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Use of fluorescent probes for ROS to tease apart Type I and Type II photochemical pathways in photodynamic therapy

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Abstract

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Photodynamic therapy involves the excitation of a non-toxic dye by harmless visible light to produce a long-lived triplet state that can interact with molecular oxygen to produce reactive oxygen species (ROS), which can damage biomolecules and kill cells. ROS produced by electron transfer (Type I) include superoxide, hydrogen peroxide and hydroxyl radical (HO•), while singlet oxygen (¹O₂) is produced by energy transfer. Diverse methods exist to distinguish between these two pathways, some of which are more specific or more sensitive than others. In this review we cover the use of two fluorescence probes: singlet oxygen sensor green (SOSG) detects ¹O₂; and 4-hydroxyphenyl-fluorescein (HPF) that detects HO•. Interesting data was collected concerning the photochemical pathways of functionalized fullerenes compared to tetrapyrroles, stable synthetic bacteriochlorins with and without central metals, phenothiazinium dyes interacting with inorganic salts such as azide.

Keywords

Photodynamic therapy; singlet oxygen; hydroxyl radical; singlet oxygen sensor green; hydroxyphenyl fluorescein; fullerenes; bacteriochlorins; phenothiazinium salts

Introduction to PDT

The discovery of the concept that would lead to the development of photodynamic therapy dates to over 100 years ago in 1900 [1]. It was observed that *Paramecia* microorganisms

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were killed when exposed to acridine dye in the presence of sunlight, but not when kept with the dye in the dark. In 1904 it was discovered that the presence of atmospheric oxygen was necessary for this photosensitization effect to occur, and the term “photodynamic” was coined [2]. In the 1960s it was observed that hematoporphyrin derivative had the property of localizing in tumors when injected intravenously and could be used for cancer diagnosis [3], and in the 1970s that the tumors could be destroyed when illuminated by red light [4, 5]. In 1976 Weishaupt et al identified the cytotoxic agent responsible for tumor destruction as singlet molecular oxygen [6]

Type 1 and Type 2 ROS

Although $^1\text{O}_2$ is generally considered to be the predominant primary reactive oxygen species (ROS) involved in PDT, there is mounting evidence that other primary ROS are also produced during PDT, and can have important roles in mediating the oxidation of important biomolecules; a process that leads to cell death and tumor destruction [7]. Christopher Foote distinguished between Type 1 and Type 2 photochemical mechanisms [8]. Absorption of a photon of light with the appropriate quantum energy (wavelength) by the photosensitizer (PS) leads to the excitation of one electron into a higher-energy orbital (LUMO) as shown in eq 1 and as illustrated by the Jablonski diagram shown in Fig. 1. This singlet excited-state PS (unpaired non-parallel electrons) is very unstable (lifetime of nsec) and loses its excess energy either as emission of light (fluorescence) or production heat (internal conversion). However the excited singlet PS may undergo a process known as “intersystem crossing” to form a more stable excited triplet state with the spin of one electron inverted (unpaired but parallel electrons) (eq 2). This long-lived excited triplet state (lifetime of μsec) can survive long enough to carry out photochemistry. One common reaction involving the PS triplet is called the Type 2 photochemical pathway, and involves energy transfer to the ground state of molecular oxygen (also a triplet) to form the ground singlet state PS and the excited state $^1\text{O}_2$ (eq 3). The Type 1 photochemical pathway is more complex and involves production of superoxide anion ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\text{HO}^\bullet$). This is thought to occur by an initial one-electron transfer step to produce the PS radical anion (one electron reduction, eq 4). The PS radical anion can react with oxygen to produce the superoxide radical anion ($\text{O}_2^{\bullet-}$) (eq 5). Dismutation of $\text{O}_2^{\bullet-}$ (H_2O_2) (eq 6) or another one electron reduction (eq 7) can both give hydrogen peroxide, which in turn can undergo yet another one-electron reduction to form the powerful oxidant, hydroxyl radical ($\text{HO}^\bullet$) (eq 8). This one electron reduction can also be carried out by the Fenton reaction (eq 9) if there is any free (non chelated) iron available. The ferric ions produced can be recycled by superoxide reduction to ferrous ions in a process called the Haber-Weiss reaction (eq 10). ROS generation via Type 2 chemistry is mechanistically much simpler than via Type 1, and most PS used for anti-cancer PDT are generally believed to operate via the Type 2 rather than the Type 1 mechanism.

