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Effect of linker length on the spectroscopic properties of bacteriochlorin – 1, 8-naphthalimide conjugates for fluorescence-guided photodynamic therapy

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ABSTRACT

Conjugation of 4-pyrazolyl-1,8-naphthalimide and propargyl-15²,17³-dimethoxy-13¹-amide of bacteriochlorin *e* by the oligo(ethylene glycol) linker resulted in the dual function system comprising the modalities of a photosensitizer and a fluorescence imaging agent. In comparison with the conjugate of the same structure but containing the shorter linker, the system demonstrated the higher emission intensity originating from the naphthalimide unit, which could be explained by the decrease in resonance energy transfer efficiency between the chromophores. It has been shown that the ability of bacteriochlorin to generate singlet oxygen remains at the same level when this fragment is introduced in the conjugate structure. 4-Pyrazolyl-1,8-naphthalimide group in the studied compounds was found to easily undergo photooxidation yielding highly emissive 4-pyrazolyl-1,8-naphthalimide derivatives.

1. Introduction

Photodynamic therapy (PDT) is a relatively novel and minimally invasive treatment that can exert a selective cytotoxic activity towards malignant cells [1,2]. The method includes three necessary elements: a drug called photosensitizer (PS), light and molecular oxygen presenting in a solubilized form in cells and tissues. When irradiated with a specific wavelength of light, PS is first transformed to S_1 state. Subsequent intersystem crossing produces the triplets $^3PS^*$, which are capable to interact with 3O_2 upon collisions resulting in the production of cytotoxic singlet oxygen (1O_2) and free radicals. This causes cell death *via* apoptosis or necrosis.

Derivatives of porphyrin, chlorin and bacteriochlorin are known to be one of the most commonly used types of photosensitizers for PDT. A significant advantage of these compounds is due to their ability to accumulate in tumors and to sensitize the formation of 1O_2 and other reactive oxygen species (ROS) with a high quantum yield. Many of these compounds have reached different stages of clinical or preclinical trials [3–7]. In addition to therapy, the difference in the concentration of PS in malignant and normal tissue can be the basis for fluorescence diagnostics. Fluorescence imaging methods are low-cost and non-

invasive. In the meantime, they can provide an accurate estimations concerning the tumor size and its localization [8,9]. Unfortunately, porphyrin-based photosensitizers have very small Stokes shifts, which significantly limits their application in fluorescence imaging. Furthermore, excitation of PS during the diagnostic mode would obviously cause related phototoxicity effects with respect to normal tissues.

To enhance the imaging capability of the PDT agents, a new approach, which consists in combining a fragment of a photosensitizer with a fragment of a fluorescent dye in one molecule, has been proposed [10–12]. Thus, it is expected that the excitation of the fluorophore would give a strong emission signal, which can be used to monitor the response of the tumor to treatment. Further excitation with a different wavelength of light corresponding to the absorption maximum of PS would lead to the formation of ROS responsible for cancer cell damage.

Recently, we have prepared conjugates of propargyl-15²,17³-dimethoxy-13¹-amide of bacteriochlorin *e* (BChl, Scheme 1) and *N*-aminobacteriopurpurinimide with 1,8-naphthalimide derivatives [13,14]. This type of fluorescent dyes exhibited high Stokes shifts, good photostability and imaging capability *in vitro* [14–16]. However, the fluorescence of naphthalimide core in the studied conjugates was found

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Scheme 1. Synthesis of Con1 and Con2.

Fig. 1. ^1H NMR spectrum (600.22 MHz) of the crude product **4a** in CDCl_3 at 19°C . Signals of the oxidized form (4-pyrazol-1,8-naphthalimide **Ox4a**, Scheme 2) are marked with blue color. Numbering of carbon atoms in the naphthalimide fragment of **4a** and **Ox4a** is shown in Scheme 2.

to be completely quenched due to resonance energy transfer (RET) to the acceptor photosensitizer fragment. Thus, the imaging was only possible after the high power irradiation, which caused photodestruction of the bacteriochlorin chromophore [14].

