Different effect of polymer-incorporated nanoparticles of Au and Ag on hematoporphyrin interaction with graft polymers

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One of the ways to improve the efficiency of photodynamic therapy is to enhance the accumulation of the photosensitizer (PS) in the tumor; for this, either polymers or metal nanoparticles (NP) could be used. Here we studied the effect of Au and Ag nanoparticles (AuNPs and AgNPs, respectively) synthesized in situ in solution of non-charged and anionic polymers on spectral properties of PS hematoporphyrin (HP) as well as on $^1O_2$ generation by this compound (revealed by $^1O_2$ emission at 1275 nm). The star-like copolymer Dextran-graft-Polyacrylamide (D-g-PAA) and its anionic form (D-g-PAAan) were used as polymer matrices for nanosystems preparation. Absorption and fluorescence spectra show that HP molecules bind to D-g-PAA and D-g-PAAan in water that leads to the destruction of HP aggregates; these changes are accompanied by increase of $^1O_2$ generation. Meanwhile, in the presence of polymers with incorporated AgNPs (D-g-PAA/Ag and D-g-PAAac/Ag) the mentioned effect is stronger as compared to corresponding polymers without incorporated NPs. Thus AgNPs affect the graft polymers interaction with HP. Contrarily, the presence of both polymers with incorporated AuNPs enhances HP aggregation; besides, in the case of non-charged polymer with gold nanoparticles D-g-PAA/Au, Au nanoparticles induce appearing of different HP form, presumably protonated one. Effect of Ag and Au nanoparticles on fluorescent properties of HP is mainly determined by the effect of these NPs on aggregation of HP (and, in the case of D-g-PAA/Au, by appearing of different HP form). As for HP-sensitized singlet oxygen luminescence, effect of AgNPs is also mainly related to aggregation destruction, while this of Au nanoparticles could have other mechanisms as well.

Keywords: photodynamic therapy, hematoporphyrin, metal nanoparticles, graft polymers, singlet oxygen.

Одним из путей улучшения эффективности фотодинамической терапии является повышение накопления фотосенсибилизатора (ФС) в опухоли; для этого можно использовать полимеры либо наночастицы (НЧ) металлов. В работе исследовано влияние наночастиц золота и серебра (АuНЧ и АgНЧ соответственно) синтезированных *in situ* в растворе незаряженных и анионных полимеров, на спектральные свойства ФС гематопорфирина (ГП), а также на генерацию синглетного кислорода ($^1O_2$) этим соединением (которую детектировали по излучению $^1O_2$ на 1275 нм). Разветвленный кополимер декстран-полиакриламид (Д-ПАА) и его анионная форма (Д-ПААан) использовались как полимерные матрицы для получения наносистем. Спектры поглощения и флуоресценции демонстрируют, что молекулы ГП в воде связываются с Д-ПАА и Д-ПААан, что приводит к разрушению агрегатов ГП; эти изменения сопровождаются усилением генерации $^1O_2$. В то же время, в присутствии полимеров с инкорпорированными АgНЧ

(Д-ПАА/Ag и Д-ПААан/Ag) упомянутые эффекты сильнее по сравнению с соответствующими полимерами без инкорпорированных НЧ. Следовательно, AgНЧ влияют на взаимодействие разветвленных полимеров с ГП. В отличие от AgНЧ, присутствие обоих полимеров с инкорпорированными AuНЧ усиливает агрегацию ГП; кроме того, в случае незаряженного полимера с наночастицами золота Д-ПАА/Au, наночастицы Au вызывают появление новой формы ГП, вероятно протонированной. Влияние AgНЧ и AuНЧ на флуоресцентные свойства ГП определяется, главным образом, влиянием этих НЧ на агрегацию ГП (и, в случае Д-ПАА/Au, появлением новой формы ГП). Что касается люминесценции синглетного кислорода, сенсибилизированной ГП, то влияние на нее AgНЧ также связано, главным образом, с разрушением агрегатов, в то время как влияние AuНЧ может также реализироваться через другие механизмы.