^IIt should be noted that the efficiency of this reaction is still under debate.

It is clear that the electron in eq (4) has to come from somewhere. However experiments that have tried to provide an artificial source of electrons have not been very successful as electron donors (or reducing agents) tend to be easily oxidized and therefore are quite good at quenching the ROS that are produced during the PDT reaction. NADH is a very good example of this, where it is an electron donor that increases the yield of superoxide, but reduces the extent of microbial killing in a PDI experiments. One source of electrons that does not suffer from this disadvantage is the use of iodide, because when iodide donates an electron it forms reactive iodine species that also kill microbial cells [9, 10].

When we attempt to distinguish which PS are good at carrying out Type I photochemical mechanisms one of the most important factors to be taken into account is how good an electron acceptor the PS is. In other words what is its redox potential? PS that have low redox potentials are good electron acceptors and can carry out Type I reactions, while PS that are bad electron acceptors (high redox potentials) can only participate in Type II reactions.

Methods of detection

Major progress has been made on better understanding of the behavior and biological effects of the individual species of ROS. However, the low concentration, high reactivity and fast kinetics make these species very difficult to detect selectively, especially *in vivo*. In the recent years, more sensitive methods of detection have been developed, with continuous efforts towards the quantification and the selective identification of the reactive pathways involved.

1280 nm luminescence

Singlet oxygen ($^1\text{O}_2$) shows a specific light emission at ca. 1280 nm. The detection of this luminescence is a frequently used technique for the detection and characterization of singlet oxygen production, both in solution and in living organisms [11-13]. It is a direct, non-invasive method that allows the detection and also quantification of the singlet oxygen produced, and therefore can be used as dosimetry tool [14]. Moreover, the time-resolved detection of the phosphorescence provides detailed information about singlet oxygen production, diffusion and interaction with the environment [15]. Thus, the kinetics of the singlet oxygen production and decay has been studied to determine the localization of the photosensitizing agent in cells and microorganisms [16-20]. The spatial resolution has been also explored in microscope-based single cell experiments by using two-photon or genetically encoded photosensitizers [21, 22]. Major drawbacks of the direct monitoring of singlet oxygen phosphorescence are (i) the intrinsic low quantum yield of the singlet oxygen emission; (ii) the high biomolecular reactivity of singlet oxygen; (iii) the sub-optimal performance (low signal-to-noise ratio) of the near-infrared (NIR) detectors. The development of new technologies such as NIR-sensitive imaging arrays [23, 24] and superconducting nanowire single-photon detectors [25] can provide more sensitive detectors of $^1\text{O}_2$ that will be eventually used for clinically feasible PDT dose monitoring.

Electron Spin Resonance (ESR) and spin traps

Electron spin resonance (ESR) spectroscopy, also known as electron paramagnetic resonance (EPR), is a powerful technique for studying chemical species or materials that have one or more unpaired electrons. Thus, free radicals such as ROS can be monitored directly if they occur in high enough concentrations and have sufficient stability. Due to the short half-life and high reactivity of the ROS themselves, spin traps are generally employed to transform them to stable or long-lived detectable spin-active species with a distinctive line splitting pattern [26-28]. Several substances have been used as spin-trapping agents, and can be classified in three groups: compounds with nitron, nitroso and piperidine/pyrrolidine

