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Comparison of water-soluble squaraine and norsquaraine as fluorescent material for biomedical applications

***I.V.Hovor*[1], *O.S.Kolosova*[1], *O.M.Obukhova*[1], *A.L.Tatarets*[1], *L.D.Patsenker*[2]**

[1]SSI "Institute for Single Crystals", STC "Institute for Single Crystals", National Academy of Sciences of Ukraine, 60 Nauky Ave., 61072 Kharkiv, Ukraine
[2]Department of Chemical Sciences, The Faculty of Natural Sciences, Ariel University, 40700 Ariel, Israel

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Spectral and photophysical properties of norsquaraine dye Nor–Sq were investigated and compared with those for 3-carboxypentyl substituted squaraine Sq in aqueous buffer solutions, organic solvents and under interactions with proteins in order to obtain fluorescent labels for proteins with improved properties. Both dyes absorb and fluoresce in the same spectral region, but Sq has higher the molar absorptivity and is much more sensitive to polarity and viscosity of the medium, as well as to the presence of proteins. The absence of a substituent at the nitrogen atom in norsquaraine molecule provides the pH-sensitivity of Nor–Sq in the range of 9.0–11.4. As a result, Sq is more preferable for using in the most of biomedical applications, where interaction with proteins increases the brightness of the dye due to changes of polarity and/or viscosity of environment, while Nor–Sq is applicable for pH monitoring and in assays, where signal should not depend on changes in dye molecule environment.

Keywords: norsquaraines, squaraines, protein conjugates, fluorescent pH-sensors.

Порівняння водорозчинних скваратного та норскваратного барвників як флуоресцентних матеріалів для медико-біологічних застосувань. *І.В.Говор, О.С.Колосова, О.М.Обухова, А.Л.Татарець, Л.Д.Паценкер*

З метою отримання флуоресцентних міток для білків з покращеними властивостями досліджено та порівняно спектральні та фотофізичні властивості норскваратного барвника Nor–Sq з властивостями 3-карбоксипентилзаміщенного скваратна Sq у водних буферних розчинах, органічних розчинниках і при взаємодії з білками. Обидва барвники поглинають і флуоресціюють в одному спектральному діапазоні, але Sq має більш високий коефіцієнт екстинкції і є набагато більш чутливим до полярності і в'язкості середовища і присутності білків. Відсутність замісника у атома азоту в молекулі норскваратну забезпечує pH-чутливість Nor–Sq у діапазоні 9,0–11,4. В результаті Sq є більш переважним для використання у більшості медико-біологічних досліджень, де взаємодія з білками збільшує яскравість барвника через зміни полярності і в'язкості середовища, в той час як Nor–Sq є прийнятним для моніторингу pH і в аналізах, де сигнал не повинен залежати від зміни оточення молекули барвника.

С целью получения флуоресцентных меток для белков с улучшенными свойствами исследовались и сравнивались спектральные и фотофизические свойства норскваинового красителя Nor–Sq со свойствами 3-карбоксипентилзамещенного сквараина Sq в водных буферных растворах, органических растворителях и при взаимодействии с белками. Оба красителя поглощают и флуоресцируют в одной и той же области спектра, но Sq имеет

более высокий коэффициент экстинкции и гораздо более чувствителен к полярности и вязкости среды и к присутствию белков. Отсутствие заместителя у атома азота в молекуле норскварайна обеспечивает pH-чувствительность Nor–Sq в диапазоне 9,0–11,4. Показано, что Sq более предпочтителен для использования в большинстве медико-биологических исследований, где взаимодействие с белками увеличивает яркость красителя из-за изменения полярности и/или вязкости среды, в то время как Nor–Sq лучше применять для мониторинга pH и в анализах, где сигнал не должен зависеть от изменения окружения молекулы красителя.

1. Introduction

Squaraines, a subclass of cyanine dyes, whose structure includes 3-oxocyclobuten-1-olate moiety (squaric acid residue) in the polymetine chain as well as the terminal heterocyclic moieties, have found a wide field of applications in biomedical investigations [1–3]. Squaraine dyes comprising indolenine based terminal end groups are of the highest interest, in particular, for biomedical research due to their increased brightness and photostability [4]. Another advantage of squaraines is their red or near infrared (NIR) absorption, which is found in biological "transparency window" and possibility of use for excitation the cheap and easily available 633 or 640 nm lasers [5]. This allows getting minimal interference with biological tissue absorption and autofluorescence, especially for in vivo application. Squaraines may be substituted with various functional groups at the indolenine nitrogens [6–8] as well as remain unsubstituted as in the case of so-called norsquaraines [9]. The last ones arouse the increased interest due to their applicability as fluorescent pH-probes. Recently there was reported the comparative analysis of squaraines versus norsquaraines [10]. According to the results of the comparison of spectral properties of these two subclasses of squaraine dyes, norsquaraines exhibit a substantially reduced sensitivity to the microenvironment and to the presence of proteins and other biomolecules in solution. Thus it was assumed that norsquaraines due to low non-specific interactions might be useful for development of assays based on a specific interaction of the dye-biomolecule conjugates with biological counterparts in heterogeneous biological media, where the presence of non-targeting proteins and other large-molecular-weight biomolecules could affect the assay. But squaraines as well as norsquaraine dyes under investigation in [10] are hydrophobic and non-reactive, so they can not be bound to proteins covalently and their strong aggregation in aqueous media does not allow using them for investigation of dye-protein interactions. The work [9] describes the water-soluble norsquaraines, some of them containing carboxy-groups were conjugated to protein, and compares their spectral properties with conventional squaraines. Due to the relevance of water-soluble pH-sensitive norsquaraines we have chosen one of them (Nor–Sq) to be the object of the present research. Nor–Sq has functionality for binding to proteins because it contains carboxypentyl groups in position 3 of indolenine moieties [9] (Fig. 1). It is known that dyes with carboxypentyl group in position 3, like pentamethinecyanine dye Alexa Fluor 647, have superior properties when used in conjugates with proteins as compared to a dye with this group in position 1, like Cy5 [11–13].

