New Trends in Photodynamic Therapy Research

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Abstract. Photodynamic therapy is well established yet still rapidly developing treatment modality for cancer and other diseases. It is based on production of reactive species, especially singlet oxygen, by energy transfer from light-excited dye molecules in target tissue. In recent years, a whole bunch of strategies was proposed and examined to enhance efficiency of photodynamic therapy. In this review paper, we provide a short survey of novel trends, approaches, and perspectives in photodynamic therapy research from the point of view of biophysics. Especially the emerging use of nanoparticles is addressed.

Introduction and Insight

Singlet oxygen ($^1O_2$) is the lowest excited state of molecular oxygen which is several orders of magnitude more reactive than ground state oxygen. $^1O_2$ efficiently oxidizes many kinds of biomolecules (e.g., lipids, nucleic acids etc.) which can lead to a destruction of tissue and cell death. $^1O_2$ shows very weak phosphorescent emission around wavelength of 1275 nm, which can used for direct monitoring of $^1O_2$. Arguably the most important way of $^1O_2$ production is the photosensitizing process: An appropriate dye molecule, so-called photosensitizer (PS), is brought to the excited singlet state (S_1) by photon absorption. The PS then passes to the triplet spin state (T_1) by intersystem-crossing (ISC). The triplet state of PS is able to transfer energy efficiently to the ground triplet state of molecular oxygen, leading to production of $^1O_2$ (see Figure 1 left) [37]. Many molecules possess the ability to produce $^1O_2$ in this way, especially natural dyes based on porphyrins and many other dye molecules usually containing a system of conjugated double bonds and aromatic cores. Some of the PSs exert quantum yield of $^1O_2$ production close to 100% [33]. Photodynamic therapy (PDT) takes advantage of the described process. The PS is administrated to the patient and selectively accumulates in target (e.g., cancerous) tissue. The target tissue is then irradiated by visible or near-infrared (NIR) light which is absorbed by PS and $^1O_2$ is produced. It is widely believed that $^1O_2$ is the main cytotoxic agent in PDT, however it was suggested that also other reactive oxygen species (ROS) produced by PS, such as superoxide radical or hydroxyl radical, may play major role in PDT. Moreover, under hypoxic conditions, the triplet states of PSs may be deactivated be electron transfer to surrounding molecules giving rise to another free radicals [16]. There are three mechanisms of cell death induced by PDT: apoptosis, necrosis and autophagocytosis. The prevalent mechanism depends strongly on the type of PS, its cellular localization, light dose administrated, and other parameters of PDT treatment. Three mechanisms for tumor eradication were described: direct destruction of tumor cells, destruction of tumor vasculature leading to ischemia of the tumor tissue, and finally subsequent cancer-targeted immune response induced by release of decay products of tumor cells [10]. It is believed that the combination of all the three mechanisms is needed for long-term elimination of cancer. Again, these processes may be strongly influenced by parameters of PDT (e.g., time between drug administration and irradiation, or fractionation of light supply etc.) [4, 10, 34].

There are several important advantages of PDT over conventional cancer therapies (chemotherapy or radiotherapy) which promote PDT as a very promising treatment modality. First of all, precise spatial and time control of light supply in PDT leads to substantially increased selectivity to cancer tissue. The selectivity is absolutely crucial for all cancer therapies due to usually severe side-effects on the healthy tissue. PDT is externally switchable—it allows to launch the therapy at the most appropriate moment with respect to pharmacokinetics of the drug. An interesting point of PDT is that the cytotoxic agent is $^1O_2$ and other ROS which are fully natural substances. As it was mentioned before, PDT may induce cancer-targeted immune response leading to long-term control of tumor, including metastases. On the contrary, conventional chemotherapy and radiotherapy usually suppress immune response by destruction of bone marrow. Many of the PSs are fluorescent which enables diagnostics and imaging of cancerous tissue together with the therapy. It is well known that cancerous cells suppress some mechanisms of signaling pathways leading to programmed cell death, which causes their resistivity to conventional treatment. It was suggested that PDT induces apoptosis by a variety of pathways thus enhancing its success [4]. Last but not least, PDT has the potential to be low-cost treatment.