groups [29]. Nitrones such as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) and 5-ethoxycarbonyl-5-methyl-1-pyrroline N-oxide (EMPO) have been extensively used for trapping hydroxyl and superoxide anion radicals in biochemical and biological systems, forming distinguishable adducts depending on the structure of the trapped radical [30]. The linear nitrones such as α -phenyl-N-tert-butyl nitron (PBN) and α -(4-pyridyl-1-oxide)-N-t-butyl nitron (POBN) are mostly used for carbon-centered radicals. Conversely, most compounds of the nitroso family present some degree of instability and high toxicity, limiting their applications to cell-free model systems [31]. To detect singlet oxygen, the oxidation product TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) has been widely used [32]. Singlet oxygen reacts with the trapping probe TEMP (2,2,6,6-tetramethylpiperidine), yielding the TEMPO free radical easily detected by ESR [26]. The ESR technique has been constantly improved and the measurement of ROS and other bioradicals has been successfully performed in living animals [30, 33-35], attracting attention for its clinical application [36].

Fluorescence probes

Fluorescence detection of ROS has high sensitivity and experimental convenience. It does not require the use of advanced techniques and it can be easily implemented and analyzed. Therefore, several fluorescent probes have been synthesized. However, there are major limitations of the use of some these probes, mainly lack of selectivity and autooxidation, which can potentially lead to misinterpreted results and inaccurate conclusions [37, 38]. Dichlorodihydrofluorescein (DCFH) is probably the most widely used probe to measure H_2O_2 and oxidative stress. Despite its popularity, this probe has been demonstrated to have no specificity for H_2O_2 and shows significant instability due to autooxidation [39]. Another probe used to measure H_2O_2 is Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine). It is oxidized by H_2O_2 in presence of horseradish peroxidase (HRP) yielding the highly fluorescent resorufin. However, some concerns have arisen regarding photooxidation and secondary reactions [40, 41]. 1,3-Diphenylisobenzofuran (DPBF) is a probe used to detect singlet oxygen by fluorescence quenching when incorporated in phospholipid vesicles [42], but also its fluorescence can be quenched by superoxide anion radicals [43]. The intracellular detection of superoxide can be monitored instead by dihydroethidium (DHE) and its derivative MitoSOX Red. The reaction yields highly specific red fluorescent product, 2-hydroxyethidium. However, nonsuperoxide-dependent processes can also oxidize DHE to ethidium, which is also red fluorescent [44]. Thus, the synthesis of robust, stable and selective probes for the detection of ROS has been a long pursued goal. DPAX, 9-[2-(3-carboxy-9,10-diphenyl)anthryl]-6-hydroxy-3H-xanthen-3-one, and DMAX, 9-[2-(3-carboxy-9,10-dimethyl)anthryl]-6-hydroxy-3H-xanthen-3-one, were the first selective fluorescence traps for detection of singlet oxygen [45, 46]. Soon after, the Singlet Oxygen Sensor Green (SOSG) fluorescent reporter was commercialized. Since then, other novel singlet oxygen-specific fluorescence probes have emerged, such as the anthracene analog diethyl-3-3'-(9,10-anthracenediyl)bis acrylate (DADB) which is cell permeable [47], or naphthoxazole-based probes that show improved sensitivity compared to other probes [48]. The development of 2-[6-(4'-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) and 2-[6-(4'-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) probes that specific

target hydroxyl radicals widened the chemical and biological applications of the use of fluorescence techniques [49]. Further description and results using the HPF and SOSG probes will be addressed below.

Quenchers

A quick and simple method to confirm the presence of ROS and their role in the oxidation process observed is the use of quenchers. Some of them are naturally occurring scavengers. The enzyme superoxide dismutase (SOD), which catalyzes the dismutation of superoxide radicals into molecular oxygen and H_2O_2 , and catalase, which breaks down H_2O_2 , are first-line cellular defense against oxidative stress. A widely used example of non-enzymatic ROS scavenger is sodium azide, which quenches singlet oxygen. It is known to inhibit the photodynamic damage of singlet oxygen in bacteria and cells, however under certain conditions, it can potentiate the photokilling by an oxygen-independent mechanism [50-52]. The use of quenchers is still a well-established method to identify the photochemical mechanism but it does not give information about the localization and quantification of the ROS produced.

