

## Effect of chlorin $e_6$ molecular form on the induced singlet oxygen luminescence

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Chlorin  $e_6$  is a known photosensitizer used in photodynamic therapy of cancer as a component of existing formulations and in development of novel ones. Upon the increase of pH, chlorin  $e_6$  is known to undergo a molecular transition (considered to be the molecule deprotonation) at about pH 6.1–6.4, and the effects of this transition on singlet oxygen generation by chlorin  $e_6$  should be considered. Here we have studied the effect of solvent (Tris-HCl buffer (pH 7.2) vs distilled water) and of the presence of dextran-polyacrylamide and dextran-polyacrylamide-co-polyacrylic acid graft polymers in distilled water on the luminescence of the singlet oxygen generated by chlorin  $e_6$ . Luminescence intensity of singlet oxygen generated in the solution of chlorin  $e_6$  in distilled water (where chlorin  $e_6$  is mostly in its protonated form) was shown to be much higher than in Tris-HCl buffer where chlorin  $e_6$  is non-protonated. At the same time, the presence of dextran-polyacrylamide and dextran-polyacrylamide-co-polyacrylic acid graft polymers in distilled water results in the transition of chlorin  $e_6$  to its non-protonated form. This is accompanied by a decrease in singlet oxygen luminescence intensity. Based on these observations, we suggest that the efficiency of singlet oxygen generation by chlorin  $e_6$  (upon excitation at the wavelength of its Soret band) is higher for protonated form of chlorin  $e_6$  as compared to non-protonated one.

**Keywords:** photodynamic therapy, chlorin  $e_6$ , graft polymers, singlet oxygen luminescence.

Хлорин  $e_6$  является известным фотосенсибилизатором, который используется в фотодинамической терапии рака в качестве составляющей существующих препаратов, и препаратов в стадии разработки. Известно, что при повышении pH происходит молекулярная трансформация хлорина  $e_6$  (считается, что имеет место депротонирование молекулы) при pH 6.1–6.4. Влияние такой трансформации на генерацию хлорином  $e_6$  молекулярного кислорода необходимо исследовать. В работе изучено влияние растворителя (Tris-HCl буфер (pH 7.2) и дистиллированная вода), и присутствия разветвленных кополимеров декстран-полиакриламид и декстран-полиакриламид-ко-полиакриловая кислота в дистиллированной воде на люминесценцию синглетного кислорода, индуцированного хлорином  $e_6$ . Показано, что интенсивность люминесценции синглетного кислорода, индуцированного в растворе хлорина  $e_6$  в дистиллированной воде (где хлорин  $e_6$  находится преимущественно в протонированной форме) значительно выше, чем в случае раствора в Tris-HCl буфере, где хлорин  $e_6$  непротонирован. В то же время присутствие разветвленных кополимеров декстран-полиакриламид и декстран-полиакриламид-ко-полиакриловая кислота в дистиллированной воде приводит к переходу хлорина  $e_6$  в непротонированную форму, что сопровождается уменьшением интенсивности люминесценции синглетного кислорода. На основании этих данных предполагается, что эффективность генерации синглетного кислорода хлорином  $e_6$  (при возбуждении на длине волны полосы Soret) выше для протонированной формы хлорина  $e_6$  в сравнении с непротонированной.

**Вплив молекулярної форми хлорину  $e_6$  на індуковану люмінесценцію синглетного кисню.** М.Ю.Лосицький, Р.А.Харченко, Ю.І.Гарагуц, П.А.Вірич, Н.В.Куцевол, В.М.Ящук.

Хлорин  $e_6$  є відомим фотосенсибілізатором, який використовується у фотодинамічній терапії раку як компонент існуючих препаратів та препаратів у стадії розробки. Відомо, що при підвищенні рН хлорин  $e_6$  зазнає молекулярне перетворення (як вважається, депротонування молекули) при рН 6.1–6.4. Вплив такого перетворення на генерацію хлорином  $e_6$  молекулярного кисню варто дослідити. У роботі вивчено вплив розчинника (Tris-HCl буфер (рН 7.2) та дистильована вода) і присутності розгалужених кополімерів декстран-поліакриламід та декстран-поліакриламід-ко-поліакриловова кислота у дистильованій воді на люмінесценцію синглетного кисню, індукованого хлорином  $e_6$ . Показано, що інтенсивність люмінесценції синглетного кисню, індукованого у розчині хлорину  $e_6$  у дистильованій воді (де хлорин  $e_6$  перебуває переважно у протонованій формі) суттєво більша, ніж у випадку розчину у Tris-HCl буфері, де хлорин  $e_6$  непротонований. У той же час, присутність розгалужених кополімерів декстран-поліакриламід та декстран-поліакриламід-ко-поліакриловова кислота у дистильованій воді призводить до переходу хлорину  $e_6$  у непротоновану форму, що супроводжується зменшенням інтенсивності люмінесценції синглетного кисню. На основі цих даних зроблено припущення, що ефективність генерації синглетного кисню хлорином  $e_6$  (при збудженні на довжині хвилі смуги Core) є вищою для протонованої форми хлорину  $e_6$  порівняно з непротонованою.

