Near-infrared photon-converting systems for bioimaging, theranostics and optogenetics

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**NEAR-INFRARED PHOTON-CONVERTING SYSTEMS
FOR BIOIMAGING, THERANOSTICS AND
OPTOGENETICS**

WANG ZHIMIN

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2020

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FOR BIOIMAGING, THERANOSTICS AND
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**SCHOOL OF PHYSICAL AND MATHEMATICAL
SCIENCES**

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

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Prof Xing Bengang

Authorship Attribution Statement

This thesis contains material from 7 papers published in the following peer-reviewed journals in which I was listed as the first or co-first author.

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- Prof Xing provided the initial topic.
- I prepared the manuscript drafts. The manuscript was revised by Prof Xing, Mr D. C. Thang, Ms Q. Han, Mr X. Zhao, Dr X. Xie, Dr Z. Wang and Prof Lin.

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- Prof Xing provided the initial project direction, gave critical advice on the experimental results and edited the manuscript drafts.
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- Dr X. Ai and I performed all the small molecules, nanoprobe synthesis and characterization, carried TEM, UV-Vis absorption spectra and upconversion fluorescence spectra, performed cells culture and fluorescence confocal imaging, conducted animal bioimaging experiments, and collected data.
- Dr R. Zhang and Prof Y. Zheng assisted the photoacoustic test of nanoprobe response in solution.
- Dr Y. Wang and Prof M. Gao provided the *in vivo* experimental facilities and assisted the MSOT and upconversion luminescence bioimaging.
- Dr L. Liang and X. Liu assisted the upconversion luminescence lifetime determinations.

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- Dr X. Ai and I performed the nanoprobe synthesis and characterization, carried TEM, UV-Vis absorption spectra and upconversion fluorescence spectra, performed cells culture and fluorescence confocal imaging, conducted animal bioimaging experiments, and collected data.
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- Dr L. Liang and X. Liu assisted the upconversion luminescence lifetime determinations.

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- Other than the *in vivo* photoacoustic tumor imaging, all the experiments were carried out by me.
- Dr X. Zhen and Prof K. Pu established tumor bearing mice model.
- Dr P. K. Upputuri and Prof M. Manojit provided the NIR-II photoacoustic imaging setup and assisted tumor imaging.
- Dr Z. Meng and Dr Y. Lu conducted the molecular simulation study.

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- Other than the *in vivo* NIR-II photoacoustic tumor imaging and photothermal tumor therapy, all the experiments were carried out by me.
- Dr X. Zhen and Prof K. Pu established tumor bearing mice model and conducted NIR-II photothermal tumor therapy. Dr X. Zhen also assisted the data analysis of photothermal therapy effects.

- Dr P.K. Upputuri and Prof M. Manojit provided the NIR-II photoacoustic imaging setup and conducted tumor imaging.

3/8/2020

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List of Abbreviations

ACQ	Aggregation caused quenching
ALT	Alanine aminotransferase
AST	Aspartate transaminase
ACN	Acetonitrile
ALP	Alkaline phosphatase
APAP	N-acetyl-para-aminophenol
APTES	(3-Aminopropyl) triethoxysilane
BCA	Bicinchoninic acid
BUN	Blood urea nitrogen
CTNs	Charge-transfer nanocomplexes
ChRs	Channelrhodopsins
CA	Contrast agent
CYP450	Cytochrome P450
CT	Computed tomography
CTAC	Cetyltrimethylammonium chloride
CREA	Creatinine
DILD	Drug-induced liver damage
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DIEA	N, N-Diisopropylethylamine
DMSO	Dimethyl sulfoxide
DSPE	1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine
ELISA	Enzyme-linked immunosorbent assay
EPA	End-point analysis
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
FA	Folic acid
FBS	Fetal bovine serum
FTIR	Fourier-transform infrared spectroscopy
GLUT-4	Glucose transporter type 4
GSH	Glutathione
GSK3 β	Glycogen synthase kinase 3 β

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPLC	High performance liquid chromatography
H&E	Hematoxylin and eosin
HRP	Horseradish peroxidase
HOBT	Hydroxybenzotriazole
4-HNE	4-Hydroxynonenal
IFE	Inner filter effect
ICG	Indocyanine green
ICT	Intramolecular charge-transfer
INF- γ	Interferon- γ
INH	Isonicotinylhydrazide
IVIS	In vivo imaging system
IR	Insulin receptor
LFTs	Liver function tests
LOD	Limit of detection
LINPs	Lanthanide-imaging nanoprobe
LRET	Luminescence resonance energy transfer
LED	Light-emitting diode
LPS	Lipopolysaccharide
LPO	Lipid peroxidation
LDPE	Low-density polyethylene
LDH	Lactic acid dehydrogenase
MSOT	Multispectral optoacoustic tomography
MS	Medial septum
MnTBAP	Mn(III) tetrakis (4-benzoic acid) porphyrin
MEG	Mercaptoethyl guanidine
MRI	Magnetic resonance imaging
NIR	Near-infrared
NAC	N-acetylcysteine
NAPQI	N-acetyl-p-benzoquinone imine
OA	Oleic acid
PCSs	Photon-converting systems
PAI	Photoacoustic imaging

PDT	Photodynamic therapy
PTT	Photothermal therapy
PMMA	Poly(methyl methacrylate)
PEI	Polyethyleneimine
PAA	Polyacrylic acid
PEG	Polyethylene glycol
PMA	Phorbol 12-myristate 13-acetate
PIP3Ks	Phosphoinositide 3-kinases
PI	Propidium iodide
PVDF	Polyvinylidene difluoride
QDs	Quantum dots
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
ROI	Region of interest
SNR	Signal-to-noise ratio
SIL	Silibinin
SPNs	Semiconducting polymers nanoparticles
SEM	Scanning electron microscope
TMB	Tetramethylbenzidine
TEM	Transmission electron microscopy
TME	Tumor microenvironment
TEOS	Tetraethyl orthosilicate
TUNEL	Terminal deoxyribonucleotide transferase-mediated nick-end labeling
UCNPs	Upconversion nanoparticles
UCL	Upconversion luminescence
UV	Ultraviolet
USFDA	U.S. Food and Drug Administration
US	Ultrasonography
UST	Ultrasound transducer
UGTs	Uridine 5'-diphospho-glucuronosyltransferases
VEGFR	Vascular endothelial growth factor receptor
VTA	Ventral tegmental area
XRD	X-ray diffraction

Abstract

Near-infrared (NIR) biophotonics-based smart photon-converting systems (PCSs) have made great process in recent academic research and biomedical applications, such as optical bioimaging and phototheranostics. Owing to the versatile controllability, superb spatiotemporal resolution and minimal tissue invasiveness in practical operations, these NIR-responsive platforms provide opportunities and possible solutions to investigate the physiopathological and therapeutic aspects from cells to the organism level. In principle, the design of NIR-biophotonic PCSs is primarily based on the photophysical process and physicochemical properties of the optical materials upon NIR photoirradiation, and the mechanisms include the radiative and nonradiative transitions-mediated photon emission, heat generation as well as the cascade energy transfer. So far various optical biomaterials have been explored and used, including small-molecule dyes, quantum dots (QDs), semiconducting polymers nanoparticles (SPNs), upconversion nanoparticles (UCNPs) and so on. These NIR-sensitive agents have their own photophysical performances and merits in biological sensing and therapeutic interventions. In particular, UCNPs with promising anti-Stokes shift emissive features show the potentials in deep-tissue luminescent imaging and photoactivation of cellular signaling. Besides, the NIR-absorbing materials with low-emissivity like charge-transfer nanocomplexes (CTNs) exhibit the distinctive capability of photothermal conversion, serving as superb biophotonic platforms for photoacoustic imaging (PAI) and photothermal therapy (PTT) with desirable therapeutic efficacy. In this thesis, I just focus on the development of NIR photon-converting systems based on UCNPs, cyanine dyes and CTNs for multipurpose biological applications, including liver pathological bioimaging, tumor phototheranostics, bacterial infection sensing and optogenetic regulation of insulin resistance, as shown in the following sections.

