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REVIEW PAPER

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Methods of singlet oxygen generation and detection for understanding photodynamic processes

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ABSTRACT

Introduction. Photodynamic therapy (PDT) is a clinically approved therapeutic procedure that exerts selective cytotoxic activity toward malignant cells.

Aim. Our goal is to present the PDT procedure which involves administration of a photosensitizing agent followed by irradiation at a wavelength corresponding to the absorbance band of the photosensitizer and energy transfer to ground state oxygen to generate cytotoxic singlet oxygen

Material and methods. Analysis of literature.

Results. In this paper we described the basics of PDT and lifetime of singlet oxygen in different media.

Keywords. Photodynamic therapy, lifetime of singlet oxygen, photosensitizing agent

Introduction

Singlet oxygen ($^1O_2$) is a highly reactive oxygen species (ROS) and is the predominant cytotoxic agent produced during photodynamic therapy (PDT).[1] Oxygen in the lowest excited electronic state is a reactive intermediate in many chemical processes.[2] In PDT, we can distinguish several main elements that must be properly selected to make the therapy effective. First, you need a suitable photosensitizing (PS) drug that is given to the patient that, after delivery, accumulates in cells of malignant tissue. The photosensitizer and the applied light in themselves cannot be toxic. The next element is exposing the area to light. The ntensity and duration of light pulses are pre-determined.[3] The luminescence emitted by $^1O_2$ during PDT is very weak, therefore the detection of this signal requires advanced measurement techniques and very sensitive devices. Measurement of the weak near infrared luminescence of $^1O_2$ is possible in cells *in vitro* and tissues *in vivo*.[4] This paper describes the principle of photodynamic therapy, the principles of choice of light sources used, photosensitizers and examples of singlet oxygen measurement methods.

Light sources in PDT

Red light (650 nm - 800 nm) for most tissues has the ability to penetrate several mm.[5] In the case of pho-

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todynamic therapy, wavelengths in this range are the most commonly chosen.[6] The exact excitation wavelength must be properly tuned to match the absorption range of the PS. The use of UV light is also applicable due to the greater absorption of some PS in this wavelength range, however, this range is outside the therapeutic window and has defects such as endogenous stimulation of the PS, as shown by studies conducted by Baier et al. 2006.[5] Short wavelength visible light penetrates tissue to a very small extent compared to red and infrared light, which is why in each case we cannot use any PS and any light source. The sources of light in PDT most often diode lasers due to their small size, price, exposure time and ease of installation.[7,8] Very often for solid tumors, PDT is performed using interstitial fiberoptic light sources illuminated with a light from a single laser.[9] For many skin lesions, non-laser light sources such as filtered lamps or more recently, light emitting diodes [10] are used. Regardless of which light source is used, the light field must be uniform to accurately calculate the dose.

Photosensitizers and synthetic dyes

Photosensitizers are an essential element in PDT. Most of them have a structure similar to that contained in the protoporphyrin prosthetic group. PSs have different absorption ranges that depends on their exact structure.[11-15] The ideal PS should have a strong absorption peak in the spectral range from 650 nm to 800 nm. This is because the absorption of photons with a wavelength > 800 nm is unable to provide sufficient energy for conversion $^3O_2$ to $^1O_2$.[16] Another required feature that an ideal PS should have is a lack of „dark" toxicity and rapid clearance from the body.[17] PSs are usually hydrophobic compounds that diffuse into cells quickly. The lifetime of $^1O_2$ is very short, as shown in Table 1. Because of the short lifetime of singlet oxygen, the distribution of $^1O_2$ in cells is limited to ca. 55 nm.[18]

As has been said before, the absorption of a photon of light having the appropriate energy and wavelength leads to the excitation of a PS electron to an orbital with higher energy. Then, with the appropriate energy transfer, ground state oxygen is converted to singlet oxygen. In Figure 1, the general principle of photodynamic therapy is schematically presented.

Table 1. Lifetime of singlet oxygen in different media

References	Applied light	Dye	Photosensitizer	Media	$^1O_2$ lifetime
Schlothauer J.*et al.*[11]	670 nm	Trypan blue	Tetra (p-sulfophenyl) porphyrin (TPPS)	cell suspensions (intracellular)	0.5 – 1.0 μs
Jiménez-Banzo A. *et al.*[12]	532 nm	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)	5,10,15,20-tetrakis(N-methyl-4-pyridyl)-21H,23Hporphine (TMPyP)	cells (Foreskin cells, ATCC CRL-1635)	1.7 μs
			5,10,15,20-tetrakis-(4-sulfonatophenyl)-21H,23H-porphine (TPPS)		1.5 μs
Hatz S. *et al.*[13]	420 nm	Rh123	5,10,15,20-tetrakis(N-methyl-4-pyridyl)-21H,23Hporphine (TMPyP)	HeLa cells in H_2O	≈ 3.2 μs
				HeLa cells in D_2O	≈ 68 μs
Niedre M. *et al.*[14]	630 – 670 nm	-	AlS_4Pc	suspensions of leukemia cells	≈ 0.6 μs
Oelckers S. *et al.*[15]	675 nm	-	pheophorbide-a (PHEO)	red cell ghost suspensions	35 – 100 μs