Протилежний вплив наночастинок Au та Ag, інкорпорованих у полімери, на взаємодію гематопорфірину з розгалуженими полімерами. *М.Ю.Лосицький, Р.А.Харченко, Ю.І.Гарагуц, Е.А.Ширінян, Ю.В.Маліновська, Н.В.Куцевол, В.М.Ящук.*

Одним зі шляхів покращення ефективності фотодинамічної терапії є підвищення накопичення фотосенсибілізатора (ФС) у пухлині; для цього можна використовувати полімери або наночастинки (НЧ) металів. В досліджено вплив наночастинок золота і срібла (AuНЧ та AgНЧ відповідно) синтезованих *in situ* у розчині незаряджених та аніонних полімерів, на спектральні властивості ФС гематопорфірину (ГП), а також на генерацію синглетного кисню ($^1O_2$) цією сполукою (яку виявляли за випромінюванням $^1O_2$ на 1275 нм). Розгалужений кополімер декстран-поліакриламід (Д-ПАА) та його аніонна форма (Д-ПААан) використовувалися як полімерні матриці для отримання наносистем. Спектри поглинання та флуоресценції демонструють, що молекули ГП у воді зв'язуються з Д-ПАА та Д-ПААан, що призводить до руйнування агрегатів ГП; ці зміни супроводжуються посиленням генерації $^1O_2$. В той же час, в присутності полімерів з інкорпорованими AgNP (Д-ПАА/Ag та Д-ПААан/Ag) згадані ефекти є сильнішими порівняно з відповідними полімерами без інкорпорованих НЧ. Отже, AgНЧ впливають на взаємодію розгалужених полімерів з ГП. На відміну від AgНЧ, присутність обох полімерів з інкорпорованими AuНЧ посилює агрегацію ГП; крім того, у випадку незарядженого полімеру з наночастинками золота Д-ПАА/Au, наночастинки Au викликають появу нової форми ГП, ймовірно протонованої. Вплив AgНЧ і AuНЧ на флуоресцентні властивості ГП визначається, головним чином, впливом цих НЧ на агрегацію ГП (та, у випадку Д-ПАА/Au, появою нової форми ГП). Що стосується люмінесценції синглетного кисню, сенсибілізованої ГП, то вплив на неї AgНЧ також пов'язаний, головним чином, з руйнуванням агрегатів, в той час як вплив AuНЧ може також реалізуватися через інші механізми.

1. Introduction

Photodynamic therapy (PDT) is a modern cancer treatment method that is intensively developed and successfully applied [1]. This method is based on administration of a non-toxic photosensitizer (PS) into organism, followed by selective PS accumulation in tumor tissue. Upon further irradiation of the accumulated PS in the presence of molecular oxygen, its reactive forms (e.g., singlet oxygen $^1O_2$) are generated, eradicating tumor cells [2].

The main advantages of PDT as compared to traditional methods of treatment (chemotherapy and radiation therapy) are high tumor specificity and low harm to healthy tissues [3]. At the same time, PDT has several drawbacks, the main one being the limited depth of tissue penetration of visible or near infrared light. One of the ways to improve the efficiency of photodynamic therapy is to enhance the PS sensitivity to light as well as its accumulation in the tumor, making cancer tissues sensitive even to the irradiation attenuated throughout a tumor volume. Thus, designing PS agents with improved photosensitizing and pharmacokinetic properties still remains a major challenge in PDT. Besides, an extensively studied approach to enhance the tumor treatment efficiency is to combine different mechanisms of treatment action [4], e.g. PDT and photothermal therapy (PTT) [5]. One of the promising ways to achieve these goals is to use metal nanoparticles and polymers.

Linear polymers are known to improve drugs delivery to specific cells or tissues, as well as to enhance efficiency of the photodynamic drugs. Particularly, a system composed of the known PS chlorin e_6 and a water-soluble and non-toxic polymer polyvinylpyrrolidone was successfully used in PDT [6]. At the same time, high efficiency of graft dextran-polyacrylamide macromolecules as vector nanocarriers in drug therapy

was demonstrated [7]. We have shown that binding with linear and graft polymers reduces the aggregation of the photosensitizer hematoporphyrin (HP) [8, 9].