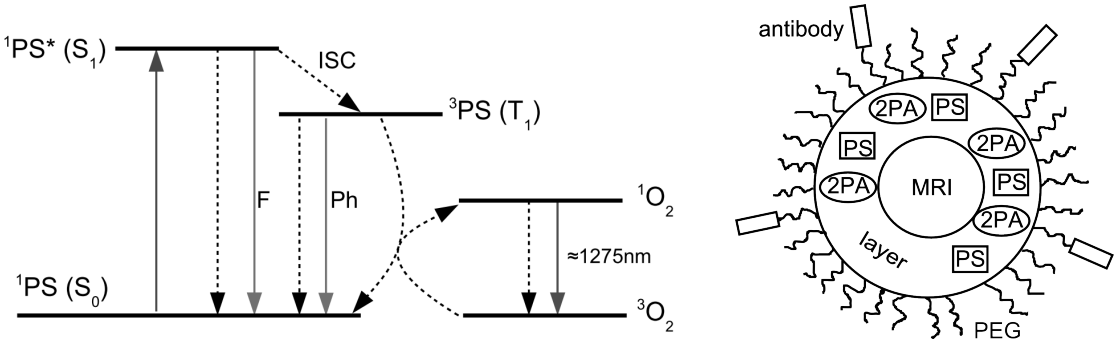

Figure 1. Left: Scheme of photosensitizing process described in Introduction. Full arrows denote radiative transitions (F: fluorescence, Ph: phosphorescence), dotted arrows nonradiative ones.
Right: Nanoparticle-based multifunctional photosensitizing assembly. MRI: magnetic core as contrast agent for magnetic resonance imaging, PS: photosensitizer, 2PA: two-photon absorbing dye, layer: oxygen permeable layer for dye encapsulation, antibody: tumor specific antibody, PEG: poly-ethylene-glycol coating.

However, there are also some shortcomings of PDT. The most obvious is the need to bring light to the treated tissue. It can be quite easily done for superficial skin lesions and some inner tumors easily accessible endoscopically, but it can be a difficult problem for deep located tumors. At the same time, the light penetration depth to tissue is limited to a few millimeters for visible light due to presence of natural absorbers as hemoglobin, melanin, or fat. Nevertheless, the light penetration depth substantially increases for red and near-infrared (NIR) light. The design of PSs absorbing in therapeutic window (approximately 650–1000 nm corresponding to penetration depths up to centimeter range [41]) is a big issue in PDT-associated research and it will be discussed later in the text.

Up to date, several PSs have been clinically approved. The first and still widely used is *Photofrin*, a not well defined mixture of haematoporphyrin derivatives which proved affinity for neoplastic tissues [28]. Photofrin was clinically approved for treatment of esophageal cancer, bladder cancer, lung cancer or cervical cancer. Photofrin is still considered a 'gold standard' of PDT, although there are many drawbacks. It has got very poor molar extinction coefficient at red region ($1170\,\mathrm{M^{-1}cm^{-1}}$ at 630 nm) and an exceptionally long half-life of about twenty days causing long lasting photosensitivity of the patient. *Photofrin* is a so-called first generation PS. Another of widely clinically used PS is *Foscan*, which is classified as a second generation PS due to approximately 30 fold increased absorptivity ($3 \times 10^4\mathrm{M^{-1}cm^{-1}}$ at 652 nm) in red region. It has been approved for palliative treatment of head and neck cancer and is under clinical trials for other types of cancer. Prolonged photosensitivity still remains to be a major drawback. *Levulan* and *Metvix* are aminolevulinic acid-based PSs indicated for treatment of actinic keratoses and other skin lesions. Aminolevulinic acid is naturally metabolized to protoporphyrin IX (PpIX), which is a precursor for heme and acts as PS. PpIX is selectively accumulated and metabolised in neoplastic tissues. PDT can by also exploited for a variety of skin diseases as psoriasis or acne vulgaris [28]. Recently it has been shown that PSs may act as efficient anti-microbial agents for treatment of antibiotic-resistant bacteria in wounds or elimination of oral bacteria, which opens a whole new field [26].