So the aim of this work was to compare the spectral and photophysical properties of norsquaraine label Nor–Sq versus 3-carboxypentyl substituted squaraine Sq (Fig. 1) to show their applicability for labeling of proteins in biomedical research. In this field the sensitivity of squaraine and norsquaraine to the microenvirenment, and their behavior towards proteins were investigated.

2. Experimental

Synthesis. The dyes Nor–Sq and Sq were synthesized according to known procedures [9, 14].

Bovine serum albumin (BSA, essentially fatty acid free, fraction V, reagent grade ≥96 %) and bovine immunoglobulin G (IgG), Sephadex G50 were purchased from Sigma and used without further purification. Solvents, salts for buffers and other materials were from *Merck* and used as is.

The phosphate buffer pH 7.4 (67 mM) (PB) was prepared by dissolving $Na_2HPO_4 \cdot 2H_2O$ (9.596 g) and KH_2PO_4 (1.743 g) in 1 L of Milli-Q water. Bicarbonate buffer of pH 9.0 (0.1 mM) was obtained by dissolving $NaHCO_3$ (8.401 g) in 1 L of Milli-Q water.

Spectral. All the absorption spectra were recorded in 1 cm quartz cells at 25°C using a PerkinElmer Lambda 35 UV/Vis spectrophotometer. Absorption maxima were determined with an accuracy of ±0.5 nm and rounded off.

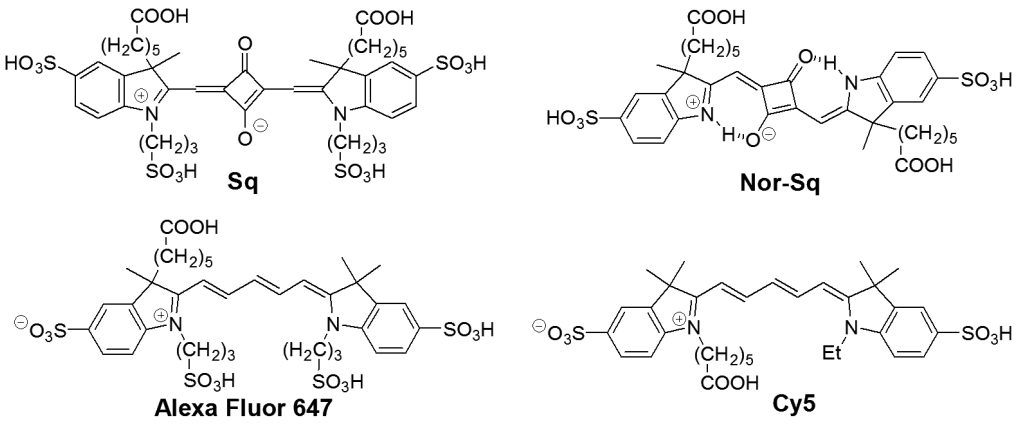

Fig. 1. The molecular structure of norsquaraine Nor–Sq, squaraine Sq, and pentamethinecyanine dyes Alexa Fluor 647 and Cy5.

The fluorescence measurements were done in 1 cm standard quartz cells at 25°C using a Varian Cary Eclipse spectrofluorometer. All emission spectra were corrected by the spectral sensitivity of the PMT. Emission maxima were determined with an accuracy of ±1.0 nm.

Quantum yields were measured in a standard 1 cm quartz cell on a Varian Cary Eclipse spectrofluorometer at 25°C for the solutions of free dyes in different solvents, for dye-protein complexes and conjugates in PB. All dyes were excited at 620 nm (λ_{ex}), with an excitation slit width of 5 nm and emission slit width of 2.5 nm. All fluorophore concentrations were adjusted to be ~ 0.5 μM.

The quantum yields (QYs) were calculated according to Eq. (1) [15] using Cy5 in PB as reference (Φ_F = 27 %) [16].

$$\Phi_F = \Phi_{FCy5} \cdot (F/F_{Cy5}) \cdot (A_{Cy5}/A) \cdot (n^2/n^2_{Cy5}), \quad (1)$$

where Φ_{FCy5} is the quantum yield of Cy5, F_{Cy5} and F are the integrated areas of the fluorescence spectra ($F = I(\lambda)d\lambda$) of Cy5 and the solution under investigation; A_{Cy5} and A are the absorbencies at the excitation wavelength (620 nm) of Cy5 and the sample under examination; n_{Cy5} and n are the refractive indices of PB and the solvent, where the dye under examination is dissolved, correspondingly.

