

Contents lists available at ScienceDirect

# Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy



journal homepage: www.elsevier.com/locate/saa

# Nanoparticles of water-soluble dyads based on amino acid fullerene C<sub>60</sub> derivatives and pyropheophorbide: Synthesis, photophysical properties, and photodynamic activity



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## HIGHLIGHTS

- Three amino acid-fullerene[60]-pyro pheophorbide a dyads (AFD-PPa) were synthesized.
- AFD-PPa dyads form nanoassociates in water as a result of self-assembly.
- Photodynamic mechanism switching from type II of dye to I type of dyad was shown.
- All dyads have a pronounced phototoxicity on HeLa cancer cells.

#### ARTICLE INFO

Article history: Received 20 November 2020 Received in revised form 22 April 2021 Accepted 25 April 2021 Available online 30 April 2021

Keywords: Fullerene Water-soluble amino acid fullerene derivatives Pyropheophorbide Fluorescence quenching Photochemical activity Photosensitizer

# 1. Introduction

# Photodynamic therapy is an actively developing field of medicine, based on the selective effect on a tumor or microorganisms of low-toxic molecules of dyes – photosensitizers when excited into a triplet state with the light of a certain wavelength. Under

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# GRAPHICAL ABSTRACT



# ABSTRACT

Synthesis, spectral properties, and photodynamic activity of water-soluble amino acid fullerene  $C_{60}$  derivatives (AFD) and four original AFD-PP*a* dyads, obtained by covalent addition of dye pyropheophorbide (PP*a*) to AFD, were studied. In aqueous solution, these AFD-PP*a* dyads form nanoassociates as a result of self-assembly. In this case, a significant change in the absorption spectra and strong quenching of the dye fluorescence in the structure of the dyads were observed. A comparison of superoxide or singlet oxygen generation efficiency of the studied compounds in an aqueous solution showed the photodynamic mechanism switching from type II (singlet oxygen generation of the native dye) to I type (superoxide generation of dyads). All dyads have pronounced phototoxicity on cells Hela with IC<sub>50</sub> 9.2  $\mu$ M, 9.2  $\mu$ M, 12.2  $\mu$ M for dyads Val-C<sub>60</sub>-PP*a*, Ala-C<sub>60</sub>-PP*a* and Pro-C<sub>60</sub>-PP*a*, respectively. Such facilitation of type I photodynamic mechanism could be perspective against hypoxic tumors.

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photoexcitation, free radicals are generated – singlet oxygen, superoxide, hydroxyl radical, and other reactive oxygen species (ROS), which suppress the growth of tumors or microorganisms [1,2]. The effectiveness of the photosensitizer depends on the chemical structure of the dye and the wavelength of the exciting light.

Dyes from the class of porphyrins, phthalocyanines, and chlorins are widely used nowadays in medical practice, which have a high triplet state quantum yield and absorb in the red spectral region since the light of these wavelengths penetrates the body tissues best [1-4].

The possibility of a significant increase in the photodynamic activity was previously demonstrated due to the formation of non-covalent complexes of the water-soluble pentacationic fullerene derivative with the photodynamic preparation Fotosens, with xanthene dyes fluorescein, eosin, erythrosine [5–8], as well as covalent conjugates of polysubstituted fullerene derivatives with the antitumor antibiotic Ruboxil, dyes fluorescein and chlorin  $e_6$  [9–12]. These works showed that the generation of superoxide anion radical was enhanced by electron transfer or excitation from a singlet-excited dye to fullerene and then to an oxygen molecule. The generation efficiency of  $O_2^{-}$  depended on the presence of electrostatic charges on the dye and fullerene. Similar effects were observed for other fullerene-dye dyads [12–21].

In addition to spectral properties, an important characteristic of compounds for photodynamic therapy is their ability to penetrate biological membranes and accumulate in tumors. There are suggestions that compounds with a minimum charge have the most effective permeability through biological membranes [22]. In this regard, in addition to studying fullerene-dye dyads based on pentasubstituted fullerenes derivatives, it seems interesting to analyze the possibility of creating photoactive water-soluble fullerene-dye dyads and nanoparticles (NPs) based on monosubstituted amino acid fullerenes derivatives (AFD) (Fig. 1).

An effective and technologically advanced method for creating such water-soluble AFDs that form stable aqueous solutions with a concentration of 10–30 mg/ml has been previously developed [23,24]. According to this technique, fullerene is modified by attaching an amino acid to the fullerene core by breaking the double bond of the fullerene and forming a bond with the amino group of the amino acid. This method allows one to obtain a wide range of biologically active derivatives of fullerene, which showed membrane-active, antioxidant, and immunomodulatory properties with low toxicity, antivirus, antitumor, and neuroprotective activities [25–32]. Theoretical calculations showed the thermodynamic stability of such amino acid fullerene derivatives [33,34].

