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Voltage-sensitive styryl dyes as singlet oxygen targets on the surface of bilayer lipid membrane

V.S. Sokolov [a,*], A.N. Gavrilchik [a], A.O. Kulagina [a], I.N. Meshkov [a], P. Pohl [c], Yu.G. Gorbunova [a,b]

[a] A.N. Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Science, Moscow, Russia
[b] N.S. Kurnakov Institute of General and Inorganic Chemistry, Russian Academy of Science, Moscow, Russia
[c] Institute of Biophysics, Johannes Kepler University Linz, Austria

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ABSTRACT

Photosensitizers are widely used as photodynamic therapeutic agents killing cancer cells by photooxidation of their components. Development of new effective photosensitive molecules requires profound knowledge of possible targets for reactive oxygen species, especially for its singlet form. Here we studied photooxidation of voltage-sensitive styryl dyes (di-4-ANEPPS, di-8-ANEPPS, RH-421 and RH-237) by singlet oxygen on the surface of bilayer lipid membranes commonly used as cell membrane models. Oxidation was induced by irradiation of a photosensitizer (aluminum phthalocyanine tetrasulfonate) and monitored by the change of dipole potential on the surface of the membrane. We studied the drop of the dipole potential both in the case when the dye molecules were adsorbed on the same side of the lipid bilayer as the photosensitizer (*cis*-configuration) and in the case when they were adsorbed on the opposite side (*trans*-configuration). Based on a simple model, we determined the rate of oxidation of the dyes from the kinetics of change of the potential during and after irradiation. This rate is proportional to steady-state concentration of singlet oxygen in the membrane under irradiation. Comparison of the oxidation rates of various dyes reveals that compounds of ANEPPS series are more sensitive to singlet oxygen than RH type dyes, indicating that naphthalene group is primarily responsible for their oxidation.

1. Introduction

Photodynamic therapy is based on the use of photosensitizers (PS) binding to cancer cells and killing them under exposure to light due to generation of reactive oxygen species, the most effective of which is singlet oxygen [1–3]. Despite extensive research on clinical and preclinical trials of several PS [4], the search for new derivatives which are capable of efficient generating of singlet oxygen continues. There are several ways to detect singlet oxygen. A direct method detects a very weak phosphorescence at 1268.7 nm wavelength associated with $^1\Delta g \rightarrow {}^3\Sigma g^-$ transformation [5]. However, this approach requires a special technique that restricts its wide use in preliminary tests of sensitizers. A more convenient approach is based on the use of chemical traps, photosensitive compounds reacting with singlet oxygen. All traps can be classified into two groups depending on solubility either in water or in organic solvents. The most common and practical trap is 1.3-diphenylisobenzofuran. It can be applied in non-aqueous environments since it is soluble in various organic solvents. There are plenty of water-soluble chemical traps, most of them containing anthracene core since it is sensitive to singlet oxygen. All these traps can be used for preliminary solution studies [6–12].

The targets of singlet oxygen in cells are lipids, proteins or nucleic acids. Therefore, for developing novel efficient photosensitizers quantitative studies of their activity in the medium similar to the cell membrane are essential. Interaction of PS with the membrane and mechanisms of primary oxidation reactions of targets of singlet oxygen can be studied in artificial bilayer lipid membrane (BLM), which proved to be a robust model of cellular membranes. It was used to study binding and auto-oxidation of PS by measuring the change of boundary potential drop on the membrane/water interface [13–16]. Irradiation of BLM containing unsaturated lipids with adsorbed PS leads to the membrane damage [17,18]. If the BLM contains no unsaturated lipids, it remains stable under irradiation and can be used to study oxidation of special molecules – targets of singlet oxygen: peptides forming ion channels [13,15,18–22] or simpler molecules [23,24].

In our previous investigation we studied the photodynamic oxidation of phloridzin by singlet oxygen generated by photoexcited aluminum phthalocyanine tetrasulfonate (AlPcS4) [24]. The advantage of such approach is the possibility to compare the rates of oxidation of phloridzin on opposite sides of the membrane. It allows us to estimate the permeability of lipid bilayer for singlet oxygen. This estimation requires verification with the use of other targets of singlet oxygen having

* Corresponding author.
E-mail address: sokolov.valerij@gmail.com (V.S. Sokolov).

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various structures differing by the location in the membrane and sensitivity to singlet oxygen. In the present study, we investigated voltage sensitive fluorescent styryl dyes (ANEPPS and RH groups) as targets of singlet oxygen. Their structures are shown in Fig. 1. Although these dyes were developed as sensors of electric field in the membrane [25–28], their molecules have considerable dipole moment, due to which they can change the dipole potential drop at the lipid-water interface [29], similarly to phloridzin we studied earlier. We used the water-soluble AlPcS4 as a photosensitizer again. We quantified the kinetics of change of the potential during irradiation and its subsequent restoration in the dark, and developed a theoretical model for accurate determination of the rate of oxidation of the targets, which is proportional to the steady-state concentration of singlet oxygen in the membrane. Comparison of the oxidation rates of the investigated dyes demonstrated that the most sensitive group, responsible for the reaction with singlet oxygen, is naphthalene ring at the end of the molecule immersed into membrane.