It is well-known that the resonance energy transfer efficiency (Φ_{RET}) in a bichromophoric system depends on the distance between the photoactive units (r); namely, Φ_{RET} is inversely proportional to r^6 if the Förster mechanism of RET (or FRET) is in operation [17]. In the present paper, we aimed to use the above-mentioned feature of FRET to improve the fluorescent characteristics of conjugates. For this purpose, two novel conjugates **Con1** and **Con2** (Scheme 1) combining 4-(1,5-diphenyl- Δ^2 -pyrazolin-3-yl)-1,8-naphthalimide and bacteriochlorin *e* units were obtained. The conjugates **Con1** and **Con2** are different in the length of the linker group **L** connecting the fluorophore and PS moieties so that their energy transfer characteristics might be also different. Furthermore, oligo(ethylene glycol) linker in **Con2** could make the whole molecule rather amphiphilic; it could self-assemble into micelles with a suitable size not only for long body circulation but also for *in vivo* accumulation in the tumor area [18].

2. Results and discussion

2.1. Synthesis of the compounds

Conjugates **Con1** and **Con2** were synthesized by the cycloaddition click reaction of the bacteriochlorin *e* derivative **Bchl** and modified 4-(1,5-diphenyl- Δ^2 -pyrazolin-3-yl)-1,8-naphthalimides **6** and **4b** bearing azide groups in the *N*-imide substituent (Scheme 1). Compound **Bchl** was afforded from the methyl ester of bacteriopheophorbide *a* and propargylamine as described earlier [14]. As a source of the former, biomass *Rhodobacter capsulatus* was used [19,20]. For the preparation of pyrazolinyl-1,8-naphthalimides **4b** and **6**, condensation of 4-acetylnaphthalic anhydride **1** with benzaldehyde was carried out. Then, the resulting product **2** was imidated with amines **7a** and **7b** to yield 4-cinnamoyl-1,8-naphthalimides **3a** and **3b**, which were further subjected to condensation with phenylhydrazine. Regarding compound **6**, introduction of the azide group was performed after the formation of pyrazolinyl ring via subsequent treatment of **4a** with POCl_3 and NaN_3 . This, however, was not the case for the compound **4b**, which was

Scheme 2. Photooxidation of pyrazolanyl group.

prepared using commercially available oligo(ethylene glycol) derivative **7b** containing the terminal azido group.

An interesting observation was found in the ^1H NMR spectra of the isolated products **4a**, **5**, **6**, **Con1** and **Con2**. Structure elucidations revealed that the signals of the naphthalimide fragment are split into two sets with the intensity ratio of about 1 : 0.12 $\div$ 0.40. As an example, Fig. 1 shows the ^1H NMR spectrum of **4a** in CDCl_3 (see Supplementary information for the NMR spectra of other compounds). At the same time, EI mass spectra of **4a** and **5** showed clear peaks of $[\text{M} - 2]^+$ species (Fig.S27,S29). Aiming to rationalize these results, we supposed that five-membered heterocyclic ring at C-4 position of the naphthalimide fragment could be oxidized to form 4-pyrazolyl group (Scheme 2). Such oxidation has been previously found for 1,3-diaryl-

and 1,3,5-triaryl-2-pyrazolines [21–24] as well as for pyrazoline-substituted coumarins [25–27] either upon irradiation in the presence of oxygen or even when irradiation in degassed solution is applied. The possible mechanism involves sensitization of singlet oxygen formation by a dye molecule or, alternatively, the electron transfer from the photoexcited chromophore to the solvent (especially for solvents like CCl_4 or C_2Cl_6). Unfortunately, we were not able to isolate pure pyrazolanyl derivatives in all cases, and the mixtures of the non-oxidized with some amount of the oxidized form (usually 11–29 mol. % as revealed from ^1H NMR spectra) were used for the subsequent synthetic steps and photophysical studies.

