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Biomedical Applications of Photothermal

Semiconducting Polymer Nanoparticles

Lyu Yan

School of Chemical and Biomedical

Engineering

2019

Biomedical Applications of Photothermal Semiconducting Polymer Nanoparticles

Lyu Yan

**School of Chemical and Biomedical
Engineering**

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

Mar. 5th, 2019

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Date

Lyu Yan

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Lyu Yan

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

Mar. 5th, 2019

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Date

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Assoc. Prof. Pu Kanyi

Authorship Attribution Statement

This thesis contains material from 4 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 3 is published as **Yan Lyu**, Yuan Fang, Qingqing Miao, Xu Zhen, Dan Ding, Kanyi Pu*. Intraparticle molecular orbital engineering of semiconducting polymer nanoparticles as amplified theranostics for *in vivo* photoacoustic imaging and photothermal therapy. *ACS Nano*, **2016**, *10*, 4472. DOI: 10.1021/acsnano.6b00168.

The contributions of the co-authors are as follows:

- Assoc. Prof. Kanyi Pu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Prof. Dan Ding.
- I co-designed the study with Assoc. Prof. Kanyi Pu and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- Yuan Fang carried out the animal work in Nankai University.
- Dr Qingqing Miao, Dr. Xu Zhen provided guidance in the experiment operation and data process.

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The contributions of the co-authors are as follows:

- Assoc. Prof. Kanyi Pu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts.
- I co-designed the study with Assoc. Prof. Kanyi Pu and performed all the laboratory work at the School of Chemical and Biomedical Engineering and School of Biological Science. I also analyzed the data.
- Asst./Prof. Yansong Miao gave me the guidance on the operation of fluorescence microscope and imaging process.
- Dr. Xu Zhen cooperated with me to carry out the animal work.

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The contributions of the co-authors are as follows:

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- I co-designed the study with Assoc. Prof. Kanyi Pu and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- Yuyan Jiang assisted in the synthesis of the polymer.
- Dr. Jianfeng Zeng, Dr. Xu Zhen and Shanshan Qiu carried out the animal work in Soochow University.

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- Assoc. Prof. Kanyi Pu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts.
- I co-designed the study with Assoc. Prof. Kanyi Pu and performed all the laboratory work at the School of Chemical and Biomedical Engineering and School of Biological Science. I also analyzed the data.
- Dong Cui assisted in the synthesis of the polymer.
- Asst./Prof. Yansong Miao and He Sun assisted me in the genetic modification of plasmids and image process.

Mar. 5th, 2019

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Date

Lyu Yan

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Lyu Yan

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Glossary of Acronyms

BSA: Bovine serum albumin

BL: Bioluminescence

CAAC: Copper(I)-catalyzed alkyne-azide cycloaddition

DSP: Dendronized semiconducting polymer

DLS: Dynamic light scattering

DPPF: Poly[3-(5-(9-hexyl-9-octyl-9*H*-fluoren-2-yl)thiophen-2-yl)-2,5-bis(2-hexyldecyl)-6-(thiophen-2-yl)pyrrolo[3,4-*c*] pyrrole-1,4(2*H*,5*H*)-dione]

DYn-2: 4-(Pent-4-yn-1-yl)cyclohexane-1,3-dione

DPPV: Poly{2,2'-[(2,5-bis(2-hexyldecyl)-3,6-dioxo-2,3,5,6-tetrahydropyrrolo[3,4-*c*]pyrrole-1,4-diyl)dithiophene]-5,5'-diyl-*alt*-vinylene}

DPPT: Poly{2,2'-[(2,5-bis(2-hexyldecyl)-3,6-dioxo-2,3,5,6-tetrahydropyrrolo[3,4-*c*]pyrrole-1,4-diyl)dithiophene]-5,5'-diyl-*alt*-thiophen-2,5-diyl}

EPR: Enhanced permeability and retention

EGF: Epidermal growth factor

EDX: Energy-dispersive X-ray spectroscopy

EGFP: Enhanced green fluorescence protein

EDC: 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride

FTIR: Fourier transform infrared spectroscopy

FBS: Fetal bovine serum

GPC: Gel permeation chromatography

HOMO: Highest occupied molecular orbital

Glossary of Acronyms

H&E: Hematoxylin and eosin

HSP: Heat shock promoter

HSF: Heat shock factor

HSE: Heat shock element

HPLC: High-performance liquid chromatography

H₂O₂: Hydrogen peroxide

HCIO: Hypochlorous acid

IR: Infrared

LOMO: Lowest occupied molecular orbital

LOD: Limit of detections

LPS: Lipopolysaccharides

MPE: Maximum permissible exposure

MPO: Myeloperoxidase

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

NIR: Near-infrared

NIR-I: First near-infrared window

NIR-II: Second near-infrared window

NMR: Nuclear magnetic resonance

N/P ratio: Nitrogen/phosphate ratio

¹O₂: Singlet oxygen

PA: Photoacoustic

PTT: Photothermal therapy

Glossary of Acronyms

PDT: Photodynamic therapy

PET: Photoinduced electron transfer

PL: Photoluminescence

PCNA: Proliferating cell nuclear antigen

PEG: Poly(ethylene glycol)

PPV: Poly(phenylenevinylene)

PES: Polyethersulfone

PCPDTBT: Poly[2,6-(4,4-bis-(2-ethylhexyl)-4*H*-cyclopenta[2,1-*b*;3,4*b'*]dithiophene)-*alt*-4,7-(2,1,3-benzothiadiazole)]

PC70BM: (6,6)-Phenyl-C71-butyric acid methyl ester

PLGA-PEG: Poly(ethylene glycol) methyl ether-*block*-poly(lactide-co-glycolide)

PAMAM3: Third-generation polyamidoamine

ROS: Reactive oxygen species

ROI: Region of interest

*r*SPN: Reaction-based semiconducting polymer nanoprobe

SBR: Signal-to-background ratio

SP: Semiconducting polymer

SPN: Semiconducting polymer nanoparticle

SON: Semiconducting oligomer nanoparticle

-SOH: Sulfenic acid

-SOOH: Sulfinic acids

-SOOOH: Sulfonic acids

Glossary of Acronyms

SD: Standard deviations

TEM: Transmission electron microscopy

TUNEL: TdT-mediated dUTP-biotin nick end labeling

TDM: Two-dimensional material

TCEP: Tris(2-carboxyethyl)phosphine

TEOS: Tetraethyl orthosilicate

Nomenclature

A_λ : Absorbance at the used laser wavelength

C_p : Heat capacity

h : Heat transfer coefficient

I : Laser power used

m : Mass

η : Photothermal conversion efficiency

$Q_{in,np}$: Photothermal energy input from nanoparticles

$Q_{in,surr}$: Heat input due to light absorption by the solvent and container

Q_{Dis} : Heat input due to light absorption by the container

Q_{out} : Heat lost to the surrounding

S : Surface area of the container

T : Temperature

t : Time

T_{max} : Maximum steady-state temperature of solvent

T_{surr} : Ambient surrounding temperature

V_o : the initial tumor volume

V : the tumor volume

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Summary

Biomedical Applications of Photothermal Semiconducting Polymer Nanoparticles

Student: Lyu Yan

Supervisor: Associate Professor Pu Kanyi

Semiconducting polymer nanoparticles (SPNs), as a new optical nanomaterial with completely organic components, show good biocompatibility and have been widely used in disease diagnosis and therapy. Specifically, beneficial from the superb photothermal conversion capability, SPNs have been applied in photoacoustic (PA) imaging, the brightness of which is closely related to the capability of photothermal conversion, and photothermal therapy (PTT). However, their translational potential to clinics is still challenging, which requires the further optimization of the SPN properties, such as brightness, specificity and biodegradability.

Until now, the methods to improve PA brightness companied by increasing PTT efficacy are limited in the synthesis of completely new polymer molecules, which is time- and labor-consuming. Thus, I design a SPN with a binary optical component nanostructure, wherein a near-infrared absorbing semiconducting polymer and an ultrasmall carbon dot (fullerene) interact with each other to induce photoinduced electron transfer upon light irradiation. Such an intraparticle optoelectronic interaction augments heat generation and

Summary

consequently enhances the PA signal and maximum photothermal temperature of SPNs by 2.6- and 1.3-fold, respectively. With the use of the amplified SPN as the theranostic nanoagent, it permits enhanced PA imaging and photothermal ablation of tumor in living mice.

In terms of specificity, the SPN-based probes to real-time detect cellular events at the submolecular level is seldom reported. Thus, I design a reaction-based SPN (*r*SPN) which comprises an optically active semiconducting polymer as the core shielded with inert silica and poly(ethylene glycol) corona. The sulfenic acid reactive group (1,3-cyclohexanedione) is conjugated to the surface of nanoparticles *via* click chemistry. Such a nanostructure enables the specific recognition reaction between *r*SPNs and protein sulfenic acids without compromising the fluorescence and PA properties. In addition to *in vitro* tracking of the production of protein sulfenic acids in cancer cells under oxidative stress, *r*SPNs permit real-time PA and fluorescence imaging of protein sulfenic acids in tumors of living mice.

In translational research, the most concerned issue to be addressed is the metabolism and clearance of the applied theranostic nanoagents. I herein develop a biodegradable SPNs with enhanced PA and PTT efficacy for cancer therapy. The design capitalizes on the enzymatically oxidizable nature of vinylene bonds in conjunction with polymer chemistry to synthesize a biodegradable semiconducting polymer (DPPV) and transform it into water-soluble nanoparticles (SPNV). As compared with its counterpart SPN (SPNT), the presence of vinylene bonds within the polymer backbone also endows SPNV with a significantly enhanced mass absorption coefficient (1.3-fold) and photothermal conversion efficacy (2.4-fold). As such, SPNV provides the PA

Summary

signals and the photothermal maximum temperature higher than SPNT, allowing detection and photothermal ablation of tumors in living mice in a more sensitive and effective way.

Beyond the optimization of the theranostic applications, I also explore the potential applications of SPNs. I synthesize a near-infrared (NIR) absorbing dendronized semiconducting polymer (DSP) and utilize it as a photothermal nanocarrier not only to efficiently deliver genes but also to spatiotemporally control gene expression in conjunction with heat-inducible promoter. DSP has a high photothermal conversion efficiency (44.2%) at 808 nm, permitting fast transduction of NIR light into thermal signals for intracellular activation of transcription. Such a DSP-mediated remote activation can rapidly and safely result in 25- and 4.5-fold increases in the expression levels of proteins in living cells and mice, respectively.

In summary, I have developed the general methods to individually improve SPN brightness, specificity and biocompatibility, which promote the translational study of SPNs utilized in PA imaging and PTT. Besides, I take full use of the high photothermal conversion capability of SPN and employ SPNs as the photothermal transducer to achieve noninvasive regulation of gene expression, which broadens its biomedical applications.

Chapter 1 Introduction

1.1 Background

Biophotonics is a recently developed field that involves the utilization of photonic technologies to detect and regulate biological events at the molecular, cellular or tissue level without disrupting the original integrity of samples.^[1] The applications of biophotonics are mainly determined by the photophysical process of the related optical materials upon irradiation, such as the emission of photons, generation of heat and transferring energy to surrounding molecules.^[2] Thus, biophotonics are widely applied in diagnosis, with the assistance of optical^[3] and photoacoustic (PA) imaging,^[4] and therapy (photothermal/photodynamic therapy^[5] or combinational therapy).^[6] Recently, with the discovery of optically sensitive biomolecules and development of optical-responsive chemical groups, biophotonics are applied in the regulation of cellular processes including protein dimerization, the regulation of ion channels, gene editing and so on.^[7]

The optical materials used in biophotonics include quantum dots,^[8] gold nanoparticles,^[9] carbon nanotubes,^[10] upconversion nanoparticles,^[11] two-dimensional nanomaterials,^[12] small molecule dyes^[3,13] and semiconducting polymers nanoparticles (SPNs).^[2,6] The inorganic nanomaterials have successfully applied in noninvasive theranostics; however, the slow excretion out of the body and high accumulation in the reticuloendothelial system potentially raise the risk of long-term toxicity.^[14] Small molecular dyes serve as a relatively safe optical agent which can rapidly cleared from the body through

urinary excretion. But the photobleach phenomenon has been widely observed in small molecular dye-based probes, which aggravates the concern on the stability issue.^[15] Compared with these materials, SPNs with superior optical properties, photo-stabilities and biocompatibilities are a promising optical nanoagent in biophotonic applications.

In this thesis, I just focus on the photothermal process of SPNs. The related biophotonic applications include PA imaging, photothermal therapy (PTT) and the regulation of cellular events.

1.2 Motivation

As a new generation of purely organic photonic nanoagent, SPNs have been proved to be effective in sensing and imaging of small molecules/biomacromolecules both *in vitro* and *in vivo*.^[16] The structural diversity of SPs makes it feasible to pair with different imaging modalities, including photoluminescence, chemiluminescence and PA imaging. Moreover, the stronger light harvest capability over inorganic nanomaterials allows SPNs to provide brighter optical signals and subsequently reduce the limit of detection (LOD); meanwhile, the efficient light harvest capability is beneficial to the photothermal conversion process. With these merits, SPNs serve as a versatile photonic nanoagent to be applied in PA imaging and PTT with desirable therapeutic efficacy.

However, challenges are still present which limits their translational applications in clinics:

1. Although SPNs show higher PA signals compared with inorganic

Chapter 1

nanoagents, the further improvement of PA intensity is still required. The increased PA signals facilitate the discrimination of real signals from the background noises. As such, the required administration dosage of SPNs can be reduced which potentially decreases the safety risk. Meanwhile, because the PA intensity is proportional to their photothermal conversion capability,^[17] the improvement of PA signals is also beneficial to PTT efficacy of SPNs. However, the design and synthesis of new SPs is the commonly used method to improve PA imaging quality and PTT efficacy of SPNs, which needs large amount of time and efforts devoted. As such, a simple and general method is highly desired.

2. Although SPNs has been fully developed as PA imaging probes to detect reactive oxygen species (ROS)^[18] and pH^[19], it is still hard to use them to recognize the subtle differences at the submolecular level which is meaningful in the exploration of disease pathology and conducting the early stage diagnosis.^[20] Therefore, a SPN-based PA probe to differentiate subtle variations at the submolecular level is desired.

3. The completely organic components of SPNs endow it biocompatibility and there is no obvious toxicity observed in cells and in mice after treated with SPNs. However, the accumulation of SPNs inside bodies may raises the risk of toxicity and its long-term safety is still uncertain.^[21] Thus, the design of biodegradable SPNs which can be degraded and excreted out of living bodies after accomplishing their theranostic duties are highly desired.

4. Given by the key merits of flexibility and excellent optical properties, it is of interest to integrate other functionalities into SPNs allowing for applications beyond sensing and imaging. Thus, the exploration of broad applications for

SPNs is needed.

1.3 Objectives of This Work

The objectives of this work are:

1. In the first part of work, I will explore the factors to affect the photothermal conversion efficacy of SPNs. Based on the related knowledge, I attempt to design a simple and general method to synthesize SPNs with improved PA imaging and PTT performance.
2. In the second part of work, I try to design a reaction-based semiconducting polymer nano-probes (*r*SPNs), combining with PA imaging modality to real-time track the dynamic variation in living systems at the submolecular level.
3. Through the molecular design, I attempt to synthesize a degradable SPN which can be degraded in physiological environment to the size less than 5 nm which can be excreted out of the body through urinary clearance.
4. In the final part of work, I take full use of the superior photothermal conversion capability of SPNs to achieve the noninvasive control of gene expression in living systems.

1.4 Organization of The Thesis

The thesis aims to optimization the PA imaging and PTT performance of SPNs including improving the PA signals and PTT efficacy, developing a PA probe with selectivity at the submolecular level and endowing SPNs with biodegradability to fulfill the requirements of clinical translation. At last, SPNs are designed to regulate cellular events, which explores more application possibilities beyond

Chapter 1

theranostics. In details, the organization of the thesis is as follows:

Chapter 2 reviews the fundamental knowledge including the definition of SPs, the preparation of SPNs and biophotonic applications of SPNs, especially the photothermal applications. In the detailed introduction of photothermal applications, I first introduce the photothermal conversion phenomenon and then review the SPN applications in PA imaging and PTT.

The following chapters report the individual work to achieve the objectives of this thesis. **Chapter 3** designs an intraparticle molecular orbital engineering approach to simultaneously enhance PA brightness and PTT efficacy of SPNs for the *in vivo* imaging and treatment of cancer. **Chapter 4** reports a reaction-based semiconducting polymer nanoprobe (*r*SPN) with near-infrared (NIR) absorption for *in vivo* photoacoustic (PA) imaging of protein sulfenic acids. **Chapter 5** reports the development of biodegradable SPNs with enhanced PA intensity and PTT efficacy for cancer therapy. **Chapter 6** synthesizes a NIR absorbing dendronized semiconducting polymer (DSP) and utilizes it as a photothermal nanocarrier, which can not only efficiently deliver genes but also spatiotemporally control gene expression.

At last, **Chapter 7** concludes all these finished works and designs the future work to suggest the further research direction. I have drawn the flowchart to indicate the frame of this thesis as **Figure 1-1**.

Figure 1-1. Flowchart of the frame for the thesis.

Chapter 2 Literature Review

2.1 Semiconducting Polymer Nanoparticles

SPNs is a new kind of optical nanomaterials transformed from hydrophobic SPs, which hold potentials in biophotonic applications. Distinct from inorganic nanoparticles such as quantum dots and gold nanoparticles, SPNs are composed of benign organic ingredients including SPs and amphiphilic agents. Thereby, SPNs avoid the issue of heavy metal ion induced toxicity and show superior biocompatibility.^[5a,22] Furthermore, the good biodistribution allows SPNs to perform theranostics in living animals after systemic administration.^[23] Beneficial from these advantages, SPNs have been widely used in optical imaging and phototherapy.^[2,5a,6,24] Recently, taking the advantages of light including safety, easy tunability and high spatiotemporal resolution, SPNs with tunable optical properties are also applied in the regulation of neuron cell behaviors.^[25]

In the following part, the basic knowledge of the main component SP is firstly introduced; then, the commonly used preparation methods to form SPNs are described; at the last part, the biophotonic applications of SPNs are concluded.

2.1.1 Main component: Semiconducting Polymer

The main component of SPNs is optically active SPs which possess both optical and electronic properties. SPs are defined as the kind of polymers with π conjugated system which is formed by at least 3 connected p -orbitals.^[26] It can

Chapter 2

be usually told from the structures with alternating single and multiple bonds. Different from the majority of polymers which are insulators, electrons of SPs are able to move along the backbone of polymers through the overlap of the delocalized *p*-electron clouds.^[24]

The delocalization of the π -electrons leads to the formation of π -band gap varying from 1.5 to 4 eV.^[27] The energy space between the band gap determines both the conductivity and absorption wavelength range.^[28] Under appropriate light irradiation, electrons of SPs are excited from the highest occupied molecular orbital (HOMO) to the lowest occupied molecular orbital (LOMO), forming the excited electrons in LOMO and leaving the holes in HOMO. Then part of the recombination of the excited electrons and free holes results in the generation of luminescent photons.^[29]

Since the first classical SP, poly(acetylene), was synthesized in 1958, the development of SPs has entered in the third generation with intrinsic anisotropy.^[30] Because of the co-existence of the electrical and optical properties, SPs, as a kind of promising materials, are widely used in producing organic electronics/electrolytic cells^[31] and developing molecular imaging probes.^[21,24]

The optical/electrical properties of SPs, which are determined by polymer molecular structures, affect the final applications and performance of SPs.^[32] With the development of molecular design strategy, a kind of SPs is designed with donor-acceptor (D-A) alternating structures (**Figure 2-1**).^[33] The bandgaps and energy levels of SPs are closely related to the choosing donor monomers (electron-rich) and acceptor monomers (electron-deficient), which ultimately determine the optical/electrical properties of the formed SPs. Thus, SPs with

Chapter 2

desired optical/electrical properties can be achieved by tuning the pairs of the donor and acceptor to find the most suitable one.

Figure 2-1. The molecular structures of some typical semiconducting polymers with D-A alternating structures.

The commonly used method to synthesize SPs including Kumada, Sonogashira, Suzuki, and Stille reaction. These reactions show the advantages of mild reaction conditions and the tolerance of functional groups.^[34] Kumada reaction is used to generate carbon-carbon bonds between a Grignard reagent and an organic halide and catalyzed by nickel or palladium.^[35] It is the first reported coupling reaction requiring transition metal catalysts. Sonogashira reaction also facilitates the formation of carbon-carbon bonds but needs the co-catalyzation by palladium and copper.^[36] Suzuki reaction is a cross-coupling reaction between a boronic acid and an organohalide requiring the catalysis of palladium(0) complex.^[37] Stille reaction is also a palladium-catalyzed coupling reaction. But different from Suzuki reaction, Stille reaction is used in the coupling between organotin compound and organic electrophiles.^[38] Beneficial from the high efficacy, Suzuki and Stille reactions are widely used in the synthesis SPs with alternating monomers. Suzuki reaction is suitable to develop benzene-containing SPs and Stille reaction is suitable for SPs with thiophene groups.

2.1.2 Preparation of Semiconducting Polymer Nanoparticles

The hydrophobicity of SPs limits their biomedical applications. As such, some methods to transform SPs to nanoparticles with hydrophilicity have been developed. There are two general ways to prepare SPNs: nano-precipitation and mini-emulsion, which are introduced as follows (**Figure 2-2**):

Figure 2-2. Illustration of the preparation methods of SPNs: mini-emulsion and nano-precipitation. Both methods need to dissolve SPs and amphiphilic agents (optional) in an organic solvent firstly and then the mixture is injected into water to form nanoparticles. The difference is that the organic solvent used for mini-emulsion is immiscible with water and that for nano-precipitation is miscible. Reproduced with permission.^[16b] Copyright 2017, WileyVCH Verlag GmbH & Co. KGaA, Weinheim.

Nano-precipitation is a very common method to prepare nanoparticles from hydrophobic polymers. SPs are firstly dissolved in an organic solvent in which they show the good solubility. Sometimes, additional amphiphilic agents are required to blend with SPs to form homogenous solutions in the organic solvent.^[39] Then the organic solutions containing SPs and amphiphilic agents

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(optional) are quickly injected into excess water under ultrasonication. Contributed by the dramatic change of the solvent polarity, the nanoparticles form driven by the hydrophobic interaction. Many researchers have reported that the original concentration of SPs in organic solvent played a critical role in the determination of the final diameters for the formed nanoparticles.^[40] Meanwhile, the size is also affected by the mass ratio of SPs to amphiphilic agents.