In various versions, this technique was reproduced by other researchers [35,36,45,37–44]. It is possible to significantly expand the possibilities of creating hybrid nanoparticles based on amino acid derivatives of fullerenes by attaching the second addendum to the fullerene core by replacing the "acidic" proton of the amino acid fullerene derivative. (Fig. 1) [28,46,47]. Thus, it is possible to create a wide range of biocompatible hybrid structures using a combination of two different addends: one of them, the amino acid, gives the fullerene core water solubility, and the second – additional biological properties, including photodynamic.

It should be noted that AFD, even with the addition of one amino acid, has a sufficiently high solubility in water, up to 30 mg/ml. However, studies show that in this case, NPs are formed, which often complicates the establishment of the molecular structure of newly synthesized compounds [48-50]. Specific interactions arise between the AFD and the solution, which leads to a change in the density of the solution, melting point, surface tension, conductivity, the dissociation constant, and the effect of dynamic light scattering [51–56]. Based on the above methods, in this work, with the aim of creating new photodynamic compounds. we investigated methods for creating covalent AFD dyads with dye pyropheophorbide a (PPa) (AFD-PPa). It was shown that these dyads form NPs in aqueous solutions as a result of self-assembly. Spectral, photophysical properties of these dyads and NPs based on them were studied. Photodynamic action was investigated in an aqueous medium with singlet oxygen and superoxide anion radical probes, as well as on cell culture HeLa.

# 2. Experimental section

# 2.1. Materials

## 2.1.1. Reagents

The following reagents were used in the studies: fullerene  $C_{60}$  (Fullerene Center, Nizhny Novgorod, 99.5%), L-Valine, L-Proline, L-Alanine (Sigma), DPBF (1,3-Diphenylisobenzofuran, Sigma), methyl iodide (Sigma, 99%), NADH (nicotinamide adenine dinucleotide, Sigma), NBT (nitro blue tetrazolium chloride, Sigma), EDTA (ethylenediamine-tetraacetic acid, Sigma), MTT (3-(4,5-dime thyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma)

Dichlorobenzene («Acros organics», 99%) and pyridine («Acros organics», 99%) were distilled and dehydrated before usage. Other solvents (purity  $\sim$  99.9%) were used without purification.

# 2.2. Synthesis of pyropheophorbide a, $C_{60}$ -amino acid derivatives and dyads

**Synthesis of pyropheophorbide a.** The synthesis of pyropheophorbide *a* (PP*a*, Fig. 1) was carried out according to the previously described method [57] from methylpheophorbide *a* (Fig. S1, Supplementary). Product yield: 87.5% mg. The ESI-ms, NMR, and UV–VIS-spectra of pyropheophorbide *a* coincide with those in [58].

Synthesis of Zn-substituted pyropheophorbide a (PPa(Zn), Fig. 1) was carried out according to the previously described method [59] (Fig. S1, Supplementary).

*Synthesis of*  $C_{60}$ *-amino acid derivatives.* Amino acid fullerene derivative (AFD) **1**, **2**, and **3** (Fig. 1) were synthesized according to the method described earlier [23] (Fig. S1, Supplementary).

**Synthesis of AFD-PPa dyads.** Compounds Val-C<sub>60</sub>-PPa, Ala-C<sub>60</sub>-PPa, Pro-C<sub>60</sub>-PPa (**6**, **7**, and **8**, Fig. 1) were synthesized by attaching PPa to AFD with replacing the "acidic" proton of the amino acid fullerene derivative according to the following procedure (Fig. S1, Supplementary): PPa (0.0288 mmol) was dissolved in 2 mL abs. chloroform, then with stirring and cooling in an ice bath, a 1.5-fold excess of thionyl chloride (0.0433 mmol, 5.15 mg) was added to it, and then it was boiled with stirring for 2 h. After cooling, 2 mL of pyridine was added and the excess thionyl chloride was distilled off. For synthesis of Val-C<sub>60</sub>-PPa (as example), a 1.5-fold excess of Val-C<sub>60</sub> methyl ester (0.0433 mmol, 38 mg) in 2 mL abs. pyridine was added to this solution and stirred at room temperature for one day.

