an increase in isomerization activation energy [15]. The correlation between isomerization activation energy and chain length in the molecule was demonstrated in a study by Akesson, E. et al., who observed that upon elongation of the alkyl tail attached to the imine nitrogen atom from 2 to 14 carbon atoms, the isomerization barrier in ethanol increased by 3 kJ/mol [16]. Therefore, Cy3 undergoes photoisomerization more easily from the first excited state than Cy5, which has a longer polymethine chain [13]. One would

expect that Cy3 is more sensitive than Cy5 to environmental factors as well as to the structure of the linker. This is especially important in the case of cyanine dyes acting as FRET donors or acceptors.

There is a number of scattered data documenting changes in the fluorescence properties of Cy3 and Cy5 upon covalent binding to biopolymers. Kretschy et al. studied the sequence dependence of Cy3 and Cy5 fluorescence in double-stranded DNA [17]. Pace et al. described the cyanine structure fixation with adenine and thymine to increase fluorescence [18]. Stennett et al. investigated Cy3-DNA constructs in which both Cy3 nitrogen atoms are attached to the DNA backbone via short linkers, but the dye molecule in this structure remains capable of photoisomerization [19].

An effective way to increase the quantum yield of fluorescence by preventing photoisomerization is the complete fixation of the Cy3 polymethinyl chain, such as in Cy3B (Fig. 3) [20, 21]. Cy3B has been shown to provide the most efficient fluorescence yield at relatively low labeling ratios, and its fluorescence lifetime is 2.9 ns, making it very valuable for research and applications [20].

Inspired and motivated by these literature data, we investigated photophysical properties of Cy3 and Cy5 in an attempt to explain the properties of cyanine dyes in solution in terms of solubility and *cis-trans*-isomerization. We assumed that due to the shorter length of the polymethine chain of Cy3, the effect of the environment on the fluorescence of Cy3 would be much greater than that of Cy5. Because of the poor solubility of the cyanine dyes themselves in water, they are extremely prone to forming aggregates in aqueous solutions. The aggregates appear to be self-quenching due to the proximity of dye molecules. Therefore, the fluorescence efficiency of the cyanine dyes can be increased to some extent by improving their solubility. Another important point is the photoisomerization induced fluorescence decrease. We observed that the cyanine dye covalently bound to oligonucleotide T10 had the highest fluorescence efficiency, and the increase in fluorescence after covalent binding was more significant for Cy3 compared to Cy5, which is consistent with our original assumption.

Experiment

The dyes used Cy3-alkine, Cy5-alkine, sCy3-NHS, sCy5-NHS and labeled oligonucleotides T_{10}-Cy3, T_{10}-Cy5 were purchased from Primetech ALC (Minsk, Belarus).

Preparation of PEG azide derivative: To a solution of 0.2 g MeO-PEG(2000)-OH in 1 mL of dichloromethane at room temperature, 0.2 mL of chlorosulfonyl isocyanate (Cl-SO_2-NCO) was added. The mixture was stirred for 20 minutes, then 2 mL of diethyl ether was added, and the PEG derivative precipitated out of the solution. The precipitate was centrifuged and washed in portions of 2 mL diethyl ether three times. The precipitate was evacuated to remove the solvent traces, then 1.0 mL of aminopropyl azide was added to the precipitate, and the mixture was incubated for 2 hours. The excess of the reagent was removed by washing with diethyl ether three times with 2 mL each, and the product was dried in vacuum. 0.21 g of the product was obtained as a white powder. Synthesis results were verified using a Thermo Scientific LTQ XL mass spectrometer.

Synthesis of polyethylene glycol (PEG) azide and the following conjugation with alkyne cyanine dyes was performed as shown in Fig. 4. The conjugates were purified by gel-exclusion chromatography using Sephadex G-25, and purity was confirmed by thin-layer chromatography with 9:1 mixture of dichloromethane and methanol.

Photophysical experiments with fluorescent substances were performed in a phosphate-saline buffer at pH 7.4 at 20°C. Absorption spectra were recorded on a Shimadzu UV 3600 Plus spectrophotometer; steady-state fluorescence spectra were recorded on a Horiba Scientific Fluorolog 3 spectrometer; fluorescence spectra were recorded when excited by continuous xenon lamp radiation (exc = 513 nm for Cy3, exc = 607 nm for Cy5) in a quartz cuvette (5x10 mm). The ratio of the fluorescence quantum yields η of the dyes was calculated by the formula:

$$\eta = \frac{I}{I_{ref}} \cdot \frac{D_{ref}}{D} \cdot \frac{n^2}{n_{ref}^2},$$

where I and I_{ref} are integral fluorescence intensities, D and D_{ref} are optical densities, and n and n_{ref} are refractive indices of the sample solvent and comparison standard, respectively.