There are more approved PSs and many more of them under investigation. Recent findings and development allow us to consider a completely new concept of third generation PS which would allow for (1) increasing the selectivity of photosensitizing drugs for cancerous tissue, (2) overcoming problems with depth of light penetration, (3) combination with other treatment modalities (e.g., radiotherapy or hyperthermia) to increase the outcome, and (4) parallel cancer diagnosis, imaging and real-time monitoring of PDT effect. To this end, a nanoparticle (NP) based PS formulation was proposed. The NP serves as a platform for multifunctional assembly of several components. An example is shown in Figure 1 right) and various implementations of such a model will be discussed further in the text.

Strategies

Tumor Selectivity. It has been mentioned that porphyrin based PSs accumulate preferentially in neoplastic tissue. Several mechanisms were proposed. The prevalent theory claims that hydrophobic porphyrins aggregate with serum proteins as human serum albumin (HSA) and low-density-lipoprotein (LDL). Cancer cells have over-expressed LDL receptors due to their fast consumption of cholesterol which is carried by LDL. Generally, cancer cells are more 'greedy' and show so-called 'Enhanced permeation and

retention effect' (EPR) [25]. Blood vessels in tumors are more leaky and allow for easier extravasation of larger objects as macromolecules, proteins, or NPs. On the other hand, the decreased lymphatics of tumor tissue leads to longer-lasting retention of these larger molecules and objects, while the healthy tissue quickly clears out the external particles. The described mechanism of selective accumulation is called passive targeting. This observation leads to the idea of using NPs as carriers of PSs and thus increase their affinity for tumor tissue. Moreover, many potent PSs are hydrophobic which means that a carrier is needed to bring them into blood stream (aqeous body-environment). Indeed, many nanocarrier-formulations of PSs have been investigated: liposomal, HSA, LDL, polymeric, ceramic or metallic [3, 5]. Nanocarriers should allow for easy functionalization of their surface. PEGylation, covalent attachment of Poly-ethylene-glycol (PEG), is a common functionalization of NPs. It increases biocompatibility, masks the agent from host's immune system (reduces immunogenicity), reduces renal clearance, and provides water solubility [40].

Functionalization also enables active targeting. Active targeting consists in antigen-antibody interaction of drug and surface receptors of cancer cells. Folate receptors or growth factor receptors are usually over-expressed in cancer cells and represent a frequent approach for active targeting [15]. Not only the surface, but also the inner environment of the cell differs between cancer and normal cells. Another sophisticated approach for enhancement of selectivity for cancer tissue is to activate the PS only in the presence of intra-cellular biomarker specific for cancer cells. So-called photodynamic molecular beacon consists of PS held in close proximity to a quencher by a linker which is cleavable by the biomarker. In normal tissue, the quencher can directly quench the excited state of PS by Förster resonant energy transfer or may act as physical $^1O_2$ quencher (e.g., carotenoids). In cancerous tissue, the quencher is unlinked and the PS starts to produce $^1O_2$. First reported molecular beacon system consisted of pyropheophorbide as PS and carotenoid as $^1O_2$ quencher held together by caspase-3 cleavable linker [6]. Another possible way is to target the higher acidity of interstitial fluid of many kinds of tumors. Hydrophobic PS meso-tetraphenylporphin together with pH indicator bromocresol purple were encapsulated in organically modified silica NPs. Under basic conditions the pH indicator absorbs competitively with PS at the excitation wavelength and acts as inner filter. Under acidic conditions, the pH indicator absorption shifts, efficient excitation of the PS is allowed and $^1O_2$ is produced in higher rate [22].

Deeper Light Penetration. A typical representative of porphyrins, meso-tetraphenylporphin (TPP), exerts strong absorption band around 415 nm and four absorption bands in 500–700 nm region, which are two orders of magnitude weaker. An efficient PS should ideally absorb in the therapeutic window (650–1000 nm). As it was mentioned before, the light penetration depth in tissue is much higher for red and NIR light. This problem was partially overcome by introduction of second generation PS strongly absorbing in red portion of spectrum, such as chlorins, bacteriochlorins, phtalocyanines, porphycenes, or hypericine. *Tookad* is a red and NIR absorbing PS presently under intensive clinical trials [1, 28].