On the other hand, since recently metal nanoparticles have been widely involved in the development of novel anticancer therapeutics. Particularly, gold nanoparticles (AuNPs) draw a special attention for biomedical applications due to their biocompatibility and low toxicity [10]. The ability of AuNPs to penetrate the endothelium of the tumor vessels provides higher selectivity of the AuNPs-containing drug accumulation in the tumor [11]. The phenomenon of a localized surface plasmon resonance in AuNPs leads to appearance of the intense plasmonic band in their absorption, allowing for application of AuNPs in plasmonic photothermal therapy (PPTT), where irradiation of AuNPs with light at the wavelength within a plasmonic absorption band results in strong localized heating that destructs the surrounding cancer cells and tissues [12–14]. Though PPTT requires high power pulsed laser irradiation and larger particles with higher concentration in the targeted site to be efficient [15], combination of PDT with tumor hyperthermia is known to increase the efficiency of PDT, causing an increase in the cytocidal activity of the PS on the tumor cells in vivo [16]. This opens the way to achieve an enhanced PDT by combining it with localized heating caused by irradiation of AuNPs delivered to cancer site alongside with PS. Possible applications of silver nanoparticles (AgNP) in cancer therapy are also studied; and cytotoxicity of AgNPs to various cancer cells was shown [17]. Besides, photothermal effect was demonstrated for AgNPs, though it was lower as compared to that of AuNPs [18].

The use of metal NP incorporated into polymers could combine the above mentioned approaches to improve the PDT efficiency with the possibility of plasmonic enhancement of PS absorption and singlet oxygen generation. Earlier, the graft dextran-polyacrylamide macromolecules were shown to be appropriate as templates for preparation of nanosystems containing both metal NP and sensitizer simultaneously, which could be very efficient in antitumor PDT [7]. Besides, graft dextran-polyacrylamide macromolecules containing AuNPs were shown to double the *in vitro* photodynamic activity of the PS chlorin e_6 in comparison with that of free chlorin e_6 [19].

In this paper, we studied the effects of AuNPs and AgNPs incorporated into dextran-polyacrylamide (PAA) and dextran-PAA-co-polyacrylic acid (D-g-PAA and D-g-PAAac, respectively) graft polymers on spectral properties of photosensitizer HP, as well as on $^1O_2$ generation by this compound.

2. Experimental

Materials

Hematoporphyrin was kindly provided by M.F. Gamaleia (R.E. Kavetsky Institute for Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine).

Polymers. Copolymers Dextran-graft-polyacrylamide (D-g-PAA) with dextran core ($M_w = 70·10^5$ g/mol) and 20 grafted PAA chains in nonionic and anionic form were used as a polymer matrix for AgNPs and AuNPs synthesis and fabrication of trial nanosystems containing both AuNPs and PS. Synthesis, molecular parameters and peculiarities of macromolecular structure of star-like copolymer D-g-PAA were discussed in details in [20]. The average molecular weight of D-g-PAA (M_w) was equal to $1.43·10^6$ g/mol, radius of gyration (R_g) — 64 nm, and polydispersity (M_w/M_n) — 1.98.

The 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 % [21]. The D-g-PAAan copolymer was purified, freeze-dried and kept under vacuum for preventing it from further hydrolysis.