2. Experimental

2.1. Materials

The styryl dyes - 4-(2-(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium (di-4-ANEPPS), 4-(2-(6-(Dioctylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS), 4-(6-(4-(Dibutylamino)phenyl)hexatrienyl)-(*N*-(4-sulfobutyl)-pyridinium (RH-237); 4-(4-(4-(Dipentylamino)phenyl)-1,3-butadienyl)-1-(4-sulfobutyl)-pyridinium (RH-421) were purchased from (Aldrich) and used without additional purification. Lipid membranes were formed from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) dissolved in *n*-decane, purchased from Avanti Polar Lipids, USA, and Sigma-Aldrich, USA, respectively. Aluminum phthalocyanine tetrasulfonate (AlPcS4) and zinc phthalocyanine (ZnPc) from Porphyrin Products, Logan, USA, were used for generation of $^1O_2$ upon irradiation. Working buffer solutions were prepared using twice distilled water with 100 mM KCl (chemically pure, Reachim, Russia) and 10 mM HEPES (Calbiochem, USA) at pH 7.5. The pH of the solution was adjusted by KOH (chemically pure, Reachim, Russia).

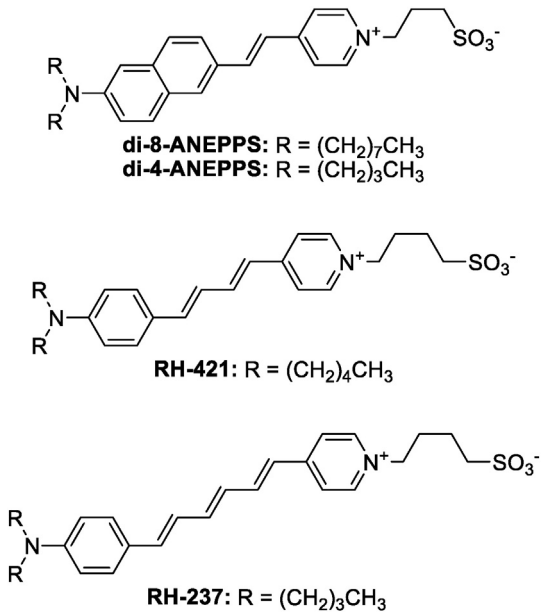

Fig. 1. Structures of di-8-ANEPPS, di-4-ANEPPS, RH-421 and RH-237.

2.2. Methods

Planar bilayer lipid membranes were formed by Mueller-Rudin method [30] on a round hole in the septum of a Teflon cell using lipid solution in the concentration of 15 mg/ml. The diameter of the hole was 0.8 mm. The cell was divided by a septum into two compartments. These compartments of equal volumes (500 μl each) were filled with working buffer solution and were continuously stirred with magnetic stirrer.

Electrical measurements were performed with the aid of silver-chloride electrodes with agar bridges. The bridges were made of standard plastic pipette tips, the bottom part of which was filled with agarose gel, and remaining volume was filled with 0.1 M KCl solution. Total electrical resistance of the electrodes with the bridges did not exceed 50 kΩ.

Membrane capacitance and conductance were continuously measured by the technique similar to that described in [24]. Triangular voltage waves were applied to the membrane, and the resulting current was recorded and analyzed with the aid of self-made software. The voltage was applied from the analog output of ADC-DAC board (Lcard L780, Russia) to one electrode in the cell. The other electrode was connected to the input of current-voltage converter Keithley-427 (USA), the output voltage of which was sampled by ADC (Lcard board "L780"). The increase of the membrane capacitance to the steady-state value of about 1–2 nF indicated that the membrane formation was complete.

The boundary potential difference of planar membrane, $\Delta\varphi_b$, was measured using the inner field compensation (IFC) method based on measurement of second harmonics of the capacitance current ([31], see also reviews [32,33]). The equipment was similar to that described in [24]. To illuminate the cell with bilayer lipid membrane, a semiconductor laser module was used (wavelength 670 nm, optical power 1 mW).

The fluorescence of di-4-ANEPPS in liposomes was measured by Panorama Fluorat 02 (Lumex, Russia). The liposomes were made of DPhPC via extrusion method; lipid concentration was 20 μg/ml. The irradiation of the liposomes with di-4-ANEPPS and AlPcS4 was performed in standard quartz cell by white light from mercury lamp (intensity of about 200 W/m^2).

The photooxidation of styryl dyes by singlet oxygen in DMSO solution was investigated using the experimental set-up described in [34]. Typically, 2 ml portion of DMSO solution containing 2 ml of phthalocyanine complex (10^{-5} M) and 0.005 ml of styryl dye, was irradiated by He-Ne laser LG-78 ($\lambda = 631$ nm, 2 mW, (http://www.laser-device.com/) for 2–8 h. The absorption was measured by UV–vis spectrometer (Ocean Optics QE-65000).

3. Results and Discussion

3.1. The Behavior of Styryl Dyes in Solutions under Irradiation in the Presence of Photosensitizer

We investigated the photooxidation of styryl dyes by singlet oxygen generated by ZnPc or AlPcS4 using UV–vis spectroscopy on the example of di-4-ANEPPS in DMSO. Red laser ($\lambda = 631$ nm) was used to irradiate the quartz cell containing the solution of PS and ANEPPS for 2.5 h, causing decrease in the intensity of ANEPPS absorption by 7% in the case of ZnPc and by 16% in the case of AlPcS4 (Fig. 2). When the same experiments were performed in the absence of oxygen (solution was saturated with argon), the intensity of the ANEPPS band did not change during 8 h of irradiation. Such a behavior can be explained by different reasons. First, the 1,3-addition of singlet oxygen with dienes and conjugated *cis*-dienes, [4 + 2] cycloaddition, is similar to the Diels–Alder reaction, where singlet oxygen is the dienophile. An example of this reaction is interaction of singlet oxygen with the most common and practical trap — 1,„3-diphenylisobenzofuran. On the other hand, naphthalene, anthracene and tetracene derivatives are widely used as water-soluble singlet oxygen traps [1,9,35] due to interaction with $^1O_2$ yielding