Mini-emulsion is very similar to nano-precipitation except for that the organic solvent used in mini-emulsion is immiscible with water. Thus, it forms two phases after the injection of organic solutions into water during the preparation of nanoparticles. Under the vigorous stirring and ultrasonication, the organic phase evenly disperses in water forming a homogeneous emulsion solution.^[41] The stable nanoparticles gradually form with the evaporation of organic solvents. Similarly, the diameter of nanoparticles can be controlled by altering the ratio of SPs to amphiphilic agents in the process of preparation.^[40b]

In conclusion, nano-precipitation and mini-emulsion are two commonly used methods to prepare SPNs. Their difference is the organic solvent used in nano-precipitation is miscible with water, while that is immiscible for mini-emulsion. The property of the chosen SPs is an important factor which needs to be taken into consideration when choosing the preparation methods. Generally, the nanoparticles prepared by mini-emulsion show larger diameters.^[23] Otherwise, the SP concentrations and the ratio of SPs to amphiphilic agents may also affect the final nanoparticle diameters.

2.1.3 Biomedical Applications of Semiconducting Polymer Nanoparticles

The biomedical applications of SPNs including imaging, therapy and the regulation of cellular events (**Figure 2-3**) are closely related to the photophysical processes of SPs upon the light irradiation.^[2,42] The following parts will introduce the outline of the correlation between applications and photophysical processes or other physical properties.

Imaging applications are firstly discussed. After the light irradiation, the ground state electrons of SPs are excited to the excited state with high energy. The instability of the excited electrons can directly relax to the ground state with radiative decay to emit photons, generating fluorescence signals. Thus, SPNs can be used in fluorescence imaging. By tuning of the corresponding polymer structures, the fluorescence range of SPNs can be redshifted to the second near-infrared window (NIR-II, 1000-1700 nm) showing the deeper penetration.^[43] The excited electrons can also directly relax through the nonradiative decay to generate heat. In this situation, SPNs can be used in PA imaging, an imaging modality related to the heat generation which will be comprehensively introduced in **Chapter 3**. Different from these two kinds of direct relaxation to the ground state, the excited electrons may be transferred to the triplet state. Then the energy stored in the excited electrons can be transferred to the surrounding oxygen to generate singlet oxygen ($^1\text{O}_2$). Recently, I report the vinylene bonds of PPV-containing SPs conduct π^2 - π^2 cycloaddition reaction with $^1\text{O}_2$ to form the high-energy intermediate PPV-dioxetane, initiating the generation of afterglow signals.^[44] Thus, SPNs can be also used in afterglow imaging, showing a 67-fold

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higher signal-to-background ratio (SBR) over fluorescent imaging. It is worth to be noticed that these three processes are possible to happen in one SPs. As such, SPNs can be used in more than one imaging modality at the same time.

Figure 2-3. The conclusion of the biomedical applications of SPNs including imaging (photoacoustic, fluorescence and afterglow imaging), therapy (photothermal, photodynamic and chemo therapy) and the potential applications in the regulation of cellular events (the regulation of ion-channels to control neuron behaviors, enzymatic activities and gene expressions).

In terms of therapy, because SPNs are capable to generate heat and $^1\text{O}_2$ upon light irradiation as introduced previously, SPNs can be applied in photothermal and photodynamic therapy. Beneficial from the hydrophobic property of SPs, hydrophobic drugs can be encapsulated in SPNs through π - π stacking during the process of preparing nanoparticles.^[45] As such, SPNs can be also used in chemotherapy to deliver drugs and control their release.

SPNs are seldom applied in the field beyond traditional imaging and therapeutic applications. However, some cellular events are regulated through

heat-responsive motifs, such as ion-channels,^[46] enzymes^[47] and promoters,^[48] which provides the opportunity to SPNs with superior photothermal conversion capability. Thus, SPNs are potential to be used in the regulation of cellular events including the regulation of ion-channels, enzymatic activities and gene expressions.

Considering that photothermal applications are the main focus of this thesis and will be introduced in the second section of this chapter. Therefore, here I just simply introduce SPNs used in fluorescence/afterglow imaging, photodynamic therapy and chemotherapy.

2.1.3.1 Fluorescence Imaging

SPNs used as fluorescence imaging agents can be further divided into non-targeting, active-targeting and activatable probes.^[49] Then enhancement of non-targeting SPN signals only depends on the permeability and retention (EPR) effect without selectivity. It is hard to achieve further amplifying signals or the selective imaging for specific species of interest on the molecular level. Signals of active-targeting SPNs are accumulated and evolved with lock-and-key molecular recognition of the overexpressed receptors in disease sites,^[50] which may be affected by the strong background noises. No matter non-targeting or active-targeting SPNs, their signals are always on. While activatable SPNs are in the “off” state at the beginning and send out signals only when encountering targets (**Figure 2-4**).^[51] As compared with the conventional “always on” SPNs, activatable SPNs usually show higher SBRs and lower LODs, permitting real-time detection of targets in living organisms.^[52] The activatable SPNs have been used in gaseous molecule sensing, ions detection pH/temperature sensing and

enzyme/calcium dipicolinate/nitroaromatic detection.^[16b]

Figure 2-4. Illustration of “always on” and activatable probes. “Always on” probes develop signal contrast through accumulation in disease site and they do not change signals upon interaction with the target of interest; in contrast, activatable probes are in “off” state at the beginning and turned “on” in the presence of the target. Reproduced with permission.^[16b] Copyright 2017, WileyVCH Verlag GmbH & Co. KGaA, Weinheim.

2.1.3.2 Afterglow Imaging

Different from fluorescence, afterglow is a luminescence process to generate light after removal of light (**Figure 2-5**), which bypasses the interference from autofluorescence.^[53] However, most of afterglow imaging applications employ inorganic nanoparticles which have the disadvantages of weak brightness, short luminescence time and potential toxicity.^[54] Recently, Pu’s group discovered that poly(phenylenevinylene) (PPV)-based SPNs can emit long-living luminescence even after cessation of light excitation.^[44,55] Such afterglow emission is associated with the production of $^1\text{O}_2$ under photoirradiation, which spontaneously reacts with the vinylene bonds of PPV to form dioxetane units. These defect units are unstable and gradually degrades to emit photons as one

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of the products. Such an excitation-free luminescence process of PPV-based SPNs eliminates the tissue background noise leading to the significantly increased SBR, and is used in the sensitive detection of cancer, lymph nodes and even disease biomarkers in living mice.

Figure 2-5. The schematic illustration of fluorescence and afterglow. Different from fluorescence, afterglow is a luminescence process to generate light after removal of light which eliminates the real-time excitation. Reproduced with permission.^[56] Copyright 2019, WileyVCH Verlag GmbH & Co. KGaA, Weinheim.

2.1.3.3 Photodynamic Therapy

Photodynamic therapy (PDT) is a treatment method to employ the photosensitizer generated radicals (Type I) and/or singlet oxygen ($^1\text{O}_2$) (Type II) to kill the surrounding cells.^[57] Through destroying blood vessels to interrupt the energy supply to cancer cells and activate immune response to kill cancer cells, PDT have been used to inhibit tumor growth.^[58]

PDT requires three components to work together to generate phototoxicity: photosensitizers, light and molecular oxygen. SPNs with strong light harvest capability have been widely used in PDT.^[6] Incorporation of other photosensitizers in SPNs have been used to further improve the therapeutic effect of PDT. However, limited by the shallow penetration of the present utilized light, PDT cannot be used to treat deep buried tumor and large tumors until now.^[59] Additionally, the hypoxic microenvironment of tumors^[60] is the other challenges for the clinical applications of PDT. Some strategies to overcome the

low oxygen concentration in tumors have been reported.^[61]

2.1.3.4 Chemotherapy

Chemotherapy is a therapeutic modality to use drugs to treat diseases which is widely used in the treatment of cancer. However, chemotherapy without the target capability take effect on both normal tissues and disease sites resulting in severe side effects.^[62] Taking the advantages of EPR effect from nanomaterials, SPNs with hydrophobicity have been used as the drug carrier to enhance the target delivery to tumors. Surface modification of SPNs with target groups which can be recognized by cancer cells can further increase the efficacy of target delivery.^[63] In some cases, SPNs are designed to respond to the microenvironment of cancers which facilitates the specific release at tumor sites to further reduce the side effects.^[5a]

Recently, chemotherapy is usually integrated with photo-therapy to treat solid tumors in preclinical studies,^[45] which not only overcomes the issue of drug resistance, but also achieves the benefits from synergistic therapeutic effect. In addition to the better therapeutic effect, the chemo-phototherapy minimized the side effect to normal tissues compared with mono-therapeutic method. Chemo-phototherapy system based on SPNs are rarely reported, most of which release drugs responsive to the lower pH in tumor microenvironments or laser irradiation.^[64]

2.2 Photothermal Biomedical Applications

Photothermal effect is a phenomenon to produce thermal energy (heat) after electromagnetic radiation which usually needs the help of photosensitizers, such

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as SPNs. After illuminated by light with sufficient energy, electron-hole pairs are generated and then, partial electron-hole pairs undergo nonradiative recombination resulting in the increase of local temperature (“photothermal effect in semiconductors”).^[42] Only part of the energy from the incident light can be converted to heat and the ratio of light energy to be transformed to heat is defines as photothermal conversion efficacy, which indicates the photosensitizer capability to generate heat upon irradiation.^[25] The photothermal conversion efficiencies were measured and calculated as follows:

Photosensitizers in solutions are first irradiated by light to reach the maximum. Then the light source is removed, and the solutions are cool down at room temperature for the same time span. From an energy balance in a system, we can describe the total energy balance as:

$$\sum_i m_i C_{p,i} \frac{dT}{dt} = Q_{in,np} + Q_{in,surr} - Q_{out} \quad (1)$$

Where m and C_p are the mass and heat capacity of the solvent (water) and T is the solution temperature. $Q_{in,np}$ is the photothermal energy input from our nanoparticles which can be described as:

$$Q_{in,np} = I(1 - 10^{(-A_\lambda)})\eta \quad (2)$$

Where I is the laser power used for the photothermal experiment. A_λ is the absorbance at the used laser wavelength, and η is the photothermal conversion efficiency.

$Q_{in,surr}$ is Q_{Dis} and stands for the heat input due to light absorption by the solvent and container, which can be measured as:

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$$Q_{in,surr} = Q_{Dis} = hS_{buff} \times (T_{Max} - T_{Surr})_{buffer} \quad (3)$$

Where hS_{buff} is the parameter which is relevant with container and solvent (h and S stand for heat transfer coefficient and surface area of the container, respectively). T_{max} is the maximum steady-state temperature of solvent. T_{surr} is the ambient surrounding temperature. Q_{out} is the heat lost to the surrounding:

$$Q_{out} = hS \times (T - T_{surr}) \quad (4)$$

The quantity hS can be determined by measuring the rate of temperature decrease after removing the light source. In the absence of any laser excitation, combining with eq. (4), eq. (1) becomes

$$\sum_i m_i C_{p,i} \frac{dT}{dt} = -Q_{out} = -hS \times (T - T_{surr}) \quad (5)$$

After rearrangement and integration, the following expression for t is obtained:

$$t = -\left(\frac{m_{buff} C_{p,buff}}{hS}\right) \ln\left(\frac{T - T_{surr}}{T_{max} - T_{surr}}\right) \quad (6)$$

Two characteristic rate constants can then be defined, $\tau_s = \frac{m_{buff} \times C_{p,buff}}{hS}$,

$$\theta = \frac{T - T_{surr}}{T_{max} - T_{surr}}, \text{ such that:}$$

$$t = -\tau_s \ln(\theta) \quad (7)$$

According to the cooling curve, τ_s and heat transfer coefficients (hS) can be determined. The parameter hS_{buff} can be calculated in the same way, when container just contains buffer. At the maximum steady state temperature, eq (1)

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equals to 0 and we can get

$$Q_{in, np} + Q_{in, surr} = I(1 - 10^{(-A_\lambda)})\eta + Q_{dis} = Q_{out} = hS(T_{max} - T_{surr}) \quad (8)$$

Where T_{max} is the maximum steady-state temperature. Therefore, the photothermal conversion efficiency can be calculated as

$$\eta = \frac{hS(T_{max} - T_{surr}) - Q_{dis}}{I(1 - 10^{(-A_\lambda)})} \quad (9)$$

In terms of therapy, the photothermal effect is widely used in biomedical applications, such as blood vessel lesions, laser resurfacing, laser hair removal and PTT to treat cancers. Besides, PA imaging as a recently appeared imaging modality is closely related to the photothermal conversion capability.^[17] As such, PA imaging is also concluded as the photothermal applications of SPNs. In the following part, I comprehensively introduce the SPN applications in PA imaging and PTT.

2.2.1 Photoacoustic Imaging Modality

PA imaging is a new non-ionizing imaging technology that integrates optical excitation with ultrasonic detection based on the PA effect. It provides deeper tissue imaging penetration with higher spatial resolution as compared with traditional optical imaging techniques (e.g. fluorescence).^[65] Given its many key merits, PA imaging as one of the fastest-growing molecular imaging technologies has been proved effective in tumor detection and molecular characterization.^[66] In addition, consistent with the principle for PTT that requires maximized energy absorption and efficient heat conversion, PA imaging is ideal and versatile to pair with PTT to develop theranostic platforms.^[67] However, the

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endogenous contrast is limited to hemoglobin, lipids and melanin.^[66b] Therefore, the full utilization of PA imaging in biological and medical fields heavily rely on the development of efficient PA agents.

2.2.1.1. Reactive Oxygen Species

Figure 2-6. SPN-based activatable probe for PA imaging of ROS. (a) The chemical structure of P17 used for preparation of the SPN-P17 probe for PA imaging of ROS. (b) Illustration of the mechanism of detection of ROS. (c) PA spectra of the SPN-P17 probe in the absence and presence of ROS. (d) PA/ultrasound overlaid images of saline-treated (i) and zymosan-treated (ii) regions in the thigh of living mice. The SPN-P17 probe was intramuscularly injected into the thigh 20 min after zymosan treatment. (e) The ratiometric PA signals (PA₇₀₀/PA₈₂₀) as a function of time post-injection of the SPN-P17 probe. *Statistically significant difference in PA₇₀₀/PA₈₂₀ between zymosan-treated and saline-treated mice at all time points starting from 10 min ($P < 0.05$). Reproduced with permission.^[18] Copyright 2014, Macmillan Publishers Limited, part of Springer Nature.

A NIR light absorbing SP, poly(cyclopentadithiophene-alt-benzothiadiazole), was doped with an organic NIR dye IR775S (**Figure 2-6a**) to form the SPN-based probe for *in vivo* PA imaging of ROS.^[18] The probe emitted strong PA signals with the maxima at 700, 735 and 820 nm. In the presence of ROS, the peaks at 735 and 820 nm corresponding to IR775S decreased because of the

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destruction of IR775S, while the peak at 700 nm remained unchanged (**Figures 2-6b&2-6c**). Thus, the ratiometric PA signals (PA700/PA820) were generated, showing specificity towards certain ROS including ONOO^- and ClO^- . The capability of the SPN probe for in vivo imaging of ROS was validated in a murine model with acute oedema induced by zymosan. After treatment of zymosan, the PA signals of the probe significantly decreased at 820 nm without obvious change at 700 nm (**Figure 2-6d**), and thus PA700/PA820 gradually increased. (**Figure 2-6e**). These data indicated that the SPN-P17 was activated by ROS produced in inflammatory region.

A semiconducting macromolecular PA probe to detect ROS was also developed which relied on the aggregation induced PA signal amplification. The presence of ROS cleaved the hydrophilic components on the surface of nanoparticles. The remaining hydrophobic cores were unstable and aggregated to nanoparticles with the significantly improved size, resulting in the increase of PA signals.^[68]

2.2.1.2. pH

Pu's group proposed a facile nanoengineering approach to develop activatable PA probes based on semiconducting oligomer nanoparticles (SONs) for pH imaging.^[19] Owing to the low molecular weight of semiconducting oligomer, the resulted SONs were ultrasmall with the diameter of ~10 nm. The sensing dye, pH-BDP (**Figure 2-7a**), was not only responsible to pH, but also well paired with semiconducting oligomer to favor efficient photoinduced electron transfer (PET) and in turn promoted nonradiative thermal deactivation. This led to amplified PA signals for the probe (**Figure 2-7b**). With increasing acidity, the

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PA signals at 750 nm decreased but that at 680 nm remained almost unchanged (**Figure 2-7c**). Thus, the ratiometric PA signals (PA680/PA750) could quantify pH, showing a good linearity between 5.5 to 7.4. Because of the good biodistribution, the SON-based probe permitted *in vivo* ratiometric PA imaging of pH in the tumor of living mice after systemic administration (**Figure 2-7d**). As compared with normal tissues, the significant stronger signals in tumor proved the effectiveness of the SON probe in sensing of subtle pH variations in physiological environment (**Figure 2-7e**).

Figure 2-7. PA imaging used in pH detection. (a) The chemical structures of the semiconducting oligomer (O1) and the organic dye (pH-BDP) used for preparation of the SON-O1 probe for PA imaging of pH. (b) Illustration of the mechanism for detection of pH. (c) PA images of the SON-O1 solution at pH 7.4, 6.4 and 5.5. A pulsed laser was turned to 680 and 750 nm for ratiometric PA imaging. (d) PA and ratiometric images (PA680/PA750) of a subcutaneous HeLa tumor in a nude mouse before and 6 h after systemic administration of the SON-O1 probe. The yellow arrows indicated one of the blood vessels of the tumor. (e) Ratiometric PA signals (PA680/PA750) of the muscle and the tumor with local administration of the SON-O1 probe. *Statistically significant difference in PA680/PA750 between the muscle and the tumor ($P < 0.001$, $n = 3$). Reproduced with permission.^[19] Copyright 2016, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.2.2 Photothermal Therapy

The traditional cancer therapy including surgery, chemotherapy and radiation therapy, but these methods are all difficult to achieve complete removal of tumors without damage. In addition, cancer cells prefer to spread to the nearby tissues limiting the therapeutic efficacy.^[69] Therefore, some noninvasive therapeutic modalities which are promising to improve the accuracy, decrease the harm and inhibit the metastasis are desired.

PTT is a noninvasive therapeutic strategy which convert optical energy (usually NIR light) to heat for thermal ablation of tumors. Upon irradiation, the cancer cells in primary tumors can be eradicated by PTT and even the metastasis can be inhibited through eliminating the local metastasis in the near lymph node.^[70] Compared with the traditional therapeutic methods, PTT shows higher specificity, minimized invasiveness and better spatial-temporal selectivity.^[71] Now metallic nanoparticles,^[72] nanocarbons^[73] and organic nanostructures^[74] have all been used in PTT and their therapeutic efficacies are heavily dependent on the heat conversion efficacy. To further improve the therapeutic outcome, PTT has also been combined with other traditional therapeutic modalities, such as radiotherapy and chemotherapy, to achieve synergistic efficacy.^[75]

As reported, SPNs show photothermal conversion efficacy ranging from 33.3-65.0%,^[6] equivalent or even higher than the common inorganic nanomaterials. Moreover, SPNs possess turnabilities by simple changing their molecular structures to achieve the enhanced capability to convert optical energy to heat^[17] and the changed absorption range.^[76] Redshift of the absorption range of SPNs

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to NIR-II is also an efficient way to improve the therapeutic results, especially for the deep-seated tumors. PTT with NIR-II light takes the inherent advantage of its deeper penetration compared with the first near-infrared window (NIR-I) light.^[77] At the same time, NIR-II light with longer wavelength carries less energy which reduces the harm to skin and normal tissues. As such, it is allowed to increase the laser power to perform PTT in NIR-II which shows higher maximum permissible exposure (MPE) power.^[78] To compare the therapeutic efficacy of NIR-I and NIR-II PTT treatment, Pu's group has synthesized a SP with absorption in both NIR-I and NIR-II.^[78b] Chicken breast tissues with depth of 5 mm are covered on the solid tumors to simulate the deep buried tumors. Although the SPNs used in this work show similar photothermal conversion efficacies at 808 (44.9%) and 1064 nm (43.4%), NIR-II PTT achieve completely ablate the tumors with more heat generation and deeper penetration; while NIR-I PTT in the same condition cannot inhibit the tumor growth. Other SPNs to perform NIR-II PTT are also reported and their photothermal conversion efficacies are further improved.^[79]

In conclusion, as a prominent noninvasive therapeutic method, PTT usually uses longer wavelength light and is able to kill cancer cells with reduced harm to normal tissues. To further improve the therapeutic results, PTT can combine with other traditional therapeutic methods to achieve synergy effect, improve photothermal conversion efficacy of photosensitizers to generate more heat or redshift the absorption range to NIR-II to achieve deeper penetration.

2.2.3 Challenges

PA imaging shows deeper tissue penetration over optical imaging, but its SBR

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is still required to be improved because the existence of endogenous absorbers to generate background noises which may sacrifice the diagnosis accuracy. To achieve this, the improvement of the PA brightness is effective, and it can also enhance the PTT efficacy of the corresponding agent because of the intrinsic association between PA imaging and PTT. However, the underlying mechanism to determine the nonradiative ratio in the total excited electrons is unclear. It was reported that, for SPs with electron donor and acceptor backbones and employing diketopyrrolopyrrole (DPP) as the electron acceptor, the change of the electron donor with the increasing donating capability improved the PA properties of the resulted SP.^[17] Except for this, seldom researches focused on the relationship between the SP structure and its PA property. As such, the improvement of PA signals of SPNs is still challenging.

Probes without specificity develop contrast signals through accumulation and they do not change signals upon interaction with the target of interest. Compared with this, probes with specificity have higher signal-to-noise ratio and lower detection of limit, demonstrating the advantage of molecular imaging to real-time detect cellular events in living organisms. However, PA probes with specificity are limited. It may because PA imaging is a recently developed imaging modality. Meanwhile, unlike fluorescence probes, it is hard to find an effective motif, such as a “quencher” or a “energy transfer partner”, to affect the PA property of a specific probe by simply changing their distance responsive to the target of interest. Thus, a general method to endow SPN-based PA probes with specificity is required.

The accumulation of agents used in PA imaging and PTT in living bodies may raise the concern on the long-term toxicity. To increase the potential to be

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applied in clinics, biodegradable agents are highly demanded. Although biodegradable polymers have been reported,^[80] simply introducing in the degradable components to the backbone of SPs may sacrifices their optical properties. As such, a general method to endow SPNs with biodegradability but without the compromise of their optical properties is challenging.

