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SINGAPORE

**Semiconducting Polymer Nanoparticles for Multimodal
Biophotonics**

YUYAN JIANG

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2020

**Semiconducting Polymer Nanoparticles for
Multimodal Biophotonics**

YUYAN JIANG

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

2020

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.

2020.5.31

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Date

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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.

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Prof Kanyi Pu

Authorship Attribution Statement

This thesis contains material from 8 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 1 and **2** are published as (i) **Y. Jiang** and K. Pu. Multimodal biophotonics of semiconducting polymer nanoparticles *Accounts of Chemical Research* **51**, 1840-1849 (2018); (ii) **Y. Jiang** and K. Pu. Advanced photoacoustic imaging applications of near-infrared absorbing organic nanoparticles. *Small* **13**, 1700710 (2017); (iii) **Y. Jiang** and K. Pu. Molecular fluorescence and photoacoustic imaging in the second near - infrared optical window using organic contrast agents. *Advanced Biosystems* **2**, 1700262 (2018).

The contributions of the co-authors are as follows:

- I prepared the manuscript drafts. The manuscript was revised by Prof Kanyi Pu.

Chapter 3 is published as **Y. Jiang**, J. Huang, X. Zhen, Z. Zeng, J. Li, C. Xie, Q. Miao, J. Chen, P. Chen, K. Pu. A generic approach towards afterglow luminescent nanoparticles for ultrasensitive in vivo imaging. *Nature Communications*, **10**, 2064 (2019).

The contributions of the co-authors are as follows:

- K. Pu. and Y. Jiang conceived the research plan.
- Y. Jiang, J. Huang, X. Zhen, Z. Zeng, J. Li, C. Xie, and Q. Miao performed the experiments.
- J. Chen and C. Peng synthesized carbon dots.
- Y. Jiang analyzed the data.
- Y. Jiang and K. Pu wrote the manuscript.
- All authors contributed to the discussion of results and comments on manuscript revision.

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The contributions of the co-authors are as follows:

- K. Pu and Y. Jiang conceived the research plan.
- Y. Jiang conducted the majority of chemical synthesis and animal work. P. K. Upputuri, C. Xie, Z. Zeng, A. Sharma, X. Zhen, J. Li, J. Huang provided powerful support for these experiments.
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- Y. Jiang analyzed the data.
- Y. Jiang and K. Pu wrote the manuscript.
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- K. Pu and Y. Jiang conceived the research plan.
- Y. Jiang conducted the majority of chemical synthesis and animal work. J. Li, Z. Zeng, C. Xie and Y. Lyu provided powerful support for these experiments.
- Y. Jiang analyzed the data.

- Y. Jiang and K. Pu wrote the manuscript.
- All authors contributed to the discussion of results and comments on manuscript revision.

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- K. Pu, R. Zhao and Y. Jiang conceived the concept and designed the research.
- Y. Jiang performed nanoparticle preparation.
- Y. Jiang, X. Zhao, P. K. Upputuri and M. Pramanik conducted PA characterization.
- Y. Jiang and H. Duan performed photothermal characterization.
- Y. Jiang, H. Sun, X. Han and Y. Miao conducted western blotting.
- Y. Jiang, J. Huang. and J. Li performed in vivo anti-cancer therapy.
- K. Pu, R. Zhao and Y. Jiang wrote the manuscript draft.
- All other authors contributed to interpretation of the results and the writing of this manuscript.

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Abbreviations

(4,8-Didodecylbenzo[1,2-b:4,5-b']dithiophene-2,6-diyl)bis(trimethylstannane): BDT
2,6-Dibromo-4,4-bis(6-bromohexyl)-4H-cyclopenta[2,1-b:3,4-b']dithiophene: CPDT-Br
2',7','-Dichlorofluorescein diacetate: DCFH-DA
3,3'-Dioctadecyloxacarboyanine perchlorate: DiO
4,9-Dibromo-6,7-bis(4-hexylphenyl)-[1,2,5]thiadiazolo[3,4-g]quinoxaline: TDQ
4',6-Diamidine-2'-phenylindole dihydrochloride: DAPI
5,5'-Dithiobis(2-nitrobenzoic acid): DTNB
Afterglow luminescent nanoparticle: ALNP
Anti-cytotoxic T-lymphocyte antigen-4: aCTLA4
Arachidonic acid: AA
Arachidonyl-CoA: AA-CoA
Benzobisthiadiazole: BBT
Benzodithiophene: BDT
Benzothiadiazole: BT
Bis(2,4,6-trichlorophenyl) oxalate: TCPO
Camptothecin: CPT
Carboxyhemoglobin saturation: SCO
Caspase-3: Cas-3
CdSe/ZnS core-shell: QD630
Chemically initiated electron exchange luminescence (CIEEL)
Chlorin e6: Ce6
Computed tomography: CT
Confocal laser scanning microscopy: CLSM
Cypate: Cy
Damage-associated molecular pattern: DAMP
Dendritic cell: DC
Diketopyrrolopyrrole: DPP
Donor-acceptor: D-A
Doxorubicin: DOX

Dulbecco's modified eagle medium: DMEM
 Dynamic light scattering: DLS
 Electrospray ionization mass spectrometry: ESI-MS
 Enhanced permeability and retention: EPR
 Ferroportin-1: FPN-1
 Food and drug administration: FDA
 Glutathione peroxidase 4: GPX4
 Glutathione: GSH
 Hematoporphyrin: HP
 Hematoxylin and eosin staining: H&E staining
 Hemoglobin oxygen saturation: SO₂
 Hepatocyte growth factor: HGF
 Hybrid semiconducting nanozyme: HSN
 Hydrogen peroxide: H₂O₂
 Hydroxyl radical: ·OH
 Hydroxyl radical: ·OH
 Hypoxia responsive element: HRE
 Immunogenic cell death: ICD
 Indocyanine green: ICG
 Institutional animal care and use committee: IACUC
 Internal conversion: IC
 Intersystem crossing: ISC
 Intramolecular charge transfer: ICT
 Lipid peroxidation: LPO
 Lipopolysaccharide: LPS
 Long-chain-fatty-acid-CoA ligase 4: ACSL4
 Magnetic resonance imaging: MRI
 Maximum permissible exposure: MPE
 Metal-organic framework: MOF
 Methoxypolyethylene glycol dibenzocyclooctyne: DBCO-mPEG2000
 Methylene blue: MB

Monocarboxylate transporter: MCT
Mononuclear phagocyte system: MPS
Myeloperoxidase: MPO
N,N-dimethylformamide: DMF
Near-infrared: NIR
Nile red: NR
Nuclear remodeling and deacetylation: NuRD
p300 and cyclic adenosine monophosphate response element binding protein: p300/CBP
Perfluorocarbon: PFC
Perylene tetracarboxylic diimide: PDI
Phospholipid: LH
Photoacoustic: PA
Photodynamic therapy: PDT
Photothermal conversion efficiency: PCE
Photothermal nanotherapeutics: PTN
Photothermal therapy: PTT
Poly(9,9' -dioctylfluorenyl-2,7-diyl): PFO
Poly(lactic-co-glycolic) acid: PLGA
Poly[(9,9' -dioctyl-2,7-divinylene fluorenylene)-alt-(9,10-anthracene)]: PFVA
Poly[(9,9' -dioctylfluorenyl-2,7-diyl)-alt-(benzo[2,1,3]thiadiazol-4,7-diyl)]: PFBT
Poly[2,7-(9,9' -dioctylfluorene)-alt-4,7-bis(thiophen-2-yl)benzo-2,1,3-thiadiazole]:
PFODBT
Poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene]: MEHPPV
Polyphenylenevinylene: PPV
Polypyrrole: PPy
Polyvinylidene fluoride: PVDF
Resorufin: Reso
Scanning transition electron microscopy-energy dispersive X-ray: STEM-EDX
Second near-infrared: NIR-II
Secondary energy transfer: SET
Semiconducting polymer amphiphile: SPA

Signal-to-noise ratio: SBR
 Signal-to-noise ratio: SNR
 Singlet oxygen sensor green: SOSG
 Singlet oxygen: $^1\text{O}_2$
 Sodium/bicarbonate cotransporter: NBC
 Squaraine dye: SQ
 Target to background ratio: TBR
 Tetrahydrofuran: THF
 The highest occupied molecular orbital: HOMO
 The lowest occupied molecular orbital: LUMO
 Transmission electron microscope: TEM
 Transport mean free path: TMFP
 Triplet state: T_n
 Tumor associated antigen: TAA
 Tumor necrosis factor- α : TNF- α
 Ultrasound: US
 Vascular cell adhesion molecule-1: VCAM-1
 Very late antigen 4: VLA-4

Abstract

Biophotonics which studies and manipulates interactions between optics and biological system plays an increasingly important role in modern biomedicine. As an emerging multidisciplinary paradigm, biophotonics converges chemistry, life sciences, optics and bioengineering, offering unique opportunities for sensitive imaging of biological or pathological events and photo-related therapeutic intervention. Despite these merits, development of biophotonics into clinical or preclinical translations is greatly hampered due to the lack of suitable photoactive biomaterials that have multimodal performance whereas minimal in vivo toxicity. With the merits of tunable photophysical properties, flexible molecular amenability and excellent biocompatibility, semiconducting polymer nanoparticles (SPNs) as a newly emerged family of optical agents make an ideal nanopatform to perform multimodal biophotonics. However, the exploitation of SPNs in biophotonics is still in its infancy and requires substantial investigation. In this regard, research in this thesis seeks to delve into the molecular engineering of SPNs so as to explore the potential of SPNs to address the critical challenges in multimodal photonics, which include afterglow imaging with ultrahigh sensitivity, photoacoustic (PA) imaging by metabolizable contrast agents, and advanced combinational phototherapies to improve anticancer efficacy.

In the first project, we design a generic approach to transforming SPNs into afterglow luminescent nanoparticles (ALNPs) for ultrasensitive in vivo imaging in deep tissue. Afterglow imaging with long-lasting luminescence after cessation of light excitation provides opportunities for ultrasensitive molecular imaging; however, the lack of versatile

afterglow agents with tunable emission channels impedes its exploitation in clinical settings. In this regard, we develop a generic approach by integrating a cascade photoreaction into a single-particle entity, enabling ALNPs to chemically store photoenergy and spontaneously decay it in an energy-relay process. Not only the afterglow profiles of ALNPs can be finetuned to afford emission from visible to near-infrared (NIR) region, but also their intensities can be predicted by a mathematical model. The representative NIR ALNPs permit rapid detection of tumor in living mice with a signal-to-background that is more than three orders of magnitude higher than that of NIR fluorescence. The biodegradability of the ALNPs further heightens their potential for ultrasensitive in vivo imaging.

In the second project, we synthesize metabolizable SPNs as contrast agents for PA imaging in second near-infrared (NIR-II) window. PA imaging in the second near-infrared (NIR-II) window (1000-1700 nm) holds great promise for deep-tissue diagnosis due to the reduced light scattering and minimized tissue absorption in this region; however, exploration of such non-invasive imaging technique is greatly constrained by the lack of biodegradable NIR-II absorbing agents. The metabolizable NIR-II SPNs are composed of a π -conjugated yet oxidizable optical polymer as the PA generator and a hydrolysable amphiphilic polymer as the particle matrix to provide water solubility. They are readily degraded by myeloperoxidase and lipase abundant in phagocytes, transforming themselves from the non-fluorescent nanoparticles (30 nm) into the NIR fluorescent ultra-small metabolites ($\sim$ 1 nm). As such, these NIR-II PA nanoagents can be effectively cleared out via both hepatobiliary and renal excretions after systematic administration, leaving no toxicity to living mice. More importantly, the nanoagents possess good photothermal conversion

efficiencies, and emit bright PA signals at 1064 nm, enabling sensitive NIR-II PA imaging of both subcutaneous tumor and deep brain vasculature shielded by intact skull in living mice at a low systematic dosage. In addition, this study provides a generalized molecular design towards organic metabolizable semiconducting materials for biomedical optical applications in the NIR-II windows.

In the third project, we make use of amphiphilic SPNs as drug delivery system to encapsulate chemotherapeutics for fluorescence/PA imaging-guided chemo-photothermal therapy. Chemo-photothermal nanotheranostics has the advantage of synergistic therapeutic effect, providing opportunities for optimized cancer therapy. However, current chemo-photothermal nanotheranostic systems generally comprise more than three components, encountering the potential issues of unstable nanostructures and unexpected conflicts in optical and biophysical properties among different components. To simplify chemo-photothermal nanotherapeutics, we synthesize an amphiphilic semiconducting polymer PEG-PCB and utilize it as a multifunctional nanocarrier. PEG-PCB has a semiconducting backbone that not only serves as the diagnostic component for NIR fluorescence and PA imaging, but also acts as the therapeutic agent for photothermal therapy. In addition, the hydrophobic backbone of PEG-PCB provides strong hydrophobic and π - π interactions with the aromatic anticancer drug such as doxorubicin for drug encapsulation and delivery. Such a trifunctionality of PEG-PCB eventually results in a greatly simplified nanotheranostic system with only two components but multimodal imaging and therapeutic capacities, permitting effective NIR fluorescence/PA imaging guided chemo-photothermal therapy of cancer in living mice. This study thus provides a

molecular engineering approach to integrate essential properties into one polymer for multimodal nanotheranostics.

In the fourth project, we synthesize an organic photodynamic nanoinhibitor (OPNi) to counteract carbonic anhydrase IX (CA-IX) for synergistic photodynamic therapy. Despite the great potential in cancer treatment, photodynamic therapy (PDT) often exacerbates hypoxia and subsequently compromises its therapeutic efficacy. To conquer such pitfall, OPNi is synthesized to additionally inhibit CA-IX, a molecular target in hypoxia-mediated signalling cascade. OPNi is composed of a metabolizable semiconducting polymer as the photosensitizer and a CA-IX antagonist-conjugated amphiphilic polymer as the matrix. Such a molecular structure allows OPNi not only to selectively bind CA-IX positive cancer cells to facilitate its tumor accumulation but also to regulate CA-IX-related pathway. The integration of CA-IX inhibition into targeted PDT process eventually has a synergistic effect, leading to the superior anti-tumor efficacy over sole PDT as well as the reduced probability of hypoxia-induced cancer metastasis. This study thus proposes a molecular strategy to devise simple yet amplified photosensitizers to improve traditional PDT.

In the fifth project, we synthesize a hybrid semiconducting nanozyme for synergistic NIR-II photothermal ferrotherapy. Despite its growing promise in cancer treatment, ferrotherapy has low therapeutic efficacy due to compromised Fenton catalytic efficiency in tumor milieu. A hybrid semiconducting nanozyme (HSN) with high photothermal conversion efficiency is thus developed for PA imaging guided photothermal ferrotherapy in second near-infrared window. HSN comprises an amphiphilic semiconducting polymer as photothermal converter, PA emitter and iron-chelating Fenton catalyst. Upon photoirradiation, HSN generates heat not only to induce cytotoxicity but also to enhance

Fenton reaction. The increased $\cdot\text{OH}$ generation promotes both ferroptosis and apoptosis, oxidizes HSN (42 nm) and transforms it into tiny segments (1.7 nm) with elevated intratumoral permeability. The non-invasive seamless synergism leads to amplified therapeutic effects including a deep ablation depth (9 mm), reduced expression of metastasis-related proteins and inhibition of metastasis from primary tumor to distant organs. Thereby, this study provides a generalized nanozyme strategy to compensate both ferrotherapy and phototherapeutics for complete tumor regression.

In summary, we make use of nano-engineering to transform SPNs into diversified nano-systems to perform multifunctional biophotonics. Molecular engineering of SPNs allows us to manipulate their bandgaps and regulate their internal photophysical processes to diversify their biophotonic responses. Meanwhile, nano-functionalization of SPNs provide the feasibility to modify their outer structures and inner compositions, enabling them as a multifunctional biophotonic nanoplatform. Such structural and functional versatility of SPNs in conjunction with their other intrinsic merits have helped advance the field of biophotonics, represented by their unprecedented applications ranging from ultrasensitive afterglow imaging and deep-tissue PA imaging, to versatile phototherapeutic interventions.

Chapter 1 Introduction

1.1 Background

1.1.1 Biophotonics

Biophotonics that deals with interactions between photons and biological matters plays a pivotal role in fundamental biology and medicine. As a multidisciplinary paradigm encompassing life sciences, optics, and engineering, biophotonics provides unique capabilities for noninvasive regulation of biological activities, ultrasensitive detection of biomarkers with spatial resolution ranging from nanometer to centimeter, and real-time tracking of pathological events with a broad temporal scale from femtoseconds to days.¹ As a result, biophotonics has been serving as an indispensable tool in diverse biomedical applications including molecular imaging, biosensors, phototherapy, laser surgery, and optogenetics.²⁻⁵

Over recent years, development in nanotechnology has led to profound improvement in biophotonics.⁶ Coalescence of both broadens the vision of existing photonic modalities and imparts future biomedical techniques related to optical stimuli. Inorganic nanoparticles including semiconductor quantum dots, plasmonic nanostructures, silica nanoparticles, carbon dots and upconversion nanoparticles have been involved in biophotonics.⁷ In these applications, they often serve as the intermediators to transduce biomolecular interactions into optical signals, augment signal readout, or convert the light energy into heat or electric current to simulate the biological activities.⁸ However, they have their own merits and demerits including toxicity issue for quantum dots and upconversion nanoparticles, poor photostability for plasmonic nanostructures, and short excitation and emission wavelengths

for carbon dots and silica nanoparticles, making them less versatile and effective in biophotonics. In contrast to inorganic nanoparticles, organic nanoparticles have been less exploited for biophotonics, although they have the advantages of easy functionalization, relatively low cost, and potentially high biosafety.⁹

Molecular optical imaging lies as an important theme in biophotonics. In general, optical imaging leverages light with wavelengths ranging from ultraviolet to NIR region as the excitation source and/or output signals to visualize biological or pathological processes.⁸

Compared with conventional biomedical imaging modalities such as magnetic resonance imaging (MRI) or X-ray computed tomography (CT), optical imaging has advantages of fast operation, low cost, minimal invasiveness, high throughput and superior sensitivity.¹

Thereby, a diversity of optical imaging modalities have emerged and are applied for imaging of cancer, vasculatures, or organs; in vivo tracking of specific biomarkers; and imaging guided therapy. One of the most broadly studied techniques in optical imaging is fluorescence imaging, which relies on the detection of emission from optically excited fluorescent molecules to map the target of interest. Because of the high sensitivity and temporal resolution, it serves as a powerful tool in biomedical research to provide versatile opportunities for in vivo labelling and tracking of specific targets, fluorescence imaging guided surgery, and so forth. Despite the significant progress, fluorescence imaging suffers from shallow penetration depth due to the loss of photons during their interactions with biological tissues.

To overcome such drawback, one approach is to develop afterglow imaging. Different from fluorescence imaging which relies on real-time excitation and detection, afterglow imaging detects luminescent signals from specific contrast agents after cessation of optical

illumination, thereby circumventing background autofluorescence from endogenous molecules. In detail, the afterglow contrast agents play as an optical battery to store incident photons during excitation, then slowly release them after removal of light source. Due to the elimination of background noise, afterglow imaging provides superior signal-to-noise ratio (SBR) than conventional fluorescence imaging, enabling deep-tissue visualization with higher spatiotemporal resolution for in vivo tracking and monitoring of biomarkers (e.g. glutathione, ascorbic acid) or drug-induced hepatotoxicity, etc. However, reported afterglow contrast agents are restricted to a limited number. And the majority of them are composed of heavy-metal ions, of which the potential leakage to cause chronic toxicity issue in vivo remains a significant challenge. In this regard, development of bio-friendly organic afterglow agents with bright and tunable emission is of high demand.

Table 1. Comparison of representative low-background optical imaging modalities

Modality	Contrast agents	Signal-to-background ratio	Life-time	Penetration depth	Ref.
NIR-II fluorescence	SBP-M13-SWNT	134	N.A.	9.7-18.2 mm	PNAS, 2014, 111, 13948-13953
	IR-12N3@Erbix	~10	N.A.	~0.5-0.8 cm	Adv. Mater. 2018, 30, 1802546
	Ag ₂ S quantum dot	14-16	N.A.	N.A.	Angew. Chem. Int. Ed. 2012, 51, 9818-9821
	CP-IRT	8.3	N.A.	N.A.	Adv. Mater. 2018, 30, 1800106
	p-FE-PTX-FA nanoparticle	20	N.A.	N.A.	Nano Res, 2018, 12, 273-279
	P-FE	~12	N.A.	1.3 mm	Nat. Commun, 2018, 9, 1171
Chemiluminescence	L012 and hemin-Pdots	18	~ 10 h	N.A.	Anal. Chem. 2018, 90, 6929-6935
	Probe 2a	20	~ 2 h	N.A.	J. Am. Chem. Soc. 2017, 139, 13243-13248
Bioluminescence	QD655-Luc8-R9	~1000	N.A.	3 mm	Nat. Biotechnol. 2006, 24, 339-343
	RET ₂ IR@cRGD	116	> 25 h	2 cm	Nat. Commun. 2012, 3, 1193.
Cerenkov luminescence	[¹⁸ F]FDG-excited fluorescein	154	N.A.	25 mm	Nat. Med. 2013, 19, 1345-1350.
	⁹⁰ Y-1	29	N.A.	N.A.	J. Label. Compd. Radiopharm 2014, 57, 558-565.
	⁹⁰ Y-PRGD ₂	3.4	~ 20 h	~ 1 cm	J. Nucl. Med. 2014, 55, 1905-1909.
Persistent luminescence	LPLNP-TAT	172	~ 72 h	N.A.	Anal. Chem. 2016, 88, 4114-4121.
	PLNP	186	> 80 min	N.A.	Nat. Mater. 2014, 13, 418-426.
	PEG-LPLNP	1826	> 15 days (powder)	N.A.	J. Am. Chem. Soc. 2013, 135, 14125-14133.
	ZGC-1	275	> 200 s	1 cm	J. Am. Chem. Soc. 2015, 137, 5304-5307.
Afterglow luminescence	SPN-NCBS	3387	hours	4 cm	Nat. Biotechnol. 2017, 35, 1102-1110

Photoacoustic (PA) imaging provides another approach to break through the depth limit of conventional fluorescence imaging. Such imaging technique is a hybrid of optical imaging and ultrasound imaging, which depends on photoacoustic effect to transform incident photons into acoustic waves through thermo-elastic expansion.¹⁰ Because acoustic waves are far less (approximately three orders of magnitude) scattered in biological tissues than photons, PA imaging has potential for deep tissue visualization at centimeter-scale with high spatial resolution while retaining high contrast of optical imaging. By virtue of these merits, PA imaging is currently one of the fastest developing optical imaging modalities and is extensively investigated for in vivo diagnosis of cancer, monitoring of hemoglobin oxygen, mapping of brain vasculature, detection of physiological or pathological biomarkers, etc.¹¹

Current PA imaging majorly utilizes NIR light (NIR-I, 650-950 nm) which has better biological transparency than visible or ultraviolet light. However, very recent studies on fluorescence imaging have identified a second NIR window above 1000 nm (NIR-II, 1000-1700 nm) that encounters even reduced photon scattering in biological tissue than NIR-I window.¹² Due to the lower energy of photons at longer wavelengths, NIR-II light has higher permissible power limit for bioimaging than NIR-I window. In light of these merits, NIR-II PA imaging is envisioned to have even superior deep-tissue imaging performance and has attracted growing research attention. Nevertheless, the clinical translation of NIR-II PA imaging is greatly hindered due to the lack of suitable contrast agents that have both good PA performance and biodegradability. Thereby, increasing efforts have been devoted to the development of metabolizable NIR-II contrast agents.

In addition to molecular imaging, photoactive therapeutic intervention (phototherapy) achieved via nano-scaled agents is another important aspect in biophotonics. Classified by therapeutic mechanism, there are two major modalities of phototherapy: photothermal therapy (PTT) and photodynamic therapy (PDT). Photothermal therapy leverages local hyperthermia (basically above 43°C) to cause DNA damage, destruction of proteins and disruption of membrane integrity, ultimately leading to cellular apoptosis and necrosis.¹³ Such local hyperthermia is normally achieved by external laser irradiation to the disease site, which could be further amplified if an exogenous photothermal agents are administered. On the other hand, PDT depends on external laser irradiation to excite photosensitizers so as to generate cytotoxic reactive oxygen species (ROS) such as superoxide anion, singlet oxygen, hydroxyl radical, and so on to cause cellular damage.¹⁴ There are two photosensitization mechanisms involved in PDT: type I and type II reactions. In type I reaction, the excited photosensitizer directly interacts with nearby substrate to produce radicals, then these radicals could further transform to oxygenated products in the presence of oxygen. Whereas, in type II reaction, the excited photosensitizer interacts with oxygen to transform it from triplet state to singlet state ($^1\text{O}_2$), which subsequently reacts with biomolecules to generate oxidized products. Owing to the minimal invasiveness and handiness of use, phototherapy has been extensively studied in scientific research of advanced cancer treatments.¹⁵⁻¹⁶ Currently, both PTT and PTT are being tested in the clinics to treat versatile types of cancer, such as prostate cancer, skin cancer, head and neck cancer, etc.

To further improve therapeutic efficacy, phototherapy is frequently combined with other modalities, such as chemotherapy, ferrotherapy and immunotherapy. Classic

chemotherapy is usually challenged by multi-drug resistance and severe adverse effects, whereas the combined chemo-phototherapy also provides additional photo-controlled tumor ablation mechanism to overcome the drug resistance limitation and to amplify therapeutic outcome. For instance, Li et al. developed a pH-switchable multifunctional micelles consisting of photosensitizer Chlorin e6 (Ce6) and chemotherapeutics doxorubicin (DOX) for combinational PTT/PDT/chemotherapy to treat drug resistant-tumor.¹⁷ Different from chemotherapy, ferrotherapy leverages iron ion ($\text{Fe}^{2+}/\text{Fe}^{3+}$)-mediated Fenton reactions to convert hydrogen peroxide (H_2O_2) into highly potent cytotoxic hydroxyl radical ($\cdot\text{OH}$) to kill cancer cells. Although ferrotherapy circumvent the common issue of drug resistance in conventional chemotherapy, it suffers from low therapeutic efficacy due to the suboptimal catalytic condition in tumor microenvironment. Thereby, it is increasingly investigated to be synergized with phototherapy, especially PTT therapy wherein hyperthermia could additionally reinforce Fenton catalytic efficiency.¹⁸ As an example, Zhang and Liu et al. reported a nanozyme $\text{PtFe}@\text{Fe}_3\text{O}_4$ with peroxidase and catalase-like activities for photo-enhanced deep pancreatic cancer therapy.¹⁹ The advent of immunotherapy has been indicated to revolutionize cancer treatment.¹³ In principle, immunotherapy makes use of host immune system to counteract cancer. Whereas immunotherapy frequently encounters critical challenges such as low response rate in patients or severe immune-related adverse effects. With the promise to improve anti-tumor immune response in an effective and safe manner, immunotherapy has been integrated with phototherapy which provides precise spatiotemporal regulation of treatment site and mediates additional immunogenic cell death to reinforce anti-tumor immunity. For instance, Liu et al. prepare a multifunctional nanoparticle composed of a photothermal agent

indocyanine green (ICG) and immune-adjuvant imiquimod (R837) to combine with checkpoint blockade anti-cytotoxic T-lymphocyte antigen-4 (aCTLA4) for potentiated cancer metastasis inhibition and stronger immunological memory effect.²⁰ Most recently, due to the deeper tissue penetration of NIR-II relative to NIR-I light, NIR-II phototherapy in combination with versatile cancer treatment modalities are attracting growing research attention and hold great promise to treat deeply seated tumors.²¹

Figure 1.1. Schematic illustration of nanoplatforms for advanced applications in biophotonics. Reprinted with permission from ref 225. Copyright 2018, American Chemical Society.

Despite the significant progress in multimodal biophotonics, further translation to preclinical and clinical use is hampered due to lack to biocompatible and multifunctional nanoagents. Although a diversity of nano-sized structures have been developed, they have their own merits and demerits including toxicity issue for quantum dots and up-conversion nanoparticles, poor photostability for plasmonic nanostructures, and short excitation and

emission wavelengths for carbon dots and silica nanoparticles, making them less versatile and effective in biophotonics. In contrast to inorganic nanoparticles, organic nanoparticles have the advantages of easy functionalization, relatively low cost, and potentially high biosafety,⁹ whereas they have been relatively less exploited for biophotonics.

1.1.2 Semiconducting Polymers

Semiconducting polymer nanoparticles (SPNs), which are made of π -conjugated semiconducting polymers (SPs) originally used in organic electronics, have emerged as one of the most competent organic agents for biophotonics.²² Because the bandgaps of SPNs are mainly determined by the chemical structures of the precursor SPs, their photophysical properties are less dependent on their sizes, which is different from metallic nanoparticles. Thus, through molecular engineering, SPNs can possess versatile light-responsive properties to meet the specific requirements for different biophotonic applications. Surface PEGylation of SPNs can lead to the good biodistribution and passive tumor targeting ability.²³ Thus, in association with their excellent optical properties, SPNs have been applied for cancer phototheranostics. Until now, SPNs have been used as fluorescent agents for imaging of biomolecules and living organisms,²⁴⁻²⁶ phototherapeutic agents for cancer therapy,²⁷ and optical regulators for bioactivation.²⁸

Figure 1.2. Potential applications of SPNs for advanced biophotonics. Reprinted with permission from ref 225. Copyright 2018, American Chemical Society.

For instance, dependent on the molecular structures of SPs, the photothermal conversion efficiencies of SPNs range from 6% to 71%.²⁹⁻³⁰ In addition to polymer screening, modulation of internal photophysical processes of SPNs can be used to amplify photothermal conversion. For instance, PCB-based SPNs were doped with fullerene to favor photo-induced electron transfer (PET).³¹ Such doping quenched the fluorescence of PCB and promoted non-radiative relaxation to augment heat generation (Figure 6b, c), consequently affording improved anti-tumor PTT efficacy. As another example, a hybrid nanocomposite consisting of PCB as the photosensitizer and nanoceria as the ROS regulator were developed for self-regulated PDT.³² Serving as ROS converter at pH 6.5 whereas ROS scavenger at pH 7.4, nanoceria induced enhanced ROS generation in the acidic tumor microenvironment but minimized ROS generation in neutral normal tissues. Such disease-correlated self-regulating theranostic nanocomposite not only reduced

nonspecific side effects, but also contributed to the enhanced anti-tumor PDT efficacy. Furthermore, a multilayered SPN (SPN-M1) coated with manganese dioxide (MnO_2) was devised to overcome the hypoxia limitation of tumor for amplified PDT.³³ In hypoxic and acidic environment of solid tumor, MnO_2 nanosheets on the surface of SPN-M1 reacted with and transformed H_2O_2 into O_2 , enabling 2.68-fold higher yield of $^1\text{O}_2$ relative to the uncoated SPN (SPN-0) upon laser irradiation at 808 nm. As such, SPN-M1 demonstrated improved PDT efficacy than SPN-0 in mouse model.

1.2 Objectives and scope

Despite the promise of SPNs, their potential of application in biophotonics lacks substantial exploitation. In this regard, the overall objective of this thesis is to develop multifunctional SPNs to advance multimodal biophotonics, especially aiming to tackle the critical problems encountered in the areas of afterglow imaging, PA imaging, and phototherapies. The first task is to transform SPNs into afterglow contrast agents with tunable bright emission via devising a generic approach. Such approach integrates a cascade photoreaction into a single-particle entity, enabling ALNPs to chemically store photoenergy and spontaneously decay it in an energy-relay process. In addition, a mathematical model is rationally proposed and examined to predict the afterglow intensities. Thereafter, afterglow imaging performance of the representative ALNPs is evaluated by in vivo tumor imaging in comparison with NIR fluorescence imaging. Further, the biodegradability and in vivo clearance of representative ALNPs are investigated.

The second task is to convert SPNs into the first series of organic metabolizable PA imaging contrast agents in second window that are capable of gradual clearance from living bodies. Such design is accomplished due to the oxidizable backbones of SPs and

hydrolyzable polymer encapsulating matrix. The obtained SPNs are expected to be biodegraded by synergistic digestion of peroxidase and lipase, followed by continuous elimination through both hepatobiliary and renal excretions. Degradation and clearance behaviors of SPNs are carefully studied both in vitro and in vivo. At last, NIR-II PA imaging capability of these metabolizable SPNs are examined by tumor and brain vasculature imaging on living mice.

The third project is to exploit SPNs as drug delivery system to load DOX for NIR fluorescence/PA imaging guided chemo-photothermal therapy. To achieve this, amphiphilic SP is prepared by conjugation with PEG through side chain grafting. As a result of p-conjugated backbone, the obtained PEG-PCB is used to serve as (i) the diagnostic component for imaging, (ii) photothermal agent by efficiently converting photon energy into heat, and (iii) drug delivery system assigned to strong hydrophobic and p-p interactions with the aromatic anticancer DOX. In the following, imaging performance and therapeutic efficacies of such nano-theranostic systems are investigated both in vitro and in vivo.

The fourth task is to develop the first organic semiconducting photodynamic nanoinhibitor (OPNi) to overcome the hypoxia limitation of conventional PDT for synergistic cancer therapy. Such a nanoinhibitor is designed to consist of SP PCVB as the biodegradable photosensitizer and an acetazolamide (CAi)-terminated amphiphilic polymer (CAi-b-PEG-b-PPG-b-PEG-CAi) as the inhibitor to counteract carbonic anhydrase IX (CA-IX), a crucial downstream gene product in the HIF-mediated signalling cascade. With CAis on the surface, OPNi is expected to not only specifically bind the cancer cells to exert targeted PDT, but also inhibit the catalytic function of CA-IX and consequently dysregulate both

pHe and pHi to suppress cancer metastasis. Afterwards, imaging performance and therapeutic performance of OPNi are substantially studied in vivo.

The last ask is to convert SPN into a hybrid semiconducting nanozyme (HSN) with photoirradiation enhanced catalytic activity for NIR-II PA imaging-guided synergistic photothermal ferrotherapy. To achieve this, an amphiphilic SP pTBCB-PEG is synthesized to serve as both the NIR-II photothermal transducer and iron chelator. Upon NIR-II photoirradiation, pTBCB mediates photothermal transduction, which is envisioned to (i) directly triggers cell death (PTT), (ii) potentiates Fenton reaction to enhance both apoptosis and ferroptosis. Furthermore, the elevated $\cdot\text{OH}$ production is expected to accelerate the decomposition of HSN to tiny fragments. Afterwards, molecular performance of HSN-mediated NIR-II PA imaging and photothermal ferrotherapy is substantially evaluated both in vitro and in vivo. Further, underlying molecular mechanisms are carefully studied.

Chapter 2 Literature Review

2.1 Molecular design of semiconducting polymers

Molecular engineering of SPs plays a crucial role in the modulation of photophysical processes of SPNs and ultimately determines their biophotonic applications.²⁴ Donor-acceptor (D-A) approach can be used to adjust the energy levels of SPs, wherein alternating electron-rich (donor) and electron-deficient (acceptor) moieties are incorporated into the π -conjugated backbones (Figure 2-1a).³⁴ In general, D-A copolymers are synthesized *via* palladium-catalyzed polycondensation (e.g. Stille or Suzuki cross-coupling reactions) from donor and acceptor monomers. Some typical donors (e.g. thiophene, cyclopentadithiophene, and dithienosilole) and acceptors (e.g. benzothiadiazole, diketopyrrolopyrrole, and thiadiazoloquinoxaline) discussed in this Account are exemplified in Figure 2.1a. After polymerization, the highest and lowest occupied molecular orbitals (HOMO and LUMO, respectively) of the donor unit fuse with the respective orbitals of the acceptor unit to afford a new set of hybridized orbitals with narrowed bandgaps. The result is the red-shifted absorption for the synthesized copolymer relative to its monomers.

Photophysical processes of SPs upon light excitation or irradiation along with the corresponding biophotonic functions are depicted in the form of Jablonski diagram (Figure 2.1b).³⁵ Upon light excitation, SP is excited from the ground state to the excited states of higher energy (S_n). The excited state of SP is unstable and subsequently undergoes non-radiative vibrational relaxation, also termed as internal conversion (IC), to the lowest vibrational level of excited state (S_1). Following this, there are two different pathways:

either returning to the ground state *via* relaxation or going through intersystem crossing (ISC) to the triplet state (T_n). As for the direct relaxation to the ground state, one way is to the radiative decay with the emission of photons defined as fluorescence; while the other way is the non-radiative IC process that leads to heat generation. Thus, in addition to fluorescence agents, SP can be used as photothermal agents for PA imaging, PTT, and thermal regulation of biological functions. On the other hand, once SP is transferred to triplet state, it might also relax *via* the emission of photons (phosphorescence); or it can transfer the energy to the surrounding molecules such as oxygen to generate 1O_2 . 1O_2 not only can be used as the cytotoxic therapeutic molecule for PDT, but also can react with the vinylene bonds in the π -conjugated backbone of polyphenylenevinylene (PPV)-based SPs to form unstable chemical defects (dioxetane units), which can spontaneously and slowly break down to release photons for afterglow imaging.

