Summary of the Doctoral Thesis

Development of stable and biocompatible sensitizers and sensors for singlet oxygen generation and detection

(一重項酸素の生成および検出のために安定性と生体適合性 を付与した増感剤およびセンサーの開発)

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This thesis encloses the preparation, characterization, photophysical studies, and applications of organic molecular photosensitizers and singlet oxygen ($^1O_2$) sensors, aiming to demonstrate a few essential aspects for advancing photodynamic therapy (PDT). This thesis is divided into five chapters. The first chapter entitled “introduction” briefly introduces PDT, photosensitizers, and $^1O_2$ detection. PDT avoids the devastating harm to the body caused by radiotherapy or chemotherapy and avoids surgery, while treating cancer accurately. Therefore, PDT has been used in laboratory and clinical research, and many countries have approved photosensitizer (PS) drugs for the treatment of tumors. Photosensitizers is classified into three. Although first-generation PSs have been used in medicine, their efficiency in treating tumors and skin diseases is limited to peripheral tissues. The second-generation PS drugs provide a variety of promising core structures that improve the water solubility of the molecules and the ability to harvest the tissue penetrating near-infrared light. Although third-stage PSs are still in the laboratory research stage, they harvest longer wavelength light. Also, their $^1O_2$ production abilities, and modified structures combined with drug delivery systems allow for the treatment to have higher tumor or organelle targeting and cancer killing efficiency.

ROS is a series of active and ephemeral substances generated from reactions of oxygen molecule (3O2), which are continuously generated and eliminated in plants and animals (1, 2). About 1% of the oxygen absorbed by plants is converted into ROS to maintain life activities (3), They are mainly produced in chloroplasts, normally at low concentrations and act as signaling molecules that mediate various reactions to assist in controlling germination, rhizome growth, stomatal closure and programmed cell death (PCD) (4–10). While at high concentrations, they cause the oxidative damages to lipids, proteins and DNA (7). An increase in the ROS concentration is related to complex environmental factors, including cold or dry climate, high salinity and nutritional deficiencies and bacterial infections (5, 11–14). In addition, as a practical case, artificial use of ROS overexpression can lead to herbicidal effects. ROS in the body is mainly generated by mitochondrial oxidative phosphorylation (OXPHOS), because electrons can be transferred to O2 during the electron transfer pathway in the respiratory chain (15). Likewise for animals or humans, low levels of ROS play a role in different cell signaling pathways. The elimination of ROS relies on enzymes like superoxide dismutase and catalase, non-enzyme macromolecules like transferrin and albumin, and small molecules like vitamin C, vitamin E, glutathione, uric acid, and coenzyme Q (16, 17). However, overproduction of ROS and dysfunction of the buffering system can lead to

the development of chronic diseases, including cardiovascular diseases, diabetes, Parkinson's disease, Alzheimer's disease, and acquired immunodeficiency syndrome (18).

$^1O_2$ is molecular oxygen in an excited state, which has higher energy and electrophilicity than 3O2. It exists in plants, algae, and animal cells and exhibits strong biological activity as one of the ROS. The electronically excited states of oxygen are denoted as 1Σ+g and 1Δg. The 1Σ+g singlet state with higher energy (31.5 kcal mol-1) quickly transforms into the more stable, lower energy 1Δg singlet state (22.5 kcal mol-1) (19–21). The 1Δg excited state with a longer lifetime plays a very important role in biological, medical, and environmental-related chemical reactions. Because $^1O_2$ has a strong ability to react with organic compounds and biomolecules. Therefore, $^1O_2$ is a highly active species with cytotoxic implications. It is most sensitive to cell membranes and mitochondria. Also, it affects a variety of biological macromolecules in the cell and causes damage to cells. This chapter further discusses the PDT aspects of $^1O_2$, classification of photosensitizers, $^1O_2$ detection using fluorescence spectroscopy, including classical examples of sensors.