Chapter 3 An Intraparticle Molecular Orbital Engineering Approach to Improve Photothermal Related Properties¹

3.1 Introduction

One of the emerging research areas at the convergence of physical science, engineering and life science is the development of theranostic nanoagents that integrate therapy and diagnosis for cost-effective precision medicine.^[81] By virtue of the real-time diagnostic capability, theranostic systems can target accurately at refined therapeutic window, administering enhanced efficacy with minimized side effect.^[82] As compared to magnetic,^[83] ultrasound^[84] and radioactive nanoparticles,^[85] optical imaging agents have been more intensively integrated into theranostics,^[86] probably because of the convenience that light is the solely-needed source not only providing the energy to afford diagnostic signals but also triggering the therapeutic processes such as PTT and PDT. Current optical theranostic systems generally rely on fluorescence as the signal readout and thereby share the drawbacks of autofluorescence background and limited light penetration depth, which unfortunately can compromise the diagnostic accuracy to some extent.^[65]

PA imaging as a new imaging modality exceeds the optical diffusion limit by detection of phonon instead of photon after light excitation, and thus provides deeper tissue penetration and higher spatial resolution for theranostics.^[87] In

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addition, because PA signal is mainly determined by photothermal conversion, the principles of selecting agents for PA imaging are naturally consistent with those for PTT, which makes PA and PTT an ideal pair to be seamlessly and synergistically combined into theranostics.^[67,88]

I employ SPNs as the nanoplatform which are resistant to ROS and much more photostable than gold nanorods and photoacoustically brighter than carbon nanotubes upon pulsed laser irradiation. Through investigation of the structure-property relationship of different diketopyrrolopyrrole-based SPs,^[89] it has been confirmed that the PA brightness of SPNs is proportional to their photothermal conversion efficiency and thus envisioned their utility in PTT. In fact, polypyrrole (PPy)^[90] and poly-(3,4- ethylenedioxythiophene) poly(4-styrenesulfonate)^[91] based SPNs have been reported for PTT, but their PTT efficacies were constrained by their weak absorption in the NIR region as their absorption maxima are located in the visible region. Moreover, PA/PTT theranostic systems based on SPNs have been less studied.^[89,92] Although synthesis of new SPs is certainly a feasible way to improve both PA imaging and PTT of SPNs, it inevitably requires a huge input of time and effort. Thereby, simple yet effective approaches toward efficient PA/PTT theranostic systems based on SPNs remain elusive.

I herein report an intraparticle molecular orbital engineering approach to enhance both PA brightness and PTT efficiency of SPNs for amplified theranostics in living mice. The designed SPNs have a binary optical component nanostructure, wherein a near-infrared absorbing SP and an ultrasml carbon dot (fullerene) interact with each other. The molecular orbitals of the optical components are carefully aligned to make the electron affinity and ionization

potential of the SP, respectively, higher than those of the dopant. Such an energy level alignment favors PET within the confined nanocompartment and, thus, results in quenched fluorescence and enhanced nonradiative heat generation after light irradiation, ultimately bringing in both amplified PA brightness and improved PTT efficiency. The synthesis and characterization of theranostic SPNs are first described, followed by *in vitro* validation of our intraparticle molecular orbital engineering concept through systematic investigation of the effect of dopant on the optical, PA, and PTT properties of SPNs. At last, the proof-of-concept application of theranostic SPNs in imaging and treatment of tumor in living mice is demonstrated.

3.2 Materials and Methods

Chemicals. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Materials Characterization. TEM images were obtained on a JEM 1400 transmission electron microscope with an accelerating voltage from 40 to 120 kV. Dynamic light scattering (DLS) was performed on the Malvern Nano-ZS Particle Sizer. UV-Vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were carried out on a Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). Fluorescence images of SPNs solutions were acquired with the IVIS spectrum imaging system. IR thermal image was recorded by the photothermal camera FLIR T420. Fluorescence confocal image was conducted using confocal laser scanning microscopy (Leica TSC SP8, Germany). An 808 nm high power NIR Laser (operating mode: CW, output power after fiber: 2.5 W, LED display: diode current,

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multimode fiber, fiber core diameter: 400 μm , fiber connector: SMA905, with tunable laser driver module: 0 - 100%) purchased from CNI Co., Ltd. was used for PTT experiments. The laser spot size is 1 cm^2 . It was used as the surface laser and position on top of the mice. The laser power was measured by laser power meter.

Preparation of SPNs. PCPDTBT (1 mg) and PC70BM (1 mg) were dissolved in THF (1 mL) individually by bath sonication to get the solution of 1 mg/mL. The resulted solutions were filtered through a polyvinylidene fluoride (PVDF) syringe driven filter (0.22 μm) (Millipore). Then, a mixed THF solution (1 mL) containing PCPDTBT (0.25, 0.24, 0.225, 0.20, 0.15 mg/mL), corresponding to PC70BM (0, 0.01, 0.025, 0.05, 0.10 mg/mL according to the doping amount) and PEG-*b*-PPG-*b*-PEG (20 mg/mL) were used to prepare SPNs by rapidly injecting the mixture into distilled-deionized water (9 mL) under continuous sonication with a microtip-equipped probe sonicator (Branson, W-150) at a power output of 6 watts RMS for 2 min. After sonication for additional 1 min, THF was evaporated at 65°C under nitrogen atmosphere. The aqueous solution was filtered through a polyethersulfone (PES) syringe driven filter (0.22 μm) (Millipore), and washed three times using distilled-deionized water and 50K centrifugal filter units (Millipore) under centrifugation at 3,500 rpm for 15 min at 4°C. The SPNs solutions were diluted in 1× PBS buffer (pH 7.4) and the concentrations were determined by UV-Vis absorption according to absorption coefficients of PCPDTBT. The SPNs solutions were finally concentrated to 0.08 mg/mL (based on the mass of PCPDTBT) by ultrafiltration and stored in dark at room temperature. The actual doping amount of PC70BM has been calculated according to the extinction coefficients of PC70BM at 404 nm and the SP

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(PCPDTBT) at 650 nm. The actual doping amount of SPN-F0, SPN-F4, SPN-F10, SPN-F20, SPN-F40 are 0 w/w%, 9.46 w/w%, 18.73 w/w%, 33.27 w/w%, and 42.95 w/w%, respectively. The actual doping amount for each SPNs is very close to the feeding amount.

PA Instrumentation. A commercial Endra Nexus128 PA tomography system (Endra Inc., Ann Arbor, Michigan) was also used in this study. The system houses a tunable nanosecond pulsed laser (7 ns pulses, 20 Hz pulse repetition frequency, 9 mJ/pulse on the animal surface, wavelength range (680–950 nm), 128 unfocused ultrasound transducers (with 5 MHz center frequency and 3 mm diameter) arranged in a hemispherical bowl filled with water, animal tray on top of the bowl, data acquisition/reconstruction console, servo motors for 3D rotation of the bowl, and a temperature monitor of the water bath.

***In vitro* PTT of SPNs.** The 1×PBS solution (pH 7.4) of SPNs (200 L, 15 g/mL) and the same amount of PBS buffer were exposure to laser of 808 nm (0.5 W/cm²). The temperature was recorded every 15 seconds and stopped until the temperature without big changes (5 minutes here).

Cell Culture. HeLa cervical adenocarcinoma epithelial cells were purchased from the American Type Culture Collection (ATCC). HeLa cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) (GIBCO) supplemented with 10% FBS (fetal bovine serum) (GIBCO) and antibiotics (10 U/mL penicillin and 10 mg/mL streptomycin). The cells were maintained in an atmosphere of 5% CO₂ and 95% humidified air at 37°C.

Cytotoxicity Test. Cells were seeded in 96 well plates (1000 cells in 100 µL per well) for 24 h, and then SPN-F0 and SPN-F20 (final concentration 5, 10, and 15 µg/mL) was added to the cell culture medium separately. Cells were incubated

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with SPN-F0 or SPN-F20 for 24 h, followed by the addition of MTT (20 μ L, 5 mg/mL) for 3 h. The media was removed and DMSO (200 μ L) was added into each well and gently shaken for 10 min at room temperature to dissolve all formed precipitates. The absorbance of MTT at 550 nm was measured by using a SpectraMax M5 Microplate/Cuvette Reader. Cell viability was expressed by the ratio of the absorbance of the cells incubated with SPN-F0 or SPN-F20 solution to that of the cells incubated with culture medium adding the same volume of saline.

Tumor Mouse Model. All animal studies were performed in compliance with the guidelines set by Tianjin Committee of Use and Care of Laboratory Animals and the overall project protocols were approved by the Animal Ethics Committee of Nankai University. To establish tumors in six-week-old BALB/c mice (obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China)), two million 4T1 cells suspended in 50 mL of 50 v/v % mixture of Matrigel in supplemented DMEM (10% fetal bovine serum, 1% pen/strep, 100 U/mL penicillin and 100 μ g/mL streptomycin) were injected subcutaneously in the shoulders of the mouse. Tumors were grown until a single aspect was $\sim$ 7 mm (approximately 10–15 days) before used for PA imaging and PTT experiments.

In Vivo PA Imaging. After nude mice were anesthetized using 2% isoflurane in oxygen, SPN-F0 (200 μ L, 100 μ g/mL) (n=4), SPN-F20 (200 μ L, 100 μ g/mL) (n=4) or saline (200 μ L) (n=4) were systematically injected through the tail vein using a microsyringe. Endra Nexus128 PA imaging system was used to acquire the PA images at 750 nm after systematically administration of SPN-F0, SPN-F20 or saline. Data was acquired through a continuous model that took 12 s to

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obtain one data set. Three-dimensional PA image was reconstructed off-line using data acquired from all 128 transducers at each view and a back-projection algorithm. The algorithm corrects for pulse-to-pulse variations in the laser intensity and small changes in the temperature that affect acoustic velocity in the water.

In Vivo Epi-fluorescence Imaging. Fluorescence whole-animal imaging was performed on a CRi Maestro whole animal imaging system (Caliper Life Sciences, MA, USA). BALB/c mice bearing 4T1 tumors were injected with SPN-F0 (200 μ L, 100 μ g/mL) (n=4) through tail vein using a microsyringe. Fluorescence imagings of mice were acquired at designated time points after nanoparticle administration. Image quantitation was performed using Nuance software.

In Vivo PTT of SPNs. BALB/c mice bearing 4T1 tumors were exposure to 808 nm laser of 0.3 W/cm², after systemic administration of saline (200 μ L) (n=4), SPN-F0 (200 μ L, 100 μ g/mL) (n=4) or SPN-F20 (200 μ L, 100 μ g/mL) (n=4) at post-injection time of 6 h. Using IR thermal camera to record the temperature change of the tumor in every 15 seconds for 5 minutes after irradiation. The tumor size was measured by caliper every the other day and calculated as the volume= (tumor length) $\times$ (tumor width)²/2. Relative tumor size was calculated as V/V_0 (V_0 was the initial tumor volume). Mice with tumors larger than 1000 mm³ should be euthanized according to the standard animal protocol.

Biodistribution Method. After 24 h post-injected with SPN-F0 (200 μ L, 100 μ g/mL) (n=4) or SPN-F20 (200 μ L, 100 μ g/mL) (n=4), the mice were sacrificed by CO₂ asphyxiation. Major organs, including liver, kidney, pancreas, heart, brain, skin and spleen were harvested and weighted at 100 mg. The organ parts

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were embedded in the agar gel and the PA signals were detected under pulsed laser (7 ns pulses, 20 Hz pulse repetition frequency, 9 mJ/pulse) of 750 nm using Endra Nexus128 PA imaging system.

Histological Studies. After 16 days of PTT, the mice treated with SPN-F0 (200 μ L, 100 μ g/mL) (n=4), SPN-F20 (200 μ L, 100 μ g/mL) (n=4) and saline (200 μ L) (n=4) were sacrificed. The liver, spleen and tumor were fixed in 4% formalin and then conducted with paraffin embedded section for H&E staining. (The superficial and deep regions for tumor tissues are defined as 1 and 4 mm away from the tumor surface, respectively) The slices were examined by a digital microscope (Leica QWin). For proliferating cell nuclear antigen (PCNA) staining, formalin-fixed tumor and organs samples were sliced and used for confocal immunofluorescent analysis using PC10 Mouse mAb (#2586, Cell Signaling Technology) following the supplier's protocol. For the TUNEL apoptosis staining, the fixed tumor sections were determined following manual instruction of In-Situ Cell Death Detection Kit (Roche Applied Science). Hoechst 33342 was used for nuclear counterstaining.

Data Analysis. PA signal intensities were measured by region of interest (ROI) analysis using the Vevo LAZR imaging system software package or OsiriX. PTT graph process was using the software FLIR Tools. Results are expressed as the mean $\pm$ SD deviation unless otherwise stated. All statistical calculations were performed using GraphPad Prism v.5 (GraphPad Software Inc., CA, USA).

3.3 Results and Discussions

The theranostic SPNs were synthesized through nanoprecipitation. Poly[2,6-(4,4-bis-(2-ethylhexyl)-4*H*-cyclopenta[2,1-b;3,4-b']dithiophene)-*alt*-4,7-(2,1,3-

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benzothiadiazole)] (PCPDTBT) and (6,6)-phenyl-C71-butyric acid methyl ester (PC70BM) (**Figure 3-1**) were chosen as the primary component and the secondary dopant, respectively. PCPDTBT is a SP with a relative narrow energy band gap, ensuring the strong absorption of NIR light.^[93] PC70BM is a fullerene with highly electrophilicity and hydrophobicity,^[94] which can be efficiently encapsulated into the nanoparticle to closely contact with PCPDTBT. The HOMO and LUMO are -4.9 and -3.5 eV for PCPDTBT and -6.5 and -4.3 eV for PC70BM, respectively.^[95] Thereby, the energy levels of these optical pairs are ideally aligned to favor intraparticle PET. The amphiphilic triblock polymer (PEG-*b*-PPG-*b*-PEG) is used to improve the hydrophilicity of SPNs as well as to provide a relatively inert particle surface.

Figure 3-1. Schematic illustration of intraparticle molecular orbital engineering design approach for amplified photoacoustic (PA) imaging and photothermal therapy (PTT). **(a)** Chemical structures of PCPDTBT, PC70BM and PEG-*b*-PPG-*b*-PEG. **(b)** Photography of the solutions of SPNs with the different doping amount of PC70BM (w/w%). [SPN] = 16 μg/mL. **(c)** Illustration of PET-induced amplified theranostic SPNs. The highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) are -4.9 and -3.5 eV for PCPDTBT and -6.5 and -4.3 eV for PC70BM.

Figure 3-2. *In vitro* characterization of SPNs. Representative TEM images (a) and DLS (b) of SPN-F20. (c) Hydrodynamic average diameters of SPNs with different doping amount of PC70BM. UV-vis absorption (d), PL (e) and photoacoustic spectra (f) of SPNs. [SPN] = 2 μ g/mL. 1 $\times$ PBS (pH 7.4). Excitation for PL: 650 nm. The arrows indicate the increase (d&f) or decrease (e) of the intensities with the increased doping amount of PC70BM in SPNs.

To optimize the doping amount for optical theranostics, SPNs with the different doping amount of PC70BM were prepared: SPN-F0, SPN-F4, SPN-F10, SPN-F20 and SPN-F40, which have the weight percentages of PC70BM equal to 0 w/w%, 4 w/w%, 10 w/w%, 20 w/w%, and 40 w/w%, respectively. As shown in **Figure 3-1b**, all the nanoparticle solutions are transparent and have the quite similar color of cyan. Dynamic light scattering (DLS) shows that the SPNs possess the similar average diameter $\sim$ 50 nm (**Figure 3-2c**). Thereby, the PC70BM doping has a negligible effect on the size of SPNs, due to the ultrasmall size of PC70BM ($\sim$ 1 nm).^[96] Using SPN-F20 as an example, transmission electron microscopy (TEM) shows a spherical morphology with the diameter of $\sim$ 38 nm (**Figure 3-2a**). The TEM-measured diameter is smaller than that measured by DLS (54.33 ± 0.55 nm) (**Figure 3-2b**), which should be attributed to the shrinkage of nanoparticles

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during the sample preparation of TEM. After being stored in the dark place at room temperature (24°C) for 44 days, the size of SPN-F20 remained nearly the same. In addition, the photoluminescence (PL) spectrum of SPN-F20 shows no obvious change after incubation in serum at 37°C for 24 h. These data prove the excellent stability of fullerene-doped SPNs.

Figure 3-3. *In vitro* fluorescence, PA and PT data of SPNs. Epi-fluorescence (a) and PA (b) images of SPN solutions in tubes; IR thermal images of SPN solutions after laser irradiation for 300 s (c). Quantification of fluorescence (d) and PA (e) intensities of SPNs in solutions (* $p < 0.01$, ** $p > 0.05$); (f) The temperature of SPN solutions as a function of laser irradiating time at the power intensity of 0.5 W/cm². [SPN] = 15 μ g/mL, 1 $\times$ PBS (pH 7.4). A single laser pulse at 680 nm with a laser fluence of 9 mJ/cm² and a pulse repetition rate of 20 Hz was used for photoacoustic imaging. The fluorescence images were acquired by excitation at 665 nm and collection of signals at 840 nm. Error bars were based on standard error of mean (SEM) ($n = 3$).

To study the doping effect on optical properties, I measured and compared the UV-Vis absorption, PL and PA spectra of these SPNs in PBS at pH 7.4. As shown in **Figure 3-2d**, the non-doped nanoparticle of SPN-F0 has two absorption peaks with the maxima at 404 and 650 nm, respectively. With increasing the doping amount of PC70BM, the peak at 404 nm gradually elevates, while the peak at 650 nm remains unchanged. Such an increase at

404 nm is caused by the contribution from PC70BM which has broad absorption spectrum from 200 to 550 nm.^[97] The PL spectrum of SPN-F0 has a maximum emission at 840 nm (**Figure 3-2e**), which is gradually quenched with increasing doping amount of PC70BM. In contrast, the physical mixture of SPN-F0 and PC70BM does not show such a fluorescence quenching and the PL intensity of SPN-F0 remains the same regardless of the concentration of PC70BM. This not only proves the doping of PC70BM into the SPN but also confirms the efficient intraparticle PET process from PCPDTBT to PC70BM. It should be noted that the PL is completely quenched at the doping amount of 20 _{w/w}%. The PA spectra of SPNs were acquired by pulsed laser irradiation ranging from 680 to 950 nm (**Figure 3-2f**). All the SPNs show detectable PA signals ranging from 680 to 825 nm. With increasing the doping amount of PC70BM, the PA intensity gradually increases while the peak at around 750 nm progressively dominates. This subtle spectral change is in line with the slightly red-shifted spectral profile and the increased vibronic shoulder at 750 nm in the absorption spectra of these SPNs. The difference between the absorption spectral profile and PA spectral profile of SPNs should be mainly caused by two facts: (i) optical absorption and PA spectra measures different photophysical processes; and (ii) optical illumination parameters are different, high-power pulsed laser for PA spectra verse low-power continuous-wave light illumination for absorption spectra.

The doping effect on the fluorescence brightness, PA brightness and PT efficiency was investigated and compared for the different SPNs in PBS (pH 7.4) at the same concentration. As shown in **Figures 3-3a-c**, epi-fluorescence, PA, and PT images of the SPNs solutions were recorded by IVIS living imaging system, Endra PA tomography system and infrared (IR) camera, respectively.

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The intensities and the temperatures were quantified and summarized in **Figures 3-3d-f**. With increasing the doping amount of PC70BM, the fluorescence intensity drops rapidly, the PA intensity increases obviously, and the solution temperature increases gradually. It should be emphasized that the fluorescence intensity of SPN-F20 is very close to the background noise, while its PA intensity and the solution temperature are as high as those of SPN-F40. The data thus points out that the optimum doping amount of PC70BM is 20_{w/w}%. As compared to SPN-F0, the PA intensity of SPN-F20 is amplified by 2.6 fold, while its solution temperature after laser irradiation for 5 min is elevated by 1.3-fold from 48 to 62°C. The tendencies of fluorescence brightness, PA brightness and PT efficiency upon increasing the doping amount of PC70BM coincide well with each other and comply with the photophysical mechanisms. As well known, the excitation energy absorbed by a chromophore is generally dissipated through the following pathways: i) fluorescence emission, ii) generation of heat through vibrational relaxation (thermal deactivation), and iii) intersystem conversion to long-lived species (e.g., phosphorescence).^[98] As these SPNs are not detectably phosphorescent, the pathways (i) and (ii) play the roles in determining their fluorescence, PA and PT efficiencies. Upon light excitation, the thermal deactivation competes with light emission in determining heat and acoustic generation. As a consequence, the loss of fluorescence intensity induced by intraparticle PET within SPNs is spontaneously accompanied by the gain of amplified PA brightness and increased solution temperature. These *in vitro* data firmly predict that as compared to SPN-F0, SPN-F20 should be a better optical theranostic system for PA imaging and PTT.

The diagnostic capability of the theranostic SPNs was validated with xenograft

4T1 tumor mouse model. Among these SPNs with the different doping amount of PC70BM, SPN-F20 was tested along with the nondoping control nanoparticle (SPN-F0) as SPN-F20 has the best PA and PT efficiencies. After systemic administration of SPN-F20 or SPN-F0 into the tumor-bearing mice through tail vein, the PA images were recorded overtime by pulsed laser excitation at 750 nm. The PA signals reach its maximum at 6 h post-injection for both SPNs, which is consistent with the *in vivo* epi-fluorescence imaging results. The PA images at t=6 h and the PA intensity as a function of post-injection time are shown in **Figure 3-4a** and **3-4b**, respectively. Generally, the SPN-F20 injected mice have much higher PA intensities in the tumor area as compared to that for SPN-F0 at all the time points. At t=6 h, the PA intensity reaches its maximum for both SPNs, where the intensity for SPN-F20 injected mice is 1.8-fold of that for SPN-F0 treated mice. This intensity difference can be obviously visualized in the PA images (**Figure 3-4a**). The amplified PA brightness of SPN-F20 allows us to carry out a 3D signal reconstruction, which clearly delineates that the PA signals are detectable both inside and outside of the blood vessels of tumor. The almost homogeneously-distributed PA signal proves the extravasation ability of SPN-F20, which is frequently observed for SPNs as reported in our previous studies.^[75] The *ex vivo* PA data in **Figure 3-4c** illustrate that SPN-F20 and SPN-F0 have quite similar dynamic biodistribution in major organs such as tumor, liver, spleen and skin *et al.*. Such a similarity for different SPNs stems from their nearly identical particle sizes (~50 nm) and the same PEG-coated surface structure, suggesting the probability of using SPNs for multiplex *in vivo* imaging provided that their PA maxima are turned to different laser wavelengths.

Figure 3-4. *In vivo* photoacoustic imaging. (a) PA images of tumor after systemic administration of SPN-F0, SPN-F20 (200 μ L, 100 μ g/mL) or saline, for 6 h. The representative PA maximum intensity projection (MIP) images with axial view for SPN-F0 and SPN-F20 and the 3D image for SPN-F20. (b) Quantification of PA intensities at 750 nm as a function of post-injection time of SPN-F0, SPN-F20 or saline. (c) *Ex vivo* PA quantification of major organs of mice 24 h after systemic administration of SPNs or saline. The PA data were acquired at 750 nm. The weigh for the part of each organ used for PA measurement is 100 mg. Error bars were based on standard error of mean (SEM). (n = 4).