Singlet oxygen sensor green (SOSG) and 4-hydroxyphenyl fluorescein (HPF)

Singlet oxygen sensor green (SOSG) was introduced as a commercial reagent in 2006 and the chemical structure and reaction with singlet oxygen are shown in Scheme 1. SOSG does have the disadvantage that on activation with shorter wavelength light (UVA, blue and green) it may suffer from instability, self-activation [53], and even produce singlet oxygen without any PS [54].

4-Hydroxyphenyl fluorescein (HPF) and the related compound 4-aminophenyl fluorescein (APF) were introduced in 2003 by Setsukinai et al [55]. The chemical structure of HPF and its reaction with hydroxyl radical are shown in Scheme 2. Both probes (HPF and APF) will react with peroxynitrite ($ONOO^-$) as well as hydroxyl radical. The difference between HPF and APF lies in the relative specificity for $HO^\bullet$ and 1O_2 , with APF being more likely to be activated by 1O_2 than HPF. Nevertheless, even with the use of all three probes (HPF, APF, SOSG), there is still some level of uncertainty remaining, when attempting to determine the relative amounts of $HO^\bullet$ and 1O_2 generated from any particular PDT or PDI experiment.

These probes are best used in aqueous solution (buffered around neutral pH), and are most suitable for studying PS that are activated with red or NIR light (rather than blue or green that can partially activate SOSG but not HPF). However some PS are not particularly water-soluble. In this case we have tried using the probes in a 50% aqueous acetonitrile solution, which seems to work quite well, although perhaps not exactly as well as pure water. Both HPF and APF are taken up into cells so they can be used to detect the intracellular generation of $HO^\bullet$ and $ONOO^-$ [56]. Although it was reported that SOSG is not taken up into cells, a subsequent report showed that it could bind to proteins and gain entry to cells [57], and it was reported to detect 1O_2 in vivo in plant leaves [58].

Our studies with HPF and SOSG

Bacteriochlorins

It is now quite accepted that bacteriochlorin BC structures in general produce substantial quantities of Type I ROS, as compared to other tetrapyrroles such as porphyrins and chlorins. This has been shown for TOOKAD (Pd-bacteriopheophorbide) [59] and TOOKAD soluble [60] that both have Pd as a centrally co-ordinated metal. Fukuzumi et al showed that a Zn-substituted BC produced more Type I ROS than the equivalent Pd-substituted BC [61]. Generation of Type I ROS has also been shown by a fluorinated sulfonamide bacteriochlorin that was a free base (no central metal) [62].

In collaboration with Jon Lindsey's laboratory in North Carolina State University we have studied a wide range of stable synthetic bacteriochlorin (BC) molecules as new PS in PDT [63]. We examined four compounds shown in Fig 2, a basic free base BC, the dicyano free base (NC)₂BC, and the zinc-substituted Zn-(NC)₂BC and the palladium-substituted Pd-(NC)₂BC [64]. For SOSG activation the order was Pd-(NC)₂BC $\gg$ Zn-(NC)₂BC > (NC)₂BC > BC. For HPF activation the order was Pd-(NC)₂BC $\gg$ (NC)₂BC > BC > Zn-(NC)₂BC. The big change was for Zn-(NC)₂BC which moved from 2nd in ¹O₂ production to last in HO[•] production. There are probably two factors affecting these results. Firstly Pd increases the triplet yield to such an extent (no detectable fluorescence) that the yields of both ¹O₂ and HO[•] are highest. Secondly changing the central metal from free-base (2H) or Pd to Zn increases the proportion of energy transfer at the expense of electron transfer. The order of phototoxicity (PDT killing of HeLa cancer cells) was Pd-(NC)₂BC $\gg$ (NC)₂BC $\gg$ Zn-(NC)₂BC > BC. This implies that there is a correlation between production of HO[•] and more efficient PDT cell killing. Similar results were obtained in a study comparing Zn and Pd substituted imidazole porphyrins [65]. Here we found that for both the phototoxicity and the activation of HPF that Pd was better than Zn.