Gold nanoparticles syhthesis. The AuNPs were synthesized by the chemical reduction of Au precursor (tetrachloroauric acid) [19]. All synthesized polymers play a role of matrices C

apable to act as nucleating, capping and stabilizing agents simultaneously. 0.012 ml tetrachloroauric acid aqueous solution ($C = 0.1$ M) was added to 0.5 ml of aqueous polymer solution ($C = 1$ g·l^{-1}) and stirred during 20 min. Then, 0.047 ml of 0.1 M aqueous solution of $NaBH_4$ was added. The final solution was stirred for 30 min. It turned ruby-red in color, thus the formation of AuNPs was indicated. The reduction process was performed at 25°C.
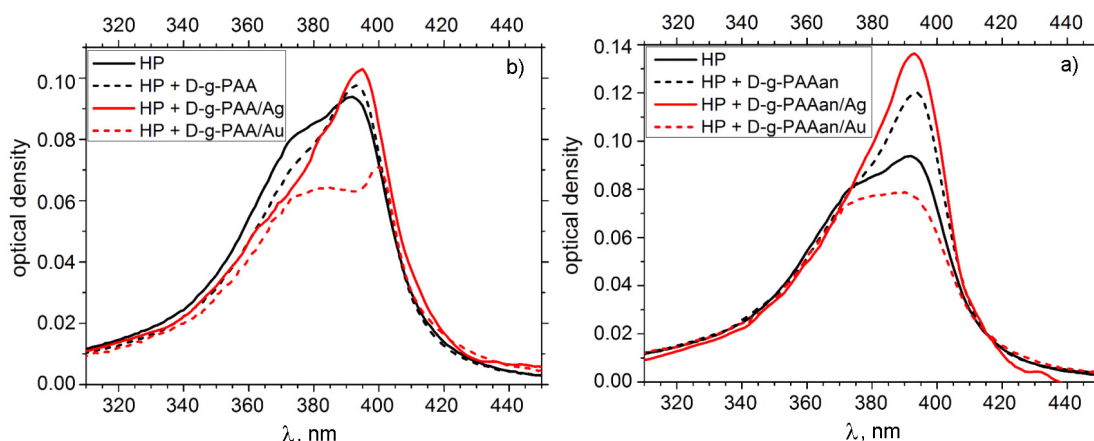
Fig. 1. Absorption spectra (Soret band) of HP solution in distilled water and in the presence of (a) D-*g*-PAA, D-*g*-PAA/Ag, D-*g*-PAA/Au, and (b) D-*g*-PAAan, D-*g*-PAAan/Ag, D-*g*-PAA/Au. Concentrations were 10^{-6} M for HP, 0.05 mg/mL D-*g*-PAA and D-*g*-PAAan, 0.049 mg/mL for Au and 0.027 for Ag. For AgNP- and AuNP-containing samples, absorption of corresponding polymer/NP systems was subtracted for better visualization of HP spectrum changes.

Silver nanoparticles synthesis. Reduction of Ag ions was performed in aqueous solutions of polymer templates [22]. Molar ratio of acrylamide groups to Ag ions was equal to 5. At first 0.01 M AgNO$_3$ aqueous solution was added to 0.5 ml of polymer solution ($C = 1$ g·l^{-1}) and stirred during 20 min at 25°C. Then, 0,1 M of NaBH$_4$ was added drop by drop at stirring. It turned reddish brown, thus the formation of AgNPs was indicated. Dimethylformamide (DMF), ethanol and distilled water were used as solvents.

Preparation of solutions

Stock solution of hematoporphyrin (10^{-2} M) was prepared in DMF, and further dissolved in ethanol to 10^{-3} M. The solutions of polymers and polymer/NP systems were further dissolved in water, and after letting them reach equilibrium for about an hour, aliquot of 10^{-3} M HP solution was added. Final concentrations were 10^{-6} M for HP, 0.05 mg/mL for D-*g*-PAA and D-*g*-PAAan, 0.049 mg/mL for Au and 0.027 for Ag (for absorption and fluorescence measurements), and $5 \cdot 10^{-6}$ M for HP, 0.1 mg/mL for D-*g*-PAA and D-*g*-PAAan, 0.098 mg/mL for Au and 0.054 for Ag (for singlet oxygen emission measurements).

Spectral measurements

Absorption spectra were registered with the UV1900PC spectrophotometer (China). In order to make clear the effect of polymers and polymer/NP systems on HP absorption, spectra of polymer/NP solutions were subtracted from the spectra of HP in the presence of corresponding solutions.