2.2 Preparation of semiconducting polymer nanoparticles

Nano-formulation is used to convert intrinsically hydrophobic SPs into water-soluble nanoparticles (SPNs).²⁷ Two approaches are commonly employed including mini-emulsion and nano-precipitation (Figure 2.1c). Amphiphilic agents (e.g. surfactants, amphiphilic polymers) are generally used to facilitate the formation of stable SPNs in aqueous solution. Of these two approaches, nano-precipitation produces smaller nanoparticles (10-50 nm) than mini-emulsion because it utilizes water-miscible solvents (e.g. tetrahydrofuran) rather than water-immiscible solvents (e.g. dichloromethane). Optical properties of SPNs in aqueous solution are often slightly different from the precursor SPs in organic solvent (hypochromic shift of absorption and reduced fluorescent

quantum yields), because of the changes of SPs from the initial extended state into a condensed state within nanoparticles.

The facile nano-formulation methods enable SPNs to be further functionalized through intra-particle doping or surface modification for a variety of biological applications (Figure 2.1d). Hydrophobic components can be incorporated into SPNs during nano-precipitation. For instance, doping of sensing molecules (e.g. bis(2,4,6-trichlorophenyl) oxalate, TCPO, ~14.5% loading capacity with long-term colloidal stability), inorganic nanostructures (e.g. nanoceria), electron acceptors, and therapeutic agents (e.g. anti-cancer drug) for smart chemiluminescence imaging,³⁶ self-regulative PDT,³² amplified PTT,³¹ and combinational therapy,³⁷ respectively. Meanwhile, surface engineering of SPNs can be conducted through chemical reactions such as click chemistry or carboxyl/amine coupling reaction. For example, targeting moieties (peptide and antibody) and reactive components (dyes and enzymes) can be linked to the SP backbones so as to direct nanoparticles to specific sites (e.g. cancer cells,³⁸ organelles,³⁹ and ion channels,⁴⁰ etc.) and make them responsive to specific biomolecule of interest (e.g. protein sulfonic acid),⁴¹ respectively. In general, SPNs are pegylated or formulated with biocompatible polymers to endow neutrally charged surface that results in ideal pharmacokinetics and biodistribution in living animals. Furthermore, the SPNs could be lyophilized and stored for later use.

Figure 2.1. Molecular designs, photophysics and functionalization. (a) Simplified mechanism of donor-acceptor engineering to reduce bandgap. Right: representative chemical structures of donors (red) and acceptors (blue). (b) Jablonski diagram of the photophysical processes of SPNs. Abs., absorption; IC., internal conversion; Fluo., fluorescence; ISC., intersystem crossing; phos., phosphorescence; PT, photothermal. (c) Scheme of preparation of SPNs. (d) Schematic illustration of components and surface modification of SPNs. Reprinted with permission from ref 225. Copyright 2018, American Chemical Society.

2.3 Molecular imaging

2.3.1 Afterglow imaging

Conventional photoluminescence imaging relies on fluorescent signals acquired upon real-time light excitation to delineate biological tissues, wherein auto-fluorescence from endogenous fluorophores is inevitable. Thereby, conventional photoluminescence imaging

has relatively low SBRs, resulting in limited sensitivity. Although different modalities of self-illuminating imaging which include chemiluminescence, bioluminescence and Cerenkov imaging have been studied to circumvent background autofluorescence, they each has their pros and cons. For instance, chemiluminescence and bioluminescence depend on chemical reactions that respectively require ROS and enzyme to trigger the initiation of luminescence, thereby the imaging sensitivity is frequently compromised due to the disturbance from cellular environment and substrate availability. Although Cerenkov luminescence imaging does not require additional substrates, it suffers from shallow penetration depth due to short-wavelength emission and potential biosafety issue owing to the use of radiotracers. Differently, the emerging self-illuminating paradigm afterglow (persistent luminescence) imaging relies on afterglow agents to serve as the optical battery that traps irradiated photoenergy in defects and then slowly releases the stored energy in the form of luminescence (Figure 2.2), thereby avoiding the need of invasive radiotracers or additional substrates.

Figure 2.2. Schematic illustration of afterglow process.

For instance, Richard and Scherman et al. reported the inorganic nanophosphor (ZGO) constructed from chromium-doped zinc gallate with red-excitable NIR persistent luminescence (Figure 2.3).⁴² Such optical characteristics enabled high-sensitivity

bioimaging on living mice. Prior to intravenous injection, ZGOs were charged ex vivo with UV irradiation. Compared with commercially available NIR-emitting QDs, ZGOs achieved far superior target to background ratio (TBR) and imaging sensitivity due to the minimized background noise (Figure 2.3a, b). Further, surface of ZGOs could be facily functionalized or adjusted to favor versatile biomedical applications. As an example, ZGO-NH₂ of higher possibility to undergo phagocytosis was utilized to label RAW 264.7 macrophages ex vivo and then track the cell fate in vivo by persistent luminescence imaging. In contrast to free ZGO-NH₂, tagged RAW cells demonstrated obvious lung homing (Figure 2.3d-e), validating the potential of ZGO as a general optical reporter for wide application in bio-related research.

Besides in vivo tracking of labeled cells, these persistent luminescent nanoparticles have been transformed to activatable probes that could only produce optical signal in the presence of target of interest for detection of biomarkers. For instance, Tan et al. developed persistent luminescence nanorods conjugated with lysozyme-binding aptamer and quencher-labeled DNA for autofluorescence-free detection of lysozyme in blood sample.⁴³ In addition, these nanoparticles have been modified for the detection of hemoglobin,⁴⁴ matrix metalloproteinase-2,⁴⁵ glutathione,⁴⁶ etc.

Figure 2.3. Persistent nanophosphors ZGO for in vivo optical imaging. (a) and (b) Comparison of imaging performance of ZGO (a) and QDs (b) after intravenous administration in living mice. (c) and (d) Biodistribution of ZGO-NH₂ and RAW cells labeled with ZGO-NH₂ after systemic injection in living mice. © Ex vivo biodistribution of ZGO-NH₂ and RAW cells labeled with ZGO-NH₂ at 24 h after systemic injection in living mice. Li, S, K, H, Lu respectively stand for liver, spleen, kidney, heart, and lung. Reprinted with permission from ref 42. Copyright 2014 Nature Publishing Group

However, persistent luminescence behavior is limited to a few inorganic nanomaterials that have the rare-earth or heavy metal ingredients. In addition to the toxic concern, their luminescence half-lives are relatively short (< 200 s) in aqueous solution.⁴⁷ Even though SPNs doped with chemiluminescent substrates have been exploited for self-illuminating imaging, endogenous ROS are required to trigger luminescence.³⁶ And their potential in afterglow imaging lacks substantial exploration.

2.3.2 Photoacoustic imaging

PA imaging, also termed optoacoustic imaging, is currently one of the fastest developing non-invasive hybrid imaging technique.⁴⁸⁻⁵⁰ Traditional optical imaging modalities depending on the propagation of photons suffer from quickly attenuated optical signals with the depth of imaging due to the strong scattering of photons within tissue. So far, imaging depth of widely used optical microscopes is limited within 1 mm, determined by the transport mean free path (TMFP) of a photon in tissue.⁵¹⁻⁵² In contrast, PA imaging is based on PA effect that incorporates optical imaging with sound, extending the limitation of imaging depth to a few centimeters.⁵³⁻⁵⁵ During the PA imaging, a light absorber (imaging agent) in tissue is irradiated by short-pulsed laser, and then it harvests the energy of the laser beam and partially converts it into heat, generating a transient rise of local temperature and subsequently a rise of pressure waves (sound waves) *via* thermos-elastic expansion. These PA waves are then collected by an ultrasound transducer outside the tissue and transformed into PA images according to their arrival times. As phonons are scattered far less than photons in biological tissues and scattered photons could as well contribute to the generation of PA waves, PA imaging is capable of high spatial resolution mapping in deep tissues (~ 6 cm) while retaining high contrast of optical imaging.⁵⁶⁻⁶¹ To

date, PA imaging has demonstrated great potential in clinical translation for early diagnosis of prostate and breast cancer,⁶²⁻⁶³ detection of tumor metastases,⁶⁴ endoscopic gastrointestinal imaging and treatment monitoring.⁶⁵⁻⁶⁶

PA imaging is able to conduct diagnosis without using contrast agents, because endogenous molecules such as hemoglobin and melanin can generate PA signals. Particularly, hemoglobin oxygen saturation (SO₂) and carboxyhemoglobin saturation (SCO) of the subcutaneous microvasculature can be noninvasively distinguished in PA imaging due to their different absorption spectra.⁶⁷⁻⁷¹ Moreover, overexpression of melanin in metastatic melanoma cells can be monitored by PA techniques for early diagnosis and treatment of cancer.⁷²⁻⁷⁶ However, endogenous contrast agents basically suffer from poor imaging depth with maximum value about 10 mm due to relatively weak optical absorption.⁷⁷⁻⁷⁸ To further improve the imaging depth, exogenous imaging agents with high sensitivity and specificity have been developed and widely studied, especially nanoparticles with strong absorption in the NIR region where biological tissues are relatively transparent and optical attenuation is weaker than visible light region.⁷⁹⁻⁸⁰ Furthermore, the high human safety limit of NIR light intensity partially contributes to the increased imaging depth.⁸¹⁻⁸³

Many optical nanomaterials have been exploited as exogenous PA imaging agents, such as gold nanoclusters,⁸⁴⁻⁸⁸ carbon nanotubes,⁸⁹⁻⁹⁴ reduced graphene oxide,⁹⁵⁻⁹⁷ up-conversion nanoparticles,⁹⁸⁻¹⁰¹ quantum dots,¹⁰²⁻¹⁰⁷ small-molecule dyes,¹⁰⁸⁻¹¹⁰ SPN.¹¹¹⁻¹¹³ Each has its own merits and drawbacks. For instance, metallic nanoparticles exhibit excellent and tunable optical properties but their safety issue in terms of clinical application remains to be addressed *via* longer studies.^{57, 114} Organic imaging agents especially small molecule dyes have long since been a research hotspot owing to their versatility of

modification, better biocompatibility and relatively low cost, but their photostability needs to be improved.⁵⁷ Most recently, SPNs with high photostability and tunable optical properties have emerged as an alternative kind of optical agents and are among the most popular organic imaging agents of PA imaging.^{112, 115-116}

Cyanine dye-based Nanoparticles

ICG has long been an FDA approved tricarbocyanine fluorophore.¹¹⁷ Recently, ICG and its analogues have been developed as PA contrast agents because of their high absorption in the NIR region.¹¹⁸⁻¹¹⁹ However, drawbacks such as poor photostability, extensive protein binding and degradation in water severely impede the biomedical application of free cyanine dye molecules.¹²⁰ As a result, cyanine dyes and their derivatives are commonly encapsulated into or grafted to biocompatible polymers to form stabilized nanoparticles.¹²¹⁻

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Cyanine dye-based nanoparticles have been utilized for imaging guided therapy of cancer, an emerging concept in cancer treatment. The combination of phototherapy, such as PTT or PDT, and PA imaging can enhance the accuracy of diagnosis and improve the efficacy of therapy *via* synergistic effects. Liu *et al.* reported a multifunctional nano-micelle system for imaging guided-combined PTT and PDT of cancer.¹²⁵ A photosensitizer Ce6 was anchored on the backbone of C18PMH-PEG *via* a short PEG linker and acted as a chelating agent to capture Gd^{3+} . An analogue of ICG, IR825, was then encapsulated into C18PMH-PEG-Ce6-Gd (Figure 2.4a). UV-Vis-NIR absorbance spectra revealed the characteristic peak of IR825 (≈ 830 nm) after self-assembly process (Figure 2.4b). The resulted IR825@C18PMH-PEG-Ce6-Gd nano-micelles served as a multifunctional contrast agent for not only PA imaging but also fluorescence and MRI. After injection of the IR825-

containing micelles, the PA signal in 4T1 tumor tissue increased over time and the entire tumor was lightened up at later time points (Figure 2.4c). Compared with single photo treatment which only affected the superficial region of tumor, the combination of PTT and PDT at the same dose of IR825@C18PMH-PEG-Ce6-Gd nano-system was able to induce significant damage to both superficial and deep parts of tumor. Similarly, Chen and Liu *et al.* developed a dual modal imaging system combined with cancer therapy taking advantage of a cyanine dye Cypate (Cy) as a PA and NIR fluorescent contrast agent.¹²⁶ Both NIR dye Cy and photosensitizer Ce6 were encapsulated in mPEG-b-Pasp(DA) micelles to achieve precise anatomical tumor localization and simultaneous PTT/PDT.

Figure 2.4. In vivo PA imaging via IR825@C18PMH-PEG-Ce6-Gd (a) Schematic illustration of self-assembly of IR825@C18PMH-PEG-Ce6-Gd nano-micelles. (b) UV-Vis-NIR absorption spectra of free Ce6, free IR825, C18PMH-PEG-Ce6, and IR825@C18PMH-PEG-Ce6. (c) In vivo PA imaging of tumor-bearing mice at different time points after intravenous injection of IR825@C18PMH-PEG-Ce6-Gd micelles.^[96]

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Porphyrin-based Nanoparticles

Porphyrin chromophores featured by their intrinsically strong absorption in Soret and Q bands are commonly exploited as sensitizers for dye-sensitized solar cells as well as PDT.¹²⁷⁻¹²⁹ Zheng *et al.* conjugated porphyrin to phospholipid and transformed porphyrin-phospholipid conjugates into nanovesicles *via* supramolecular self-assembly, which introduced a new kind of photonic nanomaterials, porphysomes.¹³⁰ The structure of porphysomes is composed of highly packed porphyrin bilayers that induced strong self-quenching *via* intermolecular interaction, giving rise to enhanced nonradiative heat generation and consequently amplified PA signals. In addition, porphyrin-lipid conjugates exhibit tunable NIR absorption, unique metal ion-chelating property, and excellent biodegradability.

Recently, Zheng's group utilized single-layered porphyrin-phospholipid conjugates to develop photonic microbubbles (MBs) as combined ultrasound (US)/PA contrast agents.¹³¹ An in-situ porphyrin microbubbles (pMBs) was synthesized to consist of a bacteriochlorophyll-lipid shell, which encapsulated perfluorocarbon gas as ultrasound contrast agent for dual modal imaging of tumor.¹³² Upon sonication with low-frequency conversion ultrasound, pMBs would burst into smaller nanoparticles (pNPs) (Figure 2.5a). pNPs retained similar optical properties (Figure 2.5b) but could be further delivered into a solid tumor because of their reduced size. The bathochromic shift of absorption peak to 824 nm and enhanced absorption intensity of pMBs revealed the formation of J-aggregates of porphyrins in the monolayer shell. After conversion by ultrasound pulses, the 824 nm

absorption peak was maintained but gradually decreased, accompanied by an increase at 750 nm assigned to monomeric BChl-lipid due to the disruption of ordered aggregates (Figure 2.5b). PA spectra of pMBs before and after ultrasound treatment correlated well with the absorption spectra, showing a peak signal at 824 nm (Figure 2.5c). This micro-to-nano conversion was further validated *in vivo* by injection of pMBs into KB xenograft-bearing mice (Figure 2.5d). When low-frequency ultrasound sonicated the tumor, the PA signal was maintained within the tumor for at least 2 h post injection compared with a sharp decrease of PA signal within 30 min without ultrasound application. This indicated the successful delivery and retention of porphyrins in the tumor via conversion to pNPs (Figure 2.5e). Porshe MBs with 3-fold porphyrin content were further generated for intrinsic US/PA/FL trimodal imaging of tumor.¹³³ Due to the PA imaging ability, porphyrin-lipid conjugates should hold great potential in theranostics.

Figure 2.5. In vivo PA imaging via porphyrin microbubbles (pMBs) (a) Scheme of pMBs and micro-to-nano conversion process under ultrasound. (b) UV-Vis-NIR absorption spectra of pMBs after applying different conversion ultrasound pulses, in contrast with BChl-lipid monomer. (c) PA spectra of pMBs before and after sonication. pNPs maintain

the PA peaks assigned to pMBs. (d) PA images of the tumor xenografts without and with sonication over time. Micro-to-nano conversion (pMBs to pNPs) prolonged the retention time of contrast agents in tumor xenografts. Scale bars, 2 mm. (e) Normalized PA amplitudes of the tumor as a function of time, with ultrasound applied (green) or not applied (blue). PA amplitudes are normalized to the peak PA amplitude. Mean $\pm$ 1 s.d. (n=3).^[103] eprinted with permission from ref 11. Copyright 2017 WILEY-VCH Verlag GmbH & Co. KgaA, Weinheim.

Melanin-based Nanoparticles

Melanin is a ubiquitous naturally-occurring pigment featured by its protective role against oxidative stress.¹³⁴⁻¹³⁵ Dark-brown melanin has broad NIR absorption, strong chelating properties and excellent stability under physiological conditions and could be used for PA imaging.¹³⁶⁻¹³⁷ Cheng *et al.* successfully developed melanin-based nanoparticles (MNP) to chelate metal ions for multimodal imaging of tumor.¹³⁸ Melanin granules were first dissolved under basic conditions and then sonicated to form ultra-small MNPs (~4.5 nm) (Figure 2.6a). After PEGylation, a targeting peptide RGD was attached to the surface. Then Fe³⁺ and ⁶⁴Cu²⁺ was chelated for PA/MRI/PET multimodal imaging. As shown in Figure 2.6b, good linearity existed between PEG-MNP concentrations and the intensity of PA signals. *In vivo* PA imaging investigation of RGD-PEG-MNP on U87MG tumor mice displayed obvious augment of PA signals at 4h post injection, due to the EPR effect and targeting ability of RGD to $\alpha_v\beta_3$ integrin (Figure 2.6c). In another study, highly water soluble melanin free acid was prepared under mild oxidative and neutralized pH conditions for dynamic contrast enhanced PA imaging of tumor vasculature.¹³⁹ PA imaging of HER2 tumor demonstrated a slow but constant uptake of PEGylated melanin free acid into the

tumor up to 15 min post-injection, and the PA signal was further enhanced after addition of tumor necrosis factor- α (TNF- α) due to the increased vascular permeability.

Figure 2.6. In vivo PA imaging via MNPs. (a) Illustration of multimodal molecular imaging of MNPs. Melanin granules were dissolved in NaOH solution, followed by neutralization under sonication to get homogenous water-soluble MNPs. After PEGylation and RGD attachment for tumor targeting, Fe^{3+} ion and $^{64}\text{Cu}^{2+}$ ion were chelated for MRI and PET respectively. (b) *In vitro* PA properties of MNPs. PA signals of PEG-MNPs as a function of concentration (0.625, 1.25, 2.5, 5.0, 10 and 20 μM). $R^2 = 0.995$ (c) Overlay of ultrasonic (gray), PA (red) and corresponding subtraction images of xenograft tumor before and after administration of ^{64}Cu -Fe-RGD-PEG-MNP (10 μM , 200 μL) in living mice via tail vein injection.^[109] Reprinted with permission from ref 11. Copyright 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Perylene-diimide-based Nanoparticles

Perylene tetracarboxylic diimide (PDI) with intense photoluminescence and photochemical stability has long been served as industrial pigment.¹⁴⁰⁻¹⁴¹ Recent applications of perylene bismides are focused on their prominent roles in organic electronic devices owing to the high n-type conductivity.¹⁴²⁻¹⁴⁴ For the first time, Cheng *et al.* successfully developed PDI based nanoparticles as an efficient contrast agent for deep brain tumor in living mice (Figure 2.7a).¹⁴⁵ PDI molecules consisting of tertiary amine groups as electron donors and diimide groups as electron acceptors were encapsulated in the amphiphilic DSPE-mPEG to obtain water-soluble nanoparticles. PDI nanoparticles had strong absorption at around 700 nm with the extinction coefficient as high as $2.00 \times 10^8 \text{ M}^{-1}\text{cm}^{-1}$, indicating its outstanding capability as NIR light absorber. The imaging performance of as-prepared PDI nanoparticles with high photo-stability and PA sensitivity were investigated via PA imaging of orthotopic glioblastoma tumor models in vivo (Figure 2.7b). At 2 h post-injection, the PA image of the skull region in tumor mice was apparently brighter than that of the control mice due to the circulating PDI nanoparticles within blood vessels. After 1 d injection, an obvious PA signal in tumor region was observed as shown by blue dotted circle in Figure 2.7b, due to the enhanced permeability of PDI nanoparticles into extravascular space caused by the disrupted blood brain barrier in tumor areas. As a result, a deeper tumor region was clearly visualized after 2 d injection. PA spectra measurements of circle areas were also conducted to further validate the successful accumulation of PDI nanoparticles in brain tumor. As shown in Figure 2.7c, the in vivo PA spectrum with two peaks at 700 and 735 nm corresponded well with the in vitro NIR absorption spectrum of PDI nanoparticles. In addition, the spatial localization of PDI nanoparticles were well

delineated through overlaying PA and US images of tumor models, suggesting the excellent ability of PDI nanoparticles for 3D PA imaging of brain tumor.

Figure 2.7. In vivo PA imaging using PDI (a) Scheme of *in vivo* PA imaging process of brain tumor using PDI nanoparticles. (b) PA coronal sections of brain tumor models before and after administration of 250 μ L of 250 nM PDI nanoparticles via tail vein injection. Skull region and tumor region were delineated by red and blue dotted circles, respectively. (c) PA spectra of PDI nanoparticles in water (black line), skull region of (b) before injection of nanoparticles (red dotted circle, 0 h) and tumor region of (b) after 2d injection of nanoparticles (blue dotted circle, 2d).^[116] Reprinted with permission from ref 11. Copyright 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Squaraine dye-based Nanoparticles

Visible-light-absorbing squaraine dyes (SQs) with high absorption coefficients are among the most promising fluorophores for optical imaging.¹⁴⁶ Recently, SQ aggregates with red-

shifted absorption in the NIR region as compared with its unimolecular form have been exploited as contrast agents for PA imaging of tumor.¹⁴⁷ For example, Wurthner and Wang *et al.* reported nano-confined SQ assemblies for PA/NIR fluorescence dual-modal imaging of tumor.¹⁴⁸ Halogenated dicyanovinyl-functionalized SQs enveloped within hydrophobic bilayers of liposomes were prone to form H-aggregates, leading to fluorescence quenching and in turn amplified PA signals. The reversible self-assembly of SQ monomers into H-aggregates in lipid bilayers could be adjusted by altering the mixing ratio of SQ and phospholipid. Ten-fold enhancement of PA signals at the tumor site were detected in vivo after administration of SQ-embedded (1w%) liposomes as compared with the control mice treated only with liposomes. Based on such aggregation-induced red-shifted absorption, Li *et al.* similarly designed a NIR-absorbing SQ-albumin nanocomplexes.¹⁴⁹ In vivo PA imaging application of such SQ nanocomplexes was successfully validated by imaging the liver of a living mouse.

Semiconducting Polymer Nanoparticles

SPs are optically active organic materials with π -conjugated backbones which are typically used for solar cells, electronic sensors and tissue engineering.¹⁵⁰⁻¹⁵² In addition to fluorescence imaging applications,^{27, 153-154} SPs lately have been developed into a new class of PA contrast agents due to their strong NIR absorption.¹⁵⁵ SPNs have many intrinsic advantages such as good biocompatibility, excellent photostability and oxidative tolerance. These features are desired for in vivo optical imaging but do not exist in other imaging agents such as small-molecule dyes and metallic nanoparticles (QDs and gold nanorods). Pu and Rao *et al.* developed a new series of SPs consisting of low-bandgap diketopyrrolopyrrole (DPP) moieties for in vivo PA imaging of tumor.¹⁵⁶ Three DPP-based

copolymers (SP2, SP3 and SP4) were synthesized and then formulated into the PEGylated SPNs via nanoprecipitation (Figure 2.8a). Poly(cyclopentadithiophene-alt-benzothiadiazole)-based SP1 (PCPDTBT) was formulated in a similar way for comparison. The UV-vis absorption spectra of SPN1-4 were shown in Figure 2.8b. Among these SPNs, SPN4 had the brightest PA signal because of the highest efficiency of photothermal conversion resulted from its strongest electron donor-acceptor backbone and narrowest bandgap (Figure 2.8c). The capability of SPN4 for tumor imaging was validated using the HeLa xenograft tumor model. The PA signal in the tumor area reached maximum at 2h after administration of SPN4 via tail vein injection, 5.3-fold of the background signal (Figure 2.8e). Real-time *in vivo* PA spectrum of tumor region correlated well with the spectrum of SPN4 in solution, further confirming the enhanced PA signal coming from its accumulation (Figure 2.8f). Furthermore, the 3D PA images at 2h post-injection distinctly depicted the PA signals were detected in the regions both inside and outside the blood vessels of the tumor, demonstrating that SPN4 was capable of effusing from the blood vessels of tumor, probably due to their relatively small size (Figure 2.8d). Pu *et al.* further designed self-quenched SPNs for amplified PA imaging of tumor in living mice.¹⁵⁷ Incorporation of benzothiadiazole (BT) as an electron-deficient structure unit into the backbone of SPs ultimately afforded 1.7-fold PA amplification compared with the corresponding SPN. In addition, Pu's group developed the first generation of structurally stable NIR absorbing semiconducting polymer amphiphiles (SPAs) which was capable of spontaneous self-assembly into homogenous NPs for *in vivo* PA imaging.¹⁵⁸ Different from the SPNs prepared from nanoprecipitation which had the highest uptake in liver, the SPAs

showed the highest accumulation in tumor. This was benefited from the self-assembly design which overcame the tissue of nanoparticle dissociation.

Since the generation of PA signal is closely related to photothermal conversion, SPNs have also been utilized in the synergistic application of PA imaging and PTT. Pu *et al* demonstrated an intraparticle molecular orbital engineering approach to develop SPNs with amplified PA brightness and PTT efficacy for in vivo imaging and treatment of cancer.¹⁵⁹ Within the nanoparticles, SP1 and the optical dopant (PC70BM) served as the electron donor and acceptor, respectively. Photoinduced electron transfer favoured by the energy level alignment between the optical components led to quenched fluorescence and augmented nonradiative heat generation upon laser irradiation, consequently affording both amplified PA brightness and enhanced PTT efficacy. As confirmed by both in vivo and ex vivo histological data, utilization of SPN-F20 (PC70BM doping amount 20 w/w%) as the optical theranostics permitted enhanced PA imaging and improved photothermal ablation of tumor in living mice. In another study, Dai *et al.* combined polypyrrole (PPy) nanoparticles with tantalum oxide (TaOx) for X-ray CT/PA bimodal imaging-guided PTT of tumor.¹⁶⁰ In this study, TaOx were encapsulated into PPy nanoparticles via a facile one-step chemical oxidation polymerization. The resulted TaOx@PPy nanoparticles exhibited an excellent photothermal efficacy due to the strong NIR absorption and could be utilized for accurate locating of cancerous tissue as well as precise guidance for PTT.

Figure 2.8. In vivo PA imaging using SPN. (a) Schematic illustration of components, fluorescence and PA mechanism of SPNs. (b) UV-Vis-NIR absorption spectra of SPN1-4. (c) PA spectra of SPN1-4 at pH = 7.4. (d) Representative 3D PA image of xenograft tumor in a living mouse at 2 h after injection of SPN4 ($30 \mu\text{g mouse}^{-1}$). PA image was acquired at 750 nm. (e) PA amplitudes at 750 nm of tumor over time post-injection of SPN4. Error bars represent standard deviations of three separate measurements. (f) Real-time *in vivo* PA spectra of tumors in living mice at 2 h after administration of SPN4 or saline.^[128] Reprinted with permission from ref 11. Copyright 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.4 Phototherapy

2.4.1 Photothermal therapy

Phototherapy depends on light-tissue interactions to achieve therapeutic effect. In principle, it includes two modalities: PTT and PDT. PTT employs light-absorbing molecules to

realize photothermal effect, which converts incident photoenergy into heat so as to cause damage to cells.¹⁶¹ Compared with traditional cancer therapies such as radiotherapy, chemotherapy and surgery, PTT demonstrates unique merits such as precise regulation, minimal invasiveness to surrounding tissues, low cost, handiness, etc. In recent years, the progress in nanoscience has inspired the convergence of both fields and a diversity of photothermal nanotherapeutics (PTN) have been developed to amplify photothermal effect so as to advance PTT of cancer (Figure 2.9). They include metallic nanostructures (e.g. constructed based on gold, silver, platinum, copper, etc.), 2D nanomaterials, magnetic nanoparticles, metal-organic hybrid nanocomposites, small molecule dyes, SPNs, and so forth.¹⁶² To improve therapeutic outcome, PTT could be guided with imaging modalities (e.g. fluorescence or PA imaging) and/or combined with other cancer treatments such as chemotherapy. In addition to ablation effect, hyperthermia could cause super-enhanced permeability and retention effect in vasculature, of which the degree is increased by up to ~24-fold relative to enhanced permeability and retention (EPR) effect.¹⁶³ When PTT is applied in combination with nanotherapeutics, such superior vascular permeability potentially contributes to promoted intratumoral delivery of nanotherapeutics to enhance overall therapeutic efficacy.

Figure 2.9. Scheme of PTT to treat cancer and inhibit cancer metastasis with PTN.

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For example, Liu et al. designed a nanovaccine to combined PTT with cancer immunotherapy (Figure 2.10).²⁰ Such nanosystem consisted of purely clinical approved components: ICG as photothermal agent, imiquimod (R837) as Toll like receptor 7 (TLR 7) agonist and poly(lactic-co-glycolic) acid (PLGA) as amphiphilic coating. Upon NIR laser irradiation, the administered PLGA-ICG-R837 nanoparticles mediated photothermal ablation of primary tumors, resulting in release of tumor associated antigens (TAAs) and damage-associated molecular patterns (DAMPs). Together with R837, these antigens and biomolecules served as the adjuvant to stimulate the maturation of dendritic cells (DCs). Applied in combination with immune checkpoint inhibitor aCTLA-4, the combinational therapy mediated potentiated immune response to combat cancer metastasis and rechallenge.

Figure 2.10. Schematic illustration of PLGA-ICG-R837-based PTT in combination with checkpoint blockade for cancer immunotherapy. Reprinted with permission from ref 20. Copyright 2016 Nature Publishing Group.

2.4.2 Photodynamic therapy

In addition to PTT, PDT provides another approach to initiating phototoxicity. PDT leverages photosensitizers which could be excited by laser irradiation to produce a deal of ROS so as to cause cellular damage. Such photo-excitable production of ROS additionally offers opportunities for photosensitizers to act as a mediator that triggers the activation of prodrug in photo-responsive therapies.¹⁶⁴ Resembling PTT, PDT has advantages of high spatiotemporal therapeutic regulation, high treatment selectivity, and minimal side effects to normal tissues. And numerous photosensitizing nanomaterials (e.g. QDs, TiO₂, porphyrin derivatives, SPNs) have been extensively studied to widen the applications of PDT in oncological intervention.¹⁴ Given the great merits, PDT has been approved for clinical use in dermatology via topical application.¹⁶⁵

Figure 2.11. Schematic illustration of summary of PDT.¹⁴ Reprinted with permission from ref 14. Copyright 2015 American Chemical Society.

As an example, Lin et al. reported a metal-organic framework (MOF)-based photosensitizer W-TBP to deliver immunostimulant CpG for synergized PDT and immunotherapy (Figure 2.12).¹⁶⁶ Nanosized MOFs have emerged as a novel family of nano-photosensitizers because of the excellent photosensitization capability assigned to the high loading of photosensitizing moieties. The cationic and porous nature of W-TBP enabled itself as a nanosized vehicle to deliver TLR9 agonist CpG oligodeoxynucleotides that were negatively charged. Upon laser irradiation, the obtained W-TBP/CpG mediated PDT to trigger immunogenic cell death (ICD), resulting in generation of TAAs and DAMPs. Meanwhile, CpG were delivered to DCs and triggered DC maturation to further enhance antigen presentation. Together with checkpoint blockade treatment via anti-PD-

L1, the combinational therapy aroused potent anti-tumor immunity and superb therapeutic efficacy in bilateral breast cancer model.

Figure 2.12. Synergistic PDT and immunotherapy via W-TBP/CpG. Reprinted with permission from ref 166. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.4.3 Photo-chemotherapy

As a conventional modality, chemotherapy is currently one of the most prevalent treatments of cancer in clinic. In addition to cell death through necrosis or apoptosis, recent

studies have indicated that some chemotherapeutic drugs (e.g. DOX, paclitaxel, oxaliplatin) are able to mediate ICD, providing opportunities to be synergized with cancer immunotherapy.¹⁶⁴ However, the therapeutic outcome of chemotherapy is frequently challenged by multi-drug resistance and severe side effects. The advent of nanotechnology has not only significantly improved the pharmacokinetics of chemotherapeutics during blood circulation but also enabled the combination of chemotherapy with other cancer treatments to optimize therapeutic efficacy. The convergence of phototherapy with chemotherapy especially demonstrates merits which not only provides additional photocytotoxicity to kill cancer cells but also enhances drug delivery to potentiate chemotherapy. For instance, Argitis et al. reported a smart nanosystem that could be degraded in response to either light or pH so as to release drug with high specificity for combinational photo-chemotherapy (Figure 2.13).¹⁶⁷ Such nanosystem is composed of polyacetal-based block copolymer as photo- and pH-labile matrix to encapsulate chemotherapeutic drug camptothecin (CPT) and photosensitizer hematoporphyrin (HP) (Figure 2.13a). Upon laser irradiation or under acidic condition, the labile polymer matrix underwent photolysis or acidolysis to liberate CPT and HP (Figure 2.13b). HP thus mediated photosensitization to produce ROS, which not only triggered cell death, but also caused endosome escape of both therapeutic molecules through photochemical internalization. Such concerted photo-chemotherapeutic modality via drug cocktail encapsulated in biodegradable polymer matrix allowed for precisely controlled intracellular drug release and ultimately increased cell death.

Figure 2.13. Chemo-phototherapy via dual biodegradable chemo-phototherapeutic nanoparticles. (a) Chemical structure and biodegradable mechanism of polymer matrix. (b) Proposed mechanism of intracellular drug liberation triggered by photoirradiation and pH. Reprinted with permission from ref 167. Copyright 2014 Nature Publishing Group.

2.4.4 Photo-ferrotherapy

Recent years have witnessed the emergence of ferrotherapy to treat cancer. Different from traditional chemotherapy, ferrotherapy relies on iron-containing therapeutics to mediate Fenton reaction which transforms mild ROS H_2O_2 to highly cytotoxic $\cdot\text{OH}$ so as to induce cell death.¹⁸ Very recent studies have indicated the FDA-approved Ferumoxytol iron oxide nanoparticles could trigger the polarization of tumor-associated macrophages from immunosuppressive M2 phenotype to pro-inflammatory M1 phenotype, thereby demonstrating great potential to immunotherapy.¹⁶⁸ Because of the distinctive cytotoxic pathway, ferrotherapy circumvents the common issues such as drug resistance or severe adverse effect that are commonly seen in chemotherapy, displaying great promise as a new

cancer treatment.¹⁶⁹ However, ferrotherapy usually suffers from relatively poor therapeutic efficiency, which is majorly due to the compromised catalytic condition in tumor microenvironment. To improve therapeutic outcome, ferrotherapy has been extensively studied to applied in combination with other cancer treatments. Because Fenton reaction could be potentiated by heat, PTT serves as an ideal partner to be synergized with ferrotherapy. And a number of photothermal ferrotherapeutic nanoagents have been developed. For instance, Liu and Zhang et al. prepared a nanozyme $\text{PtFe@Fe}_3\text{O}_4$ with both peroxidase and catalase-like activities in tumor microenvironment (Figure 2.14).¹⁹ Because of the broad absorption of the nanozyme, it could additionally serve as photothermal transducer. When NIR photoirradiation is applied, the nanozyme simultaneously mediated photothermal effect to reinforce both catalytic reactions, ultimately contributing to augmented killing effect and superior inhibition of deep tissue pancreatic cancer.

Figure 2.14. Scheme of photothermally enhanced therapeutic efficacy of $\text{PtFe@Fe}_3\text{O}_4$ nanozyme. Reprinted with permission from ref 19. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.5 Photo-regulation of biological events

Photo-regulation that makes use of light to remotely manipulate biological processes has gained increasing research attention. In comparison with other external stimuli such as magnetic field, electronic field and mechanic force, light possesses intrinsic advantages such as cost-effectiveness, convenience, and non-invasiveness. Thereby, it has been used for versatile regulatory events which include photo-induced crosslinking of tissues, photostimulation of ion channels, photo-uncaging of bio-regulators, and so forth.¹⁷⁰ Due to the shortage of endogenous photosensitive biomolecules, exogenous molecular transducers play essential roles to advance photo-regulation in living subjects. In principle, these transducers transform incident light into versatile forms such as ROS, heat, or acoustic waves to trigger biological change. Recently, photo-regulation has been applied in neuroscience to manipulate neuron activity or in visual systems to stimulate vision recovery,¹⁷¹⁻¹⁷² showing great potential to treat neurological disorders and ophthalmic diseases.