Purification of the reaction products in the synthesis of AFD-PPa dyads was carried out by gel chromatography on a 3x70 cm glass column with Bio Beats SX1 (cross-linked polystyrene) with pyridine as eluent. The fractions yield was recorded using an LKB Bromma 2151 densitometer at a wavelength of 415 nm. The analysis of the obtained chromatographic fractions was carried out by UV–VIS spectrophotometry and fluorescence spectroscopy. During chromatography, the target product AFD-PPa comes out as the first main peak, and the unattached excess of PPa comes out as a subsequent extended peak.

The synthesis of methyl ester Val-C<sub>60</sub>-PPa(Zn) (**9**, Fig. 1) was carried out using ethylene chlorohydrin according to the following procedure (Fig. S1, Supplementary): to 0.0655 g (0.1217 mmol) of PPa(Zn) dissolved in 5 mL of DMF, 0.0098 g (0.1217 mmol) of ethylene chlorohydrin, 0.0251 g (0.1217 mmol) of dicyclohexylcarbodiimide were added and left at room temperature for 2 days. To this solution was added 0.0852 g (0.1 mmol) of N-(monohydro) fullerenyl valine methyl ester in 5 mL of pyridine and stirred for 24 h at room temperature. Determination of the content of Zn atoms in the composition of nanoparticles based on Val-C<sub>60</sub>-PPa (Zn) was carried out by X-ray fluorescence spectral analysis using the VRA-30 spectrometer from Karl Zeiss, Jena (Germany).



Fig. 1. Structures of AFDs, PPa, chlorin e<sub>6</sub>, and AFD-PPa dyads.

#### 2.3. Preparation of water-soluble nanoparticles based on dyads

To obtain water-soluble dyads 10-fold excess of NaOH in water was added to dyad in pyridine and stirred for 4 h at room temperature. The solution of AFD-PPa NPs was dialyzed against aqueous solution using a 14,000 Da membrane (Sigma, D9277) for 3–4 days with frequent replacement of the aqueous solution to remove excessive pyridine and unconjugated PPa. The concentration of AFD-PPa dyads in the resulting solution was estimated from an aliquot that was dried by the known molecular weight of the dyad.

# 2.4. Computational simulation of dyads structures

The spatial structures of AFD-PP*a* dyads (Fig. 3) were geometrically optimized with a semi-empirical quantum chemistry method using the MOPAC2016 package [60] with PM7 and COSMO parameterization methods to account for the surrounding water [61].

# 2.5. Photophysical and photochemical studies

Absorption spectra were measured using a Cary-60 spectrophotometer equipped with a thermostated cell. Fluorescence steadystate spectra of the dyes under study were recorded by a Cary-Eclipse fluorescence spectrophotometer. Dynamic light scattering (DLS) measurements were performed at a detection angle of 90° with a Photocor Complex (Photocor Instruments Inc., USA) setup equipped with a TEC stabilized diode laser ( $\lambda$  = 790 nm).

The photochemical activity of the studied compounds in water was investigated with NBT and DPBF probes for the detection of superoxide anion radical and singlet oxygen by the methods described in [12]. Table 1 shows both the values of the comparative efficiency of ROS generation by all compounds normalized to the photodynamic activity of the Ce6 dye and the observed quantum yield expressed in the number of  $O_2^{\bullet}$  or  ${}^1O_2$  molecules per absorbed quantum of light detected in the model system. Normalization was carried out, taking into account the absorbance spectrum of the compound, transparency spectrum of used cut-off glass filter, and xenon lamp emission spectrum at  $\lambda > 630$  nm.

# 2.6. Phototoxicity on HeLa cell line

The influence of the compound under study on cell line HeLa viability was evaluated with staining with MTT. Conditions of cell incubation and irradiation were the same as in [12].

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#### Table 1

A comparison of the photophysical properties and photodynamic activity of covalent conjugates AFD-PPa.

	Ce <sub>6</sub>	6	7	8
Hydrodynamic radius in water, nm	27-80	20-25	23-30	32-42
Q band $\lambda_{Qmax}$ , nm	654	680	673	672
Fluorescence $\lambda_{max}$ , nm	659	667	670	660
Relative fluorescence quantum yield	0.22*	4.6·10 <sup>-3</sup>	3.5·10 <sup>-3</sup>	9.4·10 <sup>-3</sup>
Relative efficiency of $O_2^{\bullet-}$ generation in water	1	2.4	3.6	2.7
Relative efficiency of O <sub>2</sub> <sup>•-</sup> generation in water, absorption normalized	1	4.8	10.2	3.7
Relative efficiency of O <sub>2</sub> <sup>-</sup> generation in liposomes	1	3.5	4.0	3.9
Relative efficiency of O <sub>2</sub> <sup>•</sup> generation in liposomes, absorption normalized	1	6.9	11.3	5.2
Relative efficiency of <sup>1</sup> O <sub>2</sub> generation in liposomes	1	0.087 (1/11.5)	0.108 (1/9.3)	0.13 (1/7.6)
Relative efficiency of <sup>1</sup> O <sub>2</sub> generation in liposomes, absorption normalized	1	0.17 (1/5.9)	0.30 (1/3.3)	0.18 (1/5.6)
Phototoxicity on HeLa cells IC <sub>50</sub> , $\mu$ M	0.83	8.6	12	8.2