In Chapter 2 entitled “experimental,” I explored the synthesis of photosensitizers that can utilize near infrared light to penetrate deeper tissues and have higher efficacy. Also, this chapter includes the synthesis of a few rhodamine- and coumarin-based $^1O_2$ sensors. I designed and synthesized a series of new photosensitizers based on porphyrin derivatives, such as rTPA, 5 rTPA derivatives. Generally, this chapter, compiles the synthesis and characterization of novel photosensitizers and $^1O_2$ sensor molecules. Structural identification of organic compounds is confirmed by 1H-NMR, 13C-NMR and ESI-MS. I developed novel photosensitizers, rTPA derivatives which are further explored based on an original rTPA as the core structure to invent more efficient and promising compounds for PDT in the near-infrared (NIR) region. UV visible absorption spectroscopy, fluorescence spectroscopy and DFT calculations are used to compare the intrinsic photophysical properties of the derivatives with original rTPA. $^1O_2$ generation by the derivatives is evaluated with the help of a commercially available sensor, SOSG, and the lifetime of triplet state is also measured to support the results. The design strategy of the $^1O_2$ fluorescence probes is usually relies on photoinduced intramolecular electron transfer (PIET). Donor-acceptor (D-A)-based coumarin-anthracene conjugates (CA1-3) and a rhodamine 6G-anthracene (RA) conjugate have been designed and synthesized to provide more functional $^1O_2$ probes for PDT. The molecules synthesized include rTPA-NH2, rTPA-OH, rTPA-EGNH2, rTPA-EGOH, rTPA-DETA,

and coumarin- and rhodamine-anthracene conjugates (13 molecules). The $^1O_2$ sensing ability and detection mechanism of sensor are studied using fluorescence and absorption spectroscopic techniques. The procedures for the cervical cancer cell (Henrietta Lacks, Hela) and the pancreatic cancer cell (PCI55) culturing are also discussed, including the procedures for cell labeling using the Mitotracker deep red and MitoBright LT green. The images of organelle colocalization and $^1O_2$ sensing are recorded using dual-color confocal laser scanning microscopy (CLSM). This chapter further extends to various spectroscopic, electrochemical, microscopic, and computational methods, such as nuclear magnetic resonance (NMR) spectroscopy, electrospray ionization mass (ESI-MS) spectroscopy, UV-vis absorption spectroscopy, steady-state fluorescence spectroscopy, cyclic voltammetry (CV), differential pulse voltammetry (DPV) measurements, density functional theory calculations, confocal laser scanning microscopy (CLSM), dynamic light scattering (DLS) measurements, transient absorption (TA) spectroscopy, and time-resolved fluorescence spectroscopy, in the characterization of the properties and cell-based PDT applications of the above sensitizers and sensors.

In Chapter 3 entitled "modified of π-extended porphyrin-based photosensitizers for near-infrared photodynamic therapy", I compared the photophysical properties of the rTPA derivatives using absorption and fluorescence spectroscopic methods. Also, I conducted and conducted density functional theory (DFT) calculations for the molecules, which support their optical properties. I compared the abilities of these rTPA derivatives to produce $^1O_2$ using commercial singlet oxygen sensor green (SOSG). Further, transient absorption spectra were measured to determine the triplet state lifetime. These rTPA derivatives were delivered to cells using Mito-Porter, a drug delivery system. Cellular localization of these derivatives was visualized by confocal laser scanning microscopy (CLSM). Since rTPA derivatives carry different charges, I observed and studied the impact of charges on the preparation and stability of liposomes, which are new nano-carriers. Secondly, I compared the organelle localization and anti-cancer effects of Mito-Porter equipped with rTPA derivatives using cell viability assay.

Near-infrared (NIR) photosensitizers absorb NIR light and transfer the absorbed energy into nearby material. Popular applications are to generate reactive oxygen species such as $^1O_2$ for photodynamic therapy (PDT) or photocatalytic reactions (22,23). While ultraviolet and visible lights are absorbed by various molecules, NIR light is selectively absorbed by specific molecules,

thus reduces side effects or reactions and bring about an ideal medical effects in deep tissue (24). Accordingly, the exploration of high performance NIR-photosensitizers has opened up new probabilities in the fields of photocatalysis, PDT, and photothermal therapy (PTT) (25-27).

PDT for cancer utilize $^1O_2$ to selectively damage tumor cells, vasculature and tissue (22,23, 28-30). This is also one of the advantages of PDT over traditional treatment methods during surgery. Especially for PDT, which uses NIR light to target specific organelles in tumor, the development of more efficient phototherapy techniques has attracted great attention because it implements a clinical effect with low doses (27, 31-33). In recent years, several research groups have contributed NIR photosensitizers targeting mitochondria. In this field, our system consisting of a π-extended porphyrin type sensitizer, rTPA, and a mitochondrial-targeting delivery career, MITO-Porter (34), according to the previous work, proved more than one-order effective photoactivated cancer-killing capacity than those of approved drugs, such as Photophryin (35, 36).