Glycerol, the surfactant Tween-20 and raw materials used for the synthesis of PEG-azide were purchased from Merck, and solutions were prepared using Milli-Q water.

Results And Discussion

In the absorption spectra of the Cy3 derivatives (Fig. 5) we observed that the spectral absorption maximum of Cy3 was located at 543 nm, while the spectral absorption maxima of sCy3 and T10-Cy3 were shifted to a lower frequency region by 5 nm (548 nm). The same phenomenon was observed in the study of Cy5 derivatives, where the spectral absorption maximum of Cy5 was located at 641 nm and the spectral absorption maximum of sCy5 and T10-Cy5 was at 647 nm, that is shifted to the region of lower frequencies by 6 nm.

We compared the fluorescence properties of the cyanine dyes covalently bound to oligonucleotide T10 and sulfonated cyanine dyes with those of unmodified dyes (Fig. 5). The results met our expectations, with the fluorescence efficiency of the cyanine dyes covalently bound to oligonucleotide T10 being the greatest, followed by sulfonated cyanines. Specific η/η_{ref} results are shown in Table 1.

As can be seen, the improvement in the fluorescence efficiency of sCy dyes compared to Cy analogs is not as significant as the improvement after covalent binding of the dye molecule to the oligonucleotide. In the latter case, the fluorescence efficiency of T10-Cy3 is 9.2 times higher than that of Cy3, and the fluorescence efficiency of T10-Cy5 is 4.7 times higher than that of Cy5.

Table 1
Ratio of the fluorescence quantum yield (η) of cyanine dyes in conjugates with T10 and sulfonated cyanines to η_{ref} of unmodified dyes.

Conjugate or derivative	Dye	η/η_{ref} (×)
T10-Cy3	Cy3	9.23
sCy3	Cy3	2.67
T10-Cy5	Cy5	4.73
sCy5	Cy5	3.13

The extremely low solubility of the cyanine dyes in aqueous solutions impairs their fluorescence intensity. To compare the impact of solubility effects on Cy3 and Cy5 dyes, we used the surfactant Tween-20 as a solubilizing agent to study the spectroscopic properties of Cy3 and Cy5. We compared the spectral properties of Cy3 and Cy5 in PBS buffer with 0% and 0.25% Tween-20 (it was experimentally confirmed that Tween-20 at this concentration does not affect the solution viscosity). The result is shown in Fig. 6, the maximum of the Cy3 absorption spectrum in the 0.25% Tween-20 solution was located at 549 nm, shifted by 6 nm to a lower frequency region, while the maximum of the Cy5 absorption spectrum was located at 651 nm, shifted by 10 nm to the lower frequency region. It was found that in the 0.25% Tween-20 solution the fluorescence efficiency of Cy3 increased 5.2-fold and that of Cy5 increased 3.2-fold.

Another factor affecting the fluorescence efficiency of the cyanine dyes is the *cis-trans* isomerization of the dye molecule. The activation energy of isomerization of the Cy5 molecule is initially higher than that of the Cy3 molecule because the polymethine chain in Cy5 is longer. That's why we expected the environment to have a greater effect on the isomerization of the Cy3 molecule compared to Cy5. We measured the quantum yield ratio of Cy3 and Cy5 at room temperature in glycerol solution at mass concentration from 10–70%, whose viscosity and refractive index were taken from [23, 24], and found that the fluorescence efficiency improved as the solution viscosity increased. In addition, we observed that the increase in fluorescence efficiency was greater for Cy3 than for Cy5. This confirmed our preliminary assumption (Fig. 7).

We assumed that the reason for the high fluorescence efficiency of the cyanine dye covalently bound to T10 was the more notable deceleration of the intramolecular rotation, which results in the lower degree of photoisomerization along with the good solubility of the conjugate. To prove this, we bound the dye molecules to PEG using the copper-free azide-alkyne cycloaddition reaction (Fig. 4) and measured their relative quantum yield of fluorescence. As might be expected, the quantum yields increased markedly, with Cy3-PEG fluorescing 2.5-fold stronger than Cy3 and Cy5-PEG fluorescing 1.8-fold stronger than its unmodified counterpart (Fig. 8).

The experiments of Sanborn et al. showed that sCy3 fluoresces 2.4-fold stronger in single-stranded DNA than in double-stranded DNA [15]. In our study of the fluorescence intensity of the cyanine-PEG conjugates, there was not very strong increase in fluorescence. This suggests that the cyanine dye forms a more stable structure when combined with single-stranded DNA compared with other macromolecules.