In chapter 2, I presented an innovative photon-upconverting nanoprobe by tailoring the multi-emissive UCNPs with multispectral optoacoustic tomography (MSOT) technique for simultaneous sensing of diversely endogenous oxidative stress biomarkers with good spatiotemporal precision in intricate living conditions. Upon encapsulating two selective reactive free radicals-sensitive NIR cyanine dyes onto UCNPs surface, such a multifunctional nanoprobe could orthogonally respond to disparate nitrosative and oxidative stimulations, and generate opposite MSOT and luminescence variations, which thus realized compelling superiorities for tracking of multiple free radicals under dual independent wavelength channels in a reversed ratiometric manner, and precise validation and correlation of the complex dynamics of redox-mediated pathophysiological procession *in vivo*. This study also provided a possible solution for precise and dynamic profiling of the pathophysiological implications stipulated by multiple biological indicators with deep penetration and high accuracy.

In chapter 3, I further integrated the deep-tissue penetrable MSOT and upconversion luminescence (UCL) imaging into a cross-referencing technology through the combination of reactive radical-sensitive cyanine chromophore with multicolour UCNPs. Such a reversed-ratiometric and dual-modal imaging paradigm provided a novel cross-referencing platform with superb sensing accuracy, thus enabling selective and sensitive liver disease biology evaluation as well as therapeutic assessments *in vivo*. During the process of drugs treatment, the specific reactions between pathological oxidative stress and reactive oxygen species (ROS)-sensitive cyanine probes converted an absorption band shift, which can induce MSOT signal changes; meanwhile, ROS-triggered reactions also affected multispectral UCL variations, exhibiting an opposite signal response trend against MSOT. As compared to conventional liver function tests (LFTs) such as tissue biopsy-based examinations and blood assays, this NIR photon-converting nanoprobe enabled noninvasive, spatiotemporal and highly ingenious mapping of

hepatopathological status in intact organisms, especially beneficial for dynamic assessment of therapeutic responses in practice.

In chapter 4, I introduced a novel class of organic and pH-sensitive PA contrast agent (CA) working in the second near-infrared region (NIR-II, the wavelength at 1000 - 1700 nm), which was derived from tetramethylbenzidine (TMB) and its dication form (TMB^{2+}) based charge-transfer nanocomplex (CTN). Such a promising NIR-II PA nanoprobe could specifically detect pH change in the physiological environment (*e.g.*, at pH 5) in the tumor mice model with good signal-to-noise ratio (SNR). Most importantly, CTNs also demonstrated the biodegradability in the normal biological condition (*e.g.*, at pH 7.4), which provided high biosafety index for *in vivo* applications without concerns of nanomaterials accumulation. These data showed the great potential using CTNs as sensitive, biodegradable, and tumor-specific NIR-II PA imaging probes of pH.

In chapter 5, owing to the excellent photothermal-conversion capability of CTNs, I further fabricated a redox-activatable and biocompatible NIR-II nanotheranostics towards tumor phototherapy, which displayed sensitive diagnostic and therapeutic signals only upon tumor microenvironmental redox activation and acid-enhancement, while keeping silent at normal tissues. This intelligent turn-on-type NIR-II optical nanotheranostics allowed nearly zero-background PA imaging and combined PTT in living animals with negligible side effects. Considering the superior tissue-penetration depth, high sensitivity and excellent biocompatibility, such a stimuli-responsive NIR-II phototheranostics provided a wide prospect for the investigation of physiological and pathological redox and acid-associated events.

In chapter 6, I introduced a NIR small-molecule probe based on HCy5-Cy7 dyad for simultaneous and sensitive monitoring of multiple RONS variations. HCy5-Cy7 specifically turned on its fluorescence at 660 nm *via* superoxide or hydroxyl radical ($\bullet\text{O}^{2-}$, $\bullet\text{OH}$)-mediated

oxidation of reduced HCy5 moiety to Cy5, while peroxynitrite or hypochlorous species (ONOO^- , ClO^-)-induced Cy7 structural degradation led to the emission turn-off at 800 nm. Such multispectral signal responses allowed HCy5-Cy7 to multiplexly investigate the dynamic oxidative/nitrosative stress events with high spatiotemporal precision. Most importantly, this multifunctional probe molecule may be useful in the multiple radical correlations and implications during the pathogenic and defensive processes of bacteria-infected mice, providing new perspectives for infectious diseases sensing, *in vivo* studies are still ongoing.

In chapter 7, I presented a smart UCNPs-mediated optogenetic system for NIR photoactivation of Akt/insulin signaling and to further investigate the possibility of type-2 diabetes (T2D) management. Principally, the photo-inducible protein interaction module CRY2-Akt2 and Myr-CIBN (termed Opto-Akt2) were expressed in 3T3-L1 adipocytes for the optogenetic Akt2 regulation. Upon NIR light (*e.g.*, at 980 nm) irradiation, UCNPs could emit anti-Stokes shift blue emissions (*e.g.*, at $\sim 450, 480$ nm) that trigger CRY2/CIBN dimerization; meanwhile, inactive Akt2 was recruited to the cell membrane and activated by the upstream kinases (*e.g.*, PI3K). Such a NIR optogenetic platform was further proposed to enable effective manipulation of insulin/Akt2 signaling pathways, including the induction of GLUT4 vesicles trafficking and glucose uptake with deep penetration, minimum cell perturbation and high spatiotemporal resolution, which may provide experimental evidence for insulin resistance management *in vitro* and in animals, relevant studies are still ongoing.

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Chapter 1 General Introduction

1.1 NIR photon-converting systems (PCSs)

In recent years, the multidisciplinary biophotonics research has attracted tremendous attention in the diagnostics and therapies due to the advantages of non-invasiveness, high efficiency and accuracy.¹ Besides the engineering of optical devices like laser, microscopy and other optic setups, the optical contrast materials play a crucial role in the practical biophotonic studies. So far, various organic or inorganic optical agents ranging from small-molecule dyes to nanomaterials including quantum dots (QDs),² semiconducting polymers nanoparticles (SPNs),³ upconversion nanoparticles (UCNPs),⁴ gold nanoparticles⁵ and carbon nanotubes,⁶ have been widely utilized for biological regulation, imaging and phototherapy.