Drug delivery system (DDS) carriers are enables us to explore photo-drugs for organelle-targeting PDT. But the current consensus is that the good performance of photosensitizers in DDS emerges from optimizing the interaction between the DDS carrier and cargo (37, 38). The physical characteristics of the cargo may alter or destroy the carrier system. For example, the influence of encapsulant charge is crucial for improving DDS-based pharmaceutical compounds. Therefore, whether derivatized new compounds with positive and negative charges are suitable for the corresponding DDS carrier provides valuable suggestions for the development of effective therapeutic agents. However, there are limited reports revealing systemic correlations between cargo and DDS carriers. One reason for this is the limited number of available photo molecules that can change their structure and properties readily. Since rTPA has two carboxyl groups in the short axis direction, we can easily modify its structure and properties.

In photodynamic therapy (PDT) of cancer, $^1O_2$ destroys tumor cells and blood vessels, and induces an immune response. The specific wavelength light source can be focused to tumor tissue, according to the location and depth of the tumor, so that precise and targeted treatment can be achieved. Photosensitizers that harvest the near-infrared (NIR) light is attractive for advancing various aspects of phototherapy, such as photodynamic (PDT) and photothermal therapy (PTT). Especially, organelle-targeted PDT in the NIR window has attracted huge interest as a highly efficient cancer therapy method. In this regard, there have been numerous studies on drug delivery

systems (DDS) carrying photosensitizers for effective PDT at near-infrared wavelengths. but the impact of charge on cargo in DDS carriers remains unclear, such as uniformity and stability of liposome. In this study, I developed a series of NIR-photosensitizers from a π-extended porphyrin-type sensitizer (rTPA), which permits us to alter the structure and properties with a one-step operation for the systematic study. One-step amidations supply 5 derivatives of rTPA, And they were comprehensively studied on the properties of photosensitizers based on their molecular structure and charge. rTPA loaded DDS carriers are prepared using a microfluidic device. One of the combinations, rTPA NH2@MP, shows the best capability of phototherapy among the derivatives. The current study presents a guideline for inventing effective DDS-based NIR-PDT compounds for future photodynamic drugs.

Overall, I introduced two carboxylic moieties in the original rTPA by chemically modifying it. Next, I evaluated the influence of rTPA functionalization on its photophysical, electrochemical and $^1O_2$ generation properties. The rTPA derivatives have absorption spectra like the original rTPA, indicating the functionalization did not significantly affect the intrinsic π-conjugated structure and photophysical properties of rTPA. DFT calculations helped confirm the photophysical properties of the derivatives and the original rTPA. Among the derivatives, rTPA-NH2 showed the best $^1O_2$ generation ability with reasonable photostability. The confocal laser microscopic imaging of cells labeled with rTPA or its derivatives were predominantly localized on the periphery of cells. Finally, drug-carrier interaction was investigated using MITO Porter and rTPA, rTPA-NH2, and rTPA-OH. All compounds demonstrated little difference in the particle size and zeta potentials, achieving effective cell membrane penetration and remarkable cancer-killing effects under 700 nm light illumination. The present study clarifies the important aspects of the structures and properties of cargo and carrier for optimized and organelle-targeted PDT.

In Chapter 4 entitled “the toles of molecular structures of substituted coumarins on singlet oxygen sensing,” novel $^1O_2$ sensors, coumarin-anthracene conjugates based on the coumarin 7 derivatives were prepared and studied. These derivatives were expected to show efficient intramolecular electron transfer-induced fluorescence quenching of coumarin derivatives, like other sensors, commercially available or synthesized in our laboratory. Here, photoinduced electron transfer from or to anthracene, anthracene quenches the fluorescence of the fluorophore, which is recovered when $^1O_2$ oxidizes anthracene. Unlike the reported electron donor-acceptor (D-

A) $^1O_2$ sensor molecules, the polarity of the solvent showed a significant impact on intramolecular PET of these new sensors, which exhibits intense emission in low-polarity solvents. Conversely, the fluorescence is quenched in highly polar solvents, due to aggregation-induced intramolecular electron transfer. Aggregation in aqueous solutions, as observed from fluorescence lifetime and DLS data, triggers PET. Further, I applied a coumarin-based $^1O_2$ sensor, which is sensitive in the aggregate state in a polar solvent, to cells and demonstrated the localization in the cell membrane or cytoplasm.