Conclusion

In this work, we investigated the effect of solubility and *cis-trans*-isomerization of the cyanine molecule itself on the fluorescence efficiency and showed that the photophysical properties of Cy3 and Cy5 strongly depend on the environment and the specific position in the system. When the dyes are conjugated, their properties differ dramatically from those of the free dyes. These differences require great care when using Cy3 and Cy5 and their derivatives as donor-acceptors for FRET, and it is recommended to study and design in advance for different systems to avoid large errors.

Declarations

Ethical Approval

Not required for this research since it does not involve any animal or biological experiments.

Competing interests

The authors declare that they have no conflict of interests.

Authors' contributions

I.L.L., T.P.S. synthesized the compounds.

F.F., V.A.P. performed all spectrophotometric and spectrofluorimetric measurements and calculations, wrote the main manuscript text and prepared figures.

V.V.S., O.L.S., I.O.M. designed the study, analysed data, and edited the main manuscript text.

All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

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Figures

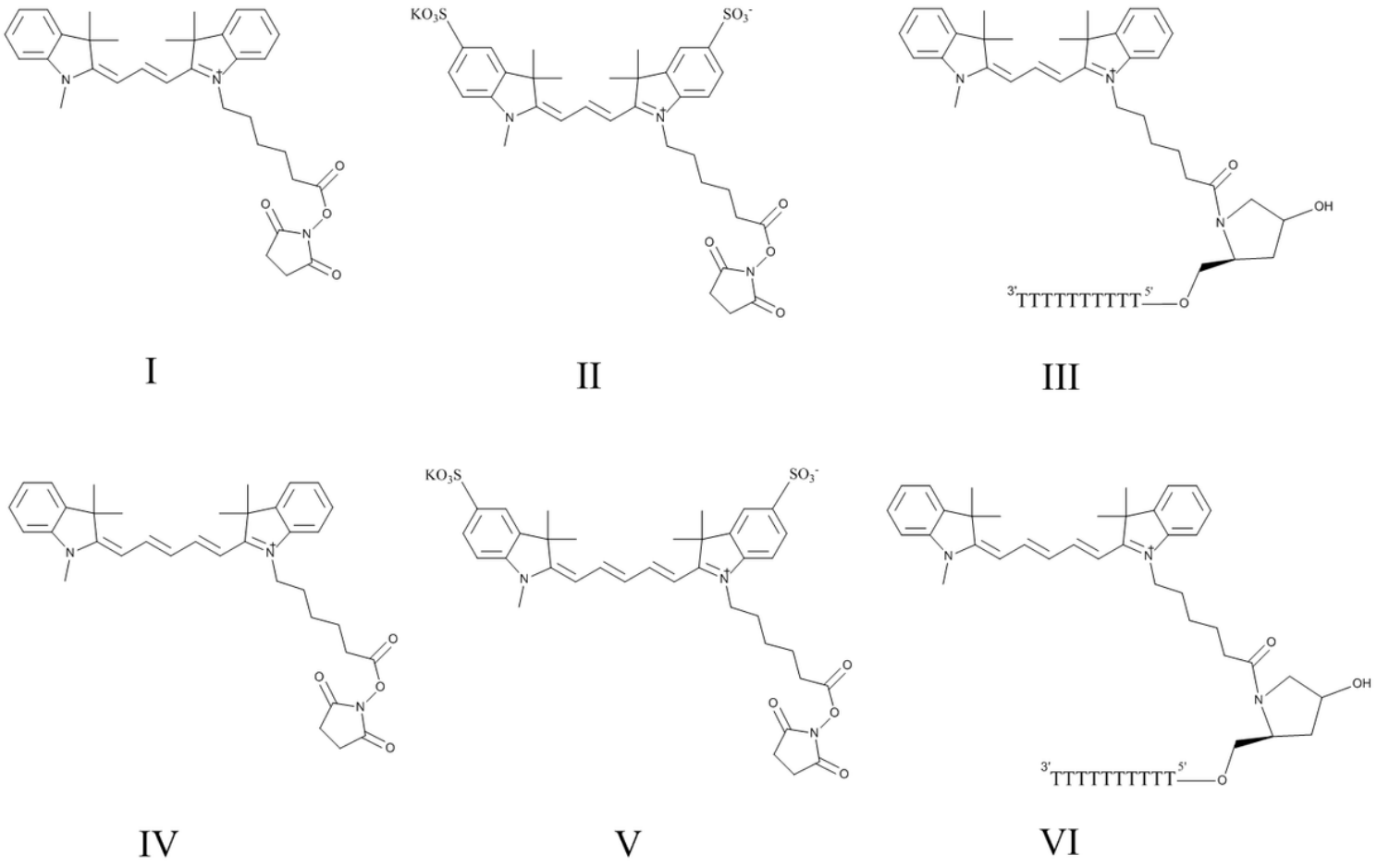

Figure 1

Structures of (I) Cy3-NHS, (II) sCy3-NHS, (III) T10-Cy3, (IV) Cy5-NHS, (V) sCy5-NHS, (VI) T10-Cy5.