Fluorescence quantum yield value of chlorin  $e_6$  trisodium salt ( $Ce_6$ ) was taken from work [4].

## 3. Approach justification

In recent years, special attention has been paid to the creation of new highly effective photodynamic compounds based on hybrid structures «fullerene-dyes». In these structures, the fullerene core generates reactive oxygen forms by transferring energy or an electron upon photoexcitation of a dye. In addition, the presence of a hydrophobic fullerene core ensures membranotronic properties of such hybrid structures, which can also enhance the photodynamic effect. Membranotropic properties of such hybrid structures can vary significantly due to the addition of polar addends to the fullerene core. Earlier, we studied the photodynamic activity of hybrid structures based on pentanionic fullerene derivatives having a solubility in water>100 mg/ml [62]. At the same time, it has been suggested that compounds with a lesser charge more effectively interact with membranes [22].

In this regard, this publication presents studies on the creation of new photodynamic hybrid structures based on monoamino acid fullerene derivatives (AFDs) and a chlorin derivative pyropheophorbide a and NPs based on them. AFDs 1, 2, and 3 were used to create photodynamic hybrid structures with one negative charge (in the form of the sodium salt of amino acid moiety) or have no charge (in the methylated form of amino acid moiety). It was shown that such amino acid derivatives are highly soluble in water, have low toxicity, and exhibit various biological activities: they have pronounced membranotropic, antioxidant, immunomodulating, and antiviral activity, as well as the high stereospecificity of interaction with membranes and proteins [25-32,63].

In this work, we propose a new method for creating hybrid photodynamic structures using the original method of attaching pyropheophorbide to amino fullerene derivatives, which was previously used to create various biologically active fullerene derivatives [28]. AFDs are known to have limited solubility in water up to 20–30 mg/mL and tend to form NPs in the aqueous phase. The addition of hydrophobic PPa should further reduce their solubility in water. In this regard, in this paper, the possibility of the formation of NPs based on new conjugates AFD-PPa by their selfassembly in an aqueous solution was investigated. To solve these problems, this paper proposes original methods for creating dyads **6**, **7**, and **8** with pronounced photodynamic activity.

## 4. Results and discussion

4.1. Synthesis and characterization of AFDs and covalent dyads AFD-PPa

The synthesis conditions were chosen to obtain AFD conjugates in which one amino acid and one dye were attached to the fullerene core, which was confirmed by ESI-MS and IR spectra (Figs. S2, S3, Table S1, Supplementary).

In the IR spectra of all synthesized AFD, there was an intense (strong, s) band near 1707–1726 cm<sup>-1</sup> (vC = O), associated with COOH group. In these spectra the group of 3 peaks exhibit near 1100–1108 cm<sup>-1</sup> (strong, s), 951–962 cm<sup>-1</sup> and 837–886 cm<sup>-1</sup> (middle, m), and weak (w) band near 1250 cm<sup>-1</sup>. These bands were found in the IR spectra of all amino acid fullerene derivatives, and they are associated with fullerene fragment vibrations of these molecules [46]. There are bands in the spectrum of methyl ether Val-C<sub>60</sub>, which could be assigned to vibrations of the amino-acid fragment: 3315 s, broad (vNH + vOH), 2972 and 2925 m (vCH), 2878 and 2851w (vCH<sub>3</sub>), 1707 s (vC = O), 1360 m (CH<sub>3</sub> deformation), 1238, 1178 – (carbon fragment vibrations), 1044 cm<sup>-1</sup> (vC-OH). The IR spectrum of methyl ether Pro-C<sub>60</sub> demonstrated the disappearance of the band near 3300 cm<sup>-1</sup> associated with the stretch mode of the NH bond in proline.

The presence of a C-N bond between the fullerene cage and the amino group of the amino acid in such derivatives was previously demonstrated by solid-state NMR [64]. The fact of monoaddition of amino acids to fullerene in AFD was also shown earlier by the method of amino acid analysis [65].

