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Photophysical properties of novel PDT photosensitizer Radachlorin in different media

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Abstract

The absorption and fluorescence spectra of recently presented photosensitizer for photodynamic therapy "Radachlorin" are studied in different pH, solvents and in the presence of human serum albumin. Radachlorin is found to efficiently generate singlet oxygen when irradiated with visible light. The formation of singlet oxygen by photolysis of Radachlorin was evidenced by HPLC trapping $^1O_2$ with furfuryl alcohol and 1,3-cyclohexadiene-1,4-diethanoate and by the histidine test. The photophysical properties of this novel photosensitizer are used to discuss its anti-tumoral photoactivity.

Key words: Anti-tumoral photoactivity; photosensitizer; radachlorin; singlet oxygen.

Propiedades fotofísicas del nuevo fotosensibilizador Radaclorin en diferentes medios

Resumen

Los espectros de absorción y fluorescencia del recientemente presentado fotosensibilizador para terapia fotodinámica "Radaclorin" se estudió en diferentes pH, solventes y en presencia de albúmina humana. El Radaclorin genera en forma eficiente oxígeno singlete cuando es irradiado con luz visible. La formación de oxígeno singlete generada por fotólisis de Radaclorin fue detectada por HPLC mediante el uso de atrapadores como furfuril alcohol y 1,3-ciclohexadien-1,4-dietanoato, como también mediante el ensayo de histidina. Las propiedades fotofísicas de este nuevo fotosensibilizador son de gran utilidad para el estudio de su fotoactividad anti-tumoral.

Palabras clave: Fotoactividad anti-tumoral; fotosensibilizador; oxígeno singlete; radaclorin.

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Introduction

Recent joint efforts of physicists, chemists, and physicians resulted in a significant progress in photodynamic therapy (PDT) of malignant tumors and some non-oncological diseases (1, 2). This therapy involves the administration of a photosensitizing agent (usually a porphyrin-based compound) followed by tissue exposure to a sufficiently powerful laser irradiation in the visible range. When the tumor with accumulated photosensitizer is illuminated by light of the appropriate wavelength, photochemical reactions occur. Most probably, a light induced excitation of the photosensitizer molecules produces a series of molecular energy transfers to ground state oxygen. This last process leads to a generation of singlet molecular oxygen ($^1O_2$), highly reactive and citotoxic species, resulting in cell death (3, 4). Rapid advances in diode laser technology have brought powerful, economical laser to the market. These lasers are specifically adapted for PDT with radiation at the required wavelength (5).

The hematoporphyrin derivatives are mainly used as clinical PDT photosensitizer: 'Photofrin I and II" (USA, Canada), "Photosan" (Germany), "Photoheme" (Russia), and others. A number of new photosensitizers based on chlorins, bacteriochlorins, benzoporphyrins, phthalocyanines, etc. (photosensitizers of "second and third generation") are actively developed and studied to increase efficiency of PDT. A number of clinical trials have been reported and PDT has been shown to have a potential role in both curative treatment of early tumors and the palliative control of advanced cancer. Hematoporphyrin and its derivatives such as N-aspartyl chlorin e_6 have proven to be effective and safe photosensitizers for PDT. However, this result came at the expense of tissue selectivity (6, 7). Many of the photosensitizers currently used in clinical or pre-clinical studies of PDT localize in (or have a major influence on) mitochondria, and PDT is a strong inducer of apoptosis in many situations in addition of the necrotic pathway produced by tumor ablation (8).