## 1. Introduction

The photodynamic therapy of cancer is based on the generation of reactive oxygen species, particularly singlet oxygen ( $^1O_2$ ), by excitation of the special photosensitizer molecules accumulated in the tumor tissue [1–3]. The process of singlet oxygen generation consists of (i) the optical excitation of photosensitizer, (ii) intersystem crossing in this molecule that brings it from the singlet excited state to the triplet one, and (iii) the transfer of the triplet excitation of photosensitizer to the oxygen molecule that brings the latter to the toxic singlet state [2,3]. One of the important properties of a successful photosensitizer should be the high efficiency of  $^1O_2$  generation. On the other hand, understanding the possible influence of molecular transformations of photosensitizer on its properties to generate  $^1O_2$  is important for the development of complex photosensitizing nanosystems.

The majority of photosensitizer molecules belong to porphyrins and related compounds [1]. Particularly, chlorin  $e_6$  is a known photosensitizer used in photodynamic therapy of cancer as a component of existing formulations [4] and in development of novel ones [5, 6]. At the same time, it is known that the change of the water solution pH makes chlorin  $e_6$  to undergo the molecular form transition at about pH 6.1–6.4; this transition is manifested as the change in the absorption and fluorescence spectra of chlorin  $e_6$  [7]. The mentioned transition is connected with the deprotonation of chlorin  $e_6$  molecule upon pH increase, which is believed to be deprotona-

tion of its hydroxyl groups [8]. Besides, this transition could also occur upon binding of protonated chlorin  $e_6$  with polymers, e.g. polyvinylpyrrolidone (PVP) [4]. Thus, for the construction of efficient photosensitizer nanosystems using chlorin  $e_6$ , it is important to study the dependence of singlet oxygen generation by chlorin  $e_6$  on the molecular form of this molecule.

Here we have studied the effect of solvent (Tris-HCl buffer (pH 7.2) vs distilled water) and of the presence of dextran-polyacrylamide (PAA) and dextran-PAA-co-polyacrylic acid (D-g-PAA and D-g-PAAc respectively) graft polymers in distilled water on the luminescence of  $^1O_2$  generated by chlorin  $e_6$ .

## 2. Experimental

Chlorin  $e_6$  (Frontier Scientific Inc.) was kindly provided by T.Y.Ohulchansky (Institute for Lasers, Photonics and Biophotonics at the State University of New York at Buffalo). Dimethylformamide (DMF), ethanol, distilled water and 50 mM TRIS-HCl buffer (pH 7.2) were used as solvent.

Dextran-graft-polyacrylamide with dextran core ( $M_w = 70 \cdot 10^5$  g/mol) and 20 grafted PAA chains in nonionic (D-g-PAA) and anionic (D-g-PAAm) form were used in the study. Synthesis, molecular parameters and peculiarities of macromolecular structure of star-like copolymer D-g-PAA were discussed in detail in [9]. The average molecular weight of D-g-PAA ( $M_w$ ) was equal to  $1.43 \cdot 10^6$  g/mol, the radius of gyration (Rg) — 64 nm, and polydispersity ( $M_w/M_n$ ) — 1.98.

Anionic form of the copolymer (referred throughout as D-g-PAAan) was obtained via alkaline hydrolysis of initial copolymer during 30 min by using sodium hydroxide. According to evaluation by potentiometric titration, the fraction of amide groups that were thus converted to carboxylate groups was equal to approximately 37 % [10]. The D-g-PAAan copolymer was purified, freeze-dried and kept under vacuum for preventing further hydrolysis.

Stock solution of chlorin e<sub>6</sub> (10<sup>-2</sup> M) was prepared in DMF, aliquot of this stock solution was added to either distilled water or 50 mM TRIS-HCl buffer (pH 7.2). The solutions of polymers were further dissolved in distilled water, and after letting them reach equilibrium for about an hour, aliquot of 10<sup>-2</sup> M chlorin e<sub>6</sub> solution was added. Final concentrations were 5·10<sup>-6</sup> M for chlorin e<sub>6</sub>, 0.1 mg/mL for D-g-PAA and D-g-PAAan.