2.6 Biophotonics in second near-infrared window

NIR light from 650 to 1000 nm, termed as NIR-I window has been extensively investigated for optical imaging of various diseases.¹² Compared with conventional visible region that undergoes significant attenuation by endogenous molecules, NIR-I light is able to achieve higher transmittance in biological tissues attributed to the reduced light-tissue interactions of photons at longer wavelengths (Figure 2.15b, c, and d). As a consequence, NIR-I imaging can be used for deeper visualization of anatomical structures, pathological changes, and metabolic processes *in vivo*.¹⁷³⁻¹⁷⁵ However, NIR-I window remains suboptimal

because of the intrinsic issues of high tissue autofluorescence and shallow tissue penetration depth.¹⁷⁶

Figure 2.15. NIR-II vs NIR-I window for in vivo imaging. (a) Schematic illustration of light-tissue interaction. (b) Reduced scattering coefficients (μ_s') of various tissues plotted as a function of wavelength covering 400-1700 nm. (c) Vis-NIR absorption spectrum of water. OD represented optical density. NIR-IIa: 1300-1400 nm; NIR-IIb: 1500-1700 nm. (d) Effective attenuation coefficients of blood, skin, and fat plotted as a function of wavelength covering 200-1800 nm. (e) Ex vivo autofluorescence spectra of mouse liver, spleen, and heart tissue. Excitation wavelength: 808 nm. (f) Quantum efficiency of cameras utilizing silicon (Si), mercury cadmium telluride (HgCdTe), or indium gallium arsenide (InGaAs) sensors as a function of wavelength (200-1800 nm).^{12,176-177} Reprinted with permission from ref 12. Copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Very recent studies have identified a new optical imaging region, the second NIR window (NIR-II, 1000-1700 nm), which has high potential to impart much superior imaging quality

than NIR-I window.¹⁷⁶ Advantages of NIR-II over NIR-I window could be well explained by the analysis of light-tissue interactions in the context of fluorescence and photoacoustic (PA) imaging.¹² As shown in Figure 2.15a, four typical processes are involved in light-tissue interactions when a laser beam penetrates through biological tissues: reflection, scattering, absorption that generates background PA signals, and autofluorescence emitted by endogenous fluorophores. Among these factors, scattering of light in biological tissues plays an explicitly crucial role in optical imaging since stray light usually contributes to background noise and also diminishes penetration depth. Note that light scattering follows an inversely proportional declining trend with the increase of wavelength in almost all kinds of tissues (Figure 2.15b).¹⁷⁸⁻¹⁷⁹ Such reduced scattering pattern affords decreased background noise and thus contributes to the improved SBR when going into tissues at longer wavelengths.

Autofluorescence caused by endogenous fluorophores significantly decays as a function of emission wavelength upon NIR excitation, reaching a nearly autofluorescence-free region above 1500 nm (Figure 2.15e).¹⁸⁰⁻¹⁸¹ The obvious decrease of autofluorescence at longer wavelengths contributes to the reduced background interference with respect to fluorescence imaging, largely improving the imaging performance of organs rich of endogenous fluorophores. Additionally, due to the lower energy of photons with longer wavelengths, much higher maximum permissible exposure (MPE) is allowed for NIR-II than NIR-I lasers, giving rise to the enhanced applicable laser power especially for promoted PA imaging performance. Despite all these merits, the increased absorbance of water above 950 nm represents the pitfall for NIR-II window imaging (Figure 2.15c). As an important endogenous absorber, water exhibits extremely high absorption centered at

around 1450 nm with a surrounding range (1400-1500 nm), which has to be excluded from optical imaging.¹⁸²⁻¹⁸³

The combination of reduced light scattering, minimized autofluorescence, and increased MPE readily outperforms the slightly higher photon absorbance in the NIR-II window. As a result, NIR-II light allows for even deeper tissue penetration than NIR-I light with improved SBRs and superior clarity.¹² By virtue of NIR-II light, tissue depth unprecedented for NIR-I laser could be readily reached to detect biological processes happening deep inside living bodies. However, limited numbers of NIR-II contrast agents have been developed in the past few years. Thus far, the majority of NIR-II fluorophores are composed of inorganic nanomaterials such as QDs and rare-earth based nanoparticles.¹⁸⁴⁻¹⁸⁷ However, issues of potential metal ion leakage and long-term safety concern need to be considered when it comes to the practical use of these inorganic agents. Carbon-based structures such as single-walled carbon nanotubes have also been utilized for NIR-II fluorescence imaging but suffer from drawbacks such as relatively low QY, limited optical tunability, and difficulty in in vivo clearance.^{180, 183, 188-192} Similar problems have also been confronted in NIR-II PA imaging, since most NIR-II PA agents are designed based on inorganic nanomaterials (copper sulfide nanoparticles, silver nanoplates, and gold nanostructures et al.) as well.¹⁹³⁻¹⁹⁷

Recent development of organic contrast agents with the emission or absorption above 1000 nm opens new opportunities to in vivo imaging in NIR-II window.¹⁹⁸⁻¹⁹⁹ Fine-tuning of energy band gaps via molecular engineering allows for tunable emission or absorption peaks covering the NIR-II region. Compared with inorganic nanomaterials, organic contrast agents eliminate the common concern of heavy metal ion-induced toxicity and are

capable of in vivo biodegradation or fast excretion after careful design, reducing the risk of unknown long-term damage caused by residual contrast agents.²⁰⁰⁻²⁰¹ More importantly, organic NIR-II agents are of similar chemical properties and pharmacokinetics with Food and Drug Administration (FDA)-approved NIR-I organic agents, highlighting their great potential towards clinical translation.

Currently, PA imaging emerging as a promising diagnostic technique has been progressively studied worldwide, pushing forward related applications from bench to bedside.²⁰²⁻²⁰⁶ Different from traditional optical imaging, PA imaging relies on PA effect to transform photons absorbed by endogenous or exogenous chromophores into acoustic waves which experience much reduced scattering in biological tissues compared with photons.¹¹ As a hybrid imaging modality, PA imaging combines the high contrast of optical imaging and high spatial resolution of ultrasound imaging, allowing for clear imaging at centimeter-scale depth in living bodies.²⁰⁷ Current PA imaging mainly capitalizes on NIR-I laser.² Nevertheless, further investigations of PA imaging in a superior NIR-II window have been far less reported.

Increasing efforts have been made to develop PA agents that potently absorb light ranging from 1000 to 1350 nm within NIR-II window in recent years. Thanks to the largely increased MPE and reduced scattering of incident photons at longer wavelengths, NIR-II PA imaging is envisioned to endow further improved imaging depth as well as enhanced SBR relative to utilizing NIR-I laser, regardless of the slightly higher endogenous absorption in NIR-II window.^{196, 208} In practice, a surprising depth of 11.6 cm in chicken breast tissue has been reached through PA imaging at 1064 nm using an organic contrast agent based on phosphorus phthalocyanine.²⁰⁹ Successive development of organic NIR-II

absorbers is of vital importance to impart noninvasive PA imaging of physiological or pathological processes in centimeters deep in living organisms, contributing to effective diagnosis of various diseases at unprecedented depths.

Figure 2.16. NIR-II PA imaging of rat brain vasculature using SPN-II. (a) Schematic illustration of SPN-II. (b) UV-Vis-NIR absorption spectrum of SPN-II (40 µg mL⁻¹ in tetrahydrofuran). (c) PA spectra of SPN-II (200 µg mL⁻¹) and water. Error bars correspond to standard deviations of 10 separate measurements. (d) In vitro deep-tissue PA imaging in NIR-I and NIR-II windows. SPN-II (1 mg mL⁻¹) spots were embedded in an agar gel phantom placed under different depths of chicken breast tissue. SNRs (dB) of SPN-II spots at 750 and 1064 nm were plotted as a function of the depth of chicken breast tissue, respectively. Energy density: 5.5 mJ cm⁻². $R^2 = 0.92549$ and 0.99172 for 750 and 1064 nm, respectively. (e) Two-dimensional NIR-II PA images of the agar gel phantom containing SPN-II spots at different depths of chicken breast tissue. Energy density: 20 mJ cm⁻² at 1064 nm. Concentrations of SPN-II spots (from the white asterisk anti-clockwise): 1, 0.5, 0.2, and 0.05 mg mL⁻¹. (f) In vivo PA imaging of rat brain in NIR-II compared with NIR-

I window. Representative PA images of rat cortex were captured at 70 min after tail vein injection of SPN-II (1.8 mg per rat) at 750 and 1064 nm, respectively. Energy density: 5.5 mJ cm⁻². (g) SNRs (dB) of brain cortex at 70 min post-injection of SPN-II at 750 and 1064 nm. Energy density, 5.5 mJ/cm⁻². *Statistically significant difference in SNR (dB) between NIR-I and NIR-II window ($p < 0.05$, $n = 3$).²⁰⁸ Reprinted with permission from ref 208. Copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

To substantially validate the advantage of NIR-II over NIR-I light for PA imaging, Pu *et al.* reported an organic PA agent SP2 with broadband absorption for the comparative study of PA performance in both NIR windows (Figure 2.16a).²⁰⁸ By virtue of the highly electron-deficient acceptor thiadiazoloquinoxaline and enhanced donor–acceptor interactions due to D–A₁–D–A₂ structure, SP2 exhibited lower band gap and subsequently strong absorption in NIR-II window. After enveloping of SP2 by PEG-b-PPG-b-PEG, the resulted water-soluble SPN-II demonstrated a broadband absorbing property with an absorption peak at 1253 nm (Figure 2.16b). More importantly, SPN-II exhibited nearly identical PA signals at 750 and 1064 nm (Figure 2.16c), enabling itself an optimal contrast agent for the fair comparison of PA performance in both NIR windows.

In vitro deep-tissue PA imaging of SPN-II solutions embedded under different depths of chicken breast tissue was performed on a homemade PA system with switchable wavelengths between 750 and 1064 nm. As shown in Figure 18d, signal-to-noise ratio (SNR) in decibels of SPN-II solution imaged in NIR-II window was higher than that in NIR-I window at each depth of chicken breast tissue when compared at the same power density. In particular, the SNR of SPN-II solution obtained at 1064 nm was 1.4-fold of that at 750 nm at a tissue depth of 3 cm. The maximum PA imaging depth was further enhanced

to 5 cm in chicken breast tissue when increasing the energy density of 1064 nm laser to 20 mJ cm⁻² (Figure 2.16e).

Such superior PA imaging performance in NIR-II window was further validated by in vivo brain vasculature imaging on living rats. At 70 min post-injection of SPN-II, the PA image captured at 1064 nm exhibited improved clarity than that at 750 nm, as indicated by the higher contrast and clearer branch vessels (Figure 2.16f). Detailed ROI analysis indicated that the SNR gleaned at 1064 nm (~27.7 dB) was 1.5-times of that at 750 nm (~18.2 dB) (Figure 2.16g). Taken together, this work provided solid evidence of the superiority of PA imaging in NIR-II window compared with traditional NIR-I window, underlining the need for developing PA agents with strong absorption beyond 1000 nm.

Figure 2.17. NIR-II PA imaging of tumor. (a) UV-Vis-NIR absorption spectra of TII-PEG and TSPN. The green arrow indicated that the convex was due to the saturated water absorption. (b) In vivo PA (depicted in red and yellow) and US (depicted in grey) imaging of mouse tumors (dashed circles) with (upper) or without (lower) injection of matrigel inclusions of TSPNs (50 μ L, 40 μ g mL⁻¹). Energy density: 800 nm, 10-26 mJ cm⁻²; 1000

nm, 19-49 mJ cm⁻²; 1100 nm, 33-40 mJ cm⁻²; 1200 nm, 37-46 mJ cm⁻²; 1300 nm, 25-35 mJ cm⁻². (c) Vis-NIR absorption spectrum of P1 NPs (10 µg mL⁻¹). (d) In vivo PA and US (depicted in grey) imaging of brain tumor post-injection of P1 NPs. Scale bar: 5 mm.²¹⁰⁻²¹¹

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Later, NIR-II PA imaging of cancer has also been carefully investigated. Mei and Cheng *et al.* developed a SP-based agent TSPN with strong NIR absorption and utilized it for multi-wavelength PA imaging of tumor in NIR-II window (Figure 2.17a).²¹⁰ Thanks to the electron deficiency and high planarity of thienoisindigo (TII) unit, TII-TEG with triethylene glycol (TEG) side chains exhibited ultra-narrow bandgap and in turn strong absorption in NIR-II region. Matching well with the absorption spectrum, the water-soluble TSPNs after formulation of TII-PEG with DSPE-PEG2000 generated strong PA signals in NIR-II region. As a result, the tube containing TSPNs with a low concentration of 40 µg mL⁻¹ was clearly visualized by NIR-II PA imaging after embedding in chicken breast tissue at a depth up to 5.3 cm. Furthermore, TSPNs injected into the tumor could be clearly depicted by PA imaging conducted at multiple wavelengths ranging from 800 to 1300 nm (Figure 2.17b). In particular, thanks to the reduced interference from background tissue, the PA signal from tumor region was promoted by ~7 fold and ~13.3 fold at 1100 and 1300 nm after administration of TSPNs, respectively. To sum up, this study disclosed the optimal wavelengths (1100 and 1300 nm) in NIR-II window for deep tissue PA imaging with enhanced contrast. Further development of TSPN was expected to afford various functionalities for NIR-II theranostics.

Similar study of NIR-II PA imaging of cancer has also been reported on a brain tumor model.²¹¹ To endow a low bandgap and far-red absorption, a strongly electron-deficient acceptor benzobisthiadiazole (BBT) was utilized to copolymerize with a well-known electron donor benzodithiophene (BDT) to obtain P1. On account of the high planarity and intramolecular charge transfer (ICT) in the conjugated backbone, P1 exhibited red-shifted absorption in the NIR-II region with a relatively high extinction coefficient ($\epsilon_{1064\text{ nm}} = 22.6\text{ mL mg}^{-1}\text{ cm}^{-1}$) after encapsulation by DSPE-PEG2000 to P1 NPs (Figure 19c). Consistent with the absorption spectrum, P1 NPs generated strong PA signals at 1064 nm so that a very low concentration of P1 NPs ($6\text{ }\mu\text{g mL}^{-1}$) could be detected by PA imaging at a limited power density (5.6 mJ cm^{-2}). More importantly, dual-modal PA/US imaging of orthotopic brain tumor was successfully performed at 1064 nm using P1 NPs on living mice. As shown in Figure 2.17d, the tumor was clearly delineated at 3 h post-injection of P1 NPs, as indicated by the strong PA signal detected under skin and skull. Notably, the tumor region at a tissue depth of $\sim 3.4\text{ mm}$ could be obviously visualized at 24 h post-injection with an unprecedented signal-to-background ratio of 59. Taken together, Liu and Zheng et al. reported the first organic contrast agent utilized for PA imaging of brain tumors in NIR-II window, highlighting the necessity to develop highly efficient NIR-II PA agents for potential applications in brain diseases.

In addition to photoacoustic imaging, NIR-II absorbing nanoagents have been leveraged for photothermal bio-applications such as photothermal ablation, photothermally controlled drug release, dilation of vessels, and so on. For instance, Han et al. utilized CuS as the NIR-II photothermal switch to regulate transient receptor potential vanilloid 1 (TRPV1) channel for precise tumor therapy.²¹² As another example, Zhao, Zhou and Sheng

et al. cooperatively reported Au/Ag nanorods as activatable NIR-II photothermal transducers to treat multidrug-resistant *Staphylococcus aureus* (MRSA) infections.²¹³ In the presence of ferricyanide, Au/Ag nanorods gradually dissolved to release cytotoxic Ag^+ and expose NIR-II absorbing Au rods to amplify photothermal effect. Such synergistic treatment ultimately led to superior bacteria elimination and wound healing.

Chapter 3 Semiconducting afterglow luminescent nanoparticles for ultrasensitive in vivo imaging

3.1 Introduction

Optical imaging that utilizes photon-electron interactions to decipher biological processes has grown into an indispensable tool in biomedical research and clinical practice.¹ Complementary to tomographic modalities such as magnetic resonance imaging (MRI), computed tomography (CT), and positron emission tomography (PET), optical imaging has the unique advantages of high spatial-temporal resolution and low cost, permitting real-time investigation of pathological processes at molecular level and sensitive detection of diseases for intraoperative imaging-guided surgery.^{8, 214-216} However, most optical techniques detect fluorescent signals generated upon real-time light excitation, wherein the background noise from endogenous molecules in biological subjects are inevitable.²¹⁷ Such a flaw challenges reliable detection of signals, giving rise to minimized signal-to-background ratio (SBR), limited penetration depth and consequently compromised imaging sensitivity.^{12, 176}

Real-time light-excitation free optical agents including chemiluminescent, bioluminescent, Cerenkov, and afterglow (or persistent luminescent) probes can circumvent the interference of tissue autofluorescence.²¹⁸ However, each has its own trade-offs. For instance, chemiluminescent and bioluminescent agents utilize chemical reactions that respectively require reactive oxygen species and enzyme to catalyze the decomposition of substrates to trigger luminescence,^{174, 219} and their imaging sensitivity is usually perturbed by cellular environment and substrate availability.²²⁰⁻²²¹ On the contrary, Cerenkov and afterglow

agents do not require particular chemical mediator or exogenous enzyme, and thus have higher versatility for imaging applications. However, Cerenkov agents are basically radioisotopes and intrinsically limited to only emit visible light,²²²⁻²²³ thus their biomedical applications are challenged by both the biosafety issue of radiotracers and shallow imaging depth due to short-wavelength emission. Differently, afterglow agents act as the optical battery to trap irradiated photoenergy in defects and then slowly release the stored energy by photonic emission upon physical (thermal, mechanical, etc.) activation, eliminating the need of invasive radiotracers or exogenous mediators.²²⁴

Despite the advantage of afterglow imaging over other excitation-free strategies,²²⁵ there are only two kinds of afterglow nanoagents, which are the rare-earth metal (e.g. Europium, Praseodymium) containing inorganic nanoparticles and poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEHPPV) based organic nanoparticles.^{47, 173, 226-229} The inorganic agents are limited to the general formulas (e.g. $\text{ZnGa}_{2(1-x)}\text{Cr}_{2x}\text{O}_4$), which may suffer from potential leakage of heavy metal ions.^{42, 230} In contrast, the MEHPPV based nanoparticles have demonstrated high biocompatibility in living mice. Because of the higher SBR of afterglow relative to fluorescence (up to ~150-fold),¹⁷³ these afterglow agents have been utilized for in vivo cell tracking,²³¹⁻²³² monitoring of biomarker (e.g. glutathione, ascorbic acid),^{46, 233} visualization of vascularization,⁴² lymph node mapping,²³⁴ monitoring of drug-induced hepatotoxicity,¹⁷³ in vivo temperature indication,²³⁵ and cancer theranostics.²³⁶⁻²⁴⁰ However, to fully explore the potential of afterglow imaging in fundamental biology and clinical practice, versatile afterglow agents with bright and tunable emission are of high demand.

Herein, we report a generic approach to transform ordinary fluorescent agents into afterglow luminescent nanoparticles (ALNPs) for *in vivo* imaging. This approach relies on an intraparticle cascade photoreaction of three key components termed as afterglow initiator, afterglow substrate, and afterglow relay unit (Figure 3.1a) to store the photoenergy as the chemical defects for delayed luminescence after cessation of light excitation. Within ALNPs, a photosensitizer serves as the afterglow initiator to absorb and convert photoenergy into signaling singlet oxygen ($^1\text{O}_2$); a $^1\text{O}_2$ -reactive molecule serves as the afterglow substrate to absorb and react with $^1\text{O}_2$, forming the unstable chemiluminescent intermediate (1,2-dioxetane); and a fluorescent agent behaves as the afterglow relay unit to accept the energy from 1,2-dioxetane *via* chemically initiated electron exchange luminescence (CIEEL), gradually releasing it in the form of photons. Depending on if there is an efficient secondary energy transfer (SET) between the fluorescent agent and the photosensitizer, the ultimate afterglow emission spectrum could be close to that of the fluorescent agent or the photosensitizer.

3.2 Materials and methods

3.2.1 Chemicals and characterization

All materials were purchased from Sigma-Aldrich Pte. Ltd. unless otherwise noted. PFVA, PFO, and PFBT were purchased from Luminescence Technology Corp. DO and SO were purchased from Aberjona Laboratories, Inc.

DLS profiles of nanoparticles were measured by Malvern Nano-ZS Particle Sizer. TEM images of nanoparticles were captured by JEOL JEM 1400 TEM with an acceleration rate of 100 kV. ^1H NMR were measured by Bruker Avance 300MHz NMR. Electrospray ionization mass spectrometry (ESI-MS) spectrum of RB was measured by ThermoFinnigan

LCQ Fleet MS equipped with Thermo Accela LC and ESI source. Absorption spectra were measured on a Shimadzu UV-2450 spectrophotometer. Fluorescence spectra and fluorescence efficiency were acquired on a Fluorolog 3 spectrofluorometer (HORIBA, Ltd.). Chemiluminescence of afterglow substrates was recorded with a Luminometer (Promega, USA). Molecular weights of PFVA ALNPs in biodegradation studies were characterized by GPC using THF as the eluent and polystyrene as the standard. White light source for afterglow luminescence was supplied by an LED Fiber Optic Illuminator (L-150A) with an output power density of 0.1 W cm^{-2} (wavelength range: 400-800 nm). Fiber coupled 808 nm laser system was purchased from Changchun New Industries Optoelectronics Tech. Co., Ltd. NIR fluorescence and afterglow images were acquired by IVIS SpectrumCT In Vivo Imaging System (PerkinElmer, Inc.).

3.2.2 Synthesis of RB

Compound 1 (1.0 g; 1.0 mmol) and 2-ethylhexyl bromide (0.5 g; 2.6 mmol) were dissolved and magnetically stirred in N, N-dimethylformamide (DMF) at 80 °C. After 6 h, excess 2-ethylhexyl bromide and DMF were removed by rotary evaporation. The residue was then dissolved in diethyl ether. To remove compound 6 and inorganic salts, the residue in diethyl ether was washed with water for 3 times followed by desiccation using anhydrous Na_2SO_4 . The obtained residue was further purified by column chromatography using ethyl acetate as the eluent to afford the final product RB in deep purple colour. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ (ppm): $\delta = 0.78\text{-}0.83$ (t, 6H), 1.24 (m, 8H), 1.45 (m, 1H), 3.86-3.88 (d, $J = 6$ Hz, 2H), 7.51-7.52 (s, 2H). ESI-MS (m/z): calculated: 1084.61; found: 1084.95.

3.2.3 Synthesis of HBA

HBA was synthesized following the method in literature.²⁴¹ ¹H NMR (300 MHz, CDCl₃, **Supplementary Fig. 4**) δ (ppm): δ = 1.78-1.96 (m, 12H), 2.65 (s, 1H), 3.24 (s, 1H), 3.32 (s, 3H), 5.35 (s, 1H), 6.76-6.79 (d, J = 9 Hz, 1H), 6.85-6.89 (m, 2H), 7.21 (t, 1H).

3.2.4 Preparation of ALNPs and SPN-NCBS5

ALNPs were prepared at the optimized doping ratios of different components. Afterglow initiator (0.0025 mg), afterglow substrate (0.05 mg), afterglow relay unit (0.1 mg), and PEG-b-PPG-b-PEG (10 mg) were dissolved in THF respectively and then mixed together under sonication. Then THF solvent was removed by rotary evaporation to afford a thin film. The obtained film was hydrated in distilled deionized water (2 mL) under vigorous sonication to prepare ALNPs. The resulted ALNP solutions were filtrated through 0.22 μ m Millipore poly(ether sulfone) syringe driven filter to remove impurities and then concentrated by ultracentrifugation. The concentrated ALNP solutions were diluted in 1 $\times$ PBS buffer (pH 7.4) and stored in dark at 4 $^{\circ}$ C.

SPN-NCBS5 nanoparticles were prepared following the reported method.¹⁷³ MEHPPV (0.25 mg), PEG-b-PPG-b-PEG (20 mg), and NCBS (0.0125 mg) were dissolved and mixed in THF (1 mL), followed by rapid injection into deionized water and removal of THF by a gentle N₂ flow. The resulted SPN-NCBS5 was then purified by filtration through the abovementioned 0.22 μ m filter and concentrated by ultracentrifugation.

3.2.5 NIR fluorescence and afterglow imaging

NIR fluorescence images of ALNPs were acquired by IVIS SpectrumCT In Vivo Imaging System under fluorescence mode with exposure time for 0.1 s. Fluorescence signals were

collected with excitation at 710 nm and emission at 780 nm. Afterglow images of ALNPs were acquired by IVIS SpectrumCT In Vivo Imaging System within 5 s after laser irradiation under bioluminescence mode with open filter (exposure time: 1 s). Afterglow spectra of ALNPs were acquired at similar conditions yet with specific emission filters. Both fluorescence and afterglow intensities were quantified by region of interest (ROI) analysis using Living Imaging 4.3 Software.

3.2.6 Definition and measurement of afterglow descriptors

Afterglow descriptors were measured and calculated using N-DO ALNPs as the reference. Relative afterglow intensity ($\Phi_{\text{Afterglow}}$) was calculated as the ratio of absolute afterglow intensity of individual ALNP (I_{ALNP}) to that of N-DO ($I_{\text{N-DO}}$) under identical mass concentration and laser condition ([afterglow initiator] = 0.75 $\mu\text{g mL}^{-1}$, [afterglow substrate] = 15 $\mu\text{g mL}^{-1}$; laser condition: 808 nm laser at 1 W cm^{-2} for NCBS doped ALNPs while white light at 0.1 W cm^{-2} for TPP and RB doped ALNPs for 5 s). Equation was listed as follows (Eq. 2):

$$\Phi_{\text{Afterglow}} = \frac{I_{\text{ALNP}}}{I_{\text{N-DO}}}$$

Chemiluminescence quantum yields (Φ_{Cl}) of SO and HBA were measured and calculated relative to that of DO ($\Phi_{\text{Cl}} = 0.021$) referring to the reported method.²⁴²

Production of $^1\text{O}_2$ by afterglow initiator (Φ_{S1}) (0.75 $\mu\text{g mL}^{-1}$, 1 mL) was calculated as the fluorescence enhancement (F/F_0) of $^1\text{O}_2$ sensor green (SOSG, 1 μM) at 524 nm after laser irradiation (808 nm at 1 W cm^{-2} for NCBS while white light at 0.1 W cm^{-2} for TPP and RB) for 5 s (Eq. 3).

$$\Phi_{S1} = \frac{F}{F_0}$$

Fluorescence efficiency of ALNPs (η_{FI}) was calculated as the ratio of integrated fluorescence intensity of particular ALNPs ($\int_{500}^{840} Fl_{ALNP} d\lambda$) to that of N-DO ($\int_{500}^{840} Fl_N d\lambda$) with identical mass concentration ([afterglow relay unit] = 5 $\mu\text{g mL}^{-1}$, [afterglow initiator] = 0.125 $\mu\text{g mL}^{-1}$; excitation: 450 nm, emission: 500-840 nm) (Eq. 4). Afterglow substrates were excluded because of their negligible influence on fluorescent emission.

$$\eta_{FI} = \frac{\int_{500}^{840} Fl_{ALNP} d\lambda}{\int_{500}^{840} Fl_N d\lambda}$$

HOMO energy levels of afterglow relay units were collected from either references or computational calculation.

3.2.7 Calculation of energy levels

HOMO and LUMO energy levels of Reso and high-energy intermediates of DO, SO, and HBA were calculated by Gaussian 09 software based on density functional theory (DFT) with B3LYP/6-31G(d) method.

3.2.8 Cell culture and cytotoxicity assay

4T1 murine mammary carcinoma cells were purchased from ATCC (American Type Culture Collection). These cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (fetal bovine serum) and 1% antibiotics (10 U/mL penicillin and 10 mg/mL streptomycin). Flasks seeded with 4T1 cells were placed in an incubator with 5% CO₂ and 95% O₂ humidified air atmosphere at 37 °C.

To examine the cytotoxicity of ALNPs, 4T1 Cells were seeded in 96-well plates (6000 cells in 200 μL supplemented DMEM per well). After culture for 24 h, PFVA-N, PFVA-

N-DO, PFVA-N-SO, and PFVA-N-HBA (final concentration [PFVA] = 5, 10, 30, 50 $\mu\text{g mL}^{-1}$) were added to cell culture medium, respectively. After incubation of nanoparticles with cells for 24 h, the culture medium was removed, and cells were gently washed with fresh sterile $1 \times$ PBS buffer for 3 times. Fresh supplemented DMEM (100 μL per well) mixed with MTS (0.1 mg mL^{-1} , 20 μL per well) was then added to cells. After 3 h incubation, absorbance of culture medium at 490 nm was recorded by SpectraMax M5 microplate/cuvette reader. Because absorbance at 490 nm is proportional to the quantity of living cells, cell viability was calculated as the ratio of absorbance of sample treated cells to that of control cells.

3.2.9 In vitro and in vivo tissue penetration study

PFVA-N-DO, PFVA-N-SO, PFVA-N-HBA ([PFVA] = 100 $\mu\text{g mL}^{-1}$, [DO] = 50 $\mu\text{g mL}^{-1}$, [NCBS] = 2.5 $\mu\text{g mL}^{-1}$, 50 μL), and SPN-NCBS5 solutions ([MEHPPV] = 100 $\mu\text{g mL}^{-1}$, [NCBS] = 5 $\mu\text{g mL}^{-1}$, 50 μL) were placed under different depths of chicken breast tissue (0, 1, 2, 3, 4, or 5 cm). NIR fluorescence images of PFVA-N-DO beneath different depths of tissue were captured (exposure time: 0.1s) with excitation and emission wavelength at 710 and 780 nm, respectively. Before capturing afterglow images, PFVA-N-DO, PFVA-N-SO, PFVA-N-HBA, and SPN-NCBS5 solutions were pre-irradiated with 808 nm laser at 1 W cm^{-2} for 5 s. Afterglow luminescence was then recorded under bioluminescence mode with open filter in IVIS SpectrumCT In Vivo Imaging System (exposure time: 1 s). As for in vivo tissue penetration study, nanoparticle solutions were placed under a living mouse with a tissue depth of 1.5 cm, and other procedures was the same as in vitro tissue penetration study.

Signal-to-background ratio (SBR) is calculated as the ratio of luminescence (fluorescence or afterglow) in region of interest with ALNPs to that of tissue background without ALNPs.

3.2.10 Tumor mouse model

Animal experiments were carried out under the guidelines of Institutional Animal Care and Use Committee (IACUC), Sing Health. To establish tumor model, 2 million 4T1 cells suspended in supplemented DMEM were subcutaneously injected to the left shoulder of female NCr nude mouse (~ 6-week-old). Tumors were allowed to grow until 7~10 mm³ before in vivo NIR fluorescence and afterglow luminescence imaging.

3.2.11 In vivo tumor imaging

NIR fluorescence and afterglow luminescence imaging of 4T1-tumor bearing mice were carried out using IVIS SpectrumCT In Vivo Imaging System. NIR fluorescence and afterglow images were at first captured before injection of ALNPs. NIR fluorescence was acquired with excitation at 710 nm and emission at 780 nm (exposure time: 0.1 s). Afterglow images were acquired (exposure time: 1 s) after 808 nm laser irradiation of tumor region at 0.3 W cm⁻² for 5 s. 4T1-tumor bearing NCr nude mice were then intravenously injected with PFVA-N-DO nanoparticles ([PFVA] = 250 µg mL⁻¹, [DO] = 125 µg mL⁻¹, [NCBS] = 6.25 µg mL⁻¹, 250 µL, n = 3). Afterglow and NIR fluorescence images of mice were longitudinally monitored at different time points (1, 2, 3, 4, and 6 h). Quantification of afterglow luminescence and NIR fluorescence were performed by ROI analysis of tumor region using Living Imaging 4.3 Software.

Signal-to-background ratio (SBR) is calculated as the ratio of luminescence (fluorescence or afterglow) in tumor region after i.v. injection of ALNPs to that of tissue background before injection.

3.2.12 Biodistribution method

After 6 h post-injection of PFVA-N-DO ([PFVA] = 250 $\mu\text{g mL}^{-1}$, [DO] = 125 $\mu\text{g mL}^{-1}$, [NCBS] = 6.25 $\mu\text{g mL}^{-1}$, 250 μL , $n = 3$), mice were euthanized by CO_2 asphyxiation. Tumors and major organs (livers, hearts, spleens, kidneys, lungs, and intestines) were collected for ex vivo NIR fluorescence imaging (exposure time: 0.1 s) with excitation and emission wavelength at 710 and 780 nm, respectively. Quantification of fluorescence signal of individual tumor/organ was performed by ROI analysis using Living Imaging 4.3 Software.

3.2.13 In vivo clearance of PFVA-N-DO

6-week-old female NCr nude mice were intravenously administered with PFVA-N-DO ([PFVA] = 250 $\mu\text{g mL}^{-1}$, [DO] = 125 $\mu\text{g mL}^{-1}$, [NCBS] = 6.25 $\mu\text{g mL}^{-1}$, 250 μL , $n = 3$). NIR fluorescence images of mice in supine position were then long-termly monitored by IVIS SpectrumCT In Vivo Imaging System (exposure time: 0.1 s) with excitation and emission wavelengths at 710 and 780 nm, respectively. Representative images were captured at $t = 1, 1/6, 3, 7, 13, 14, 18, 20, 26, 33$ day after administration of PFVA-N-DO. Fluorescence intensities were quantified by ROI analysis using Living Imaging 4.3 Software.

3.2.14 Histological studies

After 33 days post-injection of PFVA-N-DO ($[(\text{PFVA}) = 250 \mu\text{g mL}^{-1}, [\text{DO}] = 125 \mu\text{g mL}^{-1}, [\text{NCBS}] = 6.25 \mu\text{g mL}^{-1}, 250 \mu\text{L}, n = 3)$ or saline ($250 \mu\text{L}, n = 3$), female NCr nude mice were euthanized by CO_2 asphyxiation and major organs (hearts, livers, spleens, lungs, and kidneys) were collected. These organs were then fixed in 4% paraformaldehyde followed by embedment in paraffin and 10- μm sectioning. H & E staining was then performed to tissue sections referring to standard procedure. Optical images were captured by Nikon ECLIPSE 80i microscope (Nikon Instruments Inc., NY, USA).

3.2.15 Data analysis.

Statistical analysis was performed by IBM SPSS Statistics software. Results were demonstrated as mean $\pm$ SD. Statistical significance was evaluated by two-tailed Student's t test. For all tests, a P value smaller than 0.05 was regarded as statistically significant. NIR fluorescence and afterglow images were analyzed using Living Imaging 4.3 Software.

3.2.16 Data availability

The authors declare that all data related to this study are available in the article/and or its supplementary information files or from the authors upon reasonable request.

3.3 Results and discussions

3.3.1 Preparation of ALNPs

To examine the generality of the afterglow design, different kinds of substances were chosen for each component to prepare the ALNPs. Three photosensitizers including RB

(rose bengal octyl ester), TPP (meso-tetraphenylporphyrin), and NCBS (silicon 2,3-naphthalocyanine bis(trihexylsilyloxy)) were used as the representative afterglow initiators (Figure. 3.1c), which had the excitation and emission maxima at 576 and 591 nm for RB, 418 and 650 nm for TPP, and 774 and 780 nm for NCBS, respectively. Thus, the afterglow luminescence could be triggered by light irradiation at different wavelengths. Three $^1\text{O}_2$ -responsive chemiluminescent molecules including DO (*N,N*-dimethyl-4-(3-phenyl-5,6-dihydro-1,4-dioxin-2-yl)aniline), SO (*N,N*-dimethyl-4-(2-phenyl-5,6-dihydro-1,4-oxathiin-3-yl)aniline), and HBA (3-((1*r*,3*r*,5*R*,7*S*)-adamantan-2-ylidene(methoxy)methyl)phenol) were chosen as the afterglow substrates (Figure 3.1d),²⁴¹⁻²⁴² which had chemiluminescent emission ranging from 350 to 550 nm. Three kinds of commonly used fluorescent agents were tested as the afterglow relay units, (Figure 3.1e), which included semiconducting polymers (SPs), small-molecule dyes (SMDs), and inorganic fluorophores (IFs) such as semiconductor quantum dots (QDs), graphene quantum dots (GQDs) and carbon quantum dots (CQDs). The fluorescence emissions of these agents ranged from visible to NIR region, providing the feasibility to fine-tune the afterglow profiles of ALNPs.

Figure 3.1. The design approach towards afterglow luminescent nanoparticles (ALNPs) (a) Schematic illustration of detailed intraparticle photoreaction processes leading to afterglow. (b) Illustration of afterglow imaging of ALNPs. (c-e) Chemical structures of afterglow initiators (c), afterglow substrates (d), afterglow relay units (e), and amphiphilic copolymer (f). PFVA, poly[(9,9'-dioctyl-2,7-divinylfluorenylene)-*alt*-(9,10-anthracene)]; PFBT, poly[(9,9'-dioctylfluorenyl-2,7-diyl)-*alt*-(benzo[2,1,3]thiadiazol-4,7-diyl)]; PFO, poly(9,9'-dioctylfluorenyl-2,7-diyl); PFODBT, poly[2,7-(9,9'-dioctylfluorene)-*alt*-4,7-bis(thiophen-2-yl)benzo-2,1,3-thiadiazole]; DiO, 3,3'-dioctadecyloxacarbocyanine perchlorate; NR, nile red; Reso, resorufin; QD630, CdSe/ZnS core-shell.²⁴³ Reprinted with permission from ref 243. Copyright 2019 Nature Publishing Group.

The afterglow contrast agents were prepared with different combinations of afterglow initiator, substrate, and relay unit through co-nanoprecipitation with an amphiphilic copolymer PEG-*b*-PPG-*b*-PEG (Figure 3.1f). The doping ratios for each component within the ALNPs were optimized. The solutions of resulted 50 kinds of ALNPs were translucent with no obvious precipitates after preparation. Dynamic light scattering (DLS) revealed the hydrodynamic diameters of the ALNPs ranged from 80 to 180 nm (Figure 3.2a), except for CQD-based ALNPs (12 nm). This should be attributed to the intrinsic hydrophilicity and surface charge of CQD. Furthermore, transmission electron microscope (TEM) revealed the spherical morphology of these ALNPs (Figure 3.2a).