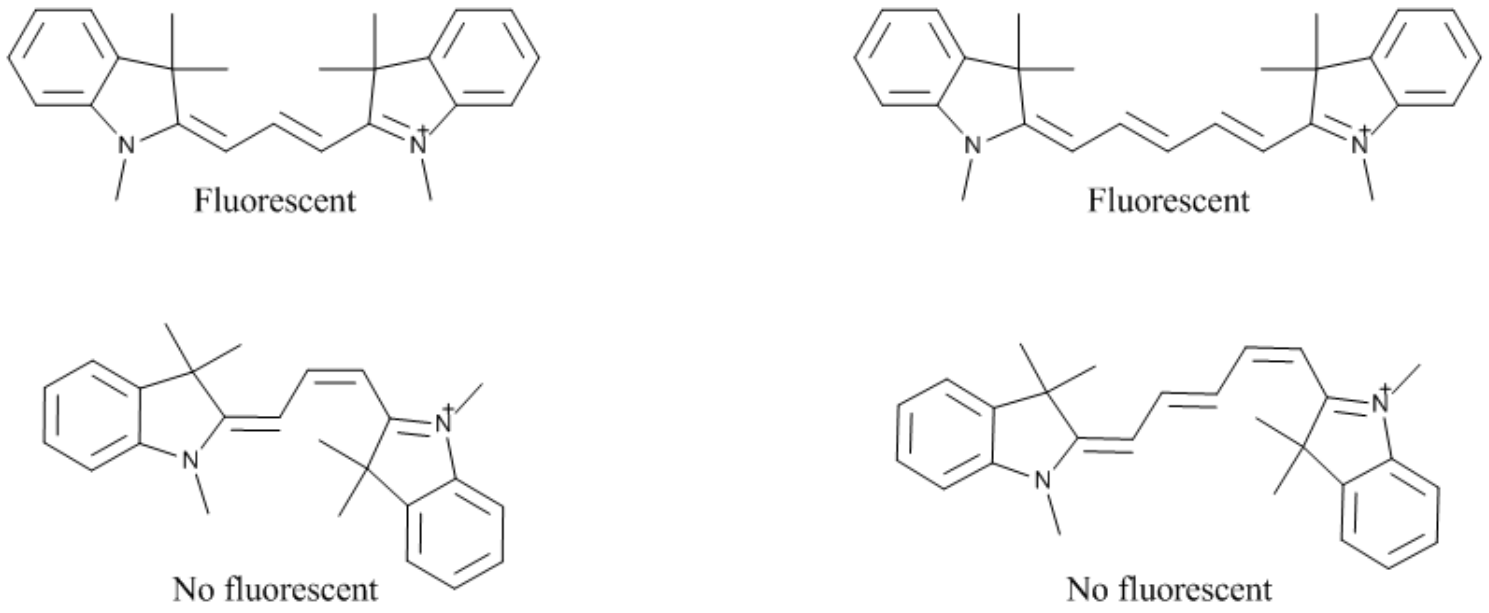

Figure 2

Structures of cis-isomer Cy3 (upper left) and Cy5 (upper right), trans-isomer Cy3 (lower left), and Cy5 (lower right).