For the measurement of fluorescence quantum yield of chlorin e<sub>6</sub> in distilled water and 50 mM TRIS-HCl buffer (pH 7.2), stock solution of chlorin e<sub>6</sub> (10<sup>-2</sup> M in DMF) was further dissolved in ethanol to 10<sup>-3</sup> M, and an aliquot of 10<sup>-3</sup> M chlorin e<sub>6</sub> solution was added to either distilled water or 50 mM TRIS-HCl buffer (pH 7.2). Final concentration of chlorin e<sub>6</sub> was 5·10<sup>-6</sup> M, with the optical density of the Soret band being less than 0.1.

Absorption spectra were registered with the UV1900-PC spectrophotometer (China). Fluorescence spectra were measured with the help of a Cary Eclipse fluorescence spectrophotometer (Varian, Australia). Fluorescence quantum yield of chlorin e<sub>6</sub> in distilled water and 50 mM TRIS-HCl buffer (pH 7.2) was measured upon excitation at 405 nm using chlorin e<sub>6</sub> in ethanol as a reference; the fluorescence quantum yield of chlorin e<sub>6</sub> in ethanol (excited in Soret peak) was taken as 0.16 [11]. For estimation of the fluorescence quantum yield, fluorescence spectra of chlorin e<sub>6</sub> were corrected for the spectral sensitivity of the fluorescent spectrophotometer. For the measurements, solutions were placed into 1cm×1cm quartz cell. All measurements were performed at room temperature.

Luminescence spectra of the molecular oxygen were registered using the homemade spectral station based on MDR-24 monochromator (wavelength range 400–1400 nm) and Hamamatsu PMT cooled module in the range 1200–1300 nm. Excitation was performed with 405 nm diode laser. For the obtained spectra, subtraction of the background, monotonously decreasing with wavelength, was performed.

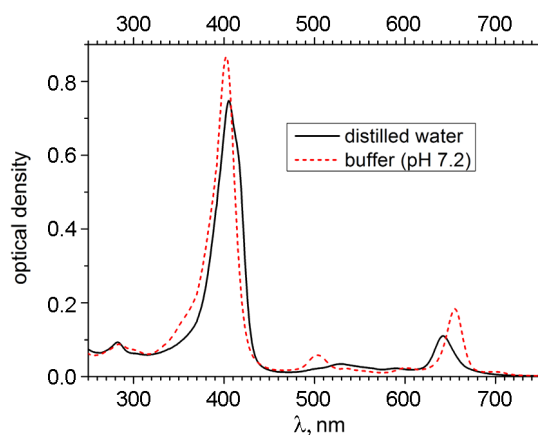


Fig. 1. Absorption spectra of chlorin e<sub>6</sub> (5 μM) in distilled water (black solid curve) and in 50 mM Tris-HCl buffer (pH 7.2) (dashed curve).

### 3. Results and discussion

First we have studied the solutions of chlorin e<sub>6</sub> in distilled water and in 50 mM Tris-HCl buffer (pH 7.2). Absorption spectra of the solutions (Fig. 1) mainly correspond to the protonated state of chlorin e<sub>6</sub> for the solution in distilled water and to the non-protonated state for the solution in buffer; this can be seen by difference in maxima of Q-bands (529 nm vs 504 nm, and 642 nm vs 655 nm respectively) [7].

Fluorescence spectra (normalized) of the mentioned solutions excited at 500 nm and 530 nm (i.e. pronounced maxima of non-protonated and protonated chlorin e<sub>6</sub>, respectively; Fig. 1) are presented in Fig. 2. These spectra demonstrate that the solution in distilled water still contains some part of non-protonated molecules (their emission is partly manifested upon excitation at 500 nm). At the same time, excitation at 405 nm (that was used for the registration of the singlet oxygen luminescence) results mainly in the emission of the protonated molecules (maximum at 650 nm; Fig. 2a); thus, in this case singlet oxygen is mainly generated by the protonated molecules of chlorin e<sub>6</sub>. As for the solution of chlorin e<sub>6</sub> in buffer (pH 7.2), excitation at 405 nm results almost completely in fluorescence of the non-protonated molecules (maximum at 662 nm; Fig. 2b).