Fluorescence spectra were measured using a Cary Eclipse fluorescence spectrophotometer (Varian, Australia). To account for the inner filter effect due to the strong extinction at the fluorescence excitation wavelength (400 nm), HP emission spectra in the presence of D-*g*-PAA/Au, D-*g*-PAAan/Au, D-*g*-PAA/Ag and D-*g*-PAAan/Ag were corrected for the factor of $10^{D/2}$, where D is the optical density of corresponding polymer/NP system at 400 nm [23]. For measurements, solutions were placed into 1 cm·1 cm quartz cell. All measurements were performed at room temperature.

Singlet oxygen luminescence measurements

Luminescence spectra of the molecular oxygen were registered with the help of the laboratory-designed 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.

3. Results and discussion

First of all, absorption spectra were measured for HP solutions in water, as well as in the presence of graft polymers and polymer/NP systems. Processes of intermolecular interactions with participation of HP molecules are manifested the most obviously in the shape and position of the Soret band (Fig. 1). Thus, for HP solution in water, maximum at 392 nm and shoulder at
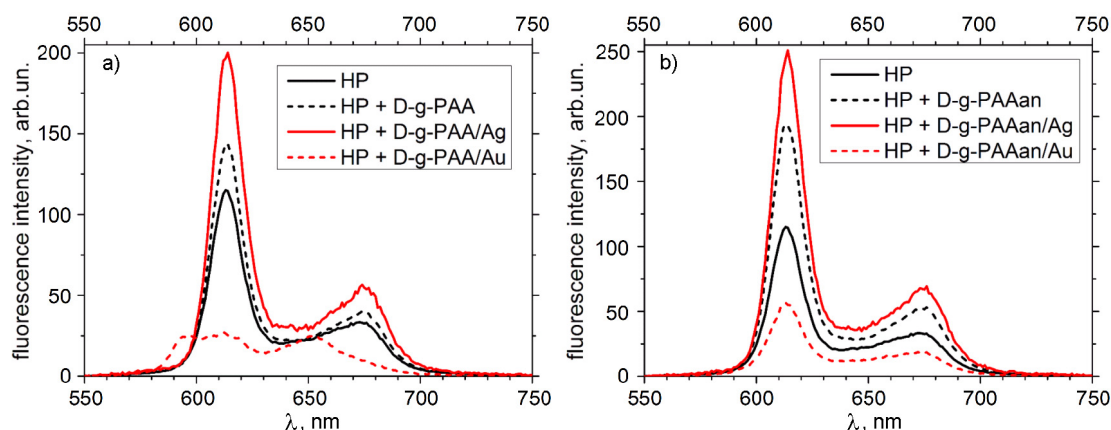
Fig. 2. Fluorescence emission spectra of HP solution in distilled water and in the presence of (a) D-g-PAA, D-g-PAA/Ag, D-g-PAA/Au, and (b) D-g-PAAan, D-g-PAAa/Ag, D-g-PAAan/Au. Concentrations were 10^{-6} M for HP, 0.05 mg/mL for D-g-PAA and D-g-PAAan, 0.049 mg/mL for Au and 0.027 for Ag. Excitation wavelength 400 nm.

372 nm are observed; these bands correspond to monomers and aggregates (dimers) of HP molecules, respectively [24]. The presence of both non-charged dextran-polyacrylamide and anionic dextran-polyacrylamide-co-polyacrylic acid graft polymers leads to a decrease in the contribution of the dimer band to the spectrum (Fig. 1a and Fig. 1b, respectively) that could be interpreted as the destruction of HP aggregates as a result of HP molecules binding with the polymers. Earlier, we observed the same effect for similar graft polymers [8]. It should be mentioned that in the case of negatively charged polymer D-g-PAAan, the destruction of aggregates is stronger than in the presence of non-charged polymer D-g-PAA.