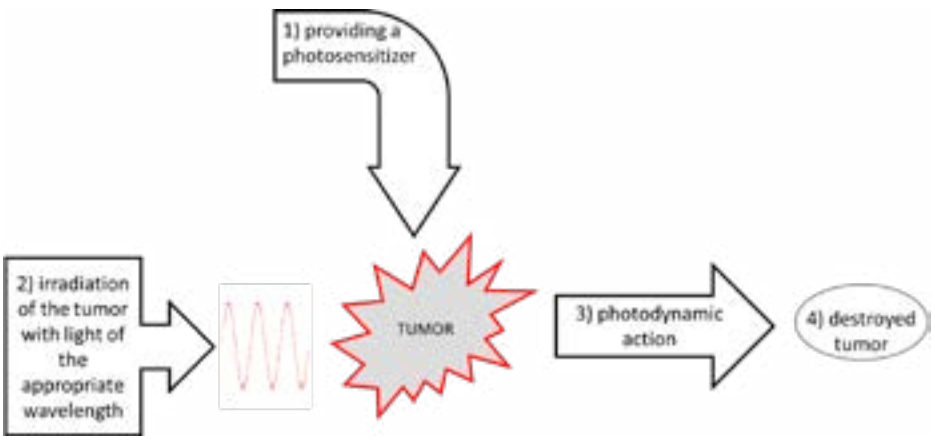

Fig. 1. Schematic of PDT treatment of a tumor

The largest group of PSs that are used in PDT include tetrapyrrole structures. In the case of this group of photosensitizers, singlet oxygen in most cases is produced via a type II mechanism that has been accurately described by Foote.[19] In this process, energy from triplet excited state (T_1) is directly transferred to $^3O_2$ forming $^1O_2$. Only when sensitizer is in the same triplet state multiplicity as ground state oxygen can energy transfer to $^3O_2$ occur.[19] Type I and type II processes occur simultaneously, however, the type II mechanism is the dominant process in photodynamic therapy and is catalytic. The tetrapyrrole structures that play an important role in PDT also include chlorins, bacteriochlorins, and phthalocyanines.[20] One of the most important tetrapyrrole compounds used as a PS in PDT are, inter alia, ALA-induced protoporphyrin IX (Phorphyrin); 5,10,15,20-Tetrakis (1-methylpyridinium-4-yl) porphyrin tosylate; Monoaspartyl chlorin (e6), talaporphin sodium; HPPH (Chlorin); Chloroaluminium sulfonated phthalocyanine (CASP) (Phthalocyanine) or Phthalocyanine RLP068.16 Haematoporphyrin derivative (HpD) and Photofrin were among the first PSs used in PDT and are still widely used.[17]

Another group of PSs is synthetic dyes. An example of such a dye used in PDT is Rose Bengal (RB). RB is a photoactive dye that efficiently generates singlet oxygen. RB has a maximum absorption in the region 540-570 nm.[21] Another example of synthetic dyes are Methylene Blue (MB) and Toluidine Blue (TB). Both of these dyes are characterized by their photobactericidal efficacy. TB exhibits a greater bactericidal activity than MB. The absorption maximum for these dyes is 660 nm and 630 nm respectively.[22,23] In this group there are also dyes that are based on the 4,4-difluoro-4-bora-3a, 4a-diaza-s-indacene (BODIPY) core. They have properties desirable in PDT such as high extinction coefficients, environment insensitivity, and resistance to photobleaching.[24,25] They have heavy halogen atoms in the pyrrole rings. Examples of such dyes are, for example, Zinc (II) -dipicolylamine di-iodo-BODIPY or DIMPy-BODIPY. The synthetic dyes group also includes transition metal compounds and phenalenones.[16] There are also natural products that can act as photosensitizers. These types of PSs are mostly of plant origin. An example of such a PS is, for example, Hypercyol with an absorption maximum at 600 nm. Hypericin is a hydrophobic molecule, which means that it requires a formulation in a drug delivery vehicle.[26, 27] Hypocrellins A and B belong to the PSs of natural origin, along with curcumin and riboflavin used as antimicrobial PS.[29,30]

Detection equipment

Different methods of measurement are used to detect $^1O_2$ luminescence. The main detection methods are an-