Chlorin e<sub>6</sub> is known to aggregate in protonated state at concentrations higher than ~ 10 μM; such aggregates are manifested in the absorption spectrum by the changes of the Soret band, where the short-wavelength aggregate band appears near 395 nm [8]. It

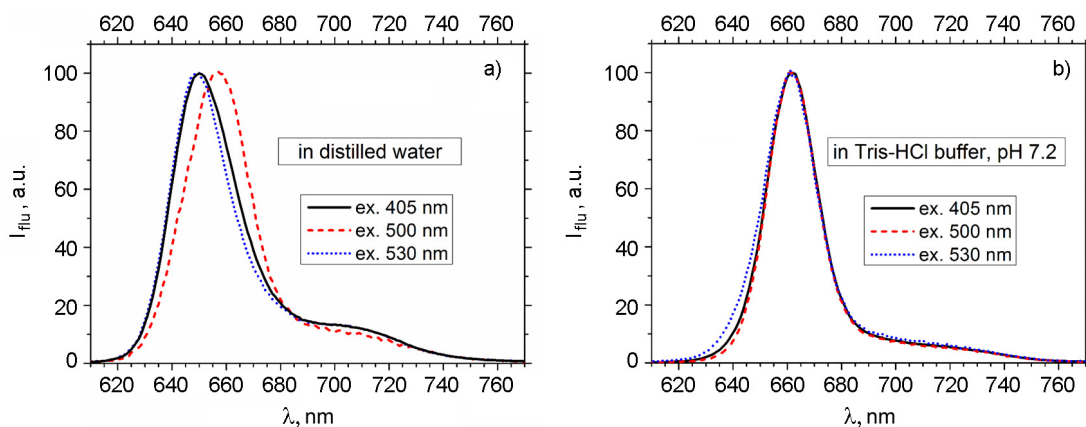


Fig. 2. Fluorescence spectra (normalized) of chlorin  $e_6$  ( $5 \mu\text{M}$ ) in distilled water (a) and in 50 mM Tris-HCl buffer (pH 7.2) (b) upon excitation at 405 nm (black solid curve), 500 nm (red dashed curve) and 530 nm (blue dotted curve). For normalization, fluorescence intensity of the spectra excited at 405, 500 and 530 nm were multiplied by the factor of 0.18, 2.4 and 1.2, respectively (a), and by the factor of 0.11, 0.5 and 1.6 respectively (b).

is seen from the absorption spectra (Fig. 1) that at the used concentration of chlorin  $e_6$  ( $5 \mu\text{M}$ ) no aggregation band is manifested. So, its molecules are predominantly monomeric in distilled water, as well as in 50 mM Tris-HCl buffer (pH 7.2). Thus, we could conclude that upon excitation (at 405 nm) of chlorin  $e_6$  solutions in distilled water and 50 mM Tris-HCl buffer (pH 7.2), singlet oxygen is mainly generated by protonated and non-protonated chlorin  $e_6$  molecules respectively.

The spectra of singlet oxygen luminescence generated by excitation (at 405 nm) of chlorin  $e_6$  ( $5 \mu\text{M}$ ) solutions in distilled water and 50 mM Tris-HCl buffer (pH 7.2) are presented in Fig. 3. It is seen that in the case of distilled water solution, the luminescence intensity of singlet oxygen generated by chlorin  $e_6$  is about two times higher as compared to buffer solution. As it is seen from Fig. 1, optical density of chlorin  $e_6$  at 405 nm is about the same (even a little higher) for its buffer solution as compared to distilled water one. Thus, the possible reason of higher luminescence intensity of singlet oxygen generated by chlorin  $e_6$  in distilled water as compared to its buffer solution could be more efficient generation of singlet oxygen by protonated chlorin  $e_6$  form as compared to non-protonated one, due to, e.g., more efficient intersystem crossing, or more efficient energy transfer from chlorin  $e_6$  to  $^1\text{O}_2$ .