The QY of each sample was independently measured 3–4 times and the average value was calculated. The quantum yields of the dye-protein conjugates were measured for various dye-to-protein ratios (D/P) and then the QY for $D/P = 1$ was determined by a non-linear interpolation.

Molar absorptivities (ε). The dye (7–10 mg) or protein was dissolved in the PB (50 mL), the obtained stock solution was diluted to the concentration c_{Dye} ~ 0.5 μM and the absorbance (A) at the absorption band maximum was measured in a 5 cm standard quartz cell. The molar absorptivities were calculated according to the Beer-Lambert law. The molar absorptivity for each compound was independently measured three times and the average value was taken. The reproducibility for determining the molar absorptivity was within ±2,000 $M^{-1}cm^{-1}$.

Spectral characteristics of dyes in presence of proteins (dye-protein complexes) were measured at c_{Dye} = 0.5 μM and BSA or IgG concentration 6 mg/mL.

Protein labeling. A dye (1 mg) was dissolved in 100 μL of anhydrous DMF. Then *N*,*N*,*N*′,*N*′-tetramethyl(succinimido)-uronium tetrafluoroborate (TSTU) (0.5 mg) and *N*, *N*-diisopropylethyl-amine (DIPEA) (3 μL) were added and the mixture was stirred at room temperature for 1 h. The progress of reaction was monitored by TLC (Silica gel 60 RP-18, water-acetonitrile). The obtained stock solution of the dye-NHS-ester was used without purification for the further conjugation with proteins. Solutions of BSA (3 mg) or IgG (1 mg) in 0.5 mL of bicarbonate buffer were prepared. Aliquots of the NHS-activated dye stock solution (in the range of 5–50 μL) were added to each protein solution and stirred for 3 h at 25°C to form the dye-protein conjugates. The conjugates were separated from the unbound dye by gel-permeation chromatography on Sephadex G50 (eluent:PB).

Determination of the dye-to-protein ratio (D/P). To calculate the dye-to-protein ratios each conjugate was diluted with PB in such a way that the absorbance at the long-wavelength absorption maxima of the dye-protein conjugate ($A_{conj(\lambda max)}$) was in the range of 0.1–0.2 when measured in a 1 cm quartz cell. Subsequently the absorbencies at the $A_{conj(\lambda max)}$ and at 278 nm ($A_{conj(\lambda 278)}$) were determined. The absorbencies of the free dye at 278 nm ($A_{Dye(\lambda 278)}$) and at the absorption maximum ($A_{Dye(\lambda max)}$) were also taken. The dye-to-protein ratio (D/P) was determined according to the Eq. 2 with the assumption that the molar absorptivities of the dyes were not changed after conjugation to protein [16, 17]:

$$D/P = A_{conj(\lambda max)} \cdot \varepsilon_{protein} / (A_{conj(\lambda 278)}) - \left(\frac{A_{Dye(\lambda 278)}}{A_{Dye(\lambda max)}}\right) \cdot A_{conj\,(\lambda max)} / \varepsilon_{Dye}, \quad (2)$$

where $\varepsilon_{protein}$ is the molar absorptivity of protein at 278 nm (BSA: 43,824 $M^{-1}cm^{-1}$ [18, 19] at 278 nm; IgG: 201,700 $M^{-1}cm^{-1}$, experimentally determined);

ε_{Dye} is the molar absorptivity of dye at the absorption maximum (Nor–Sq: 188,000 $M^{-1}cm^{-1}$; Sq: 290,000 $M^{-1}cm^{-1}$).

Determination of brightness. The brightness of conjugates was calculated according [20]:

$$\text{Total brightness} = \varepsilon \cdot QY \cdot D/P, \quad (3)$$

where ε is the molar absorptivity of dye, QY is quantum yield at the certain dye-to-protein ratio (D/P) of conjugates.

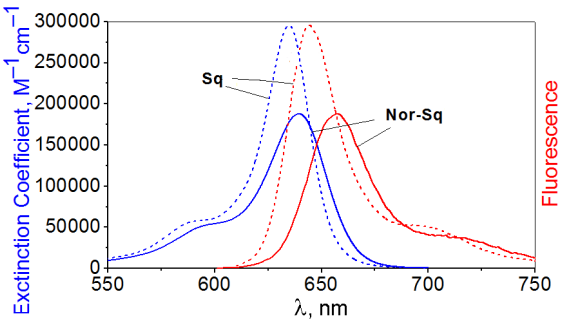

Fig. 2. Absorption and emission spectra of norsquaraine Nor–Sq (solid line) and squaraine Sq (dashed line) in PB (c_{Dye} = 0.5 μM).

3. Results and discussions

3.1. Spectral properties of free dyes

The absorption and emission maxima (λ_{max}), molar absorptivities (ε), quantum yields (Φ_F), Stokes' shifts ($\Delta\nu_{st}$) as well as the absorption and emission bands halfwidths ($\Delta\nu_{1/2}$) of Nor–Sq and Sq in PB, ethanol, methanol and glycerol are summarized in Table 1 while the absorption and emission spectra in PB are shown on Fig. 2. As the indicator of solvent polarity the normalized empirical parameter E_T^N was taken [21].