To purify the reaction products and to prove the covalent attachment of PPa and AFD, the method of gel chromatography was used in this work, followed by analysis of chromatographic fractions by spectrophotometry, luminescence, and X-ray fluorescence analysis. Since the absorption spectra of PPa moiety in the composition of the AFD-PPa conjugate vary greatly, and the PPa fluorescence is quenched, the determination of the PPa content in the AFD structure was carried out by X-ray fluorescence analysis by recording Zn atoms which were specially introduced into the PPa. Since PPa(Zn) was unstable under the conditions of the synthesis of **9** was carried out under milder conditions: ethylene chlorohydrin was used as a linker, which leads to an increase in the length of the linker (Fig. S1, Supplementary).

It was determined by X-ray fluorescence analysis that after covalent attachment of PPa(Zn) to  $Val-C_{60}$ , the Zn content of **9** is 3.8% with a calculated value of 4.16%. This proves that the AFD: PPa(Zn) ratio in the **9** conjugate is 1:1.

# 4.2. Formation and characterization of water-soluble nanoparticles of AFD-PPa

To create AFD-PPa water-soluble dyads, the carboxyl groups of AFD were converted from the methylated form to the form of sodium salt, and then AFD(Na)-PPa, were transferred to the aqueous phase by dialysis as described in section 2.3. The resulting conjugates 6, 7, and 8 exhibit amphiphilic properties; therefore, they are capable of dissolving in water but are also prone to form

nanoassociates. Water-soluble nanoparticles based on them formed a transparent colored solution and practically did not precipitate during storage of the solution for several months.

# 4.3. Estimation of the size of AFD-PPa NPs by dynamic light scattering method

As can be seen from Fig. 2.1., the dynamic light scattering method didn't show the formation of the NPs for Val- $C_{60}$ -PPa (compound **6**) in pyridine. Nevertheless, as will be discussed further, we believe that fullerene derivatives under study form NPs even in the pyridine solution, but their size less than the sensitivity of the applied method (size < 5 nm).

As discussed in a literature review, AFDs tend to aggregate in an aqueous medium due to the association of hydrophobic  $C_{60}$  core, forming nanoparticles. Using dynamic light scattering, it was found that the amino acid derivative of fullerene **1** and covalent dyads **6**, **7**, and **8** in aqueous solutions form supramolecular structures: 10–20 nm as for AFD and 20–42 nm – for the dyads (Fig. 2 and Table 1). Thus, such water-soluble NPs, obtained by dialysis method, have a small size (up to 42 nm) with a relatively narrow size distribution.

#### 4.4. Computational simulation of dyads structures

Conformations of dyad **6** as an example of AFD-PPa structures are presented in Fig. 3. It can be seen that the chlorin fragment in the dyad is located in a plane perpendicular to the surface of the fullerene spheroid. The distances between the dye and the fullerene core in dyad **6** were calculated as 3.8 Å (between the closest atoms of fullerene and dye) and 8 Å (between the centers of fullerene and dye).

Such a «perpendicular» spatial position of the fullerene core relative to the dye macrocycle plane could also enhance aggregation processes, as it was shown for another fullerene-chlorine dyad with a similar spatial configuration [66].

The goal of computational simulation of dyad structures in the present work was to make an estimation of a distance between the fullerene core and the dye and their spatial arrangement. This rough estimation was made by a relatively simple PM7/COSMO parametrization method. We want to note that it is possible to calculate absorbance spectra and electron-transfer parameters for fullerene-dye dyads, although it requires the use of significantly more complex DFT-based methods that are taking into account the solvent influence, as was shown for fullerene-porphyrin structures in [67].

#### 4.5. Photophysical properties of AFD-PPa nanoparticles

An analysis of the absorption spectra of **6** dyad dissolved in pyridine shows that upon conjugation of AFD with PPa, a significant change in the absorption spectrum of the dye fragment in the dyad occurs, accompanied by an extinction decrease in the Soret band (407 nm) and Q-band, with a small bathochromic shift of the Qband (from 673 nm for PPa to 677 nm for 6, Fig. 4, A). A broad shoulder was also observed in the dyad spectrum, increasing in the blue region and in the near UV region, which could be explained by the contribution of the fullerene fragment. In addition, in the Q-band region, a second band appears at a wavelength of 712 nm; a possible reason for this may be the formation of intermolecular aggregates of these dyads in a pyridine solution. For another fullerene-chlorine dyad with a similar spatial configuration, a similar picture of the Q-band peak broadening was shown, where it was explained by strong aggregation [66].