Owing to the light absorption and scattering effects within the biological tissues, most of *in vivo* optical imaging trials in the visible spectrum at 400-700 nm have been significantly hampered by the poor photon penetration depth and high background interference.⁷ Alternatively, recent advances validated that the medical imaging and phototherapeutic techniques in the near-infrared (NIR) region (700-1700 nm) show promising theranostic outcomes with reduced tissue scattering, minimum absorption and negligible autofluorescence.⁸ In this respect, there have been two main directions to achieve deep-tissue NIR bioimaging, including both the development of new instrumentations and contrast agents. In which, the NIR photon-converting systems (PCSs, Scheme 1) derived imaging agents with improved quantum efficiency, photostability and biocompatibility have extended the conventional NIR-I and NIR-II imaging windows,⁹ these progresses further advance the NIR phototheranostics into clinical applications.¹⁰

Scheme 1. Different NIR photon converting systems for biophotonic applications.

In light with the unique photophysical properties of these PCSs, multimodal biophotonic platforms can be fabricated through either making use of photo-converting mechanisms of optical materials or the technical integration of different phototheranostic modalities. For instance, the NIR light sensitive cyanine dyes, QDs and UCNPs have been used for luminescence imaging and photodynamic therapy (PDT) based on the photon-to-luminescence conversion; other NIR optical-absorbing PCSs (*e.g.*, SPNs, AuNRs, etc.) with the feature of photon-to-heat conversion enable the emergence of photoacoustic (PA) imaging and photothermal therapy (PTT). These biophotonic platforms have their own merits in biomedical applications, thereby greatly diversifying our choices to solve the complex issues closely related to human health.

1.2 NIR optical imaging and phototherapy

Recent advances related to imaging techniques have witnessed the significant progress in noninvasive biological detection *in vitro* and *in vivo*.^{11,12} Among different imaging techniques,

fluorescence imaging has attracted enormous attentions mainly due to their advantages of easy use, high spatiotemporal precision, and good sensitivity for biomarkers tracking. Upon light irradiation, the fluorophores are excited with fluorescence emission generation that can be detected by the optical camera (Scheme 2a).¹³ In this line, various fluorescence materials, such as organic fluorophores, nanoparticles and genetically encoded fluorescent proteins have been extensively developed for real-time diagnostic purposes.⁸ However, despite the initial success, some obvious disadvantages such as shallow tissue penetration, poor photostability and limited signal-to-noise ratio (SNR) remain the technical obstacles that would potentially hamper their further biomedical applications in living systems.

Scheme 2. Illustration of luminescence and photoacoustic imaging modalities.^{13,14}

Alternatively, photoacoustic imaging as a hybrid technology emerged in biomedical fields, has been well recognized to provide reliable sensing outcomes and superb tissue penetration depth closing to the clinical requirement.¹⁵ Principally, upon the pulse laser irradiation, the optical energy can be absorbed by either endogenous biomolecules (*e.g.* hemoglobin etc) or exogenous contrast agents, and subsequently converted to the localized thermal energy, followed by the generation of pressure changes through thermo-elastic expansion.¹⁶ Such a pressure signal will be propagated as acoustic waves, and can be captured by the ultrasonic transducers (Scheme 2b). As a contrast, the higher penetration depth of PA imaging could

overcome the limitations of conventional fluorescence imaging and low-resolution ultrasonic imaging, can therefore enable its outstanding studies in the clinical biodetection. Despite tremendous potential, PA imaging is also restricted by the significant background signal due to the comparable tissue optical absorption. Therefore, robust PA setups and sensitive contrast probes need to be developed to overcome these limitations.

Scheme 3. Illustration of tissue penetration of light and NIR phototheranostics.

The deep-tissue penetration ability of NIR light is key to clinical bioimaging, due to light–tissue interactions are wavelength-dependent.¹⁷ Recent studies proved that the optical imaging in the NIR-I and even NIR-II windows showed reduced tissue-absorption and scattering effects, allowing phototheranostics applications in living systems (Scheme 3). Therefore, in this thesis I mainly focus on the development of stimuli-responsive PCSs using UCNPs, cyanine dyes and charge-transfer nanocomplexes (CTNs) for phototheranostic studies, and relevant background information is introduced in more detail below.

1.3 UCNPs and photon upconversion

Typically, UCNPs contain three essential components, comprising host matrix (usually NaYF₄), sensitizer (usually Yb³⁺) and emitter (usually Tm³⁺, Er³⁺ or Ho³⁺ etc.), which are normally excited with 980 nm laser, and the multicolor emissions from the UV-Vis to NIR range can be manipulated by controlling the combination of lanthanide dopants and dopant concentration (Figure 1a).¹⁸ In addition, the excitation wavelength can be further transferred to ~ 800 nm by fabrication of Nd³⁺-sensitized UCNPs, in which Nd³⁺ ion takes the role of

absorbing photons at 800 nm, while the Yb^{3+} ion acts as bridging ions for the energy transfer from Nd^{3+} to the emitters (Figure 1b).¹⁹ Such an excitation shift significantly decreases of the tissue thermal effect in practical imaging applications, as the absorption coefficient of water at 800 nm is several magnitudes lower than that at 980 nm.^{20,21} However, UCNPs often suffer from low photon-upconversion efficiency and obvious quenching effects in aqueous conditions. To further enhance the quantum yield, innovative strategies through rational energy-transfer manipulation, crystal-lattice modification, fabrication of core-shell structures, as well as ingenious integration of UCNPs with other plasmonic nanomaterials and organic dyes have been developed in the past decades (Table 1).^{22,23}

Recently, a brand-new type of Er^{3+} -sensitized upconversion nanoplatfrom was reported that can transfer the absorbed wavelength at ~ 1532 nm (Figure 1c).^{24,25} Moreover, these UCNPs are capable of converting multiple emissions ranging from ~ 400 to 1200 nm that can be modulated by rational manipulation of the dopant and nanostructure (Figure 1d). Such a design opens new possible avenues for the emerging NIR-II bioimaging applications. In light with the above advances, great opportunities and achievements have been endeavored for the use of photon-upconverting nanoprob es in various biomedical and pre-clinical practices.

Figure 1. Photon upconversion of UCNPs. a) Upconversion luminescence spectra and multicolor fine-tuning through using lanthanide doped NaYF₄ nanoparticles with varied dopant ratios (Yb³⁺/Tm³⁺/Er³⁺). b) Typical energy level and energy transfer diagram under the NIR laser excitation (980 nm for normal Yb³⁺-sensitized UCNPs, 800 nm for Nd³⁺/Yb³⁺-sensitized UCNPs). c) Illustration of Er³⁺-sensitized photon upconversion under 1532 nm excitation. d) Proposed energy transfer mechanisms for Er³⁺-sensitized upconversion emissions in the NIR-II region. This figure was reprinted with permission.²⁶

Table 1. The typical strategies for enhancing photon-converting efficacy of UCNPs.