Possibility of the photooxidation was evaluated using the previously obtained model compound **8** (Scheme 2) [28] in which the presence of polar COOH group on the one hand provides good solubility and on the other makes the substituent at the N-imide nitrogen of the naphthalimide ring be resistible to oxidizing agents (in contrast to $\text{CH}_2\text{CH}_2\text{OH}$ group in **4a**). First, a solution of **8** (10 μM) in CHCl_3 was irradiated by the full light of high pressure Hg vapour lamp (120 W) during 90 s, and the absorption spectra were recorded every 3, 5 or 10 s. It was found that irradiation caused bleaching of the absorption band of the 4-pyrazolanyl-1,8-naphthalimide ($\lambda_{\text{max}}^{\text{fl}}$ 495 nm) and a concomitant appearance of the new band at 365 nm with the isosbestic point at 409 nm (Fig.S33). The oxidation product **Ox8** was isolated as a precipitate after the irradiation was ceased (see Supplementary information for characterization data of **Ox8**). The ^1H NMR spectrum of compound **8** (7.6 mM) in CDCl_3 under irradiation at the same conditions demonstrated the growth of the intensities for proton resonances of the oxidized form (**Ox8**), the signals of pyrazolynynaphthalimide **8** being completely disappeared after 40 min of exposure to the UV light (Fig.S34). Yet, compound **Ox8** was also obtained by the oxidation of **8** with $\text{K}_2\text{Cr}_2\text{O}_7$ in AcOH (see Supplementary Information).

2.2. Photophysical characterization of dyes

Steady-state absorption and emission properties of conjugates **Con1** and **Con2** as well as monochromophoric derivatives **BChl** and **4a** are shown in Fig. 2 and Table 1. As it can be seen from Fig. 2a,c, absorption spectra of **Con1**, **Con2** and equimolar mixture of **BChl** and **4a** are very similar, each showing the presence of four bands of almost the same intensity at around 355, 480, 515 and 750 nm corresponding to electronic transitions in naphthalimide (480 nm) and bacteriochlorin (355,

515 and 750 nm) moieties. Thus, the lack of π , π -interaction between the covalently linked chromophores could be suggested in the ground state.

The long wavelength absorption band of compound **4a** with maximum at 482 nm can be attributed to intramolecular charge transfer (ICT) from the pyrazolanyl fragment to carbonyl groups of dicarboxyimide moiety [29]. Due to rather strong ICT nature of the lowest energy transition such type of chromophores could be excited by two-photon absorption with an IR laser. Indeed, in our recent paper [28], we have shown that fluorescence output from the carboxylic derivative **8** (the analogue of **4a**) can be obtained *in vitro* by irradiation with 880 nm light which perfectly fits the phototherapeutic window (600–1200 nm).

Emission spectra of all compounds presented in Table 1 (except **BChl**) were recorded using 470 nm excitation wavelength (λ_{ex}). Considering the changes in the UV/Vis absorption spectrum of compound **8** induced by the irradiation (Fig.S33), one can suppose that such excitation light would not be absorbed by the oxidized form and, therefore, all the collected spectra would arise from the direct excitation of the 4-pyrazolanyl-1,8-naphthalimide chromophore. To supply this assumption, we have measured the fluorescence emission spectra of the crude product **4a** at λ_{ex} 360 and 470 nm (Fig.S36b, Fig.1b). Indeed, the spectrum at λ_{ex} 360 nm showed the intense peak at 475 nm, whereas the long wavelength excitation produced only one band with $\lambda_{\text{max}}^{\text{fl}}$ 651 nm. Excitation spectra obtained at 480 and 680 nm clearly show different emission peaks centered at 367 and 480 nm respectively (Fig.S36a). These observations are consistent with the highly emissive character of the oxidized form¹ that absorbs at around 350–370 nm fluoresces in the blue region ($\lambda_{\text{max}}^{\text{fl}}$ 475 nm).

Fluorescence maxima of **BChl** appears at 755 nm (Fig. 2b). When the solution containing the mixture of equal amounts of **BChl** and **4a** was excited at 470 nm, we observed the broad-band fluorescence which was very similar to that of **4a**. This can be understood by the fact that the most part of the 470 nm light is absorbed by the naphthalimide fragment and only minor direct excitation of the bacteriochlorin unit occurs leading to the low intensity long wavelength shoulder in the spectrum (Fig. 2d). The spectrum of **Con1** containing the short linker between the chromophores was found to be completely different (Fig. 2d). Following the excitation at 470 nm, this compound exhibited the characteristic peak of the bacteriochlorin and no emission signal from the naphthalimide was detected. Such result could be consistent with the possible resonance energy transfer from the fluorophore to the PS in the system.