At the same time, it can be seen from the absorption spectra that metal nanoparticles incorporated into the polymers affect the spectra of HP. Namely, the presence of AgNP in D-g-PAA/Ag and D-g-PAAan/Ag polymer/NP nanosystems leads to still stronger enhancement of the monomer band and reduction of the dimer one as compared to corresponding polymers without NP. Thus, the presence of AgNP incorporated into polymers results in stimulating the interaction of HP monomer molecules with the polymer.

Meanwhile, the presence of AuNPs in both D-g-PAA/Au and D-g-PAAan/Au polymer/NP systems results in quite different effect on HP spectra. It was shown (Fig. 1 a, b) that the presence of both D-g-PAA/Au and D-g-PAAan/Au leads to the decrease of monomer band of HP even stronger than for the free HP. Besides, a narrow band at 400

nm appears in the spectrum of HP in the presence of D-g-PAA/Au, which should correspond to some different form of HP.

Fluorescence spectra of HP in water and in the presence of polymers and polymer/NP systems are presented in Fig. 2. It can be seen that the shapes of the spectra are close for HP in water and for HP in the presence of D-g-PAA, D-g-PAAan, D-g-PAA/Ag, D-g-PAAan/Ag and D-g-PAAan/Au and contain two bands with maxima at 613 and 675 nm that correspond to emission of monomeric form of HP in water [8, 9]. As for the intensities of the mentioned spectra, fluorescence intensity of HP is higher in the presence of D-g-PAA and D-g-PAAan as compared to free HP, and the presence of D-g-PAA/Ag and D-g-PAAan/Ag leads to still higher intensity growth (Fig. 2a, b). As for the D-g-PAAan/Au, its presence decreases the fluorescence intensity of HP as compared to free HP (Fig. 2b). The fluorescence intensities of the mentioned samples correlate with the relative quantities of monomeric HP form in the solution. Thus, the influence of D-g-PAA, D-g-PAAan, D-g-PAA/Ag, D-g-PAAan/Ag and D-g-PAAan/Au on HP emission intensity could be explained by influence of these polymers and nanosystems on the ratio of fluorescent HP monomers and its nonfluorescent aggregates; no metal enhancement of fluorescence was observed.

At the same time, the case of D-g-PAA/Au polymer/NP system is quite different. It was shown that its presence resulted in appearing of a different HP form, characterized by relatively narrow absorption Soret band at 400 nm (Fig. 1). At the same
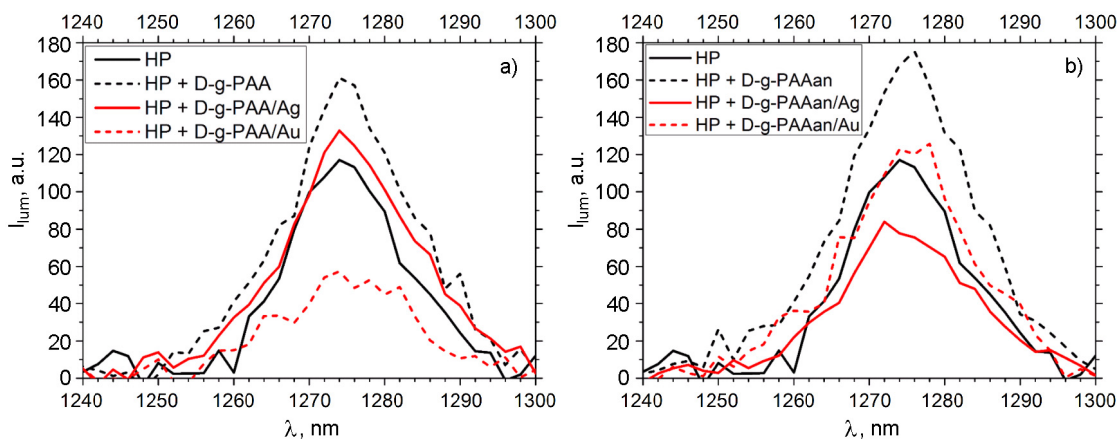
Fig. 3. Luminescence spectra of $^1O_2$ obtained by excitation (at 400 nm) of HP solution in distilled water and in the presence of (a) D-g-PAA, D-g-PAA/Ag, D-g-PAA/Au, and (b) D-g-PAAan, D-g-PAAan/Ag, D-g-PAAan/Au. Concentrations were $5 \cdot 10^{-6}$ M for HP, 0.1 mg/mL D-g-PAA and D-g-PAAan, 0.098 mg/mL for Au and 0.054 for Ag.