This chapter provides the synthesis, spectroscopic properties, $^1O_2$ sensing and cell labeling characteristics of CA1 sensor. The structural and photophysical properties, related to $^1O_2$ sensing, of CA1 are compared with two other CA-based molecules. After screening by fluorescence spectroscopic and lifetime studies, the probe with the ability to detect $^1O_2$ was selected from three D-A molecules. The screening helped reveal the unique mechanism of a coumarin-anthracene donor-acceptor system (CA1) to show intramolecular electron transfer through aggregation effect. Fluorescence lifetime and DLS measurements prove aggregation-induced intramolecular transfer and $^1O_2$ sensing abilities of CA1 in highly polar solvents such as aqueous buffers. CA1 absorbs below 480 nm, without any self-photosensitized fluorescence intensity enhancement under 530 nm excitation, and can be used as an ideal fluorescent probe to detect $^1O_2$ during PDT under wavelength exceeding 500 nm. I found that CA1 forms a stable intermediate, which enables intense fluorescence under a weak 365 nm LED, making it a time controllable emitter. I also explored and explained the problems faced by two similarmolecules, CA2 and CA3, through calculating the redox potentials, energy levels and molecular structures, providing a theoretical basis for the development of functional molecules for $^1O_2$ detection. Finally, I evaluated the intracellular localization and cell membrane binding nature of CA1 by fluorescence co-localization experiments.

Chapter 5 entitled “biocompatible singlet oxygen fluorescence probes for mitochondrial localization,” focuses on $^1O_2$ sensors based on rhodamine 6G-anthracene conjugates. Recently, the electron donating property of the aminomethyl anthracene moiety reported by Kohara et al., Sasikumar et al., and Sobhanan et al. helped further develop D-A-based $^1O_2$ detecting methodology (39-41). Coumarin-aminomethyl anthracene conjugates, show weak fluorescence after reacting with $^1O_2$ and forming the endoperoxide, and an apparent fluorescence increase triggered by weak

photoexcitation of the endoperoxide is an interesting sensing method. This phenomenon originates from the unique energy levels of photoexcited states and the nitrogen atom of the molecule, which accelerates the intersystem crossing by the spin-orbit charge transfer mediated intersystem crossing (SOCT-ISC) (42). However, Coumarin-aminomethyl anthracene derivatives needs UV irradiation for phototriggered fluorescence activation and have low solubility in aqueous solvents, which limits further applications of the probes, including to cell based studies.

Here I explored the reaction mechanism between RA, a rhodamine-anthracene conjugate, and $^1O_2$ by recording the fluorescence enhancement of RA under photosensitization at different wavelengths and separating the final product by HPLC. In cell experiments, RA show excellent solubility in aqueous buffers, enabling me to apply this sensor for intracellular $^1O_2$ detection. When applied to cultured cells, the sensor passed the cell membrane and localized to the mitochondria. Fluorescence enhancement in labeled cells was also observed by CLSM. In addition, the charge separated state calculated by DFT helps to understand the ability of the sensor molecule to generate $^1O_2$.

Overall, in this chapter, a rhodamine 6G-anthracene-conjugate (RA) is synthesized and characterized by 1H NMR and ESI-mass spectra, demonstrating unique wavelength-dependent functionalities to sense and generate $^1O_2$. RA acts as an ordinary fluorogenic $^1O_2$ sensor molecule like a commercially available SOSG under UV, blue, or green light illumination. In contrast, RA acts as the temporally controlled $^1O_2$ sensing reagent under longer wavelength illumination, in which RA waits to become strongly fluorescent until it is exposed to UV, blue, or green light. Moreover, RA generates $^1O_2$ much more efficiently than its original rhodamine 6G via the spin-orbit charge transfer mediated intersystem crossing (SOCT-ISC) process. The observed results and the DFT calculations indicate that all these features are achieved by the unique photoexcited states in RA. Moreover, photoexcitation of RA is achievable in living cells using focused laser light. The presented wavelength-switchable functionalities in RA offer promising molecular tools for the full use of $^1O_2$.