In this research we focus on the photophysical properties of one of such photosensitizer, "Radachlorin" (9), recently presented on "Photonics West 2002" (1). The sensitizer is a chlorophyll *a* derivative, including mainly sodium chlorin e_6. It already passed a complete pre-clinical investigations, and its clinical trials demonstrated significant advantages: very low toxicity in dark, high contrast of tumor accumulation, much more rapid body evacuation (only two days), intensive absorption band at relatively large wavelength where tissues are more transparent, and finally high phototoxicity. Information on photophysical properties obtained via optical studies, such as electronic absorption and fluorescence spectra, is of vital importance to optimize the photosensitizer application. This prompted us to carry out an extensive study of fluorescence and absorption of Radachlorin in different homogeneous solvents and in aqueous solutions at different pHs. We explored the effect of the human serum albumin on optical spectra and also give evidences of the role of Type II reactions involving the photoactivated drug, its triplet state reacting with molecular oxygen to generate singlet oxygen.

Methods and Materials

All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany). Human serum albumin (HSA), histidine, rose bengal and p-nitrosodimethyl aniline were purchased from Sigma (St. Louis, MO, USA).

UV-Vis spectrophotometry of the Radachlorin solution was followed using a Milton-Roy Spectronic 3000 array instrument (Milton Roy Company-USA). The fluorescence spectra were registered with a Shimadzu RF 1501 spectrofluorophotometer. These spectrophotometers were used to characterize the properties of Radachlorin in diffe-

rent pH solutions and solvents, as the titration with HSA.

Radachlorin (aqueous solution, 0.35%) was obtained from RADA-FARMA Ltd., Russia.

Titration of Radachlorin solution with HSA was performed by addition of appropriate aliquots of an aqueous HSA stock solution at 1.0 mM concentration directly to the absorbance or fluorescence cell so that the final protein concentration was in the range from 0 to 5 x 10^{-4} M.

Singlet oxygen generation

Indirectly, photosensitized degradation of histidine was measured in the presence of 0.25, 0.50, 1.0, and 1.5 x 10^{-5} M solution of Radachlorin (10). These solutions were mixed with an equal quantity of L-histidine solution at 0.60 to 0.74 mM in phosphate buffer 0.01, pH 7.4. Samples of this mixture were irradiated with an Osram HQL 250 W medium pressure Hg lamp through a Pyrex filter (radiation dose 4.5 J/cm^2) at time intervals from 60 to 180 min. with the respective controls being protected from light. The concentration of histidine was determined by a colorimetric reaction using phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol as reagents. The optic density was read on a spectrophotometer at 530 nm against a blank reagent, a modified Pauly reaction and by bleaching of p-nitrosodimethylaniline (11, 12).

Another "trap" method has been successfully used to detect generated $^1O_2$ in a variety of samples (13, 14). This method is based upon following the consumption of a chemical trap (Furfuryl alcohol, FFA) that react with singlet oxygen. The comsumption of FFA was followed by HPLC using a 90:10 H_20/CH_3CN mobile phase composition. The detection wavelength used for monitoring FFA consumption was λ=222 nm. Rose bengal, a well known $^1O_2$ sensitizer, was used as a standard for comparison with Radachlorin for $^1O_2$ formation, under identical conditions of photolysis.

To verify the possible reaction of singlet oxygen with Radachlorin, the irradiation of Radachlorin was carried out in the presence of rose bengal or also of tetraphenyl porphine (TPP) using a potassium chromate solution (100 mg/L) as a filter allowing λ > 400 nm and maintaining all other conditions. No photodegradation of Radachlorin under this investigation was observed.

A new method for the determination of the quantum yield of singlet oxygen formation was used with more precision and with smaller ambiguity than others chemical trapping process. A water solution of sodium 1,3-cyclohexadiene-1,4-diethanoate (0.01 M) and Radachlorin (0.04 M) was irradiated under oxygen atmosphere in the same condition previosly described and the determination of the $^1O_2$ quantum yield was carried out by HPLC as the procedure according to Nardello et al. (15).

Results and Discussion

Photolability of Radachlorin in aqueous media of different pHs has been also studied. This effect is due to the acid-base catalyzed photodegradation of the anionic radachlorin. The positions of the principal absorbance bands are given in Table 1. At low pH values, we observe a displacement of the band (of interest for PDT) to higher wavelength values. However, the spectra do not change appreciably for pH≥5. The absorption spectra of Radachlorin for pH 3 and 7 are shown in Figure 1.