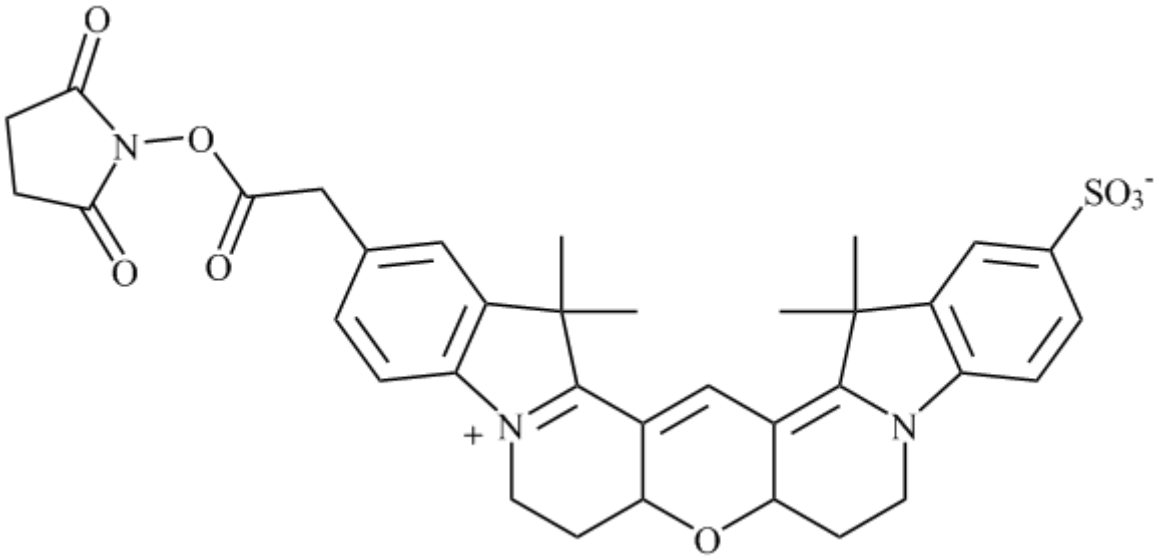

Figure 3

Structure of Cy3B.

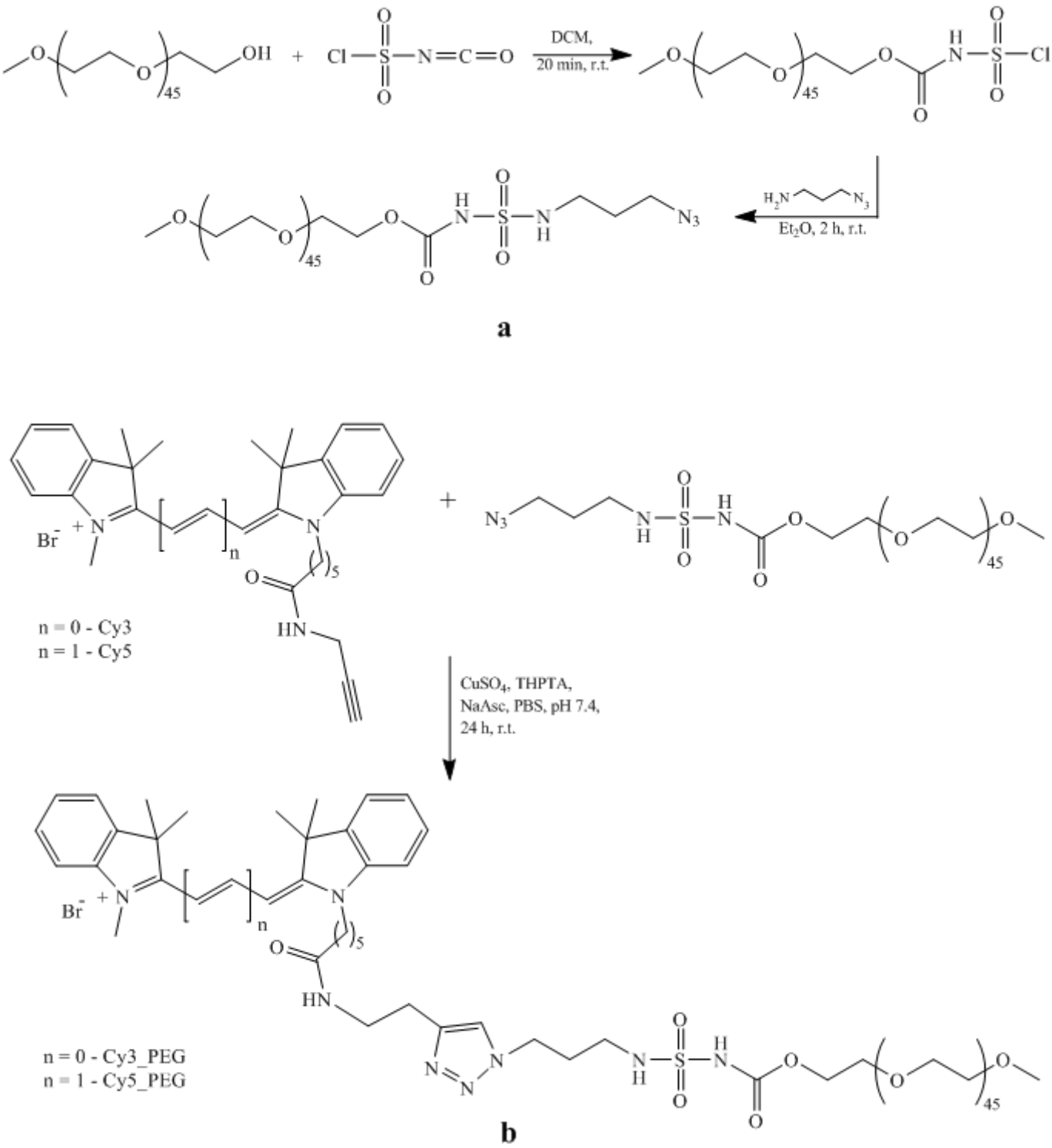

Figure 4

a) Synthesis of azide-modified polyethylene glycol; b) Click-modification [22] of alkyne derivatives of cyanine dyes with azide-modified polyethylene glycol.

