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Water soluble indodicarbocyanine dyes based on 2,3-dimethyl-3-(4-sulfobutyl)-3*H*-indole-5-sulfonic acid

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ABSTRACT

A series of mono-reactive, water-soluble, indodicarbocyanine dyes based on 2,3-dimethyl-3-(4-sulfobutyl)-3*H*-indole-5-sulfonic acid was synthesized. These dyes contain two aromatic sulfo groups and in addition to (up to 3) sulfoalkyl groups in the indolenine end groups. The impact of the number and position of sulfobutyl groups on the spectral properties and photostabilities of the dyes free in solutions and after binding to an antibody (IgG) was investigated. These dyes have absorption maxima between 649 and 653 nm, molar absorptivities between 240,000 and 256,000 M^{-1} cm^{-1}, emission maxima from 668 to 672 nm, and fluorescence quantum yields between 28 and 32% in free form and up to 43% when bound to IgG. Independent of the number of sulfo groups, these dyes exhibit no aggregation in water at concentrations up to 5×10^{-5} M. The number of sulfoalkyl groups was found to directly correlate with the fluorescence quantum yields and photostabilities of the IgG conjugated dyes.

1. Introduction

Water soluble indodicarbocyanine dyes containing reactive groups are widely used in biomedical applications as fluorescent labels [1,2]. The carboxylic groups in these dyes can be converted to *N*-hydroxysuccinimide ester (NHS) or maleimide for covalent binding under mild conditions to amino or thiol groups of biomolecules [3]. To avoid cross-linking, labels containing only one reactive group are preferred [4,5]. The widely used, commercially available indodicarbocyanine labels such as **Cy5** (*GE Healthcare*) [6] or **Alexa 647** (*Life Technologies*) [3] contain only one reactive carboxylic group and, respectively, two and four sulfo groups rendering the dyes soluble in aqueous media.

There are many reports on improving the quantum yields [7], fluorescence lifetimes [8], and photostabilities [9,10] of long-wavelength cyanine labels. An increased number of sulfo groups was reported to have a positive effect on the quantum yields [11], fluorescence lifetimes [12], and photostabilities [13] mostly attributed to a reduced dye aggregation tendency in aqueous media [14]. The quantum yield of **Cy5** in phosphate buffer was reported to be 27% [6], while for **Alexa 647** it is 33% [3,15] and the difference is even more pronounced when the dyes are bound to proteins. Further the photostability of **Alexa 647** is also increased compared to **Cy5** [16].

There are several positions in the indocyanine dye molecules which are available for introduction of sulfo, sulfoalkyl and/or carboxyalkyl groups. Most commonly the sulfo groups were introduced in the position 5 of indolenine moiety, while sulfoalkyl and carboxyalkyl groups are in the positions 1 and/or 3. Although selected indodicarbocyanine dyes containing carboxyalkyl and sulfoalkyl groups in different positions of the indolenine moiety have been reported in several papers [6] and patents [17,18], a systematic study of such compounds has not been done.

In this work we synthesized a series of unsymmetrical mono-reactive indodicarbocyanine dyes based on quaternized 2,3-dimethyl-3-(4-sulfobutyl)-3*H*-indole-5-sulfonic acid, investigate the spectral properties, quantum yields, fluorescence lifetimes, and photostabilities of these dyes free in aqueous solutions and after binding to IgG.

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2. Experimental

2.1. General

^{1}H NMR spectra were measured on a *Varian Mercury-VX-200* (200 MHz) spectrometer in DMSO-d_6 using TMS as an internal standard. Chemical reactions were monitored by TLC (Silica gel 60 RP-18, Merck) and spectrophotometrically. The purities of the dyes were determined by HPLC (column: Discovery® Bio Wide Pore C18, 250 mm × 4.6 mm; mobile phase A: water + 0.05% TFA; mobile phase B: acetonitrile–water (50:50) + 0.05% TFA; gradient: 0% B–80% B in 30 min). The dyes purities were 85–98%.

Absorption spectra for the dye concentrations (c) ranging between 1×10^{-8} M and 5×10^{-5} M in water and phosphate buffer (PB, pH 7.4) were recorded at room temperature on a *PerkinElmer Lambda 35* UV/Vis spectrophotometer. The molar absorptivities (ε) were measured in water for the same dye concentrations. Each dye (2–4 mg) was dissolved in 50 mL water and the absorbance (A) was measured. Then the solution was diluted (1:2) and the absorbance was measured again. The dilution followed by the absorbance measurement was repeated 11–13 times. The measurements were done using standard 10, 5, 1, and 0.1-cm quartz cells. The molar absorptivities were calculated according to the Lambert–Beer's law. The molar absorptivities (ε) of each dye were independently measured 3–4 times, corrected by the dye purity, and the average value was taken. The reproducibility of the ε values was no less than ± 2000 M^{-1} cm^{-1}.

Emission spectra and quantum yields were measured in water or phosphate buffer (PB, pH 7.4) at room temperature on a *Varian Cary Eclipse* spectrofluorometer. The dye concentrations measured were in the range of 1×10^{-8} M to 5×10^{-5} M. The emission spectra were corrected. To reduce the inner filter effect, the quantum yields were measured at $c \sim 1–3 \times 10^{-7}$ M in 0.1-cm quartz cells.

Absorption and emission maxima were determined at $c \sim 1–3 \times 10^{-7}$ M with accuracy ±0.3 nm and ±0.5 nm, respectively, and rounded off.

For the determination of the quantum yields (Φ_F), the integrated relative intensities of the dyes were measured against **Cy5** ($\Phi_F = 27\%$ [6] at $c \sim 1–3 \times 10^{-7}$ M) as the reference. Optical density of the dye solutions at the excitation wavelength (610 nm) was between 0.04 and 0.11 measured in a 5-cm cell. The emission spectra of the solutions were recorded and the absolute quantum yields of the dyes were determined as described in [19,20]. The quantum yield of each sample was independently measured 3–5 times and the average value was calculated. The reproducibility was no less than 7%.

Fluorescence lifetime measurements were acquired using a *ChronosFD* (ISS, Champaign, IL) frequency domain fluorometer with a 635-nm laser diode as the excitation light source. In all lifetime measurements the dye concentrations in PB pH 7.4 were in the range of 10^{-6}–10^{-7} M. Instrument control, data acquisition and data analysis were performed using Vinci-Multidimensional Fluorescence Spectroscopy software (ISS, Champaign, IL). The analysis of the time-resolved fluorescence data was carried out using the traditional non-linear least squares method. The Marquardt–Levenberg algorithm was utilized for the minimization routine of the χ^2-function.

Photostability measurements were performed in water solutions at $c \sim 5 \times 10^{-7}$ M. The optical density of the long-wavelength maximum was controlled to be between 0.10 and 0.20, and all measurements were carried out in 1-cm cells. These solutions were placed at the distance of approximately 30 cm from a 500 W halogen lamp with heat filter and irradiated with occasional stirring. The absorption and emission spectra of the solutions were taken before irradiation and during light exposure. The relative photostabilities were calculated as the ratio between (i) the measured absorbencies at the long-wavelength maximum before and after exposure (A/A_0) and (ii) the relative fluorescence intensities before and after exposure (I/I_0), and the corresponding plots were generated. The photostability was estimated *via* half-life ($\tau_{1/2}$), which is the period of time it takes for the absorption and emission intensity to decrease by half.

The formation energy (ΔH_f) and electronic charge distribution on dye molecules were calculated by the PM3 method using standard parameterization [21,22]. Full geometry optimization was undertaken.

Cy5 was from GE Healthcare. Immunoglobulin G (IgG) from bovine serum (reagent grade ≥95%) and Sephadex G50 were purchased from Sigma. All other reagents, materials and solvents were from Aldrich, Merck, and Acros and were used as is.

2.2. Synthesis

2.2.1. 5-(Ethoxycarbonyl)-5-methyl-6-oxoheptane-1-sulfonic acid (**1**)

To a solution of potassium *tert*-butoxide (9.5 g, 0.085 mol) in *tert*-butanol (140 mL), ethyl 2-methyl-3-oxobutanoate (10 mL, 0.071 mol) and then 1,4-butane sultone (7.2 mL, 0.071 mol) were added dropwise. The mixture was refluxed for 2 h. After cooling, the solvent was removed under reduced pressure using a rotary evaporator. The residue was dissolved in 50 mL water, impurities and co-products were extracted with hexane on a separatory funnel, the aqueous layer was collected and acidified using 1 M hydrochloric acid until pH = 1. The solvent was removed under reduced pressure and residue was dried. The product was dissolved in methanol (170 mL) and the insoluble precipitate was filtered and the obtained methanolic solution was used in the next step without purification. A sample for analysis was obtained after removal of methanol. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 4.12 (2H, q, J = 7.1 Hz, OCH_2), 2.38 (2H, t, J = 7.7 Hz, CH_2SO_3H), 2.10 (3H, s, $COCH_3$), 1.78–1.44 (4H, m, CH_2), 1.27–1.01 (2H, m, CH_2), 1.22 (3H, s, CCH_3), 1.17 (3H, t, J = 7.3 Hz, CH_3).