A weak fluorescence signal of the dyad **6** was registered; its intensity was 21 times lower than the PP*a* signal (Fig. 4B and Table 1). The observed effect indicates effective quenching of the

excited singlet state of the PPa dye in the structure of the dyad as a result of electron or excitation transfer to the fullerene core.

Analysis of the absorption spectra of water-soluble nanoparticles based on AFD-PPa dyads in an aqueous solution showed that all dyads had pronounced Soret bands in the range of 408– 415 nm and Q-band maximum in 673 – 680 nm range (Fig. 5A and Table 1). It should be noted that in the wavelength range of 350 – 500 nm, the absorption can also be due to the significant contribution of the AFD absorption spectrum and light scattering of nanoparticles (Fig. 5A and Table 1). Given this contribution, we can conclude that in all dyads, the intensities at the absorption Q-bands reduced by 1.5–3 times as compared with the spectrum of the water-soluble analog of PPa – chlorin  $e_6$  sodium salt (C $e_6$ ). This effect was previously observed in dyads based on fullerene and C $e_6$ [12,68]. Moreover, the absorption intensity of AFD-PPa NPs in the Q band region is close to the individual dyes (Fig. 5A).

A study of the steady-state fluorescence spectra of covalent AFD-PPa NPs in comparison with the Ce<sub>6</sub> spectra in an aqueous solution showed that the fluorescence quantum yields of conjugates **6**, **7** and **8** were significantly lower compared to Ce<sub>6</sub> by 90, 233, and 30 times, correspondingly (Fig. 5B and Table 1). It is known that the quantum yield of fluorescence of chlorin  $e_6$  in water is 0.22 [4]; for PPa in dimethylformamide it is 0.31 [69]. Based on these data, the fluorescence quantum yield of these conjugates could be calculated (Table 1). The observed result indicates effective quenching of the excited singlet state of the dye as a result of electron transfer or excitation to the fullerene core.

It is well known that fluorescence quenching of the dye in the structure of the dyads occurs due to electron or the energy transfer from the excited dye to the fullerene. Due to the low overlap integral between donor fluorescence and acceptor absorbance spectrum, according to Forster's theory, it can be expected that energy transfer is highly unlikely in the fullerene-chlorin system [66,70].

Based on steady-state fluorescence measurements, we can calculate the fluorescence decay lifetime of PP*a* moiety in the dyads, given that the lifetime of the original PP*a* is 1.66 ns [71]. Thus, fluorescence decay lifetime of PP*a* moiety in **6**, **7** and **8** were, correspondently, 18, 7.1, and 55 ps. As a result, we can estimate the electron transfer constants  $k_{et}$  in these conjugates, which are in the range  $(1.8-14) \cdot 10^{10} \text{ s}^{-1}$ .

On the other hand, we can estimate the electron transfer constant  $k_{et}$  from the formula

$$k_{et} = k_0 \exp(-\alpha R) \tag{1}$$

which was obtained as a result of generalizing the electron transfer data in various molecular structures, including photoexcited molecules [72–76]. Here,  $k_0 = 10^{13} \text{ s}^{-1}$ ,  $R(\text{\AA})$  is the distance between the donor and acceptor (between the  $\pi$ -orbitals of the dye and fullerene core),  $\alpha$  is the parameter characterizing the overlap of the donor and acceptor wave functions due to the superexchange interaction. Depending on the type of matrix separating the donor and acceptor (e.g., a saturated hydrocarbon chain, protein structure, or water molecules), the value  $\alpha$  can be different: 0.9 Å<sup>-1</sup> for saturated hydrocarbon chains and 1.8 Å<sup>-1</sup> for water molecules [74].

The distances between structures of PP*a* and fullerene core in dyads, as determined from dyad **6** structure (Fig. 3), are equal to 3.8 A (between the nearest atoms of the conjugated structures) and 4.2 A (along the linker). Using the formula (1), we can calculate  $k_{et} = 2.8 \ 10^{11} \ s^{-1}$  in the case of electron transfer along saturated hydrocarbon chains and  $k_{et} = 10^{10} \ s^{-1}$  for electron transfer through the aqueous medium.

Based on these considerations, we can conclude that experimentally estimated electron transfer constants are comparable to theoretical one, and in dyads under study, electron transfer most likely occurs, both along the linker chain between PPa moiety



Fig. 2. The hydrodynamic radius of NPs: dyad 6 in pyridine (1); AFD 1 in water (2); dyad 6 in water (3); dyad 7 in water (4); dyad 8 in water (5).



Fig. 3. Calculated conformation of dyad 6.