Afterglow luminescence of these ALNPs were recorded with optimized light irradiation time (5 s). Moreover, the afterglow intensity of ALNPs was independent of their particle size. Besides, bright afterglow luminescence of ALNPs could be repeatedly recharged (up to 6 cycles) by optical irradiation or restored after preservation of pre-irradiated ALNPs at -20 °C. Because of different absorption, NCBS-doped ALNPs were irradiated with 808 nm laser (1 W cm⁻²) while RB- or TPP-doped ALNPs were irradiated with white light (0.1 W cm⁻²). As expected, luminescence was detected from ALNPs after cessation of laser irradiation (Figure 3.2d), which was barely detectable for the nanoparticles consisting of only afterglow initiator and substrate or relay unit (Figure 3.2e, f). This validated the proposed afterglow mechanism and the collaborative roles of three components. Dependent on the compositions of ALNPs, the afterglow luminescence spectra ranged from visible to NIR region (Figure 3.2b, c). In general, the afterglow spectra of ALNPs were similar to the corresponding fluorescence spectra. If there was energy transfer from afterglow relay unit to initiator, the shape of afterglow spectrum could be more like that of

the initiator. Otherwise, the afterglow emission was closer to that of the relay unit. Thereby, the full width at half maximum (FWHM) of afterglow emission should majorly depend on either the relay unit or the initiator. For instance, ALNPs consisting of PFO, NCBS, and DO (termed as PFO-N-DO) had a strong NIR afterglow emission at 780 nm because of the secondary energy transfer from PFO to NCBS; whereas ALNPs consisting of PFO, RB, and DO (termed as PFO-R-DO) only had the afterglow emission from PFO due to the inefficient energy transfer from PFO to RB. Discrepancy in the fluorescence and afterglow spectral profiles was observed for several ALNPs (especially TPP doped ones) such as GQD-N-DO, DiO-TPP-DO (termed as DiO-T-DO), CQD-R-DO, etc. This should be ascribed to the fact that the afterglow photophysical process was different from that of fluorescence: in the fluorescence process, light excitation only led to the emission of the fluorescent agent, which was followed by the potential energy transfer to the photosensitizer; whereas, in the afterglow process, in addition to such a potential energy transfer, the photosensitizer could be directly excited through the energy released from the high-energy intermediate (1,2-dioxetane). Thus, the photophysical interplay between the afterglow initiator and the relay unit offered additional space to finetune the afterglow profiles of ALNPs, allowing for potential multiplexed afterglow imaging.

Figure 3.2. Afterglow luminescence of ALNPs composed of varying afterglow initiators, substrates, and relay units. (a) Representative TEM image of PFVA-N-DO ALNPs (upper panel) and representative hydrodynamic diameters of various ALNPs measured by DLS (bottom panel) in $1 \times$ PBS buffer (pH = 7.4). Scale bar: 200 nm. (b) Normalized fluorescence spectra of representative ALNPs with the emission ranging from visible to NIR region in $1 \times$ PBS buffer (pH = 7.4). (c) Normalized afterglow luminescence spectra of ALNPs in (b) in $1 \times$ PBS buffer (pH = 7.4). (d) Fluorescence and afterglow images of ALNPs with various components. Fluorescence images of NCBS based ALNPs were acquired at 780 nm upon excitation at 710 nm. Afterglow images were captured after light pre-irradiation (1 W cm^{-2} 808 nm for NCBS based ALNPs while 0.1 W cm^{-2} white light for RB or TPP based ALNPs) for 5 s. For all ALNPs, [afterglow initiator] = $0.75 \mu\text{g mL}^{-1}$ (2.5 w/w%), [afterglow substrate] = $15 \mu\text{g mL}^{-1}$ (50 w/w%), [afterglow relay unit] = $30 \mu\text{g mL}^{-1}$. (e) Quantification of afterglow intensities of ALNPs with the same afterglow initiator

(NCBS) whereas varying substrates and relay units in (d). (f) Quantification of afterglow intensities of ALNPs with the same afterglow substrates (DO) whereas varying afterglow initiators and relay units in (d). Error bars indicated standard deviations of 3 separate measurements. Reprinted with permission from ref 243. Copyright 2019 Nature Publishing Group.

The afterglow intensities of ALNPs were different from each other (Figure 3.2d-f). Comparison of the nanoparticles with the same afterglow initiator (NCBS) and relay unit revealed that DO-doped nanoparticles had the brightest afterglow luminescence among three afterglow substrates (Figure 3.2e), which was followed by SO and HBA doped ones. Moreover, among all the tested fluorescence agents, PFVA-based nanoparticles had the highest afterglow intensities provided that other two components were the same. For instance, PFVA-N-DO gave the brightest afterglow luminescence among all NCBS-doped ALNPs, which was 12- and 123-fold higher than that of PFVA-N-SO, and PFVA-N-HBA nanoparticles, respectively. Variation of photosensitizer also impacted the afterglow luminescence of ALNPs, because the ability to generate $^1\text{O}_2$ was different. NCBS-doped ALNPs generally had brighter afterglow luminescence than TPP- and RB-doped ones when other components were the same (Fig. 3.2f). However, this was not the case when the fluorescent agent was PFVA. For instance, afterglow intensities of PFVA-R-DO and PFVA-T-DO were 4.6- and 4.1-fold higher than that of PFVA-N-DO, respectively. This was because of that the additional amount of $^1\text{O}_2$ generated by PFVA under white light irradiation but not 808 nm irradiation. Thus, the afterglow intensities were determined by all three components.

3.3.2 Quantitative analysis and prediction of afterglow intensity

To quantitatively analyze the factors governing the afterglow intensity of ALNPs, a mathematic model was proposed. Relative afterglow intensity ($\Phi_{\text{Afterglow}}$) was defined as the ratio of the afterglow luminescence of individual ALNP to that of the control ALNP which consisted of NCBS and DO without afterglow relay units (termed as N-DO). Based on the detailed afterglow process, four descriptors were retrieved and defined for simulation. First of all, because afterglow process started from photosensitization, the production of $^1\text{O}_2$ by afterglow initiator (Φ_{S1}) was defined as the first descriptor (Figure 3.3a). As previously reported,^{36, 244} chemiluminescent property of afterglow substrate and the oxidation potential of fluorescent agent (afterglow relay unit) are important to CIEEL. Therefore, the chemiluminescent quantum yield of afterglow substrate after reaction with $^1\text{O}_2$ (Φ_{Cl}) was selected as the second descriptor (Figure 3.3b); moreover, corresponding to oxidation potential, the energy level of highest occupied molecular orbital (HOMO) of afterglow relay unit (E_{HOMO}) with respect to frontier molecular orbital theory (Figure 3.3c) was defined as the third descriptor. Because the afterglow emission in this generic approach involved the potential SET between the afterglow relay unit and the afterglow initiator, the relative fluorescence efficiency of ALNP (η_{FI}) was defined as the last descriptor. This descriptor was determined by both initiator and relay unit and was quantified by the ratio of the integrated total fluorescence intensity of individual ALNP to that of the control ALNP N-DO. After supervised learning analysis,²⁴⁵⁻²⁴⁶ a simulated equation which correlated four descriptors with $\Phi_{\text{Afterglow}}$ was generated as shown below (See Methods for calculation details):

$$\Phi_{\text{Afterglow}} = \Phi_{\text{S1}}^{2.76} \times \Phi_{\text{Cl}}^{0.46} \times e^{[2.70 \times E_{\text{HOMO}} + 13.13]} \times$$

According to statistical results, all descriptors were strongly correlated with $\Phi_{\text{Afterglow}}$ ($P < 0.05$). The calculated Eq.1 showed an impressive coefficient of determination (R^2) of 0.944 (adjusted $R^2 = 0.936$), and the measured and simulated $\Phi_{\text{Afterglow}}$ values involved in quantitative analysis showed close proximity to each other (Figure 3.3d), both suggesting the excellent fitness of Eq.1 to this afterglow model. Based on Eq.1, it was apparent that $\Phi_{\text{Afterglow}}$ demonstrated non-linear increment with four descriptors related to the cross-talk of three major afterglow components. Basically, increased production of $^1\text{O}_2$, chemiluminescence of afterglow substrate, HOMO energy level of afterglow relay unit, or fluorescent efficiency of fluorescent units in ALNP could contribute to brightening afterglow. Such a pattern corresponded well with the experimental data in Figure 3.2. Thereby, these statistical data demonstrated the rationality of descriptor selection for quantitative analysis and implied the feasibility of Eq.1 for afterglow prediction.

Figure 3.3. Mechanistic study of descriptors in the estimation of afterglow intensity of ALNPs. (a) Quantification of $^1\text{O}_2$ generating capability for the different afterglow initiator doped nanoparticles at identical mass concentration ($0.75 \mu\text{g mL}^{-1}$) in $1 \times \text{PBS}$ ($\text{pH} = 7.4$). NCBS nanoparticles were pre-irradiated at 808 nm for 5 s (at 1 W cm^{-2}), while TPP and RB nanoparticles were pre-irradiated with white light for 5 s (0.1 W cm^{-2}). Production of $^1\text{O}_2$ was defined as the fluorescence enhancement (F/F_0) of $^1\text{O}_2$ sensor green (SOSG, $1 \mu\text{M}$) at 524 nm. (b) Chemiluminescent intensities (RLU s^{-1}) of DO, SO, and HBA in the presence of NCBS in tetrahydrofuran after pre-irradiation at 808 nm for 5 s (at 1 W cm^{-2}). $[\text{DO}] = [\text{SO}] = [\text{HBA}] = 15 \mu\text{g mL}^{-1}$; $[\text{NCBS}] = 0.75 \mu\text{g mL}^{-1}$. RLU, relative light unit. (c) Schematic illustration of energy levels of afterglow substrates and afterglow relay units.^{36, 247-248} LUMO, lowest unoccupied molecular orbitals. DO/SO/HBA-IMD, DO/SO/HBA-based dioxetane intermediate. (d) Measured (measurement) and estimated (simulation) afterglow intensities of ALNPs. Estimated afterglow intensities were calculated from Eq. 1. (e) Prediction of afterglow intensities of a new afterglow relay unit CPV by Eq. 1. P values were calculated by Student's two-sided t -test. Inset: chemical structure of CPV. Error bars indicated standard deviations of 3 separate measurements. Reprinted with permission from ref 243. Copyright 2019 Nature Publishing Group.

To test the predictive capability of the proposed equation, another fluorescent agent, CPV, was used as the afterglow relay unit. Note that CPV hardly emitted afterglow luminescence by itself.¹⁷³ However, after the nanoformulation through doping CPV with afterglow initiator (NCBS) and substrate (DO, SO, or HBA), intense afterglow luminescence was detected. The comparison of experimentally measured and Eq.1 simulated $\Phi_{\text{Afterglow}}$ of CPV-based ALNPs was shown in Figure 3.3e. Impressively, no significant difference was

observed between the measured and estimated $\text{Ln}[\Phi_{\text{Afterglow}}]$ ($P > 0.05$), validating the prediction reliability of Eq.1 to estimate afterglow luminescence for this generic afterglow approach.

3.3.3 Tissue penetration of afterglow luminescence

To assess the imaging capability of ALNPs, we examined the imaging performance of ALNPs in comparison with NIR fluorescence at different tissue depths. Moreover, the afterglow performance was benchmarked against the reported afterglow agent SPN-NCBS5. SPN-NCBS5 was similarly prepared via nanoprecipitation, wherein MEHPPV was doped with 5 _{w/w}% NCBS using PEG-*b*-PPG-*b*-PEG as the matrix.¹⁷³ Considering strong afterglow luminescence and NIR emission at 780 nm, PFVA-N series with long-term stability (in PBS and plasma-mimicking cell culture medium supplemented with fetal bovine serum) and good cytocompatibility were selected as the representative ALNPs. With the increase of tissue depth, both NIR fluorescence and afterglow luminescence intensities from the buried ALNPs significantly decreased (Figure 3.4a). Because of the minimized background noise of afterglow imaging, the SBRs of afterglow images were remarkably higher than those of NIR fluorescence images at all tissue depths (Figure 3.4b). Notably, the NIR fluorescence was almost undetectable at 3 cm (SBR close to 1), whereas the afterglow luminescence could still be clearly visualized (SBR: 61 ± 8 ; SBR is expressed as mean $\pm$ standard deviation of 3 independent measurements). These data suggested the superior imaging performance of afterglow luminescence over NIR fluorescence. Moreover, depending on afterglow substrate, PFVA-N ALNPs showed similar (PFVA-N-HBA) or even higher (PFVA-N-DO/SO) afterglow SBRs than SPN-NCBS5 at the same tissue depth. For instance, at a tissue depth of 2 cm, PFVA-N-HBA showed similar SBR

(122 ± 1) to SPN-NCBS5 (116 ± 1), whereas the SBRs of PFVA-N-DO (248 ± 10) and SO (191 ± 36) were respectively 2.1 and 1.6-fold higher than that of SPN-NCBS5, mainly attributed to the much higher afterglow brightness (Figure 3.4a). In particular, PFVA-N-DO (or SO) reached a maximum imaging depth of 5 cm (SBR: 26 ± 1 for PFVA-N-DO and 6 ± 1 for PFVA-N-SO), which was deeper than 4 cm by SPN-NCBS5 (SBR: 3 ± 1). These results not only indicated the advantage of PFVA-N ALNPs over SPN-NCBS5 for afterglow imaging, but also emphasized the design flexibility of ALNPs to further promote penetration depth and imaging sensitivity.

Figure 3.4. Tissue penetration study of ALNPs. (a) Afterglow and NIR fluorescence images of PFVA-based ALNPs ([PFVA] = $100 \mu\text{g mL}^{-1}$, [DO], [SO], or [HBA] = $50 \mu\text{g mL}^{-1}$, [NCBS] = $2.5 \mu\text{g mL}^{-1}$, $50 \mu\text{L}$) and SPN-NCBS5 nanoparticles ([MEHPPV] = $100 \mu\text{g mL}^{-1}$, [NCBS] = $5 \mu\text{g mL}^{-1}$, $50 \mu\text{L}$) under different depths of chicken breast tissues. (b) SBRs of afterglow or fluorescence images in (a) at different tissue depths. (c) Schematic

illustration of optical imaging through a living mouse. Afterglow and NIR fluorescence images of PFVA-based ALNPs or SPN-NCBS5 were captured after penetrating through a living mouse. CCD, charged coupled device. (d) Afterglow and fluorescence images of PFVA-based ALNPs or SPN-NCBS5 nanoparticles penetrating through a living mouse. Concentrations of nanoparticles were identical to those in (b). (e) Quantification of the SBRs of afterglow or fluorescence images in (d). Both the PFVA-based ALNPs and SPN-NCBS5 were pre-irradiated at 808 nm for 5 s (at 1 W cm^{-2}) before imaging. Fluorescence images were captured at 780 nm upon excitation at 710 nm. Error bars indicated standard deviations of 3 separate measurements. Reprinted with permission from ref 243. Copyright 2019 Nature Publishing Group.

The deep-tissue imaging capability of PFVA-N ALNPs was further validated in living animals. As illustrated in Figure 3.4c, nanoparticle solutions were placed beneath a living mouse wherein the tissue depth was measured to be 1.5 cm. Because of the strongest afterglow intensity and low background noise (Figure 3.4d), the afterglow of PFVA-N-DO had the highest SBR (1136 ± 19), followed by PFVA-N-SO (272 ± 3) and PFVA-N-HBA (138 ± 1) (Figure 3.4e). Remarkably, the afterglow SBR of PFVA-N-DO exceeded that of SPN-NCBS5 (131 ± 3) by 8.7 times. On the other hand, the NIR fluorescence from PFVA-N-DO could hardly be differentiated from the background (tissue autofluorescence). These data thus corresponded well with in vitro tissue penetration study, validating the ability of ALNPs for ultrasensitive deep-tissue afterglow imaging.

3.3.4 In vivo tumor imaging and biodegradation study

To evaluate afterglow performance of ALNPs for in vivo imaging, PFVA-N-DO was selected as the representative ALNP to image tumor in living mice in comparison with NIR

fluorescence. After systemic administration of PFVA-N-DO, the afterglow SBR in tumor region dramatically increased, leading to the visualization of tumor at 1 h post-injection (Figure 3.5a). In contrast, the NIR fluorescence SBR slightly increased (Figure 3.5b), and thus the tumor was only detectable at 4 h post-injection. Note that at 1 h post-injection, the afterglow SBR in tumor region (2922 ± 121) was three orders of magnitude higher than that of NIR fluorescence (~ 1). Such a afterglow SBR was not only higher than fluorescence imaging in both first and second NIR window (SBRs up to ~ 135),²⁴⁹ but also significantly exceeded the SBRs of other excitation-free imaging modalities including chemiluminescence (up to ~ 20),²⁵⁰ bioluminescence (up to ~ 1000),²¹⁹ and Cerenkov luminescence imaging (up to ~ 154).²⁵¹ This should be mainly attributed to the fact that chemiluminescence, bioluminescence, and Cerenkov luminescence imaging usually rely on visible emission. Ex vivo data revealed that the uptake of PFVA-N-DO in tumor was 0.58-fold of that in liver, further confirming its ability to passively target tumor. These data highlighted that by PFVA-N-DO mediated afterglow imaging allowed for rapider detection of tumor with the superior contrast and sensitivity over other optical agents.

in (d). (f) Quantification of the NIR fluorescence intensities of liver region in living mice as a function of time after intravenous injection of PFVA-N-DO ([PFVA] = 250 $\mu\text{g mL}^{-1}$, [DO] = 125 $\mu\text{g mL}^{-1}$, [NCBS] = 6.25 $\mu\text{g mL}^{-1}$, 250 μL). NIR fluorescence images were acquired at 780 nm upon excitation at 710 nm. (g) Hematoxylin & eosin staining of major organs from mice after tail vein injection of PFVA-N-DO ALNPs ([PFVA] = 250 $\mu\text{g mL}^{-1}$, [DO] = 125 $\mu\text{g mL}^{-1}$, [NCBS] = 6.25 $\mu\text{g mL}^{-1}$, 250 μL) or saline (250 μL) for 33 days. Scale bar: 50 μm . Error bars indicated standard deviations of 3 separate measurements ($n = 3$). Reprinted with permission from ref 243. Copyright 2019 Nature Publishing Group.

Biodegradation and in vivo clearance study were subsequently performed to examine the biosafety of PFVA-DO-N. To mimic in vivo environment, myeloperoxidase (MPO) abundantly expressed in phagocytes was used as the oxidative enzyme for in vitro biodegradation (Figure 3.5c). In the presence of hydrogen peroxide (H_2O_2), MPO catalyzes the production of hypochlorous acid (HClO) to digest foreign substances.²⁵² After overnight incubation of PFVA-N-DO with MPO and H_2O_2 , the absorbance at 450 nm assigned to PFVA significantly dropped (Figure 3.5d), suggesting the fragmentation of PFVA by MPO. The biodegradation was further confirmed by gel permeation chromatography (GPC), as indicated by the evidently decreased molecular weight of PFVA after MPO treatment (Figure 3.5e). Such an efficient degradation should be ascribed to the oxidation induced cleavage of double bonds in the conjugated backbones of PFVA (Figure 3.5c), which was previously reported.^{173, 234}

To monitor the in vivo clearance of PFVA-N-DO, they were systemically administered into mice followed by long-term NIR fluorescence recording (Figure 3.5f). After

administration, NIR fluorescent signals from liver increased over time and reached the maximum at 3 days post-injection. Later, the NIR fluorescence from liver continuously decreased to almost undetectable level at 33 days post-injection. These results indicated the long-term clearance of PFVA-N-DO via hepatobiliary excretion in living animals. Furthermore, no noticeable histological damage was observed in the major organs of living mice after systemic administration of PFVA-N-DO for 33 days (Figure 3.5g), suggesting the good biocompatibility of PFVA-N-DO.

3.4 Conclusions

In summary, we reported a generic approach that transformed traditional fluorescent agents into a new library of afterglow agents (ALNPs). By virtue of an efficient intraparticle cascade photoreaction of three key components (afterglow initiator, substrate, and relay unit), ALNPs were able to chemically store the photoenergy and spontaneously emit long-lived luminescence after cessation of optical excitation. Such a facile approach was applicable to a wide range of compositions: RB, TPP, or NCBS for the initiator, DO, SO, or HBA for the substance, and inorganic or organic fluorophores for the relay units. The sophisticated but fairly controllable photochemical interactions within the nanoparticles allowed to finetune the afterglow emission from visible to NIR region by adjusting the ALNP compositions. To elucidate the factors involved in this generic afterglow process, a 4-descriptor based mathematical model (Eq.1) was generated from supervised learning analysis, which accurately predicted the afterglow intensities of unknown ALNP composition. Using PFVA-N-DO ALNPs as an example, the afterglow achieved a maximal imaging depth at 5 cm in biological tissue, deeper than the reported afterglow agents (4 cm). As compared with NIR fluorescence, the afterglow of ALNPs exhibited three orders

of magnitude higher SBR (2922 ± 121), allowing for rapider detection of tumor in living mice after systematic administration. To the best of our knowledge, this is the highest SBR achieved so far for in vivo optical imaging regardless of their optical modalities and detection wavelengths. In conjunction with the heavy-metal-free benign nature, the representative PFVA-N-DO ALNPs were enzymatically biodegradable and clearable with a good long-term biocompatibility, further ensuring their in vivo applications. Thus, our study showed a controllable nanoengineering approach nearly applicable to all kinds of fluorophores regardless of their composition for background-free molecular optical imaging.

Chapter 4 Metabolizable Semiconducting Polymer Nanoparticles for Second Near-Infrared Photoacoustic Imaging

4.1 Introduction

Optical imaging plays an essential role in modern biomedical science.⁸ Particularly, optical imaging in the second near-infrared (NIR-II) window (1000-1700 nm) has aroused growing interest over the past few years,^{12, 176} because NIR-II light has reduced tissue scattering and increased maximum permissible exposure (MPE) limits to living matters as compared with the light in the first (NIR-I) window (650-900 nm).²²⁵ Thus, NIR-II optical imaging provides opportunities to monitor biological or pathophysiological processes at imaging depths deeper than commonly adopted NIR-I imaging.^{217, 253} Among current optical imaging modalities, photoacoustic (PA) imaging emerged as a hybrid strategy to further reduce signal scattering in tissues by detecting thermoacoustic waves rather than light emission upon light excitation.^{202, 207, 254} Specifically, PA imaging in the NIR-II window has been proved to render the ability to detect at centimeter-scale depth in living animals.²⁰⁸⁻²¹⁰ However, because few endogenous biological molecules have the ability to absorb in the NIR-II window, development of exogenous NIR-II PA agents are essential.

Imaging agents administered in vivo are required complete clearance from living bodies for clinical translation according to guidelines of US Food and Drug Administration (FDA).²⁵⁵⁻²⁵⁶ However, only a limited number of contrast agents have been reported to emit NIR-II PA signals,^{195-196, 257-261} and the majority of them (e.g. gold nanostructures, silver

nanoplates) are intrinsically resistant to biodegradation. Furthermore, the long-term retention of these non-metabolizable agents (especially for heavy-metal containing nanomaterials) potentially poses a chronic toxicity issue,²⁶² casting a shadow over their translation into clinics. Although few of NIR-II PA agents were claimed to be biodegradable (charge-transfer nanocomplex and CuS perfluorocarbon nanodroplets),²⁶³⁻²⁶⁴ their in vivo clearance behaviors were not fully studied. Thereby, to overcome clinical translation barriers of NIR-II PA imaging, biodegradable NIR-II absorbing agents capable of in vivo clearance over a period of time are highly desired.

Semiconducting polymer nanoparticles (SPNs) as a new family of optical agents have provided a promising strategy to addressing the toxicity issue.^{24, 218, 265-266} Composed of highly π -conjugated backbones, SPNs demonstrate overall excellent photothermal conversion efficiency as well as photostability.^{112, 267} Moreover, molecular versatility of SPNs not only favors facile tuning of optical spectra independent of dimension or morphology, but also allows for convenient modification to backbones and/or side chains for on-demand functionalization.²⁷ Taking advantage of these merits, we and others have demonstrated SPNs as powerful PA agents for detection of biomarkers and cancer theranostics.^{11, 268} Furthermore, our preliminary results have shown the potential of SPNs as degradable NIR-I PA agents.^{29, 203} However, SPNs as metabolizable NIR-II PA agents have yet to be exploited.

We herein report the first series of NIR-II absorbing SPNs that are metabolizable and capable of gradual clearance from living bodies after PA imaging. By virtue of oxidizable backbones of SPs and hydrolysable polymer encapsulating matrix, the obtained SPNs could be biodegraded in the presence of biologically abundant enzymes (peroxidase and

lipase) into ultrasmall NIR fluorescent segments (~ 1 nm), followed by continuous elimination through both hepatobiliary and renal excretions. In the following, the design and synthesis of biodegradable NIR-II PA SPNs are first described, followed by the characterization of their optical and PA properties. Next, the degradation processes of these SPNs are carefully investigated in biomimicking in vitro conditions, and the clearance behaviors and pathways of the representative metabolizable SPNs are further detected in vivo. At last, the proof-of-concept applications of metabolizable NIR-II SPNs are demonstrated in the PA imaging of both tumor and brain vasculature in living mice.

Figure 4.1. Molecular design and mechanism of metabolizable SPNs for NIR-II PA imaging. a) Chemical structures of PBBT-OT, PBBT-PT, and PBBT-OT. b) Scheme of preparation of NIR-II PA SPNs via nanoprecipitation. c) Scheme of NIR-II PA imaging of brain and tumor as well as in vivo biodegradation mechanism of SPNs. NADPH: reduced nicotinamide adenine dinucleotide phosphate; NOX: NADPH oxidase.²⁶⁹ Reprinted with permission from ref 269. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.2 Materials and methods

4.2.1 Chemicals

2,6-Bis(trimethyltin)-4,8-didodecylbenzo[1,2-b;4,5-b']dithiophene, (4,8-bis((2-ethylhexyl)oxy)benzo[1,2-b;4,5-b']dithiophene-2,6-diyl)bis(trimethylstannane), 2,5-bis(trimethylstannyl)thieno[3,2-b]thiophene, and benzobisthiadiazole (CS10361) were purchased from Luminescence Technology Corp (Lumtec). Myeloperoxidase was purchased from Planta Natural Products. The other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

4.2.2 Characterization

Absorption spectra of SPs and nanoparticles were measured on Lambda 950 spectrometer. Proton nuclear magnetic resonance (^1H NMR) spectra were measured on Bruker Avance II 300 MHz NMR spectrometer. Hydrodynamic sizes were measured by dynamic light scattering on Malvern Nano-ZS Particle Sizer. Transmission electron microscope (TEM) images of SPNs were obtained on JEOL JEM 1400 transmission microscope. FLIR T420 thermal camera was utilized to monitor photothermal temperatures. Fluorescence spectra were recorded on Fluorolog 3-TCSPC spectrofluorometer produced by Horiba Jobin Yvon. Gel permeation chromatography was carried out on Shimadzu LC-VP system using polystyrene as the standard and tetrahydrofuran as the eluent. Confocal images were captured on Zeiss LSM 710. Flowcytometry was carried out on Fortessa X20. In vivo fluorescence imaging was performed on an IVIS-CT machine (PerkinElmer).

4.2.3 Synthesis of PBBT-OT, PBBT-PT, and PBBT-DT

To synthesize PBBT-PT, 2,6-Bis(trimethyltin)-4,8-didodecylbenzo[1,2-b;4,5-b']dithiophene (21 mg, 0.025 mmol), and benzobisthiadiazole (25 mg, 0.023 mmol), $\text{Pd}_2(\text{dba})_3$ (1.0 mg, 0.001 mmol), and tri(*o*-tolyl)phosphine (2.0 mg, 0.006 mmol) were dissolved in chlorobenzene (4 mL) and then placed in a 50 mL Schlenk, followed by 3 circles of freeze-pump-thaw for degassing. After degassing, the mixture was heated by oil bath at 100 °C protected by nitrogen atmosphere to carry out Still polymerization for 75 mins. After reaction, the obtained mixture was added drop by drop into methanol and dark precipitates were collected by centrifuge. The obtained precipitates were washed by methanol for 3 times to remove impurities and then dried under vacuum overnight to remove methanol. PBBT-OT and PBBT-DT were synthesized following the same procedure, whereas PBBT-DT was obtained with a shorter reaction time (30 min). ^1H NMR for PBBT-PT (CDCl_3 , σ ppm): 6.98 (s, 4H), 2.48-2.27 (m, 8H), 1.43-1.25 (m, 106H), 0.88 (m, 18H). ^1H NMR for PBBT-OT (CDCl_3 , σ ppm): 6.99-6.91 (s, 4H), 4.16 (d, 4H), 2.35 (d, 4H), 1.43 (m, 84 H). ^1H NMR for PBBT-DT (CDCl_3 , σ ppm): 7.61 (s, 1H), 6.98 (s, 2H), 6.91 (s, 1H), 2.27 (d, 4H), 1.25 (m, 66H), 0.88 (m, 12H).

4.2.4 Preparation of NIR-II SPNs

PBBT-PT (0.1 mg) and PLGA-PEG (4 mg) were dissolved in THF (2 mL). The mixture was rapidly injected into deionized water (8 mL) during vigorous ultrasonication. THF was then removed by evaporation under N_2 flow for 40 mins. Then, the aqueous solution was filtered through a 220 nm polyvinylidene fluoride (PVDF) syringe driven filter (Millipore) to remove large precipitates. The obtained SPN-PT nanoparticles was concentrated by

ultrafiltration followed by dilution in $1 \times \text{PBS}$ ($\text{pH} = 7.4$). SPN-OT and SPN-DT were prepared using the same procedure.

4.2.5 In vitro photothermal study

To evaluate photostability, 200 μL of SPN solution was irradiated by 1064 nm laser at 1 W cm^{-2} for 6 mins followed by a 6-min cooling period. Such heating-cooling process was repeated for 5 cycles. Temperature of SPN solutions were monitored by IR thermal camera. Photothermal conversion efficiencies of SPNs were measured and calculated according to previous report.²⁵⁷

To measure photothermal conversion efficiency, SPN (2 mL, optical density = 1) is added to a quartz cuvette (5.66 g). Then SPN is exposed to 1064 nm laser irradiation at 1 W/cm^2 for 30 minutes to reach equilibrium, followed by cooling down to room temperature. Solution temperature is recorded by a thermometer. Photothermal conversion efficiency is calculated as follows.

Total energy input and dissipation of the abovementioned measurement system is expressed as:

$$\sum_i m_i C_{p,i} \frac{dT}{dt} = Q_{SPN} + Q_{sys} - Q_{diss}$$

where m_i and $C_{p,i}$ are respectively the mass and specific heat capacity of individual component in the measuring system (e.g. water and quartz). Q_{SPN} is the energy input from SPNs while Q_{sys} is the energy input from the other components in the measuring system except for SPNs. Q_{diss} is heat loss from measuring systems to surroundings.

Q_{SPN} as a laser-induced source term can be expressed as:

$$Q_{SPN} = I (1 - 10^{-A_{1064}}) \eta$$

Where I is the laser power. A_{1064} is the optical density of SPNs at 1064 nm ($A_{1064} = 1$).

And η is the photothermal conversion efficiency.

Q_{diss} as the heat dissipation of system is linear with temperature of system

$$Q_{diss} = hS(T - T_{surr})$$

where h is the heat transfer coefficient and S is the surface area of the cuvette. T is the temperature of measurement system and T_{surr} is the temperature of surroundings.

When the SPN solution irradiated by laser reaches steady temperature (T_{max}), the heat input to the measurement system should be equal to the heat dissipation:

$$Q_{SPN} + Q_{sys} = Q_{diss} = hS(T_{max} - T_{surr})$$

During the cooling process when laser is shut off, there is no heat input. And Eq. 1 can be expressed as:

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

After rearrangement and integration, Eq.5 can be expressed as:

$$t = - \frac{\sum_i m_i C_{p,i}}{hS} \ln \frac{T - T_{surr}}{T_{max} - T_{surr}}$$

System time constant τ_s is defined as:

$$\tau_s = \frac{\sum_i m_i C_{p,i}}{hS}$$

And a dimensionless parameter θ is defined as:

$$\theta = \frac{T - T_{surr}}{T_{max} - T_{surr}}$$

Thus, Eq. 6 could be expressed as:

$$t = - \tau_s \ln \theta$$

Therefore, τ_s could be determined by linear regression of time in cooling period versus $\ln\theta$, which is calculated using temperatures of measurement system and surroundings. Afterwards, hS can be obtained according to Eq.7.

Q_{sys} can be measured following the same procedure by replacing SPN solutions with pure solvent (H_2O):

$$Q_{sys} = hS(T_{max,H_2O} - T_{surr})$$

Thus, η could be calculated as:

$$\eta = \frac{hS(T_{max} - T_{surr}) - Q_{sys}}{I(1 - 10^{-A_{1064}})}$$

4.2.6 Cytotoxicity test

4T1 mammary carcinoma cells (purchase from American Type Culture Collection, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin). Cells were cultured in an incubator providing an atmosphere containing 5% CO_2 and 95% humidified air at 37 °C. To evaluate cytotoxicity of NIR-II SPNs, 4T1 cells were seeded in 96-well plates (5000 cells per well) for 24 h, and SPN solutions of different concentrations were respectively added to the cells. After co-incubation for another 24 h, the culture medium was removed, and cells were gently washed by fresh PBS. 120 μ L fresh DMEM containing with 20 μ L MTS solution was then added to each well followed by incubation for 2 h. Afterwards, absorbance of each well at 490 nm was measured by a SpectraMax M5 microplate reader. Cell viability was calculated as the ratio of the absorbance from 4T1 cells incubated with SPNs to that of cells without SPN treatment (control).

4.2.7 In vitro biodegradation studies

Biodegradability of SPNs in solutions were evaluated by incubation of SPNs (3 $\mu\text{g/mL}$) with MPO (50 $\mu\text{g/mL}$), H_2O_2 (300 mM) in 0.1 M phosphate buffer containing 0.1 M chloride ion at 37 °C. Absorption of SPNs was longitudinally recorded. Fluorescence spectra of the mixture were measured before and after degradation. The degradation products were dissolved in THF for GPC and LCMS characterization. To evaluate the biodegradability of SPNs in phagocytes, RAW264.7 cells purchased from ATCC were seeded in glass bottom dishes and cultured with the abovementioned supplemented DMEM for 24 h. Afterwards, SPNs (20 $\mu\text{g/mL}$) were added to the dish and confocal fluorescence images of cells were captured at different time points. Before confocal imaging, culture medium containing SPNs were removed and cells were washed with PBS followed by fixation using 4% paraformaldehyde for 10 min. After fixation, cells were stained with Hoechst33342 (purchased from Thermo Fisher Scientific) and then imaged under confocal microscope (excitation: 405 nm for Hoechst33342 and 633 nm for degradation products). For flowcytometry, RAW264.7 cells were seeded in 12-well plates and incubated with SPNs (20 $\mu\text{g/mL}$). After incubation for 48 h, cells were digested and fluorescent signals were measured on Fortessa X20 using APC-Cy7 channel.

4.2.8 In vivo biodegradation studies

All animal experiments were performed according to the guidelines established by the Institutional Animal Care and Use Committee (IACUC), Sing Health. To study in vivo biodegradation behavior, SPNs were intravenously injected into female NCr living mice. Then, mice were placed in metabolic cages to collect urine and feces. After collection of excrement, urine was transferred to Eppendorf tube for fluorescence quantification by IVIS-CT

machine. Feces were gently washed with DI water, dried, and then transferred to Eppendorf tube for fluorescence quantification by IVIS-CT machine. Blood samples were collected from tail followed by centrifugation (3000 rpm, 10 min) to obtain serum (supernatant) for fluorescence quantification. Fluorescent signals from mice, the obtained serum, urine, and feces were measured on IVIS-CT machine with excitation at 710 nm and emission at 820 nm. Quantification of fluorescent signals were performed by Living Image 4.0 software. At designated time points after administration of SPNs, mice were euthanized, and major organs (hearts, livers, spleens, lungs, and kidneys) were harvested. These organs were fixed in 4% paraformaldehyde for 10 h followed by dehydration in 30% sucrose solution overnight. Afterwards, organs were cut into 10- μ m frozen sections on a cryostat (Leica, CM1950) according to standard protocols. These sections were then washed with PBS, stained with DAPI, and mounted by Fluoromount Aquaous Mounting Medium. Later, tissue sections were images under confocal microscope with excitation at 405 nm for DAPI while 633 nm for ultra-small metabolites.

4.2.9 Histological studies

After 30 days post-administration of SPNs or PBS, mice were sacrificed and major organs including hearts liver, spleens, lungs, and kidneys were harvested and fixed in 4% paraformaldehyde. Afterwards, these organs were processed by paraffin embedding and sectioning, followed by hematoxylin and eosin (H&E) staining according to standard protocols. After staining, H&E images of these tissue sections were captured under a Nikon ECLIPSE 80i microscope.