In fluorescence spectra of Radachlorin in solvents with differents pHs, we observe a broad band with maximum at 646 nm which is probably an overlap of two emissions (Figure 2, curve a). As the pH value increases, a low wavelength shoulder appears and develops into a distinct feature at pH between 7 and 9, and almost disappears at pH = 11. We conclude that changes of a solvent lead to

Table 1
Positions (nm) of principal bands in the absorption and fluorescence spectra for different pH and solvents

pH	Absorbance				Fluorescence
3	283	405	526	641	646
5	284	403	504	655	658
7	285	402	502	655	660
9	285	403	502	655	659
11	284	402	502	655	660
Ethanol	290	404	504	663	665
Acetone		403	506	664	668
HSA	210	405	505	661	665

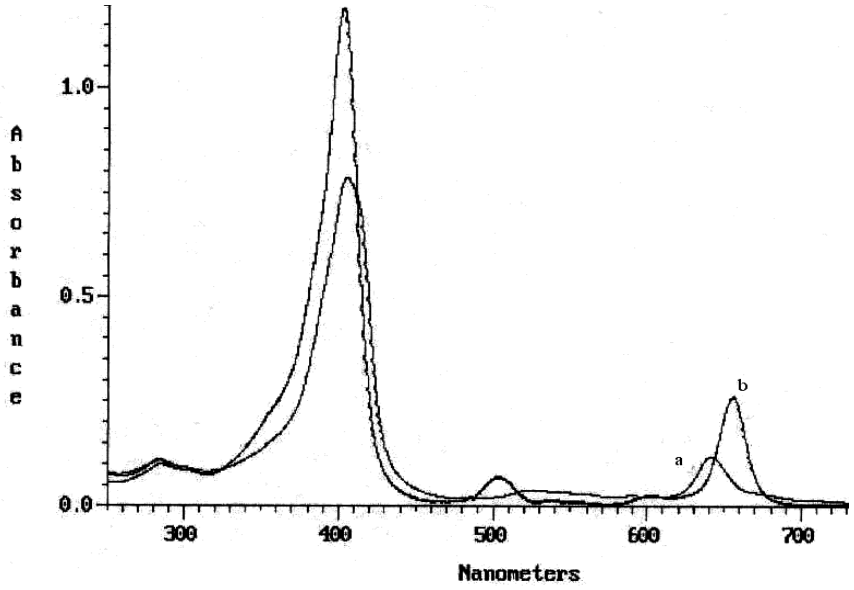

Figure 1. Absorbance spectra of Radachlorin in solvents with pH = 3 (a) and 7 (b).

important modifications of both absorbance and fluorescence.

In Figure 3, we observe a variation in the shape and significantly of the wavelength (see table 1) of the absorption band in water, ethanol and acetone.

Similar band displacement in the fluorescence spectra from 646 nm at pH 3 to 660 nm at pH 7 in water, ethanol and acetone (Figure 4), suggest a formation of hydrogen-bonded associated aggregates in Radachlorin (16). Such aggregation is favored in aprotic solvents and pH > 3. But the nature of the medium and concentration of Radachlorin are very important factors that affect the acid-base and monomer-aggregate equilibria in the ground and excited state.

A presence of HSA also induces changes in the UV-spectra of Radachlorin. At pH 7.2, we initiallly observe a slight de-