Sq and Nor–Sq absorb and fluoresce in the red region of spectra where biological objects are mostly transparent [22]. In PB, which is the most commonly used buffer in biological research, absorption and fluorescence maxima are 639/657 nm for norsquaraine Nor–Sq and 635/644 nm for squaraine Sq with molar absorptivities ε = 188,000 $M^{-1}cm^{-1}$ and ε = 290,000 $M^{-1}cm^{-1}$, respectively (Fig. 2). A slight red-shift of Nor–Sq as compared to

Table 1. Spectral characteristics of Nor–Sq and Sq in PB, ethanol, methanol and glycerol (c_{Dye} = 0.5 μM)

Dye	Media	E_T^N	λ_{max}(Ab), nm	λ_{max}(Em), nm	$\Delta\nu_{st}$, cm^{-1}	$\Delta\nu_{1/2}$(Ab), cm^{-1}	$\Delta\nu_{1/2}$(Em), cm^{-1}	ε, $M^{-1}cm^{-1}$	Φ_F, %
Nor–Sq	PB pH 7.4	~ 1	639	657	430	840	810	188,000	33
	Glycerol	0.812	655	672	390	800	750	n/d*	45
	Methanol	0.762	652	668	370	780	740	n/d	37
	Ethanol	0.654	656	673	390	770	730	n/d	36
Sq	PB pH 7.4	~ 1	635	644	220	620	700	290,000	12
	Glycerol	0.812	646	657	260	600	670	n/d	80
	Methanol	0.762	640	649	220	590	640	n/d	40
	Ethanol	0.654	643	653	240	580	660	n/d	48

* not determined

Sq by 4 and 13 nm for absorption and emission respectively (Fig. 2) is perhaps due to the more planar molecular structure of norsquaraine [10]. The absorption and fluorescence spectra of the dyes exhibit a well-defined shoulder (Fig. 2), which can be attributed to the vibrational transitions [23–25]. It is known that squaraines exhibit a strong tendency to form nonfluorescent aggregates, which leads to increase of shoulder in the absorbance spectrum [26] and mirror symmetry breaking. At concentration 0.5 μM of the investigated dyes the absorption and emission bands demonstrate no violation of the mirror symmetry rule, which evidences for no aggregates formation as well as no substantial conformational changes or solvation effects occur in the excited state (Fig. 2). Due to the more rigid and planar structure of norsquaraines as compared to squaraines [10] the decrease of Stokes shift ($\Delta\nu_{st}$) for Nor–Sq was expected, but actually it happens the other way around: Stokes shift of Nor–Sq (430 cm^{-1}) in PB is higher than that for Sq (220 cm^{-1}) (Table 1). The increase of Stokes shift for Nor–Sq is likely due to its molecule higher polarity evaluated from the dipole moment as compared to Sq molecule. The dipole moments of norsquaraines and squaraines of similar structures were investigated in [10] and it was shown, that norsquaraines are presented by more polar conformer. But another and maybe more substantial reason of the increased $\Delta\nu_{st}$ of Nor–Sq is supposedly solvation shell rearrangement or a change in the intramolecular H-bonds between oxygens of squarylium moiety and hydrogens of NH-groups in the excited state.

The half-width ($\Delta\nu_{1/2}$) of the fluorescence band of Nor–Sq is lower by 30 cm^{-1} than that for the absorption band, in the case of Sq the situation is quite opposite — the absorption band half-width is narrower by 80 cm^{-1}. This fact leads us to assume that the rigid structure of norsquaraine molecule becomes even more rigid and planar in the excited state, while squaraine molecule contrariwise becomes less rigid in the excited state resulting in the broadening of emission band half-width.

The quantum yield of Nor–Sq in PB is 2.7 times higher than for Sq and are 33 % and 12 %, respectively.

Sensitivity to microenvironment of the dye is the significant factor to be taken into account when the dye is used for biomedical applications. Interaction with bioobjects usually leads to change in local polarity, hindering the vibrational motion of the dye molecule, while the presence of amino and carboxy-groups in the binding site affects its local acidity. Thus we investigated the influence of media polarity, viscosity and acidity on the spectral properties of Nor–Sq and Sq.

The influence of solvent polarity on the spectral properties of the dyes was investigated by measurements in PB, methanol and ethanol (Table 1). The absorption maxima of dyes are red shifted by 17 nm for Nor–Sq and 8 nm for Sq with a decrease in solvent polarity from water to ethanol. Thereby, both dyes exhibit negative solvatochromism, but in case of Nor–Sq it is more pronounced. As it can be seen from the Table 1, the Stokes shifts as well as the absorption and fluorescence bands half-widths are decreased accordingly to the decrease of the solvent polarity for the both dyes. It is worth to note that all aforementioned solvents are polar and protic, capable to form hydrogen and other intermolecular bonds forming the solvate shell around the dye molecule. The both dye should have higher polarity in the ground state, which is stabilized by polar solvents, as compared to the excited state [27], which is expressed by the negative solvatochromism, while for Nor–Sq changes in polarity (dipole moment) in the excited state is more vivid and leads to more substantial solvate shell rearrangement. As stated above this can be also the reason for the larger Stokes shift of Nor–Sq. Another possible explanation for the broadening of Nor–Sq spectra and almost twice as large Stokes shift can be the intramolecular proton transfer between indolenine nitrogen and squarate oxygen, which happens in the excited state.