4.2.10 Tumor mouse model

Female NCr nude mice were utilized to establish tumors. 2 million 4T1 cells suspended in 0.2 mL supplemented DMEM were subcutaneously injected into the right shoulder of the mice. Tumors were allowed to grow until a single aspect was longer than 6 mm for PA imaging.

4.2.11 PA instrumentation and in vivo PA imaging

In vitro PA characterization and in vivo PA imaging were performed on a home-made single ultrasound transducer (UST) scanning based PAT system. The excitation source was 1064 nm Nd:YAG laser (Continuum, Surelite Ex) that can generate 5 ns pulses at 10 Hz repetition rate. The 1064 nm beam was delivered to the single-UST circular scanner, and expanded to illuminate the inspection area using an optical diffuser (OD). The UST was immersed in water medium for coupling the acoustic signal to the transducer. A non-focused transducer (V323-SU/2.25 MHz, Olympus NDT) with a 13 mm active area and 70% nominal bandwidth was used to receive the acoustic signals from the sample. The PA signals thus collected were subsequently amplified, band-pass filtered (1-10 MHz) by an ultrasound receiver unit (URU), (Olympus NDT, 5072PR) and then digitized and recorded by the computer with a data acquisition (DAQ) card (25 Ms/s, GaGe, compuscope 4227). For cross-sectional imaging, the A-lines from the sample were acquired by continuously rotating the UST in a circular motion. The A-lines were used to reconstruct the PA image of the sample using a delay-and-sum back projection algorithm. For tumor imaging, female NCr nude mice bearing 4T1 xenograft tumor were intravenously administered with SPN-PT (250 $\mu\text{g/mL}$, 200 μL per mouse). For brain imaging, healthy female rat (NTac: Sprague Dawley® SD®) was intravenously administered with SPN-PT (1.1 mg/mL, 1 mL per rat).

PA images were recorded at designated time points before and after administration of SPN-PT.

A signal-to-background ratio (SBR) is calculated as the ratio of PA amplitude in tumor region at designated time point post-administration of nanoparticles to that of tumor background before injection.

4.2.12 Biodistribution Method

After 30 h post-injection of SPN-PT (250 $\mu\text{g/mL}$, 200 μL per mouse) for tumor imaging, mice were sacrificed. Tumors, livers, spleens, intestines, lungs, kidneys, and hearts were harvested and embedded in 1% agar gel phantom for ex vivo PA measurement in the abovementioned PA system to quantify the biodistribution of SPN-PT.

4.2.13 Data analysis

Fluorescence signals were quantified by ROI analysis using Living Image 4.0 (PerkinElmer Inc.). PA signals were analyzed by MATLAB. The other statistical analysis was conducted by GraphPad Prism 7 (GraphPad Software, Inc., CA, USA).

4.3 Results and discussions

4.3.1 Preparation and characterization of NIR-II PA SPNs.

To extend the absorbance into NIR-II region, a strong electron-withdrawing monomer benzobisthiadiazole (BBT) was selected as the acceptor to narrow the band gap of precursor polymers. Particularly, the labile thiophene-based moieties of BBT are susceptible to oxidation,^{203, 270} which potentially endows SPs with biodegradability. Through palladium catalyzed-Stille polycondensation of the BBT monomer with three different electron-donating monomers, (4,8-bis((2-ethylhexyl)oxy)benzo[1,2-b :4,5-

b']dithiophene-2,6-diyl)bis(trimethylstannane), 2,6-bis(trimethyltin)-4,8-didodecylbenzo[1,2-b;4,5-b']dithiophene, and 2,5-bis(trimethylstannyl)thieno[3,2-b]thiophene, three SPs, PBBT-OT, PBBT-PT, and PBBT-DT with strong absorption in NIR-II window were obtained, respectively (Figure 4.1a). PBBT-PT and PBBT-OT shared similar absorption spectra ranging from 900 to 1200 nm; in contrast, the absorption of PBBT-DT is broader, extending above 1500 nm (Figure 4.2a).

To prepare water-soluble nanoparticles, these hydrophobic SPs were respectively encapsulated into the FDA-approved biodegradable amphiphilic copolymer matrix, poly(ethylene glycol) methyl ether-block-poly(lactide-co-glycolide (PLGA-PEG), via nanoprecipitation (Figure 4.1b). All the resulted SPNs (termed as SPN-OT, SPN-PT, and SPN-DT) demonstrated the absorption spectra similar to their corresponding SPs, showing the peak absorption at ~1079 nm (Figure 4.2b). As revealed by dynamic light scattering (DLS) (Figure 4.2c) and transmission electron microscope (TEM) (Figure 4.1d), these SPNs had similar hydrodynamic diameter averaged at ~30 nm with a spherical morphology. In addition, the SPN solutions remained translucent in aurantia or violet color and demonstrated negligible size change after storage at 4 °C for 2 months in dark (Figure 4.2d, Figure S5, Supporting Information), indicating the good aqueous stability of these SPNs. Moreover, cell viability test indicated that these SPNs had minimal cytotoxicity to 4T1 cells (Figure 4.2e), suggesting their good cytocompatibility for biomedical applications.

As the PA signal is generally associated with heat generation, the photothermal conversion efficiencies (η) of these SPNs were first measured and compared. Among them, SPN-PT had the highest η (53%), followed by SPN-DT (49%) and SPN-OT (36%) (Figure 4.2f). Notably, the η s of SPN-PT and SPN-DT were higher than the majority of reported NIR-II

photothermal nanoagents (Figure 4.2f), and η of SPN-PT was in particular the second to the highest among all the reported organic NIR-II contrast agents. Besides, the maximal temperatures of these SPN solutions remained unchanged after 5 heating-cooling cycles under irradiation of 1064 nm laser at 1 W/cm^2 , demonstrating their excellent photostability for PA imaging application (Figure S6, Supporting Information). The PA spectra of these SPNs were measured to range from 680 nm in the NIR-I window to 1064 nm in the NIR-II window, showing similar trend with their respective absorption spectrum (Figure 4.2g). To highlight the potential advantage of PA imaging in NIR-II over NIR-I window, the PA spectra of biological tissues including fresh blood, brain cortex, skin, and muscles were recorded. The PA amplitude of blood at 1064 nm had a significant decrease to $\sim 30\%$ of that in NIR-I region (Figure 4.2h), while the intrinsic PA amplitudes for other endogenous matters were fairly low and no obvious difference was observed between two NIR windows. These spectral profiles clearly validated that red-shifting PA imaging from NIR-I to NIR-II window can significantly reduce the background noise from blood, potentially leading to increased signal to background ratio. In addition, all SPNs demonstrated good linearity between concentration and PA amplitude at 1064 nm (Figure 4.2i, Figure S7, Supporting Information), indicating their suitability for signal quantification.

Figure 4.2. In vitro characterization of SPNs. a) Normalized absorption spectra of PBBT-OT, PBBT-PT, and PBBT-DT in tetrahydrofuran. b) Normalized absorption spectra of SPN-OT, SPN-PT, and SPN-DT in $1 \times$ PBS buffer. c) DLS profiles of SPN-OT, SPN-PT, and SPN-DT in $1 \times$ PBS buffer. d) TEM images and photographs of (i) SPN-OT, (ii) SPN-PT, and (iii) SPN-DT. e) Cytotoxicity assay of SPN-OT, SPN-PT, and SPN-DT in 4T1 cells. f) Comparison of photothermal conversion efficiencies (η) of SPN-PT (C), SPN-DT (E), and SPN-OT (M) (optical density = 1) with other reported NIR-II photothermal agents. (A) AuPB²⁵⁷; (B) PBTPBF-PT²⁷¹; (C) SPN-PT; (D) TBDOPV-DT NPs²⁵⁹; (E) SPN-DT; (F) H-SiO_x NPs²⁷²; (G) Nb₂C²⁷³; (H) SPN_{I-II}²⁷⁴; (I) Cu₃BiS₃ NR²⁷⁵; (J) (NH₄)_xWO₃²⁷⁶; (K) Au-Cu₉S₅ NPs²⁷⁷; (L) SPN-OT; (M) Bi-LyP-1²⁷⁸; (N) PtNP²⁷⁹; (O) Fe₃O₄@CuS-PEG²⁷⁹.

g) PA spectra of SPN-OT, SPN-PT, and SPN-DT in $1 \times$ PBS buffer ($10 \mu\text{g/mL}$). h) PA spectra of blood, brain cortex, skin, and muscle in NIR-I and NIR-II windows. i) PA amplitudes of SPN-PT at 1064 nm as a function of concentration. Error bars indicated standard deviation of 3 independent experiments. Reprinted with permission from ref 269. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.3.2 In vitro biodegradation of NIR-II PA SPNs

Because administered nanoparticles often undergo continuous clearance by phagocytes in mononuclear phagocyte system (MPS), the biodegradation potential of these NIR-II SPNs was first tested in phagocyte-mimicking in vitro conditions (Figure 4.3a). Myeloperoxidase (MPO), which was commonly expressed in phagocytes to catalyze the generation of hypochlorous acid (HClO) in the presence of H_2O_2 and chloride ion,²⁵² was used as the representative enzyme to perform oxidative stress on backbones of NIR-II SPs (Figure 4.1c). After incubation with MPO in bio-mimicking conditions, SPN-PT, SPN-DT, and SPN-OT respectively demonstrated gradually decreased absorbance concomitant with a hypsochromic shift (Figure 4.3b), in agreement with the degradation pattern in HClO titration (Figure S8, Supporting Information). Although the intact SPNs were barely fluorescent, the NIR fluorescence ($\sim 810 \text{ nm}$) was detected in biodegradation products (Figure 4.3c, Figure S10, Supporting Information), suggesting the production of segments resembling the optical properties of BBT ($\sim 820 \text{ nm}$ NIR emission). Such oxidation-induced backbone cleavage in backbone of SPs was further validated by gel permeation chromatography (GPC), which indicated significantly decreased molecular weights of degradation products relative to original SPs (Figure 4.3d). Furthermore, liquid chromatography-mass spectrometry was performed to analyze these fluorescent segments

after biodegradation. The possible degradation mechanism of the SPNs was proposed, suggesting the thiophene unit as the vulnerable moiety towards oxidation.²⁸⁰⁻²⁸²

Despite the efficient MPO-mediated degradation of the SPs, the hydrodynamic sizes of the SPNs remained nearly unchanged after MPO treatment (Figure 4.3e), probably because the degradation products were still retained in the PLGA-PEG matrix. To further mimic *in vivo* degradation conditions, lipase as a ubiquitous enzyme subclass to esterase was employed to catalyze the hydrolysis of ester linkages of PLGA-PEG.²⁸³ As expected, after combinational treatment of MPO and lipase, the hydrodynamic sizes of the SPNs dramatically decreased from ~ 30 nm to ~ 1 nm, below the kidney filtration threshold (~ 5.5 nm).²⁸⁴ By contrast, single treatment of excess lipase failed to break down the SPNs into the residues smaller than 10 nm, validating the necessity to involve both enzymes for efficient degradation.

Encouraged by degradation results in biomimicking solutions, we then examined the metabolism of the SPNs in macrophages which were prevalent in MPS system responsible for clearance of nanoparticles.²⁸⁵ Because the degradation products were fluorescent, confocal laser scanning microscopy was utilized to monitor the metabolic behavior of the SPNs in macrophages. After incubation of SPN-PT as the representative NIR-II SPN with lipopolysaccharide (LPS) stimulated macrophages, NIR fluorescent signal was clearly observed in cytoplasm of cells (Figure 4.3f), suggesting the efficient degradation of SPN-PT in phagocytes. In addition, semi-quantification of fluorescence signals in confocal images indicated decreased mean fluorescence intensity per cell for an incubation time longer than 48 h, implying the loss of fluorescent products probably due to exocytosis (Figure 4.3g).²⁸⁶⁻²⁸⁷ Flow cytometry further validated degradation of three NIR-II SPNs in

macrophages. After 48 h incubation, mean fluorescence intensity of macrophages cultured with SPN-PT, SPN-DT, and SPN-OT was 2.9-, 2.0-, and 3.7-fold higher than that of control cells, respectively (Figure 4.3h). Collectively, these data suggested the potential of the NIR-II PA SPNs to be degraded by MPS system in living animals.

Figure 4.3. In vitro biodegradation of NIR-II PA SPNs. a) Scheme of in vitro biodegradation of NIR-II SPN in cells. b) Monitoring of absorption spectra of SPN-DT (3 $\mu\text{g/mL}$) after treatment with MPO (50 $\mu\text{g/mL}$) and H_2O_2 (300 mM) in 100 mM phosphate buffer containing 100 mM NaCl. c) Fluorescence spectra of SPN-PT before and after biodegradation by MPO. d) GPC results of SPN-PT, SPN-DT, and SPN-OT before and after treatment with H_2O_2 or MPO in the presence of H_2O_2 and Cl^- . e) DLS profiles of SPN-PT before and after treatment with MPO, lipase, and combination of MPO and lipase. f)

Confocal images of RAW 264.7 cells after incubation with SPN-PT (20 $\mu\text{g/mL}$) at different time points. Nuclei were depicted in blue. Biodegradation products detected by NIR channel were indicated in red. g) Quantification of mean fluorescence intensity of single macrophage after incubation with SPN-PT (20 $\mu\text{g/mL}$) for different time periods. h) Flowcytometric results of RAW 264.7 cells after incubation with SPN-PT, SPN-DT, and SPN-OT (20 $\mu\text{g/mL}$) for 48 h, respectively. Error bars indicated standard deviation of 3 independent experiments. Reprinted with permission from ref 269. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.3.3 In vivo biodegradation and clearance of NIR-II PA SPNs

We next investigated clearance behaviors of the NIR-II SPNs in living mice. SPN-PT as the representative NIR-II SPN was systematically administered into living mice. Because metabolites were fluorescent (Figure 4.3c), NIR fluorescence signals from liver, blood, urine, and feces were longitudinally recorded to trace the metabolism of administered nanoparticles. Fluorescence signal from blood reached maximum earliest at day 1 post-injection of SPN-PT (Figure 4.4c), followed by urine at day 2 (Figure 4.4d), liver region at day 4 (Figure 4.4a, b), and finally feces at day 5 (Figure 4.4e). Such order of fluorescence enhancement not only validated successful degradation of SPN-PT in vivo, but also indicated that the degradation products could be cleared out from living animals via both hepatobiliary and renal excretion. As well known that nanoparticles larger than 20 nm tend to accumulate in liver and spleen,²⁸⁸ urinary excretion of SPN-PT metabolites should be attributed to the ultra-small degradation products (~ 1 nm) that favored the filtration through glomerular capillary walls (2-8 nm) (Figure 4.3e).²⁸⁴ Besides, the time lapse among peak fluorescent signals from blood, urine, followed by feces suggested that renal

clearance was the predominant pathway in the beginning, while hepatobiliary excretion gradually took over as the primary clearance route later on. Such phenomenon was in accordance with the previous reports that urinary excretion usually has faster clearance rate than hepatobiliary excretion,²⁵⁵ whereas longer retention of nanoparticles might reduce the permeability of glomerular basement membrane and podocytes to slow down the rate.²⁷⁸ At 15 days post-administration of SPN-PT, fluorescent intensities from blood, urine, feces, and liver region decreased to the normal levels before injection, suggesting the nearly complete clearance of SPN-PT in living mice.

To further evaluate the clearance efficiency of SPN-PT, histological sections of major organs from mice at designated time points post-injection of SPN-PT were examined using confocal laser scanning microscopy. As shown in Figure 4.4f, strong NIR fluorescence was detected in tissue sections from liver, kidney, and spleen collected from mice at day 5 post-administration of SPN-PT, implying the presence of degradation products in these organs. Such phenomenon should be attributed to the existence of macrophages (e.g. Kupffer cells in liver, resident red pulp macrophages in spleen) in MPS organs and/or retention due to slowed flow speed.²⁸⁹⁻²⁹⁰ In line with real-time monitoring of excrement, NIR fluorescence signals from tissue sections of these organs significantly dropped to nearly undetectable level under identical imaging conditions at day 15 post-injection of SPN-PT, verifying the in vivo clearance of SPN-PT at organ levels. In addition to SPN-PT, SPN-OT and SPN-DT demonstrated similar in vivo clearance behaviors. Furthermore, hematoxylin and eosin (H&E) staining was performed to examine major organs of mice at 15 days after administration with SPN-OT, SPN-PT, or SPN-DT (Figure 4.4g). No noticeable histopathological lesions were observed in SPN treated mice in comparison with saline

treated group. Moreover, blood smears of SPN-PT, SPN-DT, and SPN-OT demonstrated their negligible hematotoxicity. Thereby, these data demonstrated that the efficient biodegradability and high biosafety of the NIR-II PA SPNs.

Figure 4.4. In vivo biodegradation and clearance of NIR-II PA SPNs. a) Time-course fluorescence images of living mice after intravenous injection of SPN-PT (250 μ g/mL, 200 μ L per mouse). (n = 3) Excitation: 710 nm, Emission: 820 nm. b) Quantification of fluorescence intensities of liver region in living mice as a function of post-injection time. (n = 3) Fluorescence intensity of blood (c), urine (d), and feces (e) from living mice at representative time points after intravenous injection of SPN-PT. (n = 3) f) Fluorescent

images of histological sections of major organs from mice after injection with SPN-PT at designated time points. Nuclei were depicted in blue and biodegradation products were indicated in red. g) Representative hematoxylin and eosin staining of major organs of mice after respective systemic administration of saline (200 μ L), SPN-OT, SPN-PT, and SPN-DT (250 μ g/mL, 200 μ L) for 1 month. Error bars indicated 3 independent experiments ($n = 3$). Reprinted with permission from ref 269. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.3.4 In vivo NIR-II PA imaging of brain and tumor using SPN-PT

NIR-II PA imaging capability of SPNs was examined on a home-made NIR-II PA imaging system (Figure 4.5a). A Q-switched Nd:YAG laser source was adopted to offer incident 1064 nm pulse laser, and the induced acoustic waves were coupled into an ultrasonic transducer using water as the coupling medium. SPN-PT with the highest η was used as the representative contrast agent. The PA performance of SPN-PT was first investigated on a superficial imaging model, nude mice bearing subcutaneous 4T1 xenograft tumor. After systemic administration of SPN-PT, the average PA amplitude in tumor region gradually increased (Figure 4.5b). At 2 h post-injection, tumor was clearly visualized, implying the efficient accumulation of SPN-PT into tumor probably because of enhanced permeation and retention (EPR) effect due to its small size (~ 30 nm) and PEGylated surface. At 8 h post-injection of SPN-PT (Figure 4.5c), the average PA amplitude of tumor reached the plateau (0.51 ± 0.05) with a signal-to-background ratio (SBR, see calculation details in Supporting Information) of 4.6. Although the PA amplitude decreased after 8 h post-administration, the overall PA amplitude retained higher than background until at least 30 h. At 30 h post-injection, the mice were euthanized for biodistribution study. Spleens

had the highest uptake of SPN-PT, followed by livers, tumors, intestines, lungs, kidneys, and hearts. Besides, the injection dose of SPN-PT (2.5 mg kg^{-1}) for NIR-II PA imaging of tumor is largely reduced in comparison with many NIR-II contrast agents reported so forth ($\geq 10 \text{ mg kg}^{-1}$).^{260 193, 209, 257}

Furthermore, SPN-PT was utilized for deep transcranial NIR-II PA imaging to observe brain vasculature through intact scalp and skull. Although brain as an important part in central nervous system is of great significance to neuron-related pathological study, brain imaging using optical contrast agents remains challenging due to the strong scattering of light by skull. However, light scattering of skull in NIR-II window is largely reduced so that emerging NIR-II PA imaging provides a powerful solution to address this concern.¹² After intravenous administration of SPN-PT, the PA amplitude of brain vasculature instantly increased (Figure 4.5d), leading to a good contrast between brain vasculature and background parenchyma. At 2 min post-injection, the SBR of superior sagittal sinus ((i) in Figure 4.5d) reached 2.3. The transverse sinus (ii), vascular branches (iii) and middle cerebral arteries (iv) were also clearly visualized, and the pattern matched well with the open-skull anatomical image of brain cortex after the imaging experiments. The average PA amplitude remained nearly unchanged for at least 180 mins (Figure 4.5e), suggesting the long blood circulation of SPN-PT in agreement with tumor imaging. These results thus highlighted SPN-PT as excellent NIR-II PA contrast agent for both superficial and deep tissue imaging.

Figure 4.5. In vivo NIR-II PA imaging of brain and tumor using SPN-PT. a) Schematic illustration of home-made NIR-II PA imaging system. DM, dichromic mirror; RP, right angle prism; CRD, circular rotating disc; MPS, motor pulley system; SM, stepper motor; DAQ, data acquisition card; URU, ultrasound receiver unit; UST, ultrasound transducer. OD, optical diffuser. b) PA images of tumor at designated time points at 1064 nm after intravenous injection of SPN-PT (250 $\mu\text{g/mL}$, 200 μL per mouse) to living mouse. c) Quantification of PA amplitudes of region of interests (ROIs, yellow circles) in (b) as a function of time. d) PA images of brain vasculature at designated time points at 1064 nm after intravenous administration of SPN-PT (1.1 mg/mL, 1 mL per rat) to living rat. (i) superior sagittal sinus; (ii) transverse sinus; (iii) vascular branches; (iv) middle cerebral artery. e) Quantification of PA amplitudes of major blood vessels in (d) as a function of

post-injection time. Error bars indicated 3 independent experiments ($n = 3$). Reprinted with permission from ref 269. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.4 Conclusions

In summary, we reported the first series of SPN-based biodegradable NIR-II PA contrast agents with efficient *in vivo* clearance. By virtue of strong electron-withdrawing donor BBT, the obtained SPNs (SPN-PT, SPN-DT, and SPN-OT) had strong absorbance at ~ 1079 nm and high η at 1064 nm. Taking advantage of oxidizable thiophene moiety in conjugated backbones and FDA-approved hydrolysable polymer matrix (PLGA-PEG), three NIR-II SPNs could be effectively degraded in the presence of biologically abundant MPO and lipase, leading to the remarkable decomposition from 30-nm nanoparticles into NIR fluorescent ultra-small nanoparticles (~ 1 nm). Because of the ultra-small hydrodynamic sizes of degradation products, these NIR-II SPNs could be effectively cleared from living mice via both renal and hepatobiliary excretion within 15 days post-administration. As a representative agent, SPN-PT demonstrated excellent NIR-II PA imaging performance, achieving the high SBRs of 4.6 and 2.3 respectively for tumor and brain vasculature in living mice at a systematic dosage lower than other reported agents. Because *in vivo* metabolization of SPN-OT took much longer time than *in vivo* imaging, the *in vivo* clearance of SPN-OT had no noticeable influence on its imaging performance.

To the best of our knowledge, our study not only reported the first organic biodegradable NIR-II PA contrast agents, but also unveiled the underlying cellular and *in vivo* clearance pathways of these agents. We believe this study provides a generalized strategy to construct organic metabolizable semiconducting materials applicable to other biomedical

applications such as NIR-II activatable molecular probes and phototheranostics. The current findings should advance further development of organic semiconducting materials as promising optical agents towards clinical translations

Chapter 5 Amphiphilic Semiconducting Polymer as Multifunctional Nanocarrier for Fluorescence/Photoacoustic Imaging Guided Chemo-Photothermal Therapy

5.1 Introduction

Development of nanotechnology in the past few decades has stimulated the emergence of nanotheranostics for simultaneous diagnosis and therapeutics of diseases.²⁹¹ Nanotheranostics has the ability to monitor disease status before and during treatment, providing optimized therapeutic window and personalized treatment strategy.²⁹²⁻²⁹⁴ In particular, chemo-photothermal nanotheranostics integrating anticancer drug with thermal ablation not only overcomes the multidrug resistance of conventional chemotherapy, but also brings in synergistic therapeutic effect to improve therapeutic outcome.²⁹⁵⁻²⁹⁷ Such combined nanotheranostics thus has the potential advantages of reduced risk of cancer recurrence and minimized side effects relative to monotherapy.²⁹⁸

Current chemo-photothermal nanotheranostic systems often use fluorescence for diagnosis mainly due to its low cost and high sensitivity for whole-body imaging.^{50, 201} However, fluorescence imaging often encounters the issue of shallow penetration depth as a result of tissue autofluorescence and light scattering.^{12, 176} Thus, photoacoustic (PA) imaging that breaks the optical diffusion limit has recently been integrated into chemo-photothermal nanotheranostics, which has the advantage of increased imaging depth theoretically up to 7 cm.^{2, 10, 207, 254, 299} Till now, numerous theranostic nanoagents have been exploited for chemo-photothermal therapy, such as 2D nanomaterials,³⁰⁰⁻³⁰³ gold nanostructures,³⁰⁴⁻³⁰⁵ carbon-metal composites,³⁰⁶ small-molecule dye loaded

nanoparticles,³⁰⁷ and so on.³⁰⁸ However, because most chemo-photothermal nanotheranostic systems comprise more than three components (imaging agents, drug, photothermal agents and encapsulating polymers et al.), clinical translation of these nanotheranostics faces many challenges, which include complex and unstable nanostructure as well as unexpected conflicts in optical and biophysical properties among different components. As a result, rational design of nanotheranostics with minimal components and simple nanostructure is highly desired.

Semiconducting polymer nanoparticles (SPNs) have been recently transformed to organic theranostic nanoagents owing to their higher absorption and photothermal conversion efficiencies in near-infrared (NIR) region as compared with other inorganic nanoparticles such as carbon nanotubes and gold nanorods.^{27, 174, 205, 309-312} Because their optical and photothermal properties come from long π -electron delocalized backbones, SPNs generally possess better photostability than small-molecule dyes.³¹³ Recently, we have applied SPNs for fluorescence and PA imaging applications as well as photothermal therapy and control of neural activity.^{31, 36, 39-41, 203-204, 314-315} Despite their promise in nanotheranostics, SPN-based chemo-photothermal systems are rarely developed,³¹⁶⁻³¹⁸ which are complicated and composed of more than three components.

Figure 5.1. Schematic illustration of DSPN. (a) Schematic illustration of preparation of DSPN. (b) and (c) Synergistic effect of chemotherapy and PTT of cancer by DSPN.³⁷ Reprinted with permission from ref 37. Copyright 2017 Elsevier.

In this study, we report a simple dual-component nanotheranostic system based on SPNs for NIR fluorescence/PA imaging guided chemo-photothermal therapy (Figure 5.1a). Such a nanotheranostic design takes advantage of an amphiphilic poly(ethylene glycol) (PEG) grafted poly(cyclopentadithiophene-*alt*-benzothiadiazole) (PEG-PCB), which is a highly integrated multifunctional component that self-assembles into homogenous nanoparticles in aqueous solution. As a result of π -conjugated backbone, PEG-PCB not only serves as the diagnostic component for NIR fluorescence and PA imaging, but also acts as the photothermal agent by efficiently converting photon energy into heat. In addition, the hydrophobic π -conjugated backbone of PEG-PCB provides strong hydrophobic and π - π interactions with the aromatic anticancer drug such as doxorubicin (DOX) for drug

encapsulation and delivery. Such a unique trifunctional feature of PEG-PCB leads to the greatly simplified nanotheranostic system with only dual components but multimodal imaging and therapeutic capacities. In the following, we first describe the synthesis and characterization of PEG-PCB and drug-loaded PEG-PCB (DSPN), followed by *in vitro* studies of the cellular internalization and chemo-photothermal therapeutic efficacy. At last, the proof-of-concept application of theranostic DSPN in fluorescence/PA imaging guided chemo-photothermal therapy of tumor in living mice is demonstrated.

5.2 Materials and methods

5.2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise mentioned.

5.2.2 Synthesis of PCB-Br

2,6-dibromo-4,4-bis(6-bromohexyl)-4H-cyclopenta[2,1-b:3,4-b']dithiophene (50 mg, 0.075 mmol), 4,7-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzo[c][1,2,5]thiadiazole (28 mg, 0.075 mmol), Pd(PPh₃)₄ (5.5 mg, 0.052 mmol) and K₂CO₃ (122.49 mg, 0.75 mmol) were placed in a 50 mL Schlenk tube. Then toluene (2.5 mL) with methyltriocetylammmonium chloride (1 mg) and water (1.25 mL) were added to the Schlenk tube subsequently. The mixture was degassed by freeze-pump-thaw circles for three times. The reaction was carried out at 100 °C under nitrogen atmosphere for 2 h. Then the solvent was removed under reduce pressure. The obtained solid was re-dissolved by excess dichloromethane and washed with water for three times. The organic phase was concentrated and precipitated into excess methanol. The obtained solid was washed three times by methanol and then dried under vacuum to obtain product. ¹H NMR (300 MHz,

CDCl_3) δ 8.12 (s, 1H), 7.89 (s, 1H), 7.61 (s, 2H), 3.33 (s, 3H), 1.90 (d, $J = 93.0$ Hz, 8H), 1.25 (s, 12H), 0.87 (d, $J = 7.7$ Hz, 4H).

5.2.3 Synthesis of PCB- N_3

SP-Br (10 mg) and sodium azide (4 mg) were dissolved in a mixture of THF (12 mL) and DMF (6 mL). The mixture was stirred at 25 °C overnight. After that the solvent was removed under reduced pressure, an excess of dichloromethane was added to the residue. The resulting solution was washed three times with water and the organic phase was collected to dry over Na_2SO_4 . The dichloromethane was removed and SP- N_3 was obtained. ^1H NMR (300 MHz, CDCl_3) δ 8.11 (s, 1H), 7.89 (d, $J = 8.5$ Hz, 1H), 7.72 – 7.41 (m, 2H), 3.18 (s, 3H), 2.02 (s, 4H), 1.28 (d, $J = 17.3$ Hz, 18H), 0.87 (d, $J = 6.9$ Hz, 3H).

Synthesis of PEG-PCB: SP- N_3 (10 mg) was dissolved in THF (12 mL). CuBr (11 mg), PMDETA (66.8 mg) and methoxy-PEG-alkyne ($M_n = 2000$, 150 mg) were added into previous solution subsequently. The reaction was carried out at room temperature under nitrogen atmosphere for 48 h. After that the solvents were removed under reduced pressure and the remaining residue was dissolved into water. The resulting solution was dialysis against DI water to remove the salt and excess methoxy-PEG-alkyne in the system. The SP-PEG was obtained after lyophilization. ^1H NMR (300 MHz, CDCl_3) δ 7.71 (s, 1H), 7.61 (s, 1H), 7.53 – 7.42 (m, 2H), 4.00 – 3.39 (m, 320H), 1.76 (d, $J = 53.7$ Hz, 32H), 1.31 (d, $J = 33.2$ Hz, 32H), 0.87 (d, $J = 7.3$ Hz, 24H).

5.2.4 Preparation of PEG-PCB Nanoparticles

PEG-PCB (1 mg) was dissolved in 1 mL THF by bath sonication. The obtained solution (1 mg/mL) was rapidly injected into a mixture of deionized water (9 mL) and THF (1 mL)

solution under continuous sonication at a power output of 110 W for 1 min. THF in the solution was then removed by a nitrogen flow. The obtained solution was filtrated through a 0.22 μm PCDF syringe driven filter (Millipore). The PEG-PCB solution was then concentrated by centrifugation at 2600 rpm for 25 min and subsequently diluted to different concentrations by $1 \times \text{PBS}$ ($\text{pH} = 7.4$) for further use.

5.2.5 Preparation of DSPN₅

DSPN₅ was prepared via nanoprecipitation of DOX and PEG-PCB under controlled pH condition. DOX hydrochloride ($\text{DOX} \cdot \text{HCl}$, 2 mg) was first dissolved in dimethyl sulfoxide (DMSO) (0.2 mL). Triethylamine (5 μmL) was then added to the $\text{DOX} \cdot \text{HCl}$ DMSO solution (10 mg/mL) under bath sonication for 10 min. The resulted solution was subsequently mixed with the PEG-PCB solution in THF (1 mL, 1mg/mL). The obtained mixture was dialyzed in $1 \times \text{PBS}$ buffer ($\text{pH} = 7.4$) for 48 h to remove excess free DOX. The resulted DSPN₅ solution was purified by filtration through a 0.22 μm PCDF syringe driven filter (Millipore) and then concentrated by ultrafiltration. Concentration of DOX encapsulated into nanoparticles was quantified by the absorbance of DSPN₅ at 500 nm after subtracting the absorbance of PEG-PCB at this wavelength.

5.2.6 Characterization of Nanoparticles

^1H NMR spectra were obtained by Bruker Avance II 300 MHz NMR with CDCl_3 as the solvent. DLS was conducted on a Malvern Nano-ZS Particle Sizer. TEM images were acquired by a JEM 1400 transmission electron microscope with the accelerating voltage ranging from 40 to 120 kV. UV-Vis absorption spectra were recorded by Shimadzu UV-2450 spectrometer. Fluorescence spectra were obtained by Fluorolog 3-TCSPC

spectrofluorometer (Horiba Jobin Yvon). An 808 nm high power NIR laser (operating mode, CW; output power after fiber, 2.5 W; LED display; diode current, multimode fiber; fiber core diameter, 400 μm ; fiber connector, SMA905, with tunable laser driver module of 0-100%) purchased from CNI Co., Ltd. was used for PTT treatments. The laser power output was measured by power meter. IR thermal images was captured by photothermal camera FLIR T420.

5.2.7 PA Instrumentation

An optical parametric oscillator (OPO) (Continuum, Surelite) pumped by a Q-switched 532 nm Nd: YAG laser was used as the laser source to generate tunable laser pulses within 680-900 nm wavelength range with 100 mJ/pulse energy at 10 Hz repetition rate. Nanoparticle solution was placed inside a low-density polyethylene (LDPE) tube with an inner diameter (i.d.) of 0.59 mm and an outer diameter (o.d.) of 0.78 mm. The LDPE tube containing samples and the ultrasound transducer (UST) (V323-SU/2.25MHz, 13 mm active area, and 70% nominal bandwidth, Panametrics) were both immersed in water to couple PA signals to UST. The LDPE tube was irradiated with laser pulses of wavelengths ranging from 680 to 920 nm with 10 nm increment. PA signals were collected by UST, amplified with a gain of 50 dB, and then band pass filtered (1-10 MHz) by a pulse/receiver unit (Olympus-NDT, 5072PR).

5.2.8 In Vitro PTT of Nanoparticles

200 μL of PEG-PCB (PCB, 10 $\mu\text{g/mL}$), DSPN₅ (PCB, 10 $\mu\text{g/mL}$; DOX, 50 $\mu\text{g/mL}$), free DOX (DOX, 50 $\mu\text{g/mL}$), and PBS solutions were exposed to 808 nm laser with the power

intensity of 1 W/cm², respectively. Temperature of solution was recorded every 20 s by an IR thermal camera until it reached the maximum (6 min).

5.2.9 In Vitro Drug Release Property of DSPN₅

1 mL DSPN₅ solution (PCB, 0.2 mg/mL; DOX, 1 mg/mL) was filled into dialysis tubing (MW, 3000) which was then sealed and immersed in 5 mL PBS buffer of different pH conditions (pH = 5.5, 6.5, and 7.4), respectively. The dialysis reservoir was incubated in 37 °C water bath with constant shaking. At designated time points, the PBS buffer was removed to quantify the amount of released DOX while another 5 mL fresh PBS buffer was added to the dialysis reservoir for further study. The concentration of released DOX was measured by UV-Vis absorption spectroscopy.

5.2.10 Cell Culture and Cell Imaging

4T1 mammary carcinoma cells were purchased from the American Type Culture Collection (ATCC). 4T1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (10 U/mL penicillin and 10 mg/mL streptomycin) in an atmosphere of 5% CO₂ and 95% humidified air at 37 °C. The cells were seeded in confocal dishes and incubated with PBS, PEG-PCB (PCB, 10 µg/ml), and DSPN₅ (PCB, 10 µg/ml; DOX, 50 µg/ml) respectively for 12 h before replacement of medium with fresh DMEM and nuclei staining by DAPI. Confocal images were captured by a LSM710 confocal laser scanning microscope (Carl Zeiss, Germany) with the excitation wavelength of 405 nm for DAPI, 488 nm for DOX and 633 nm for PCB.

5.2.11 In Vitro Chemo-Photothermal Therapy:

Cells were seeded in 96-well plates (6000 cells in 200 μ L DMEM per well) for 24 h, and then free DOX, PEG-PCB, or DSPN₅ (final PCB concentration 2, 5, 7, 10, and 15 μ g/mL; final DOX concentration 10, 25, 35, 50, and 75 μ g/mL) was added to the culture medium of cells separately. After incubation for 12 h, cells were treated with or without laser irradiation of 1 W/cm² for 6 min. After PTT treatment, cells were incubated for another 12 h. Then the medium was removed and fresh DMEM with MTS (0.1 mg/mL, 20 μ L/well) was added to cells. After incubation for 4 h, the absorbance of MTS at 490 nm was measured by a SpectraMax M5 microplate/cuvette reader and the cell viability was quantified by the ratio of the absorbance of cells incubated with samples to that of control cells incubated with DMEM only.

5.2.12 Tumor Mouse Model

All animal experiments were performed in compliance with the guidelines established 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, two million 4T1 cells suspended in 50 mL of 50 v/v% mixture of Matrigel in supplemented DMEM (10% FBS, 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 $\approx$ 7 mm (10-15 days) before used for *in vivo* imaging and chemo-photothermal treatments.