Strategies	Representative Example	Enhancement Factor ^{a)}	Ref.
A	Lithium ion or other transition-metal ions doping to improve the coordination environment and crystal field splitting of lanthanides	$\text{Na}_x\text{Li}_y\text{YF}_4: \text{Yb}^{3+}, \text{Er}^{3+}$	~30 (540 nm) 27
		$\text{Y}_2\text{O}_3: \text{Er}^{3+}, \text{Li}^+$	~60 (410 nm), ~25 (540 nm) 28,29
		$\text{NaGdF}_4: \text{Yb}^{3+}, \text{Er}^{3+}, \text{Li}^+$	~47 (540 nm), ~23 (660 nm) 30
B	Incorporating transition metals to control the energy transfer processes	$\text{Er-Mo}: \text{Yb}_2\text{Ti}_2\text{O}_7$	~250 (540 nm) 31
		$\text{Er-Mn}: \text{Yb}_3\text{Al}_5\text{O}_{12}$	~260 (540 nm), ~20 (660 nm) 32
	Optimizing Tm doping concentration	$\text{NaYF}_4: \text{Yb}^{3+}, \text{Tm}^{3+}$	~70 (450 nm) 33
	Increasing Yb doping concentration	$\text{NaTm}_{0.02}\text{Lu}_{0.98-x}\text{Yb}_x\text{F}_4$	13.6 (340 nm), 23.3 (450 nm) 34
C	Encapsulating Au or Ag for plasmonic enhancement of upconversion luminescence	$\text{NaYF}_4: \text{Yb}^{3+}, \text{Tm}^{3+}@\text{Au}$	~50 (450 nm), ~30 (470 nm) 35
		$\text{NaYF}_4: \text{Yb/Er/Tm}@\text{Ag}$	~21 (450 nm), ~22 (540 nm) 36

D	Amorphous shell coating	$\text{Y}_2\text{O}_3: \text{Tm}^{3+}, \text{Yb}^{3+} @ \text{SiO}_2$	~200 (480 nm)	37
	Crystalline-shell coating	$\text{KYF}_4: \text{Yb}^{3+}, \text{Er}^{3+} @ \text{KYF}_4$	>20 (540 nm, 660 nm)	38
		$\text{NaGdF}_4: \text{Yb}^{3+}, \text{Tm}^{3+} @ \text{NaGdF}_4$	~450 (480 nm)	39
E	Incorporation of broadband sensitizers	$\text{NaYF}_4: \text{Yb/Tm} @ \text{NaYF}_4: \text{Nd}$	~7	40
		$\text{NaYF}_4: \text{Yb/Er} @ \text{NaYF}_4: \text{Nd}$	(450/480 nm, 540 nm, 650 nm)	
		$\text{NaYF}_4: \text{Yb/Ho} @ \text{NaYF}_4: \text{Nd}$		
		$\text{NaYF}_4: \text{Yb/Er} @ \text{IR806}$	3300 (550 nm, 650 nm)	41
F	Adding UCNPs to photonic crystals	PMMA OPCs / $\text{NaYF}_4: \text{Yb}^{3+}, \text{Tm}^{3+}$	~32 (450nm, 480 nm)	42

^{a)} Fold enhancement of the upconversion luminescence at specific wavelength. This table was reproduced with permission.⁴³

1.4 Luminescence imaging with upconversion nanoprob es

Molecular functionalization of UCNPs as sensitive nanoprob es remarkably promotes their applicability in precise detection of biomarkers and biological events in living organisms. Similar to small-molecule fluorescent reporters, upconversion nanoprob es can also be mainly divided into two types, termed as “always-on” and activatable prob es (Scheme 4).^{44,45}

Generally, “always-on” prob es rely on the specific contrast accumulation while the signals remain unchanged upon interaction with the target of interest. Given the continuous luminescence of UCNPs, the effective approach for enhancing the SNR of these nanoprob es is by modification of the outer layer with targeting ligands or antibodies. For example, introducing folic acid (FA), RGD peptide, anti-vascular endothelial growth factor receptor (VEGFR) antibodies or other tumor targeting moieties onto the surface of UCNPs that can trigger selective accumulation and uptake in the tumor area, such target-specific nanoprob es have been widely applied in tumor theranostics.⁴⁶ However, despite the great promise, current “always-on” upconversion nanoprob es remain restricted by the pseudo signal readout and a lack of quantitative sensing capability.

In contrast, activatable nanoprob es with the intrinsic signal variation that can only be activated by the sensing targets or environments, thereby exhibiting higher SNR and lower limit of detection (LOD) in comparison with conventional “always-on” nanoprob es. Such kind of techniques are also capable of correlating the signal variations with the quantity and activity of analytes, further providing the feasibility of quantitative sensing and imaging.^{44,47}

Scheme 4. Design and principles of different types of lanthanide-imaging nanoprobess (LINPs). The analytes include nucleic acid, protein, enzyme, temperature, pH, redox, and so on.

So far, activatable upconversion nanoprobess typically undergo two development strategies based on either stimuli-responsive “turn-on-type” emission enhancement or ratiometric emission color change. The design principles of such nanoprobess usually involve fluorescence or luminescence resonance energy transfer (FRET or LRET) and inner filter effect (IFE) mechanisms.^{45,48} Briefly, LRET indicates nonradiative energy transfer process from the luminescence donor UCNPs to the nearby modified energy receptor, which results in the quenching effect of lanthanide luminescence and consequently lights up the receptor molecules; similarly, the luminescent photon reabsorbed by the acceptor is termed as inner filter effect. Notably, the efficient energy transfer of LRET nanoprobess only happens when the quenching molecules are closer enough to the surface of UCNPs; while the IFE systems depend on the tuning of appropriate chromophores.⁴⁹ Therefore, it is essential to rationally design the sensitive

UC naoprobes according to the analytes. To achieve the effective detection of target species, two types of UCNP-based nanoprobes can be fabricated such as “turn on” and ratiometric, in view of the intensity changes in the single emission band or multiple emission bands. Accordingly, the typical routes to improve the responses are available *via* manipulating either the spectra overlap or the distance between UCNPs and the energy acceptors.

Upconversion-based nanoprobes benefit from the advantages of high photostability, good biocompatibility, unique NIR anti-stokes shift and enhanced tissue penetration, which have been widely used for the imaging of biological processes and disease indicators.^{50,51} The representative upconversion nanoprobes and their multipurpose bioimaging applications have been briefly summarized in Table 2.

1.5 NIR upconversion phototherapeutics

Light-mediated drug-uncaging approaches have attracted considerable interest in the healthcare management, due to the fact that light can be tightly focused and manipulated in highly spatiotemporal accuracy.^{66,67} The mechanisms of most photo-triggered drug delivery systems are based on ultraviolet (UV) light-induced chemical structures breaking or conformational switch, which raise the concerns of potential phototoxicity and unsatisfied penetrability in living conditions.⁶⁸ Gratifyingly, recent advances in NIR light-controlled theranostic release have greatly overcome the disadvantage of UV light, enabling the possibility of noninvasive, biocompatible and precise diseases therapy in deep tissues.^{69,70} Among the methodologies of NIR photo-controlled drug delivery, the utilization of biophotonic nanomaterials, especially UCNPs as versatile NIR-to-UV/vis optical transducers to generate in situ high intensity light source, achieving on-demand therapeutics photorelease with less adverse effects and better tissue penetration.^{71,72}

Table 2. Summary of representative LINPs for bioimaging applications.