The therapeutic capability of the theranostic SPNs was testified on xenograft 4T1 tumor mouse model using SPN-F20 and SPN-F0 as examples. According to the PA imaging results, PTT was conducted at 6 h post-injection of SPNs as this time point shows the highest PA signals (**Figure 3-4b**). After systemic administration of SPN-F20, SPN-F0 or saline for 6 h, the 4T1-tumor bearing mice were irradiated under the laser of 808 nm with the power of 0.3 W/cm² for 5 min. The thermal images of mice and the surface temperature of tumor as a function

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of laser irradiation time are shown in **Figure 3-5a** and **3-5b**, respectively. It is clear that the tumor temperature of SPN-F20 injected mice is higher than that for both SPN-F0 and saline injected mice at all the time points. The tumor temperature reaches plateau after laser irradiation for 2 min. At this time point, the tumor temperature of SPN-F20 injected mice is 62°C, which is 1.3- and 1.7-fold of that for SPN-F0 (48°C) and saline (37°C) injected mice, respectively. Such a difference in the tumor temperature under laser irradiation is consistent well with the *in vitro* data (**Figure 3-3f**), which also shows 1.3-fold temperature enhancement when changing from SPN-F0 to SPN-F20 (**Figure 3-3f**).

To qualitatively evaluate the ablating effect of theranostic SPNs, the tumor sizes were continuously monitored for 16 days after PTT (**Figure 3-5c**). Without laser irradiation, the tumors of SPNs injected mice grew as quickly as that of saline injected mice, indicating that both SPNs themselves do not have the ability to inhibit tumor growth. In contrast, after laser irradiation, both SPNs effectively inhibited the tumor growth for the first 8 days. However, only SPN-F20 successfully inhibited the tumor growth, while SPN-F0 failed to do so at later time points. The better PTT outcome for SPN-F20 can be contributed to the fact that the tumor temperature of SPN-F20 injected mice increased to 62°C, while SPN-F0 injected mice was only able to reach 48°C, which was not high enough to effectively ablate the cancer cells *in vivo* under our experiment conditions. Moreover, the body weights of the mice for all groups were continuously monitored for 16 days after PTT, and no significant weight loss was observed (**Figure 3-5d**), suggesting the low toxicity of all treatments. This is consistent with the *in vitro* data showing the high cytocompatibility of SPNs.

Figure 3-5. *In vivo* photothermal therapy. (a) IR thermal images of 4T1 tumor-bearing mice under 808 nm laser irradiation (0.3 W/cm²) after systemic administration of saline, SPN-F0 or SPN-F20 (200 µL, 100 µg/mL) at post-injection time of 6 h. (b) Mean tumor temperature as a function of laser irradiation time after systemic administration of saline, SPN-F0 or SPN-F20 at post-injection time of 6 h. Error bars were based on standard error of mean (SEM). (n = 4). (c) Tumor growth curves of different groups of mice after systemic treatment with saline, SPN-F0 or SPN-F20 with and without laser irradiation. Error bars were based on standard error of mean (SEM). (* indicate $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p > 0.05$, n = 4). (d) Body weight data of mice after systemic treatment with saline, SPN-F0 or SPN-F20 with and without laser irradiation. Error bars were based on standard error of mean (SEM) (n = 4).

To further verify the therapeutic outcome of theranostic SPNs, all the groups were sacrificed at day 16 and histological analysis was performed for different organs using hematoxylin and eosin (H&E) staining as well as fluorescent proliferating cell nuclear antigen (PCNA) and TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining. As shown in **Figure 3-6 a**, no noticeable histopathological abnormalities or lesions can be found for the liver and spleen of

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both SPN-F20 and SPN-F0 injected mice after PTT. In contrast, typical nucleus dissociation is observed for the tumor tissues for SPNs-injected mice after PTT. Besides, both superficial and deep tumor tissues of SPN-F20 injected mice show more obvious necrosis as compared to those of SPN-F0 injected mice. The analysis of large H&E images shows that the percentage of necrosis area of SPN-F20 in the whole image is 77.2%, which is significantly higher than that for SPN-F0 (42.9%) and saline (2.1%).

Figure 3-6. *Ex vivo* studies. (a) Histological H&E staining for liver, spleen and tumors (superficial & deep regions) at Day 16 after the photothermal treatment with saline, SPN-F0 or SPN-F20. (b) Fluorescence PCNA and TUNEL staining of tumor slices (superficial & deep regions) at day 16 after the photothermal treatment with saline, SPN-F0 or SPN-F20. Green fluorescence indicates the signal from PCNA and TUNEL staining while the blue fluorescence is from the nucleus staining.

The PCNA fluorescence staining in **Figure 3-6b** indicates that the reproduction capacity is obviously reduced for SPNs-injected mice after PTT; however, some green fluorescent spots can still be found in the deep tumor tissue of SPN-F0 injected mice. Similarly, the TUNEL fluorescence staining shows that apoptosis is clearly detectable for SPNs-injected mice after PTT,

while SPN-F20 causes severer apoptosis than SPN-F0 does as witnessed by the stronger green fluorescence from the TUNEL staining for both superficial and deep tumor tissues of SPN-F20 injected mice. It should be noted that no obvious apoptosis and necrosis as well as reduced reproduction capacity are observed for all the control groups (without laser irradiation or saline injected mice with laser irradiation). These histological data verify at cellular level that SPN-F20 has a better PTT efficacy than SPN-F0 does, which is in perfect accordance with the *in vivo* observation from the tumor growth curves in **Figure 3-5c**.

3.4 Conclusions

I have demonstrated an intraparticle molecular orbital engineering approach to develop organic optical theranostics with amplified PA brightness and PTT efficacy. Our design capitalizes on the structural flexibility and synthetic simplicity of SPNs to create intraparticle PET by incorporation of an electron-withdrawing carbon dot (fullerene: PC70BM) into SPNs. Such an intraparticle electronic interaction between SP and PC70BM results in enhanced heat generation upon light irradiation, subsequently amplifying both PA brightness and PTT efficacy for the theranostic SPNs. As compared to the non-doped SPN (SPN-F0), the *in vitro* PA brightness of the doped SPN (SPN-F20) is amplified by 2.6-fold, while its maximum photothermal temperature increases by 1.3-fold from 48 to 62°C both *in vitro* and *in vivo*. Due to the ultrasmall size of PC70BM, doping has no obvious impact on the particle size, and thus the theranostic SPNs have similar bio-distribution. As confirmed by both *in vivo* and *ex vivo* histological data, utilization of SPN-F20 as the optical theranostics permits enhanced PA

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imaging and improved photothermal ablation of tumor in living mice. By virtue of their completely organic ingredients, the theranositc SPNs show no sign of toxicity both *in vitro* and *in vivo* and thus hold great promise for other therapeutic applications.

As the energy levels of optically active components within nanoparticles can be easily paired and aligned to favor PET, the design concept of amplified optical theranositcs should be applicable to other systems. For instance, integration of graphene and SP should have the similar synergistic outcome due to the widely observed PET-induced fluorescence quenching for them.⁶² Thus, our study not only brings in a new generation of organic optical theranostics based on SPNs but also provides new molecular design guidelines to seamlessly and synergistically merge light-intensive imaging with therapeutic applications.

Chapter 4 A Reaction-Based Photoacoustic Probe for Sulfenic Acids²

4.1 Introduction

Development of PA imaging agents has attracted significant attention because PA imaging offers higher penetration depth as compared with fluorescence imaging.^[99] Many PA agents including small molecular dyes,^[100] gold nanorods,^[101] carbon nanotubes,^[102] two-dimensional materials^[103] and porphyrins^[104] have been developed. However, their signal-to-noise ratios are usually achieved *via* EPR effect or lock-and-key molecular recognition between the recognition groups (such as small-molecule ligand, peptide and antibody) and the overexpressed receptors in disease site.^[105] In contrast, the reaction-based PA probes, which undergo the specific chemical reaction with the target of interest *in situ* and subsequently enhance signal intensity in disease site, are seldom developed.^[106] However, the reaction-based probes have been proved effective for optical imaging, because the reaction-based probes are able to identify the specific variant with the subtle difference from its analogues.^[49,107] Such capability provides spatial and temporal information on the sub-molecular level to track the dynamic biological and pathological events inside living organisms. Thus, the development of reaction-based PA probes is highly demanded to further facilitate the application of PA imaging in biology and medicine.

In this study, I report a reaction-based SPN probe (*r*SPN) for *in vivo* PA

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imaging of protein sulfenic acids. Protein sulfenic acids, as the reversible oxide of cysteine in proteins, are recognized as a center post-translational modification when proteins encounter with oxidative stress.^[108] The vital role of protein sulfenic acids in oxidative signal transduction places them at the forefront of redox biology,^[109] relating to many pathological conditions such as cancer,^[110] cardiac dysfunction,^[111] ischemia/reperfusion injury and neurodegenerative diseases.^[112] However, current chemical probes for the detection of protein sulfenic acids rely on visible fluorescence signals, limiting their utility to *in vitro* assays.^[108,113] In contrast, the reaction-based probes (*r*SPNs) designed here have NIR fluorescence and PA signals, allowing for real-time imaging of protein sulfenic acids in both living cells and small animals. This nanoprobe comprises 1,3-cyclohexanedione groups on the particle surface that enable specific reaction and covalent binding to the sulfenic acid sites of oxidative proteins.^[114] To minimize the perturbation of such reaction on the fluorescence and PA properties, the optically active SP core is shielded with silica and poly(ethylene glycol) (PEG) corona. As follows, I first describe the synthesis and characterization of *r*SPNs, followed by *in vitro* validation of the specific reaction of *r*SPNs with protein sulfenic acids. I next discuss the utility of *r*SPNs as the fluorescence probes for imaging of protein sulfenic acids in cancer cells under oxidative stress. At last, I demonstrate the proof-of-concept application of *r*SPNs for real-time PA imaging of protein sulfenic acids in the tumor of living mice.

4.2 Materials and Methods

Chemicals. DYn-2 was purchased from Cayman Chemical Company. Silane-PEG molecules were purchased from Nanocs. All chemicals were purchased

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from Sigma-Aldrich unless otherwise stated.

Materials Characterization. Fluorescence images were conducted using fluorescence microscopy (Leica, Germany). The other characterization methods are similar with those described in **Chapter 3.2**.

Preparation of SPNs. SP1 (1 mg) or SP2 (1 mg) was dissolved in THF (1 mL) by bath sonication and filtered through a polyvinylidene fluoride (PVDF) syringe driven filter (0.22 μ m) (Millipore) to get the solution (1 mg/mL). Then, a mixed THF solution containing SP1 or SP2 (500 μ L, 1 mg/mL) and PEG-*b*-PPG-*b*-PEG (1 mL, 50 mg/mL) were stirred at room temperature for 3 h to reach homogeneous. After evaporation of the THF solvent with the nitrogen flow, the solid thin film was redispersed in hydrochloride solution (1.6 mL, 0.85 M) with ultrasonication to form a homogeneous suspension. Then the silica condensation was barked on by adding TEOS (90 μ L) and subsequently stirring for 2 h at room temperature before adding silane-PEG-azide (Mw 5000)/ mPEG-silane (Mw 2000) mixture at the weight percentage of 20 w/w% as terminators (50 μ L, 2000 mg/mL in ethanol solution). The termination should last 24 hours at room temperature with stirring. To remove hydrochloride and ethanol, the product was washed and centrifuged four times at 3500 rpm for 30 min using an ultracentrifuge tube of 50 KDa (Millipore). The aqueous solution was filtered through a polyethersulfone (PES) syringe driven filter (0.22 μ m) (Millipore). The SPNs solutions were diluted in 1 $\times$ PBS buffer (pH 7.4) and the concentrations were determined by UV-Vis absorption according to absorption coefficient of SP1 or SP2.

Synthesis of rSPNs. One batch of SPNs prepared with SP (500 μ g) possesses

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azide group about 2 μmol . The solid powder of CuSO_4 (1 mg) and sodium ascorbate (2 mg) were added into a round-bottomed flask firstly. After excluding the oxygen, one batch SPNs solution in PBS buffer (10 mM, pH 7.4) and DMF solution of DYn-2 (400 μL , 1 mg/mL) were added into the sealed flask drop by drop using syringe individually. The solution was stirring under N_2 for 2 days at room temperature. The resulted *r*SPNs were dialyzed to exclude the copper ions which may be toxic to cells.

***In vitro* study of the reactivity of *r*SPNs.** The reactivity between 1,3-cyclohexanedione and protein sulfenic acids has been conducted as follows: bovine serum albumin (BSA) powder (8 mg) was dissolved in Tris-HCl buffer (1 mL, 50 mM, pH 7.4). To pre-reduce protein, tris(2-carboxyethyl)phosphine (TCEP) solution (20 μL , 50 mM) was added in the BSA solution and incubated at room temperature for 30 min. Then the reduced BSA was washed with Tris-HCl buffer to remove TCEP using ultracentrifuge tube of 50 KDa and made the final volume to 1 mL. The reaction system of total volume of 100 μL was made up as follows: *r*SPN1/BSA-SOH was prepared by mixing with *r*SPN1 (50 μL , 40 $\mu\text{g/mL}$), pre-reduced BSA (25 μL) and Tris-HCl buffer (to the final volume of 100 μL) and adding H_2O_2 (5 μL , 1 mM) at last. The *r*SPN1/BSA (without H_2O_2) and *r*SPN (without BSA and H_2O_2) solutions were used as negative controls. The reaction systems were incubated at 37 $^\circ\text{C}$ for 1 h. The resulted reaction solutions were used for further characterization. For DLS measurement, the final reaction system was diluted in the acetate buffer (100 mM, pH 4.7) reaching isoelectric point of BSA to avoid nonspecific absorption firstly and then measured as usual.

Agarose Gel Electrophoresis. Agarose gels (4%) were prepared using

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agarose powder purchased from Bio-Rad (Certified™ Molecular Biology Agarose) and immersed in 1× Tris/boric acid/EDTA (TBE) buffer, containing sodium dodecyl sulfate (0.1%, SDS). Before loading the gel with the reaction solution of *r*SPN1, SDS (2 µL, 20%) solution was mixed with the final reaction solution (20 µL). All the samples were then loaded into the wells of the gel and electrophoresed on a horizontal electrophoresis system (Mini-Sub Cell GT; Bio-Rad; electrode spacing 10 cm) for 1.5~2 h at 120 V. At last, an IVIS living imaging system was used to image the locations of nanoparticles in the agarose gel with an excitation at 530 nm and the emission at 670 nm.

***In vitro* PA Instrumentation.** *In vitro* PA spectrum was measured by an optical parametric oscillator, OPO (Continuum, Surelite), generating pulses with duration 5 ns and pulse energy 100 mJ/pulse at 10 Hz repetition rate, which was pumped by a Q-switched 532 nm Nd:YAG laser. The output was tunable from wavelength 680 nm to 860 nm. The SPNs solution (23 µg/mL) was placed inside a low-density polyethylene (LDPE) tube with an inner diameter (ID) of 0.59 mm and outer diameter (OD) of 0.78 mm. After irradiation with wavelengths ranging from 680 - 860 nm with 10 nm increment, the PA signals produced in LDPE tube was coupled to the single-element ultrasound transducer, UST (V323-SU/2.25 MHz, 13 mm active area, and 70% nominal bandwidth, Panametrics). Both the tube and UST were immersed in water medium. PA signals at individual wavelength were collected using the UST, subsequently amplified with a gain of 50 dB. Band pass was filtered (1-10 MHz) by a pulser/receiver unit (Olympus-NDT, 5072PR) and the output was digitized with a data acquisition card (GaGe, compuscope 4227) operated at 25 MHz. The data were finally stored in the computer. Peak-to-peak voltage of the PA signals for each wavelength were

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then normalized with the laser power. The normalized signals were plotted as a function of wavelengths to generate the PA spectra.

Cell imaging. HeLa cells (human epithelial carcinoma cell line; American Type Culture Collection) were cultured as mentioned. After seeded cells reaching 70-90% confluency, the cells were washed 3 times using prewarmed PBS buffer (10 mM, pH 7.4) and incubated with medium containing different concentration of H₂O₂ (0, 10, 100 μ M) for 5 min at 37°C. Then the cells were washed 3 times using PBS buffer and medium containing *r*SPN2 or SPN2 with the final concentration of 12 μ g/mL were added. The cells were incubated with nanoparticles in an atmosphere of 5% CO₂ and 95% humidified air at 37°C for 24 h. Then the cells were stained with pHrodo® Red epidermal growth factor (EGF) Conjugate (ThermoFisher) for the endo-/lysosome and with Hoechst 33342 (NucBlue Live ReadyProbes Reagent, Thermo Fisher) for the nuclei as protocol. Fluorescence images of the live cells were taken with the wide-field fluorescence microscope Leica DMI8 (Leica, Germany) equipped with scientific CMOS (sCOMS, Hamamatsu, Japan). Excitation/emission wavelengths were 390 $\pm$ 22/440 $\pm$ 25 nm for Hoechst, 560 $\pm$ 25/607 $\pm$ 25 nm for endo-/lysosome indicator, and 648 $\pm$ 20/694 $\pm$ 25 nm for the nanoparticles.

Histological Studies. After 48 h post-injection of nanoparticles, the mice treated with *r*SPN2 or SPN2 (200 μ L, 100 μ g/mL) (n = 3) were sacrificed. The tumors were fixed in 4% formalin and dehydrated with 30% sucrose solution. Then, cryostat sectioning was conducted for immunofluorescence staining. The sliced tumor samples were blocked with PBS solution (including 3% BSA and 0.1% TritonX-100) and stained with anti-cysteine sulfenic acid antibody (#07-2139,

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Merck, 1:3000) for 1 h at 37°C. The slices were then washed three times with PBST and stained by donkey anti-rabbit IgG (H+L) secondary antibody (#A21206, Thermo Fisher, 1:1000) for 30 min at 37°C in the dark. Then the slices were washed with PBS for three times and stained with DAPI (D9542, Sigma) following wash with PBS for three times. At last, the slices were mounted with Fluoromount G (Southern Biotech) and used for immunofluorescence analysis.

Data Analysis. The *in vivo* and *ex vivo* PA signal intensities and real-time PA spectra were measured by region of interest (ROI) analysis using OsiriX software. Fluorescence signal intensities of cells were measured by ROI analysis using ImageJ. Colocalization coefficients of *r*SPN2 and pHrodo® Red EGF Conjugate were calculated using the function of Coloc2 of ImageJ as protocol (<http://imageJ.net>). Fluorescence signal intensities of mice were measured by ROI analysis using Living Image software for IVIS imaging system. Results were expressed as the mean $\pm$ SD deviation unless otherwise stated.

4.3 Results and Discussions

Two SPs, poly[3-(5-(9-hexyl-9-octyl-9*H*-fluoren-2-yl)thiophen-2-yl)-2,5-bis(2-hexyldecyl)-6-(thiophen-2-yl)pyrrolo[3,4-*c*] pyrrole-1,4(2*H*,5*H*)-dione] (DPPF, SP1) and PCPDTBT (SP2) (**Figure 4-1a**), were employed to synthesize the surface-modifiable SPNs (**Figure 4-1b**). The THF mixture of SP and an amphiphilic triblock copolymer (PEG-*b*-PPG-*b*-PEG) (**Figure 4-1a**) at the mass ratio of 1:100 was first evaporated to yield a thin film. After reconstituting in the HCl solution under ultrasonication, the silicate condensation of tetraethyl orthosilicate (TEOS) efficiently occurred, leading to the formation of a thin silica

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shell for SPNs. The presence of silica element in the nanoparticles was detected by the energy-dispersive X-ray spectroscopy (EDX), which showed the peak of silica element. This was also confirmed by the characteristic peak at $\sim 1100\text{ cm}^{-1}$ for Si-O-Si bond in Fourier transform infrared spectroscopy (FTIR) analysis.^[115] The surface structure of SPNs was constructed by adding silane-PEG molecules as the terminators to the reaction. Utilization of silane-PEG-azide/silane-PEG (Figure 4-1a) mixture at the weight percentage of 20 w/w% not only conferred SPNs with a PEG passive layer for biocompatibility and aqueous stability but also yielded the azide reaction groups on the nanoparticle surface for post-conjugation.

Figure 4-1. Synthesis and characterization of SPNs. (a) Chemical structures of SP1, SP2, PEG-*b*-PPG-*b*-PEG, silane-PEG-azide, silane-PEG and DYn-2 used for preparation of rSPNs. (b) Schematic illustration of the preparation of rSPNs.

(c) DLS profile of *r*SPN2. Inset: photos of SPNs and *r*SPNs solutions (15 $\mu\text{g/mL}$) in $1 \times \text{PBS}$ (pH 7.4). (d) Average diameters of SPNs and *r*SPNs in PBS (pH 7.4). (e) Representative TEM image of *r*SPN2.

Figure 4-2. *In vitro* validation of the specific reaction between *r*SPN1 and the sulfenic acids in BSA (BSA-SOH). (a) Illustration of the mechanism of the specific reaction between *r*SPNs and BSA-SOH. (b) Agarose gel electrophoresis of *r*SPN1, *r*SPN1 in the presence of BSA or BSA-SOH. (c) Average diameters of BSA, *r*SPN1 and *r*SPN1 in the presence of BSA or BSA-SOH. (d) Epi-fluorescence of *r*SPN1 and *r*SPN1 in the presence of BSA or BSA-SOH. UV-vis absorption (e) and fluorescence (f) spectra of *r*SPN1 and *r*SPN1 in the presence of BSA or BSA-SOH. [*r*SPN1] = 2 $\mu\text{g/mL}$; $1 \times \text{PBS}$ (pH 7.4). Excitation for fluorescence at 530 nm. Emission for epi-fluorescence images at 670 nm.

Click chemistry was used to conjugate the sulfenic acid reactive group, 4-(pent-4-yn-1-yl)cyclohexane-1,3-dione (DYn-2), to the surface of SPNs (**Figure 4-1b**). The resulted reaction-based SPNs (*r*SPNs) were transparent and homologous with cyan color in PBS solution, the same as their precursors

(**Figure 4-1c**, inset). The optical properties of SPNs including absorption and fluorescence spectra before and after conjugation with DYn-2 were the same. DLS showed that all SPNs had the similar hydrodynamic diameters of ~18 nm (**Figures 4-1c&4-1d**). The spherical morphology of *r*SPN2 was observed by TEM (**Figure 4-1e**). The good aqueous stability was proved by the unchanged diameters of SPNs after stored in the dark at room temperature (24°C) for more than 1 month. In addition, *r*SPN2 was stable in serum at room temperature for 24 h at least. These data confirmed the weak non-specific interaction of *r*SPNs with serum proteins, suggesting the suitability of *r*SPNs for biological application.