5.2.13 In Vivo Fluorescence Imaging

Whole-body fluorescence imaging of mice was conducted by a Cri Maestro whole animal imaging system (Caliper Life Sciences, Hopkinton, MA). BALB/c mice bearing xenograft 4T1 tumors were administered with PEG-PCB (PCB, 30 μg per mouse) ($n = 3$) or DSPN₅ (PCB, 30 μg ; DOX, 150 μg per mouse) ($n = 3$) through tail vein injection. Fluorescence images of mice were acquired at different time points (2, 4, 6, 8, 24 h) post-injection of nanoparticles. Quantification of fluorescence in each image was performed by Nuance software.

5.2.14 In Vivo PA Imaging:

PA imaging of tumor region were performed by the Endra Nexus128 PA imaging system to acquire images at 680 nm. 4T1 tumor xenografted BALB/c mice were first anesthetized by 2% isoflurane in oxygen and then placed in the imaging system to determine the endogenous PA signal of tumor region before administration of samples. PEG-PCB (PCB, 30 μg per mouse) ($n = 3$), DSPN₅ (PCB, 30 μg ; DOX, 150 μg per mouse) ($n = 3$), or saline (150 μL) ($n = 3$) were then systematically administered through tail vein injection. Data were acquired through a continuous model that took 12 s to obtain one data set. 3D PA images were reconstructed offline using data acquired from all 128 transducers at each view and 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.

5.2.15 In Vivo Chemo-Photothermal Therapy

4T1 tumor xenografted BALB/c mice were systemically administered with PEG-PCB (PCB, 30 μg per mouse) ($n = 3$), DSPN₅ (PCB, 30 μg ; DOX, 150 μg per mouse) ($n = 3$), free DOX (DOX, 150 μg per mouse) ($n = 3$), or saline ($n = 3$) via tail vein injection. At 6 h post injection, tumor regions of living mice were exposed or without exposure to 808 nm laser of 0.3 W/cm². IR thermal camera was used to record the temperature change of the tumor every 15s for 5 min after irradiation. The tumor size was measured by caliper every other day and calculated as follows: 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.

5.2.16 Biodistribution Method

After 24 h post-injection of PEG-PCB (PCB, 30 μg per mouse) ($n = 3$) or DSPN₅ (PCB, 30 μg ; DOX, 150 μg per mouse) ($n = 3$), the mice were sacrificed by CO₂ asphyxiation and tumor, heart, liver, spleen, lung, kidney, skin, muscle, and bones were harvested for *ex vivo* fluorescence imaging to quantify the biodistribution of nanoparticles.

5.2.17 Histological Studies

After 16 days of PTT, the mice treated with PEG-PCB (PCB, 30 μg per mouse) ($n = 3$), DSPN₅ (PCB, 30 μg ; DOX, 150 μg per mouse) ($n = 3$), or saline ($n = 3$) were sacrificed. The tumor, liver, and spleen were fixed in 4% formalin. Paraffin embedded sectioning was then performed for H&E staining. The obtained slices were next examined under a digital microscope (Leica QWin). As for PCNA staining, sliced formalin-fixed tumor samples were used for confocal immunofluorescent analysis by PC10 Mouse mAb (#2586, Cell

Signaling Technology). Fixed tumor sections were treated following the protocol of In Situ Cell Death Detection Kit (Roche Applied Science) for TUNEL apoptosis staining and Hoechst 33342 was used for nuclear counterstaining.

5.2.18 Data Analysis

In vivo PA signal intensities were measured by region-of-interest (ROI) analysis using OsiriX. Fluorescence signal intensities were measured by ROI analysis using ImageJ. Results were 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).

5.3 Results and discussions

5.3.1 Synthesis and characterization of PEG-PCB and DSPNs

The amphiphilic PEG-PCB was synthesized via a grafting-on approach (Figure 5.2a). Monomers 1 and 2 were copolymerized via Suzuki polycondensation reaction to afford the polymer with bromide side groups (PCB-Br). The bromide groups were converted to azide, allowing for copper(I)-catalyzed azide-alkyne click reaction (CuAAC) to graft hydrophilic PEG chains onto highly hydrophobic polymer backbones. The resulted polymer (PEG-PCB) could be directly dissolved water and self-assemble into nanoparticles. Drug-loaded nanoparticles (DSPNs) were prepared via nanoprecipitation of deprotonated DOX and PEG-PCB under controlled pH condition, followed by dialysis to remove excess free DOX. The solutions of nanoparticles kept translucent before and after drug loading (Figure 5.2b), while the color of solutions changed from cyan to crimson owing to the incorporation of DOX. Dynamic light scattering (DLS) revealed the diameter increased from 40 nm for

PEG-PCB to 100 nm for DSPN with the highest drug loading rate ($W_{\text{DOX}}/W_{\text{PCB}} = 5$, DSPN₅) (Figure 5.2c). Spherical morphology was observed for both PEG-PCB and DSPN₅ by transmission electron microscopy (TEM) (Figure 5.2d). In addition, no precipitation or obvious change in diameter was observed for both PEG-PCB and DSPN₅ after storage in $1 \times \text{PBS}$ (pH = 7.4) for two months, suggesting their excellent aqueous stability.

Figure 5.2. Synthesis and characterization of PEG-PCB and DSPN₅. (a) Synthetic route of PEG-PCB. (i) Pd(0), K₂CO₃, methyltriocetylammmonium chloride, Toluene/H₂O; (ii) NaN₃; (iii) mPEG-alkyne, CuBr, PMDETA, THF. (b) Photographs of PEG-PCB and DSPN₅ solution. (PCB 10 μg/ml, DOX 50 μg/ml). (c) Size distribution of PEG-PCB and DSPN₅ by DLS. (d) TEM images of PEG-PCB (left) and DSPN₅ (right). Reprinted with permission from ref 37. Copyright 2017 Elsevier. Optical properties of DSPN were studied and compared with PEG-PCB under physiological conditions. PEG-PCB had one absorption peak at 670 nm assigned to the absorbing PCB (Figure 5.3a), while DSPN showed an additional absorption peak at 500 nm attributed to DOX. The absorbance of DSPN at 500 nm gradually increased with the doping ratio of DOX. Although the fluorescence spectra

of SPN and DSPN had the emission maximum at 825 nm (Figure 5.3b), the fluorescence was quenched after the loading of DOX. Compared with PEG-PCB, a 75% decrease of fluorescence intensity at 825 nm was observed for DSPN₅. Such fluorescence quenching should be attributed to the charge transfer between the π -conjugated polymer backbones and the anthraquinone part of DOX. Furthermore, both SPN and DSPN emitted PA signals ranging from 680 to 900 nm with the peak signal at 680 nm (Figure 5.3c), correlated well with their absorption spectra (Figure 2a). As the fluorescence of DSPN₅ was quenched and that part of energy was converted into heat,³¹ the PA amplitudes of DSPN were slightly higher than those of PEG-PCB.

Figure 5.3. *In vitro* optical, PA, photothermal, and drug release properties of PEG-PCB and DSPN in $1 \times \text{PBS}$. (a) UV-Vis-NIR absorption and (b) Fluorescence spectra of PEG-PCB and DSPN with different loading ratio ($W_{\text{DOX}}/W_{\text{PCB}} = 1, 1.6, \text{ and } 5$ for DSPN₁, DSPN_{1.6}, and DSPN₅, respectively). Excitation wavelength, 670 nm. (c) PA spectra of PEG-PCB (PCB, 100 $\mu\text{g}/\text{ml}$) and DSPN₅ (PCB, 100 $\mu\text{g}/\text{ml}$; DOX, 500 $\mu\text{g}/\text{ml}$). (d) Photothermal

profiles, thermal images of PBS, free DOX (DOX, 50 $\mu\text{g/ml}$), PEG-PCB (PCB, 10 $\mu\text{g/ml}$), and DSPN₅ (PCB, 10 $\mu\text{g/ml}$; DOX, 50 $\mu\text{g/ml}$) under irradiation of 808 nm laser (laser intensity, 1 W/cm^2). (e) Stability study of DSPN₅ under the photothermal heating and natural cooling cycles (laser intensity, 1 W/cm^2 ; PCB, 10 $\mu\text{g/ml}$; DOX, 50 $\mu\text{g/ml}$). (f) Cumulative drug release of DSPN₅ under different pH conditions at 37 °C (PCB, 0.2 mg/ml ; DOX, 1 mg/ml). Error bars indicate standard deviations of 3 separate measurements. Reprinted with permission from ref 37. Copyright 2017 Elsevier.

Photothermal properties of PEG-PCB and DSPN₅ were subsequently investigated and compared. Under laser irradiation at 808 nm with the power intensity of 1 W/cm^2 , the solution temperatures for both PEG-PCB and DSPN₅ gradually increased, which was not observed for PBS and free DOX solutions (Figure 5.3d). After continuous laser irradiation for 6 min, the solution temperatures of both PEG-PCB and DSPN₅ reached a plateau. At this time point, the solution temperature of DSPN₅ (60.9 °C) was about 4.1 °C higher than that of PEG-PCB (56.8 °C). Such slightly enhanced maximum photothermal temperature for DSPN₅ should be attributed to the additional heat generated by DSPN₅ as a result of quenched fluorescence. The reversible heating-cooling operation showed that the maximum solution temperatures of DSPN₅ retained almost unchanged throughout at least 5 heating-cooling cycles, indicating its good photostability (Figure 5.3e). Besides, the photothermal conversion efficiency of DSPN₅ at 808 nm was calculated to be 30.8% according to the previously reported procedure.⁴⁰ In addition, drug release property of DSPN₅ was studied by dialysis of DSPN₅ in 1 × PBS buffer under different pH conditions (pH = 5.5, 6.5, and 7.4) at 37 °C (Figure 5.3f). After 48 h of dialysis, ~35.5% of DOX was released from DSPN₅ at pH 5.5, followed by 22.7% at pH 6.5 and 16.7% at pH 7.4. This

pH-dependent drug release behavior should be attributed to the protonation of amine group of DOX under acidic conditions, which enhanced its hydrophilicity and facilitated its escape from the hydrophobic core of DSPN₅. These results indicated that DSPN₅ had suitable photothermal and drug release properties for chemo-photothermal therapy.

5.3.2 In vitro characterization of combination therapy by DSPN₅

To study the *in vitro* cellular uptake and drug release properties of nanoparticles, confocal laser scanning microscopy (CLSM) was conducted on murine breast cancer cells (4T1). After incubation with free DOX, PEG-PCB, or DSPN₅ for 12 h, cells were washed by PBS buffer and then stained with nuclear indicator DAPI (depicted in blue). NIR fluorescence (depicted in red) was observed for PEG-PCB or DSPN₅ treated cells (Figure 5.4a), showing the cellular internalization of both nanoparticles. Moreover, the fluorescence of DOX was detected for DSPN₅ treated cells. Although the strongest signal from DOX was observed in cytoplasm, there was a signal overlap between the fluorescence of DOX and of DAPI (Figure 5.4a). This data proved that DOX was released from DSPN₅ and diffused to nuclei, probably attributed to the protonation of amine group of DOX under acidic conditions in lysosomes (Figure 5.1c), which enhanced the hydrophilicity of DOX and facilitated its escape from the hydrophobic core of DSPN₅.

Figure 5.4. *In vitro* characterization of combination therapy by DSPN₅. (a) Confocal fluorescence images of 4T1 cells after incubation with PBS, PEG-PCB (PCB, 10 μg/ml), and DSPN₅ (PCB, 10 μg/ml; DOX, 50 μg/ml) for 12 h. (b) Cell viabilities of 4T1 cells after incubation with PBS, free DOX, PEG-PCB, and DSPN₅ with different concentrations. (c) Cell viabilities of 4T1 cells after incubation with PBS, PEG-PCB, and DSPN₅ with different concentrations after laser irradiation for 6 min (laser intensity, 1 W/cm²). Error bars indicate standard deviations of 3 separate measurements. Reprinted with permission from ref 37. Copyright 2017 Elsevier.

In vitro therapeutic efficacy of combination therapy vs monotherapy was evaluated and compared by cell viability tests. Without laser irradiation, PEG-PCB showed negligible toxicity to 4T1 cells while DSPN₅ had a significantly lower cell viability as compared with PEG-PCB at each concentration (Figure 5.4b). This verified the increased cytotoxicity of DSPN₅ due to the presence of DOX (Figure 5.4b). After irradiation of 808 nm laser for 6

min, cells incubated with PEG-PCB or DSPN₅ showed obvious decrease in viability at each concentration as compared to those without laser irradiation (Figure 5.4c vs 5.4b), indicating the enhanced cytotoxicity owing to PTT. It was noted that DSPN₅ mediated PTT treatment caused the highest cytotoxicity among all the treatments. In particular, at the concentration of 2 $\mu\text{g/mL}$ for PCB and 10 $\mu\text{g/mL}$ for DOX, cell viability after DSPN₅ mediated PTT treatment was about 19%, which was much lower than that by DSPN₅ alone (62%) and SPN-mediated PTT (83%). Furthermore, PEG-PCB was only able to achieve similar cytotoxicity at a much higher concentration (7 $\mu\text{g/mL}$ of PCB) (Figure 5.4c), and DSPN₅ without PTT treatment was insufficient to cause such high cytotoxicity within experimental range (Figure 5.4b). These results demonstrated the overall enhanced therapeutic efficacy of combination therapy relative to monotherapy, highlighting the synergistic antitumor effect of DSPN₅ for cancer therapy.

Figure 5.5. *In vivo* dual-modal NIR fluorescence/PA tumor imaging. (a) Fluorescence images of living mice bearing xenograft 4T1 tumors at 0, 4, 6, 8 and 24 h after tail-vein injection of PEG-PCB (PCB 30 μg per mouse) or DSPN₅ (PCB, 30 μg ; DOX, 150 μg per mouse). (b) Fluorescence intensities extracted from tumor regions as a function of time after systemic administration of PEG-PCB or DSPN₅. (c) *Ex vivo* quantification of average fluorescence intensity of major organs of mice after systemic administration of PEG-PCB or DSPN₅ for 24 h (n = 3). (d) Representative 3D images of tumor on a living mouse at 0 and 8 h after injection of DSPN₅ (PCB, 30 μg ; DOX, 150 μg per mouse). (e) *In vivo* real-time PA spectra extracted from tumor regions in living mice after systemic administration of DSPN₅ or saline for 24 h. Error bars indicate standard deviations of 3 separate measurements. Reprinted with permission from ref 37. Copyright 2017 Elsevier.

5.3.3 *In vivo* dual-modal NIR fluorescence/PA tumor imaging.

Dual-modal NIR fluorescence/PA imaging were conducted on living mice bearing 4T1 xenograft tumors to identify the optimum therapeutic window. After systemic administration of PEG-PCB or DSPN₅ *via* tail vein injection, both fluorescence and PA signals of living mice were continually monitored. As shown in Figures 4a and b, the fluorescence intensities from the tumor region for both PEG-PCB and DSPN₅ treated mice increased over time and reached the maximum at 6 h post-injection. At this time point, the fluorescence intensity of tumor region on DSPN₅ treated mice was 0.87-fold of that on PEG-PCB treated mice. The lower fluorescence intensity for DSPN₅ treated mice should be attributed to the quenching of fluorescence by DOX (Figure 5.3b). Moreover, the decreased accumulation of DSPN₅ in tumors as indicated by the lower tumor-to-liver intensity ratio (TLR) of DSPN₅ vs PEG-PCB (1.0 vs 1.6) treated mice should be responsible,

which could be ascribed to the relatively larger size of DSPN₅ than PEG-PCB. Similarly, the PA signal of tumor region reached the maximum at 8 h post-injection of DSPN. At this time point, the PA amplitude of tumor region in DSPN₅ treated mice was 3.8-fold higher than that of tumor background, which was consistent with the fluorescence imaging. As a result of the high optical contrast and spatial resolution of PA technique, 3D PA image after injection of DSPN₅ was able to clearly delineate the tumor region of living mouse (Figure 5.5d). Real-time *in vivo* PA spectrum extracted from tumor regions of DSPN₅ treated mice (Figure 5.5e) resembled that of DSPN₅ in aqueous solution (Figure 5.3c), confirming that the PA signal resulted from the accumulation nanoparticles. *Ex vivo* data further revealed that liver had the strongest fluorescence for both PEG-PCB and DSPN₅ treated mice, followed by tumor, and other organs (Figure 5.5c). No obvious change was measured between the biodistribution of PEG-PCB and DSPN₅ treated mice, probably due to their similar PEG surface. These *in vivo* and *ex vivo* imaging data proved that both PEG-PCB and DSPN₅ could passively target the tumors in living mice *via* enhanced permeability and retention (EPR) effect due to their idea sizes (within 100 nm) and PEGylated surface (Figure 5.1b).

Figure 5.6. *In vivo* combination therapy. (a) IR thermal images of 4T1-bearing mice under 808 nm laser irradiation (0.3 W/cm^2) at 6 h after systemic administration of saline, PEG-PCB (PCB $30 \mu\text{g}$ per mouse), or DSPN₅ (PCB, $30 \mu\text{g}$; DOX, $150 \mu\text{g}$ per mouse). (b) Mean tumor temperature as a function of laser irradiation time at 8 h post-injection of DSPN₅, PEG-PCB, or saline. (c) Tumor growth curves and (d) body weight data of different groups of mice after systemic administration of DSPN₅, PEG-PCB, or saline with and without laser irradiation ($n = 3$). Error bars indicate standard deviations of 3 separate measurements. Reprinted with permission from ref 37. Copyright 2017 Elsevier.

Therapeutic efficacy of PEG-PCB and DSPN₅ was tested and compared in 4T1 xenograft tumor mouse model. According to the *in vivo* imaging data, PTT treatment was applied at

6 h post-injection of PEG-PCB or DSPN₅ when the accumulation of nanoparticles in the tumor reached the maximum. After systemic administration of DSPN₅, PEG-PCB, or saline for 6 h, the tumor region in living mice was irradiated by 808 nm laser with the power intensity of 0.3 W/cm² for 5 min. Upon laser irradiation, the temperature of tumor region for DSPN₅ or PEG-PCB treated mice gradually increased (Figure 5.6a, b), which was significantly higher than that for saline treated group at all time points. The temperature of tumor for PEG-PCB or DSPN₅ treated mice reached a plateau after laser irradiation for 2 min, and the mean temperature of tumor for SPN (59.6 °C) or DSPN₅ treated mice (60.5 °C) was about 1.5-fold of that for saline treated mice (39.3 °C). Despite the higher in vitro photothermal maximum temperature of DSPN₅ relative to PEG-PCB (Figure 2d), no significant difference of tumor temperature was observed between PEG-PCB and DSPN₅ treated mice, which might be attributed to the reduced accumulation of DSPN₅ in tumor region as indicated by the lower TLR of DSPN₅ than PEG-PCB (Figure S6, supporting information).

To evaluate and compare the antitumor effect of PEG-PCB and DSPN₅, the volumes of tumors in living mice were continuously measured for 16 days (Figure 5.6c). Without laser irradiation, tumors in DSPN₅ treated mice grew much slower than those for saline or PEG-PCB treated mice, indicating the certain level of antitumor effect of DSPN₅ by chemotherapy. With laser irradiation, PEG-PCB inhibited the growth of tumors for the first 6 days while failed at later time points, suggesting the limited antitumor effect of PEG-PCB mediated PTT. In comparison, only DSPN₅ mediated PTT completely inhibited the growth of tumors throughout the experimental period. The superior antitumor capability of DSPN₅ over PEG-PCB should be attributed to the synergistic effect resulted from the combination

of chemotherapy and PTT. No significant weight loss was observed for all groups (Figure 5d), indicating the safety of all treatments to mice.

Figure 5.7. Histological analysis. (a) H & E staining of livers, spleens and tumors for 4T1 tumor-bearing mice at day 16 after photothermal treatment with saline, free DOX, PEG-PCB, or DSPN₅. (b) Fluorescence PCNA and (c) TUNEL staining of tumors on 4T1 tumor-bearing mice at 16 d after photothermal treatment with saline, free DOX, PEG-PCB, or DSPN₅. Green fluorescence indicated signals of PCNA and TUNEL staining while blue

fluorescence indicated nucleus staining. Reprinted with permission from ref 37. Copyright 2017 Elsevier.

To further validate the enhanced therapeutic effect of DSPN₅, all the mice were sacrificed at day 16 and histological analysis was performed on tumors and different organs by hematoxylin and eosin (H & E) staining, fluorescent proliferating cell nuclear antigen (PCNA) and TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining. No noticeable histopathological damage was found in the liver or spleen of mice treated with free DOX, PEG-PCB, and DSPN₅ after PTT, while typical nuclear dissociation was clearly observed in tumor tissues of the same groups, with the overall severest dissociation found in DSPN₅ injected mice after PTT treatment (Figure 5.7a). Besides, PCNA staining of tumor regions on mice demonstrated that the reproduction capacity of cancer cells was reduced in free DOX, PEG-PCB, or DSPN₅ treated mice after PTT (Figure 5.7b), which was not observed saline treated mice without laser irradiation. In comparison with the sparse green fluorescent dots observed in free DOX or PEG-PCB treated mice, no fluorescent dots could be found in DSPN₅ treated group, which implied the minimum proliferation capability of cancer cells caused by chemo-photothermal therapy. Similarly, TUNEL staining of tumor region of mice showed the evident apoptosis of cancer cells observed in free DOX, PEG-PCB, or DSPN₅ treated mice after PTT (Figure 5.7c). As indicated by the stronger green fluorescence in the tumor tissue, DSPN₅ caused severer apoptosis than PEG-PCB and free DOX. The outcome of histological analysis validated at cellular level the superiority of DSPN₅ over PEG-PCB and free DOX for antitumor effect, which was consistent with the result of *in vivo* antitumor experiments (Figure 5.6c).

5.4 Conclusions

We have demonstrated a simplified theranostic nanoagent DSPN₅ for NIR fluorescence/PA imaging guided chemo-photothermal therapy. Such a concise design was based on an amphiphilic polymer PEG-PCB synthesized by PEGylation of semiconducting polymer via click chemistry. By virtue of the hydrophobic π -conjugated backbone, PEG-PCB not only acted as the contrast agent for both NIR fluorescence and PA imaging, but also served as the therapeutic agent for PTT as well as the drug delivery system for chemotherapy. Taking advantage of such trifunctionality, PEG-PCB was utilized to encapsulate DOX to obtain the greatly simplified nanotheranostic system (DSPN₅) with only dual components but multimodal theranostic capabilities. Due to the small size of nanoparticle, DSPN₅ was able to passively accumulate in the tumor site of living mice via EPR effect, allowing for effective NIR fluorescence and PA imaging of tumor. After systemic administration of DSPN₅, the PA amplitude of tumor region on DSPN₅-treated mice could be 3.8-fold higher than that of the tumor background before systemic administration. Moreover, both *in vitro* and *in vivo* characterization of combination therapy revealed the superior antitumor efficacy of DSPN₅ over PEG-PCB or free DOX owing to the synergistic effect of chemotherapy and PTT. Superior to PEG-PCB mediated PTT or DSPN₅ mediated chemotherapy, DSPN₅ mediated chemo-photothermal therapy totally inhibited the tumor growth during experimental period, which was further validated at cellular level by histological analysis.

Our study thus provides a molecular engineering approach to integrate desired properties into one polymer for multimodal nanotheranostics. Because the side chains of amphiphilic SPs can be easily modified with Fluorine-19, further integration of magnetic resonance

imaging (MRI) into the system could be feasible. In addition to chemotherapy drugs, other therapeutic agents such as immunoadjuvants can also be encapsulated into such polymer for other therapeutic purpose.

Chapter 6 Semiconducting nanoinhibitor for synergistic photodynamic therapy

6.1 Introduction

Photodynamic therapy (PDT) that utilizes photosensitizers to transform oxygen (O_2) into cytotoxic reactive oxygen species (ROS) to trigger cellular apoptosis holds great promise for cancer therapy.³¹⁹⁻³²⁰ Unlike other electromagnetic radiation therapy such as radiotherapy, PDT offers merits of non-invasiveness and minimal adverse effects.³²¹ The crucial role of photosensitizer in PDT has stimulated the exploration of various optical agents for such application, which include porphyrin derivatives,³²² small-molecule dyes,¹⁴ metallic oxides,³²³ quantum dots,³²⁴ upconversion nanoparticles,³²⁵ and scintillation nanomaterials.³²⁶ However, PDT efficacy is not only related to the ROS generation ability of photosensitizers, but also intrinsically limited by its reliance on O_2 . The consumption of O_2 in tumor microenvironment can exacerbate tumor hypoxia, trigger the signaling cascade mediated by hypoxia inducible factor (HIF) and subsequently activate a series of target genes (such as glucose transporters, and vascular endothelial growth factor) responsible for cancer cell survival, tumorigenesis, and distant metastases.³²⁷ Thereby, it is highly desired to bypass the side effect of PDT-exacerbated hypoxia so as to improve its therapeutic efficacy.

Although several approaches have been reported to conquer tumor hypoxia, each has its own pros and cons.³²⁸ For instance, direct O_2 supplement using MnO_2 or perfluorocarbon (PFC) nanodroplets has been exploited to focally elevate oxygen level at tumor sites,³²⁵ which has poor temporospatial regulation of O_2 release. Moreover, effort to shift PDT from

oxygen-dependent type II to H₂O-dependent type I pathway has been devoted, which often requires the use of inorganic photosensitizers containing heavy metal ions with high toxic potential.³²⁶ Another approach is to use bio-reductive prodrugs with specific activation under hypoxic conditions,³²⁹ whose anti-hypoxia activity is largely constrained by the distribution of corresponding reductases. More recently, utilization of small molecule inhibitors against molecular targets in HIF-mediated pathway is emerging as a promising approach to mitigate the probability of hypoxia-induced metastasis, which however has been less exploited in PDT.

Semiconducting polymer nanoparticles (SPNs) comprising π -conjugated macromolecules have been validated as a versatile class of biophotonic nanoagents.^{26-27, 218, 225, 265} By virtue of their high optical activity, chemical flexibility, and bio-inert nature, SPNs have been applied as phototheranostic agents for superficial/deep-tissue cancer treatment,^{269, 274} as activatable probes for molecular imaging,^{173, 204} and as light-sensitive transducers for bio-regulation.⁴⁰ Particularly, SPNs has been designed to improve PDT by doping with nanocerias to afford pH-switchable self-regulating nano-photosensitizer with reduced damage to normal tissues³², or coating with MnO₂ nanosheet to improve the hypoxia condition in tumor.³³ Additionally, we have demonstrated the utilization of SPNs as hypoxia-activatable nano-prodrug and as photodynamic pro-nanoenzyme for complete-remission cancer therapy.³³⁰⁻³³¹ Nevertheless, synergistic cooperation of SPNs with small-molecule enzyme inhibitors to improve PDT efficacy has not been explored.

We herein report the first organic semiconducting photodynamic nanoinhibitor (OPNi) to overcome the hypoxia limitation of conventional PDT for synergistic cancer therapy (Figure 6.1a). Such a nanoinhibitor consists of a custom-designed NIR absorbing SP

(PCVB) as the biodegradable photosensitizer and an acetazolamide (CAi)-terminated amphiphilic polymer (CAi-*b*-PEG-*b*-PPG-*b*-PEG-CAi) as the inhibitor to counteract carbonic anhydrase IX (CA-IX), a crucial downstream gene product in HIF-mediated signalling cascade. CA-IX is over-expressed on cell membranes of many aggressive breast cancer cells and responsible for the reversible hydration of carbon dioxide (CO_2) into proton (H^+) and bicarbonate (HCO_3^-), regulating both intra- (pHi) and extracellular (pHe) pH balance to promote cancer cell survival and invasive migration (Figure 6.1b).³³² With CAis on the surface, OPNi not only can specifically bind the cancer cells to exert targeted PDT, but also can inhibit the catalytic function of CA-IX and consequently dysregulate both pHe and pHi to suppress cancer metastasis. Furthermore, the biodegradability of OPNi enable its efficient metabolization and clearance in living animals.

Figure 6.1. Schematic illustration of OPNi. (a) nanoprecipitation of OPNi and (b) mechanism of OPNi mediated synergistic phototherapy. p300/CBP: p300 and cyclic adenosine monophosphate response element binding protein; HRE: hypoxia responsive element; NBC: sodium/bicarbonate cotransporter; MCT, monocarboxylate transporter.³³³ Reprinted with permission from ref 333. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KgaA, Weinheim.

6.2 Materials and methods

6.2.1 Chemicals

All the chemicals used in this paper were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. Acetazolamide was purchased from Tokyo Chemical Industry Co., Ltd. 2,6-Dibromo-4,4-diethylhexyl-4H-cyclopenta[2,1-b;3,4-b']dithiophene and 4,7-dibromo-2,1,3-benzothiadiazole were purchased from Luminescence Technology Corp (Lumtec). Singlet Oxygen Sensor Green was purchased from ThermoFisher Scientific. Myeloperoxidase was purchased from Planta Natural Products. Anti-VCAM-1, anti-caspase-3, anti-HGF antibodies and Alexa Fluor 488 conjugated donkey anti-rabbit secondary antibody were purchased from Abcam Inc. (Cambridge, CA, USA).

6.2.2 Characterization

Nuclear magnetic resonance (NMR) spectra were measured on Bruker Avance II 300 MHz NMR spectrometer. Absorption spectra were measured by Lambda 950 spectrometer. Fluorescence spectra were measured on Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). Hydrodynamic sizes and zeta potential were measured by Malvern Nano-ZS Particle Sizer. Transmission electron microscope (TEM) images were captured on JEOL JEM 1400 transmission microscope. Gel permeation chromatography was performed on a Shimadzu LC-VP system with tetrahydrofuran as the eluent and polystyrene as the standard. Confocal images were captured on Zeiss LSM 800. Flowcytometry was carried out on Fortessa X20 (BD Biosciences). IR thermal images were captured by FLIR T420 thermal camera. In vivo NIR fluorescence imaging of mice was performed on an IVIS-CT machine (PerkinElmer).

6.2.3 Synthesis of PCVB

2,6-dibromo-4,4-diethylhexyl-4H-cyclopenta[2,1-b;3,4-b']dithiophene (7 mg, 0.0125 mmol), and 4,7-dibromo-2,1,3-benzothiadiazole (3.7 mg, 0.0125 mmol), trans-1,2-bis(tributylstannyl)ethene (15.2 mg, 0.025 mmol), Pd₂(dba)₃ (1.0 mg, 0.001 mmol), and tri(o-tolyl)phosphine (2.0 mg, 0.006 mmol) were dissolved in 4 mL of chlorobenzene (4 mL) and sealed in 50 mL round-bottom flask. After 3 circles of freeze-pump-thaw for degassing, the mixture was stirred at 100 °C in oil bath to carry out Stille polymerization under nitrogen atmosphere for 24 h. After reaction, the mixture was precipitated in methanol. The dark precipitates were collected by centrifugation, washed 3 times with methanol, and then dried under vacuum overnight to obtain PCVB solids. ¹H NMR for PCVB (CDCl₃, σ ppm): 8.56 (d, 1H), 8.30 (d, 1H), 7.82 (d, 1H), 7.56 (d, 1H), 7.08 (s, 1H), 7.00 (d, 2H), 6.87 (s, 1H), 1.88 (s, 4H), 1.02-0.78 (m, 30H).

6.2.4 Synthesis of Alkyne-CAi

Acetazolamide (4.5 g, 20 mmol) was dissolved in ethanol (40 mL) and hydrolyzed by HCl (32%, 12 mL, 125 mmol) in a 100 mL round-bottomed flask. The mixture was refluxed at 95 °C for 16 h to afford a colorless solution. After reaction, the mixture was cooled down to room temperature and solvent was removed by rotary evaporation. The obtained concentrate was dissolved in deionized (DI) water and neutralized by NaOH (5.0 M). The white precipitates (compound 2) were collected via centrifugation followed by freeze-drying to remove excess water. Afterwards, the obtained compound 2 (350 mg, 2 mmol) and triethylamine (417 μ L, 3 mmol) were dissolved in anhydrous DMF followed by addition of 4-(chlorosulfonyl)benzoic acid (600 mg, 3 mmol) on ice bath. Then the reaction

was carried out at room temperature for 12 h. After reaction, DMF was removed by rotary evaporation. The precipitates were washed 3 times with NaHCO_3 solution and DI water, followed by freeze-drying to obtain pure compound 3. Then, compound 3 (50 mg, 0.14 mmol), propargyl bromide (30 μL , 0.4 mmol), and K_2CO_3 (50 mg, 0.36 mmol) were dissolved in DMF (2 mL) and stirred at room temperature for 5 h. After reaction, DMF was removed by rotary evaporation and the concentrates were purified by column chromatography (DCM/MeOH 10:3) to afford a pale yellow solid (Alkyne-CAi). $R_f = 0.5$ (DCM/MeOH 10:2). ^1H NMR for Alkyne-CAi (CD_3OD , σ ppm): 8.12-7.97 (s, 4H), 4.94 (s, 2H), 2.99 (s, 1H). ESI-MS (m/z): calcd: 401.98, found $[\text{M} - \text{H}]^-$: 401.14.

6.2.5 Synthesis of CAi-PEG-b-PPG-b-PEG-CAi

PEG-b-PPG-b-PEG (1.2 g) was dissolved in distilled THF and placed in a 50-mL round bottomed flask, followed by addition of NaH (200 mg). After stirring for 1 h, p-toluenesulfonyl chloride (120 mg) dissolved in distilled THF (2 mL) was added to the flask and the mixture was stirred at room temperature for 24 h. After reaction, the mixture was precipitated in cold diethyl ether and collected via centrifugation at 9000 rpm for 10 min at $-5\text{ }^\circ\text{C}$. The obtained solids were dried under vacuum to afford compound 5. Then, compound 5 (650 mg) and NaN_3 (40 mg) were dissolved in DMF (11 mL) and stirred at $60\text{ }^\circ\text{C}$ for 24 h. After reaction, the mixture was cooled down to room temperature and DMF was removed by rotary evaporation. The obtained concentrates were dialysed in DI water (3000 Da) followed by freeze-drying to afford compound 6. Afterwards, alkyne-CAi (25 mg), compound 6 (560 mg), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (30 mg), and sodium ascorbate (180 mg) were dissolved in DMF (10 mL) and stirred at room temperature for 24 h to perform click reaction. After reaction, DMF was removed by rotary evaporation and the concentrates

were dialysed in DI water (3000 Da) and then freeze-dried to obtain CAi-PEG-b-PPG-b-PEG-CAi. ^1H NMR for CAi-PEG-b-PPG-b-PEG-CAi (CDCl_3 , σ ppm): 8.13 (d, 2H), 7.99 (d, 2H), 3.71-3.60 (m, 800H), 3.47-3.33 (m, 65H), 1.29-1.04 (m, 195H).

6.2.6 Preparation of OPNi and OPNc

To prepare OPNi nanoparticles, PCVB (0.25 mg) and CAi-PEG-b-PPG-b-PEG-CAi (20 mg) were dissolved in THF (1.25 mL). The mixture was quickly injected into DI water (9 mL) under vigorous sonication. Next, THF was removed by a gentle N_2 flow for 40 mins. The obtained aqueous solution was filtered through a 220 nm polyvinylidene fluoride (PVDF) syringe driven filter (Millipore) to remove large precipitates followed by ultracentrifugation to afford concentrated solutions. OPNc was prepared following the same procedure whereas using PEG-*b*-PPG-*b*-PEG as the polymer matrix.

6.2.7 Agarose gel electrophoresis

1% agarose gel in $1\times$ tris/boric acid/ethylenediaminetetraacetic acid (TBE) buffer was prepared and cooled down to room temperature. OPNis (50 $\mu\text{g/mL}$, 40.0 μL) with or without CA-IX treatment were mixed with 10.0% SDS solution (4.0 μL) and $10\times$ loading buffer (5.0 μL). The obtained mixtures were then loaded in separate wells on agarose gel and electrophoresed at 120 V for 1 h. After electrophoresis, gel image was captured on an IVIS-CT machine with excitation and emission wavelength at 680 and 820 nm, respectively.

6.2.8 In vitro cellular uptake

MDA-MB-231 human breast cancer cells (purchased from American Type Culture Collection, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM)

supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) and placed in an incubator providing 5% CO₂ and 95% humidified air at 37 °C. To study cellular uptake, 231 cells were seeded in confocal cell culture dishes (4×10^4 cells/dish) and respectively incubated with OPNi, OPNc ([PCVB] = 50 µg/mL), or PBS buffer at 37 °C for 1 h. After co-incubation, nanoparticles were removed and cells were stained with Hoechst 33342 (purchased from Thermo Fisher Scientific). Then confocal images of cells were captured on a LSM 800 confocal laser scanning microscope.

Flow cytometry was performed on Fortessa X20 (BD Biosciences). 231 cells, HeLa human cervical cancer cells (purchased from ATCC), NIH/3T3 fibroblast cells (purchased from ATCC), and MCF-7 human breast cancer cells (purchased from ATCC) were seeded in 12-well dish (1×10^5 per well) and incubated with OPNis ([PCVB] = 50 µg/mL) or PBS at 37 °C for 1 h. Then, cells were washed, trypsinized, collected, and resuspended in PBS for flow cytometry measurement using APC-Cy7 channel.

6.2.9 In vitro photodynamic study

Commercially available ¹O₂ probe SOSG (1 µM) was added to OPNi (25 µg/mL, 1 mL) and PBS solution (1 mL), respectively. These solutions were then respectively irradiated by 680 nm laser at 0.2 W/cm² for 30 min. Fluorescence intensities at 520 nm (F) of samples were measured before laser irradiation (F₀) and every 5 min during laser irradiation (F). Production of ¹O₂ was thus expressed as fluorescence enhancement (F/F₀) at 520 nm.