2.2.2. 5-Methyl-6-oxoheptane-1-sulfonic acid (**2**)

A solution of sodium hydroxide (9.5 g) in water (95 mL) was added to the methanolic solution of 5-(ethoxycarbonyl)-5-methyl-6-oxoheptane-1-sulfonic acid (**1**). The mixture was heated at 50 °C for 10 h. Methanol was removed under reduced pressure and residue was acidified using 1 M hydrochloric acid until pH = 1. The solvent was removed under reduced pressure and the residue was dried. The product was dissolved in acetone, insoluble precipitate was filtered, and acetone was removed. Yield: 13.2 g (90% for the two stages starting from ethyl 2-methyl-3-oxobutanoate). ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 2.40 (2H, t, J = 7.8 Hz, CH_2SO_3H), 2.47–2.34 (1H, m, CH), 2.08 (3H, s, $COCH_3$), 1.64–1.42 (4H, m, CH_2), 1.32–1.14 (2H, m, CH_2), 0.97 (3H, d, J = 7.1 Hz, CH_3).

2.2.3. 2,3-Dimethyl-3-(4-sulfobutyl)-3H-indole-5-sulfonic acid (**3**)

A mixture of 5-methyl-6-oxoheptane-1-sulfonic acid (1 g, 4.8 mmol), 4-hydrazinobenzenesulfonic acid (0.71 g, 3.8 mmol), and acetic acid (10 mL) was refluxed for 6 h. The solvent was removed under reduced pressure using a rotary evaporator and the residue was dried. The product was triturated with hot *iso*-propanol and the obtained precipitate was filtered and dried. Yield: 1.17 g (85%). λ_{abs} 260 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 7.83 (1H, s, arom H), 7.53 (1H, d, J = 7.8 Hz, arom H), 7.54 (1H, d, J = 7.9 Hz, arom H), 2.58 (3H, s, 2-CH_3), 2.36 (2H, t, J = 7.5 Hz, CH_2SO_3H), 2.09–1.86 (2H, m, CH_2), 1.53–1.36 (2H, m, CH_2), 1.43 (3H, s, 3-CH_3), 0.88–0.47 (2H, m, CH_2).

*2.2.4. Dipotassium 2,3-dimethyl-3-(4-sulfonatobutyl)-3H-indole-5-sulfonate (**4**)*

To a solution of 2,3-dimethyl-3-(4-sulfobutyl)-3*H*-indole-5-sulfonic acid (1.17 g, 3.24 mmol) in methanol (10 mL), a solution of potassium hydroxide in *iso*-propanol was added dropwise until pH = 8. The mixture was stirred for 10 min and the precipitate was filtered and dried. Yield: 1.1 g (77%). ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 7.55 (1H, s, arom H), 7.53 (1H, d, J = 9.1 Hz, arom H), 7.31 (1H, d, J = 8.0 Hz, arom H), 2.23 (2H, t, J = 8.2 Hz, CH_2SO_3K), 2.17 (3H, s, 2-CH_3), 1.91–1.70 (2H, m, CH_2), 1.50–1.31 (2H, m, CH_2), 1.21 (3H, s, 3-CH_3), 0.74–0.37 (2H, m, CH_2).

*2.2.5. Dipotassium 2,3-dimethyl-1,3-di(4-sulfonatobutyl)-3H-5-indoliumsulfonate (**5a**)*

A mixture of dipotassium 2,3-dimethyl-3-(4-sulfonatobutyl)-3*H*-indole-5-sulfonate (1 g, 2.28 mmol), 1,4-butane sultone (930 mg, 6.85 mmol) and 1,2-dichlorbenzene (10 mL) was stirred at 150 °C under argon atmosphere for 8 h, cooled down to room temperature, and 40 mL ether was added. The raw product was filtered, washed with ether, dried, and column purified (RP-18, H_2O). Yield: 660 mg (50%). λ_{abs} 275 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.00 (1H, d, J = 6.6 Hz, arom H), 7.98 (1H, s, arom H), 7.82 (1H, d, J = 8.0 Hz, arom H), 4.49 (2H, t, J = 6.8 Hz, NCH_2), 2.87 (3H, s, 2-CH_3), 2.44–2.22 (4H, m, CH_2SO_3K), 2.01–1.69 (4H, m, CH_2), 1.65–1.35 (4H, m, CH_2), 1.53 (3H, s, 3-CH_3), 0.90–0.41 (2H, m, CH_2).

*2.2.6. Potassium 1-(5-carboxypentyl)-2,3-dimethyl-3-(4-sulfonatobutyl)-3H-5-indoliumsulfonate (**5b**)*

A mixture of dipotassium 2,3-dimethyl-3-(4-sulfonatobutyl)-3*H*-indole-5-sulfonate (2.4 g, 5.48 mmol), 6-bromohexanoic acid (3.38 g, 17.32 mmol) and 1,2-dichlorbenzene (18 mL) was stirred at 150 °C under argon atmosphere for 6 h, cooled down to room temperature, and 50 mL ether was added. The raw product was filtered, washed with ether, dried, and column purified (RP-18, H_2O). Yield: 1.24 g (44%). λ_{abs} 276 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 7.99 (1H, s, arom H), 7.94 (1H, d, J = 8.2 Hz, arom H), 7.83 (1H, d, J = 8.2 Hz, arom H), 4.47 (2H, t, J = 7.1 Hz, NCH_2), 2.87 (3H, s, 2-CH_3), 2.38–2.15 (2H, m, CH_2SO_3K), 2.23 (2H, t, J = 7.1 Hz, CH_2COOH), 1.92–1.74 (2H, m, CH_2), 1.64–1.32 (8H, m, CH_2), 1.53 (3H, s, 3-CH_3), 0.86–0.42 (2H, m, CH_2).

*2.2.7. Potassium 1-ethyl-2,3-dimethyl-3-(4-sulfonatobutyl)-3H-5-indoliumsulfonate (**5c**)*

A mixture of dipotassium 2,3-dimethyl-3-(4-sulfonatobutyl)-3*H*-indole-5-sulfonate (1 g, 2.28 mmol), iodoethane (2 mL) and acetonitrile (20 mL) was stirred at 90 °C for 40 h. Additional portions of iodoethane (0.3 mL) were added every 10 h. The solvent was removed under reduced pressure and the residue was column purified (RP-18, H_2O). Yield: 310 mg (32%). λ_{abs} 275 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 7.99 (1H, s, arom H), 7.93 (1H, d, J = 8.2 Hz, arom H), 7.84 (1H, d, J = 8.3 Hz, arom H), 4.50 (2H, q, J = 7.1 Hz, NCH_2), 2.86 (3H, s, 2-CH_3), 2.27 (2H, t, J = 6.3 Hz, CH_2SO_3K), 2.41–2.03 (2H, m, CH_2), 1.53 (3H, s, 3-CH_3), 1.42 (3H, t, J = 6.9 Hz, CH_3), 1.57–1.35 (2H, m, CH_2), 0.89–0.41 (2H, m, CH_2).

*2.2.8. Dipotassium 2,3-dimethyl-1-(4-sulfonatobutyl)-3-(5-carboxypentyl)-3H-5-indoliumsulfonate (**5d**) was synthesized according to Ref. [23]*

Yield: 49%. λ_{abs} 274 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 7.99 (1H, d, J = 6.8 Hz, arom H), 7.97 (1H, s, arom H), 7.82 (1H, d, J = 8.2 Hz, arom H), 4.50 (2H, t, J = 6.8 Hz, NCH_2), 2.87 (3H, s, 2-CH_3), 2.44–2.34 (2H, m, CH_2SO_3K), 2.08 (2H, t, J = 7.2 Hz, CH_2COOK), 2.02–1.69 (4H, m, CH_2), 1.67–1.28 (4H, m, CH_2), 1.53 (3H, s, 3-CH_3), 1.21–1.01 (2H, m, CH_2), 0.82–0.37 (2H, m, CH_2).

*2.2.9. Dipotassium 2,3-dimethyl-1-(4-sulfonatopropyl)-3-(5-carboxypentyl)-3H-5-indoliumsulfonate (**5e**) was synthesized according to Ref. [18]*

Yield: 53%. λ_{abs} 274 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 7.99 (1H, d, J = 6.8 Hz, arom H), 7.97 (1H, s, arom H), 7.82 (1H, d, J = 8.2 Hz, arom H), 4.50 (2H, t, J = 6.8 Hz, NCH_2), 2.87 (3H, s, 2-CH_3), 2.44–2.34 (2H, m, CH_2SO_3K), 2.08 (2H, t, J = 7.2 Hz, CH_2COOK), 2.02–1.69 (4H, m, CH_2), 1.53 (3H, s, 3-CH_3), 1.21–1.01 (2H, m, CH_2), 0.82–0.37 (2H, m, CH_2).

*2.2.10. Potassium 2,3,3-trimethyl-1-(4-sulfonatobutyl)-3H-indolium-5-sulfonate (**5f**) was synthesized according to Ref. [6] with almost quantitative yield*

λ_{abs} 273 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.01 (1H, s, arom H), 7.96 (1H, d, J = 8.4 Hz, arom H), 7.81 (1H, d, J = 8.0 Hz, arom H), 4.46 (2H, t, J = 6.9 Hz, NCH_2), 2.84 (3H, s, 2-CH_3), 2.53–2.38 (2H, m, CH_2SO_3K), 2.10–1.84 (2H, m, CH_2), 1.84–1.65 (2H, m, CH_2), 1.54 (6H, s, 3-$(CH_3)_2$).

*2.2.11. Potassium 2,3,3-trimethyl-1-(3-sulfonatopropyl)-3H-indolium-5-sulfonate (**5g**) was synthesized according to Ref. [18] with almost quantitative yield*

λ_{abs} 273 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 7.98 (1H, s, arom H), 7.93 (1H, d, J = 8.4 Hz, arom H), 7.81 (1H, d, J = 8.0 Hz, arom H), 4.60 (2H, t, J = 6.9 Hz, NCH_2), 2.80 (3H, s, 2-CH_3), 2.61 (2H, m, CH_2SO_3K), 1.84–1.65 (2H, m, CH_2), 1.52 (6H, s, 3-$(CH_3)_2$).