To validate the reactivity towards protein sulfenic acids (**Figure 4-2a**), the reaction between *r*SPN1 and BSA was studied in the absence and presence of hydrogen peroxide (H₂O₂). Incubation of BSA with H₂O₂ is a common way to generate sulfenic acids in BSA (BSA-SOH), because sulfenic acids formation in proteins is mainly recognized as the direct product of the reaction between cysteine of proteins and H₂O₂.^[116] However, sulfenic acids are a transient oxidative state and likely to be further oxidized into hyperoxidized forms, sulfinic acids (-SOOH) or sulfonic acids (-SOOOH), by excessive H₂O₂. Thereby, in order to capture this transient form, *r*SPN1 was mixed with BSA prior to addition of H₂O₂. The agarose gel electrophoresis showed *r*SPN1/BSA-SOH band migrated much less as compared with *r*SPN1/BSA and *r*SPN1 (**Figure 4-2b**). The DLS data also verified the diameter of *r*SPN1/BSA-SOH (56 nm) was 3.3-fold larger than that of *r*SPN1 itself (17 nm) (**Figure 4-2c**). The diameter of *r*SPN1/BSA (19 nm) remained almost same with *r*SPN1. Both the electrophoresis and DLS data proved the occurrence of the specific reaction between *r*SPN1 and BSA-SOH, giving rise to the increased size of nanoparticles.

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The same phenomenon was observed using *r*SPN2 when incubation with BSA and H₂O₂, but not for the control nanoprobe (SPN2). Note that in the excessive oxidative environment (H₂O₂: higher than 100 μM) the formed BSA-SOH can undergo over-oxidation to form -SOOH or -SOOOH,^[117] and thus the reaction is inhibited, resulting in the unchanged size of *r*SPN2. Moreover, the absorption and fluorescence spectra of *r*SPN1/BSA-SOH, *r*SPN1/BSA and *r*SPN1 were nearly identical (**Figures 4-2e&4-2f**), suggesting that the covalent bonding of BSA-SOH onto the surface of *r*SPN1 had no impact on the optical properties of the SP core. Such a surface-inert optical feature of *r*SPNs should benefit from the silica shell that effectively blocked the direct contact between the optically active SP core and molecules in the external environment of nanoparticles.

To facilitate *in vivo* imaging of protein sulfenic acids, *r*SPN2 with the strong absorption and emission in the NIR region was used as the PA and fluorescence probe. *r*SPN2 had the absorption spectrum ranging from 680 to 860 nm and the fluorescence spectrum with the emission maximum at 840 nm, both of which were ideal for *in vivo* PA and fluorescence imaging (**Figures 4-3a&4-3b**). Under pulsed laser irradiation, *r*SPN2 emitted PA signals with the maximum close to 680 nm which was consistent with the absorption (**Figure 4-3c**) and similar to that of its counterpart SPN2. In addition, the good linearity between PA signal and the concentration of *r*SPN2 existed from 15 to 100 μg/mL with $R^2 = 0.997$ (**Figure 4-3d**), implying the feasibility for quantification of the concentration of *r*SPN2. Similar to *r*SPN1, no change of optical properties was observed for *r*SPN2 after covalent binding with BSA-SOH (**Figures 4-3a&4-3b**). In addition, the PA brightness at 680 nm was nearly identical for *r*SPN2 with and without BSA or BSA-SOH (**Figure 4-3e**). Similar to *r*SPN1, no obvious cytotoxicity was

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observed for *r*SPN2 using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, showing its potential for biological applications (**Figure 4-3f**).

To demonstrate the reactivity of *r*SPN2 towards protein sulfenic acids in cells, the fluorescence imaging was conducted on the cervical cancer cells (HeLa) with and without the treatment of H₂O₂. H₂O₂ has been proved to be an effective way to simulate acute oxidative stress and produce R-SOH in cells.^[110,114b] After incubation with *r*SPN2 for 24 h, the cells were washed and stained with the endo-/lysosome indicator and the nuclei indicator, which were respectively depicted in pseudo green and blue colors in the fluorescence cell images. The overlaid images showed that the fluorescence from *r*SPN2 overlapped well with that from the endo-/lysosome indicator regardless of H₂O₂ treatment (**Figure 4-4a**). Statistical analysis using Manders' colocalization coefficient^[118] ranging from 0 (non-overlapping) to 1 (100% colocalization) revealed that the colocalization coefficients for all cell groups were higher than 0.58 (**Figure 4-4b**). This indicated *r*SPN2 was mainly located in the endo-/lysosome of cells. In addition, the fluorescence intensity of *r*SPN2 increased obviously along with increased concentration of H₂O₂ (**Figure 4-4a**). As compared with the untreated cells, the fluorescence intensities were respectively increased by 3.3- and 8.4-fold for the cells treated with 10 and 100 μ M H₂O₂ (**Figure 4-4c**). In contrast, the fluorescence intensities of the corresponding nanoparticles without DYn-2 groups on the surface (SPN2) remained nearly the same in the cells regardless of the treatment concentrations of H₂O₂ (**Figure 4-4c**).

Figures 4-3. *In vitro* optical and PA characterization of the specific reaction between rSPN2 and the sulfenic acids in BSA (BSA-SOH). UV-vis absorption (a) and fluorescence (b) spectra of rSPN2 and rSPN2 in the presence of BSA or BSA-SOH. (c) PA spectrum of rSPN2 (23 $\mu\text{g/mL}$) in PBS (pH 7.4). (d) PA intensities as a function of the concentration of rSPN2 from 10 to 100 $\mu\text{g/mL}$ ($R^2 = 0.997$). (e) PA intensities at 680 nm of rSPN2 and rSPN2 in the presence of BSA or BSA-SOH. (f) Cell viability of HeLa cells after incubation with rSPN2 solutions at various concentrations. Excitation for fluorescence is at 660 nm. A single laser pulse with energy of 100 mJ/pulse (duration of 5 ns) and a pulse repetition rate of 10 Hz was used for PA intensity. Error bars were based on standard error of mean (SEM) ($n=3$). *No statistically significant difference in PA intensities for rSPN2 in the absence and presence of BSA or BSA-SOH ($p > 0.1$, $n = 3$).

The previous studies have shown that protein sulfenic acids as the early oxidative products are mainly accumulated in endosomes of cells,^[118] which can develop to lysosomes in the late stage. The NIR fluorescence of rSPN2 in endo-/lysosomes thus implied the specific covalent bond between rSPN2 and protein sulfenic acids formed, leading to the selective localization of nanoparticles in cells. Furthermore, with increasing concentration of H_2O_2 , the oxidative stress was elevated and in turn the formation of protein sulfenic acids was facilitated, ultimately giving rise to the promoted reaction between rSPN2 and protein sulfenic acids. These cell image data proved that rSPN2 was able to track the dynamic concentration changes of protein sulfenic acids in cancer cells along

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with increased oxidative stress.

Figures 4-4. Fluorescence imaging of protein sulfenic acids in cancer cells using *r*SPN2. **(a)** Fluorescence microscopy of HeLa cells treated with different concentration of H₂O₂, incubated with *r*SPN2 and stained with nucleus indicator (DAPI) and endo-/lysosome indicator. **(b)** Colocalization coefficients of endo-/lysosome indicator and *r*SPN2 channel in HeLa cells treated with different concentration of H₂O₂. **(c)** Quantification of fluorescence intensities of HeLa cells incubated with *r*SPN2 or SPN2 and treated with different concentration of H₂O₂. [SPNs] = 12 μg/mL used in cell imaging. The error bars represent the standard deviation from three separate measurements. *No statistically significant difference in SPN2 incubation group treated with different concentration of H₂O₂ ($p > 0.5$, $n = 3$); ** Statistically significant difference in *r*SPN2 incubation group treated with different concentration of H₂O₂ ($p < 0.05$, $n = 3$).

Oxidative stress is a hallmark of cancer that distinguishes tumor from normal tissues.^[119] As the first step oxide of cysteine in protein, it was observed that the sulfenic acid modifications were more prevalent in tumor tissues taken from

patients, relative to matched normal tissues.^[110] *In vivo* activity of *r*SPN2 towards protein sulfenic acids was thus investigated using xenograft HeLa tumor mouse model. After systemic administration of *r*SPN2 or the control probe (SPN2) into the tumor-bearing living mice through tail vein injection, both PA and fluorescence images were longitudinally recorded and quantified. The PA and fluorescence intensities gradually increased for both nanoparticles over time (**Figures 4-5**). At $t = 36$ h, the PA intensity of tumor for *r*SPN2 injected mice was 1.3-fold higher than that for the control probe (SPN2), which was 2.2-fold higher than the PA background of tumor. Similarly, the fluorescence intensity of tumor for *r*SPN2 injected mice was 1.4-fold higher than that for SPN2, which was 74-fold higher than the fluorescence background of tumor. The observed higher signal-to-noise ratio of fluorescence imaging relative to PA imaging should be mainly attributed to the higher sensitivity of fluorescence; moreover, the substantial PA background at 680 nm from hemoglobin was partially responsible. However, PA imaging had higher resolution and thus allowed for delineating the vascular structures of tumors. As shown in the PA images (**Figure 4-5a**), the PA signals were evenly distributed throughout the tumor, implying the efficient extravasation of *r*SPN2 due to their small size (~18 nm).

The real-time *in vivo* spectra of tumor (**Figure 4-5e**) showed that the PA signals at 680 nm for *r*SPN2 was 1.5-fold higher than that for SPN2. Moreover, the PA spectral profiles resembled that in solution (**Figure 4-3c**), further confirming that the increased PA signals came from the accumulation of nanoparticles. Because both PA and fluorescence signals of *r*SPN2 were not affected by the surface reactions (**Figures 4-3b&4-3e**), the stronger signals of *r*SPN2-injected mice relative to SPN2-injected mice should result from the specific

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reaction of *r*SPN2 to sulfenic acids that facilitated nanoparticles accumulation in tumor. The formation of covalent bonds between *r*SPN2 and protein sulfenic acids in tumor led to its slower clearance from the tumor as compared with that for the control probe (SPN2) after 36 h (**Figures 4-5c&4-5d**). Thereby, the signal difference between *r*SPN2- and SPN2-treated mice was further increased to 1.5- and 2-fold at 48 h for PA and fluorescence images, respectively. The *ex vivo* PA and fluorescence data illustrated that *r*SPN2 and SPN2 had quite similar dynamic biodistribution in major organs: the highest liver uptake, followed by spleen, tumor and other organs. It was probably because of their similar sizes and surface structures. This ruled out the organ preference caused by the post-conjugation for *r*SPN2. The *in vivo* and *ex vivo* data thus validated the effectiveness of *r*SPN2 in real-time imaging of protein sulfenic acids in the oxidative microenvironment of tumor of living mice.

Figures 4-5. *In vivo* PA and fluorescence imaging of protein sulfenic acids using *r*SPN2. The representative PA maximum intensity projection (MIP) images with axial view (a) and fluorescence images (b) of tumor at representative time-points after systemic administration of *r*SPN2 or SPN2 *via* tail vein injection. The tumor was under the armpit of the right forelimb, indicated with the white dashed circles

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and arrows. Quantification of PA (**c**) and fluorescence intensities (**d**) as a function of time post-injection of *r*SPN2 or SPN2. (**e**) Real-time *in vivo* PA spectra of tumor at the post-injection time of 48 h. The PA data were acquired at 680 nm. The fluorescence data were excited at 660 nm and the signals were collected at 810 nm. Error bars were based on standard error of mean (SEM) (n = 3). *Statistically significant difference in PA and fluorescence signals between *r*SPN2 injected mice and SPN2 injected mice at post-injection of 36 and 48 h ($p < 0.01$, n = 3).

To verify the specific bonding of *r*SPN2 to protein sulfenic acids in the oxidative environment of tumor, the mice injected with *r*SPN2 or SPN2 were sacrificed at 48 h post-injection of nanoparticles and conducted the immunofluorescence imaging. The anti-sulfenic acid antibody labeled with a green fluorescence dye was used to co-stain the tumor tissue samples, which was able to specifically bind to the conjugates of the active groups (1,3-cyclohexanedione derivatives) and protein sulfenic acids but nearly show no reactivity towards both proteins and protein sulfenic acids (**Figure 4-6a**).^[110,114b] Consistent with the *in vivo* data, the NIR fluorescence for the tumor tissues of *r*SPN2-treated mice was stronger than that for SPN2-treated mice (**Figures 4-6b vs 4-6c**). In addition, the red signals were well overlaid with the green signals from the anti-sulfenic acid antibody for the tumor tissues of *r*SPN2-treated mice (**Figure 4-6b**); in contrast, weak green signals were detected for the tumor tissues of SPN2-treated mice (**Figure 4-6c**). These data proved that the specific covalent bonding between *r*SPN2 and protein sulfenic acids occurred to facilitate the nanoparticle accumulation in tumors.

Figures 4-6. Histological analysis for tumors using immunofluorescence staining with anti-sulfenic acid antibody. (a) The illustration of the mechanism of immunofluorescence staining with anti-sulfenic acid antibody, which selectively recognizes the conjugates of the active groups (1,3-cyclohexanedione derivatives) and protein sulfenic acids, showing no reactivity to proteins and protein sulfenic acids. Fluorescence microscopy of tumor slices for mice treated with *r*SPN2 (b) and SPN2 (c) at 48 h of post-injection. Red fluorescence indicates the signals from *r*SPN2 or SPN2 and green fluorescence indicates the signals from the staining with anti-sulfenic acid antibody, while the blue fluorescence is from the nucleus staining.

4.4 Conclusions

In summary, I have developed reaction-based PA nanoprobe (*r*SPNs) for imaging of protein sulfenic acids using SPNs as the platform. A templated method was used to synthesize *r*SPNs with the silica/PEG coating for the SP core so as to conjugate the sulfenic acid reactive 1,3-cyclohexanedione group onto the nanoparticle surface. Such a nanoparticle design resulted in the inert optical and PA features for *r*SPNs, enabling qualitative correlation between the signal enhancement and the extent of the sulfenic-acid-induced reaction of *r*SPNs. *In vitro* studies showed that *r*SPN2 served as an effective fluorescent probe for real-time tracking of the dynamic changes in the concentration of protein sulfenic acids under elevated oxidative stress in cancer cells. By virtue of the strong NIR absorption and fluorescence, *r*SPN2 permitted real-time PA and fluorescence imaging of protein sulfenic acids in the tumor of living mice. As

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compared with its non-reactive control probe (SPN2), substantial enhancement in the PA and fluorescence signals of tumors were observed for *r*SPN2, proving its capability to specifically react and then covalently bind to protein sulfenic acids in the oxidative microenvironment of tumor. With its good biodistribution, the reaction-based SPNs should be promising for imaging of protein sulfenic acids in other diseases such as cardiac dysfunction, ischemia/reperfusion injury and neurodegenerative diseases.

To the best of our knowledge, *r*SPNs represent the first reaction-based PA probes that recognize the sub-molecular variation of biomolecules and subsequently undergo specific chemical reaction with the target sites to form covalent bonds. The structural flexibility of SPNs will open up inspiring opportunities to develop various reaction-based PA probes for advanced molecular imaging in biology and medicine.

Chapter 5 Development of a Biodegradable Photoacoustic/Photothermal Theranostic Nanoagent³

5.1 Introduction

Theranostic nanoagents that integrate real-time diagnosis with therapeutic capability can accurately target diseased tissues and optimally exert therapy,^[42,120] representing a way for cost-effective precision medicine.^[121] In comparison with radioactive,^[122] magnetic^[123] and ultrasound^[124] imaging agents, optical nanoagents that only utilize light to generate diagnostic signals and trigger therapeutic process have attracted more attention for cancer therapy.^[86,125] In particular, photothermal nanotheranostics not only can induce localized hyperthermia in tumor to directly eradicate cancer cells but also could potentially generate anti-tumor immunological effects.^[126] Till now, most photothermal nanotheranostic systems rely on fluorescence as the signal readout, and thus share the drawbacks of shallow penetration and *tissue* autofluorescence, partially compromising imaging sensitivity.^[65b,127] In contrast, PA nanoagents that detect ultrasound after light excitation overcome such limitations and thus offer deeper tissue penetration and higher spatial resolution to guide PTT.^[87,128] Different from fluorescent nanotheranostics that has two competing process (fluorescence vs nonradioactive decay), both PA signals and PTT effect are related to photothermal conversion, making PA/PTT

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nanotheranostics easier to design in principle.^[129]

Many NIR light absorbing nanoagents including small molecule dyes,^[130] porphyrins,^[131] peptide-/protein-based nanomaterials,^[132] metallic nanoparticles,^[133] carbon nanotubes,^[134] and two-dimensional materials (TDMs)^[129,135] have been used for PA imaging or PTT. Among many other issues in their translation research, biodegradability of these nanoagents is one of the most essential concerns.^[14] Apart of this, reducing the nanoagent size below 5 nm to improve the fast clearance through urinary excretion is also feasible. Here we just focus on the first manner, to develop the biodegradability of nanoagents. In fact, there are some inorganic nanoparticles have been designed to undergo stimuli-responsive degradation.^[136] However, the metal ions released from degradation of inorganic nanoparticles could still be a threat to biological systems. Thus, development of organic nanoagents with biodegradability is highly desired for PA/PTT nanotheranostics.

SPNs have emerged as a class of optical agents for molecular imaging and phototherapy. Despite their great potential in PA/PTT nanotheranostics, SPNs with high biodegradability have not been reported. Although biodegradable polymers such as polyesters and polyamides are available,^[80] simple introduction of these hydrolyzable units (ester, carbonate, amide et al.) into the backbone of SPs will certainly hamper electron delocalization and thus compromise the optical properties of SPNs. Thereby, the design for biodegradable SPNs remains to be revealed.

I herein report the synthesis of a biodegradable SPN with enhanced PA and PTT efficiencies for cancer therapy. The molecular design involves the

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incorporation of vinylene bond as a structural unit into the backbone of SP. Because we recently found that the vinylene bonds tend to undergo π^2 - π^2 cycloaddition with oxidants followed by hydrolysis,^[44] the SPNs can be gradually broken down into small fragments in the biological environment abundant of oxidative species. In addition, the presence of vinylene bonds not only facilitates chain packing but also increases the mass absorption coefficients, leading to enhanced photothermal conversion and thus amplified PA and PTT efficiencies. In the following, the synthesis of the SPNs is described first, followed by studies of their optical and photothermal properties. Then, the biodegradability of the SPNs is studied under the *in vivo* mimetic conditions. At last, the proof-of-concept application of these SPNs as PA/PTT nanotheranostic agents is demonstrated in xenograft tumor mouse model.

5.2 Materials and Methods

Chemicals. 3,6-Bis(5-bromothiophen-2-yl)-2,5-bis(2-hexyldecyl)pyrrolo[3,4-c]pyrrole-1,4(2*H*,5*H*)-dione was purchased from Luminescence Technology Corp. All chemicals were purchased from Sigma-Aldrich unless otherwise declared.

Materials Characterization. Nuclear Magnetic Resonance (NMR) spectroscopy was recorded on a BRUKER Avance 300 NMR (¹H: 300MHz) system with CDCl₃ as the solvent. The spectra were internally referenced to the tetramethylsilane signal at 0 ppm. The other characterization methods are similar with those described in **Chapter 3.2**.

Synthesis of DPPV. 3,6-Bis(5-bromothiophen-2-yl)-2,5-bis(2-

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hexyldecyl)pyrrolo[3,4-*c*]pyrrole-1,4(2*H*,5*H*)-dione (75 mg, 0.083 mmol), trans-1,2-bis(tributylstannyl)ethene (50 mg, 0.083 mmol), Pd₂(dba)₃ (0.6 mg, 0.00065 mmol), and tri(*o*-tolyl)phosphine (1.2 mg, 0.0039 mmol) were placed in a Schlenk tube (50 mL). The Schlenk tube was then sealed, degassed and refilled with nitrogen by three vacuum-nitrogen cycles. Chlorotoluene (3 mL) was then injected into the tube. The mixture was degassed by three freeze-pump-thaw circles. The Stille polycondensation reaction was subsequently conducted at 100 °C by vigorous stirring under N₂ atmosphere for 24 h. The resulted dark viscous mixture was precipitated and washed three times by excess methanol. The residue was collected by centrifugation and then dried under vacuum to obtain the final product. ¹H NMR (300 MHz, CDCl₃, δ): 9.29 – 8.64 (m, 4H), 6.83 – 6.59 (m, 2H), 4.30-3.85 (m, 4H), 2.72-2.47 (m, 2H), 1.46-1.08 (m, 41H), 0.90-0.73 (m, 19H).

Preparation of SPNs. DPPV, DPPT and PLGA-PEG (Mn=4500) were dissolved in THF to prepare stock solutions individually. Then the THF solution (1 mL) containing DPPV or DPPT (0.25 mg) and PLGA-PEG (10 mg) were mixed and rapidly injected into the homogeneous mixture of distilled-deionized water and THF (11 mL) at the ratio of 10:1 under sonication. After sonication for additional 10 min, THF was evaporated under nitrogen atmosphere. The aqueous solution was filtered through a PES syringe driven filter (0.22 μm) (Millipore) and washed three times using distilled-deionized water and centrifugal filter units (Millipore, MWCO 50 kDa) with the speed of 3,500 rpm for 15 min. The SPN solutions were diluted in 1×PBS buffer (pH 7.4) and the concentrations were determined by UV-Vis absorption according to the mass absorption coefficient. The SPN solutions were finally concentrated by ultrafiltration and stored in dark at 4°C.

***In vitro* PA Instrumentation.** *In vitro* PA spectra were measured by an optical parametric oscillator, OPO (Continuum, Surelite), generating pulses with duration 5 ns and pulse energy 100 mJ pulse⁻¹ at 10 Hz repetition rate, which was pumped by a Q-switched 532 nm Nd:YAG laser. The output wavelength was tuned from 680 to 900 nm. The SPNV or SPNT solution was placed inside a low-density polyethylene (LDPE) tube with an inner diameter (ID) of 0.59 mm and outer diameter (OD) of 0.78 mm. After irradiation with laser wavelengths ranging from 680 to 900 nm with 10 nm increment, the PA signals produced in LDPE tube was coupled to the single-element ultrasound transducer, UST (V323-SU 2.25 MHz⁻¹, 13 mm active area, and 70% nominal bandwidth, Panametrics). Both the tube and UST were immersed in aqueous medium. PA signals at individual wavelength were acquired using the UST, which were subsequently amplified with a gain of 50 dB. Band pass was filtered (1-10 MHz) by a pulser/receiver unit (Olympus-NDT, 5072PR) and the output was digitized with a data acquisition card (GaGe, compuscope 4227) operated at 25 MHz. Peak-to-peak voltages of the PA signals for each wavelength were then normalized relative to the laser power. The normalized signals were plotted as a function of wavelengths to generate the PA spectra. The PA signals with different concentrations of SPNs were measured in the same way at the laser wavelength of 810 nm.