To verify our assumption if resonance energy transfer is a reason of quenching of naphthalimide fluorescence in **Con1**, we calculated Φ_{RET} using the Förster theory [17]. In this treatment, Φ_{RET} can be expressed by Eq. (1):

$$\Phi_{\text{RET}} = \frac{R_0^6}{R_0^6 + r^6} \quad (1)$$

where r is the distance between the donor and the acceptor and R_0 is the critical Förster radius at which 50 % of the excited donors deactivate through the RET pathway (i.e. $\Phi_{\text{RET}} = 0.5$). The value r was found from the optimized ground state geometry of **Con1** (Fig. 3, $r = 11.3 \text{ \AA}$), and R_0 was calculated according to the Eq. (2):

$$R_0^6 = \frac{9000 \ln 10 \kappa^2 \varphi_{D,0}^{\text{fl}}}{128 \pi^2 N_A n^4} \int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \quad (2)$$

where κ^2 is an orientation factor which depends on the mutual disposition of the donor and acceptor transition dipole moments, $\varphi_{D,0}^{\text{fl}}$ is the fluorescence quantum yield of the donor in the absence of the acceptor,

¹ The assumption about highly emissive character of the oxidized forms of the pyrazolanyl-1,8-naphthalimide derivatives is based on the calculation of the fluorescence quantum yield for the compound **Ox8**, which was found to be 0.49 in MeCN

Fig. 2. UV/Vis absorption (a,c) and fluorescence emission (b, d) spectra of compounds **BChl** (1), **4a** (2), **Con1** (3), **Con2** (4) and equimolar mixture of **BChl** and **4a** (5) in acetonitrile. Excitation wavelength is 470 nm for **4a**, **Con1**, **Con2**, **Mix** and 515 nm for **prop**. Concentration of all compounds – $5.0 \cdot 10^{-6}$ M.

Table 1

. Photophysical characteristics^a of compounds **4a**, **BChl**, **Con1** and **Con2** in acetonitrile.^b

	$\lambda_{\max}^{\text{abs}}/\text{nm}$	$\lambda_{\max}^{\text{fl}} (\lambda_{\text{ex}})/\text{nm}$	φ^{fl}	τ_{D} (or $\tau_{\text{D},0}$)/ns	$R_0/\text{\AA}$	Resonance energy transfer efficiency Φ_{RET}			$\Phi_{\Delta} (\lambda_{\text{ex}}/\text{nm})$
						Förster theory	Steady-state experiment	Time-resolved experiment	
4a	482	651 (470)	0.071	0.93	–	–	–	–	–
BChl	353, 515, 746	755 (515)	0.016	–	–	–	–	–	0.79 (510)
Con1	355, 515, 749	757 (470)	0.028	not determined	30.8	0.998	not determined	not determined	0.67 (510)
Con2	355, 515, 749	753 (470)	0.021	0.065	30.8	0.065	0.929	0.930	0.83 (510)

^a $\lambda_{\max}^{\text{abs}}$ and $\lambda_{\max}^{\text{fl}}$ denote the maxima positions of absorption and emission bands respectively; λ_{ex} – excitation wavelength; φ^{fl} – fluorescence quantum yield; τ_{D} and $\tau_{\text{D},0}$ – excited state lifetime of the naphthalimide chromophore in the presence and absence of energy acceptor respectively; R_0 – critical Förster distance; Φ_{Δ} – quantum yield of singlet oxygen generation.

^b Φ_{Δ} values were measured in acetone.

n is the refractive index of the solvent, N_A is Avogadro constant and the integral corresponds to the extent of overlap between the normalized emission spectrum of the donor ($F_D(\lambda)$) and the absorption spectrum of the acceptor ($\epsilon_A(\lambda)$, see Fig.S37a for the graphical representation of this overlap). Taking into account that $\kappa^2 = 2/3$ (for a random orientation) and $\varphi_{\text{D},0}^{\text{fl}} = 0.071$ (the quantum yield of **4a** in acetonitrile), we found the RET efficiency Φ_{RET} to be as high as 0.998. Exactly the same value of Φ_{RET} was found both from theoretical calculations (0.995) and the analysis of the transient absorption spectra (0.999) for the previously reported conjugate of similar structure [14], in which 4-*N,N*-dimethylaminostyryl group is presented instead of 1,5-diphenyl- Δ^2 -pyrazolin-3-yl one.