*2.2.12. 1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-indolium-5-sulfonate (**5h**) was synthesized according to Ref. [6]*

Yield: 77%. λ_{abs} 270 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.02 (1H, s, arom H), 7.93 (1H, d, J = 8.4 Hz, arom H), 7.81 (1H, d, J = 7.8 Hz, arom H), 4.44 (2H, t, J = 6.3 Hz, NCH_2), 2.84 (3H, s, 2-CH_3), 2.22 (2H, t, J = 7.1 Hz, CH_2COOH), 1.95–1.73 (2H, m, CH_2), 1.69–1.26 (4H, m, CH_2), 1.54 (6H, s, 3-$(CH_3)_2$).

*2.2.13. General procedure for synthesis of 1,3-butadienyl-N-phenylacetoamides (**6b**, **6d**, **6f**–**6h**)*

A mixture of 2.5 mmol of indolenine quaternary salt, 3.75 mmol *N,N′*-prop-1-en-1-yl-3-ylidenedianiline hydrochloride, 15 mL of acetic anhydride and 15 mL of acetic acid was refluxed for 4.5 h. The solvent was removed under reduced pressure and the residue was triturated with ethyl acetate. The obtained precipitate was filtered, washed with ethyl acetate until colorless filtrate, and dried to yield product as orange powder. The products obtained with almost quantitative yield were used for further syntheses without purification.

*2.2.13.1. Potassium 1-(5-carboxypentyl)-3-methyl-2-[4-methyl(phenyl)carboxamido-1,3-butadienyl]-3-(4-sulfonatobutyl)-3H-5-indoliumsulfonate (**6b**).* λ_{abs} 453 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.82 (1H, d, J = 13.1 Hz, δ CH), 8.33 (1H, t, J = 13.2 Hz, β CH), 7.79 (1H, s, arom H), 7.61–7.16 (7H, m, arom H), 7.03 (1H, d, J = 7.9 Hz, α CH), 5.86 (1H, t, J = 12.1 Hz, γ CH), 4.27–4.03 (2H, m, NCH_2), 2.34–2.12 (4H, m, CH_2SO_3K, CH_2COOH), 1.91 (3H, s, $COCH_3$), 1.79–1.20 (10H, m, CH_2), 1.65 (3H, s, 3-$(CH_3)_2$), 0.97–0.42 (2H, m, CH_2).

*2.2.13.2. Dipotassium 6-[3-methyl-2-[4-methyl(phenyl)carboxamido-1,3-butadienyl]-5-sulfonato-1-(4-sulfonatobutyl)-3H-3-indoliumyl]hexanoate (**6d**).* λ_{abs} 455 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.94 (1H, d, J = 12.5 Hz, δ CH), 8.59 (1H, t, J = 13.7 Hz, β CH), 7.73 (1H, s, arom H), 7.66–7.17 (7H, m, arom H), 7.03 (1H, d, J = 7.7 Hz, α CH), 5.57 (1H, t, J = 12.2 Hz, γ CH), 4.39–4.22 (2H, m, NCH_2), 2.22–1.95 (4H, m, CH_2SO_3K, CH_2COOK), 1.90 (3H, s, $COCH_3$), 1.83–1.48 (10H, m, CH_2), 1.68 (3H, s, 3-$(CH_3)_2$), 0.80–0.29 (2H, m, CH_2).

*2.2.13.3. 1-(4-Sulfonatobutyl)-3,3-dimethyl-2-[4-methyl(phenyl)carboxamido-1,3-butadienyl]-3H-5-indoliumsulfonate (**6f**).* λ_{abs} 445 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.94 (1H, d, J = 12.5 Hz, δ CH), 8.58 (1H, t, J = 12.0 Hz, β CH), 7.97 (1H, s, arom H), 7.84–7.33 (7H, m, arom H), 6.89 (1H, d, J = 14.5 Hz, α CH), 5.54 (1H, t, J = 12.2 Hz, γ CH), 4.40–4.17 (2H, m, NCH_2), 2.28–2.10 (2H, m, CH_2SO_3H), 1.91 (3H, s, $COCH_3$), 1.77–1.24 (6H, m, CH_2), 1.70 (6H, s, 3-$(CH_3)_2$).

*2.2.13.4. 1-(4-Sulfonatopropyl)-3,3-dimethyl-2-[3-methyl(phenyl)carboxamido-1,3-butadienyl]-3H-5-indoliumsulfonate (**6g**).* λ_{abs} 450 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.94 (1H, d, J = 12.5 Hz, δ CH), 8.58 (1H, t, J = 12.0 Hz, β CH), 7.97 (1H, s, arom H), 7.84–7.33 (7H, m, arom H), 6.89 (1H, d, J = 14.5 Hz, α CH), 5.54 (1H, t, J = 12.2 Hz, γ CH), 4.40–4.17 (2H, m, NCH_2), 2.28–2.10 (2H, m, CH_2SO_3H), 1.91 (3H, s, $COCH_3$), 1.77–1.24 (4H, m, CH_2), 1.70 (6H, s, 3-$(CH_3)_2$).

*2.2.13.5. 1-(5-Carboxypentyl)-3,3-dimethyl-2-[4-methyl(phenyl)carboxamido-1,3-butadienyl]-3H-5-indoliumsulfonate (**6h**).* λ_{abs} 452 nm. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.94 (1H, d, J = 12.5 Hz, δ CH), 8.58 (1H, t, J = 12.0 Hz, β CH), 7.97 (1H, s, arom H), 7.84–7.33 (7H, m, arom H), 6.89 (1H, d, J = 14.5 Hz, α CH), 5.54 (1H, t, J = 12.2 Hz, γ CH), 4.40–4.17 (2H, m, NCH_2), 2.28–2.10 (2H, m, CH_2COOH), 1.91 (3H, s, $COCH_3$), 1.77–1.24 (6H, m, CH_2), 1.70 (6H, s, 3-$(CH_3)_2$).

*2.2.14. General procedure for synthesis of cyanine dyes **7**–**16***

A solution of 0.3 mmol of 1,3-butadienyl-*N*-phenylacetoamide in 7 mL of acetic anhydride was stirred at 50 °C for 30 min. Then 0.365 mmol of indolenine quaternary salt and 7 mL of dry pyridine were added. In case of synthesis of cyanines **10**–**12**, 7 mL of acetic acid were added. The mixture was stirred at 110 °C for 30 min. After cooling, dry ether was added and the mixture was kept at 0 °C for 2 h. The obtained precipitate was filtered and washed with ether. Then the ion exchange resin Dowex 50WX8-100 was prepared as described in Ref. [6]. The raw product was dissolved in water, passed through column with Dowex 50WX8-100, and column purified (RP-18, water–acetonitrile) to give the title dye as blue powder.

*2.2.14.1. 3-(5-Carboxypentyl)-2-5-[1-ethyl-3-methyl-5-sulfo-3-(4-sulfobutyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3-methyl-1-(4-sulfobutyl)-3H-5-indoliumsulfonate (**7**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6d** and quaternary salt **5c**. Yield: 16%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.34 (2H, t, J = 13.3 Hz, β H), 7.75 (2H, s, arom H), 7.63 (2H, d, J = 8.5 Hz arom H), 7.35 (1H, d, J = 8.6 Hz, arom H), 7.30 (1H, d, J = 8.2 Hz, arom H), 6.55 (1H, t, J = 12.4 Hz, γ H), 6.42 (1H, d, J = 13.8 Hz, α H), 6.34 (1H, d, J = 13.7 Hz, α H), 4.21–4.03 (4H, m, NCH_2), 2.43–2.28 (4H, m, CH_2SO_3H), 2.06 (2H, t, J = 7.1 Hz, CH_2COOH), 1.66 (6H, s, 3-CH_3), 1.87–1.04 (14H, m, CH_2), 1.24 (3H, t, J = 7.2 Hz, CH_3), 0.95–0.36 (4H, m, CH_2).

*2.2.14.2. 1-(5-Carboxypentyl)-2-5-[1-ethyl-3-methyl-5-sulfo-3-(4-sulfobutyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3-methyl-3-(4-sulfobutyl)-3H-5-indoliumsulfonate (**8**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6b** and quaternary salt **5c**. Yield: 7%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.38 (2H, t, J = 12.9 Hz, β H), 7.77 (2H, s, arom H), 7.63 (2H, d, J = 8.1 Hz, arom H), 7.31 (2H, d, J = 8.2 Hz, arom H), 6.58 (1H, t, J = 12.2 Hz, γ H), 6.33 (2H, d, J = 12.4 Hz, α H), 4.21–4.02 (4H, m, NCH_2), 2.46–2.34 (4H, m, CH_2SO_3H), 2.20 (2H, t, J = 7.3 Hz, CH_2COOH), 1.65 (6H, s, 3-CH_3), 1.80–1.32 (14H, m, CH_2), 1.25 (3H, t, J = 7.1 Hz, CH_3), 0.94–0.38 (4H, m, CH_2).

*2.2.14.3. 2-5-[1-(5-Carboxypentyl)-3-methyl-5-sulfo-3-(4-sulfobutyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3,3-dimethyl-1-(4-sulfobutyl)-3H-5-indoliumsulfonate (**9**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6b** and quaternary salt **5f**. Yield: 42%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.35 (2H, t, J = 13.5 Hz, β H), 7.80 (1H, s, arom H), 7.76 (1H, s, arom H), 7.62 (2H, d, J = 8.4 Hz, arom H), 7.36 (1H, d, J = 7.9 Hz, arom H), 7.29 (1H, d, J = 8.4 Hz, arom H), 6.59 (1H, t, J = 12.9 Hz, γ H), 6.38 (1H, d, J = 12.9 Hz, α H), 6.34 (1H, d, J = 12.9 Hz, α H), 4.18–3.91 (4H, m, NCH_2), 2.45–2.25 (4H, m, CH_2SO_3H), 2.20 (2H, t, J = 7.0 Hz, CH_2COOH), 1.85–1.30 (14H, m, CH_2), 1.68 (6H, s, 3-$(CH_3)_2$), 1.66 (3H, s, 3-CH_3), 0.93–0.37 (2H, m, CH_2).