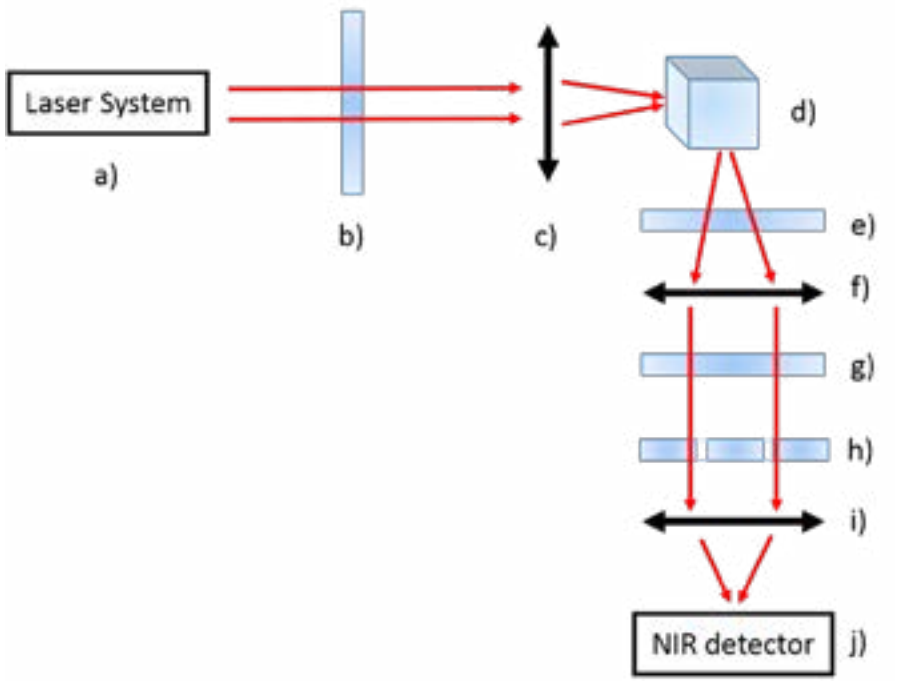

Fig. 2. An optical excitation and detection system scheme for measurement singlet oxygen lifetime; a) laser system (light source); b) bandpass filter centered at the excitation wavelength; c) lens used to focus light onto the sample; d) cuvette with a sample (made of quartz); e) and g) long pass filters to remove unwanted scattered excitation light and fluorescence from the sample (typical 1000 nm and 800 nm); f) lens to collimate light from the sample; h) bandpass filters (1270 nm corresponds to the peak of the $^1O_2$ luminescence spectrum; typically 1200 nm and 1330 nm filters which lie outside the $^1O_2$ band used to determine the background fluorescence); i) lens used to focus light onto the detector window; j) detector allows extremely sensitive detection in the 1200 - 1330 nm ranges.

alogue detectors, which are usually based on semiconductor diodes and photon counting techniques using near-infrared photomultipliers (NIR-PMT). Detection of singlet oxygen luminescence (around 1270 nm) is very difficult due to the very weak signal caused by the low quantum yield of the transition.[31] Cryogenic germanium diodes were used often to detect singlet oxygen luminescence, however, it is even more important to provide quantitative information, even in water where $^1O_2$ lifetime is 3.8×10^{-6} s. [32] To improve the sensitivity of measurements using semiconductor detectors, a differentiation technique is used. The use of two photodiodes enabled simultaneous measurement of the sample and the background, giving a signal as a difference, as shown in studies carried out by Kiryu et al. in 1999.[32] Currently, one of the most accurate methods of singlet oxygen detection is measurement using near infrared photomultipliers whose sensitivity in the 1270 nm region is almost an order of magnitude greater than that of Ge diodes (germanium).[6] The experimental setup for the lifetime of singlet oxygen using NIR detector is shown in Figure 2.

In vitro measurements of singlet oxygen luminescence require the use of not only sensitive detectors, but also a range of optical elements such as lenses, optical filters and cuvettes. Lenses are used for focusing light onto the sample and collimating light from the sample. Optical filters are used, among others, to remove unwanted scattered excitation light and fluorescence from the sample. When testing near-infrared luminescence emission from singlet oxygen using optical detectors, it is required that the detector be characterized by high sensitivity with a low signal-to-noise ratio. In addition, the signal obtained requires careful analysis to separate the true $^1O_2$ signal from scattered light and from phosphorescence and delayed fluorescence emissions from other molecules.[33] Due to its high reactivity, $^1O_2$ is characterized by having the shortest lifetime in the aquatic environment of all the reactive oxygen species (ROS).[34] For this reason, designing sensors to detect it is one of the most difficult tasks. Researchers are increasingly working on a singlet oxygen detection system based on a photomultiplier, which uses optical fibers to direct and collect signals directly from the PDT site.[35,36,37] The power of the signal received in this way is much smaller compared to the configuration of the free NIR space of the detector.[36] However, the use of a fiber optic enables, for example, the provision of a suitable dose of PDT by collecting signals through a tip placed directly in the tissue of the patient (interstitial). Recently, many researchers have proposed detectors based on superconducting single-photon detectors (SNSPDS) working at cryogenic temperatures.[36] However, this technique has disadvantages such as high cost, complexity and size.

Conclusions

PDT has the potential to meet many currently unmet medical needs in cancer treatment. The ability to detect singlet oxygen luminescence is critical in any prediction of appropriate dosimetry.

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