***In vitro* Biodegradability Studies.** SPNV or SPNT solutions (3 µg mL⁻¹) were incubated with H₂O₂ (300 µM) in the present or absent of MPO (50 µg mL⁻¹) at 37 °C in phosphate buffer (50 mM, pH 7.0) with NaCl (150 mM). The absorption spectra of SPNs were measured after the incubation for 0, 24 and 48 h until the peak of SPNV at 819 nm disappeared. The MPO enzyme was purchased from

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Bio-Techne China Co. Ltd. with specific activity $>50,000 \text{ pmol min}^{-1} \mu\text{g}^{-1}$. The average MPO concentration in neutrophils was reported to be $5.9 \times 10^{-7} \text{ U}$ per neutrophil.⁶⁰ There are around 3000-7000 neutrophils μL^{-1} in blood. The specific activity of MPO enzyme I used is $5 \times 10^{-2} \text{ U } \mu\text{g}^{-1}$. Subsequently the average MPO concentration can be translated to be $35.4\text{-}82.6 \mu\text{g mL}^{-1}$.

***In vitro* Photothermal Characterizations.** The $1 \times \text{PBS}$ solution (pH 7.4) of SPNV or SPNT with the concentration of $60 \mu\text{g mL}^{-1}$ and the same amount of PBS buffer were exposed to laser irradiation at 808 nm. The temperatures were recorded every 20 s and the laser was turned off until the temperature reaching plateau (8 minutes here). Then the temperatures were continuously monitored every 20 s for another 8 min. The heating and cooling process were repeated five times to test the photothermal stability of SPNV or SPNT. The photothermal conversion efficiencies were calculated according to our previous report.⁵⁴

Cell Culture. 4T1 and RAW264.7 cells were purchased from the American Type Culture Collection (ATCC). 4T1 cells were cultured in RPMI-1640 medium supplemented with fetal bovine serum (FBS, 10%) and Penicillin/Streptomycin antibiotics (1%). RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS (10%) and Penicillin/Streptomycin antibiotics (1%). The cells were maintained in an atmosphere of 5% CO_2 and 95% humidified air at 37°C .

Biodegradability Studies in Cells. The RAW264.7 Cells were subcultured in flask. When the cell confluency reached 60%, the cells were incubated with SPNV ($38 \mu\text{g mL}^{-1}$) for 24 hours. Then the cells were washed with PBS buffer (10 mM, pH 7.4) and treated with lipopolysaccharides (LPS, $1 \mu\text{g mL}^{-1}$) for 30

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hours. The cells without LPS-treatment were served as the control group. Then the cells were harvested, counted the number and broken by ultrasonication. The absorptions from cells ($276,000 \text{ cells mL}^{-1}$) treated with or without LPS were measured to indicate the SPNV concentrations inside cells.

Cytotoxicity Test. Cells were seeded in 96 well plates (3000 cells in 100 μL medium per well) for 12 h, and then were cultured with fresh medium containing SPNV or SPNT solutions with different concentrations of 10, 20, 60, 100 and 150 $\mu\text{g mL}^{-1}$ for 24 h. Next, the per well medium was changed to the mixture of 120 μL fresh medium with MTS solution (Promega) (20 μL). After incubation for another 4 h, the absorbance measurement at 490 nm was performed using a SpectraMax M5 microplate/cuvette reader. Cell viability was expressed as the ratio of the absorbance of the cells treated with SPNV or SPNT to that of untreated cells.

***In vitro* PTT of SPNs.** Cells were seeded in 96 well plates (10,000 cells in 100 μL medium per well) for 12 h, and followed by incubation with fresh medium containing SPNV or SPNT solutions with different concentrations of 20, 60 and 100 $\mu\text{g mL}^{-1}$ for 3 h. After it, the cells were irradiated with 808 nm laser with different power densities of 0.3 and 0.5 W cm^{-2} for 8 min and followed by incubation with fresh medium for another 12 h. At last, the cell viabilities were measured and calculated as described in the Cytotoxicity Test section.

Tumor Mouse Model. All animal studies were performed under protocols approved by Laboratory Animal Center of Soochow University. To establish tumors in eight-week-old BALB/c, ten to twenty million 4T1 cells were suspended in 1 mL fresh medium and each mouse was subcutaneously injected with 0.1

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mL cell suspension under the armpit. Tumors were allowed to grow to a single aspect ~7 mm (approximately 10–15 days) before *in vivo* experiments.

In Vivo PA Imaging. After mice bearing 4T1 tumors were anesthetized using 2% isoflurane in oxygen, SPNV or SPNT solutions were systematically injected through the tail vein using a microsyringe (6 mg kg⁻¹ body weight) (n=3). A Multispectral Optoacoustic Tomography scanner (MSOT, iThera medical, Germany) was used to acquire the PA images at 810 nm. *In vivo* PA spectra with excitation light from 680 to 850 nm were acquired from the tumor regions after 6 h systemic administration of SPNs.

In Vivo PTT. Mice bearing 4T1 tumors were exposed to 808 nm laser with the power density of 0.3 W cm⁻² after systemic administration of saline (200 µL) (n=3), SPNV (6 mg kg⁻¹ body weight) (n=3) or SPNT (6 mg kg⁻¹ body weight) (n=3) for 6 h. IR thermal camera was utilized to record the temperature change of the tumor in every 20 seconds during the laser irradiation of 5 min. The tumor sizes were measured by caliper every other day and calculated as the volume = (tumor length) × (tumor width)²/2. Relative tumor sizes were calculated as V/V₀ (V₀ was the initial tumor volume).

Biodistribution Method. After 24 h post-injection of SPNV or SPNT, the mice were sacrificed by CO₂ asphyxiation. Major organs as indicated were harvested for the PA measurements.

Histological Studies. The mice treated with saline (200 µl) (n=3), SPNV (6 mg kg⁻¹ body weight) (n=3) or SPNT (6 mg kg⁻¹ body weight) (n=3) and followed by PTT treatment were sacrificed. The kidney, liver, spleen and tumor were fixed in 4% formalin and then conducted with paraffin-embedded section for H&E

staining. The slices were examined by a digital microscope (Leica).

Data Analysis. *In vivo* and *ex vivo* PA intensities were measured by region of interest (ROI) analysis using post-processing software ViewMSOT. The histological analysis was carried out using Image J. Results were expressed as the mean $\pm$ standard deviations (SD) unless otherwise stated.

5.3 Results and Discussions

The biodegradable SP, poly{2,2'-[(2,5-bis(2-hexyldecyl)-3,6-dioxo-2,3,5,6-tetrahydropyrrolo[3,4-c]pyrrole-1,4-diyl)dithiophene]-5,5'-diyl-*alt*-vinylene} (DPPV), was synthesized through palladium-catalyzed Stille polymerization between 3,6-bis(5-bromothiophen-2-yl)-2,5-bis(2-hexyldecyl)pyrrolo[3,4-c]pyrrole-1,4(2*H*,5*H*)-dione (monomers 1) and trans-1,2-bis(tributylstannyl)ethene (monomers 2) (**Figure 5-1a**). In comparison, its analog poly{2,2'-[(2,5-bis(2-hexyldecyl)-3,6-dioxo-2,3,5,6-tetrahydropyrrolo[3,4-c]pyrrole-1,4-diyl)dithiophene]-5,5'-diyl-*alt*-thiophen-2,5-diyl} (DPPT, **Figure 5-1b**) without vinylene bonds was also synthesized according to the previous report. DPPV and DPPT were respectively co-precipitated with a biodegradable amphiphilic polymer, poly(ethylene glycol) methyl ether-*block*-poly(lactide-*co*-glycolide) (PLGA-PEG), to endow the nanoparticles with good water solubility without interfering their biodegradability (**Figure 5-1c**). The nanoparticle core was formed by the hydrophobic interaction between the PLGA segments and DPPV or DPPT, while the nanoparticle surface was covered by the PEG segments. The resulting nanoparticles (SPNV&SPNT) showed similar average hydrodynamic diameters of ~36 nm and spherical morphologies, as indicated by DLS and TEM, respectively (**Figures 5-1d, e**). After storage in the dark at 4 °C,

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the sizes of SPNV and SPNT remained almost the same for the first 15 days and slightly reduced by ~8 nm on day 22, which should be attributed to the hydrolysis of PLGA-PEG in aqueous solution.^[137] Nevertheless, the solutions of both SPNV and SPNT remained transparent and the sizes did not further change (Figure 5-1f).

Figure 5-1. Synthesis and characterization of SPNs. (a) Synthetic route of DPPV. (i) Pd₂(dba)₃ and tri(*o*-tolyl)phosphine, toluene, 100 °C, 24 h. (b) Chemical structure of DPPT. (c) Chemical structure of PLGA-PEG and schematic illustration of the preparation of SPNs. DLS profiles (d), TEM images (e), photographs of the solutions (f) and absorption spectra (g) of SPNV and SPNT. (h) PA spectra of SPNV, SPNT and PBS. (i) PA intensities at 810 nm as a function of the concentrations of SPNV and SPNT from 0 to 100 μg mL⁻¹ (R² = 0.992 and 0.995 for SPNV and SPNT respectively). The SPN solutions used in characterization were prepared in PBS buffer (pH 7.4). The solutions with the concentration of 60 μg mL⁻¹ were used in photography and PA spectra measurements. A single laser pulse with energy of 100 mJ pulse⁻¹ (duration of 5 ns) and a pulse repetition rate of 10 Hz was used for PA intensity. Error bars were based on the standard deviations (SD) of three parallel samples.

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*Statistically significant difference in PA intensities for SPNV and SPNT with the concentration ranging from 0 to 80 $\mu\text{g mL}^{-1}$ ($p < 0.01$, $n = 3$).

To study the effect of vinylene bonds on optical properties, the absorption and PA spectra of SPNV and SPNT were measured and compared under physiological conditions. Both SPNs showed broad absorption in the NIR region ranging from 600 to 900 nm (**Figure 5-1g**), allowing NIR light to generate both PA and photothermal signals. Due to the presence of vinylene bonds, the absorption maximum of SPNV was located at 819 nm, which was similar to that of SPNT at 828 nm. Besides, because the repeat unit of DPPV had the lower molecular weight ($803.31 \text{ g mol}^{-1}$) than DPPT ($859.39 \text{ g mol}^{-1}$), the mass absorption coefficient of SPNV at 819 nm ($40 \text{ cm}^{-1} \text{ mg}^{-1} \text{ mL}$) was 1.3-fold higher than that of SPNT ($30 \text{ cm}^{-1} \text{ mg}^{-1} \text{ mL}$ at $\lambda=828 \text{ nm}$). Based on the molecular weight of the repeat unit, SPNV ($32.1 \text{ cm}^{-1} \text{ mol}^{-1} \text{ mL}$) also showed 1.2-fold higher molar extinction coefficient than SPNT ($25.8 \text{ cm}^{-1} \text{ mol}^{-1} \text{ mL}$). This was due to its more planar conformation in the presence of vinylene bonds that facilitated the electron delocalization.^[138] The PA spectra of SPNs were acquired at the same concentration ($60 \mu\text{g mL}^{-1}$) by pulsed laser irradiation ranging from 680 to 900 nm (**Figure 5-1h**). Overall, the PA signals of SPNV were stronger than those of SPNT. Particularly, the PA signal of SPNV at 810 nm was 2.2-fold higher than that of SPNT. The good linear correlation between the PA signals and the concentrations of SPNs was observed for both SPNs (**Figure 5-1i**), indicating the feasibility for signal quantification. Note that the PA signal of SPNV was oversaturated above $80 \mu\text{g mL}^{-1}$. These data confirmed that the presence of vinylene bonds in SPNV led to the higher mass absorption coefficient and stronger PA signals as compared to SPNT.

Figure 5-2. Photothermal characterization of SPNs and *in vitro* PTT cell experiments. (a) Photothermal heating and natural cooling cycles under the 808 nm laser irradiation with power density of 0.3 W cm⁻². [SPN]= 60 μg mL⁻¹ in PBS buffer (pH 7.4). (b) Thermal images of SPNV, SPNT and PBS at their respective maximum temperatures. The maximum temperature values and photothermal conversion efficacies are indicated below the respective images. Relative viabilities of 4T1 cells after incubation with different concentrations (20, 60, 100 μg mL⁻¹) of SPNV (c) or SPNT (d) and irradiation under the 808 nm laser with different power densities (0.3, 0.5 W cm⁻²) for 8 min. Error bars were based on the SD of three parallel samples. *Statistically significant difference in cell viabilities treated with and without light irradiation (p<0.01, n=3).

The photothermal properties of SPNV were studied and compared with SPNT at the same concentration under the NIR laser irradiation at 808 nm with the power density of 0.3 W cm⁻², which was lower than the MPE for skin (0.33 W cm⁻²). The temperatures of both SPN solutions gradually increased upon under laser irradiation and reached maximum at t=480 s (**Figure 5-2a**). However, the temperature of SPNV was higher than that of SPNT at each time point, while PBS buffer just showed maximum temperature change by 1°C because of the

low power density applied. The maximum photothermal temperature of SPNV was 53°C, 1.2-fold higher than that of SPNT (43°C) (**Figure 5-2b**). The photothermal conversion efficacy of SPNV was calculated to be $71 \pm 2\%$, 2.4-fold higher than that of SPNT ($29 \pm 1\%$) and also significantly higher than other inorganic photothermal agents such as gold nanorods, phosphorus quantum dots and TDMs.^[129] The better photothermal performance of SPNV relative to SPNT should be ascribed to the presence of vinylene bonds within DPPV which resulted in more planar chain conformation and thus more compact chain packing within the nanoparticles. 59 Both SPNs had good photothermal stability as verified by the nearly unchanged maximum temperatures after 5 circles of heating and natural cooling (**Figure 5-2a**).

The PTT efficacies of both SPNs were firstly studied *in vitro*. 4T1 cells were incubated with different concentrations of SPNs for 3 hours before exposed to NIR laser. Then the cells were irradiated at 808 nm with the different power densities for 8 min and the cell viabilities were measured after incubation for another 12 h. With no laser irradiation, both SPNs did not affect the cell viabilities even at the concentration up to $150 \mu\text{g mL}^{-1}$, indicating their good cytocompatibility (**Figure 5-3**). Generally, for both SPNs, the cell viabilities decreased with increasing the nanoparticle concentrations or illumination power densities, while irradiation alone without SPN treatment had little effect on the cell viabilities (**Figures 5-2c, d**). However, SPNV was more effective in cell ablation than SPNT. When comparing at the concentration of $60 \mu\text{g mL}^{-1}$, SPNV-mediated laser irradiation led to the cell viability as low as 13%, which was 4.3-times lower than SPNT (57%). These data further confirmed SPNV was a better

PTT agent than SPNT as a result of its higher photothermal conversion efficacy.

Figure 5-3. Cytotoxicity studies of SPNV and SPNT. *In vitro* viability of 4T1 cells treated with SPNV or SPNT solutions at concentrations of 10, 20, 60, 100 and 150 $\mu\text{g mL}^{-1}$ for 24 h. The cell viability of treated cells was calculated relative to that of cells without treatment (viability was arbitrarily defined as 1.0). Error bars represented SD of three separate measurements.

Biodegradability of both SPNs was studied under the solution conditions that mimic *in vivo* environment (**Figure 5-4a**). Myeloperoxidase (MPO), a peroxidase enzyme abundantly expressed in immune cells with the concentration as high as $\sim 82.6 \mu\text{g mL}^{-1}$ in blood,^[139] was chosen to study if SPNV and SPNT could be digested. In living animals, the reaction of MPO and H_2O_2 can generate strong oxidant, hypochlorous acid (HClO).^[140] Upon incubation with MPO and H_2O_2 , the absorption of SPNV gradually decreased (**Figure 5-4b**), while it remained the same without MPO (**Figure 5-4c**). After incubation for 48 h, the absorption peak of SPNV at 819 nm completely disappeared, indicating the full scission of π -conjugated backbone (**Figure 5-4b**). In contrast, SPNT could not be broken down by MPO as witnessed by the unchanged absorption (**Figure 5-4c**). These data demonstrated that the presence of vinylene bonds within DPPV made SPNV much easier to be degraded as compared to SPNT.

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The biodegradability of SPNV was further verified in macrophage RAW264.7 cells. As the expression of MPO enzyme in RAW264.7 cells can be induced by stimulation with lipopolysaccharides (LPS), 62 the RAW264.7 cells were treated with LPS after incubation with SPNV and washing with PBS to remove the free SPNV nanoparticles. After stimulation with LPS for 30 hours, the cells were harvested. The concentrations of SPNV inside cells were characterized by absorption measurement. The absorption maximum of SPNV at 819 nm from cells ($276,000 \text{ cells mL}^{-1}$) treated with LPS were 0.081, which was only 12% of that without LPS treatment (0.674) (**Figure 5-4d**). This further demonstrated the biodegradability of SPNV at cellular level.

Figure 5-4. *In vitro* biodegradability study of SPNVs. **(a)** Schematic illustration of the degradation of SPNV in the presence of MPO and H_2O_2 . **(b)** Absorption spectra of SPNV in the presence of H_2O_2 ($300 \mu\text{M}$) and MPO ($50 \mu\text{g mL}^{-1}$) at 37°C for 0, 24, and 48 h in phosphate buffer (50 mM, $\text{pH} = 7.0$) containing NaCl (150 mM). **(c)** Absorption decrease (Abs/Abs_0) of SPNV at 819 nm and SPNT at 828 nm in the absence or presence of MPO ($50 \mu\text{g mL}^{-1}$) and H_2O_2 ($300 \mu\text{M}$) as a function of incubation time. $[\text{SPNVs}] = 3 \mu\text{g mL}^{-1}$. **(d)** Absorption maximum of SPNV at 819 nm from RAW264.7 cells ($276,000 \text{ cells mL}^{-1}$) treated with or without LPS. Error bars were based on the SD of three parallel samples. *Statistically significant difference in cells treated with and without LPS ($p < 0.005$, $n = 3$).

The PA imaging capability of SPNV was studied and compared with SPNT in

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living mice bearing xenograft 4T1 tumors. After systemic administration of SPNV or SPNT into the mice through tail vein, the PA images were longitudinally recorded and quantified under the excitation of pulse laser at 810 nm. The PA intensities gradually increased for both SPNs treated mice and reached its maxima at 6 h post-injection. At this time point, the PA intensity for SPNV-treated mice was 1.5-fold higher than that for SPNT-treated mice (**Figures 5-5a, b**). The real-time *in vivo* PA spectra extracted from the tumor regions for both SPNs showed the similar profiles to those in solutions (**Figure 5-5c**), confirming that the increased PA signals were generated from the nanoparticle accumulation. Because SPNV and SPNT had the same surface structure and size, their biodistribution were similar. Thus, the stronger signals from the SPNV-treated mice relative to SPNT should result from the relatively higher PA brightness of SPNV.

Figure 5-5. *In vivo* PA imaging of tumor in living mice. (a) PA images of living

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mice bearing xenograft 4T1 tumors at 0, 6 and 24 h after systemic administration of SPNV or SPNT (6 mg kg^{-1} body weight). The PA images were acquired at 810 nm. The tumors are indicated with the white dashed circles. **(b)** Quantification of PA intensities of tumor regions as a function of post-injection time of SPNV or SPNT ($n = 3$). **(c)** Real-time *in vivo* PA spectra of tumors at the post-injection time of 6 h. Error bars were based on the SD of three parallel samples.

The PTT capability of SPNV was tested and compared with SPNT. Because the PA signals reached maximum at 6 h post-injection of SPNs (**Figure 5-5b**), laser irradiation at 808 nm was conducted at this time point for 5 min with the power of 0.3 W cm^{-2} . The thermal images of mice and surface temperatures of tumors were recorded as a function of laser irradiation time (**Figures 5-6a, b**). The tumor temperatures gradually increased along with laser irradiation and reached plateau after irradiation for 4 min. At each time points, the maximum tumor temperatures of SPNV-treated mice were significantly higher than that of SPNT- or saline-treated mice. In particular, the maximum tumor temperature of SPNV-treated mice was 50°C , 1.2-fold and 1.3-fold higher than that of SPNT- (42°C) and saline-treated (39°C) mice, respectively. Such a higher tumor temperature for SPNV-treated mice relative to SPNT-treated mice was consistent with the solution data, which was due to the higher photothermal conversion efficacy of SPNV.

To evaluate the antitumor effect, the tumor volumes of SPNV- or SPNT-treated mice were continuously monitored for 16 days after laser irradiation. The tumors of SPNT-treated mice grew as fast as that of saline-treated mice (**Figure 5-6c**), which was caused by the fact that the tumor temperature was below the threshold temperature (43°C) to induce cell apoptosis. In contrast, the tumor growth for SPNV-treated mice was effectively inhibited during the whole tested period, indicating the effective *in vivo* ablation of cancer cells. The body weights

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for all groups of mice were measured at the same time and no obvious weight loss was observed, suggesting the low toxicity of all SPN treatments (**Figure 5-6d**).

Figure 5-6. *In vivo* photothermal therapy of tumor. **(a)** IR thermal images of 4T1 tumor-bearing mice under laser irradiation at 808 nm with the power of 0.3 W cm^{-2} after systemic administration of saline, SPNV, or SPNT (6 mg kg^{-1} body weight) at the post-injection time of 6 h. The tumors are indicated with the white dashed circles. **(b)** Mean tumor temperature as a function of laser irradiation time after systemic administration of saline, SPNV or SPNT at the post-injection time of 6 h. **(c)** Tumor growth curves of mice after systemic treatment with saline, SPNV or SPNT with laser irradiation. **(d)** Body weight data of mice after systemic treatment with saline, SPNV or SPNT with laser irradiation. Error bars were based on the SD of three parallel samples (*p > 0.05, **p < 0.005).

To further verify the enhanced therapeutic outcome of SPNV, all the mice treated with SPNs or saline were sacrificed and different organs and tumors were collected for histological analysis using H&E staining. No significant

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histopathological abnormalities or lesions were observed for kidney, liver and spleen of all the groups (**Figure 5-7**). In contrast, different extent of necrosis was observed for the tumor tissues of SPN-treated mice followed by PTT treatment. Large areas of necrosis were found on the tumor slides for SPNV-treated mice, which were not that obvious for SPNT-treated mice. The percentage of neurosis area on the tumor slide for SPNV-treated mice was calculated to be 54%, 9-times higher than that for SPNT-treated mice (6%). The histological analysis again proved the superior PTT outcome of SPNV over SPNT, which was consistent with the *in vivo* antitumor data.

Figure 5-7. Histological H&E staining for kidney, liver, spleen and tumors at day 16 after the photothermal treatment with saline, SPNV or SPNT. The tumor regions with necrosis are indicated with the dashed circles and arrows.