*2.2.14.4. 1-(5-Carboxypentyl)-3,3-dimethyl-2-5-[3-methyl-5-sulfo-1,3-di(4-sulfobutyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3H-5-indoliumsulfonate (**10**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6h** and quaternary salt **5a**. Yield: 31%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.36 (2H, t, J = 12.9 Hz, β H), 7.80 (1H, s, arom H), 7.77 (1H, s, arom H), 7.61 (2H, d, J = 7.6 Hz, arom H), 7.36 (1H, d, J = 8.2 Hz, arom H), 7.31 (1H, d, J = 8.4 Hz, arom H), 6.61 (1H, t, J = 12.4 Hz, γ H), 6.41 (1H, d, J = 14.0 Hz, α H), 6.31 (1H, d, J = 13.7 Hz, α H), 4.17–4.02 (4H, m, NCH_2), 2.44–2.34 (4H, m, CH_2SO_3H), 2.20 (2H, t, J = 7.0 Hz, CH_2COOH), 1.69 (6H, s, 3-$(CH_3)_2$), 1.66 (3H, s, 3-CH_3), 1.87–1.27 (14H, m, CH_2), 0.94–0.39 (2H, m, CH_2).

*2.2.14.5. 3-(5-Carboxypentyl)-3-methyl-2-5-[3-methyl-5-sulfo-1,3-di(4-sulfobutyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-1-(4-sulfobutyl)-3H-5-indoliumsulfonate (**11**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6d** and quaternary salt **5a**. Yield: 19%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.34 (2H, t, J = 12.8 Hz, β H), 7.75 (2H, s, arom H), 7.62 (2H, d, J = 8.2 Hz, arom H), 7.35 (2H, d, J = 8.5 Hz, arom H), 6.60 (1H, t, J = 12.2 Hz, γ H), 6.41 (2H, d, J = 13.4 Hz, α H), 4.21–4.00 (4H, m, NCH_2), 2.51–2.32 (6H, m, CH_2SO_3H), 2.06 (2H, t, J = 6.6 Hz, CH_2COOH), 1.66 (6H, s, 3-CH_3), 1.93–1.02 (18H, m, CH_2), 0.96–0.33 (4H, m, CH_2).

*2.2.14.6. 1-(5-Carboxypentyl)-3-methyl-2-5-[3-methyl-5-sulfo-1,3-di(4-sulfobutyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3-(4-sulfobutyl)-3H-5-indoliumsulfonate (**12**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6b** and quaternary salt **5a** Yield: 11%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.36 (2H, t, J = 12.7 Hz, β H), 7.76 (2H, s, arom H), 7.63 (2H, d, J = 8.0 Hz arom H), 7.35 (1H, d, J = 8.6 Hz, arom H), 7.30 (1H, d, J = 8.6 Hz, arom H), 6.60 (1H, t, J = 12.1 Hz, γ H), 6.40 (1H, d, J = 13.7 Hz, α H), 6.33 (1H, d, J = 13.4 Hz, α H), 4.21–4.00 (4H, m, NCH_2), 2.45–2.31 (6H, m, CH_2SO_3H), 2.20 (2H, t, J = 7.4 Hz, CH_2COOH), 1.67 (6H, s, 3-CH_3), 1.83–1.31 (18H, m, CH_2), 0.94–0.40 (4H, m, CH_2).

*2.2.14.7. 1-(5-Carboxypentyl)-2-5-[3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3,3-dimethyl-3H-5-indoliumsulfonate (**13**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6h** and quaternary salt **5f**. Yield: 5%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.35 (2H, t, J = 13.2 Hz, β H), 7.80 (2H, s, arom H), 7.63 (2H, d, J = 8.4 Hz, arom H), 7.36 (1H, d, J = 8.4 Hz, arom H), 7.31 (1H, d, J = 8.3 Hz, arom H), 6.60 (1H, t, J = 12.7 Hz, γ H), 6.39 (1H, d, J = 14.4 Hz, α H), 6.31 (1H, d, J = 13.8 Hz, α H), 4.18–3.97 (4H, m, NCH_2), 2.63–2.49 (2H, m, CH_2SO_3H), 2.20 (2H, t, J = 7.0 Hz, CH_2COOH), 1.85–1.46 (8H, m, CH_2), 1.69 (12H, s, 2 × [3-$(CH_3)_2$]), 1.46–1.29 (2H, m, CH_2).

*2.2.14.8. 2-5-[3-(5-Carboxypentyl)-3-methyl-5-sulfo-1-(3-sulfopropyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3,3-dimethyl-1-(3-sulfopropyl)-3H-5-indoliumsulfonate (**14**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6g** and quaternary salt **5e**. Yield: 14%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.36 (2H, t, J = 13.2 Hz, β H), 7.80 (1H, s, arom H), 7.75 (1H, s, arom H) 7.61 (2H, d, J = 8.4 Hz, arom H), 7.39 (2H, d, J = 8.3 Hz, arom

H), 6.58 (1H, t, $J = 12.7$ Hz, γ H), 6.43 (2H, d, $J = 14.4$ Hz, α H), 4.26 (2H, t, NCH_2), 4.09 (2H, t, NCH_2), 2.64 (4H, t, $J = 7.0$ Hz, CH_2SO_3H), 2.04 (2H, t, $J = 7.0$ Hz, CH_2COOH), 1.85–1.46 (8H, m, CH_2), 1.68 (9H, s, 3-CH_3, 3-$(CH_3)_2$), 1.31–0.46 (4H, m, CH_2).

*2.2.14.9. 2-5-[3-(5-Carboxypentyl)-3-methyl-5-sulfo-1-(3-sulfopropyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3,3-dimethyl-1-(4-sulfobutyl)-3H-5-indoliumsulfonate (**15**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6f** and quaternary salt **5e**. Yield: 5%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.38 (2H, t, $J = 13.2$ Hz, β H), 7.80 (1H, s, arom H), 7.75 (1H, s, arom H) 7.60 (2H, d, $J = 8.4$ Hz, arom H), 7.37 (2H, d, $J = 8.3$ Hz, arom H), 6.58 (1H, t, $J = 12.7$ Hz, γ H), 6.43 (2H, d, $J = 14.4$ Hz, α H), 4.26 (4H, m, NCH_2), 2.60 (4H, m, $J = 7.0$ Hz, CH_2SO_3H), 2.04 (2H, t, $J = 7.0$ Hz, CH_2COOH), 1.85–1.46 (8H, m, CH_2), 1.68 (9H, s, 3-CH_3, 3-$(CH_3)_2$), 1.32–0.48 (6H, m, CH_2).

*2.2.14.10. 2-5-[3-(5-Carboxypentyl)-3-methyl-5-sulfo-1-(4-sulfobutyl)-2,3-dihydro-1H-2-indolyliden]-1,3-pentadienyl-3,3-dimethyl-1-(4-sulfobutyl)-3H-5-indoliumsulfonate (**16**).* The dye was synthesized starting from 1,3-butadienyl-*N*-phenylacetoamide **6f** and quaternary salt **5d**. Yield: 15%. ^{1}H NMR (200 MHz, DMSO-d_6, ppm): δ 8.35 (2H, t, $J = 13.2$ Hz, β H), 7.80 (1H, s, arom H), 7.74 (1H, s, arom H) 7.60 (2H, d, $J = 8.4$ Hz, arom H), 7.37 (2H, d, $J = 8.3$ Hz, arom H), 6.59 (1H, t, $J = 12.7$ Hz, γ H), 6.40 (2H, d, $J = 14.4$ Hz, α H), 4.10 (4H, m, NCH_2), 2.60 (4H, m, $J = 7.0$ Hz, CH_2SO_3H), 2.03 (2H, t, $J = 7.0$ Hz, CH_2COOH), 1.85–1.46 (8H, m, CH_2), 1.68 (9H, s, 3-CH_3, 3-$(CH_3)_2$), 1.31–0.43 (8H, m, CH_2).

2.3. General procedure for synthesis of cyanine dyes NHS-esters

1 mg of the dye was dissolved in anhydrous DMF (100 μL). Then 0.5 mg of TSTU and 4 μL of DIPEA were added and the mixture was stirred at room temperature for 1 h. The reaction was monitored by TLC (RP-18, water–acetonitrile). The solution without purification was used for preparation of dye–IgG conjugates.

2.4. General protein labeling procedures

A stock solution of 1 mg of the NHS-activated dye in 100 μL of anhydrous DMF was prepared as described above. A series of solutions containing 1.5 mg of IgG dissolved in 0.5 mL of 50 mM bicarbonate buffer pH 9.0 were prepared. Then appropriate aliquot of the dye stock solution (in the range of 5–50 μL) was added slowly to each IgG solution. The mixtures were allowed to stir for 2 h at 25 °C to form the dye–IgG conjugates.

2.5. Purification of the dye–IgG conjugates

Separation of the dye–IgG conjugate from unconjugated dye was performed using gel permeation chromatography on a 1.5 cm × 25 cm column (Sephadex G50, 67 mM PB, pH 7.4). The fraction with the shortest retention time containing the blue dye–IgG conjugate was collected.