Upconversion nanoprobe		Ex./Em. ^{a)} (nm)	Principle	Application	Ref
“Always-on”	Antibody-UCNPs (NaYF ₄ :Yb/Er)	980/540, 650	Specific targeting of tumor carcinoembryonic antigen	In vitro HeLa cells immunolabeling, imaging	35
	RGD-UCNPs (NaYF ₄ :Yb/Er/Tm)	980/540, 650	Specific targeting of integrin $\alpha\beta_3$ -overexpressing tumors	In vitro and in vivo tumor imaging	36,37
	Antibody-UCNPs (NaGdF ₄ :Yb/Tm/Ca@NaLuF ₄) (NaGdF ₄ :Yb/Er@NaGdF ₄)	980/480, 800 980/540, 650	Specific targeting of lymph node	In vitro and in vivo lymph node metastasis and tumor detection	38
Activatable	NaGdF ₄ :Yb/Er@NaYF ₄ :Er@NaYF ₄ -GFP	980/540, 650	Protein-protein interactions induced LRET effects	In vitro GSH protein-protein interactions imaging	39
	NaYF ₄ :Yb/Er NaGdF ₄ :Yb/Er@NaYF ₄	980/520, 540	Thermal effect induces energy transfer change	Temperature sensing	40
	pHrodo-UCNPs (NaYF ₄ :Yb/Er)	980/540, 650	pH sensitive molecule induces FRET change	In vitro pH sensing, imaging	41
	HCy7-UCNPs (NaYF ₄ :Yb/Er/Tm)	980/540, 650, 800	MeHg ⁺ sensitive dye induces LRET change	In vitro and in vivo MeHg ⁺ sensing, imaging	42
	MnO ₂ -UCNPs (NaYF ₄ :Yb/Tm@NaYF ₄)	980/480	GSH destructs MnO ₂ -induced FRET quenching effect	In vitro GSH detection	43
	Cyanine-UCNPs (NaYF ₄ :Yb/Tm@NaYF ₄)	980/800	ONOO ⁻ destructs Cy7-induced LRET quenching effect	In vitro and in vivo ONOO ⁻ imaging	44
	NaErF ₄ :Ho(or Nd)@NaYF ₄	1530/1180, 980	Fenton catalysis induced LRET change	In vivo dynamic NIR-II inflammation sensing	45
Multimodal optical nanoprobe	NaGdF ₄ :Yb/Tm/Er@NaGdF ₄	980/540, 650 680, 800 ^{b)}	UCL and ICG dye-based PA effects	In vitro and in vivo UCL/PA tumor imaging	46
	HydroCy5/Cy7/UCNPs (NaGdF ₄ :Yb/Tm/Er@NaGdF ₄)	980/650, 800 680, 800 ^{b)}	ROS and RNS specific responses induce LRET changes	In vitro and in vivo ROS, RNS sensing, imaging	47
	CyB/NaGdF ₄ :Yb/Tm/Er@NaGdF ₄	980/650, 800 680, 800 ^{b)}	UCL and cyanine-based LRET and PA effects	In vitro and in vivo ratiometric UCL/PA ROS imaging	48

^{a)} Excitation and maximum emission wavelength (nm) used for bioimaging.

^{b)} Excitation wavelength (nm) of PA imaging.

Scheme 5. Upconversion nanocomposites based NIR photocontrolled therapeutics.⁷³

Despite the superb photonic feature of anti-Stokes shift UCL, it should be noted that UCNPs for practical therapeutic purposes usually require further functionalization to be biocompatible nanocarriers for drugs or bioactive molecules loading. The design of phototheranostic upconversion nanocomposites (UCNCs) also covers three main components, (i) UCNP-based nanocore, which is used as both optical transducers and nanocarriers; (ii) surface coatings, comprising silicon or various polymers (*e.g.* polyethylenimine (PEI), polyacrylic acid (PAA) and polyethylene glycol (PEG) etc.) that play the role in improving the biocompatibility, and enabling biomolecules encapsulation, tissue-targeting/accumulation as well as light-sensitive release; (iii) therapeutic agents, including clinical drugs, peptides, antibodies, nucleic acids (DNA or RNA) and photosensitizers, these molecules can be precisely released or activated *via* UCL-triggered upconversion photochemistry under NIR laser irradiation. To achieve photo-responsive delivery capabilities, various upconversion nanocomposites (UCNCs) through combining conventional UV-Vis-sensitive chemical structures or supramolecular polymeric biomaterials have been reported in the past decade.^{74,75}

These multifunctional UCNCs enable simultaneous diseases (or bioindicators) sensing and NIR photocontrolled therapeutics release.

One typical approach used in upconversion photorelease is photocleavage, in which molecular payloads are covalently caged or conjugated on the surface of functionalized UCNPs. Briefly, there are three main design strategies: (i) NIR light illumination towards photoresponsive linkers (*e.g.*, O-nitrobenzyl derivatives, coumarins etc.) that can directly uncage linked biomolecules, such as nucleic acids, proteins, and drugs; (ii) photocleavage induced degradation of the carrier layer on UCNPs that can enable release of loaded molecules; (iii) photocleavage of bioactive donor molecules (*e.g.*, gaseous donors). Besides, structural or conformational switching in the nanocarriers undergoes a physical change upon light irradiation that is also feasible for photocontrolled therapeutics release.

Photosensitization mediated cytotoxic singlet-state $^1\text{O}_2$ or other reactive oxygen species (ROS) generation has been used as a clinical treatment modality against tumor and microbial infections, which is also termed photodynamic therapy (PDT).⁷⁶ In PDT, light, oxygen and photosensitizer (PS) are three major components, where light-triggered PS molecules excitation leads to energy transfer to neighboring O_2 or other substrates, generating ROS and subsequently inducing cells damage.^{77,78} In general, there are two types of photoreactions involving the PDT mechanisms. As indicated in Scheme 6a, the PS molecules absorb photons from light and cause excitation to the singlet excited state ($^1\text{PS}^*$). The singlet excited PS^* can decay back to the ground state with release of energy in the form of fluorescence. It is also possible for the singlet to be converted into the long-lived triplet excited state ($^3\text{PS}^*$), which is able to transfer energy to another triplet (ground-state oxygen) or alternatively carry out electron transfer to oxygen producing a range of ROS *via* superoxide.⁷⁹ Such a paradigm has high therapeutic precision and efficiency, but less invasiveness and side effects in the comparison with that of commonly used chemo- and radiotherapy. However, owing to most PS molecules can only be sensitized

by visible or even UV light, traditional PDT has difficulty in the practical treatment of tumors and other diseases in deep tissues. In light with the unique NIR photon-upconverting feature of UCNPs, NIR light-triggered tissue-penetrable PDT platforms could be successfully fabricated for the practical phototherapy.^{80,81}

Scheme 6. (a) Jablonski diagram illustrating the mechanisms of PDT including type I and type II photoreactions. (b) UCNCs with representative photosensitizers for NIR photodynamic therapy.^{73,79}

To build UCNCs-based PDT systems, those widely used organic or inorganic PS molecules need to be loaded onto UCNCs thereby allowing effective photosensitization (Scheme 6b). Generally, there are several common approaches to functionalize UCNCs as nanocarriers comprising silica encapsulation, polymers (PEG, PAA, PEI, PVP, chitosan and dextran etc.) encapsulation and covalent conjugation. To improve the efficacy of upconversion PDT, core-shell or core-shell-shell nanostructured UCNCs with enhanced UCL were also developed.