In contrast to **Con1**, excitation of **Con2** with 470 nm light produced the broad emission band at around 650 nm attributed to the

fluorescence of the naphthalimide unit (Fig. 2d), thus showing that the RET is less effective. Yet, the relaxation of naphthalimide excited state in **Con2** was long enough ($\tau_{\text{D}} = 65$ ps, Table 1), so we could measure it using our time-resolved fluorescence setup (see the description in [16]). Comparison of the obtained lifetime τ_{D} with that of the individual donor chromophore $\tau_{\text{D},0}$ taken apart of the energy acceptor (0.93 ns, the

lifetime of **4a**, see Table 1) allowed to estimate the experimental value of RET efficiency (Eq. (3))². As found from Eq. (3), Φ_{RET} for **Con2** was 0.930, which is obviously lower than Φ_{RET} in the case of **Con1**. Essentially the same Φ_{RET} value (0.929) was also obtained by the Eq. (4)

² In the case of **Con1**, calculation of Φ_{RET} by the Equation [3] was not done because the lifetime of the excited naphthalimide fragment appears to be lower compared to temporal resolution in the experiment.

Fig. 3. Ground state geometry of **Con1** (left) and **Con2** (right) calculated using PM6 method. Hydrogen atoms are omitted for the sake of simplicity.

where the steady-state emission intensities of the energy donor in the presence (I_D) and absence ($I_{D,0}$) of the acceptor are used instead of corresponding lifetimes (see Fig.S37b for the non-normalized fluorescence spectra of **Con2** and equimolar mixture of **BChl** and **4a** showing the I_D and $I_{D,0}$ values).³

$$\Phi_{\text{RET}} = 1 - \frac{\tau_D}{\tau_{D,0}} \quad (3)$$

$$\Phi_{\text{RET}} = 1 - \frac{I_D}{I_{D,0}} \quad (4)$$

Further analysis revealed that the presented experimental estimations of Φ_{RET} in the case of **Con2** did not match the result of the theoretical calculation by the Förster model like in the case of **Con1**. According to Fig. 3, the distance between naphthalimide and bacteriochlorin units in **Con2** molecule could be as high as 48.0 Å. This in turn should result in only 6.5 % of RET efficiency in the system, provided that the critical distance R_0 in the Eq. (1) is the same. The higher experimental value of Φ_{RET} (~93 %) can be explained by a rather high sterical flexibility of the oligo(ethylene glycol) linker, which could lead to a situation when the majority of **Con2** molecules adopt the conformations where the distance between the naphthalimide and bacteriochlorin chromophores is lower than R_0 and, hence, an efficient RET could occur.

In order to test the applicability of **Con1** and **Con2** as bioimaging agents, we also measured their absorption and fluorescence spectra in rabbit blood serum (Fig.S38). Such a model medium makes it possible to roughly estimate how the complex biological microenvironment in which the fluorescent probe is immersed may affect its optical characteristics. As for **Con2**, there is no significant difference in the relative fluorescence intensities of naphthalimide fragment and bacteriochlorin core upon going from the acetonitrile solution (Fig. 2d) to rabbit blood serum (Fig.S38d). However, an obvious increase in the fluorescence intensity of naphthalimide unit can be deduced from the same comparison in the case of **Con1**. This observation could be a result of conformational changes in the **Con1** structure upon its possible interactions with serum components, which might cause a decrease in the orientation factor κ^2 value (see Eq.s (1) and (2)) thereby reducing the RET efficiency. In general, the data presented allow us to conclude that both conjugates will not impair their fluorescent characteristics when replacing an organic solvent with an aqueous biological medium.