2.6. Determination of the dye-to-protein ratios (D/P)

A series of labeling reactions were performed and the corresponding conjugates were purified and isolated as described above. The absorption spectra of the conjugates were measured and the dye-to-protein ratios (D/P) were calculated using the following equation with the assumption that the molar absorptivities for the free dye and the dye–IgG conjugate are the same [24,25]:

$$\mathrm{D/P} = \frac{A_{\mathrm{Conj}(\lambda_{max})}\,\varepsilon_{\mathrm{IgG}}}{\left(A_{\mathrm{Conj}(278)} - xA_{\mathrm{Conj}(\lambda_{max})}\right)\varepsilon_{\mathrm{Dye}}},$$

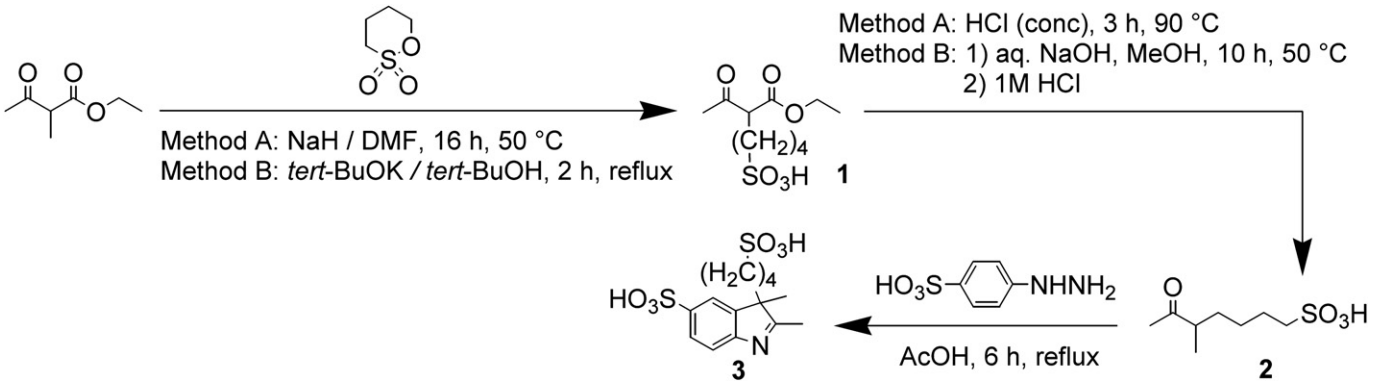
where $\varepsilon_{\mathrm{Dye}}$ is the molar absorptivity of the dye at the long-wavelength maximum, $\varepsilon_{\mathrm{IgG}} = 201{,}700\ \mathrm{M^{-1}\ cm^{-1}}$ is the molar absorptivity of IgG at 278 nm, $x = A_{\mathrm{Dye}(278)}/A_{\mathrm{Dye}(\lambda_{max})}$, $A_{\mathrm{Conj}(\lambda_{max})}$, $A_{\mathrm{Conj}(278)}$, $A_{\mathrm{Dye}(\lambda_{max})}$ and $A_{\mathrm{Dye}(278)}$ are the absorbances of the dye–IgG conjugate and free dye at the long-wavelength maximum and at 278 nm, respectively.

3. Results and discussion

3.1. Synthesis

A synthetic pathway to indole sulfonic acid **3**, a key intermediate for these cyanine dyes, is shown on Scheme 1. In accordance with Ref. [17], alkylation of ethyl 2-methyl-3-oxobutanoate by 1,4-butane sultone with sodium hydride in DMF followed by hydrolysis and decarboxylation of the intermediate sulfoalkyl derivative **1** under reflux in concentrated hydrochloric acid gives ketone **2**, which requires purification by column chromatography (Method A). By this way ketone **2** could be synthesized with a 79% yield starting from ethyl 2-methyl-3-oxobutanoate.

In our attempt to synthesize intermediate **1**, potassium *tert*-butoxide was used in the above alkylation step (Method B). As a result the reaction time was reduced from 16 to 2 h. The hydrolysis of **1** with alkaline followed by decarboxylation in acidic medium gives 5-methyl-6-oxoheptane-1-sulfonic acid 2 with 90% yield. Importantly, the obtained ketone **2** can be introduced in the next step without



Scheme 1. Synthesis of 2,3-dimethyl-3-(4-sulfobutyl)-3*H*-indole-5-sulfonic acid.

purification and reacts with 4-hydrazinobenzenesulfonic acid in acetic acid according to Fisher's method [17,26] in high yield.

The indole sulfonic acid **3** was transformed to the dipotassium salt **4** and quaternized by an excess of 1,4-butane sultone and 6-bromohexanoic acid in 1,2-dichlorbenzene to quaternary salts **5a** and **5b**, respectively, while the same salt **4** being refluxed with iodoethane in acetonitrile yielded quaternary salt **5c** (Scheme 2). The products **5a**–**5c** were obtained from dipotassium salt **4** with 50%, 44%, and 32% yields, respectively. These compounds can be synthesized also by melting of reagents without solvent (**5a**) or using tetramethylene sulphone as the solvent (**5b** and **5c**) [17]. The quaternary salts **5d**–**5h** were obtained according to Refs. [6,18,23].

The synthesis of the mono-reactive unsymmetrical cyanine dyes is presented in Scheme 3. In the first step the quaternized indolenine is reacted with *N*,*N*′-prop-1-en-1-yl-3-ylidenedianiline hydrochloride and the obtained 1,3-butadienyl-*N*-phenylacetoamide is condensed with a 2nd quaternized indolenine. We introduced the sulfoindolenines **5a**–**5h** in the reaction in such order to obtain the widest range of dyes by using the smallest number of 1,3-butadienyl-*N*-phenylacetoamide precursors. The condensation of indolenines **5b**, **5d** and **5f**–**5h** with *N*,*N*′-prop-1-en-1-yl-3-ylidenedianiline hydrochloride in a mixture of acetic acid with acetic anhydride yielded the corresponding 1,3-butadienyl-*N*-phenylacetoamides **6b**, **6d** and **6f**–**6h** (Scheme 3, Table 1). Synthesis of compounds **6d** and **6f**–**6h** was described also in Ref. [18].

The substituents in both the 1,3-butadienyl-*N*-phenylacetoamides **6b**, **6d** and **6f**–**6h** and indolenines **5a** and **5c**–**5f** were found to have a strong influence on the reaction conditions to the final cyanine dye: 1,3-butadienyl-*N*-phenylacetoamides were reacted with indolenines **5c**–**5f** containing two sulfo groups at reflux in acetic anhydride with pyridine (1:1) as described in Ref. [6] and yielded cyanines **7**–**9** and **13**–**16** with satisfactory yields. However, our attempts to synthesize cyanines **10**–**12** *via* reaction of 1,3-butadienyl-*N*-phenylacetoamides **6b**, **6d** and **6h** with indolenine **5a** containing three sulfo groups failed under these conditions. Moreover, addition of DMF, which was supposed to help in certain cases [18] had no effect on the reaction. No detectable amounts of the aimed products were obtained under both conditions.

The fact that the indolenine **5a** did not react under these conditions is most likely due to its low solubility in the reaction mixture. In contrast to the potassium disulfoindolenines **5c**–**5f** and sulfoindolenine **5h**, the dipotassium salt **5a** is totally insoluble in a mixture of acetic anhydride—pyridine even upon addition of DMF. At the same time indolenine **5a** is highly soluble in acetic acid. Indeed, adding acetic acid to the reaction mixture facilitated the reaction of 1,3-butadienyl-*N*-phenylacetoamides **6b**, **6d** and **6h** with the indolenine **5a** and cyanines **10**–**12** were synthesized with satisfactory yields. The acetic acid concentration also was found to have a strong impact on the reaction rate: cyanine products were only isolated when the reaction mixture contained at least equal amounts of acetic acid compared to acetic anhydride.

Importantly the solubility of the starting materials can be increased also by protonation of the sulfonic acid group using ion exchange chromatography. Indolenine **5a** with protonated sulfo groups showed much higher solubility in the reaction mixture (acetic anhydride/pyridine) compared to the dipotassium salt of **5a** and cyanines **10**–**12** were obtained with satisfactory yields even in absence of acetic acid.

Thus in this paper we synthesized new cyanine dyes **7**, **9**–**11**, **15** and **16**. The structures of the cyanines were confirmed using ^{1}H NMR (DMSO-d_6). The pentamethine chain is characterized by the triplet signal of two β,β′-hydrogens at 8.34–8.38 ppm, triplet signal of γ-hydrogen at 6.55–6.61 ppm, and two doublet signals of α,α′-hydrogens at 6.33–6.43 ppm. The triplet signals of two α-methylene hydrogens nearby to carboxylic groups are at 2.20 ppm for cyanines **8**–**10**, **12**, and **13** and 2.06 ppm for **7**, **11** and **14**–**16**.