5.4 Conclusions

I have developed an organic nanoagent (SPNV) with good biodegradability and enhanced PA and PTT efficacies relative to its counterpart. Our design took advantage of the oxidizable nature of vinylene bonds to endow SPNV with the ability to be efficiently digested in the presence of peroxidase meanwhile without comprising the optical and photothermal properties. In fact, because of the low molecular weight and planar conformation of vinylene bonds, SPNV showed mass absorption coefficient, and photothermal conversion efficacy (71%) that are respectively 1.3- and 2.4-fold higher than its counterpart nanoparticle without vinylene bonds (SPNT). It should be emphasized that the photothermal conversion efficacy of SPNV was also significantly higher than most reported photothermal agents. Thus, SPNV acted as a much better PA/PTT theranostic nanoagents than SPNT, providing amplified PA signals and maximum photothermal temperature in living mouse. Moreover, both *in vivo* tumor growth data and *ex vivo* histological data validated the superior antitumor efficacy of SPNV over SPNT. By virtue of its good biodegradability and organically benign composition, SPNV should hold great promise for translational medicine.

In summary, our study provides a molecular design approach to enhance the biodegradability of optically-active polymer nanoparticles meanwhile dramatically elevating their photothermal capability. Such a design can be generalized for other SPs with different structures and applied for other imaging and therapy applications such as NIR fluorescence imaging guided photodynamic therapy.

Chapter 6 Dendronized Semiconducting Polymer as Photothermal Nanocarrier for Remote Activation of Gene Expression⁴

6.1 Introduction

Regulation of cellular behaviors and transgene systems can provide useful tools to understand their physiological roles and potentially lead to innovative therapies. In this regard, gene therapy that utilizes viral or non-viral vectors to deliver exogenous nucleic acids into specific cells has become a promising therapeutic approach to treat inherited genetic diseases and cancers.^[141] However, it is challenging to precisely regulate gene expression at designated locations and times so as to minimize off-target expression.^[142] Encoding tissuespecific promoters in therapeutic nucleic acids is one feasible solution,^[143] which however, is less likely to be generalized for various diseases. Recently, gas molecules,^[144] radio waves,^[145] and light^[146] have been used to remotely switch on gene expression. Of these, light is the most ideal inducer owing to its low toxicity, easy tunability, and high spatiotemporal resolution. However, current methods are generally limited to ultraviolet^[147] and visible light^[146a,146b] with shallow tissue penetration, preventing them from *in vivo* applications; moreover, optical responsive components are knocked in through synthetic biology to trigger the complex cascade response,^[146b,146c] partially complicating their implementation.

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SPNs have also been revealed to efficiently convert photon energy into heat, making them applicable for photoacoustic imaging and photothermal therapy.^[18] Particularly, they often exhibit higher absorption and photothermal conversion efficiencies as compared with other nanoparticles, such as carbon nanotubes and gold nanorods, enabling faster heating.^[148] Based on such excellent photothermal properties, I recently developed SPN-based bioconjugates that specifically targeted and rapidly activated the protein thermosensitive ion channels in neurons.^[25] These studies imply that SPNs could serve as photothermal nanomodulators to optically regulate cellular behaviors.^[149]

I herein report the development of a NIR absorbing dendronized semiconducting polymer (DSP) and demonstrate its proof-of-concept application as a photothermal nanocarrier and intracellular nanotransducer for remote activation of gene expression. DSP comprises three key components (Figure 1a): the hydrophobic semiconducting backbone, the cationic third-generation polyamidoamine (PAMAM3) side chains, and the neural poly(ethylene glycol) (PEG) blocks, these components serve as the NIR photothermal nanotransducer, the gene vector and the water-solubility enhancer, respectively. The conversion of photothermal signal generated from DSP into the cue for gene expression is realized by simple incorporation of plasmids with the gene of interest cloned downstream of a heat shock promoter (HSP70). Thus, gene expression can be regulated by HSP70 and activated by photothermal signals. The working mechanism starts with the electrostatic attraction induced formation of nanocomplexes between DSP and HSP70-regulated gene (**Figure 6-1b**). After cellular internalization, the gene is released from the complex and enters into cellular nucleus, while DSP retains in cytoplasm. Upon laser irradiation at

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808 nm, DPS generates heat, stimulating the transformation of heat shock factor (HSF) from monomers to trimers; HSF trimers then translocate to the cellular nucleus, bind to heat shock element (HSE) in the HSP70 and activate transcription.^[150] As such, DSP sequentially acts as the gene nanocarrier and the intracellular photothermal nanotransducer to efficiently deliver and remotely control transgene expression. Note that although carbon nanotubes have been used to activate HSP70 in the previous studies, they are simply used as the heater and gene delivery need to be conducted in a separate step.^[151]

Figure 6-1. DSP as a photothermal nanocarrier and nanotransducer for remote activation of gene expression. **a)** Chemical structure and selfassembly of DSP. **b)** Illustration of DSP-mediated gene delivery and remote photothermal activation of gene expression under NIR laser irradiation at 808 nm, see text for details.

6.2 Materials and Methods

Chemicals and Other Materials. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Dialysis membrane with 500 Da MWCO was purchased from Spectrum Labs. *D*-luciferin (sodium salt) was purchased from Gold Biotechnology. The constitutive plasmids, pCMV-*EGFP* (enhanced green fluorescence proteins, EGFP, as the reporter proteins) and pSV40-*Luc* (luciferase as the reporter proteins), were purchased from AddGene (pEGFP-N1 vector) and Promega (pGL3-control vector) respectively.

Characterization. Gel permeation chromatography (GPC) was conducted by a Shimadzu LC-VP system with polystyrenes as the standard and high-performance liquid chromatography (HPLC) grade THF as the eluent. NMR spectroscopy was recorded on a BRUKER Avance 300 NMR (^1H : 300MHz) system with CDCl_3 as the solvent. The spectrum was internally referenced to the tetramethylsilane signal at 0 ppm. The gel was run in a horizontal electrophoresis system (Mini-Sub Cell GT; Bio-Rad, Hercules, CA, USA, electrode spacing 10 cm). The other characterization methods are similar with those described in Chapter 3.2.

Synthesis of PAMAM2.5. To a solution of propargylamine (220 mg, 4.00 mmol) in methanol (10.0 mL) was added dropwise methyl acrylate (1.30 mL, 14.4 mmol). The reaction mixture was stirred under argon for 48 h at 30°C. The

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reaction solution was then evaporated, and the residue was purified by column chromatography on silica gel with hexane/EtOAc (2/1), yielding PAMAM0.5 (0.9 g, 85 % yield, 95% purity) as a colorless oil. ^1H NMR (300 MHz, CDCl_3) δ 3.53 (d, J = 9.8 Hz, 6H), 3.30 (d, J = 2.3 Hz, 2H), 2.71 (t, J = 7.0 Hz, 4H), 2.34 (t, J = 7.0 Hz, 4H), 2.19–2.11 (m, 1H).

To a solution PAMAM0.5 (0.9 g, 4 mmol) in methanol (2.00 mL) was added ethylenediamine (5.00 mL, 75.0 mmol). The reaction mixture was stirred under argon for 48 h at 30°C. The reaction solution was evaporated, and then the residue was purified by precipitation with methanol/ Et_2O three times, yielding PAMAM1 (1.13 g, 97.7 % yield, 97% purity) as a pale viscous oil. To a solution of PAMAM1 (1.1 g, 4 mmol) in methanol (10.0 mL) was added dropwise methyl acrylate (2.50 mL, 27.6 mmol). The reaction mixture was stirred under argon for 72 h at 30°C. The reaction solution was evaporated, and then the residue was purified by column chromatography on silica gel with EtOAc/methanol (10/1), yielding PAMAM 1.5 (1.93 g, 79.1 % yield, 97% purity) as colorless oil. ^1H NMR (300 MHz, CDCl_3) δ 7.16 (s, 2H), 3.66 (s, 12H), 3.44 (t, J = 6.6 Hz, 2H), 3.29 (q, J = 5.7 Hz, 4H), 2.84 (t, J = 6.6 Hz, 4H), 2.75 (t, J = 6.7 Hz, 8H), 2.54 (t, J = 5.9 Hz, 4H), 2.48 – 2.28 (m, 12H), 2.19 (s, 2H).

To a solution of PAMAM1.5 (1.35 g, 2.15 mmol) in methanol (5.00 mL) was added ethylenediamine (2.80 mL, 42.0 mmol). The reaction mixture was stirred under argon for 48 h at 30 °C. The reaction solution was evaporated, and then the residue was purified by precipitation with methanol / Et_2O three times, yielding PAMAM2 (1.3 g, 81.7% yield, 98% purity) as pale viscous oil. To a solution of PAMAM2 (1.3 g, 1.85 mmol) in methanol (10.0 mL) was added dropwise methyl acrylate (2.50 mL, 27.6 mmol). The reaction mixture was stirred

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under argon for 72 h at 30°C. The solvent was evaporated, and then the residue was purified by column chromatography on silica gel with EtOAc/MeOH (2/1), yielding 1.98 g PAMAM 2.5 (1.98 g, 78.9 % yield, 99% purity) as yellow oil. ^1H NMR (300 MHz, CDCl_3) δ 7.80 (t, J = 5.2 Hz, 2H), 7.13 (t, J = 5.2 Hz, 4H), 3.671 (s, 24H), 3.45 (d, J = 1.9 Hz, 2H), 3.28 (dd, J = 11.6, 5.8 Hz, 12H), 2.91 – 2.71 (m, 28H), 2.56 (tt, J = 15.7, 7.7 Hz, 12H), 2.48 – 2.30 (m, 28H), 2.21 (d, J = 2.1 Hz, 1H).

Scheme 6-1. The synthesis route of PAMAM2.5. **i)** Methyl acrylate, methanol, 30°C, 48 h. **ii)** Ethylenediamine, methanol, 30 °C, 48 h. **iii)** Methyl acrylate, methanol, 30°C, 72 h. **iv)** Ethylenediamine, methanol, 30 °C, 48 h. **v)** Methyl acrylate, methanol, 30°C, 72 h.

Synthesis of 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl succinate polyethylene glycol ester (HMPBA-pin-SA-PEG, monomer 4). 4-(Hydroxymethyl) phenylboronic acid (compound 1) (1 g, 0.65 mmol) was added into anhydrous THF followed by the addition of pinacol (0.93 g, 7.9 mmol). MgSO_4 (0.15 g) was then added into the mixture. The mixture was stirred at room temperature for 24 h and then filtered to remove the precipitate. The filtrate was evaporated by rotary evaporation and 40 mL of chloroform was added to dissolve the residue. The obtained solution was then washed with water (3×30 mL) and dried over Na_2SO_4 overnight. After filtration and evaporation, 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-

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2-yl) phenyl methanol (compound 2) (1.13 g, 58.6% yield) was obtained. ^1H NMR (300 MHz, CDCl_3) δ 7.79 (d, J = 8.0 Hz, 2H), 7.34 (d, J = 8.0 Hz, 2H), 4.68 (s, 2H), 1.34 (s, 12H)

Compound 2 (0.5 g, 2.11 mmol), succinic anhydride (0.75 g, 4.2 mmol), DMAP (10 mg, 0.1 mmol) were dissolved in anhydrous THF (10 mL). The mixture was stirred at room temperature for 36 h. Methanol (2 mL) was then added and continuously stirred for 1 h to hydrolyze the excess succinic anhydride. THF were evaporated under vacuum and chloroform (20 mL) was added to dissolve the residue. The solution was washed with HCl (0.1 M, 20 mL) and water (30 mL). The organic phase was dried over Na_2SO_4 overnight and then evaporated to obtain 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzyl succinate (compound 3) as colorless oil (0.68g, 71% yield). ^1H NMR (300 MHz, CDCl_3) δ 7.82 (d, J = 8.0 Hz, 2H), 7.36 (d, J = 7.9 Hz, 2H), 5.27–5.10 (m, 2H), 2.79–2.64 (m, 4H), 1.36 (s, 12H).

Compound 3 (0.7 g, 2.1 mmol), methoxy polyethylene glycol (mPEG, M_n = 2000) (3.4 g, 1.75 mmol) and DMAP (0.13 g, 1.05 mmol) were dissolved in anhydrous CH_2Cl_2 (10 mL), then DCC (0.86 g, 4.2 mmol) was added to the solution dropwise. The reaction was conducted at room temperature for 48 h. And then filtered to remove the precipitate. The solvent was evaporated and the crude product was dissolved in water and purified by dialysis. After vacuum freeze drying, monomer 4 as white solids was obtained. ^1H NMR (300 MHz, CDCl_3) δ 7.81 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 8.0 Hz, 2H), 5.15 (s, 2H), 3.78–3.58 (m, 190H), 2.70 (s, 4H), 1.35 (s, 12H).

Scheme 6-2. The synthesis route of DSP. **i)** Pinacol, magnesium sulfate (MgSO_4), tetrahydrofuran (THF), 25°C , 24 h. **ii)** Succinic anhydride, 4-dimethylaminopyridine (DMAP), THF, 25°C , 36 h. **iii)** Poly(ethylene oxide) ($M_n = 2000$), dicyclohexylcarbodiimide (DCC), DMAP, THF, 25°C , 48 h. **iv)** Palladium tetrakis (triphenylphosphine) ($\text{Pd}(\text{PPh}_3)_4$), potassium carbonate (K_2CO_3), methyltrioctylammonium chloride, toluene/ H_2O , 100°C , 2 h. **v)** Sodium azide (NaN_3), dimethylformamide (DMF). **vi)** Copper(I) bromide (CuBr), *N, N, N', N'', N'''*-pentamethyldiethylenetriamine (PMDETA), THF, 25°C , 48 h. **vii)** Ethylenediamine, methanol (MeOH).

Synthesis of (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) succinate polyethylene glycol ester end-capped poly{4-(4,4-bis(6-bromohexyl)-6-methyl-4H-cyclopenta[2,1-b:3,4-b'] dithiophen-2-yl)-7-methylbenzo[c][1,2,5]thiadiazole} (P1). 4,4'-Bis-(5-bromopentyl)-4H-cyclopenta[2,1-b:3,4-b']dithiophene (monomer 5) (50 mg, 0.075 mmol), 2,1,3- benzothiadiazole-4,7-bis (boronic acid pinacol ester) (monomer 6)

(27 mg, 0.068 mmol), Pd(PPh₃)₄ (3 mg, 0.03 mmol) and K₂CO₃ (122.5 mg, 0.69 mmol) were added in a 50 mL Schlenk tube. Then toluene (1.5 mL) with methyltrioctylammonium chloride (1 mg) and water (1.25 mL) were added to the reaction tube. The reaction vessel was degassed by freeze-pump-thaw circles for three times. The reaction was conducted at 100°C, when the solution turning to blue, monomer 4 (16.9 mg, 0.0075 mmol) (dissolved in 0.4 mL of toluene, also degassed by freeze-pump-thaw) solution was added into the tube. The mixture was stirred at 100°C overnight. The product was extracted with CH₂Cl₂ and washed by water (20 mL) and brine (30 mL). The organic phase was concentrated and powdered into excess methanol. The polymer was filtered and washed with methanol and acetone and then dried under vacuum for 24 h. ¹H NMR (300 MHz, CDCl₃) δ 8.11 (s, 2H), 7.95–7.83 (m, 1H), 7.61 (s, 3H), 6.91 (s, 2H), 3.64 (s, 12H), 3.35 (d, *J* = 16.6 Hz, 4H), 1.74 (s, 6H), 1.25 (s, 28H), 0.86 (d, *J* = 7.5 Hz, 6H). GPC data: *M*_n = 8414, PDI = 1.791.

Synthesis of (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) succinate polyethylene glycol ester end-capped poly{4-(4,4-bis(6-azidohexyl)-6-methyl-4H-cyclopenta[2,1-b:3,4-b'] dithiophen-2-yl)-7-methylbenzo[c][1,2,5]thiadiazole} (P2). P1 (30 mg) and sodium azide (12 mg) were dissolved in DMF (5 mL)/ THF (10 mL). The mixture was stirred at 25°C for 24 h. The solvent was evaporated and CH₂Cl₂ was added to dissolve the residue. The solution was washed with water and brine. The organic phase was collected and dried with Na₂SO₄. The organic phase was concentrated and powdered into excess methanol. The polymer was filtered and washed with methanol and acetone and then

dried under vacuum for 24 h. ^1H NMR (300 MHz, CDCl_3) δ 8.09 (s, 2H), 7.96–7.82 (m, 1H), 7.59 (s, 2H), 6.91 (s, 2H), 3.64 (s, 11H), 3.21 (d, J = 16.6 Hz, 4H), 1.73 (s, 6H), 1.26 (s, 28H), 0.88 (d, J = 7.5 Hz, 6H).

Synthesis of (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) succinate polyethylene glycol ester end-capped poly{4-[4,4-bis(4-PAMAM 3-1H-1,2,3-triazol-1-yl)hexyl]-6-methyl-4H-cyclopenta[2,1-b:3,4-b']dithiophen-2-yl}-7 methylbenzo[c] [1,2,5]thiadiazole} (DSP).

P2 (30 mg, 0.05 mmol), propargylamine core PAMAM 2.5 (0.3 g, 0.2 mmol) and CuBr (31.2 mg, 0.2 mmol) were dissolved in THF (10 mL), then PMDETA (233 μL , 1 mmol) was added to this solution. The mixture was stirred at 25°C for 24 h. The solvent was evaporated. Excess of CH_2Cl_2 was added to the residue, and washed with water and brine. The organic phase was collected and dried with Na_2SO_4 . The organic phase was concentrated and powdered into excess methanol. The polymer P3 was filtered and washed with methanol and acetone and then dried under vacuum for 24 h. Then, P3 (60 mg, 0.03 mmol) was dissolved in methanol (20 mL)/ THF (5 mL). Ethylenediamine (36 mg, 0.6 mmol) was added to this solution dropwise and slowly. The mixture was stirred at 60°C for 48 h. The solvent was evaporated, and the residue was dissolved in water, and purified by dialysis. After vacuum freeze-drying, DSP was obtained. ^1H NMR (300 MHz, MeOD) δ 8.06 (d, J = 26.7 Hz, 2H), 7.72 – 7.47 (m, 3H), 7.03 (d, J = 14.5 Hz, 3H), 6.61 (d, J = 3.6 Hz, 1H), 3.62 (d, J = 19.1 Hz, 106H), 3.01 (d, J = 4.2 Hz, 16H), 2.95 – 2.69 (m, 135H), 2.64 – 2.52 (m, 36H), 2.36 (dd, J = 20.1, 12.0 Hz, 72H), 1.72 (s, 6H), 1.30 (dd, J = 28.9, 17.8 Hz, 34H), 0.91 (s, 6H).

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Preparation of DSP nanoparticles. DSP (237 mg) was dissolved in methanol (7.9 mL) by bath sonication. The resulted DSP solution (30 mg/mL) was centrifuge with 300 g for 5 min to exclude the precipitate. Then, the SP solution (0.25 mL) was quickly injected into distilled-deionized water (10 mL) to form nanoparticles by self-assembly under continuous sonication with a bath sonicator (Branson 250). After sonication for additional 1 min, the DSP nanoparticle solution was dialyzed with dialysis membrane of 500 Da MWCO against distilled-deionized water for 48 h to exclude methanol. The dialyzed solution was concentrated by a rotate evaporation apparatus and the concentration of the final solution was determined by UV-Vis absorption according to its absorption coefficients. The DSP nanoparticle solution was concentrated to 1.26 mg/mL (based on the mass of DSP) and stored in dark at room temperature.

***In vitro* Photothermal Studies.** PBS (1x, pH 7.4) and PBS containing DSP (200 μ L, 132 μ g/mL) were exposed to laser irradiation at 808 nm (0.247-1.522 W cm⁻²). The temperature was monitored using a photothermal camera (F30W) every 30 s until reaching maxima after approximating 8 min. After the laser exposure, the temperature was continuously monitored every 30 s for 8 min. The heating and cooling were repeated six times to test the photothermal stability of DSP. The photothermal conversion efficiencies were calculated according to our previous report.^[25]

The construction of HSP70 modified-plasmids. HSP70 promoter sequence was synthesized by Intergrated DNA Technologies (IDT DNA, Inc). pHSP70-*Luc* plasmid was constructed through inserting the HSP70 promoter fragment (amplified with the primers pHSP70-*Luc*-F/R) in the front of luciferase reporter

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gene ORF of pGL3-Basic Vector (Promega Cat. No. E1751) by Kpn I/Hind III digestion and ligation method; pHSP70-*EGFP* plasmid was constructed through integrating the HSP70 promoter fragment (amplified with the primers pHSP70-*EGFP*-F/R) into the front of EGFP reporter gene ORF of pEGFP-N1-FLAG (Addgene Cat. No. 60360) construct by Gibson assembly (NEB, USA). The information of promoter and PCR primers was provided as follows:

Table 6-1. The Sequence of HSP70 and PCR primers.

Name	Sequence (5'-3')
HSP70 promoter	GCTGCGACAGTCCACTACCTTTTTTCGAGAGTGACTCC CGTTGTCCCAAGGCTTCCCAGAGCGAACCTGTGCGG CTGCAGGCACCGGCGCGTCGAGTTTCCGGCGTCCG GAAGGACCGAGCTCTTCTCGCGGATCCAGTGTTCCG TTTCCAGCCCCCAATCTCAGAGCGGAGCCGACAGAG AGC AGGGAACCCGGC
pHSP70- <i>Luc</i> -F	ATC <u>GGTACCG</u> CTGCGACAGTCCACTAC
pHSP70- <i>Luc</i> -R	TTCA <u>AGCTT</u> GCCGGGTTCCCTGCTCTC
pHSP70- <i>EGFP</i> -F	<u>CCGGA</u> CTCAGATCTCGAGCTGCTGCGACAGTCCACT ACCTTTTT
pHSP70- <i>EGFP</i> -R	<u>CACCATGGTGGCGACCGGTG</u> GCCGGGTTCCCTGCT CTC

Plasmid Preparation and Complexation. Plasmids were amplified using *Escherichia coli* DH5 α and extracted using EndoFree Plasmid Maxi Kit (Qiagen, Cat. No.12362) following the supplier's protocol. The final concentration was

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measured by NanoDrop 2000c (Thermo Fisher). Then the plasmids (10 μ L, 0.1 mg/mL) were mixed with DSP with indicated N/P ratios and supplemented to the final volume of 20 μ L with distilled-deionized water of nucleus and endotoxin free. The complex mixture was incubated at room temperature for 30 min. Then the CaCl_2 solution (20 μ L, 100 mM) was added into the complex solution and the resulted solution was incubated for another 30 min to form DSP/plasmid complex used to do *in vitro* characterization or following transfection.