Generation of $^1\text{O}_2$ was investigated using 1,3-diphenylisobenzofuran (DPBF) as a singlet oxygen trap. The reaction between DPBF and $^1\text{O}_2$ results in the formation of endoperoxides exhibiting the absorption in the more short wavelength region compared to DPBF [30,31]. Thus, formation of singlet oxygen leads to a decrease in absorption at the main maximum of DPBF (414 nm). Rapid photobleaching of DPBF was detected in solutions of **Con1**, **Con2** and free bacteriochlorin **BChl**

(Fig.S38a–c). In the solution of the free naphthalimide dye **4a**, photobleaching of the trap was not found (Fig.S38d). Quantum yields of singlet oxygen sensitization (Φ_Δ) of **Con1**, **Con2** and **BChl** under the excitation at 510 nm were found to be 0.67, 0.83 and 0.79 respectively (Table 1). Such close values indicate that photosensitizing activity of the bacteriochlorin is not affected by the presence of naphthalimide fragment in **Con1** and **Con2**.

3. Conclusion

In summary, two novel conjugates bearing bacteriochlorin photosensitizer and 4-pyrazolyl-1,8-naphthalimide fluorophore have been synthesized. Regardless of the linker length, the presence of the naphthalimide fragment does not decrease the ability of PS core to generate $^1\text{O}_2$. Using the steady-state and time-resolved spectroscopic data, we have demonstrated that introduction of the flexible oligo(ethylene glycol) linker between the chromophores suppress the RET to some extent and, thus, leads to an increase in the emission intensity of the naphthalimide fragment. Thus, the studied conjugate **Con2** could be considered as a promising candidate for the application as photosensitizing and imaging agent.

4. Experimental part

4-Acetylnaphthalic anhydride **1** was obtained from acenaphthene according to the known method [32]. Propargyl-152,173-dimethoxy-131-amide of bacteriochlorin e (**BChl**) was synthesized according previously published procedure [14]. All other reagents were purchased from commercial sources and were of the highest grade. Synthetic procedures used for the preparation of compounds **2**, **3a**, **3b**, **4a**, **4b**, **5**, **6**, **Con1** and **Con2** as well as characterization data are listed in Supplementary information.

The absorption spectra were taken on a Varian-Cary 5 G spectrophotometer. The fluorescence quantum yield measurements were performed using a Varian-Cary 5 G spectrophotometer and a FluoroLog-3-221 spectrofluorimeter. Spectral measurements were carried out in air-saturated acetonitrile solutions (acetonitrile of spectrophotometric grade, water content < 0.005 %, Aldrich). All measured fluorescence spectra were corrected for the nonuniformity of detector spectral sensitivity. Fluorescence quantum yields were determined using Coumarin 481 in acetonitrile ($\phi^{\text{fl}} = 0.08$) [33] as a reference. The quantum yields of singlet oxygen (Φ_Δ) were estimated in acetone by using tetraphenylporphyrin (TPP) as a reference compound ($\Phi_\Delta^{\text{TPP}} = 0.7$) [30,31] and DPBF as a $^1\text{O}_2$ trap following the described method [14].

The three dimensional structure of **Con1** and **Con2** were built with MOPAC 2016 program package using PM6 semiempirical method [34]. The calculations were performed at optimized geometries, which reached gradient variations less than 0.01 kcal/mol. The solvent effect was included in geometry optimizations following the «Conductorlike Screening Model» (COSMO) implemented in MOPAC. A dielectric constant of $\epsilon = 20$ and a refraction index of solvent (n) such that $n^2 = 2$ were used.

³ Similar estimation of Φ_{RET} for **Con1** using Equation [4] is impossible due to the lack of emission signal at 650 nm in the spectrum of **Con1** (Fig. 2d) corresponding to donor chromophore fluorescence.

CRediT authorship contribution statement

Pavel A. Panchenko: Writing - original draft, Methodology, Formal analysis. **Marina A. Zakharko:** Investigation, Visualization. **Mikhail A. Grin:** Conceptualization. **Andrey F. Mironov:** Supervision. **Dmitriy A. Pritmov:** Investigation, Visualization. **Gediminas Jonusauskas:** Methodology, Investigation, Formal analysis. **Yuri V. Fedorov:** Methodology, Writing - review & editing. **Olga A. Fedorova:** Conceptualization, Supervision.

Declaration of Competing Interest

There are no conflicts to declare

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