3.2. Spectral properties

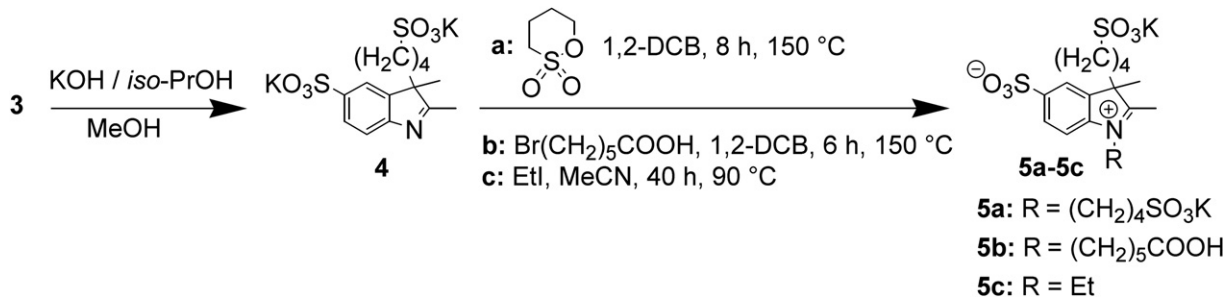
All investigated cyanine dyes **7**–**16** contain the same 5-sulfoindolenine moieties as the end groups but differ by the number and position of sulfoalkyl groups and also in regards to the position of carboxypentyl substitution. The majority of cyanines (dyes **7**–**13** and **16**) contain sulfobutyl groups while cyanines **14** and **15** differ by the length of the sulfoalkyl chains: **14** contains two sulfopropyl and **15** one sulfopropyl and one sulfobutyl group.

The spectral properties of the cyanine dyes such as the absorption and emission maxima (λ_{max}), molar absorptivities (ε), and quantum yields (Φ_F) measured free in aqueous phosphate buffer (pH 7.4) and after binding to IgG at different dye-to-protein ratios (D/P = 1 and 4) are given in Table 2. The dyes in the table are listed by order of increasing numbers of sulfoalkyl groups, which was found to correlate well with the long-wavelength shift of the absorption and emission maxima.

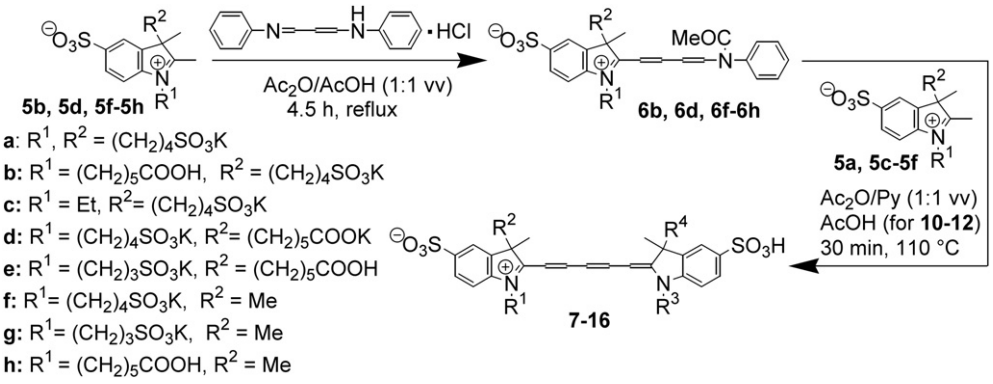
3.2.1. Free dyes

The concentration dependent spectral characteristics of the free dyes were investigated in the concentration range between 1×10^{-8} M and 5×10^{-5} M. Within the measured concentration range a linear correlation of the absorbances *vs.* concentrations was found, which evidenced no aggregation tendency within the investigated concentration range for the investigated cyanines. As a result the data in the Table 2 measured for the free dyes at $c \sim 1-3 \times 10^{-7}$ M can be attributed to non-aggregated cyanines.

The absorption and emission maxima ranged between 647–653 nm and 665–672 nm, respectively, are slightly dependent on the dyes structure: a tendency of the red-shift of the absorption and emission maxima in the order of **Cy5** < **13** < **16** < **9** ≈ **10** < **7** ≈ **8** < **11** ≈ **12** was found to correlate well with the increase of the number of sulfobutyl groups. The most pronounced red-shift (6–7 nm) was noted for cyanines **11** and **12** containing the three sulfobutyl groups. The position of sulfobutyl and carboxypentyl



Scheme 2. Quaternization of 2,3-dimethyl-3-(4-sulfobutyl)-3*H*-indole-5-sulfonic acid.



Scheme 3. Synthesis of cyanine dyes.

groups in the dyes pairs (**9** and **10**), (**7** and **8**), and (**11** and **12**) as well as the shortening of the sulfoalkyl chain in the order of **16** > **15** > **14** have almost no effect on the spectral maxima.

The molar absorptivities (ε) are in the range of 240,000–260,000 M^{-1} cm^{-1} and constant for each dye within the concentration at least between 1×10^{-8} M and 5×10^{-5} M. The molar absorptivities have demonstrated no clear correlation on the cyanine dye structures.

The absorption and emission spectra exhibit a well-defined shoulder (Fig. 1), which is known to be attributed to vibrational transitions [27,28]. The shapes of the absorption and emission spectra of the investigated dyes measured in a 0.1 cm cell are unchanged within the concentrations between 1×10^{-8} M and 5×10^{-5} M regardless of the number and position of sulfoalkyl groups, which indicates no aggregation in this concentration range. Fig. 1 shows that the shapes of the spectra of cyanine **12** with three sulfobutyl substituents are about the same as for **Cy5** containing no sulfoalkyl groups. Independent of the number and position of sulfoalkyl groups, the shape and position of the absorption and emission spectra of all the investigated cyanines measured at $c \sim 1 \times 10^{-8}$ M – 5×10^{-5} M is very similar (Fig. 1). This is evidence that the dye molecules do not aggregate but also exhibit no noticeable conformational changes and solvation effects in the excited state. The Stokes' shifts are between 18 and 19 nm ($\sim$400 cm^{-1}) and independent on the positions of carboxypentyl and sulfobutyl substituents on the indolenine ring and the sulfoalkyl chain length.

Due to the observed high inner filter effects in both the excitation and the emission paths at concentrations above $\sim 3 \times 10^{-7}$ M that can be attributed to the small Stokes shift of the dyes, and the pronounced overlap between the absorption and emission bands the emission spectra and quantum yields (Φ_F) were measured using 0.1 cm cuvettes and at low dye concentrations.

Increasing the number of sulfo groups from two (**Cy5**) to five (cyanine **12**) (Table 2) in general leads to an increase of the quantum yields from 27% to 32%, while the reduction of the sulfoalkyl chain length in the order of **16** > **15** > **14** did not have a noticeable impact on the quantum yield.

Table 1
The substituents in the dyes **7**–**16**.

Dye	R^1	R^2	R^3	R^4
7	$(CH_2)_4SO_3H$	$(CH_2)_5COOH$	Et	$(CH_2)_4SO_3H$
8	$(CH_2)_5COOH$	$(CH_2)_4SO_3H$	Et	$(CH_2)_4SO_3H$
9	$(CH_2)_5COOH$	$(CH_2)_4SO_3H$	$(CH_2)_4SO_3H$	Me
10	$(CH_2)_5COOH$	Me	$(CH_2)_4SO_3H$	$(CH_2)_4SO_3H$
11	$(CH_2)_4SO_3H$	$(CH_2)_5COOH$	$(CH_2)_4SO_3H$	$(CH_2)_4SO_3H$
12	$(CH_2)_5COOH$	$(CH_2)_4SO_3H$	$(CH_2)_4SO_3H$	$(CH_2)_4SO_3H$
13	$(CH_2)_5COOH$	Me	$(CH_2)_4SO_3H$	Me
14	$(CH_2)_3SO_3H$	Me	$(CH_2)_3SO_3H$	$(CH_2)_5COOH$
15	$(CH_2)_4SO_3H$	Me	$(CH_2)_3SO_3H$	$(CH_2)_5COOH$
16	$(CH_2)_4SO_3H$	Me	$(CH_2)_4SO_3H$	$(CH_2)_5COOH$

Due to the minimal aggregation tendency of the investigated cyanine dyes in aqueous solutions at dye concentrations below 5×10^{-5} M, the increase in quantum yields with increasing number of sulfo groups cannot be explained by the increased solubility of the dye molecules but is more likely due to other reasons. The electron withdrawing effect of sulfo groups could be considered as one of these reasons: simulations of the electron density in a model diethyl (**C1**), dibutyl (**C2**), and di-(sulfobutyl) (**C3a**) cyanines, carried out by the PM3 method (Fig. 2), demonstrate that substitution of the ethyl and butyl groups with sulfobutyl groups results in a rearrangement of the electron density in such a way that the positive charge at indolenine nitrogen increases from +0.300 (**C1**) and +0.303 (**C2**) up to +0.351 (**C3a**). The simulations indicate two possible reasons for this effect: protonation of aromatic sulfo groups and a direct electron withdrawing effect of the aliphatic sulfo-group on the indolenine nitrogen by inductive ($-I$) mechanism, which is further enhanced by the field effect through space.

While the aromatic sulfo group of the diethyl and dibutyl derivatives **C1** and **C2** more likely exists in the deprotonated state, the protonated form **C3b** seems to be the dominant form for di-sulfobutyl-cyanines. According to our calculations form **C3a** with deprotonated aliphatic sulfo groups is substantially more stable (42 kcal/mol) compared to that of **C3b** (Fig. 2). The positive charge on indolenine nitrogen in **C3a** (+0.321) increases to +0.351 in **C3b**. Furthermore the aliphatic sulfo group has a direct electron withdrawing effect on the indolenine nitrogen. The value of this effect can be estimated *via* the positive charge on the indolenine nitrogen, which increases from +0.300 to +0.303 in the diethyl (**C1**) and dibutyl (**C2**) derivatives, respectively, to +0.321 in the di-(sulfobutyl) cyanine **C3b**. It seems that the above electron withdrawing effect propagates not only inductively ($-I$), evidenced by the electronic charge redistribution on the carbon atoms within the alkyl chain (Fig. 2), but also due to the electrostatic interaction (attraction) between negatively charged sulfonyl oxygen and positively charged indolenine nitrogen. The "field" interaction is supported by the fact that the straight conformation of sulfobutyl chain with *s-trans* allocation of all alkyl chain bonds in **C3a** and **C3b** (just this conformation realizes in dibutyl derivative **C2**) becomes energetically unstable. At the same time **C3a** and **C3b** were found to exist in the folded conformation, where the sulfonyl oxygen is approaching the indolenine nitrogen at a distance of about 3.8 Å in **C3a** and 4.0 Å in **C3b** (Fig. 2).