Two-photon absorbing (2PA) dyes represent another promising approach for design of PS absorbing in therapeutic window. Simultaneous absorption of two photons of lower energy can be used for excitation of the dye. The dye can then directly produce $^1O_2$ or, if encapsulated in a NP, transfer energy by FRET to neighboring PS [18]. 2PA photosensitizers reduce the excitation volume which can be of benefit in applications requiring precise spatial control, such as neurology or opthalmology. Usual porphyrins are very poor 2PA dyes (2PA cross-section $\delta_{\max} < 50\,\mathrm{GM}$). However, acetylene-linked conjugated porphyrin dimers were shown to possess much larger 2PA cross-section ($\delta_{\max} \approx 17000\,\mathrm{GM}$). Porphycenes, structural isomers of porphyrins, show relatively high 2PA cross-section ($\delta_{\max} \approx 2300\,\mathrm{GM}$ for tetraphenyl-porphycene) together with reasonable $^1O_2$ quantum yield which promotes them as promising PS [2, 30]. Lanthanide containing NPs (e.g., $NaYF_4$:Yb^{3+},Er^{3+}) were reported to be very efficient 2PA (up-converting) phosphors. Upon excitation at 974 nm, strong emission bands at 537 nm and 635 nm are observed. The NPs were covered by a thin layer of porous silica with merocyanine-540 incorporated as PS and functionalized with tumor targeting antibody [42]. Merocyanine absorption overlaps with emission of the NP and produces $^1O_2$.

Combined therapies. Combination of PDT with other cancer therapies may provide a synergistic effect and may help to overcome some of their shortcomings. A combination of PDT with radiotherapy and thermotherapy seems to be especially useful. In both cases NPs play a key role, especially golden NPs (GNP). Unlike light used in PDT, γ-rays used in radiotherapy are able to penetrate deep into tissue.Therefore, use of scintillating NPs is another promising perspective. Scintillator absorbs ionizing radiation and converts it in visible (or NIR) photons. Emission spectrum of the scintillating NP can be tuned by adjusting its size. Such NPs are sometimes called self-lighting ones. Liu et al. [23] have reported $^1O_2$ generation by X-ray irradiated LaF_3:Tb^{3+} scintillating NPs conjugated with meso-tetra(4-

carboxyphenyl) porphine as PS and folic acid as tumor targeting antibody. An employment of several doped NPs or semiconductor NPs (e.g., ZnO or TiO_2) has been proposed [7]. As well as PDT, radiotherapy is less effective in hypoxic environment of inner-parts of larger solid tumors. The radioresistance of hypoxic tissue may be challenged by NPs as well.

Thermotherapy is a long-known cancer treatment modality. Localized hyperthermia (41–47 °C) induced by laser light causes protein denaturation, membrane disintegration and subsequent cell death. Poor heat-exchange in badly vascularized inner-parts of solid tumor reduces their heat-tolerance. Thermotherapy is therefore inherently selective for such tissue [11, 14]. As PDT attacks tumor vascularization and is less effective in hypoxic conditions, thermotherapy may act synergeticaly and complementarily. Gold nanoparticles (GNP) proved to be excellent agent for photo-induced thermal therapy. The phenomenon of localized surface plasmon resonance (collective electron oscillations) in GNPs causes great optical absorption (several order higher than organic dyes) and subsequent dissipation of energy as heat. It was suggested that a rapid formation of bubbles (microscopic underwater explosion) around GNPs is involved in tumor cell damage causing mechanical stress to the cells [14]. Structures as gold nanorods, nanospheres, nanoshells, hollow nanospheres, or nanocages have been reported [11, 35]. GNP plasmon resonance band can be finely tuned into NIR region by adjusting its size or aspect ratio. GNP have been recognized as generally excellent vector for drug delivery [12] which can be exploited also in PDT [8]. GNP can be readily functionalized and are essentially non-toxic and biologically inert. Kuo et al. [20] prepared gold nanorods conjugated with indocyanine green as PS for dual photodynamic and photothermal therapy and showed enhanced killing of cancer cells. It was also shown that GNP are able to act as radiosensitizers possibly due to generation of ROS by mechanism of photo- or Auger-electron charge transfer and emission of fluorescence X-rays [27].