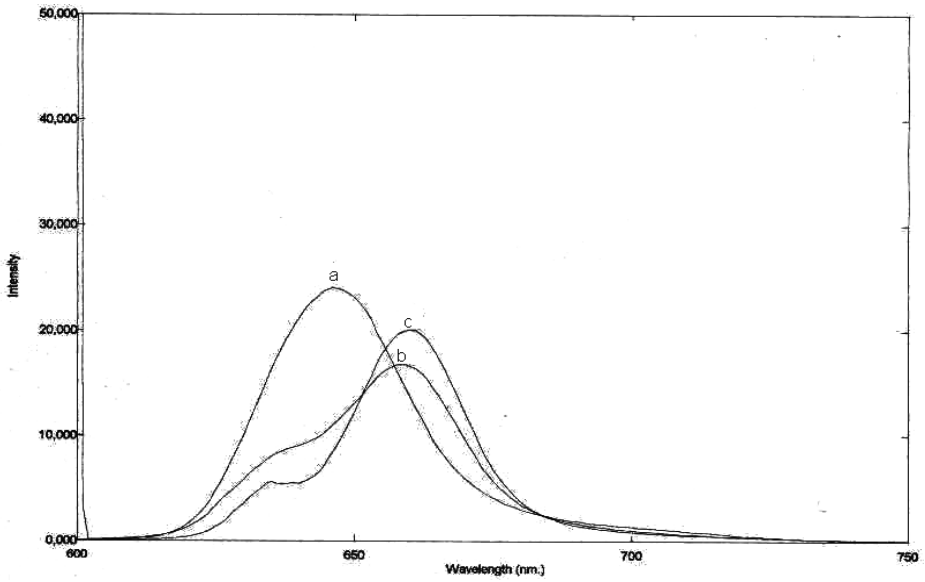

Figure 2. Fluorescence spectra of Radachlorin in water with pH = 3 (a), 5 (b) and 7 (c).

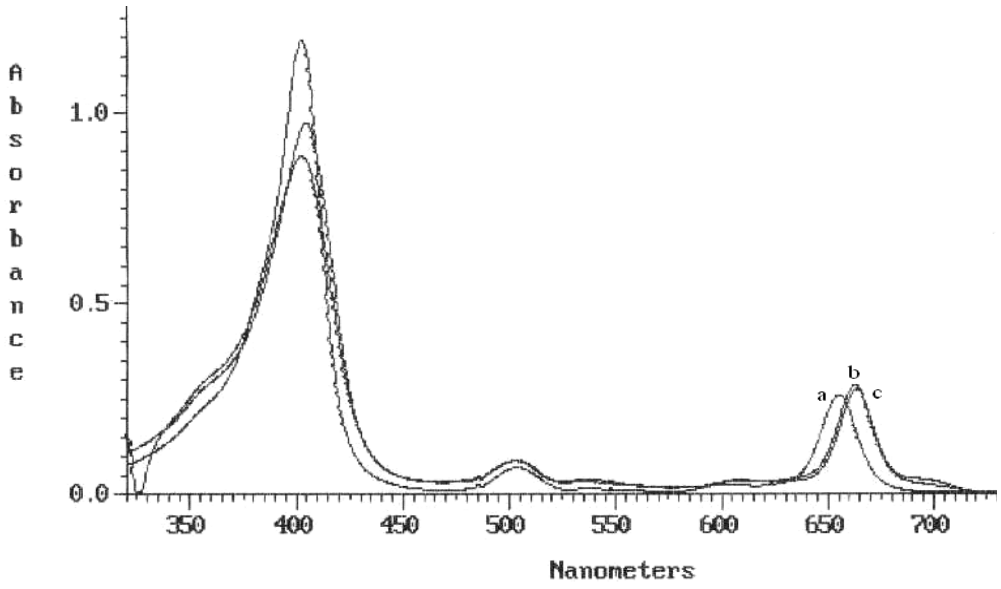

Figure 3. Absorbance spectra of Radachlorin in solvents: water pH 7 (a), ethanol (b) and acetone (c).

crease in absorption intensity followed by a red shift of the spectrum (Figure 5). Our results demonstrate the ability of Radachlorin to interact with protein. Such binding has a physiological significance as this protein could be the carrier of the drug upon its administration *in vivo* in the organism (17).