Aliphatic sulfo groups are considered electron withdrawing groups and expected to reduce the electronic charge on indolenine nitrogen. In general electron withdrawing substituents at the

Table 2
Spectral characteristics of cyanine dyes ($c \sim 1–3 \times 10^{-7}$ M) and dye–IgG conjugates in phosphate buffer pH 7.4.

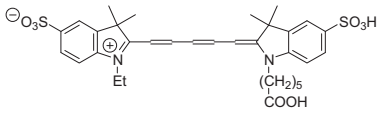
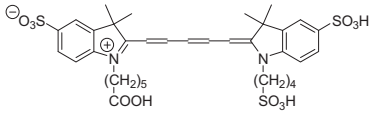
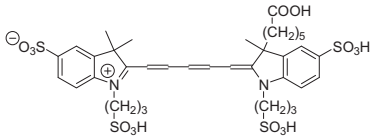
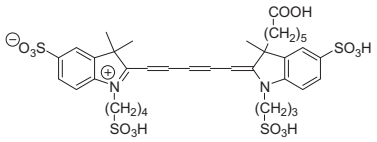
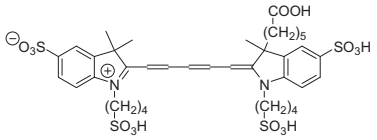
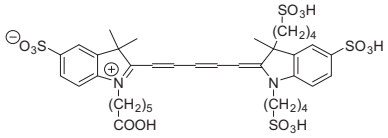
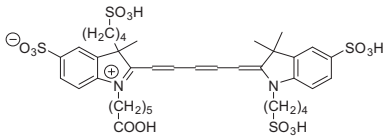
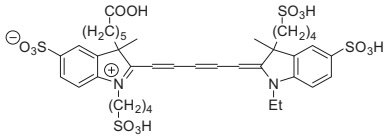
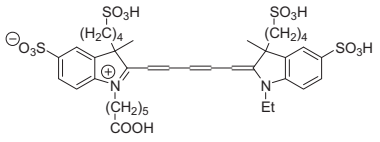
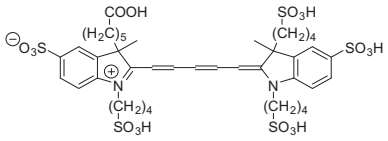
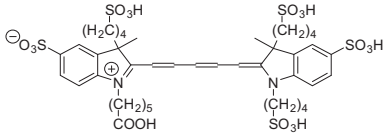
Dye		Free dye				Dye IgG conjugate			
						D/P = 1			D/P = 4
		λ_{max} (Abs), nm	$\varepsilon \times 10^{-3}$, M^{-1} cm^{-1}	λ_{max} (Em), nm	Φ_F, %	λ_{max} (Abs), nm	λ_{max} (Em), nm	Φ_F, %	Φ_F, %
Cy5		647	250	665	27	651	670	28	9
13		648	260	667	28	651	670	33	15
14		649	240	668	32	652	670	35	22
15		649	240	668	32	652	670	40	25
16		649	240	668	32	653	671	38	25
10		650	250	669	30	654	672	38	25
9		650	256	669	32	654	672	39	25
7		652	252	670	28	653	672	35	25
8		652	242	670	28	655	673	35	28

Table 2 (*continued*)

Dye		Free dye				Dye IgG conjugate			
		λ_{max} (Abs), nm	$\varepsilon \times 10^{-3}$, M^{-1} cm^{-1}	λ_{max} (Em), nm	Φ_F, %	D/P = 1			D/P = 4
						λ_{max} (Abs), nm	λ_{max} (Em), nm	Φ_F, %	Φ_F, %
11		653	250	672	31	655	673	38	32
12		653	248	672	32	655	674	43	36

heterocyclic end groups are known to increase the quantum yields of cyanine dyes [29,30], which is in good agreement with our observations (Table 2). The reduced quantum yields of cyanines **7** and **8** could be explained by electron-donating effect of ethyl group on indolenine nitrogen.

The fluorescence lifetimes (τ) of cyanines **7**–**16** were measured to be in the range between 1.00 and 1.24 ns (Table 3). There is a trend of increased lifetimes with an increasing number of sulfobutyl groups in the molecules in the order of **Cy5** < **13** < **16** ≈ **10** ≈ **9** < **11** < **12**. The sulfoalkyl chain length in cyanines **14**–**16** does not have a noticeable effect on the fluorescence lifetime.

The photostabilities of cyanine dyes were estimated *via* their photobleaching half-life ($\tau_{1/2}$), which is the time to decrease the absorption and emission intensity by half upon irradiation. Increasing the number of sulfobutyl groups noticeably increases photostability in the order: **Cy5** < **13** ≤ **10** ≤ **9** < **16** ≤ **8** ≈ **7** < **12** ≈ **11**. The photostability increases by factor 2.2–2.3 (Table 4), when the total number of sulfo groups increases from two (**Cy5**) to five (cyanines **11** and **12**). The position of sulfobutyl and carboxypentyl groups on the cyanine molecule seems to have less of an effect on the photostability.

Importantly a shortening of the sulfoalkyl chain from sulfobutyl in cyanine **16** to sulfopropyl (**14**) remarkably increases photostability by about 30% (Table 4, Fig. 3). The effect of the alkyl chain length on the photostability can be explained by different electron withdrawing influence of sulfobutyl and sulfopropyl groups on the electron distribution on cyanine dye molecules. The photostability of cyanine dyes is known to increase upon introduction of electron withdrawing substituents into the heterocyclic end groups [9,31]. Indeed quantum chemical simulations show that the sulfopropyl group in the model cyanine **C4** has a stronger electron withdrawing effect in particular on indolenine nitrogen and polymethine chain α-carbon atom as compared to sulfobutyl group in **C3a** (Fig. 2).

The electron withdrawing effect and the different impact of sulfopropyl and sulfobutyl substituents is revealed also from the ^{1}H NMR data (200 MHz, DMSO-d_6): The hydrogen signals of α-methylene NCH_2 group at indolenine nitrogen exhibit different chemical shifts (δ) depending on how far they are from the electron-withdrawing sulfo group: 4.25 ppm for sulfopropyl cyanine **14** and 4.10 ppm for sulfobutyl cyanine **16**, while cyanine **15** has the two signals: 4.26 ppm and 4.10 ppm. The same effect of the sulfoalkyl chain length on the hydrogen chemical shifts of the indolenines precursors **5g**/**5f**: NCH_2, 4.60/4.46 ppm, triplet; 2-CH_3, 2.80/2.84 ppm, singlet; 3-$(CH_3)_2$, 1.52/1.54 ppm, singlet.

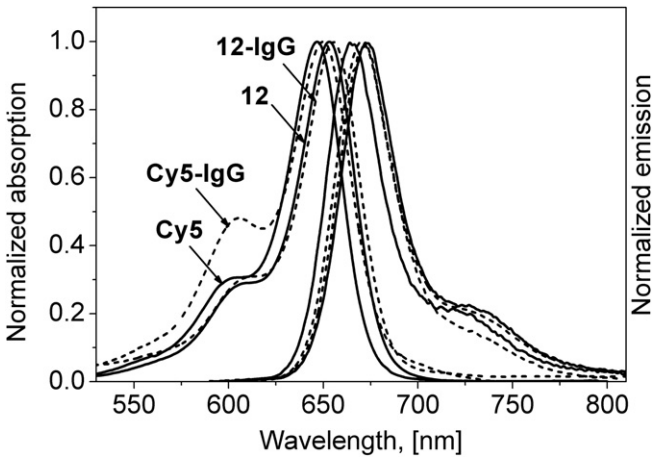


Fig. 1. Absorption and emission spectra of **Cy5** and cyanine **12** free in solution at concentrations 1×10^{-8} M and 5×10^{-5} M (solid line) and bound to IgG at D/P = 4 (dashed line).

3.3. Dye IgG conjugates

Upon conjugation of cyanine dyes to antibodies (IgGs) the absorption and emission maxima are typically shifted by up to 5 nm. This trend is more pronounced for less hydrophilic cyanines such as **Cy5** and **13** containing lower number of sulfoalkyl groups (Table 2). Similar to free dyes, an increase in the number of sulfobutyl groups results in a red-shift of up to 4 nm. The dye-to-protein (D/P) ration seems to have almost no effect on the spectral maxima.

The absorption spectra of cyanines show *absorption of the dye aggregates* which are typically blue-shifted compared to the absorption maximum (Fig. 1) and its intensity increases with increasing D/P ratios. These aggregation bands with maxima around 605 nm are well-defined for **Cy5**–IgG conjugates at D/P = 4, but are strongly reduced for more hydrophilic cyanines such as **12**.