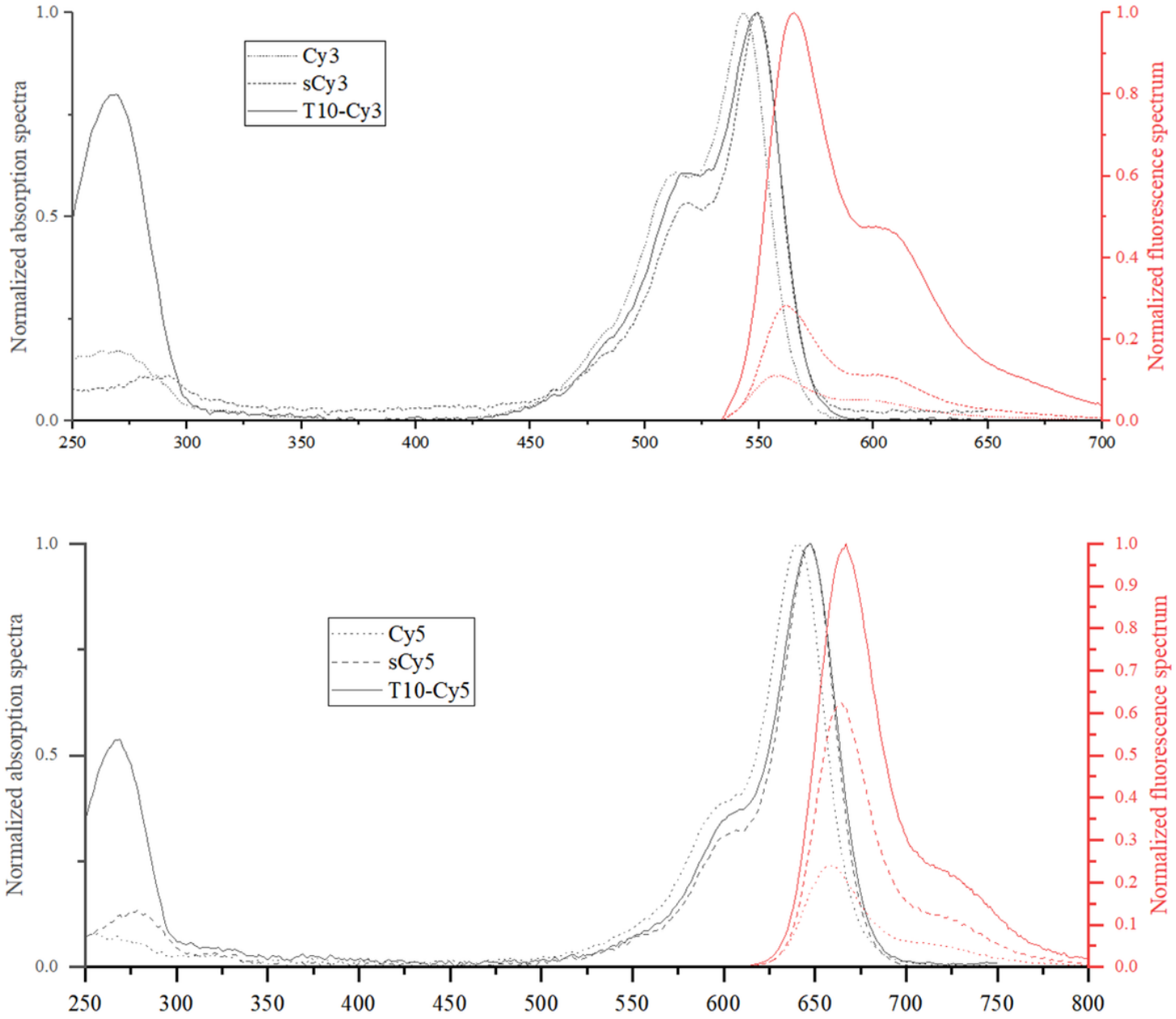

Figure 5

Normalized absorption and fluorescence spectra of Cy3 and its derivatives (Above) and Cy5 and its derivatives (Below) in PBS phosphate-salt solution, pH = 7.4.