1.6 NIR upconversion optogenetics

Using light to control biomolecules and biological processes have also gained much attention based on its unsurpassable flexibility and spatiotemporal precision.⁸² One typical technology, termed as “optogenetics”, has been extensively utilized for optical manipulation neural activity in neuroscience with high specificity and temporal resolution (millisecond-

timescale).⁸³⁻⁸⁵ Most importantly, the rapid evolution of light-sensitive microbial opsins also opens up opportunities for driving optogenetic ion channels manipulation into more complex systems, ranging from single cell level *in vitro* to brain circuitries-mediated behavioral analyses in freely moving animals.^{86,87} However, despite the remarkable achievements, currently established opsins or other optogenetic tools for membrane channels modulation are mainly sensitized in visible window (Figure 2a), which significantly limits their uses for *in vivo* applications due to the unsatisfactory light penetration and less controlling efficacy caused by biological tissues absorption and scattering (Figure 2b and c).^{88,89} Although the approach by optical fiber or LED implantation could partly achieve deep tissue stimulations, the highly invasive procedure raises tremendous safety concerns.^{90,91} Hence, the engineering of novel optogenetic technique that allows minimized invasion in deep tissues for controlling of membrane channels is of great importance.

Figure 2. Optogenetic tools-channelrhodopsins for membrane ion channels manipulation and spectral working properties. a) channelrhodopsin-2 from *Chlamydomonas reinhardtii* (ChR2) and channelrhodopsin-1 *Volvox carteri* (VChR1) from nonselective cation channels leading to depolarization of target cells; Archaeorhodopsin-3 (Arch) from *Halorubrum sodomense* works as a proton pump and leads to hyperpolarization of the target cell such as the chloride pump NpHR (NpHR) from *Natronomonas pharaonis*.⁸⁶ b) Reduced scattering coefficients of various

tissues plotted as a function of wavelength covering 400 – 1700 nm.⁹² c) Tissue penetration depth of light with different wavelengths.⁹³ This figure was reprinted with permission.⁴³

Notably, extensive efforts have been devoted to achieving the goal for *in vivo* optogenetic stimulation, in which shifting the excitation wavelength to the NIR window is considered to be favorable for deeper tissue penetration.^{94,95} Although significant progress has been acquired in genetically engineering of red-shifted ChRs,^{96,97} current optogenetics is still constrained within the visible wavelength window (*e.g.*, < 650 nm). Alternatively, by employing a combination of NIR light responsive nanomaterials and optogenetic tools encourages the possibility of enhanced penetration, where the nanomaterials mainly serve as photon-transformer in optogenetic stimulation.⁹⁸⁻¹⁰⁰ To this end, UCNPs as emerging photonic nanomaterials with their fascinating capability to convert NIR light (*e.g.* at 980 nm or 808 nm) into multiple emissions ranging from UV-Vis to NIR region, have attracted numerous interests in bioimaging and nanomedicine due to the less scattering and deeper penetration depth into tissues.¹⁰¹⁻¹⁰⁴ It is noted that the development of NIR light-triggered optogenetics though incorporating multiple emissive UCNPs into various light-sensitive opsins, terms as upconversion optogenetics, have been witnessed in the past few years, relevant progress and results in membrane ion channels control have already been widely reported (Table 3).¹⁰⁵

Scheme 7. Upconversion optogenetics with various chromophores of photoreceptor proteins.

Table 3. Summary of current applications of upconversion optogenetics.

Optogenetic tools	UCNPs	Ex./UCL ^{a)} (nm)	Power density ^{b)} mW/mm ²	Applications	Ref
LOV2- STIM1/ORAI1	NaYF ₄ :Yb/Tm@NaYF ₄	980/480	15 - 50	Optogenetic activation Ca ²⁺ signaling for immunomodulation <i>in vitro</i> and <i>in vivo</i>	106
	NaYF ₄ :Yb/Tm@NaYF ₄		1 W (Power)	Optogenetic activation of Ca ²⁺ channel <i>in vitro</i>	107
	NaYF ₄ :Yb/Tm		4 W (Power)	Optogenetic activation of Ca ²⁺ channel <i>in vitro</i> and in <i>C. elegans</i>	108
ChR2	NaYbF ₄ :Tm@NaYF ₄	808/480	50	Optogenetic activation of ChR2 <i>in vitro</i>	109
	NaYF ₄ :Yb/Tm		22.6		110
	NaYF ₄ :Yb/Tm		20	Optogenetic stimulation of the spinal cord in behaving animals	111
	NaYF ₄ :Yb/Tm/Nd@ NaYF ₄		8	Optogenetic activation of Ca ²⁺ channel <i>in vitro</i> and in zebrafish	112
C1V1, mChR1, PsChR	NaYF ₄ :Yb/Sc/Er or NaYF ₄ :Yb/Sc/Tm@NaYF ₄	980/550 or 480	41	Optogenetic activation of various ChRs <i>in vitro</i>	105
ChR2, C1V1	NaYF ₄ :Yb/Tm@NaYF ₄ NaYF ₄ :Yb/Er@NaYF ₄	980/480 or 550	7 or 4	Multiplexed optogenetic activation or inhibition of neurons <i>in vitro</i> and <i>in vivo</i>	113,114
ReaChR	NaYF ₄ :Yb/Er@NaYF ₄ @Dye	800/550	20	Optogenetic activation of hippocampal neurons <i>in vitro</i>	115
eNpHR	NaYF ₄ @NaYF ₄ :Yb/Er@NaYF ₄	980/550	6	Optogenetic inhibition of neurons <i>in vitro</i> and <i>in vivo</i>	116
ChR2, Arch	NaYF ₄ :Yb/Tm NaYF ₄ :Yb/Er	980/480 or 550	1400	Optogenetic deep brain activation and inhibition	117
Chrimson	NaGdF ₄ :Yb/Er/Ca@ NaYbF ₄ :Gd/Ca@NaNdF ₄ :Gd	808/550	640	Optogenetic manipulation of the <i>caenorhabditis elegans</i> motor circuit	118
Jaw, VChR1	NaErF ₄ :Yb/Tm@ NaYF ₄ :Yb@ NaNdF ₄ :Yb	980/660 808/550	4 W (Power)	Programmable photoactivation of ion channels <i>in vitro</i>	119
Cry2-FADD/Cib- Fas	NaYF ₄ :Yb/Tm@NaGdF ₄ :Yb	980/480	4 W (Power)	Optogenetic activation of apoptosis signaling for cancer therapy	120

^{a)} UCL indicates upconversion luminescence.