As it is known, squaraines are quenched in polar solvents [26, 28], so fluorescence quantum yields of the dyes increase from polar aqueous buffer to solutions in less polar environment like methanol and ethanol. In the case of Sq the fluorescence quantum yield value increases by 3.3 times in methanol and by 4 times in ethanol, compared to its quantum yield in PB. While the quantum yield of Nor–Sq upon moving to a less polar medium is changed only by ~ 1.1 times, thus it remains almost unchanged. In this way, the polarity of the medium, unlike in case of squaraines, does not affect the quantum yield of norsquaraines.

Thus we can conclude that Nor–Sq is more sensitive to polarity of the media by

absorption and emission band maxima shifts, while Sq shows more significant changes in the quantum yields.

Viscosity-dependent spectral behavior of the dyes was investigated in glycerol, which has similar polarity as methanol (0.812 *vs.* 0.762) [21] but much higher viscosity — 1400 mPa·s *vs.* 0.6 mPa·s for methanol, and 0.9 mPa·s for aqueous buffer. The increase in the viscosity of the solvent causes the increase in the quantum yields of both dyes, but Sq revealed to be more sensitive to viscosity changes than Nor–Sq: the quantum yield of Sq in glycerol is 80 %, which is twice higher than in methanol, while the quantum yield of Nor–Sq in glycerol increases only in 1.2 times as compared to methanol. The lower increase of Nor–Sq quantum yield in highly viscous media could be related to the presence of intramolecular H-bonds between the proton of NH-group and the oxygen atom of the squaric acid moiety, which makes molecule more rigid and conformationally stable in the solvents (Fig. 1a) [10]. Taking into account that the increase in the quantum yield of Nor–Sq in glycerol relative to PB is not as significant as for Sq (1.4 *vs.* 6.7 times), squaraine dye is more preferable for biological applications, such as monitoring of changes in membrane, nuclear and cytoplasmic viscosity.

Nor–Sq is known to be sensitive to the media acidity in the range of pH about 9–12 [9]. It does not show noticeable changes in spectral properties in pH range between 2–8.5 either, but long-wavelength absorption band (639 nm) at higher pH decreases and a new band with maximum at 519 nm appears, whereby the fluorescence consistently quenches and becomes undetectable above pH 12 (Fig. 3). The absorption and emission characteristics of squaraine Sq in their turn exhibit no noticeable sensitivity to the media pH within the range between 2–11 (Fig. 3 insert a, insert b). Thus, the absence of a substituent at the nitrogen atom in norsquaraine molecule provides the pH-sensitivity of Nor–Sq due to deprotonation of terminal heterocyclic nitrogen [9].

The values of pK_a of Nor–Sq calculated from the absorption and emission titration curves are 10.15 and 10.11, respectively, as it was shown in [9]. This range of pH is rarely reached in living systems, thus Nor–Sq can be used to label the majority of proteins for biomedical investigations without any risk to be transformed into deprotonated form. Care should be taken only for conjugation with proteins which have high isoelectric point.

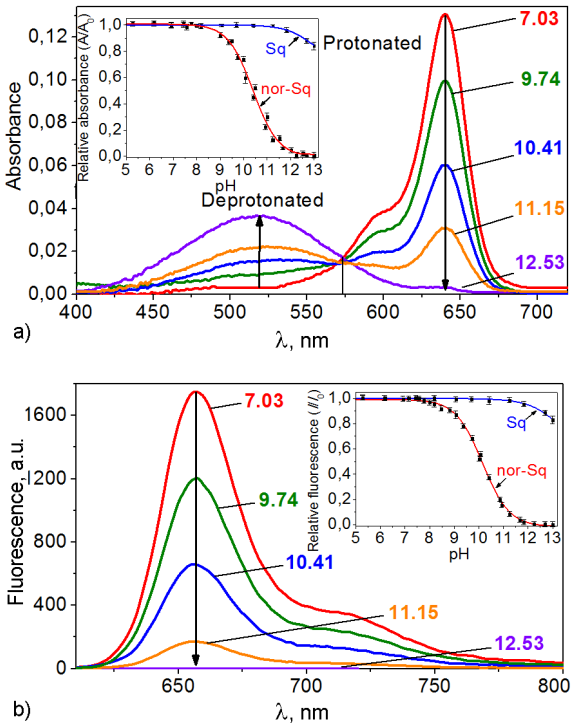

Fig. 3. Absorption (a) and emission (b) spectra of norsquaraine Nor–Sq *vs.* pH and the absorption (insert a) and emission (insert b) titration curves of norsquaraine Nor–Sq and squaraine Sq (excitation wavelength was 620 nm).

3.2. Dye-protein interaction

3.2.1. Non-covalent complexation with proteins (BSA and IgG)

The spectral and photophysical properties of squaraines are known to be substantially affected by the presence of proteins [29–31]. The absorption and emission maxima, molar absorptivity, fluorescence intensities, and quantum yields of Nor–Sq and Sq dyes investigated in this work in the presence of BSA and IgG are shown in Table 2. Comlexation with BSA (c_{BSA} = 6 mg/mL) results in red-shifts of norsquaraine and squaraine absorption and emission bands relative to spectra in PB by 17 and 13 nm for Nor–Sq, 8 and 10 nm for Sq for absorption and emission, respectively (Table 2). It can be explained by binding of the dye molecules with the hydrophobic areas of proteins [32, 33], which are inaccessible to water molecules. As a result, polarity of local environment of the dye molecules decreases and spectral behavior of the dyes change. As it is shown in the Fig. 4, with an increase in the concentration of BSA, the fluorescence spectra are red-shifted for the

Table 2. Spectral characteristics of the dyes (c_{Dye} ~ 0.5 μM), non-covalent dye-BSA and dye-IgG complexes ($c_{protein}$ = 6 mg/mL) and conjugates with BSA and IgG (D/P = 1).