To get additional information about the photophysics of both molecular forms of chlorin  $e_6$ , we have estimated the values of fluorescence quantum yield of chlorin  $e_6$

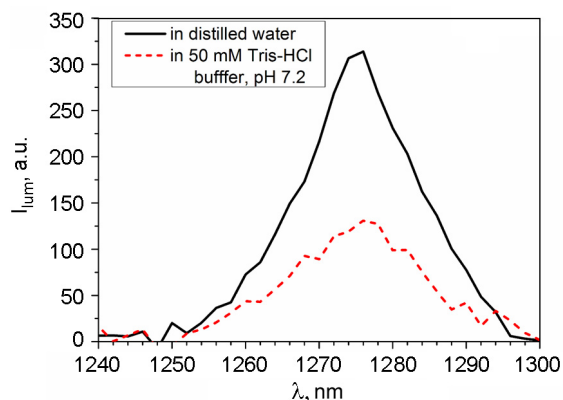


Fig. 3. Luminescence spectra of  $^1\text{O}_2$  obtained by excitation (at 405 nm) of chlorin  $e_6$  solution ( $5 \cdot 10^{-6} \text{ M}$ ) in distilled water (black solid curve) and in 50 mM Tris-HCl buffer (pH 7.2) (red dashed curve).

when dissolved in distilled water and 50 mM Tris-HCl buffer (pH 7.2) upon excitation at 405 nm; these values turned out to be equal to 0.11 and 0.13, respectively (Table). A sharp decrease in the fluorescence quantum yield of chlorin  $e_6$  upon decreasing pH value from 7 to 6 was also reported [12]. The obtained higher fluorescence quantum yield of non-protonated form is consistent with the possible lower yield of intersystem crossing for the same molecular form. At the same time, rather small values of fluorescence quantum yield leave place for various ratios between the yields of intersystem crossing of protonated and non-protonated forms of chlorin  $e_6$ .

The possible reason of higher luminescence intensity of  $^1\text{O}_2$  generated by protonated chlorin  $e_6$  molecules as compared to non-protonated ones could be also stronger

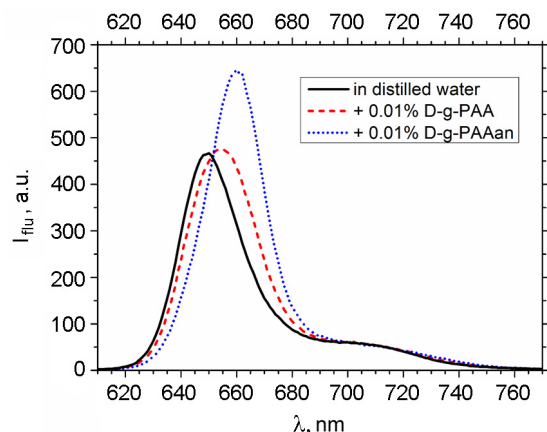


Fig. 4. Fluorescence spectra of chlorin  $e_6$  ( $5 \mu\text{M}$ ) in distilled water (black solid curve) and in the presence of D-g-PAA (red dashed curve) and D-g-PAAAn (blue dotted curve) upon excitation at 400 nm. Concentrations were  $5 \cdot 10^{-6}$  M for chlorin  $e_6$ , and 0.1 mg/mL (i.e. 0.01 %) for D-g-PAA and D-g-PAAAn.

quenching of  $^1\text{O}_2$  in buffer as compared to distilled water. This could be due to lower content of various ions in the latter as compared to the former. To clarify this point, we have also studied the effect of the presence of D-g-PAA and D-g-PAAAn graft polymers on the luminescence of  $^1\text{O}_2$  generated by chlorin  $e_6$  solution in distilled water. Earlier we found that the presence of these [13] or relative [14] polymers in distilled water resulted in destruction of hematoporphyrin aggregates due to binding its monomers to the polymers.

Fluorescence emission spectra of chlorin  $e_6$  in distilled water as well as in the presence of D-g-PAA and D-g-PAAAn graft polymers are presented in Fig. 4. It is seen from Fig. 4 that the presence of the polymers results in the long-wavelength shift of emission spectrum of chlorin  $e_6$ . This shift corresponds to deprotonation of at least a part of chlorin  $e_6$  molecules. Such molecular change is also manifested in absorption spectra of chlorin  $e_6$  in the presence of both polymers (data not presented). At the same time, the study of  $^1\text{O}_2$  luminescence gener-

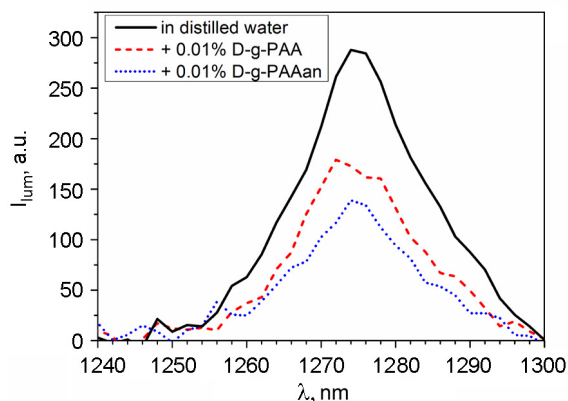


Fig. 5. Luminescence spectra of  $^1\text{O}_2$  obtained by excitation (at 405 nm) of chlorin  $e_6$  solution in distilled water (black solid curve) and in the presence of D-g-PAA (red dashed curve) and D-g-PAAAn (blue dotted curve). Concentrations were  $5 \cdot 10^{-6}$  M for chlorin  $e_6$ , and 0.1 mg/mL (i.e. 0.01 %) for D-g-PAA and D-g-PAAAn.