Functionalized fullerenes

Fullerenes are closed cage carbon nanostructures entirely composed of sp² hybridized carbon atoms and have a substantial absorption in the visible range.

They have been observed to carry out photoinduced-electron transfer reactions and when used as PS in PDT to produce a substantial proportion of Type I ROS [66]. Pristine fullerenes are insoluble in water and prone to aggregation, but when functionalized with polar groups become water-soluble and can be used for PDT [67]. If the functional groups are cationic then the fullerenes can become selective, broad-spectrum antimicrobial PS [68]. We compared two different polycationic antimicrobial PS, one based on a conjugate between the tetrapyrrole chlorin(e6) and the cationic polymer polyethylenimine called PEI-ce6, and the other a tri-cationic fullerene BB6 (Figure 3). Use of SOSG showed that PEI-ce6 produced more ¹O₂ than BB6, and that as expected both PS had the activation of SOSG inhibited by addition of sodium azide, a well-known quencher of ¹O₂. Interestingly when HPF was tested it was found that BB6 produced much more HPF activation than PEI-ce6, and moreover that azide did not inhibit HPF activation but rather potentiated it (almost double). Azide markedly reduced bacterial killing by PEI-ce6, but had almost no effect on

killing by BB6. A similar unexpected potentiation of activation of APF by addition of azide was reported by Price et al [69].

It should be noted, that it could be argued that the inhibitory effect of azide on SOSG activation might have been expected to be more pronounced. The explanation might be that the unknown product formed from azide during Type I photochemistry that activates HPF, might also activate SOSG to some extent, because of the relative lack of specificity of the two probes.

Phenothiazinium salts

Phenothiazinium salts such as methylene blue (MB) are one of the most widely-used type of PS, especially for antimicrobial applications [71]. The non-toxic nature of MB, its regulatory approval and wide-availability in pharmaceutical grade, has encouraged its use in many clinical studies. Although it has been recommended as a standard to be used in determination of singlet oxygen quantum yields [72], in reality MB also produces substantial quantities of Type 1 ROS.

When we tested whether or not azide inhibited the killing of bacteria by photoactivated MB we were somewhat surprised to find that in actual fact bacterial and fungal killing was increased by up to 2 logs when azide (10mM) was added, rather than being inhibited [50]. This paradoxical potentiation of microbial killing was attributed to formation of azide radicals as demonstrated by spin-trapping and electron paramagnetic resonance. We could envisage two potential mechanisms how azide radicals could be formed. The first involved oxidation of azide anions to azide radicals by hydroxyl radicals formed in a Type 1 photoprocess. The second possible mechanism involved the action of azide anions to reduce the excited MB triplet state to the radical anion by a 1-electron transfer at the same time producing azide radicals. We then went on to test a group of six different phenothiazinium dyes (see Figure 4) to see how general this phenomenon was and what was the effect on the activation of SOSG and HPF probes [73]. The data in Figure 4 was obtained in 50% aqueous acetonitrile because the solubility of some dyes in water was less than perfect, and moreover these dyes tend to dimerize in pure water which reduces their photoactivity [74]. It can be seen that the addition of azide inhibited the SOSG activation in the order NMB > DMMB > TBO > AA > AB > MB. In the case of HPF, the addition of azide potentiated the activation in the order TBO > AB > NMB > MB > AB, while only DMMB had its activation of HPF inhibited by addition of azide. The data on whether azide potentiated the aPDI-mediated killing depended on whether we looked at Gram-positive or Gram-negative bacteria, and whether the dye was washed away from the bacterial cells before light delivery so only bound dye would remain. With Gram-negative *Escherichia coli* and a wash, azide potentiated the killing with all six compounds, while other combinations showed occasional potentiation.