Dye	Media	λ_{max}(Ab), nm	λmax(Em), nm	$\Delta\nu_{st}$, cm^{-1}	$\Delta\nu_{1/2}$(Ab), cm^{-1}	$\Delta\nu_{1/2}$(Em), cm^{-1}	ε, $M^{-1}cm^{-1}$	ΦF, %
Nor–Sq	PB pH 7.4	639	657	430	840	810	188,000	33
	Complex with BSA pH 7.4	656	670	320	870	820	158,000	41
	Complex with IgG pH 7.4	639	657	430	840	810	178,000	33
	Conjugate with BSA pH 7.4	655	670	340	900	820	n/d*	14
	Conjugate with IgG pH 7.4	646	663	400	970	860	n/d	21
Sq	PB pH 7.4	635	644	220	620	700	290,000	12
	Complex with BSA pH 7.4	643	654	260	800	710	234,000	40
	Complex with IgG pH 7.4	635	646	270	620	700	278,000	13
	Conjugate with BSA pH 7.4	643	654	260	730	680	n/d	65
	Conjugate with IgG pH 7.4	639	650	270	670	690	n/d	33

both dyes and at concentration of BSA 6 mg/mL its maxima almost coincide to the maxima of dyes in ethanol. With an increase of BSA concentration the fluorescence intensity of nor-Sq decreases by a factor of 1.8, when excited at 620 nm, since its absorption spectra shift significantly (by 17 nm) to the red region. Despite the fluorescence intensity of complexes decreases, the quantum yield of the Nor–Sq increases by 1.2 times to 41 % at concentration of 6 mg/mL of BSA. The absorption spectra of Sq shifts only by 8 nm and its quantum yield is more sensitive to the polarity of the environment, therefore Sq reveals at 3 times higher fluorescence intensity and 3.3 fold increase of the quantum yield when complexed with BSA, which are more significant than for norsquaraine (Fig. 4b, Table 2). Upon non-covalent complexation with BSA the molar absorptivities of dyes are slightly decreased compared to the free dyes in PB and amounts to 158,000 $M^{-1}cm^{-1}$ and 234,000 $M^{-1}cm^{-1}$ for Nor–Sq and Sq, respectively. The Stokes' shift of Nor–Sq decreases in the presence of BSA (Table 2), while Sq-BSA complex Stokes' shift, contrariwise, is slightly increased upon complexation.

Since IgG is less hydrophobic protein than BSA [34], in the presence of IgG (c_{IgG} = 6 mg/mL) only a slight decrease of molar absorptivity indicates the formation of complexes. Other spectral characterestics

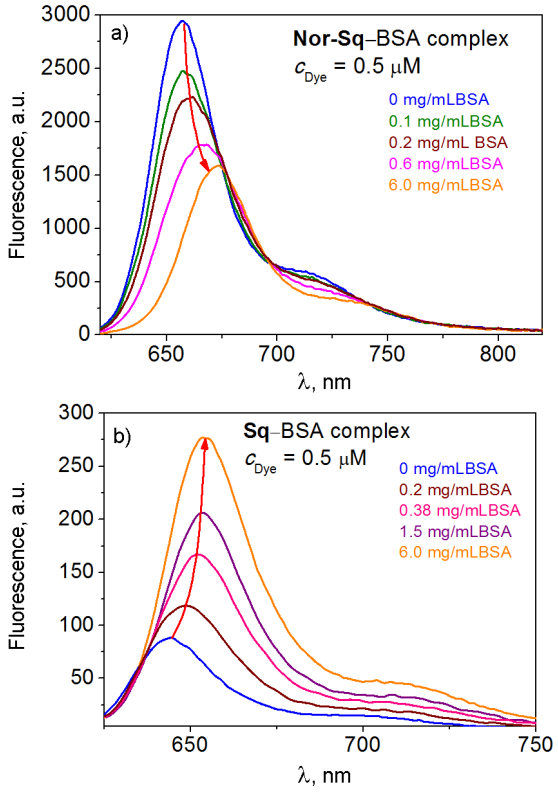

Fig. 4. Fluorescence spectra of Nor–Sq (a) and Sq (b) (c_{Dye} = 0.5 μM) in presence of different BSA concentrations (c_{BSA} = 0–6 mg/mL) in PB pH 7.4.

for Nor–Sq do not change, while for Sq they change only slightly (Table 2).