The quantum yields (Φ_F) of dye-conjugates are known to be strongly dependent on the dye-to-protein ratio (D/P): in general an increased D/P is accompanied with a decrease in Φ_F (Fig. 4) but the decrease in quantum yields becomes less pronounced for dyes with an increased number of sulfobutyl groups (Table 2). The decrease in Φ_F is most likely due to the interaction of dye molecules on the protein, which can lead to the formation of non-fluorescent or low-fluorescent aggregates and or homo-FRET (fluorescence resonance

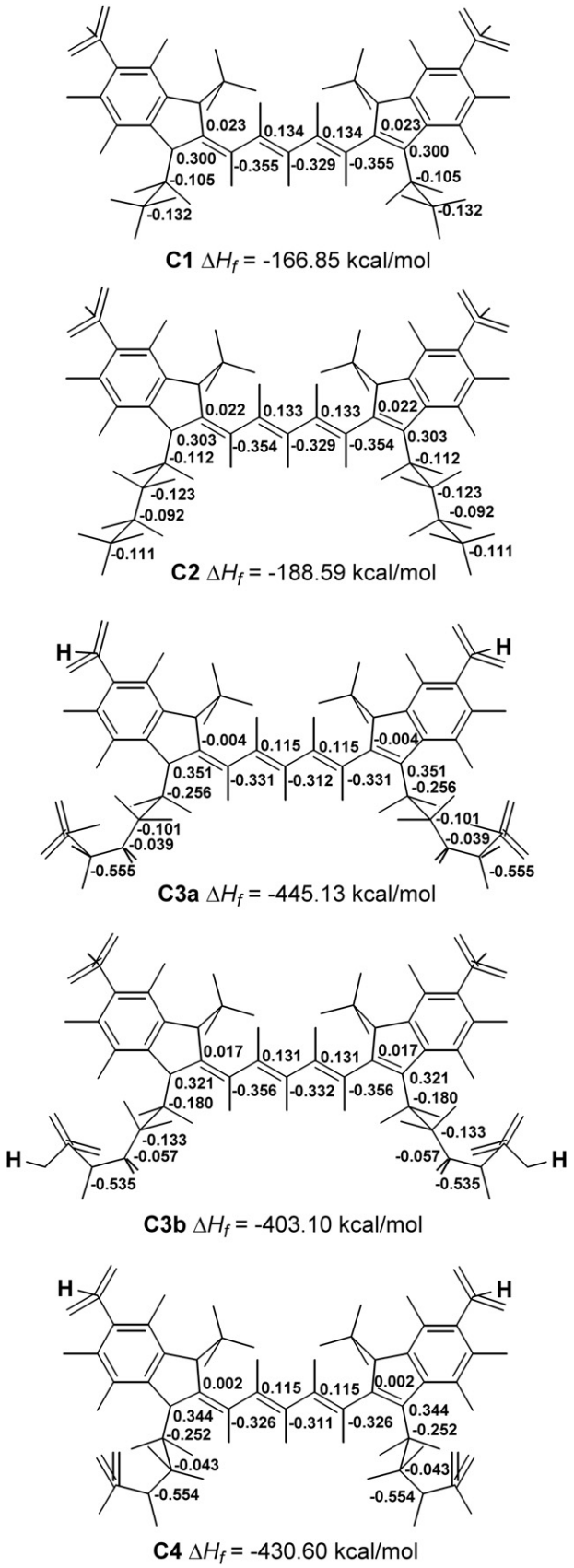


Fig. 2. The PM3 calculated conformational structures, electron charges, and enthalpies of formation (ΔH_f) of the model cyanine molecules.

Table 3
Fluorescence lifetimes (τ) of cyanine dyes in phosphate buffer (pH 7.4).

Dye	τ, ns	χ^2
Cy5	1.00	1.20
13	1.04	1.07
14	1.11	1.33
15	1.10	1.38
16	1.11	0.91
10	1.11	1.48
9	1.12	1.17
7	1.05	1.62
8	1.06	0.92
11	1.18	1.22
12	1.24	1.13

Table 4
Photostability ($\tau_{1/2}$) of cyanine dyes ($c \sim 10^{-7}$ M) in aqueous solutions.

Dye	($\tau_{1/2}$), min	
	Absorption	Emission
Cy5	103	114
13	132	152
14	247	235
15	270	237
16	185	176
10	144	152
9	144	170
7	190	210
8	185	204
11	237	263
12	230	258

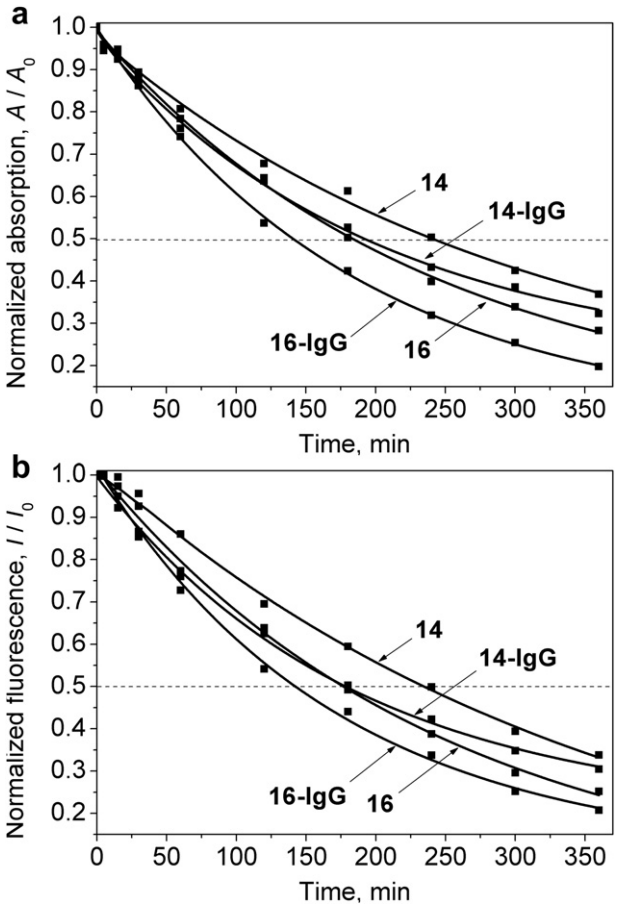


Fig. 3. Decrease of the long-wavelength absorption (a) and emission (b) band of cyanine dyes and their IgG conjugates under light exposure *vs.* the sulfoalkyl chain length.

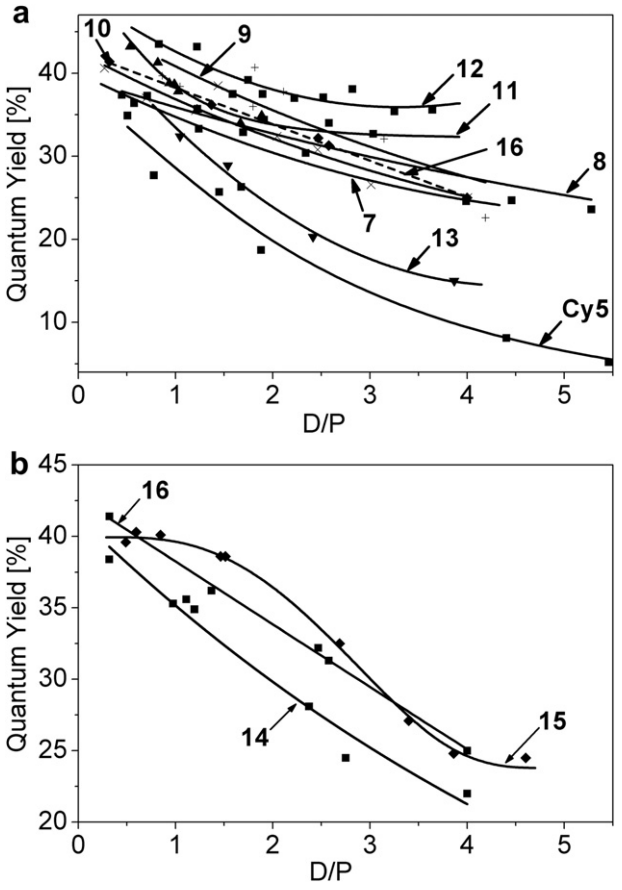


Fig. 4. Quantum yields of the IgG conjugated cyanines with different dye-to-protein ratio *vs*. the number (a) and length (b) of sulfoalkyl groups.

energy transfer) between the fluorophores that are covalently attached to the antibody molecule [32–34].

While *the quantum yields* of cyanine dyes when free in solutions are only slightly dependent on the number and position of sulfobutyl groups, the quantum yields of the IgG conjugates seem to have a more pronounced dependency on the number of sulfo groups (Table 2). This effect is more pronounced at high D/P. As a result, the plots of the Φ_F *vs*. D/P for more hydrophilic cyanines are less steep as compared to the less hydrophilic cyanines (Fig. 4a). The quantum yields of the IgG conjugated cyanines **11** and **12** are increased by a factor of 1.4 and 1.5 compared to **Cy5**–IgG at D/P = 1 but increased by a factor of 3.6 and 4.0, at D/P = 4. The IgG conjugated cyanines **7** and **8** containing ethyl group at indolenine nitrogen have lower Φ_F compared to other cyanines with the same number of sulfo groups. The highest quantum yields were obtained for cyanines **11** and **12** containing three sulfobutyl groups. *The quantum yields* of the IgG conjugates increase in the order of **14** < **16** ≤ **15** (Table 2, Fig. 4b).

The quantum yield increase of immobilized cyanine dyes is influenced by a change of the dyes microenvironment due to the decreased hydrophilicity and polarity of protein microenvironment [28,35,36] but also the reduced conformational flexibility and the *cis–trans*-isomerization tendency [37,38].

4. Conclusion

A series of monoreactive, unsymmetrical indodicarbocyanine dyes containing two aromatic and up to three aliphatic sulfo groups was synthesized and investigated. The increase in the number of sulfobutyl groups has a strong influence on the aggregation tendency of the dyes upon covalent labeling to IgG resulting in increased quantum yields and photostabilities of the IgG-conjugates.

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