Conclusions and future perspectives

We believe that in recent years Type 1 ROS have become more often investigated and discussed in PDT and aPDI studies and protocols. For decades the PDT community has concentrated primarily on Type 2 mechanisms and singlet oxygen as the primary cytotoxic

mediator. This observation particularly applies to antimicrobial PDI studies. The interesting question is why this should be? Is it because the PS structures that are most often used for antimicrobial applications are more likely to undergo Type 1 reactions? Or is it because Type 1 ROS are more effective in destroying microorganisms than they are in killing cancer cells? Certainly hydrophobic porphyrins, chlorins and phthalocyanines are more likely to generate $^1\text{O}_2$ and these PS are often used as anti-cancer PS. Bacteriochlorins are more likely to generate Type 1 ROS due to their lower HOMO-LUMO energy gap [61], and lower redox potentials [64], and some of these compounds are highly effective as anti-tumor PS [63]. By contrast non-tetrapyrrole-based PS such as phenothiazinium dyes, fullerenes, titania photocatalysis etc [75] that generally produce Type 1 ROS including hydroxyl radicals [76] are more likely to be studied in antimicrobial applications. As further studies looking at Type 1 and Type 2 photochemical mechanisms in killing cancer cells and inactivating microorganisms are reported in the future, the validity of this broad generalization will become clearer.

It is a regrettable fact that no fluorescence probes are 100% specific for either $^1\text{O}_2$ or for $\text{HO}^\bullet$. The scientific interest in investigating Type 1 and Type 2 photochemical mechanisms coupled with the relative ease of using fluorescence plate reader-based assays, will encourage synthetic chemists to synthesize even more specific novel fluorescence probes for different ROS.

Acknowledgments

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Highlights

Photodynamic therapy produces hydroxyl radical via Type I, and singlet oxygen via Type II photochemical mechanisms.

Fluorescence probes, hydroxyphenyl fluorescein and Singlet Oxygen Sensor Green can be used to tease apart these two pathways.

Factors that encourage Type I include a low redox potential allowing acceptance of electron transfer, a central metal such as palladium.

Microbial cells may be more susceptible to Type I photosensitizers, while cancer cells are susceptible to Type II.

Figure 1. Jablonski diagram showing excited singlet and triple state PS, Type II energy transfer to form singlet oxygen, and Type I electron transfer to form superoxide anion, hydrogen peroxide and hydroxyl radical.

Figure 2.

Chemical structures of the four BC compounds. (A & B) Reactive oxygen species generation measured by fluorescence generated from ROS probes in solution (10 μ M), singlet oxygen sensor green, SOSG (A), or 3'-(4-hydroxyphenyl)fluorescein, HPF (B). The bacteriochlorins were directly diluted to a final concentration of 5 μ M per well in PBS and excited with NIR light. Data adapted from [64].

Figure 3.

Chemical structures of polyethylenimine-chlorin(e6) conjugate PEI-ce6, and tri-cationic fullerene BB6. Fluorescence generated from probes (10 μ M) and PS (2 μ M) in PBS with and without addition of 10 mM NaN₃. PEI-ce6 was excited by 660-nm light and BB6 was excited by white light. (A) SOSG (B) HPF. Data adapted from [70].

Figure 4.

Chemical structures of six phenothiazinium dyes. Probes were used at $5\mu\text{M}$ and dyes at $10\mu\text{M}$. Excitation light was 660 nm. The fluorescence value from each point in the absence of azide (10mM) was subtracted from the fluorescence value of the same point in the presence of azide. (A) SOSG in 50:50 PBS acetonitrile; (B) HPF in 50:50 PBS acetonitrile.

Scheme I. Chemical structure of SOSG and its reaction with singlet oxygen

Scheme II. Chemical structure of HPF and its reaction with hydroxyl radical