Fig. 4. Absorption (A) and fluorescence (B) spectra of obtained compounds: PPa (1), 6 (2), and 1 (3). All in pyridine, concentration 10  $\mu$ M,  $\lambda_{ex}$  = 417 nm.



**Fig. 5.** Absorption (A) and fluorescence (B) spectra of obtained compounds: Ce<sub>6</sub> (1), **1** (2), **6** (3), **7** (4), **8** (5). All compounds dissolved in water, concentration 10  $\mu$ M,  $\lambda_{ex} = 410$  nm.

and fullerene core and along the direct shortest line connected the  $\pi$ -orbitals of the dye and fullerene.

# 4.6. Photodynamic activity of nanoparticles in water and in the structure of liposomes

The photochemical activity study of AFD-PPa NPs in water was evaluated by the generation of superoxide anion  $O_2^-$  and singlet oxygen  ${}^{1}O_2$  using the NBT and DPBF probes, correspondingly. The activity of generating ROS conjugates was compared with the corresponding activity of chlorin  $e_6$ , a commonly used PS. PPa and Ce<sub>6</sub> both have a pronounced absorption in the Q-band region of the spectrum and a high quantum yield of singlet oxygen generation (0.52 for PPa in DMF [69] and 0.7 for Ce<sub>6</sub> in water [77]).

It has been shown that water-soluble nanoassociates exhibit photochemical activity that significantly exceeds the generation activity of  $O_2^{-}$  by  $Ce_6$ , while the conjugate **7** is most effective. Its effect is 3.6 times greater than the effect of  $Ce_6$  (Fig. 6). The photochemical activity of the dyads **6** and **8** also exceeds the activity of free chlorin by 2.4 and 2.7 times (Table 1). It should be noted that the photochemical activity was compared at the same concentration of compounds, while their absorption in the excitation region (at  $\lambda > 630$  nm) is slightly different for dyads under study.

In this regard, the relative photochemical activity was estimated, calculated per one quantum of absorbed light, taking into account the absorption of compounds in the band 630–800 nm. It was found that the activity of **7** exceeds the activity of Ce<sub>6</sub> 10.2 times by one absorbed quantum, while the activity of **6** and **8** – exceeds 4.8 and 3.7 times (Table 1).

A comparison was also made of the photochemical activity of  $O_2^{--}$  generation for these nanoparticles upon their introduction into the liposome solution. In this case, due to the lipophilicity of the dyads, they can enter the liposome structure in the form of individual compounds, while their structure and the polarity of the envi-

ronment can change, which will significantly change the conditions of charge transfer and the efficiency of the photochemical reaction as a whole [78,79]. It was found that all dyads in liposomes had an almost equal activity of  $O_2^{--}$  generation, exceeding that of Ce<sub>6</sub> by 3.5–4.0 times, or 5–11 times per absorbed quantum of light (Fig. 6B and Table 1).

A study of the singlet oxygen  ${}^{1}O_{2}$  generation in liposome solutions showed that the dyad's photoactivity was significantly lower (by 8–12 times) than that of Ce<sub>6</sub>. However, due to the low absorption in the red region, the normalized photoactivity of **6**, **7**, and **8** dyads were only 3.3–5.9 times lower (Fig. 7 and Table 1).

Comparing the activity of  $O_2^{-} \bowtie {}^1O_2$  generation in liposome medium by dyads under study, we can conclude that the transfer of energy from an excited molecule to oxygen occurs mainly by the electron transfer mechanism, realizing I type of photochemical reaction. Thus, for all dyads under study, photodynamic mechanism switching from type II (singlet oxygen generation by Ce<sub>6</sub>) to I type (superoxide generation by dyads) was demonstrated. Facilitation of the type I photodynamic mechanism could be perspective against hypoxic tumors.

# 4.7. Photodynamic action of NPs on tumor cells HeLa

The photodynamic effect of NPs **6**, **7**, **8**, and Ce<sub>6</sub> as a reference was studied in HeLa cells, as it was described in Section 2.6. It was found that irradiation in the region > 630 nm alone does not cause cell death, due to the absence of compounds with a photodynamic effect, both in the cells themselves and in the DMEM medium.

The test substances were applied to a plate with HeLa cells under a lamp with an orange filter that cuts off light in the region of the Soret band of dye absorption (<420 nm) in order to eliminate the manifestation of an additional photodynamic effect from sunlight or fluorescent lamps. Amino acid fullerene derivative did



Fig. 6. Kinetics of superoxide O<sub>2</sub><sup>--</sup> formation sensitized by compounds in water (A) and in the liposomes (B): 6 (1), 7 (2), 8 (3), Ce<sub>6</sub> (4), 1 (5), control (6). Concentration 2 µM.