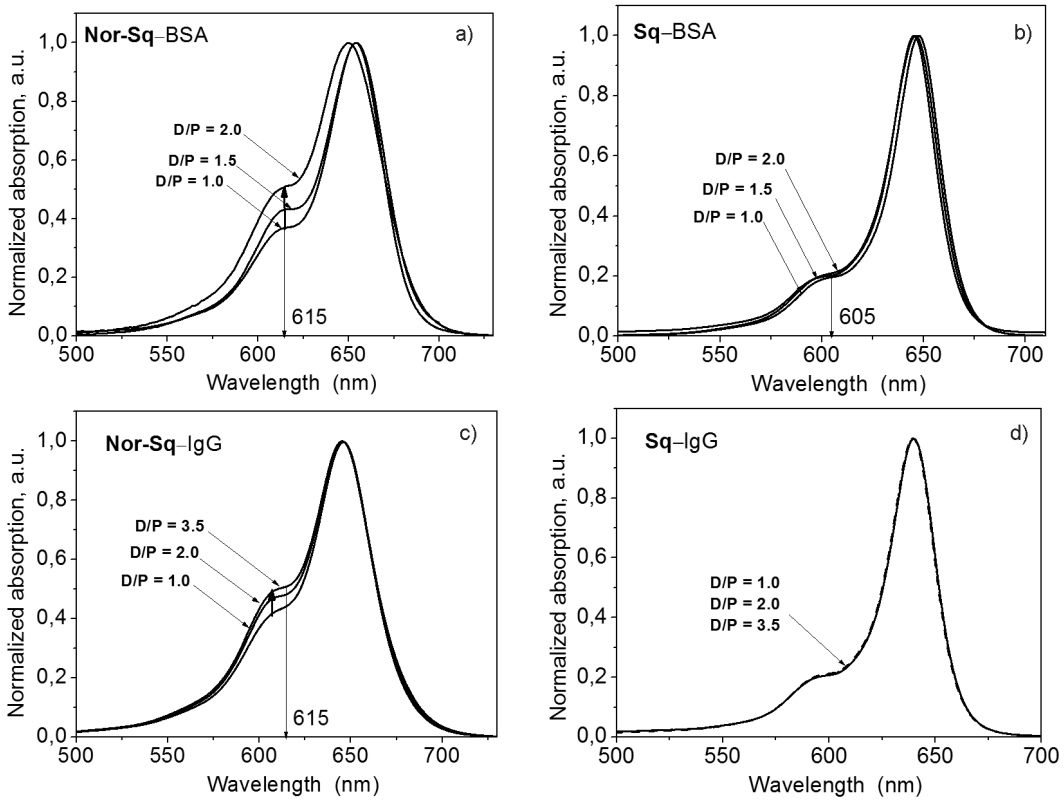

Fig. 5. The normalized absorption spectra of conjugates Nor–Sq-BSA (a), Sq-BSA (b), Nor–Sq IgG (c), and Sq-IgG (d) at different D/P.

Thus, Sq is more preferable for application in biomedical studies which deal with non-covalent complexation of a dye with protein, since a low sensitivity of Nor–Sq to proteins could significantly reduce the reliability of the results. Increasing of the hydrophobicity of proteins allows obtaining the more noticeable changes in spectral properties of dyes.

3.2.2. Covalent dye-protein conjugates

The studies of the interaction of Nor–Sq and Sq with biomolecules were also performed by covalent binding of the dyes with BSA and IgG molecules. The spectral properties of dyes after conjugation with proteins (D/P = 1) are shown in Table 2.

The covalent labeling to protein changes the media polarity around the dye creating more hydrophobic environment than the surrounding. Thus, it leads to red-shifted absorption and emission maxima of Nor–Sq and Sq conjugates as compared to spectra in PB (Table 2). In case of BSA conjugates for both dyes this shifts are more pronounced (8–16 nm) than for IgG (4–7 nm). As well as in the case of complexes with proteins, Nor–Sq in conjugates is more sensitive by its red-shift (6–16 nm) to binding with proteins than Sq (4–10 nm). The Stokes' shifts of norsquaraine conjugates with proteins have a trend to decrease (Table 2), while the Stokes' shifts of Sq upon conjugation are increased.

The quantum yields of squaraine-protein conjugates are known to be higher both for BSA- and IgG-conjugates than those for free dyes [14], which is a consequence of a decrease in polarity after labeling of dye to protein. And this trend is the same for squaraine Sq: the quantum yield is 5.4 fold increased for BSA-conjugate and 2.8 fold for IgG-conjugate. Oppositely, the quantum yield of Nor–Sq-conjugates decreased in 2.4 times for BSA-conjugate and 1.6 times for IgG-conjugates as compared to quantum yields in PB.

The decrease in the quantum yields of Nor–Sq-conjugates with proteins we attribute to the formation of the non-fluorescent dye-aggregates on protein surface. It should be taken into account that conjugation is a statistical process, and conjugate with the average D/P value of 1 really contains a series of protein molecules with different quantities of dye (i.e. 0, 1, 2...). Conjugates with 2 or more dye molecule on the one protein molecule can form aggregates. To