^{b)} NIR laser power density used in practice, especially for *in vivo* stimulation.

In recent years, the utilization of UCNPs for classical optogenetic activation has provided great opportunities for NIR manipulation of neuronal or non-neuronal functions.¹²¹ Moreover, upconversion optogenetics allow highly spatial and temporal control of biological signaling pathways both *in vitro* and in deep tissues. So far, various optogenetic tools with different chromophores-based photo-actuators are developed (Scheme 7). These light-sensitive systems including microbial light-gated ion channels (channelrhodopsins, ChRs) and other soluble photoreceptor proteins that have enabled specific regulation of biomolecular reactions localized in the cell membrane, cytoplasm and nucleoplasm.¹²² In light with the large anti-stokes shift and multicolor-turning capabilities of UCL, current progresses of UCNPs-mediated optogenetics have achieved precise photocontrol of neuronal excitability, behaviors, immunomodulation and tumor phototherapy. Some reviews have discussed the basic design principle and recent applications of upconversion optogenetics.^{123,124} Therefore, in this thesis we mainly highlight the technical improvements of UCNPs functionalization and representative NIR optogenetic manipulations *in vivo*.

For instance, Zhou and Han et al. demonstrated another type of UCNPs-based optogenetic platform, termed “Opto-CRAC”. Such unique optogenetic system was capable of remote and selective control Ca^{2+} influx, subsequent Ca^{2+} -dependent gene expression and further photo-modulate of immune-inflammatory responses to specifically destruct tumor cells both *in vitro* and *in vivo*.¹⁰⁶ Zhang et al. utilized quasi-continuous wave NIR excitation approach to improve the efficacy and applicability of upconversion optogenetic neuromodulation and manipulation of touch-akin reversal behavior in *C. elegans* model expressing ChR2.¹⁰⁸ Shi et al. reported an all-optical method by utilizing UCNPs based micro-devices to remote control neurons expressing different opsin proteins (ChR2 or C1V1) in rat brain.^{113,114} Upon NIR illumination with the robotic laser projection system, this neural stimulation technique was able to reliably trigger spiking activity and spatiotemporally modulate brain activity in various regions,

including the striatum, ventral tegmental area, and visual cortex of behaving rats. McHugh and Liu explored UCNPs as optogenetic actuators to mini-invasively stimulate deep-neurons in the mouse brain.¹¹⁷ Moreover, the NIR-mediated upconversion optogenetics could evoke dopamine release from genetically tagged neurons in the ventral tegmental area (VTA) and induce brain oscillations through activation of inhibitory neurons in the medial septum (MS). Most importantly, this system can also silence seizure by inhibition of hippocampal excitatory cells, and thus trigger memory recall. Such a promising technology will enable NIR manipulation of neuronal activity with the great potential for remote therapy of the neurological disease.

1.7 Cyanine dyes for phototheranostic applications

Cyanine dyes, also called as polymethine dyes are small molecules with two nitrogen-containing aromatic heterocycles linked by a polymethine bridge.¹²⁵ The cyanine derivatives including Cy3, Cy5, HemiCy and Cy7 (Figure 3a) show tunable excitation/emission windows from visible to NIR regions (Figure 3b), which also have high molar absorption coefficient and good fluorescence quantum yield. Such kinds of organic-based optical imaging agents have shown great potentials for clinical translations, of which indocyanine green (ICG) has been approved by USFDA and clinically used in medical diagnostics.¹²⁶ Moreover, recent studies proposed that some cyanine-based molecules, such as IR-1048 and IR-1061 (Figure 3c) could be applied for the emerging NIR-II bioimaging with higher tissue penetration and good spatial resolution as compare with the common NIR-I imaging (Figure 3d).¹²⁷ The broadband NIR absorption of these molecules also enable efficient photo-to-heat conversion for photothermal therapy.

However, most of the cyanine fluorophors suffer from low photostability, undesired aggregation caused quenching (ACQ) effect and high plasma protein binding rate. Continuous efforts have been made to overcome these limitations.¹²⁸ For example, the replacement of the middle of polymethine linker with a rigid cyclohexenyl substitution in Cy7 can remarkably

increase the photostability and fluorescence quantum yield.¹²⁹ The fluorescence efficiency could be also greatly increased upon binding of cyanine dyes with proteins or nucleic acids due to the rigidization of the fluorophores. Besides, the introduction of sulfonic acid or carboxylic groups in the cyanine structures result in a large Stokes shift, and great improvement of water solubility.¹³⁰ Other than the refinements of photophysical properties, numerous studies were reported in recent decades to further diversify the biosensing applicability of cyanine dyes. On the one hand, the dye molecules exhibit feasible conjugation with different kinds of specific molecules, such as chemical molecules, proteins, amino acids, nucleotides, DNA, RNA and antibodies, which have been applied in molecular imaging of various bioanalytes. On another hand, the NIR cyanine dyes are available to conjugate with disease-targeted ligands or disease biomarker-responsive moieties, which has proved to be very promising approach for diseases detection (*e.g.*, tumor, bacterial infection).¹²⁸ These advances have significantly promoted the optical imaging and hold promises to the development of precision medicine.

Figure 3. Representative NIR cyanine dyes for phototheranostic applications. (a, b) Typical structures and excitation/emission spectra of Cy3, Cy5 and Cy7. (c) Representative NIR-II cyanine dyes IR-1048 and IR-1061. (d) Brain vasculature imaging in mice with either NIR-I or NIR-II dye molecules. Reproduced from Ref¹³¹.

1.8 CTNs-based photothermal conversion and phototheranostics

Charge-transfer nanocomplex (CTN) materials are organic cocrystals or assemblies of the charge-donating (D) and charge-accepting (A) molecules, which have been exhaustively studied due to their wide range of physicochemical properties, especially in the ferroelectricity, photoconductivity and photothermal effects.¹³² Extensive investigations demonstrated that the intramolecular charge-transfer (ICT) could induce the formation of strong charge-transfer (CT) optical bands ranging from UV/Vis to the NIR window in the electromagnetic spectrum,¹³³ this results in the intense light harvesting and photothermal conversion.

A variety of charge-transfer-based photothermal materials working in both NIR-I and NIR-II optical windows have been exploited and applied in optical imaging and phototheranostics (Figure 4).¹³⁴ For example, Pu et al. reported the development of organic SPNs as photothermal nanoagents with the broadband and tunable absorption in both NIR-I and NIR-II regions, which enable superior deep-tissue photothermal conversion for enhanced PA imaging and tumor therapy.¹³⁵ Haam and Huh et al formulated the organic polyaniline nanomaterial for cancer PTT.¹³⁶ Besides, Li et al developed CTN-based photothermal immunoassay platforms through the Fe_3O_4 -TMB- H_2O_2 colorimetric system.¹³⁷ These PCSs not only show good photothermal converting efficiency, but also possess the superb biocompatibility and potential biodegradability in theranostic utilizations.

Figure 4. Representative organic charge-transfer based photothermal materials excited by NIR-I and NIR-II lasers.