6.2.10 In vitro synergistic cancer therapy

231 cells were seeded in 96-well plate (1×10^4 cells per well) followed by incubation with OPNi or OPNc at different concentrations for 24 h. Then cells were treated with or without

680 nm laser irradiation at 0.2 W/cm² for 30 min. After PDT treatment, cells were incubated for another 24 h, then the culture medium was replaced with fresh DMEM with 20% MTS. After another incubation for 2 h, the absorbance of MTS at 495 nm was measured on a SpectraMax M5 microplate reader. Cell viability was calculated as the absorbance ratio of cells treated with samples or laser irradiation to that of control cells.

6.2.11 In vitro biodegradation studies

To evaluate in vitro biodegradability, OPNis (5 µg/mL) were respectively incubated with H₂O₂ (300 mM), or MPO (50 µg/mL) and H₂O₂ (300 mM) in 0.1 M phosphate buffer containing 0.1 M chloride ion at 37 °C. After incubation for 24 h, absorption spectra of sample solutions were measured. Degradation products were freeze-dried and dissolved in HPLC grade THF for GPC analysis.

To evaluate the biodegradation of OPNis in phagocytes, RAW264.7 cells (purchased from ATCC) were seeded in confocal cell culture dishes (4 × 10⁴ cells/dish). RAW264.7 cells were stimulated with LPS (1 µg/mL) before incubation with OPNis (25 µg/mL). After incubation with OPNis for 24 h, DMEM containing OPNis were replaced with fresh culture medium. At designated time points post-incubation with OPNis, cells were washed with fresh PBS and nuclei were stained with Hoechst33342. NIR fluorescence from cells were then monitored on Zeiss LSM 800 microscope. Fluorescence intensity from cells were quantified by ImageJ.

6.2.12 Tumor mouse model

All animal experiments were performed in compliance with the guidelines established by the Institutional Animal Care and Use Committee (IACUC), Sing Health. To establish

tumor xenograft models, 231 cells suspended in DMEM were subcutaneously implanted (2×10^6 cells/mouse) to the right side on the back of four-week-old female NCr nude mice (purchased from InVivos, Singapore). Tumors were allowed to grow for 2 weeks before in vivo imaging and synergistic cancer therapy.

6.2.13 In vivo tumor imaging

231 xenograft tumor-bearing mice were anesthetized by 2% isoflurane in oxygen and then intravenously injected with OPNis or OPNcs ([PCVB] = 250 $\mu\text{g/mL}$, 0.2 mL) ($n = 3$). At designated time points post injection, whole-body fluorescence images of mice were captured on an IVIS-CT machine with excitation and emission at 680 and 820 nm, respectively. Quantification of fluorescent signals were performed on Living Image 4.0 (PerkinElmer Inc.).

6.2.14 Ex vivo biodistribution

At 72 h post-injection of nanoparticles, 231 xenograft tumor-bearing mice were euthanized. Tumors and major organs including hearts, livers, spleens, lungs, kidneys, and intestines were harvested and imaged on IVIS-CT machine with excitation and emission at 680 and 820 nm, respectively. Fluorescent signals from these organs were analyzed by Living Image 4.0 software (PerkinElmer Inc.).

6.2.15 In vivo synergistic therapy

231 tumor-bearing mice were respectively administered with PBS (200 μL), OPNc ([PCVB] = 250 $\mu\text{g/mL}$, 200 μL), and OPNi ([PCVB] = 250 $\mu\text{g/mL}$, 200 μL) via tail vein injection ($n = 3$). At 48 h post-injection, tumors on mice from 3 groups were irradiated with 680 nm laser at 0.2 W/cm^2 for 30 min. During laser irradiation, tumor temperature was

monitored by an IR thermal camera. Afterwards, tumor volumes and body weights of mice were measured by caliper every other day for 18 days. The tumor volume was calculated as follows:

$$V = (\text{tumor length}) \times (\text{tumor width})^2/2.$$

Relative tumor volume was expressed as V/V_0 (V_0 indicated the initial tumor volume).

6.2.16 Histological analysis

After 18 days of in vivo cancer therapy, 231 xenograft tumor-bearing mice were sacrificed. Tumors, livers, and spleens from mice were harvested and fixed in 4% paraformaldehyde, followed by paraffin embedding and sectioning into thin slices (10 μm). H & E staining was then performed according to standard protocol. After staining, tissue sections were imaged by a Nikon ECLIPSE 80i microscope (Nikon Corporation).

6.2.17 Immunofluorescence staining

After 2 days of in vivo cancer therapy, mice from each group were sacrificed, and tumors were harvested for immunofluorescence staining. After fixation in 4% paraformaldehyde, tumors were dehydrated in 30% sucrose, followed by embedding in optimal cutting temperature (O.C.T.) medium and sectioning into thin slices (10 μm) on a cryostat (Leica, CM1950). Then, the tumor sections were thawed to room temperature and washed with PBS containing 0.1% Triton X-100. Afterwards, tumor sections were incubated with 3% bovine serum albumin (BSA) solution at room temperature for 1 h and washed with fresh PBS. For staining of caspase-3, the obtained tumor sections were incubated with anti-caspase-3 antibody at 4 °C overnight, washed with PBS, and then incubated with Alexa Fluor 488 conjugated donkey anti-rabbit IgG at room temperature for 1 h. Afterterwards,

tumor sections were washed with PBS, stained with DAPI (for 10 min at room temperature), mounted by Fluoromount Aquasous Mounting Medium, and then imaged under Zeiss LSM 800 microscope. Immunofluorescence staining of VCAM-1 and HGF in tumor sections was conducted following the abovementioned protocol with their respective first antibodies.

6.2.18 Statistical analysis

Experiments were conducted in triplicate independent measurements. And results are expressed as the mean $\pm$ standard deviations. Statistical analysis is performed on GraphPad Prism 7 (GraphPad Software, Inc., CA, USA).

6.3 Results and discussions

6.3.1 Synthesis and in vitro characterization of OPNi

Figure 6.2. In vitro characterization of OPNi. (a) Synthetic routes of PCVB and CAi-PEG-b-PPG-b-PEG-CAi. (i) $\text{Pd}_2(\text{dba})_3$, tri(o-tolyl)phosphine, toluene, 24 h, 100 °C; (ii) HCl, ethanol, 16 h, 95 °C; (iii) 4-(chlorosulfonyl)benzoic acid, triethylamine, dimethylformamide (DMF), r.t., 12 h; (iv) propargyl bromide, K_2CO_3 , DMF, r.t., 5 h; (v) sodium hydride, p-toluenesulfonyl chloride, r.t., DMF, 24 h; (vi) sodium azide, r.t., DMF, 24 h; (vii) alkyne-CAi, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sodium ascorbate, r.t., DMF, 24 h. (b) DLS profiles of OPNi and OPNi after binding to CA-IX. Inset: TEM image of OPNi. (c) Normalized

absorption (blue) and fluorescence emission (red) spectra of OPNi in $1 \times$ PBS buffer. (d) Agarose gel electrophoresis of OPNi before (i) and after (ii) incubation with CA-IX. Error bars indicated standard deviations of triplicate independent experiments. Reprinted with permission from ref 333. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

The NIR-absorbing polymer photosensitizer (PCVB) was synthesized via palladium catalysed Stille polycondensation of 2,6-dibromo-4,4-diethylhexyl-4H-cyclopenta[2,1-b;3,4-b']dithiophene, 4,7-dibromo-2,1,3-benzothiadiazole, and trans-1,2-bis(tributylstannyl)ethene (Figure 6.2a). The vinylene linker of PVB was biodegradable because of its susceptibility to oxidation-induced cleavage.²⁹ To ensure sufficient recognition and inhibition, CAis were conjugated to the hydrophilic terminals of the amphiphilic polymer. Firstly, CAi was hydrolyzed and linked to 4-(chlorosulfonyl)benzoic acid, followed by alkylation by propargyl bromide to obtain alkyne-CAi. On the other hand, hydroxyl groups on both terminals of the amphiphilic polymer were transformed to azide groups (compound 6), enabling click reaction with alkyne-CAi to afford CAi-PEG-*b*-PPG-*b*-PEG-CAi. The chemical structures of the intermediates and final products were confirmed by proton nuclear magnetic resonance (¹H NMR).

Nanoprecipitation of PCVB and CAi-*b*-PEG-*b*-PPG-*b*-PEG-CAi led to OPNi, whose aqueous solution was translucent in navy color. Dynamic light scattering (DLS) indicated the average hydrodynamic diameter of OPNi as 47 nm and transmission electron microscope (TEM) revealed the uniform spherical morphology of OPNi (Figure 6.2b). Additionally, no significant change in diameter could be observed after storage of OPNi solutions for 8 weeks (Figure S4, Supporting Information), demonstrating its excellent

colloidal stability. In phosphate buffered saline (PBS), OPNi had peak absorption at 630 nm and peak emission at 820 nm (Figure 6.2c), showing the capability of NIR excitation and a large Stokes shift (~190 nm) for fluorescence imaging. The binding between OPNi and CA-IX was examined by the increased hydrodynamic diameter (82 nm) after incubation of OPNi with CA-IX (Figure 6.2b). Besides, the surface potential of OPNi was neutralized from -19.1 to -10.13 mV. Similarly, agarose gel electrophoresis revealed that OPNi showed remarkably retarded migration after treatment with CA-IX (Figure 6.2d).

6.3.2 In vitro study of OPNi and OPNc on cells

The CA-IX-targeting ability of OPNi was studied against MDA-MB-231 cells, an aggressive human breast cancer cell line with a high expression level of CA-IX.³³² After incubation of 231 cells with OPNi for 1 h, intense NIR fluorescence of OPNi was clearly observed from both membrane and cytoplasm of 231 cells (Figure 6.3a), which was not possible for the non-targeting control, OPNc (PCVB encapsulated by an amphiphilic polymer without CAi conjugation). Furthermore, flowcytometry analysis indicated that CA-IX-negative cell lines (HeLa, MCF-7, and NIH/3T3) had 5.8-12.4 times lower fluorescence intensity than CA-IX-positive MDA-MB-231 cells. The high cellular uptake of OPNi by MDA-MB-231 was attributed to the receptor-mediated endocytosis driven by the efficient binding between OPNi and CA-IX on the membrane of MDA-MB-231.

NIR photodynamic property of OPNi was measured by a $^1\text{O}_2$ indicator Singlet Oxygen Sensor Green (SOSG). To minimize laser-induced cellular damage, a low power density (0.2 W/cm^2) within the maximum permissible exposure limit of 680 nm laser was used. After continuous irradiation for 30 min, the fluorescence enhancement of OPNi was 10-fold higher than that of the control PBS solution, validating the production of $^1\text{O}_2$ mediated

by OPNi. In vitro synergistic therapeutic effect of OPNi was substantially studied on 231 cells, and cell viabilities were quantified by [3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay. Without laser irradiation, OPNi induced higher cytotoxicity than OPNc due to the catalytic inhibition of CA-IX by CAIs. With laser irradiation, OPNi had the highest cytotoxicity among all groups. In contrast, single treatment of either nanoinhibitor or PDT (indicated by OPNc treatment with laser irradiation) failed to achieve so. These data thus validated the superior therapeutic efficacy of OPNi over monotherapy owing to the cooperative effect of PDT and CA-IX inhibition.

Figure 6.3. In vitro study of OPNi and OPNc on cells. (a) Confocal microscopic images of MDA-MB-231 cells incubated with OPNi, OPNc ([PCVB] = 50 $\mu\text{g/mL}$), or PBS buffer at 37 $^{\circ}\text{C}$ for 1 h. Nuclei were indicated in blue. (b) Flow cytometry analysis of different cell lines after incubation with OPNi ([PCVB] = 50 $\mu\text{g/mL}$) at 37 $^{\circ}\text{C}$ for 1 h. (i) 231 cells with OPNi; (ii) 231 cells without OPNi; (iii) MCF-7 cells with OPNi; (iv) MCF-7 cells

without OPNi; (v) HeLa cells with OPNi; (vi) HeLa cells without OPNi; (vii) NIH/3T3 cells with OPNi; (viii) NIH/3T3 cells without OPNi. (c) Fluorescence enhancement (F/F_0) of SOSG at 520 nm in OPNi (25 $\mu\text{g/mL}$) or PBS solutions under 680 nm laser irradiation (0.2 W/cm^2). (d) Relative cell viabilities of 231 cells after incubation with different concentrations of OPNc or OPNi with or without 680 nm laser irradiation (0.2 W/cm^2 , 30 min). (e) Absorption spectra of OPNi (5 $\mu\text{g/mL}$) after respective incubation with H_2O_2 (300 mM), or H_2O_2 (300 mM) and MPO (50 $\mu\text{g/mL}$) for 24 h. Inset: confocal microscopic images of RAW264.7 macrophages at 0 and 24 h after pre-activation by LPS and pre-incubation with OPNi (25 $\mu\text{g/mL}$). Error bars indicated standard deviations of triplicate independent experiments. Reprinted with permission from ref 333. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Biodegradation of OPNi was evaluated in both phagocyte-mimicking myeloperoxidase (MPO) solution and macrophages, because nanoparticles administered in vivo are often sequestered by mononuclear phagocyte system (MPS).²⁵² MPO is abundant in phagocytes, which catalyses the conversion of hydrogen peroxide to hypochlorous acid to exert digestion of foreign substances. After incubation of OPNi with MPO and H_2O_2 , the absorbance of OPNi dramatically declined concomitant with a bathochromic shift (Figure 6.3e). This was caused by the cleavage of vinylene bonds in PCVB, which was further confirmed by gel permeation chromatography showing the significantly decreased molecular weight after MPO treatment. The biodegradability of OPNi was further examined on macrophages RAW264.7 activated by lipopolysaccharide (LPS). At 24 h after pre-incubation of OPNi with stimulated macrophages, NIR fluorescence from intact OPNi

declined to undetectable level in cytoplasm (Figure 6.3e), validating its efficient biodegradation in phagocytes.

6.3.3 In vivo NIR fluorescence imaging guided synergistic cancer therapy

The in vivo targeting ability of OPNi was tested in MDA-MB-231 xenograft mouse model. NIR fluorescence imaging was longitudinally conducted after intravenous injection of OPNi or the non-targeting OPNc into living mice. Fluorescence intensity in tumor regions from OPNi or OPNc injected group continuously increased until reaching a plateau at 48 h post-injection of nanoparticles (Figure 6.4a). At this time point, the fluorescence signal in tumor region from OPNi-treated group was 1.3-fold higher than that for OPNc-treated mice. This was consistent with in vitro results and attributed to the specific binding between OPNi and CA-IX on the cell membrane of MDA-MB-231. At 72 h post-injection of nanoparticles, mice were euthanized, and organs were harvested for biodistribution studies. Liver had the highest uptake of OPNi or OPNc, followed by tumor and the other organs. Notably, OPNi-treated mice showed 1.6-fold higher tumor-to-liver uptake ratio (TLR) than OPNc, which coincided with imaging outcome and further validated the superior tumor accumulation of OPNi due to the targeting ability of CA-IX.

Figure 6.4. In vivo NIR fluorescence imaging guided synergistic cancer therapy. (n = 3)

(a) Fluorescence images of 231 xenograft tumor bearing living mice after intravenous administration of OPNi and OPNc ([PCVB] = 250 $\mu\text{g/mL}$, 200 μL), respectively. Fluorescence images were acquired with excitation and emission at 680 and 820 nm, respectively. Tumor regions were indicated with white circles. (b) Quantification of fluorescence signals in tumor regions in (a). (c) Tumor growth curves of mice after

intravenous administration of OPNi, OPNc, or PBS ([PCVB] = 250 $\mu\text{g/mL}$, 200 μL) with or without 680 nm laser irradiation (0.2 W/cm^2 , 30 min) at 48 h post-injection. (d) H&E staining of tumors of mice after different treatments. (e) Immunofluorescent staining of caspase-3 (green fluorescence) of tumors from mice after different treatments. Blue fluorescence indicated staining of nuclei by Hoechst. (f) Immunofluorescent staining of VCAM-1 and HGF (green fluorescence) in tumors from mice administered with PBS, OPNc, or OPNi after laser irradiation. *Statistically significant difference in relative tumor volume between OPNc and OPNi injected mice after laser irradiation ($p < 0.001$, $n = 3$). Error bars indicated standard deviations of triplicate independent experiments. Reprinted with permission from ref 333. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

The potential of OPNi for synergistic cancer therapy was evaluated. OPNi, OPNc, or saline was intravenously administered into 231 xenograft tumor bearing mice, and NIR laser irradiation (0.2 W/cm^2 , 30 min) was applied to mice at 48 h post administration according to the in vivo imaging data. During the irradiation process, the tumor temperature was monitored and controlled below the threshold (43 $^{\circ}\text{C}$) to avoid hyperthermia-induced ablation. The tumor volumes of mice were monitored to quantitatively evaluate the therapeutic effect after treatment. Without laser irradiation, OPNi-treated mice showed a slower growth rate than OPNc- or PBS-treated mice, implying the partial inhibition of tumor growth attributed to the presence of CAIs in OPNi. With laser irradiation, OPNc-treated mice showed reduced tumor size for the first 10 days, followed by a gradual increase as an indication of tumor recurrence. In contrast, tumor growth for OPNi-treated mice was totally inhibited and no recurrence was observed for 18 days. Such superior therapeutic

performance was further confirmed by hematoxylin and eosin (H&E) staining. Tumor after OPNi-mediated PDT showed more obvious cellular necrosis and apoptosis than those after monotherapy. Furthermore, immunofluorescent staining of caspase-3 indicated that massive apoptosis was only detected in tumor tissues after OPNi-mediated PDT. These data confirmed the superior therapeutic efficacy of OPNi-mediated cancer therapy over monotherapy.

To examine the potential ability of OPNi to inhibit cancer metastasis, expression of metastasis-related proteins vascular cell adhesion molecule-1 (VCAM-1) and hepatocyte growth factor (HGF) in tumor tissues after treatments was evaluated by immunofluorescent staining. Negligible green fluorescence from staining of either VCAM-1 or HGF was observed in tumors after OPNi-mediated PDT (Figure 6.4f). In contrast, high expression levels of both proteins were found in the other groups. These data further confirmed the advantage of OPNi for synergistic cancer therapy.

6.4 Conclusions

In conclusion, we synthesized organic photodynamic nanoinhibitors (OPNi) that can specifically interfere with CA-IX for synergistic cancer therapy to conquer hypoxia pitfall of conventional PDT. By virtue of the binding between CAis and CA-IX, OPNi selectively targeted to CA-IX positive cancer cells, devoted to enhanced accumulation of OPNi in tumor with a 1.6-fold increase of TLR than the counterpart nanoparticle (OPNc); more importantly, the catalytic function of CA-IX was inhibited to cause pH dysregulation. Thereby, the synergistic PDT of OPNi led to the superior therapeutic effect over monotherapy, which was indicated by the inhibited tumor growth and downregulated metastasis-related proteins. Furthermore, OPNi could be gradually metabolized by

phagocytes in MPS, highlighting its translation potential for cancer therapy. To the best of our knowledge, this study not only presents the first biodegradable photodynamic nanoinhibitor, but also sheds light on molecular design of photosensitizers to overcome the drawbacks of PDT in cancer therapy.

Chapter 7 Transformable hybrid semiconducting polymer nanozyme for second near-infrared photothermal ferrotherapy

7.1 Introduction

Ferrotherapy that utilizes iron ions to catalytically disproportionate H_2O_2 to cytotoxic hydroxyl radical ($\cdot\text{OH}$) holds promise for cancer treatment¹⁸. Because ferrotherapy mechanistically bypasses the drug resistance issue of classical chemodrugs, increasing endeavors are devoted to development of ferro-therapeutic agents^{168, 334}. However, due to unfavorable catalytic conditions in tumor microenvironment, current ferro-therapeutic agents suffer from poor therapeutic efficacy and thus are often combined with other therapeutic modalities¹⁶⁹. For instance, iron-based nanomaterials have been delivered with chemotherapeutics (e.g. cisplatin)³³⁵, sonosensitizers (e.g. porphyrin)³³⁶, genetic materials (e.g. p53 plasmid)³³⁷, or immune checkpoint inhibitors (e.g. Nivolumab)³³⁸ for combinational chemotherapy, sonodynamic therapy, gene therapy, or immunotherapy, respectively. Despite their overall improved therapeutic outcomes, these combinational strategies lack synergy to overcome the intrinsic drawback of ferrotherapy to promote suboptimal catalytic efficiency in tumor milieu. In this regard, glucose oxidase has been employed in ferrotherapy to enhance Fenton reaction by elevating H_2O_2 supply³³⁹, whereas the non-specific H_2O_2 production in other tissues could cause side effects such as systemic inflammation.

Photothermal therapy (PTT) that utilizes chromophores to convert incident light into hyperthermia provides a non-invasive way to eradicate tumors³²⁰. For instance, PTT mediated by gold nanoshell has been utilized for high-precision ablation of prostate cancer

in clinical trial without causing deleterious effects to organ function³⁴⁰. In addition, photoirradiation was reported to enhance delivery of doxorubicin to centimeter-scale depth in large solid tumor³⁴¹. Because light in the second near-infrared (NIR) (NIR-II, 1000-1300 nm) window has higher maximum permissive energy to skin (1 W/cm² for 1064 nm while 0.33 W/cm² for 808 nm) and reduced tissue attenuation than that in the first NIR window (NIR-I, 650-950 nm)^{12, 176}, NIR-II absorbing PTT agents have been actively under development such as plasmonic gold blackbodies²⁵⁷, copper sulfide nanostructures²⁷⁷, platinum-derived nanoparticles²⁷⁹, niobium carbide²⁷³ etc. According to Arrhenius equation, exogenous heat can supply as a driving force to accelerate chemical reaction³⁴². Specifically, kinetics studies indicate that Fenton reaction rate is enhanced by up to 4-fold upon increasing the temperature from 20 to 50 °C³⁴³. Thereby, PTT is promising to promote intratumoral Fenton reaction efficiency during ferrotherapy³⁴⁴. However, nanoagents that synergize NIR-II photothermal effect to augment ferro-therapeutic effect have been rarely reported³⁴⁵.

The major challenge for NIR-II photothermal ferrotherapy lies in the development of delivery systems that can act as both the iron chelator and NIR-II chromophore. Semiconducting polymer nanoparticles (SPNs) composed of highly π -conjugated backbones have emerged as a family of optical materials^{265, 346-347}. By virtue of the tunable photophysical property, chemical flexibility and good biocompatibility, SPNs have been extensively exploited to convert light for therapeutic and biological interventions^{28, 218}, or developed into activatable probes for ultrasensitive molecular imaging^{173, 243}. In particular, band gaps of SPNs can be facilely modulated to afford excellent photothermal performance even in the NIR-II window²²⁵, allowing for non-invasive deep-tissue photoacoustic (PA)

imaging of brain vasculature^{208, 269}, in vivo PA tracking of human mesenchymal stem cells³⁴⁸, phototherapeutics of deep tumor²⁷⁴, etc. Moreover, due to the abundant existence of sulfur and nitrogen atoms in the semiconducting backbone that have binding affinity towards metal ions³⁴⁹⁻³⁵⁰, certain structure units of SPs are known to be able to chelate metal ions, which have been utilized for metal ion sensing³⁵⁰⁻³⁵². These previous studies clearly testify that rational design of SPNs is a promising approach towards NIR-II photothermal ferrotherapy.

Herein, we report the first hybrid semiconducting nanozyme (HSN) with photoirradiation enhanced catalytic activity for NIR-II PA-imaging guided synergistic photothermal ferrotherapy. An amphiphilic semiconducting polymer, poly[(thiadiazoloquinoxaline-*alt*-benzodithiophene)-*ran*-(cyclopentadithiophene-*alt*-benzodithiophene)] (pTBCB-PEG), is synthesized to serve as both the NIR-II photothermal transducer and iron chelator. The chelation ability of pTBCB-PEG is originated from the backbone sulfur and nitrogen atoms that have high binding affinity towards ferrous ions (Figure 7.1a). Upon NIR-II photoirradiation, pTBCB mediates photothermal transduction, which not only triggers PTT but also potentiates Fenton reaction to enhance both apoptosis and ferroptosis. Furthermore, the elevated $\cdot\text{OH}$ production accelerates decomposition of HSN and thus transforms it to tiny fragments (~ 1.7 nm), favoring deep permeation into solid tumor for elevated antitumor effect in a photothermal-depth-independent manner (Figure 7.1b). As such, the seamless synergism of NIR-II photothermal ferrotherapy leads to tumor elimination at a remarkably deep tissue depth in a non-invasive manner, contributing to complete cancer remission and metastasis inhibition.

Figure 7.1 Schematic illustration of HSN for NIR-II photothermal ferrotherapy. (a) Chemical structure of pTBCB-PEG, preparation of HSN and proposed mechanism of iron chelation. (b) Comparison of HSN-mediated NIR-II photothermal ferrotherapy with monotherapies (ferrotherapy and PTT).³⁵³ Reprinted with permission from ref 353 Copyright 2020 Nature Publishing Group.

7.2 Materials and methods

7.2.1 Chemicals.

All the chemicals were purchased from Sigma-Aldrich or Tokyo Chemical Industry unless otherwise stated. 2,6-Dibromo-4H-cyclopenta[2,1-b:3,4-b']dithiophene and 2,6-bis(trimethyltin)-4,8-didodecylbenzo[1,2-b;4,5-b']dithiophene were purchased from Luminescence Technology Corp (Lumtec). 4,9-Dibromo-6,7(4-hexylphenyl)-[1,2,5]thiadiazolo[3,4-g]quinoxaline was purchased from Brilliant Matters. Secondary antibodies for immunoblotting which include IRDye 800 CW goat anti-mouse (1:10000; 925-32210) and IRDye 680 CW goat anti-rabbit (1:10000; 925-68071) were purchased from LI-COR Biosciences. Anti-HGF (1:500; ab83760), anti-MTA2 (1:100; ab8106), anti-VCAM-1 (1:250; ab134047) antibodies were purchased from Abcam. Secondary antibody Alexa Fluor 488 conjugated goat anti-rabbit IgG H&L (1:500; ab150077) for immunofluorescent staining was purchased from Abcam.

7.2.2 Material characterization.

Absorption spectra were measured on a Lambda 950 spectrometer. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a Bruker Avance II 300 MHz NMR spectrometer. Fluorescence spectra were measured on a Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). Dynamic light scattering and zeta potential were recorded on a Malvern Nano-ZS Particle Sizer. Transmission electron microscope (TEM) images were captured on a JEOL JEM 1400 transmission microscope. Scanning transition electron microscopy-energy dispersive X-ray (STEM-EDX) element mapping was performed on JEOL 2100F with ultra-high resolution (UHR) configuration (accelerating

voltage: 200 kV). Solution temperature for photostability study was measured by a FLIR T420 thermal camera. Gel permeation chromatography was performed on a Shimazu LC-VP system (standard: styrene; eluent: tetrahydrofuran). Confocal images were captured on Zeiss LSM 800. Flowcytometry was performed on Fortessa X20 (BD Biosciences). Western blot images were captured on Licor Odyssey CLx fluorescence imaging system. 1064 nm laser was purchased from Shanghai Connet Fiber Optics Co., Ltd. (Shanghai, China).

7.2.3 Synthesis of monomer 1.

2,6-Dibromo-4H-cyclopenta[2,1-b:3,4-b']dithiophene (1g, 3 mmol), 50 % NaOH solution (freshly prepared, 64 mL) and tetrabutylammonium bromide (TBAB, 200 mg) were weighed and placed in a 250 mL round-bottom flask. The mixture was then degassed via freeze-thaw cycles, followed by reflux at 75 °C for 20 min under the protection of N₂ atmosphere. 1,6-Dibromohexane (3 mL, 7.5 mmol) was then dropwise added to the flask via a syringe and the reaction was allowed to carry out for 80 min at 75 °C. After reaction, the mixture was cooled down to room temperature, extracted with ethyl acetate, washed 3 times with water, and dried with anhydrous sodium sulfate. Thereafter, the crude monomer 1 was concentrated via rotary evaporation followed by purification via silica gel column chromatography using hexane as the eluent. ¹H NMR (300 MHz, CDCl₃) δ: 6.93 (s, 2H), 3.34 (t, 4H), 1.74 (m, 8H), 1.34-1.26 (m, 4H), 1.19-1.09 (m, 4H), 0.92-0.84 (m, 4H).

7.2.4 Synthesis of pTBCB-PEG.

To synthesize pTBCB-Br, 2,6-bis(trimethyltin)-4,8-didodecylbenzo[1,2-b:4,5-b']dithiophene (32 mg, 0.0375 mmol), 4,9-dibromo-6,7(4-hexylphenyl)-

[1,2,5]thiadiazolo[3,4-g]quinoxaline (20 mg, 0.03 mmol), monomer 1 (4.74 mg, 0.0075 mmol), Pd₂(dba)₃ (1.2 mg, 0.0012 mmol), and tri(o-tolyl)phosphine (3.6 mg, 0.0108 mmol) were weighed and placed in a 50 mL Schlenk flask. After addition of chlorobenzene (4 mL), the mixture was degassed by 3 freeze-pump-thaw cycles. Thereafter, the reaction was carried out at 100 °C under the protection of nitrogen atmosphere for 4 h. After reaction, the mixture was cooled down to room temperature, followed by dropwise addition to cold methanol. After vigorous stirring, the dark precipitates in methanol was collected via centrifugation, washed 3 times with cold methanol to remove impurities, and then dried under vacuum to afford dark solids of pTBCB-Br.

To synthesize pTBCB-N₃, pTBCB-Br (23 mg) and sodium azide (20 mg) was dissolved in a mixture (12 mL) of tetrahydrofuran (THF) and N,N-dimethylformamide (DMF) (THF: DMF 2:1). After stirring at room temperature for 24 h, the mixture was concentrated by rotary evaporation, re-dissolved in dichloromethane, washed 3 times with water, and dried over anhydrous sodium sulfate. The obtained pTBCB-N₃ (23 mg) and methoxypolyethylene glycol dibenzocyclooctyne (DBCO-mPEG2000) (100 mg) were dissolved in THF (6 mL) and stirred at room temperature for 24 h. After reaction, the solvent was removed by rotary evaporation and the obtained solids were re-dissolved in deionized water. Thereafter, the excess DBCO-mPEG2000 was removed by ultracentrifugation (MWCO 50 kDa) at 5780 xg at 4 °C. After purification, the concentrated aqueous solution was lyophilized to obtain pTBCB-PEG powder. ¹H NMR (300 MHz, CDCl₃) δ: 8.10-7.30 (m, 8.14H), 7.26-6.78 (m, 4.12H), 4.04-3.81 (m, 1.94H), 3.78-3.53 (m, 72.07H), 3.51-3.25 (m, 3.82H), 2.08-1.86 (m, 9.64H), 1.73-1.63 (m, 4.09H), 1.57-1.09 (m, 47.58H), 0.94-0.78 (m, 11.63H).

7.2.5 Preparation of HSN₀ and HSN.

To prepare HSN₀, lyophilized pTBCB-PEG powder (1 mg) was dissolved in THF (1 mL), followed by rapid injection into deionized water (8 mL) under vigorous sonication. Excess THF was then removed under a gentle N₂ flow, and the remaining aqueous solution was filtered through a 220 nm polyvinylidene fluoride (PVDF) syringe driven filter (Millipore), followed by concentration via ultracentrifugation at 5780 xg for 30 min at 4 °C to afford stock solution.

As for the preparation of HSN, freshly prepared ferrous sulfate solution (0.1 M, 0.5 mL, pH 4.0) was dropwise added to HSN₀ solution (300 µg mL⁻¹, 2 mL) under vigorous stirring. After continuous stirring overnight, the excess ferrous ion was removed by 3 cycles of ultracentrifugation at 3260 xg for 25 min at 4 °C followed by water rinse. The purified HSN solution was then diluted and filtered via a 220 nm PVDF syringe driven filter and ultra-centrifuged to afford HSN stock solution.

7.2.6 In vitro photothermal study.

To measure photostability, HSN₀ and HSN solution (12 µg mL⁻¹, 200 µL) was irradiated with 1064 nm laser (1 W cm⁻²) for 6 mins followed by natural cooling for another 6 mins after removal of photoirradiation. 5 cycles of heating-cooling process were carried out and solution temperature was monitored by IR thermal camera.

7.2.7 Calculation of photothermal conversion efficiency.

Photothermal conversion efficiency was measured and calculated according to literature.²⁵⁷ HSN or HSN₀ solution (2 mL, optical density at 1064 nm = 1) was placed in a 3.5-mL quartz cuvette (5.66 g, Sangon Biotech, Shanghai, China), followed by 1064 nm laser

irradiation (1 W cm^{-2}) for 30 mins to reach the thermal equilibrium and subsequent natural cooling process in the absence of laser irradiation. During the measurement, solution temperature was monitored by a dual input J/K type thermometer (TM300, Extech Instruments, Waltham, MA).

7.2.8 Cell culture and ROS detection.

4T1 murine mammary carcinoma cell line, MCF-7 human breast adenocarcinoma cell line, HepG2 human hepatocellular carcinoma cell line, NIH/3T3 murine fibroblast cell line, NDF normal human dermal fibroblast cell line, MDA-MB-231 human breast adenocarcinoma cell line, PC12 rat pheochromocytoma cell line, HeLa human cervical adenocarcinoma cell line, and SKOV3 human ovarian adenocarcinoma cell line (purchased from American Type Culture Collection, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) and placed in a humid CO_2 incubator providing an atmosphere containing 5% CO_2 at 37°C .

To detect the intracellular ROS aroused by HSN, cells were seeded in confocal cell culture dishes at a density of 1×10^4 cells per dish. After culture in incubator for 12 h, HSN (final concentration: $[\text{pTBCB}] = 50 \mu\text{g mL}^{-1}$) or PBS were added to the cells and incubated with cells for 24 h. Thereafter, cells were gently washed 3 times with fresh PBS to remove excess nanoparticles, and then fixed with 4% paraformaldehyde. After fixation, cells were successively stained with DAPI and DCFH-DA. Then cells were imaged under a LSM800 confocal microscope.

7.2.9 Western blotting.

Cells were lysed via vigorous sonication on ice bath and protein concentration was determined by Bradford protein assay. Thereafter, immunoblotting of cell lysates was performed using antibodies to ACSL4 (1:1000; ab205199; Abcam), FPN-1 (1:1000; NBP1-21502SS; Singlab Technologies Pte Ltd), GPX4 (1:100; sc-166120; Axil Scientific Pte Ltd), cleaved cas-3 (1:500; 9661L; Cell Signaling Technology), ferritin (1:500; MA532244; Life Technologies Holdings Pte Ltd), and GAPDH (1:500; sc-32233; Axil Scientific Pte Ltd) according to standard protocols. Source data of scans of blots were provided in Source Data File.

7.2.10 In vitro examination of apoptosis and ferroptosis.

4T1 cells were seeded in confocal cell culture dishes at a density of 1×10^4 cells per dish. After culture in incubator for 12 h, HSN (final concentration: [pTBCB] = $50 \mu\text{g mL}^{-1}$), HSN with DEVD ($100 \mu\text{M}$), HSN with DFO ($100 \mu\text{M}$), and PBS were respectively added to the cells. After incubation for 24 h, cells were gently washed 3 times with fresh PBS and then fixed with 4% paraformaldehyde. After fixation, cells were incubated with PBS containing 0.1% Triton X-100 (PBST) and then washed 3 times with ice-cold PBS. Afterwards, cells were incubated with 3% bovine serum albumin (BSA) in PBST for 30 min. Then cells were washed 3 times in PBS and incubated with primary cleaved caspase-3 antibody (Cell Signaling Technology) (1:1000 in PBST) in a humidified chamber at 4°C overnight. After incubation, cells were washed 3 times in PBS and incubated with secondary antibody Alexa Fluor 488 conjugated donkey anti-rabbit IgG (Thermo Fisher Scientific) (1:1000 in PBST) at room temperature for 1 h. Then cells were washed with PBS and sequentially stained with BODIPY 665/676 (Thermo Fisher Scientific) ($10 \mu\text{M}$,

30 min) and DAPI. After mounted by Fluoromount aqueous mounting medium, cells were imaged under a LSM800 confocal microscope.

7.2.11 In vitro cancer therapy.

4T1 cells were seeded in 96-well plates (1×10^4 cells per well) and cultured in incubator for 24 h. Then cells were treated with HSN0 or HSN at different concentrations (0, 5, 10, 20, 50 $\mu\text{g mL}^{-1}$). After incubation for 24 h, cells were treated with or without 1064 nm laser irradiation (1 W cm^{-2} , 6 min). After incubation for another 24 h, cells were gently washed 3 times with fresh PBS. Thereafter, 120 μL freshly prepared working solution (containing 100 μL DMEM and 20 μL MTS reagent) was added to each well. After incubation for 3 h, absorbance at 490 nm was measured on a SpectraMax M5 microplate reader. And cell viability was determined as the ratio of the absorbance of cells with nanoparticle and/or laser treatment to that of blank cells without any treatments.