Radachlorin is capable of producing singlet oxygen when it is irradiated with UV-A and visible light in the presence of molecular oxygen. This fact can be confirmed by trapping with histidine. We use a simple and sensitive spectrophotometric method for the detection of $^1O_2$ as produced by different sensitizing dyes in neutral air satu-

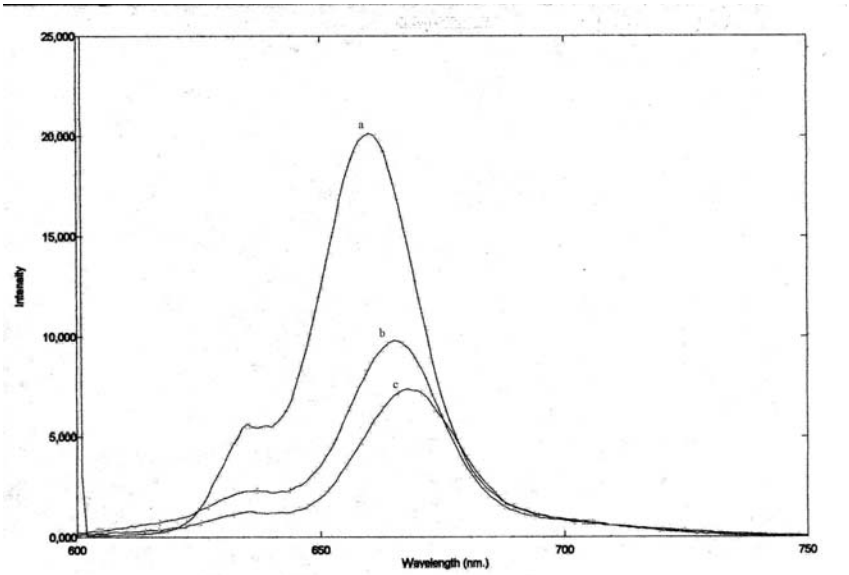

Figure 4. Fluorescence spectra of radachlorin in solvents water pH 7 (a), ethanol (b) and acetone (c).

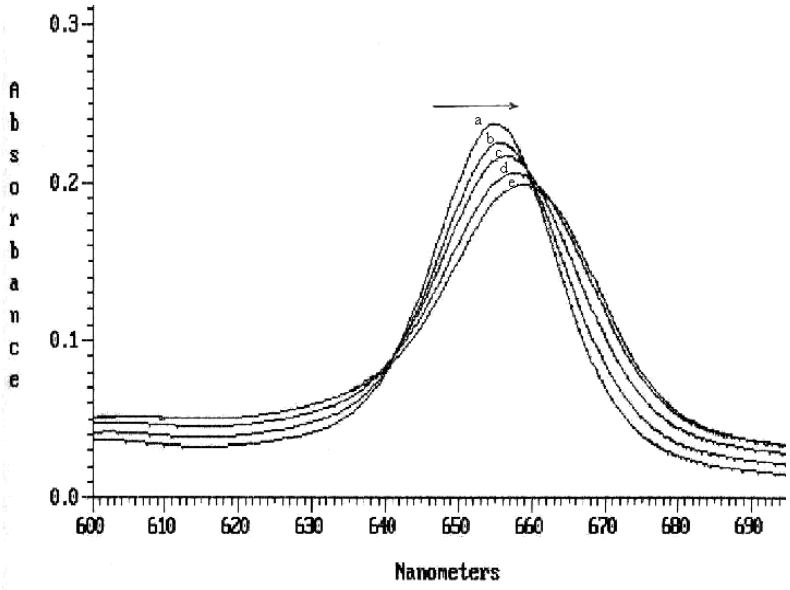

Figure 5. Absorbance spectra of Radachlorin in the presence of HSA with (a) 0, (b) $1x10^{-4}$, (c) $2x10^{-4}$, (d) $3x10^{-4}$, and (e) $5x10^{-4}$ mM concentration.