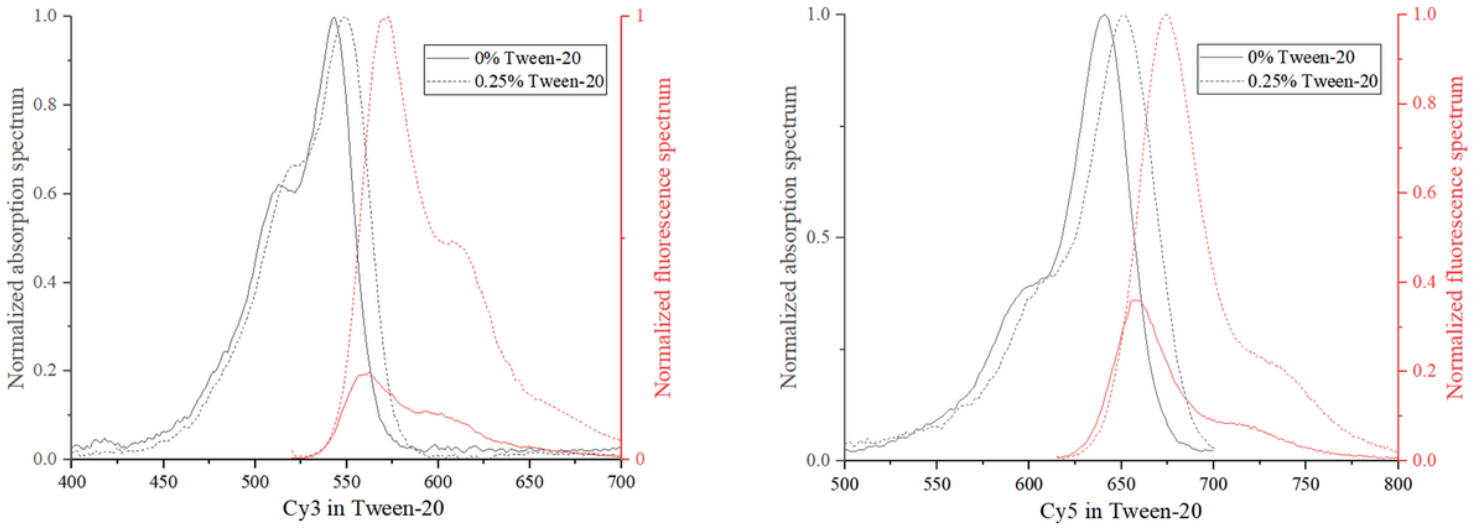

Figure 6

Normalized absorption and fluorescence spectra of Cy3 (left) and Cy5 (right) in 0% and 0.25% Tween-20 solutions.