7.2.12 DTNB Assay and DCFH-DA assay.

DTNB assay was utilized to measure GSH level of 4T1 cells in in vitro cancer therapy. After various treatments of 4T1 cells, cells were gently washed 3 time with fresh ice-cold PBS, followed by sonication in ice bath to afford cell lysate. Thereafter, Ellman's reagent (DTNB) was added to cell lysate (final concentration: 200 μM) and incubated at room temperature for 30 min. Later, the absorbance at 412 nm was measured on a SpectraMax M5 microplate reader and the relative level of GSH was determined by the ratio of absorbance for experimental group to that for control group (blank cells without any treatment).

DCFH-DA assay was utilized to measure intracellular ROS level of 4T1 cells in in vitro cancer therapy. After various treatment of 4T1 cells, cells were gently washed 3 time with fresh PBS and incubated with DCFH-DA reagent. Then, cells were washed 3 times in ice-cold PBS, trypsinized, and resuspended in fresh PBS for flow cytometry test.

7.2.13 Tumor mouse model.

Animal experiments in Singapore were performed in compliance with Guidelines for Care and Use of Laboratory Animals of the Nanyang Technological University-Institutional Animal Care and Use Committee (NTU-IACUC) and approved by the Institutional Animal Care and Use Committee (IACUC) for Animal Experiment, Singapore. Animal experiments in China were performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985) and approved by the Institutional Animal Use and Care Committee of Shan Xi Medical University (Approval No, 2016LL141, Taiyuan, China). 2 million 4T1 cells suspended in 0.2 mL supplemented DMEM were subcutaneously injected into the right flank of the female NCr nude mice (6-week-old). Tumors were allowed to grow for 2 weeks before in vivo cancer therapy.

7.2.14 NIR-II PA tomography system.

PA characterization was conducted on a home-made NIR-II PA tomography system. A 1064 nm Nd:YAG pulse (5 ns, 10 Hz) laser (Continuum, Surelite Ex) was utilized as excitation source. Briefly, the 1064 nm beam was directed to the single-ultrasound transducer (UST) (V323-SU/2.25 MHz, Olympus NDT) scanner, and expanded by an optical diffuser to the inspection area. Water was utilized as the medium and UST was

immersed in water so as to couple the acoustic signal to the transducer. The collected PA signals were then amplified and band-pass filtered (1-10 MHz) by ultrasound receiver unit (Olympus NDT, 5072PR). Later, PA signals were subsequently transmitted to a computer with a data acquisition card (25 Ms/s, GaGe, compuscope 4227) for digitalization and recording. PA images of samples or mice were reconstructed via a delay-and-sum back projection algorithm.

For *in vivo* PA imaging, NCr nude mice bearing 4T1 xenograft tumor were anesthetized and administered with HSN or HSN₀ ([pTBCB] = 250 $\mu\text{g mL}^{-1}$, 200 μL per mouse, $n = 3$) via tail vein injection. PA images were captured at designated time points before and after sample administration.

7.2.15 Ex vivo biodistribution.

At 24 h post-injection of HSN or HSN₀, mice were sacrificed. Tumors, livers, spleens, lungs, skin, hearts, intestines, and kidneys were harvested and embedded in 1% agar gel phantom. PA signals from embedded organs were measured on the home-made PA tomography system.

7.2.16 In vivo anti-cancer therapy.

4T1 tumor-bearing mice were intravenously injected with PBS (200 μL), HSN₀, or HSN ([pTBCB] = 250 $\mu\text{g mL}^{-1}$, 200 μL per mouse). At 4 h post-injection, tumors on mice for PTT and synergistic therapy were irradiated with 1064 nm laser at 1 W cm^{-2} for 6 min. Tumor surface temperature was monitored by an IR thermal camera throughout irradiation process. Afterwards, tumor sizes and body weights of mice were monitored every two days for 14 days. The tumor volume was expressed as follows:

$$V = (\text{tumor length}) \times (\text{tumor width})^2/2.$$

Thus, relative tumor volume was calculated as V/V_0 (V_0 indicated the initial tumor volume).

7.2.17 Histological studies, immunofluorescence and iron staining.

After 2 days of in vivo anti-cancer therapy, mice were euthanized, and tumors were excised for histological examination. Briefly, harvested tumors were fixed in 4% paraformaldehyde, followed by paraffin-embedding according to standard protocol and dissection to 10- μ m tissue sections at different depths (2, 6, 7, 9 mm) in the direction of laser irradiation (photothermal depth). Then tissue sections were stained with H&E according to standard protocol and imaged under a Nikon ECLIPSE 80i microscope. Area of cell death was quantified using ImageJ.

As for immunofluorescent staining, the fixed tumors were dehydrated overnight in 30% sucrose, embedded in optimal cutting temperature (O.C.T.) medium, and sectioned into 10 μ m- slices on a cryostat (Leica, CM1950) at different photothermal depths (2, 6, 7, 9 mm). Thereafter, immunofluorescent staining of cas-3 and ACSL4 as well as LPO staining was performed following the same procedure as the above-described apoptosis/ferroptosis staining. For iron staining, cryostat tissue sections at each photothermal depths (2, 6, 7, 9 mm) were stained according to standard Prussian blue stain protocol and then imaged under LX71 inverted microscope (Olympus).

7.2.18 Ex vivo lung and liver metastasis examination.

After 14 days of in vivo anti-cancer therapy, 4T1 tumor-bearing mice were sacrificed and major organs including hearts, livers, spleens, lungs, and kidneys were harvested.

Metastatic nodules in lungs were counted. Thereafter, organs were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to thin slices (10 μm), and stained with H & E according to standard protocols. Organ sections were then imaged under Nikon ECLIPSE 80i microscope and metastatic area in livers were quantified using ImageJ. Immunofluorescent staining of metastasis-related proteins was performed according to the above-described procedure.

7.2.19 Data analysis.

Results of experiments were presented as mean $\pm$ standard deviation unless otherwise stated. PA signals were analyzed using MATLAB. Statistical differences between two groups were calculated by two-tailed Student's t-test using GraphPad Prism 7 (GraphPad Software, Inc., CA, USA). For all statistical analysis, $**P < 0.05$ was regarded as statistically significant.

7.3 Results and discussions

7.3.1 Synthesis and in vitro characterization.

The NIR-II light-harvesting polymer precursor pTBCB(-Br) was synthesized via Stille polycondensation of 3 monomers: 4,9-dibromo-6,7-bis(4-hexylphenyl)-[1,2,5]thiadiazolo[3,4-g]quinoxaline (TDQ), (4,8-didodecylbenzo[1,2-b:4,5-b']dithiophene-2,6-diyl)bis(trimethylstannane) (BDT), and 2,6-dibromo-4,4-bis(6-bromohexyl)-4H-cyclopenta[2,1-b:3,4-b']dithiophene (CPDT-Br). Among these monomers, TDQ with strong electron-withdrawing ability was the acceptor to narrow the band gap; PT was the donor to finetune the absorption spectrum; whereas CPDT-Br was a modifiable moiety for side chain post-functionalization. pTBCB-Br was converted to

pTBCB-N₃ through substitution of bromide with azide, and then grafted with methoxypolyethylene glycol dibenzocyclooctyne (DBCO-mPEG2000) via copper-free click chemistry to obtain pTBCB-PEG. The ideal amphiphilicity of pTBCB-PEG allowed it to spontaneously self-assemble into nanoparticle termed as HSN₀ in aqueous solution. Meanwhile, due to the presence of ion binding sites of pTBCB-PEG, the nanozyme HSN was facilely prepared by simple addition of ferrous ion into pTBCB-PEG solution during self-assembly.

Both HSN and the iron-free control (HSN₀) had maximum absorbance at 964 nm (Figure 7.2a), showing that iron chelation had negligible effect on light-harvesting property. However, dynamic light scattering (DLS) indicated a larger hydrodynamic diameter for HSN (42 nm) relative to HSN₀ (32 nm) (Figure 7.2b). Transition electron microscope (TEM) further confirmed the larger dimension of HSN and indicated the homogenous sphere-like morphology for both nanoparticles (Figure 7.2b). There are negligible changes in diameter for both nanoparticles after storage for 2 months, implying their excellent colloidal stability. Additionally, zeta potential measurement indicated the neutralization of surface charge from -17 mV for HSN₀ to -7 mV for HSN (Figure 7.2c). Scanning transition electron microscopy-energy dispersive X-ray (STEM-EDX) element mapping illustrated that iron was distributed evenly across HSN (Figure 7.2d). Quantitative analysis further indicated the respective atomic ratio of Fe (9.79%), N (11.49%), and S (7.14%).

Photothermal transduction capability of HSN was evaluated and compared with HSN₀. Upon photoirradiation at 1064 nm, both nanoparticles induced significant aqueous temperature rise (Figure 7.2e). After continuous irradiation for 360s, the maximal solution temperature of HSN (62.3 °C) was slightly higher than that of HSN₀ (60.5 °C). However,

HSN had a higher photothermal conversion efficiency (PCE) (98.9%) than HSN₀ (83.3%), which to the best of our knowledge is the highest among all the NIR-II photothermal agents reported so forth²⁵⁷. The higher PCE for HSN was probably because of the enhanced heat transfer rate assigned to its larger size and increased intermolecular interactions due to iron chelation³⁵⁴⁻³⁵⁷. Correspondingly, HSN emitted higher PA amplitude than HSN₀ at identical concentration (e.g. 1.3-fold at 6.5 $\mu\text{g mL}^{-1}$) upon irradiation of pulsed 1064 nm laser (Figure 7.2f). Furthermore, both HSN₀ and HSN showed nearly unchanged photothermal performance during 5 heating-cooling cycles, indicating their excellent photo-stabilities.

The catalytic activities of HSN to generate $\cdot\text{OH}$ were studied in pH conditions mimicking both physiological condition (pH 7.4) and tumor milieu (pH 6.8 at tumor microenvironment and pH 5.5 at lysosomes), respectively. Based on the well-known $\cdot\text{OH}$ -induced bleaching of methylene blue (MB), the catalytic efficiency of HSN was quantified by measurement of the absorption decrease of MB at 664 nm. In the presence of H₂O₂ (0.5 mM), the decomposition of MB at pH 5.5 mediated by HSN was the fastest, followed by pH 6.8 and 7.4 (Figure 7.2g). Whereas HSN₀ hardly induced MB degradation under the same condition (Figure 7.2h). After 3 min of photoirradiation, MB bleaching by HSN was respectively enhanced from 5 to 32% at pH 7.4, 35 to 53% at pH 6.8, and 55 to 65% at pH 5.5, suggesting that HSN-mediated photothermal heating significantly accelerated Fenton reaction rate. Along with $\cdot\text{OH}$ generation, decreased absorbance of pTBCB-PEG at 960 nm was observed. DLS measurement indicated a decreased diameter of HSN from 42 to 1.7 nm after photoirradiation. Consistently, TEM illustrated the decomposition of HSN to form tiny segments (named as HSNS) (Figure 7.2i). Further, gel permeation

chromatography (GPC) proved much smaller molecular weight of HSN relative to original polymer.

Figure 7.2 In vitro characterization of HSN. (a) Absorption spectra of HSN and HSN₀ in 1 × PBS ([pTBCB] = 3 μg mL⁻¹). (b) DLS profiles of HSN and HSN₀ in 1 × PBS. Inset: TEM images of HSN₀ and HSN. Scale bar: 200 nm. (c) Zeta potential profiles of HSN and HSN₀. (d) STEM-EDX element mapping of HSN. Scale bar: 20 nm. (e) Photothermal heating-cooling cycles of HSN and HSN₀ ([pTBCB] = 12 μg mL⁻¹) under 1064 nm photoirradiation (1 W cm⁻²). (f) Linear fits of PA amplitudes of HSN ($R^2 = 0.96809$) and HSN₀ ($R^2 = 0.97314$) as a function of concentration at 1064 nm, respectively. (g) ·OH generation of HSN under different pH conditions (pH = 7.4, 6.8, 5.5) with or without 1064 nm photoirradiation (1 W cm⁻²). ·OH generation was quantified by the decrease of

absorbance of MB at 664 nm. [pTBCB] = 23 $\mu\text{g/mL}$, $[\text{H}_2\text{O}_2]$ = 0.5 mM, [MB] = 1 mM.

(h) $\cdot\text{OH}$ generation by HSN_0 at pH 6.8 with or without 1064 nm photoirradiation (1 W cm^{-2}). (i) Scheme of NIR-II photoirradiation enhanced self-degradation of HSN to HSNS in the presence of H_2O_2 . Error bars indicated standard deviations of 3 independent measurements. Reprinted with permission from ref 353. Copyright 2020 Nature Publishing Group.

7.3.2 In vitro NIR-II photothermal ferrotherapy and therapeutic mechanism.

Catalytic activity of HSN was characterized against both cancer and normal cell lines. A fluorescent turn-on probe, 2',7',7'-dichlorofluorescein diacetate (DCFH-DA), was used as the ROS indicator. After treatment with HSN, cancer cells showed much stronger green fluorescence than normal cells (Figure 7.3a), revealing the catalytic specificity of HSN in cancerous cells due to their higher basal H_2O_2 level³⁵⁸. Because ferroptosis marked by lipid peroxidation (LPO) is known to play a vital role in ferrotherapy, ferroptosis sensitivity was screened over a panel of cancer cell lines through evaluation of expression levels of hallmark regulators^{359,360}. They included: long-chain-fatty-acid-CoA ligase 4 (ACSL4), ferroportin-1 (FPN-1) and glutathione peroxidase 4 (GPX4). ACSL4, an enzyme which regulates arachidonic acid (AA) esterification to arachidonyl-CoA (AA-CoA) for phospholipid (LH) formation is a pivotal ferroptosis contributor³⁶¹. FPN-1 is identified as the sole iron exporter of which the down-regulation has been indicated to increase intracellular iron retention³⁶². While GPX4 serves as a major ferroptosis suppressor by reducing phospholipid peroxides at the expense of glutathione (GSH)³⁶³. After western blotting analysis, 4T1 cells with upregulated ACSL4 yet downregulated FPN-1 and GPX4

levels were selected as the highly ferroptosis-sensitive cell line for subsequent studies (Figure 7.3B).

In vitro therapeutic capability of HSN was investigated against 4T1 cells. After treating cells with HSN, cellular apoptosis was indicated by immunofluorescent staining (green fluorescence) of cleaved caspase-3 (Cas-3), while ferroptosis was indicated by LPO staining via a red-fluorescent probe BODIPY 665/676. As revealed in Fig. 3C, much stronger green and red fluorescence was observed in HSN treated cells than control group, suggesting that endocytosed HSN triggered both apoptosis and ferroptosis in 4T1 cells. Further, addition of an apoptosis inhibitor (DEVD) ameliorated HSN-triggered apoptosis but had negligible effect on ferroptosis inhibition. However, both apoptosis and ferroptosis were inhibited after addition of a potent iron chelator deferoxamine (DFO), confirming that cell deaths were due to ferrous ions within HSN. Next, cell viabilities after in vitro cancer therapy were examined (Figure 7.3D). In the absence of photoirradiation, HSN-mediated ferrotherapy caused slightly higher toxicity to 4T1 cells than the control treatment by HSN₀ due to the catalytic activity of ferrous ion. With 1064 nm photoirradiation, HSN-mediated photothermal ferrotherapy induced the highest cytotoxicity among all treatments. For instance, at 50 $\mu\text{g mL}^{-1}$, photothermal ferrotherapy induced a minimal cell viability of 8.7%, which was 3.4-and 9.3-fold lower than that for HSN₀-mediated PTT (29.6%) or sole ferrotherapy (80.6%), respectively.

The underlying molecular mechanism of superior therapeutic efficacy of HSN-mediated photothermal ferrotherapy was studied. Intracellular GSH level as the representative of oxidative stress was measured by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) assay after various treatments (Figure 7.3E). A most significant drop of GSH level was observed in

cells after photothermal ferrotherapy, followed by PTT or ferrotherapy. Consistently, flowcytometry analysis indicated the maximal ROS generation in 4T1 cells after photothermal ferrotherapy than sole PTT or ferrotherapy. Further, western blotting analysis indicated the most down-regulated ACSL4 expression after NIR-II photothermal ferrotherapy (Figure 7.3F), suggesting enhanced ferroptosis due to the presence of negative feedback loop possibly mediated by AA³⁶⁴⁻³⁶⁵. Besides, NIR-II photothermal ferrotherapy induced the highest Cas-3 expression, suggesting that cellular apoptosis was further enhanced. Because ferritin is the major intracellular iron storage protein, expression level of ferritin was also examined in cells after various treatments. Akin to ferrotherapy, photothermal ferrotherapy triggered more significant ferritin degradation than PTT, implying potentiated oxidative damage ascribed to the liberation of reactive iron from ferritin to replenish labile iron pool. The molecular mechanism of HSN-mediated photothermal ferrotherapy was summarized in Figure 7.3G.

Figure 7.3 In vitro NIR-II photothermal ferrotherapy. (a) Confocal laser scanning microscopy (CLSM) images of different types of cancer cells and normal cells after incubation with HSN ($[p\text{TBCB}] = 50 \mu\text{g mL}^{-1}$) or PBS for 24 h. ROS was indicated by green fluorescence from DCFH-DA staining. Nuclei were stained with 4',6-diamidino-2'-

phenylindole dihydrochloride (DAPI) and indicated by blue fluorescence. 4T1: murine mammary carcinoma cell line; MCF-7: human breast adenocarcinoma cell line; HepG2: human hepatocellular carcinoma cell line; NIH/3T3: murine fibroblast cell line; NDF: normal human dermal fibroblast cell line. (b) Western immunoblot analysis of expression levels of ferroptosis-related proteins (ACSL4, FPN-1, GPX4) in a panel of cancer cell lines. MDA-MB-231 (231): human breast adenocarcinoma cell line; PC12: rat pheochromocytoma cell line; HeLa: human cervical adenocarcinoma cell line; SKOV3: human ovarian adenocarcinoma cell line. Source data were provided in Source Data File. (c) CLSM images of 4T1 cells after incubation with PBS, HSN, HSN with apoptosis inhibitor DEVD (100 μ M), HSN with ferroptosis inhibitor deferoxamine (DFO) (100 μ M) for 24 h, respectively. [pTBCB] = 50 μ g mL⁻¹. Expression of Cas-3 was indicated by immunofluorescence staining (green fluorescence), and lipid peroxidation was stained with a red fluorescent probe BODIPY 665/676. Cell viabilities (d) and relative GSH levels (e) of 4T1 cells after incubation with HSN₀ or HSN at various concentrations for 24 h with or without 1064 nm photoirradiation (1 W cm⁻², 6 min). [pTBCB] = 50 μ g mL⁻¹. (f) Western immunoblots analysis of expression levels of ferroptosis and apoptosis related proteins in 4T1 cells in (D) and (E). + and - indicated with and without photoirradiation, respectively. Source data were provided in Source Data File. (g) Proposed molecular mechanisms of HSN-mediated NIR-II photothermal ferrotherapy. GSSG: glutathione disulfide; AA: arachidonic acid; AA-CoA: arachidonyl-CoA. LH: phospholipid. Error bars indicated standard deviations of 3 independent measurements. Reprinted with permission from ref 353. Copyright 2020 Nature Publishing Group.

7.3.3 In vivo NIR-II PA imaging guided photothermal ferrotherapy.

To identify the optimal therapeutic window for in vivo therapy, NIR-II PA imaging was conducted on 4T1-tumor bearing mice on a home-made PA system equipped with 1064 nm pulse laser. After systemic administration of HSN or HSN₀, PA signals in tumor regions gradually increased and reached the maxima at 4 h post-injection (Figure 7.4a), suggesting the passive targeting of both nanoparticles in solid tumor probably through enhanced permeability and retention (EPR) effect due to their small hydrodynamic sizes and PEGylated surfaces (Figure 7.2b, c). At this time point, the PA amplitude of tumor for HSN-treated mice was 3.1- and 1.2-fold higher than that of background and that for HSN₀-treated mice (Figure 7.4b), respectively. Such phenomenon should be mainly attributed to the superior PA property of HSN over HSN₀ (Fig. 2f). Besides, ex vivo PA data at 24 h post injection revealed that the residual injected HSN or HSN₀ mainly accumulated in liver, followed by spleen, tumor and other organs (Figure 7.4c).

Therapeutic potential of HSN-mediated NIR-II photothermal ferrotherapy was evaluated on 4T1 tumor-bearing mice and compared with monotherapies. According to PA imaging results, NIR-II photoirradiation was applied to tumor at 4 h post-administration of HSN or HSN₀. Under photoirradiation, tumor temperatures for HSN and HSN₀-treated mice gradually increased to 60.8 and 59.5 °C, respectively (Figure 7.4d). After different treatments, tumor growth was monitored over time (Figure 7.4e). Compared to PBS-treated group, mice treated with ferrotherapy showed minor retardation in tumor growth. Although PTT remarkably impeded tumor growth during the first few days, accelerated re-growth of tumor was observed 4 days after treatment. However, tumors treated with photothermal ferrotherapy were totally eradicated, and no sign of tumor regrowth was observed.

Furthermore, negligible changes in body weights were found for mice during different treatments. And no significant physiological abnormalities could be found in hearts, spleens, and kidneys of living mice after either photothermal ferrotherapy or monotherapies, showing the acceptable biocompatibility of HSN and HSN₀.

To unveil the mechanism of complete tumor elimination by HSN-mediated NIR-II photothermal ferrotherapy, tumors after various treatments were dissected at different depths (2, 6, 7 and 9 mm) in the direction of photoirradiation (termed as photothermal depth) (Figure 7.4f). Hematoxylin and eosin (H & E) staining was performed followed by quantitative analysis (Figure 7.4g, h). Overall, sole ferrotherapy displayed a relatively depth-independent therapeutic pattern (cell death decreased by 12.5% from 2 to 9 mm), whereas the therapeutic efficacy was inadequate (maximum 56.6% cell death). On the contrary, PTT demonstrated a depth-dependent therapeutic manner, showing largely compromised cell death with the increase of photothermal depth (cell death decreased by 63.3% from 2 to 9 mm) (Figure 7.4h). And ablation depth in PTT was restricted to 6 mm. However, NIR-II photothermal ferrotherapy presented a relatively photothermal-depth-independent therapeutic pattern akin to ferrotherapy (cell death only decreased by 18.8% from 2 to 9 mm), achieving efficient ablation at 9 mm that remarkably broke the record of reported therapeutic limitation in NIR-II window (~ 4 mm)²⁷³. Further, such ablation depth was almost comparable to the ablation radius (at centimeter scale) of laser-induced thermal therapy, which relies on invasive surgery to directly deliver high-power laser into tumor interstitium for efficient ablation³⁶⁶.

At molecular levels, expression of apoptosis/ferroptosis biomarkers (Cas-3, LPO and ACSL4) were evaluated at each photothermal depth after different treatments (Figure 7.4g,

4i-k). Consistent with H&E results, NIR-II photothermal ferrotherapy displayed a photothermal-depth-independent apoptosis/ferroptosis-inducing modality similar to ferrotherapy rather than PTT. For instance, from 2 to 9 mm, Cas-3 expression in photothermal ferrotherapy decreased by 30.1%, whereas that in PTT decreased by 83.6% and ferrotherapy decreased by 49.4%; LPO extent in photothermal ferrotherapy decreased by 18.6%, while that in PTT decreased by 74.5% and ferrotherapy merely decreased by 7.4%. Moreover, iron staining of tumor sections indicated evenly distributed iron stain in tumor tissue after photothermal ferrotherapy, in sharp comparison with ununiform bulky iron stains found after ferrotherapy. This was possibly because NIR-II photoirradiation potentiated self-degradation of HSN to tiny HSNS which possessed a much favorable size (~1.7 nm) to escape high interstitial fluid pressure and small intratumoral cutoff pore for elevated convective permeation in tumor interstitium³⁶⁷.

Figure 7.4 In vivo NIR-II PA imaging guided photothermal ferrotherapy. (a) Time-course NIR-II PA images of tumor region on living mice bearing 4T1-xenograft tumor after intravenous administration of HSN or HSN₀ ([pTBCB] = 250 $\mu\text{g mL}^{-1}$, 200 μL per mouse, $n = 3$). Wavelength: 1064 nm. (b) Quantification of PA amplitudes in (A) ($n = 3$). (c) Biodistribution study of mice in (a) at 24 h post-administration of HSN or HSN₀ ($n = 3$). (d) Surface tumor temperature of 4T1-tumor bearing mice upon 1064 nm photoirradiation (1 W cm^{-2} , 6 min) at 4 h after intravenous administration of HSN or HSN₀ ([pTBCB] = 250 $\mu\text{g mL}^{-1}$, 200 μL per mouse, $n = 3$). (e) Tumor growth curves of mice after photothermal ferrotherapy and monotherapies ($n = 3$). P values were calculated by

Student's two-sided *t*-test. ** $P < 0.01$, *** $P < 0.001$ ($n = 3$). (f) Scheme of photothermal depths of tumor. (g) H & E staining and immunofluorescent staining (Cas-3, LPO and ACSL4) images of tumor sections at different photothermal depths after monotherapies or photothermal ferrotherapy. Cas-3, LPO, and ACSL4 staining was indicated with green, red, and yellow false colors, respectively. (h-k) Quantification of cell death percentage (h), Cas-3 expression (i), LPO extent (j), and ACSL4 expression (k) of tumor sections at different photothermal depths after monotherapies or photothermal ferrotherapy. Upwards or downwards arrows indicated the increased or decreased percentage at 9 mm relative to 2 mm. Error bars indicated standard deviations of 3 independent measurements. Reprinted with permission from ref 353. Copyright 2020 Nature Publishing Group.

7.3.4 Inhibition of lung and liver metastasis.

Given that distant metastasis is widely observed in clinical settings of breast cancer, anti-metastasis performance of HSN-mediated NIR-II photothermal ferrotherapy was evaluated and compared with monotherapies (Figure 7.5a, b). After 14 days of different treatments, pulmonary metastases of living mice were examined using H&E staining. Fewer pulmonary metastatic nodules were counted in PTT or ferrotherapy-treated mice than PBS-treated group, whereas hardly any nodule could be found in photothermal ferrotherapy-treated mice (Figure 7.5c, d). Further, metastatic status in liver was examined (Figure 7.5e, f). Consistent with the trend of pulmonary metastasis, minor extent of hepatic metastatic lesions was observed in monotherapy-treated mice than control group; whereas almost no noticeable hepatic metastatic lesions were found in photothermal ferrotherapy-treated mice. Together, these data emphasized that HSN-mediated photothermal ferrotherapy almost

completely arrested distant cancer metastasis, which was not possible for either monotherapy.

To unveil the molecular mechanism of efficient metastasis inhibition by HSN-mediated NIR-II photothermal ferrotherapy, expression levels of metastasis-related proteins in tumor were evaluated after different treatments. These proteins included: hepatocyte growth factor/scatter factor (HGF) which activates tyrosine kinase Met signaling to promote cancer cell growth and motility for metastatic spread³⁶⁸; metastasis-associated protein 2 (MTA2) which is a crucial transcriptional repressor in nuclear remodeling and deacetylation (NuRD) complex to regulate cytoskeletal and motility pathways for metastatic dissemination³⁶⁹; and vascular cell adhesion molecule-1 (VCAM-1) of which the specific binding to very late antigen 4 (VLA-4) expressing leukocytes is responsible for metastatic invasion of malignant breast cancer cell to distant organs³⁷⁰. Immunofluorescence staining was performed to tumor sections followed by quantitative analysis. Compared with PBS-treated tumors, much weaker green fluorescence assigned to HGF, MTA2 and VCAM-1 staining could be found in tumors after monotherapies. However, green fluorescence could be hardly observed in tumors after photothermal ferrotherapy, showing the most downregulated expression of these proteins. Further, quantitative analysis indicated that HGF expression in photothermal ferrotherapy dropped to 35% and 49% of that in ferrotherapy and PTT, respectively. Similarly, MTA2 expression dropped to 40 and 54%; and VCAM-1 expression dropped to 31 and 29% (Figure 7.5g). Together, molecular mechanism of photothermal ferrotherapy-mediated metastasis inhibition was summarized in Figure 7.5h.

Figure 7.5 Cancer metastasis inhibition by HSN-mediated NIR-II photothermal ferrotherapy. (a) and (b) Schematic illustration of treatment schedule of monotherapies and

photothermal ferrotherapy. H & E images of lung (c) and liver (e) metastasis of living mice after various treatments. Metastatic area was indicated by dotted lines. (d) Number of metastasis nodules per lung in mice after various treatments. (f) Area of metastatic lesions in liver per mice after various treatments. *P* values were calculated by Student's two-sided *t*-test. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 (*n* = 3). (g) Mechanistic study and (h) illustration of molecular mechanism of metastasis inhibition by HSN-mediated NIR-II photothermal ferrotherapy. Quantification of expression levels of HGF, MTA2 and VCAM-1 in tumor tissues (at 9 mm) in living mice. T.I.: tumor interstitium. Error bars indicated standard deviations of 3 independent measurements. Reprinted with permission from ref 353. Copyright 2020 Nature Publishing Group.

7.4 Conclusions

We synthesized the first semiconducting nanozyme (HSN) based on the complexation of an organic semiconducting polymer (pTBCB-PEG) and Fe²⁺ for NIR-II PA imaging guided photothermal ferrotherapy (Figure 7.1a). Different from ordinary iron delivery systems (such as phenolic acid^{337, 344}, oxidized starch³⁷¹, leukocyte membrane³³⁸, etc.) that have no optical properties, pTBCB-PEG possessed extremely narrow band gaps for efficient photothermal transduction in the NIR-II window (Figure 7.2a). Probably because of iron-chelation-enhanced intramolecular interactions, HSN demonstrated almost the highest PCE (98.9%) among all the reported NIR-II photothermal agents. Under NIR-II photoirradiation, such excellent photothermal performance of HSN enabled improved PA delineation of tumor, enhanced Fenton reaction to elevate ·OH production (Figure 7.2g) and promoted self-degradation to tiny fragments for increased intratumoral penetration.

Molecular mechanism of NIR-II photothermal ferrotherapy was unveiled using HSN as the model system (Figure 7.3). Because of the upregulated expression of ACSL4 yet down-regulated level of and FPN-1 and GPX-4, 4T1 cell line demonstrated high ferroptosis sensitivity and was utilized for anti-cancer study. Under NIR-II photoirradiation, HSN mediated photothermal heating, which (i) triggered severe cellular apoptosis, (ii) promoted the degradation of ferritin to further replenish labile iron pool, (iii) amplified the catalytic activity of HSN/HSNS to promote $\text{OH}\cdot$ production for enhanced apoptosis/ferroptosis to cause overwhelming cell death. Thereby, HSN-mediated NIR-II photothermal ferrotherapy augmented the therapeutic effect of monotherapies via a sophisticated molecular network involving interlaced synergism of both programmed cell death modalities.

Despite the recent progress in NIR-II PTT, inadequate photothermal ablation in deep tumor due to exponential attenuation of photons greatly limits its clinical applications. The maximum therapeutic depth was restricted to 4 mm in bulky solid tumor via intravenous administration of 2D niobium carbide²⁷³, or to 5 mm through intratumoral injection of the reported SPN²⁷⁴. However, HSN-mediated NIR-II photothermal ferrotherapy presented a relatively photothermal-depth-independent therapeutic paradigm, realizing tumor elimination at an unexplored depth of 9 mm. Such superior therapeutic depth was attributed to the seamless synergy of PTT and ferrotherapy: (i) HSN-mediated photoinduced hyperthermia and photothermally-enhanced Fenton reaction; (ii) elevated intratumoral diffusion induced by self-degradation of HSN. Such deep-tissue eradication effectively impeded tumor regrowth, preventing distant metastasis to lung and liver. At cellular level, mechanistic study uncovered that HSN-mediated NIR-II photothermal ferrotherapy downregulated an array of metastasis-related proteins.

In summary, we reported a hybrid polymeric nanozyme (HSN) that compensates the limitations of both ferrotherapy and PTT for NIR-II PA imaging guided combination cancer therapy. To the best of our knowledge, our study not only pushes the therapeutic depth of non-invasive PTT to an unprecedented level, but also uncovers the biological mechanism of photothermal ferrotherapy. More broadly, the nanozyme strategy presented here can be generalized to develop photothermal ferro-therapeutic agents for other deep-tissue-seated diseases such as neurodegenerative diseases.

Chapter 8 Conclusions and future work

8.1 Conclusions

In summary, we have developed a diversity of SPNs to respectively address the critical challenges existing in 5 aspects of biophotonics: afterglow luminescence imaging, NIR-II PA imaging, PDT, photo-chemotherapy and photo-ferrotherapy. Details were listed below. In the first project, we have proposed a generic nano-engineering approach to converting nfluorescent SPNs into afterglow luminescent nanoparticles. Such design was based on cascade photoreaction among three components: photosensitizer as afterglow initiator, $^1\text{O}_2$ -sensitive molecule as afterglow substrate and fluorescent SP as afterglow relay unit. Through rational combination of 3 components, a series of afterglow nanoparticles with tunable emission spectra and bright luminescence were obtained. In addition to SPNs, such approach is applicable to other fluorescence molecules, broadly widening the scope of afterglow contrast agents.

In the second project, we have synthesized the first series of SPN-based NIR-II PA contrast agents that could be metabolized in vivo and gradually cleared out of living bolides. The biodegradability of SPN originated from the molecular design that an oxidizable thiophene moiety was incorporated into conjugated backbones and the obtained SP was encapsulated with FDA-approved hydrolysable polymer matrix. Because of the intense absorption in second window, the representative SPN demonstrated excellent NIR-II PA imaging capability when utilized in tumor or brain vasculature imaging. In addition, both in vitro and in vivo clearance pathways of the representative SPNs were substantially investigated.

And the proposed molecular design could be generalized to prepare other metabolizable organic contrast agents.

In the third project, we have synthesized an amphiphilic SP as drug delivery system to encapsulate DOX for dual-modal (NIR fluorescence and PA) imaging-guided photo-chemotherapy of cancer. Besides chemotherapeutics, the hydrophobic backbone of amphiphilic SPN was envisioned to serve as a simplified yet multifunctional drug carrier for other therapeutic agents such as immunostimulants etc.

In the fourth project, we have synthesized a SPN-based nano-photosensitizer that was covalently conjugated with CA-IX inhibitor to minimize hypoxia pitfall of conventional PDT. The integration of CA-IX inhibition mediated pH regulation into the targeted photodynamic process enables amplified cancer therapy, leading to minimized probability of cancer metastasis.

In the fifth project, we have synthesized an NIR-II absorbing amphiphilic SP which captured ferrous ion to form hybrid polymeric nanozyme for cancer treatment, aiming to conquer the limitations of both ferrotherapy and NIR-II PTT. During photoirradiation, the prepared hybrid nanozyme mediated photothermal ablation, potentiated Fenton reaction to augment ROS production, and enhanced self-decomposition to favor intratumoral permeation, ultimately resulting in efficient tumor elimination at unprecedented depth (8 mm) and superior inhibition of cancer metastasis. Broadly speaking, such molecular design could be generalized to prepare NIR-II PTT related therapeutics to treat other deeply seated diseases.

8.2 Future work

In the thesis, we have demonstrated the leverage of SPNs in versatile applications of biophotonics through rational molecular engineering. Despite these advances, the current state of art of SPNs for biophotonic applications remains in the primitive stage. And development of SPNs with advanced chemical or biological complexity to address other long-standing issues in biophotonics or to push forward the translation to clinical practice is worth pursuing. Future work is proposed in the following aspects.

(1) In the previous work, we have demonstrated the utilization of SPNs as afterglow contrast agents for background-free in vivo imaging. However, the luminescence of these afterglow nanoparticles is restricted in NIR-I region, wherein scattering of emitted photons in biological tissue is not minimized, leading to suboptimal SBR and imaging depth. To overcome this drawback, development of afterglow contrast agents with afterglow emission in a more biologically transparent NIR-II window is of high demand. The major difficulty to develop such nano-agents lies in the chemical synthesis of SPs with bright NIR-II fluorescence, which requests special molecular design.

(2) We have demonstrated NIR-II PA imaging of tumor and brain vasculature using metabolizable SPN-based contrast agents. Although NIR-II PA imaging possesses overall superior spatial resolution in deep tissue, the imaging sensitivity largely remains to be improved. Developing activatable NIR-II PA probes that could emit NIR-II PA signals only after reaction with physiological indexes or pathological biomarkers is envisioned to provide a useful solution to improve imaging sensitivity. Whereas the chemical synthesis of such activatable NIR-II absorbing contrast agents requires delicate molecular engineering and substantial preliminary investigation.

(3) In this thesis, we have demonstrated the synergy of phototherapy with chemotherapy or ferrotherapy for augmented therapeutic efficacy. Apart from these two modalities, immunotherapy that boosts host immune system to fight against tumor has revolutionized cancer treatment. With the promise to improve immune response, cancer nano-immunotherapy has been recently under extensive development. However, the majority of current nano-immunotherapeutic strategies lack regional specificity to ensure potent antitumor immunity whereas minimized systemic adverse effects. In light of the excellent chemical flexibility and photochemical property of SPNs, the integration of NIR-II absorbing SPNs with immunotherapy provides promising opportunities for the spatiotemporal photo-regulation of antitumor immunity. Whereas the specific molecular design requires substantial exploration. In addition to cancer therapy, the excellent NIR-II photothermal property of SPNs enabled their broader application to treat other life-threatening diseases such as neurodegenerative diseases, cardiac diseases, pulmonary diseases, autoimmune diseases and so forth.

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