Agarose Gel Electrophoresis. Agarose gels (1%) were prepared using agarose powder purchased from Bio-Rad (Certified™ Molecular Biology Agarose) and immersed in 1× Tris/boric acid/EDTA (TBE) buffer, prepared by diluting 10×stock solutions, Bio-Rad). Before loading the gel, DNA loading buffer (1 μ L, 10×) solution was mixed with the sample solutions (9 μ L). All the samples were then loaded into the wells of the gel and electrophoresed on a horizontal electrophoresis system (Mini-Sub Cell GT; Bio-Rad; electrode spacing 10 cm) for 40 min at 120 V. At last, a gel imaging system (SynGene G: Box) was used to image. The DNA loading buffer was prepared by mixing glycerol (3.9 mL), SDS (500 μ L, 10 w/v%), EDTA (200 μ L, 0.5 M), bromophenol blue (0.025 g), xylene cyanol (0.025 g), Gel Red nucleic acid gel staining (10 μ L, Biotium Inc. Cat. No. 41003) and supplementing the total volume to 10 mL with distilled-deionized water.

Cell Culture. HeLa cervical adenocarcinoma epithelial cells were purchased from the American Type Culture Collection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Cat. No. 10569-010) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat. No. 10270-106) and penicillin-streptomycin antibiotics (Gibco, Cat. No. 15140-122). The cells

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were maintained in an atmosphere containing 5% CO₂ and 95% humidified air at 37°C.

Cells transfection. Cells were seeded in 24-well plates (50,000 cells/well in 500 µL) and incubated overnight. The entire 40 µL DSP/plasmids complexes were added to cells cultured on 24-well plates. Given that each well contained 0.4 mL DMEM (with 100 mM chloroquine), the addition of the complex mixture resulted in a final Ca²⁺ concentration of 4.5 mM. For EGFP as the reporter proteins, the cells (100,000 cells/well in 1 mL) were seeded in the dishes for confocal imaging (Dia. 35 mm). After overnight incubation, 80 µL DSP/plasmids complexes were added into dishes containing 0.8 mL DMEM (with 100 mM chloroquine). After incubation for another 6 h at 37°C, the transfection mixture containing residual DSP/plasmids complexes was removed. The transfected cells were washed with PBS buffer and cultured with fresh media. The transfected cells were incubated for more 36-48 h and used for gene expression analysis or image studies.

Cytotoxicity Test. Cells were seeded in 96-well plates (3000 cells/well in 100 µL) and incubated for 12 h. DSP were added to the cell culture medium and incubated with cells for 6 h. The medium was then removed and added with fresh media to incubate for another 24 h. The MTS solution (20 µL, Promega Cat. No. G3581) was then added to the wells. After incubation for 4 h, the absorbance measurement at 490 nm was performed using a SpectraMax M5 microplate/cuvette reader. Cell viability was expressed as the ratio of the absorbance of the cells incubated with DSP to that of untreated cells.

***In vitro* Photothermal Activation.** After incubation with fresh media for 48 h, the transfected cells (with luciferase as reporter proteins) were suspended in the

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fresh culture medium and divided into two dispersions. Once reaching the balance with the room temperature, one of the suspensions were irradiated with 808 nm laser light (0.75 W cm^{-2} , 808 nm high power NIR Lasers purchased from CNI Co., Ltd.) for 30 min at room temperature. The 808 nm laser illuminated in the tubes with cells suspensions vertically. Part of cells were taken out to measure the expression level of luciferase and cell viability (MTS used as described in Cytotoxicity Test) at the time interval of 5 min, taking the cells without laser irradiation as the control. After irradiation, the cells were incubated at 37°C containing 5% CO_2 and continuously monitored. During the irradiation process, the temperatures of cell suspensions were longitudinally monitored by thermal camera (F30W, Nippon Avionics Co., Ltd.). The luciferase expression was measured with Luminometer system (Promega GloMAX, Cat. No. E5311) after mixing cell pellets (50 μL) with *D*-luciferin (50 μL , 468 μM in TMCA buffer as supplier's protocol) and reaction for 5 min at room temperature. For cells transfected with EGFP as reporter proteins, the laser irradiation was performed after incubation for 36 h. After laser irradiation of 30 min, the media were replaced with fresh media and cells were incubated overnight. Then cells were stained with Hoechst 33342 (NucBlue Live ReadyProbesReagent, Thermo Fisher) for the nucleus, imaged using the confocal microscope (Zeiss, LSM 800) for EGFP expression and analyzed using analysis software (Zeiss, Zen microscope software). The excitation and emission wavelength for cell imaging were 640/655-710 nm for DSP, 488/500-550 nm for EGFP and 405/410-470 nm for Hoechst.

***In vivo* Photothermal Activation.** All animal studies were performed in compliance with the Guidelines established by the Institutional Animal Care and

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Use Committee (IACUC), Sing Health. After transfected with luciferase as reporter proteins and incubation for 48 h, cells (~ 5,040,000 cells, 300 μ L) were suspended in fresh media and mixed with *D*-luciferin (8 mg/mL, 300 μ L) in TMCA buffer. Then the mixtures were injected at two positions on the back under the skin of a nude 8-week-old female mouse with equal volume (300 μ L with 2,520,000 cells, $n = 3$). The injection site on left was irradiated with 808-nm laser light at the power of 0.42 W cm⁻² with the frequency as indicated (Figure S10, Supporting Information). The 808 nm laser (808 nm high power NIR Lasers purchased from CNI Co., Ltd.) illuminated at the skin of mice vertically. During laser irradiation, temperature of the mouse skin and luciferase expression were longitudinally monitored by thermal camera (F30W, Nippon Avionics Co., Ltd.) and IVIS imaging system (exposure time: 60 s, IVIS-CT machine, PerkinElmer), respectively.

Data Analysis. BL signal intensities of mice were measured by ROI analysis using Living Image software for IVIS imaging system. Thermal images were analyzed using NS9205 viewer program. Results were expressed as the mean $\pm$ SD deviation unless otherwise stated. And the average calculations were based on the data obtained from separate experiments conducted on different days.

6.3 Results and Discussions

DSP was synthesized via a graft-on approach (**Schemes 6-1** and **6-2**). Palladium-catalyzed Suzuki polymerization of PEG-functionalized endcapping agent, 4,4'-bis-(5-bromopentyl)-4*H*-cyclopenta[2,1-b:3,4-b']dithiophene, and 2,1,3- benzothiadiazole-4,7-bis (boronic acid pinacol ester) led to the PEG-

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endcapped triblock copolymer (P1). The bromide groups of P1 were converted into the azide groups, allowing it to undergo the copper(I)-catalyzed alkyne-azide cycloaddition (CAAC) reaction to graft PAMAM2.5 onto it. At last, the resulted grafted SP (P3) reacted with ethylenediamine *via* aza-Michael addition reaction to convert PAMAM2.5 into the third-generation form (PAMAM3), affording the final polymer (DSP). The intermediate polymers and DSP were characterized by NMR spectroscopy. DSP could be directly dissolved in aqueous solution and buffer, making it feasible for gene delivery.

Figure 6-2. *In vitro* characterization of DSP and its transfection capability. **a)** Absorption and fluorescence spectra of DSP in 1×PBS at pH 7.4. **b)** Solution temperatures of DSP (132 mg mL⁻¹) as a function of laser irradiating time. The laser wavelength was 808 nm and its power intensity was 0.67 W cm⁻². Inset: thermal images of DSP and PBS solutions at their respective maximum temperatures. **c)** Agarose gel electrophoresis of DNA, DSP, and DSP/DNA nanocomplexes at various N/P ratios. **d)** DLS profiles of DSP and DSP/DNA nanocomplex with the N/P ratio of 5:1. Inset: representative TEM images of DSP and DSP/DNA complex; scale bars: 100 nm. **e)** Hydrodynamic diameters and zeta potential of DNA, DSP, and DSP/DNA nanocomplexes at various N/P ratios. **f)** Transfection efficacy of DSP/DNA nanocomplexes monitored as BL at various N/P ratios. Error bars represented SD of three separate measurements.

DSP had a broad absorption peak in the NIR region between 500 to 900 nm with a maximum at 635 nm, and its fluorescence maximum was located at 791 nm (**Figure 6-2a**). Such a NIR optical feature is beneficial for *in vivo* applications, enabling utilization of NIR light excitation to generate both fluorescence and photothermal signals. Upon continuous laser irradiation at 808 nm, the temperature of DSP solution gradually increased and reached plateau of 58.8°C at $t=480$ s (**Figure 6-2b**). The photothermal conversion efficiency of DSP was calculated to be $44.2 \pm 2.8\%$, which was higher than gold nanorods (ca. 17%).^[25] Moreover, the maximum temperature of DSP could be adjusted using different laser powers and it remained nearly unchanged after 6 reversible heating and natural cooling cycles, proving its excellent photothermal stability (**Figure 6-3**).

Figure 6-3. Photothermal performance of DSP. **(a)** Stability study of DSP under

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the photothermal heating and natural cooling cycles. **(b)** Solution temperatures of DSP as a function of laser irradiating time with different laser power. The laser wavelength was 808 nm with the power intensity of 0.67 W cm^{-2} . The concentrations of DSP were $132 \mu\text{g mL}^{-1}$ in $1\times$ PBS (pH 7.4).

To evaluate the complexation of DSP with genes, the pSV40-Luc plasmid (luciferase as the reporter protein) was used as an example, and the gel retardation, diameters, zeta potentials of the DSP/plasmid nanocomplexes were studied at different nitrogen/phosphate (N/P) ratios. Agar gel electrophoresis revealed that plasmids were fully loaded by DSP at a low N/P ratio of 5:1, because nearly all the plasmids were retarded in the slots and no migration band was observed (**Figure 6-2c**). DLS and TEM were conducted to analyze the condensation between DSP and plasmids. DSP itself had a spherical morphology with the average hydrodynamic diameter of about 15 nm (**Figure 6-2d**), remaining nearly unchanged after storage for 42 days. After complexation with plasmids, the morphology remained spherical. However, the diameters dramatically decreased from 396 to 38 nm with increasing the N/P ratio from 0.54:1 to 7.5:1 (**Figure 6-2e**). This was well documented for non-viral vectors,^[152] and could be attributed to enhanced DSP/plasmid condensation with increased N/P ratios. The diameters slightly changed when the N/P ratio was larger than 7.5:1, indicating the formation of compact and stable nanocomplexes. The zeta potential of the nanocomplexes increased from 3 to 29 mV with increasing the N/P ratio and reached maximum at the N/P ratio of 5:1 (**Figure 6-2e**). These data proved that DSP was an ideal carrier with the efficient gene loading and condensation capabilities.

The ability of DSP for *in vitro* gene transfection was studied in HeLa cells using luciferase genes (pSV40-Luc) as the reporter gene. The transfection efficacy

was evaluated by the expression level of luciferase, which can be quantified with bioluminescence (BL). With increased N/P ratio, the BL intensity gradually increased, and reached maximum at the N/P ratio of 7.5:1 (**Figure 6-2 f**). This N/P ratio was consistent with the gene loading and condensation data, wherein the compact and stable DSP/plasmid nanocomplexes formed. When the N/P ratios were above 7.5:1, the BL intensity slightly decreased, probably caused by the over condensation that blocked plasmid release. In addition to the excellent performance in transfection, DSP showed good cytocompatibility even at the high concentration.

To test the photothermal ability of DSP to remotely control transgene expression, the gene of enhanced green fluorescence protein (EGFP) or luciferase was cloned downstream of HSP70, and activation was conducted on HeLa cells. The HSP70 sequences were designed according to the previous literature.^[153] HeLa cells were incubated with the DSP/pHSP70-EGFP plasmid nanocomplexes at the optimal N/P ratio of 7.5:1, recovered with fresh media and treated with or without laser irradiation at 808 nm for 30 min. With the NIR fluorescence of DSP, confocal fluorescence microscopy could be applied to track the location of DSP and the expression of EGFP in cells. After incubation, the NIR fluorescence from DSP was observed in the cytoplasm for both laser treated or non-treated cells, indicating the efficient cellular internalization of DSP/pHSP70-EGFP plasmid nanocomplexes (**Figure 6-4a**). The location of DSP was also ideal to trigger HSP70-regulated gene, as associated HSF monomers located in cytoplasm.^[150] Only upon laser irradiation, green fluorescence was observed, showing the remotely controlled expression of EGFP. Such a laser-triggered expression was not observed in the absence of

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HSP70, confirming that the activation was triggered by DSP-mediated photothermal transduction.

Figure 6-4. *In vitro* DSP-mediated photothermal activation of gene expression. **a)** Fluorescence images of HeLa cells transfected with DSP/pHSP70-EGFP nanocomplexes with a N/P ratio of 7.5:1 with (+Laser) and without (-Laser) laser irradiation at 808 nm (0.75 W cm^{-2}) for 30 min. Quantification of changes in the BL intensities (**b**) and temperature (**c**) of HeLa cells transfected with DSP/pHSP70-Luc nanocomplexes with the N/P ratio of 7.5:1 with and without laser irradiation at 808 nm (0.75 W cm^{-2}) for 30 min. The excitation and emission wavelengths were 640/655–710 nm for DSP, 488/500–550 nm for EGFP and 405/410–470 nm for Hoechst. Error bars represented SD of three separate measurements.

To quantify the DSP-mediated photothermal activation of gene expression, similar cell experiments were conducted on HeLa cells using the DSP/pHSP70-Luc plasmid nanocomplexes at the optimal N/P ratio of 7.5:1. The luciferase expression level was longitudinally monitored for approximately 70 000 cells and

quantitatively described as the incremental percentage of BL intensities ($\Delta BL/BL_0$; **Figure 6-4b**). Meanwhile, the solution temperature was monitored along with the laser irradiation (**Figure 6-4c**). The highest expression appeared after laser irradiation for only 5 min. At this time point, $\Delta BL/BL_0$ was 25-fold higher than that without irradiation, indicating the significantly enhanced gene expression level triggered by DSP in a rapid manner. Note that during the laser irradiation, the solution temperature was increased by about 2.4°C at most. Such a temperature increase did not cause obvious cytotoxicity. These cell data verified that DSP served as the cytocompatible photothermal nanocarrier and nanotransducer to remotely control gene expression. Note that light irradiation of cellular medium alone could trigger transgene expression due to the heat generation from water absorption.^[154] However, due to the weak photothermal effect of water, it requires high power and long irradiation time, which may cause cytotoxicity issue.

To validate the potential of DSP for *in vivo* activation of gene expression, the HeLa cells pellets (ca. 2520000 cells) transfected with DSP/pHSP70-Luc nanocomplexes were subcutaneously injected into the back of living mice (**Figure 6-5a**). The BL images were longitudinally recorded and quantified for the areas with or without laser irradiation. To minimize the temperature increase, the laser treatment was set in a discontinuous manner (**Figure 6-6**). BL intensity gradually increased along with laser irradiation and reached maximum at 19 min (**Figures 6-5b,c**), while it slightly changed without laser irradiation. Such a phenomenon was not observed for the cells transfected with luciferase gene cloned downstream of the constitutive promoter (SV40). This proved that the expression of luciferase was selectively controlled by the photothermal

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generation of DSP under high tissue penetrating NIR light irradiation at 808 nm. At $t = 19$ min, $\Delta\text{BL}/\text{BL}_0$ was 4.5-fold higher than that without laser irradiation. Note that the temperature increased only by 5.2°C at most during laser irradiation (**Figure 6-5d**). The highest temperature was 39.5°C below the threshold temperature to induce apoptosis (43°C). These *in vivo* data clearly validate the feasibility of using DSP for remotely controlled gene expression at desired location and time in living animals.

Figure 6-5. *In vivo* DSP-mediated photothermal activation of gene expression. **a)** Illustration of laser irradiation in living nude mice. **b)** whole-animal BL images at representative times with (left side of animal) and without (right side of animal) laser irradiation at 808 nm (0.42 W cm^{-2}). The red/white dashed circles indicated the respective regions subcutaneously implanted with the HeLa cells pellets (2520000 cells) transfected with DSP/pHSP70-Luc nanocomplexes and treated with/without laser irradiation. Quantification of changes in the BL intensities (**c**) and temperature (**d**) as a function of time with (red) and without (black) laser irradiation. Error bars represented SD of three separate measurements.

Figure 6-6. *In vitro* viability of HeLa cells treated with DSP at different concentrations. The percentage cell viability of treated cells was calculated relative to that of cells without treatment (viability was arbitrarily defined as 100%, * $p < 0.05$). Error bars represent SD of five separate measurements.

6.4 Conclusions

In conclusion, I have synthesized a multifunctional water-soluble polymer (DSP) that can not only efficiently deliver genes into cells but also convert NIR light into heat for photothermal applications. In association with heat inducible promoter regulated genes, DSP acts as the intracellular photothermal nanotransducer to remotely activate gene expression upon NIR light irradiation at 808 nm. Real-time monitoring of luminescence indicates that gene expression can be rapidly triggered and significantly enhanced by 25- and 4.5-fold within minutes in living cells and mice, respectively. Thus, this study provides an innovative polymeric approach to noninvasively control gene expression at a designated location and time. To our knowledge, this is the first polymer system that integrates the functionalities of gene delivery and photothermal activation. In view of its completely benign organic components and NIR absorption with high-tissue penetration, such polymers hold promise to be developed into nanomedicines for controlled therapeutic transgene dosing.

Chapter 7 Conclusions and Future Work

7.1 Conclusions

Firstly, I successfully develop an intraparticle molecular orbital engineering approach to induce the PET process between the two components inside nanoparticles upon light irradiation, which results in the enhancement of PA brightness and PTT efficacy at the same time. This study not only introduces a category of purely organic optical theranostics but also highlights a molecular guideline to amplify the effectiveness of light intensive imaging and therapeutic nanosystems.

Secondly, I successfully design a reaction-based PA probes by simply conjugated the reactive groups (1,3-cyclohexanedione) on the SPN surface. The resulted SPNs are able to real-time monitor the ROS level inside cells or tumor sites through recognizing the oxidative products, sulfenic acids, at the very beginning state of the appearance oxidative stress. This study not only demonstrates the first reaction-based PA probes with submolecular level recognition ability but also highlights the opportunities provided by hybrid nanoparticles for advanced molecular imaging.

Thirdly, I successfully develop a biodegradable SPNs by introducing the vinylene bonds in the backbone of the polymers which can be degraded by the enzyme, myeloperoxidase, existing in blood. Meanwhile, the appearance of vinylene bonds enhances photothermal conversion capability of SPNs resulting in the improved PA brightness and PTT efficacy. Our study thus reveals a

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general molecular design to enhance the biodegradability of optically active polymer nanoparticles while dramatically elevating their imaging and therapeutic capabilities.

Finally, I successfully develop a dendrimer structure (PAMAM) modified SPN with gene loading capabilities. Upon light irradiation, this SPNs play the role of photothermal transducer to generated heat signals and trigger the expression of genes under the control of the inducible promoter (HSP70). This study provides a promising approach to optically regulate transgene systems for on-demand therapeutic transgene dosing. It broadens the biomedical applications of SPNs beyond imaging and phototherapy.

In this thesis, I successfully develop the general strategies to improve SPN properties for their better utilization in photothermal related theranostic applications. These improvements shorten the distance between researches and clinics in terms of the biomedical applications of SPNs. Additionally, I achieve the noninvasive regulation of gene expression with the utilization of SPNs. Inspired by this, it is promising to explore the feasibility to employ SPNs as the regulation motifs to control other cellular events, which may provide the hints to uncover the pathology mechanism leading to the development of new therapeutic methods.

7.2 Future Work

I have verified the successful application of SPNs to regulate the gene expression at cellular and mice level. However, our work is just a proof-of-concept application without employing any therapeutic genes to demonstrate the

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controllable therapeutic effect. Thus, the future work can be conducted as the following directions:

(1) The general methods to improve the brightness, specificity and biodegradability of SPNs have been developed. They are not just efficient in my work and can be used to develop SPNs with different optical properties. In the future, these methods can be combined together to develop a “super-SPN” with improved PA signals, specificity to the sub-molecular variation and biodegradability under biological oxidative environment.

(2) The genes of tumor suppresser proteins, such as P53 which is downregulated in tumor cells,^[155] can be incorporated in the system and under the regulation of HSP70. After complexed with SPNs to form nanocomplex, the oncogenes are more likely to be delivered to the tumor site because of the widely observed EPR effect. Appropriate modification of target groups on the surface of nanocomplexes can further enhance the accumulation of oncogenes in tumors.^[63] Moreover, the gene expression is only activated by the light irradiation which double confirms that the therapeutic proteins are present and take effect in disease sites. Thus, it may appear as a powerful therapeutic method with excellent selectivity to avoid the severe harm to the normal tissues.

(3) Cancer is a disease with multiple genetic disorders. Meanwhile, the upregulation or downregulation of one kind of proteins is usually ineffective because tumor cells can bypass the affected metabolic pathways and maintain their normal growth.^[156] As such, the delivery of microRNA to tumors sites appears as a better way as it can disrupt the expression of multiple proteins. SPNs can also design to deliver microRNA and regulate its release at tumor

sites. For example, the SPNs can be prepared with amine groups on the nanoparticle surface which can be reacted with phosphate group of DNA through the 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) mediated conjugation. Then, the single strand DNA, which is partially complementary with the therapeutic microRNA, can be conjugated to SPNs. After that, the therapeutic microRNA is loaded through the hybridization to form hydrogen bonds with the single strand DNA on nanoparticles. Upon light irradiation, microRNA is released because the local temperature increases to be higher than the annealing temperature resulting in the dehybridization of the therapeutic microRNA and single strand DNA. As such, therapeutic microRNA can be controllable release responsive to the light irradiation.

(4) SPNs also show photodynamic properties and can be used in afterglow imaging and photodynamic therapy. The fundamental studies can be carried out to explore the basic mechanism to determine the ratio of excited electrons to facilitate the generation of singlet oxygen ($^1\text{O}_2$). The general methods to improve the photodynamic applications of SPNs can also be developed.

(5) The property of SPNs to generate ROS upon light irradiation can also be employed to regulate the gene expression. For example, the low molecular PEI is designed to be conjugated to the branches of SPs with a ROS responsive linker between themselves. The initial low molecular PEI is incapable to load genes; while after conjugation, the increasing density of amine groups endows the formed polymers (SP-PEI) with the gene carrying capability. After light irradiation, the generated ROS breaks the linker between the PEI and SP backbone resulting in the release of genes and subsequent gene expression. Thus, the gene release responsive to the ROS generation can be used as a

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strategy to control gene therapy at designated time and location, which significantly reduce the side effects resulted from the lack of selectivity.

In conclusion, I have developed the rational molecular and nanoparticle designing strategies to improve the features of SPNs including brightness, specificity and biodegradability beneficial to the traditional photothermal applications. In addition, I have broadened the applications beyond imaging and therapy to control the cellular events. In the future, photodynamic applications of SPNs are worth to be well studied. The improvement of photothermal and photodynamic properties of SPNs is good for the clinical translation and facilitates the development of novel applications.

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