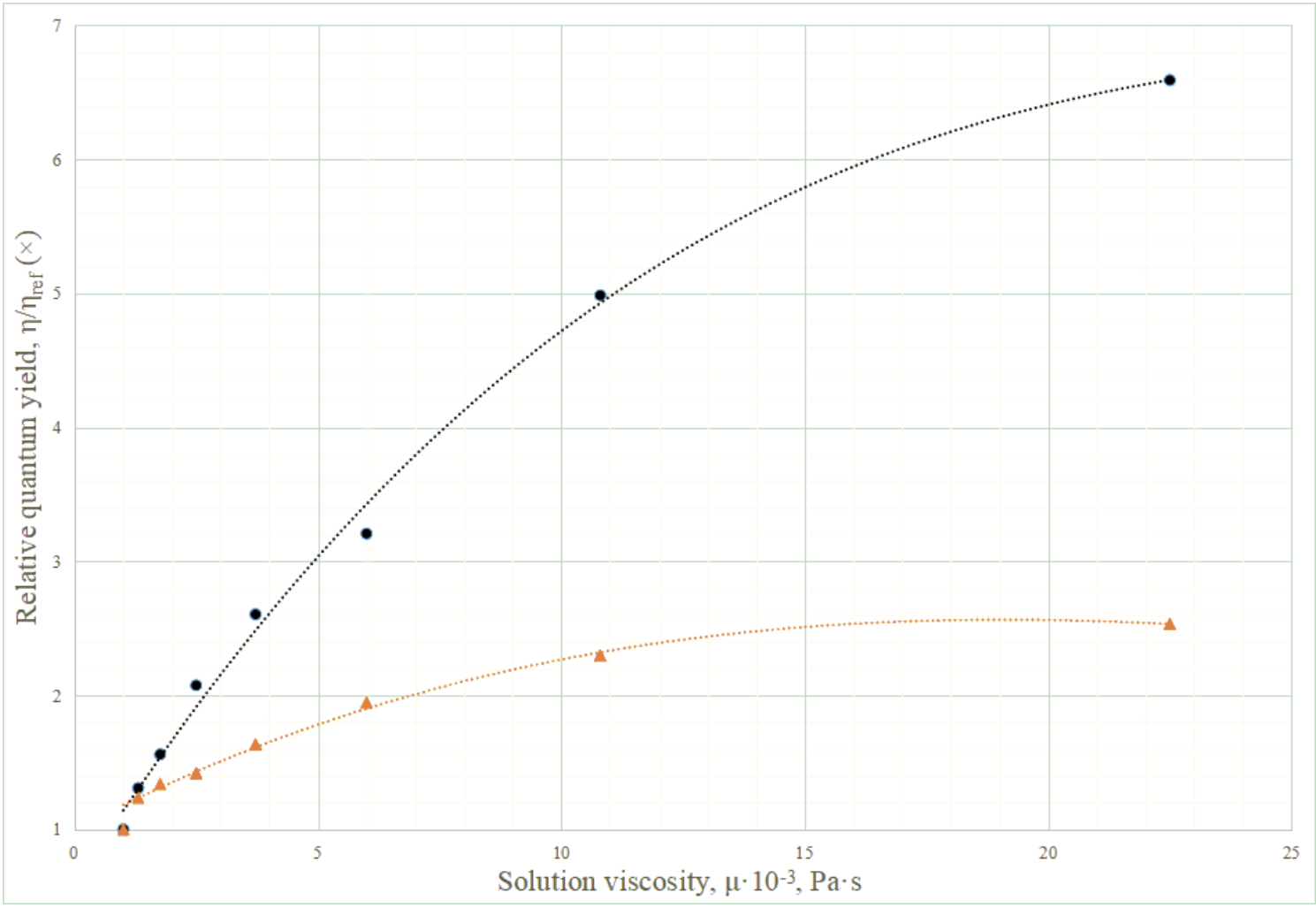

Figure 7

Dependence of the relative quantum yield on solution viscosity, Cy3 (●) and Cy5 (▲) in aqueous glycerol solutions with different concentrations.

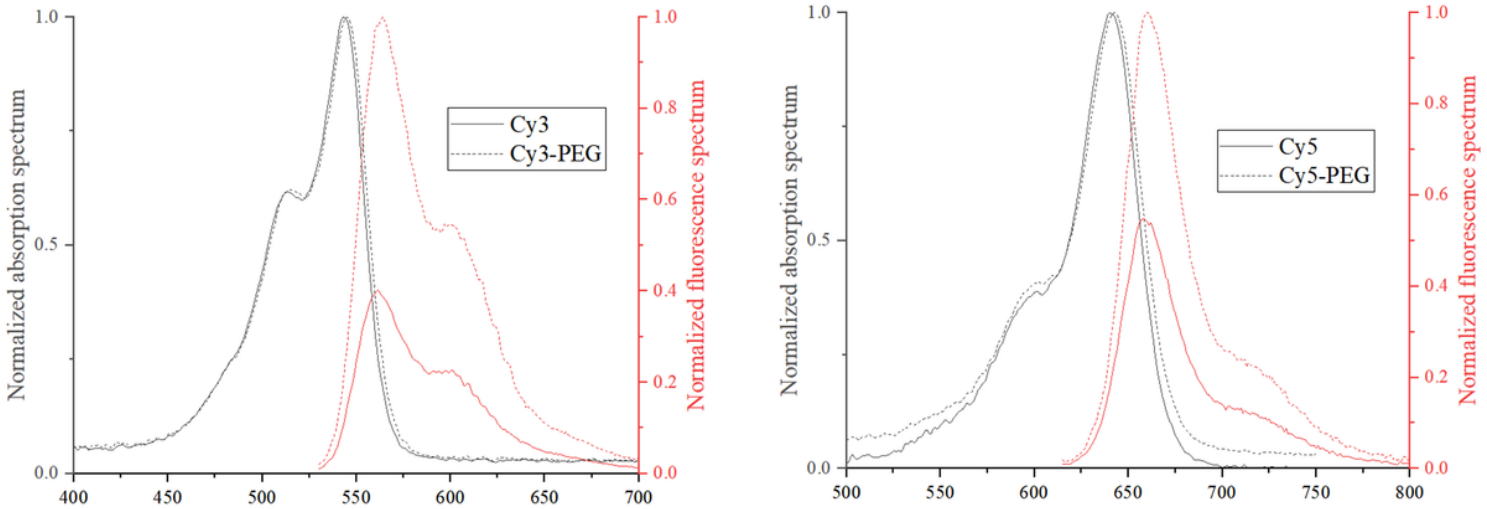

Figure 8

Normalized absorption and fluorescence spectra of Cy3 (left) and Cy5 (right) after covalent binding to PEG.