rated aqueous solutions (Figure 6). The reaction between histidine and $^1O_2$ results in the formation of a trans-annular peroxide. The presence of the latter compound may be detected by bleaching the p-nitrosodimethylaniline at 440 nm. Singlet oxygen alone can not cause the bleaching of the latter compound. No bleaching occurs in the mixture of histidine and p-nitrosodimethylaniline without singlet oxygen (12). In order to control the reaction, we observe no measurable loss of the p-nitrosodimethylaniline in the absence of histidine. We conclude that an oxidation of

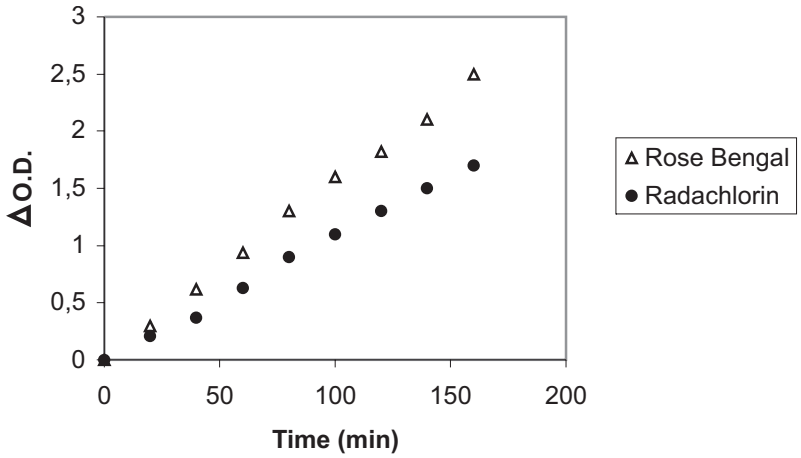

Figure 6. Influence of the time of irradiation on the bleaching of p-nitrosodimethylaniline in the histidine – Radachlorin system at 440 nm. Δ O. D. represents the difference in optical density of irradiated and non-irradiated sample.

histidine (which is susceptible to singlet oxygen attack) is produced through photoexcitation of Radachlorin acting as a singlet oxygen sensitizer (type II mechanism) (12). This particular reaction with histidine can be regarded as a model for damage to cellular protein inflicted by photoexcited Radachlorin via formation of singlet oxygen.

For an estimate of the efficiency singlet oxygen formation by Radachlorin photoexcitation, we compare it with the reported data for the same reaction using Rose Bengal: see Fig. 6. The quantum yield of singlet oxygen generation for Rose Bengal is $\phi(^1O_2) = 0.76$ (18, 19). This value can be used as a standard to determine the quantum yield singlet oxygen for Radachlorin: $\phi(^1O_2) = 0.52 \pm 0.01$.

The hystidine model should be regarded simply as a test for oxygen dependent photosensitized damage to cellular protein. Radachlorin at severals concentration was efficient for photooxidation of histidine which is susceptible to singlet oxygen attack. The formation of $^1O_2$ was confirmed also by trapping with furfuryl-alcohol (determined by HPLC) (13). A innovative method to measure the amount of singlet oxygen photogenerated in aqueous solution was necessary due to the ambiguity about the specifity of the simple trap used as furfuryl alcohol and histidine (15). The measurement with 1,3-cyclohexadiene-1,4-diethanoate (a specific chemical $^1O_2$ trapping) was determined in water with a value of 62% of singlet oxygen formation.

Conclusions

Our studies have demonstrated that Radachlorin can form aggregates in different media and pHs and also has the ability to interact with human serum albumin. We have proven that Radachlorin produces singlet oxygen under irradiation with visible light. Radachlorin have the advantages such as sufficient strength to generate $^1O_2$ with a quantum yields of 0.52, good hydrophilicity and potential specific affinity for malignant tumors. These facts are of major significance for the study of its photodynamic action and make Radachlorin a promising candidate as PDT agents in the medical field. *In situ* production of the singlet oxygen could be the principle mechanism for tumor destruction in application of photodymanic therapy employing the novel water soluble drug Radachlorin. Their photobiological properties are deserving of our further investigation.

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