Fig. 7. Kinetics of singlet oxygen  $^{1}O_{2}$  generation, sensitized by the following compounds in liposome medium: Ce<sub>6</sub> (1), **6** (2), **7** (3), **8** (4), control (5). Concentration 2  $\mu$ M.

not exhibit dark toxicity, as well as recorded photodynamic effects under the experimental conditions, which is associated with its extremely low absorption in the irradiation region ( $\lambda$  > 630 nm), and, therefore, the weak ability to generate both superoxide and singlet oxygen or other types of ROS when irradiated.

It is important to note that all conjugates **6**, **7**, and **8** do not have any noticeable dark toxicity (Fig. 8), which is quite typical for most fullerene derivatives [41,80].

All the studied dyads have similar phototoxicity, which was significant, 10–14.5 times lower than that of Ce<sub>6</sub> (Fig. 8 and Table 1). Given the lower absorption of dyads in comparison with Ce<sub>6</sub> in the excitation region, the decrease in phototoxicity is 4–7 times. Under the experiment conditions, the photodynamic effect on the cells could provide only such compounds that either penetrate the cells or interact with their membranes. If the studied conjugates penetrate and accumulate in cells weaker than the comparison compound Ce<sub>6</sub>, this will significantly reduce their observed photodynamic activity.

Based on the data on the photodynamic activity of dyads in cells and the structure of liposomes (Table 1), it can be concluded that singlet oxygen, rather than superoxide anion radical, exhibits the greatest cytotoxic effect. As a result, AFD-PPa dyads under study, that generate ROS predominantly by type I mechanism were less effective under conditions of oxygen saturation; however, they could be effective under conditions of hypoxia (e.g. low oxygen concentration), where type II photochemical reaction is significantly hampered.



**Fig. 8.** Phototoxicity of compounds under study in HeLa cancer cell with 30 min irradiation of the red light (solid line) and in the dark (dash line): Ce<sub>6</sub> (1); **6** (2), **7** (3), **8** (4).

# 5. Conclusion

A number of water-soluble covalently-linked dyads were synthesized by the original inexpensive method based on the conjugation of amino acid fullerene  $C_{60}$  derivatives with the dye – pyropheophorbide *a*.

With the formation of dyads, a significant decrease in the absorption of the dye in its structure is observed, as well as strong fluorescence quenching of the dye. It is shown that these dyads form photoactive water-soluble nanoparticles.

It was found that, depending on the structure, NPs in liposomes generate superoxide anion radical 5–11 times more effective, calculated per quantum of absorbed light, than chlorin e<sub>6</sub>, and at the same time, their quantum yield of singlet oxygen decreases by 3–6 times compared to chlorin e<sub>6</sub>. Various attached amino acids give the structures a slight water solubility and do not significantly affect the photophysical properties of nanoparticles.

The obtained nanoparticles have low toxicity in the dark; at the same time, the phototoxicity of dyads is inferior to the activity of chlorin 10–14.5 times. Based on the obtained data, we can conclude that the newly synthesized dyads predominantly enhance photodynamic reactions by type I mechanism (superoxide generation), while under normal conditions, cells are damaged by type II mechanism (singlet oxygen generation).

Further development of such type of photoactive fullerene-dye dyads should be aimed at the enhancement of type I mechanism, reducing the degree of aggregation of the photoactive molecules in water-soluble nanoparticles and maintaining their absorption in the Q-band excitation region. We believe that the proposed Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 260 (2021) 119885

approach could be used for the creation of inexpensive type I photosensitizers, highly effective for the treatment of hypoxic tumors.

# **CRediT authorship contribution statement**

**A.Yu. Belik:** Supervision, Validation, Writing - original draft, Writing - review & editing. **A.Yu. Rybkin:** Investigation, Methodology, Writing - original draft. **N.S. Goryachev:** Investigation, Data curation, Formal analysis. **A.P. Sadkov:** Investigation. **N.V. Filatova:** Investigation. **A.G. Buyanovskaya:** Investigation. **V.N. Talanova:** Investigation. **Z.S. Klemenkova:** Investigation, Validation. **V.S. Romanova:** Conceptualization, Investigation. **M.O. Koifman:** Supervision, Investigation. **A.A. Terentiev:** Investigation, Validation. **A.I. Kotelnikov:** Conceptualization, Methodology, Validation.

# **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

# Acknowledgments

Studies were funded by the Ministry of Science and Education of the Russian Federation (State task No AAAA-A19-119112590105-7).

# Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2021.119885.

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