Theranostics. Theranostics stands for fusion of therapy and diagnosis/treatment monitoring. The fusion makes it possible to optimize and tailor the treatment regime according to the individual needs of a particular patient [32]. Such a 'see and treat' approach is of special interest in PDT where an excellent spatial and temporal control over therapeutic effect can be achieved. The fluorescence of many PS which selectively accumulate in cancerous tissue has been used for diagnosis and tumor imaging. Intensity of fluorescence from the tumor can provide a valuable information about PS concentration and help to set an appropriate light dose for PDT. A moderate fluorescence quantum yield is a favorable feature of a PS. Nevertheless, various PS designs enabling more sophisticated monitoring methods were proposed. PS conjugated with magnetic NPs (e.g., iron oxide) serving as contrast agent for magnetic resonance imaging (MRI) were prepared and characterized [13, 17]. It was shown that agents containing paramagnetic transition metal ions as Gd^{3+} or Mn^{2+} may be used as MRI contrast agents due to efficient alteration of spin relaxation time [32]. On the other hand, a PDT agent containing a positron emitting radioactive isotope may be used to enable positron emission tomography (PET) imaging. ^{124}I seems to be one of the most appropriate isotopes due to its suitable lifetime of approximately four days [29].

Absolutely new and rapidly developing field is surface enhanced phosphorescence, especially with use of GNPs. It has been long known that metal surface can enhance Raman scattering (SERS) or absorption of adjacent molecules. Enhancement of fluorescence was reported as well [21]. A close proximity to metal surface (in order of tens of nanometers) is needed for the enhancement to take place. At the same time, fluorescence is quenched for the distances smaller than a few nanometers. It was shown that both quantum yields of emission from singlet (fluorescence) and triplet (phosphorescence) states can be enhanced by coupling to surface plasmon [43]. $^1O_2$ exerts very weak phosphorescence around 1275 nm with quantum yields of 10^{-5}–10^{-7} [37]. As it was mentioned before, wavelength of plasmon resonance of a metal NP can be tuned by adjusting its size and aspect ratio. Enhancement of $^1O_2$ phosphorescence by a plasmonic NP may enable direct observation and dosimetry of $^1O_2$ produced during PDT. To this end, $^1O_2$ phosphorescence enhancement by GNPs was reported. Golden nanodiscs were coated by 100 nm thick polystyrene layer doped with tetra-phenyl-porphin as PS. The enhancement factor was 3.5, but the authors suggest that it may increase to several hundreds if all the PS molecules were in appropriate distance from the surface of GNPs [39]. Enhancement of $^1O_2$ phosphorescence by factor of 35 was reported for silver-island film coated with fullerene [31].

Contribution of Our Lab. Our lab is working on several specific problems outlined above. A setup for time- and spectral-resolved near-infrared luminescence spectroscopy is our main tool which provides phosphorescence kinetics of either $^1O_2$ or PS with 5 ns time-resolution [9]. Study of phosphorescence kinetics and lifetimes brings valuable information about processes of $^1O_2$ and PS-triplet formation and deactivation. A non-trivial triplet dynamics of porphycenes, promising second generation PSs, is currently being investigated. Porous silicon NPs can serve as PS nanocarrier and nanoplatform for multifunctional PDT. Pores of nanometric size enable oxygen to diffuse in and out. Interestingly, it has been

shown that silicon NPs themselves are able to generate $^1O_2$ upon illumination [19, 24, 36]. Silicon NPs prepared by laser ablation technology are being examined as prospective photosensitizing agents. The phenomenon of metal enhanced phosphorescence is being addressed in our lab by investigating phosphorescence emission of porphyrins in presence of golden nanorods in solution (collaboration with the group of Jan Proška, FJFI, ČVUT). Construction of setup for microscopic observation of singlet oxygen luminescence directly from living cells with subcellular resolution is also a topic pursued by our lab. So far, the group of professor Ogilby has managed to make a singlet oxygen based image of neurons. They used scanning by InGaAs linear array and cells were incubated in D_2O which increases lifetime of $^1O_2$ by order of magnitude [38].

Summary

There are many new perspectives emerging in PDT research. Especially interesting topic is design of third-generation NP based multifunctional photosensitizing constructs. Better selectivity for tumor tissue, deeper light penetration, combination with other therapies as radio- or thermo-therapy, and fusion of PDT with imaging are addressed. New findings will hopefully promote PDT as a strong cancer treatment modality thus further enriching our range of tumor-fighting tools.

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