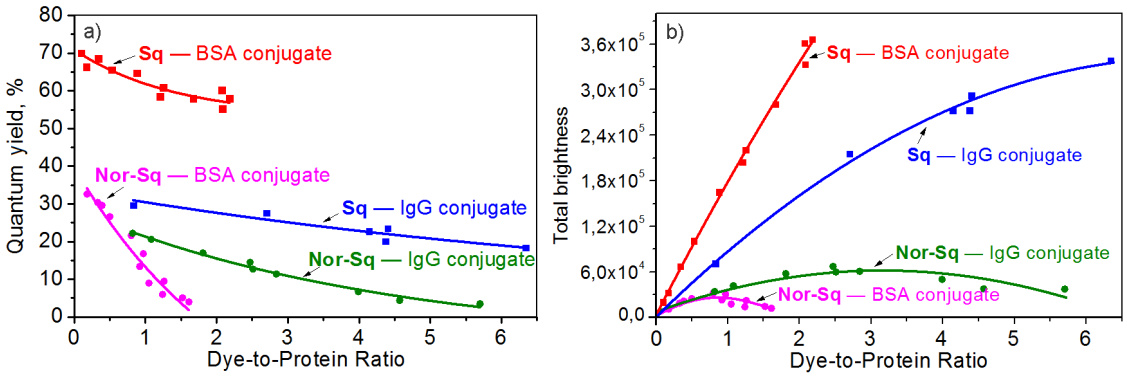

Fig. 6. The relation of QY *vs.* *D*/*P* of dye-protein conjugates (a) and total brightness *vs.* *D*/*P* of dye-protein conjugates (b).

confirm this hypothesis we have obtained and compared the conjugates with *D*/*P* 1, 1.5, 2 for BSA and 1, 2, 3.5 for IgG. The appearance of the new, shorter-wavelength band with a maximum at about 615 nm for Nor–Sq-BSA conjugates is demonstrated on Fig. 5a. The shoulder associated with the dye aggregation [35], can be found in the same spectral range as the short-wavelength vibrational band in non-aggregated molecules. The intensity of the aggregation band increases with the increasing of *D*/*P*. At the same time no any evidence of shoulder-increase for Sq-BSA conjugates is observed (Fig. 5b). Low tendency of Sq to form aggregates is related to higher quantity of sulfo-groups in its structure as compared to Nor–Sq. So, aggregation of Nor–Sq plays a critical role and reduces the quantum yield of BSA-conjugates with increasing of *D*/*P* and fluorescence is almost quenched at *D*/*P* ~ 2 (Fig. 6a). The labeling of Nor–Sq with IgG allowed getting the conjugates with higher *D*/*P* due to the larger size and higher hydrophilicity of IgG molecule (Fig. 6a), but aggregation in the case of Nor–Sq-conjugates with IgG is also observed (Fig. 5c) unlike the Sq–IgG conjugates (Fig. 5d). One of the most important parameters for practical use of dye-protein conjugate in biomedical applications is the total brightness, which is calculated by multiplying the molar absorptivity, quantum yield and *D*/*P* ratio of the conjugate (see Eq. 3). This parameter characterizes the fluorescence response, which can be obtained from the one labeled protein molecule. The optimal value of brightness is estimated by testing of the conjugates with *D*/*P* ratios in a range as wide as it is possible. The plots of total brightness of BSA and IgG conjugates of Nor–Sq and Sq vs. the *D*/*P* ratios of these conjugates are given on Fig. 6b.

As it can be seen from the Fig. 6b Nor–Sq conjugates have the maxima of brightness at *D*/*P* ~ 0.8 and *D*/*P* ~ 3 for BSA- and IgG-conjugates respectively. Squaraine Sq forms much brighter conjugates, which even do not reach the maximum value of brightness at the investigated *D*/*P* range. We associate this with the higher tendency of Nor–Sq to aggregate which leads to quenching and consequently brightness decrease when D/Ps are increased. Thus we can assume that presence of higher quantity of sulfo-groups in Sq structure allows the preparation of conjugates with higher quantum yields, brightness and *D*/*P*, which are more preferable for biological applications.

4. Conclusions

In summary, we have compared the spectral and photophysical properties of norsquaraine Nor–Sq *vs.* those for 3-carboxypentyl substituted squaraine dye Sq in water, organic solvents and under interactions with proteins. In organic solvents spectral maxima of Nor–Sq are red shifted as compared to Sq, while the molar absorptivity of squaraine is higher providing the improved accuracy of fluorescent measurements using Sq. The both dyes exhibit negative solvatochromism, but spectral maxima of Nor–Sq are more significantly blue-shifted in polar media than those for Sq. However, the quantum yield of Nor–Sq is almost unchanged with decrease of the solvent polarity, while for Sq it increases significantly (by 3–4 times), which is the same more preferable for fluorescent methods. Squaraine dye also is more sensitive to the viscosity of the medium, than Nor–Sq. Meanwhile, the absence of a substituent at the nitrogen atom in norsquaraine molecule provides the pH-sensitivity of Nor–Sq in the range of 9.0 –11.4, as

a result, norsquaraine can be applicable for pH monitoring by fluorescence methods. Non-covalent binding to BSA results in more significant changes of Sq spectral properties than for Nor–Sq, but the properties of both dyes in complex with IgG remain almost unchanged. The covalent labeling with proteins leads to the increase of quantum yield of Sq, while the quantum yield of Nor–Sq decreases, so the conjugates of Nor–Sq possess the lower brightness. Thus, it is more preferable to use Sq for the most of biomedical applications, where probing increases the brightness of the dye due to changes of polarity and viscosity of environment, while Nor–Sq is applicable for pH monitoring and in assays, where signal should not depend on changes in dye molecule environment.

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