ated in these samples (Fig. 5) reveals that the presence of both graft polymers results in the decrease of the singlet oxygen luminescence intensity as compared to analogous solution without polymers. These results support the above assumption about more efficient generation of the singlet state of  $\text{O}_2$  molecules by the protonated form of chlorin  $e_6$  as compared to non-protonated one. It should be noticed that for the solutions of hematoporphyrin in distilled water, the presence of both D-g-PAA and D-g-PAAAn graft polymers increased the luminescence intensity of  $^1\text{O}_2$ ; this was caused by the destruction of hematoporphyrin aggregates due to binding of its molecules in monomer form with D-g-PAA and D-g-PAAAn [13].

Summarizing, we have observed higher intensity of  $^1\text{O}_2$  luminescence when generated in the solution of chlorin  $e_6$  in its protonated form (in distilled water without polymers) as compared to solutions of the same compound in its non-protonated form (in Tris-HCl buffer (pH 7.2), and in distilled

Table. Fluorescence quantum yield values of chlorin  $e_6$  and luminescence intensities of generated singlet oxygen (estimated as the area under the spectrum, in arbitrary units) for protonated and non-protonated forms of chlorin  $e_6$  obtained in distilled water and 50 mM Tris-HCl buffer (pH 7.2) respectively.

Solvent	Anticipated molecular form	Fluorescence quantum yield (ex. 405 nm)	$^1\text{O}_2$ emission intensity (ex. 405 nm), arb.un.
Distilled water	Protonated	0.11	6500
50 mM Tris-HCl buffer (pH 7.2)	Non-protonated	0.13	3000

water in the presence of graft polymers). The possible explanation could be the better efficiency of singlet oxygen generation by protonated chlorin e<sub>6</sub> form as compared to non-protonated one. In this case, the former molecular form should be characterized by higher rate of either intersystem crossing in chlorin e<sub>6</sub>, or excitation energy transfer from chlorin e<sub>6</sub> to <sup>1</sup>O<sub>2</sub> as compared to the latter. It was shown in [8] for chlorin e<sub>6</sub> solutions in buffers with pH 6.3 and pH 7.4 that the lifetimes of both singlet oxygen and chlorin e<sub>6</sub> triplet state do not depend on pH. Thus we could suggest that the protonation of chlorin e<sub>6</sub> affects the rate of intersystem crossing in this molecule. This assumption is consistent with the protonation influence on the maxima of absorption spectra and thus on the positions of singlet energy levels of chlorin e<sub>6</sub>. It should be also mentioned that, basing on the measured values of singlet oxygen yield, it was assumed in [8] that the efficiency of <sup>1</sup>O<sub>2</sub> generation does not depend on protonation. At the same time, it should be pointed that the studies described in [8] were performed upon excitation at 532 nm (i.e. in the Q-band absorption), while in our experiment Soret band was excited (at 405 nm). Meanwhile, for the representatives of chlorins [15] and porphyrins [16] it was shown that intersystem crossing efficiency significantly depends on the excitation wavelength.

#### 4. Conclusions

Luminescence intensity of singlet oxygen generated in the solution of chlorin e<sub>6</sub> in distilled water (where chlorin e<sub>6</sub> is mostly in its protonated form) was shown to exceed noticeably the intensity in Tris-HCl buffer (pH 7.2) where chlorin e<sub>6</sub> is predominantly non-protonated. At the same time, the presence of dextran-polyacrylamide and dextran-polyacrylamide-co-polyacrylic acid graft polymers in distilled water results in the transition of chlorin e<sub>6</sub> to its non-protonated form; this is accompanied with a decrease in singlet oxygen luminescence intensity. Basing on these observations, we suggest that the efficiency of singlet oxy-

gen generation by chlorin e<sub>6</sub> (upon excitation at the wavelength of its Soret band) is higher for protonated form of chlorin e<sub>6</sub> as compared to non-protonated one.

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