time, in the fluorescence spectrum of HP in the presence of D-g-PAA/Au, together with the monomer band at 613 nm, there are bands at 596 nm and 652 nm, which correspond to the mentioned different HP form. Earlier we have observed the HP form with such absorption and fluorescent properties for the case of HP solution in acidic medium (pH 3.1); this form was thus attributed to protonated HP monomer [9]. Thus we can assume that the presence of AuNPs in electrically neutral polymer D-g-PAA possibly causes a decrease in pH of the local medium. It should be noted that the presence of AuNPs in negatively charged polymer D-g-PAAan does not cause such effect.

Finally, the luminescence of singlet oxygen upon excitation of HP in water and in the presence of the polymers and polymer/NPs systems was studied (Fig. 3). Excitation at Soret band (405 nm) was used as the most efficient for singlet oxygen generation [25].

For all the studied compounds, the luminescence band of $^1O_2$ with maximum at 1275 nm was detected. The presence of D-g-PAA and D-g-PAAan polymers leads to enhancement of singlet oxygen emission that correlates with HP fluorescence enhancement in the presence of these polymers. We can suppose that this increase of $^1O_2$ emission is due to the increase in the fraction of monomer HP molecules.

At the same time, for HP in the presence of polymer/NPs systems such correlation between HP-sensitized $^1O_2$ luminescence and HP fluorescence intensities is less evident. First of all, contrarily to HP emission intensity (Fig. 2), HP-induced $^1O_2$ lumines-

cence intensity is lower in the presence of AgNP-containing polymers as compared to corresponding polymers without NPs. This result could be explained by the inner filter effect due to strong absorption of AgNPs at the excitation wavelength (405 nm). To support this assumption, we also studied the samples containing the same HP concentration but ten times lower concentration for polymer and polymer/NPs system. For such samples where inner filter effect was small, both HP intensities and HP-induced $^1O_2$ intensities were higher in the presence of polymer-AgNPs systems as compared to polymers without NPs (data not presented).

Further, in the case of D-g-PAA/Au, the HP-induced $^1O_2$ intensity is lower than for the free HP as well as for HP in the presence of D-g-PAA (Fig. 3a) that correlates with the behavior of HP fluorescence (Fig. 2a). Meanwhile, the presence of D-g-PAAan/Au polymer/NPs system does not change the HP-induced $^1O_2$ intensity comparing to that for free HP (Fig. 3b), while fluorescence intensity of HP in the presence of D-g-PAAan/Au is about two times lower as compared to that of free HP (Fig. 2b). Since several factors influence the HP-induced $^1O_2$ intensity, including the rate of intersystem crossing in HP molecule, the rate of HP-to-O_2 energy transfer and the luminescence quantum yield of $^1O_2$ itself, the observed result could be explained by the effect of D-g-PAAan/Au on any of the mentioned factors.

4. Conclusions

HP molecules interact with graft polymers of both types (non-charged and in anionic form) in water, resulting in decrease of HP aggregation.

Ag and Au nanoparticles incorporated into polymers affect the binding of HP molecules in different ways. While AgNPs enhance the destruction of HP aggregates, AuNPs enhance aggregation. Besides, in the case of neutral polymer D-g-PAA, Au nanoparticles induce appearing of different HP forms, presumably protonated ones.

Effect of Ag and Au nanoparticles on fluorescent properties of HP is mainly determined by the effect of these NP on aggregation of HP (except for the case of D-g-PAA/Au where different form of HP appears). As for HP-sensitized singlet oxygen luminescence, the effect of AgNPs is also mainly related to aggregate destruction, while that of Au nanoparticles could have other mechanisms as well.

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