This thesis demonstrates $^1O_2$ generation, sensing, and applications. The first chapter provides a detailed introduction to photosensitized $^1O_2$ generation and sensing with some examples of applications. The second chapter focuses on the materials and methods, where the synthesis and characterization of various sensors and sensitizers are the focus. In chapter 3, two carboxylic

moieties in the original rTPA were successfully modified by amino-condensation, and the influence of the derivatization of the rTPA core was systematically investigated. The rTPA derivatives exhibited photophysical properties similar to the original rTPA, indicating that the chemical modifications do not significantly affect the intrinsic π-conjugated structure of rTPA. DFT calculations further supported the similarities of the optical absorption properties among the derivatives and the original rTPA. In $^1O_2$ sensing experiment, rTPA-NH_2 showed the best $^1O_2$ generation ability with reasonable photostability in an aqueous solution. The confocal laser scanning microscope observations demonstrate the localization of rTPA and its derivatives predominantly on the outside the cell membrane. Finally, drug delivery system carrier interaction was investigated using MITO-Porter and rTPA, rTPA-NH_2, and rTPA-OH. All compounds demonstrated little difference in the particle size or zeta potentials; they formed uniform and stable particles. Confocal laser microscopic observations verified that the rTPA enveloped in the Mito-porter system was successfully internalized into the cells and mostly localized in mitochondria. Phototoxicity of rTPA on PCI55 cells determined by WST-8 assay. All rTPA derivatives with mito-porter showed cancer-killing effects, and rTPA-NH_2 showed the highest cancer-killing ability under 700 nm light illumination. The present study clarifies the important aspects of the optimized choice of cargo and carrier for excellent performance in organelle-targeting PDT compounds.

In chapter 4, I explored the feasibility of a newly synthesized coumarin 7 derivatives-anthracene conjugates for singlet oxygen sensing. I synthesized 3 coumarin-anthracene conjugates (CA1-3). I found the unique mechanism of CA1, showing excellent fluorescence quenching only in highly polar solutions due to the aggregation feature of anthracene in an aqueous solution. I demonstrate CA1 can effectively detect $^1O_2$ released by the photosensitizer in aqueous solutions, with >60 fold fluorescence quantum yield enhancement. Nowadays, many $^1O_2$ fluorescent probes have been proven to produce endoperoxide during the detection process and then completely release the fluorescence in a two-step reaction. The co-localization of CA1 and cells was observed by CLSM. CA1 passes through the cell membrane, and most of the CA1 was located in lysosomes. In chapter 5, the reaction process between the donor-accepter fluorescent probe and $^1O_2$ was investigated, proving the two-step reaction mechanism of a rhodamine anthracene conjugate (RA). The $^1O_2$ sensing ability of RA is studied using fluorescence spectroscopy. $^1O_2$ is generated using a photosensitizer, TCPP. RA showed a 24-fold enhancement of the fluorescence intensity after 30 min 430 nm blue LED illumination, which indicates an effective $^1O_2$ sensing by RA. Then, I used

700nm NIR irradiation for $^1O_2$ generation to avoid RA absorbing the light when it senses $^1O_2$. The fluorescence shows two-step enhancements, verifying RA acts as a time-controlled $^1O_2$ fluorescent probe under NIR photosensitization (~700 nm): RA forms an intermediate state after reacting with $^1O_2$, which does not emit strong fluorescence till excited by weak UV or green irradiation. DFT calculation was performed for RA, indicating that it generates $^1O_2$ under green light excitation, which indicates its anti-cancer effects, and the quantum yield of $^1O_2$ generation of RA was verified as 0.33. The spin-orbit charge transfer intersystem crossing (SOCT-ISC) process helps explain the RA has the property to be an efficient photosensitizer under green light. Its $^1O_2$ generation and sensing abilities were also revealed by CLSM, a mitochondria-targeting localization in HeLa cells and pancreatic cancer cells were observed. The molecular design guidelines and the wavelength-switching activity of RA pave the way for the creation of numerous and promising molecular tools to fully utilize $^1O_2$ in a spatiotemporal manner.

In summary, I prepared five photosensitizer derivatives, out of which rTPA-NH_2 has been found to be the most efficient $^1O_2$ producer. Also, two classes of $^1O_2$ sensors for the visible-light-based sensing in solutions and cells are introduced, demonstrating the photosensitized cancer cell death and $^1O_2$ detection.