1.9 Research objectives

NIR photon-converting UCNP and CTNs show promising photophysical properties, which have been widely used as effective phototheranostics. On the one hand, the upconversion luminescence enables deep-tissue optical bioimaging, photocontrolled therapeutics release and NIR optogenetic regulation of biological processes. On the other hand, the broadband absorbing CTNs with high photothermal conversion capability allow PA imaging and PTT in the both NIR-I and NIR-II optical windows. However, there are still some opportunities to further diversify the biomedical applications of these NIR PCSs, and significant efforts need to be made to overcome the challenges for future clinical translations.

Therefore, the research objectives and detailed organizations (Scheme 8) of this thesis are listed as follows:

Scheme 8. The conceptual framework for this thesis.

➤ Firstly, I will present intelligent NIR upconversion luminescence (UCL) and PA nanoprobes based on UCNPs and cyanine dyes for highly specific and sensitive sensing of biological indicators in living animals. To achieve this goal, chapter 2 introduces a multifunctional imaging nanoprobe through the combination of different radical species-responsive cyanine molecules with multi-emissive UCNPs, such a nanocomposite allows simultaneously sense oxidative and nitrosative stress in drug-induced liver damage (DILD) models; additionally, chapter 3 presents a novel UCL and PA cross-referencing nanoprobe for dynamic manifestation of oxidative status in the hepatopathological process, which further enables earlier detection of liver failure, optical feedback of therapeutic response and *in vivo* pharmaceutical screening.

➤ In the second part, I attempt to develop biocompatible and environment-activated NIR-II PA probes for the effective detection and phototherapy against life-threatening disease (*e.g.*, tumor). Accordingly, two organic TMB/TMB⁺⁺-based CTNs as promising NIR-II phototheranostics are introduced in chapter 4 and 5, which are further applied for tumor

microenvironmental redox and acid PA imaging, as well as combined photothermal therapy. Moreover, these nanoplateforms with the biodegradable feature in normal physiological conditions endow good biosafety for potential translational research.

➤ In chapter 6, considering that bacterial infection-associated multiple free-radical dynamics is critical to accurately profile the pathogenesis and host defense mechanisms, however, few techniques currently exist can fully achieving this goal in complex living conditions. I present a NIR small-molecule probe (HCy5-Cy7) for simultaneously and sensitively monitoring of multiple reactive oxygen and nitrogen species variations. Such multispectral signal responses allow HCy5-Cy7 to multiplexly investigate the dynamic oxidative/nitrosative stress events. Most importantly, this multifunctional probe molecule is proposed to reveal the multiple RONS correlations and implications during the pathogenic and defensive processes of bacteria-infected living models.

➤ At last, in chapter 7, to further explore the biological applications of UCNPs, I will introduce a novel optogenetic technology based on CRY2-Akt2 and Myr-CIBN (Opto-Akt2). Herein UCNPs act as light nanotransducers that emit blue emissions upon NIR light irradiation, the upconversion luminescence subsequently triggers the CRY2-CIBN dimerization and Akt2 photoactivation. Such a NIR optogenetic platform further enables effective upregulation of insulin/Akt2 signaling pathways through enhancing the GLUT4 vesicles translocation and glucose uptake, providing the oppurtunaties for insulin resistance management.

1.10 References

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Chapter 2 Multispectral optoacoustic imaging of dynamic redox correlation and pathophysiological progression utilizing upconversion nanoprobes

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2.1 Introduction

Currently, early diagnosis and treatment of diseases in clinics demand the capability to comprehensively understand the intricate signaling pathways, and to precisely track their physiological and pathological processes in real-time.¹ Owing to the heterogeneous and complex nature of living systems, the bioassays or sensing reporters addressing only one single biological indicator may not be able to fully reveal the complications. In addition, lack of sufficient sensitivity and specificity to represent multiple pathophysiological variations may greatly restrict their effective validation of the pathogenesis at the different stages.² Development of specific and unified strategies that allow multiplex screening of various biomarkers, and importantly, precisely reflect the dynamic correlations of different signaling bio-regulators associated with diseases etiology and procession remains challenging in the fields.

As the essential signaling mediators in human beings, multiple reactive oxygen and nitrogen species (RONS), have been extensively authenticated as significant functional regulators involved in many physiological processes such as cellular communication, signal transduction, intermediary metabolism and immune- or inflammatory response.³ The altered redox balance may cause severe oxidative or nitrosative stress that could be closely implicated in the pathologies of a diverse range of diseases.⁴ Moreover, mounting investigations have indicated that the generation of ROS or RNS are not static, but rather, their excess or shortage, or even spatiotemporal distribution and correlation are always processing with a highly dynamic and programmed precision. Such biological diversities of free radicals provide the possibility to make them act as reliable endogenous biomarkers for precise profiling of the pathophysiological implications in complicated living systems.

Conventional strategies based on individual radical sensing encountered technical concerns that may critically prevent their implementation for direct determination of multiple

free radicals within a programmed and longitudinal resolution.⁵⁻⁸ Although monitoring of both oxidative and nitrosative stress could be initially achieved through combination of different sensing modalities,^{9,10} *in vivo* imaging of the dynamic variations of different redox species orthogonally and real-time tailoring of their close correlation with pathophysiological processing remain critical concerns. The lack of smart and unified tools toward concurrent recognition of various radicals in deep-seated tissue is still an obvious impediment, and relevant investigations are thus highly desired.

Recently, UCNPs have been extensively applied in bioimaging, molecular sensing and nanomedicine, due to their extraordinary capability to convert near-infrared (NIR) photonic excitations (*e.g.* 808 or 980 nm) into multiple emissions ranged from UV to NIR windows.¹¹⁻¹⁴ Such unique tissue-penetrable, emission-tunable and remarkably multiplexing optical properties, featured by a single photonic excitation, can ideally realize a precise interrelation and meet the complex biological demands by fitting different sensing moieties into one rationally integrated particle matrix, thus rendering UCNPs as superior multispectral reporters to simultaneously read out multiple analytes (*e.g.* ROS and RNS) in highly complex and dynamic living environments.¹⁵⁻¹⁷

As an amazing imaging modality, multispectral optoacoustic tomography (MSOT), which can supply reliable anatomy information to the diseases diagnosis *in vivo* and pre-clinical trials, has recently attracted considerable attention in biomedical research.¹⁸⁻²⁰ MSOT could construct accurate tomographic images *in vivo* by utilizing the non-ionizing radiation in the NIR window to generate ultrasonic waves, which provides promising high-resolution exquisite images at depths in living animals that are hardly accessible by conventional optical imaging approaches.²¹⁻²³ Importantly, MSOT demonstrated multi-wavelength option that could be selectively performed to concurrently exploit different absorbing agents with a well-defined contrast response, thus providing more information-rich feasibility for real-time monitoring of

dynamic phenomena through multiple sensing channels, which can promote the exploration of novel sensing techniques for the investigation of dynamic pathophysiological processes.²⁴⁻²⁷

In this work, an innovative approach for simultaneous screening of multiple radical species, and significantly, dynamic profiling of their intricate correlations with pathophysiological implications *in vivo* was presented by using NIR light mediated UCNPs as optoacoustic nanoprobes. Typically, by taking advantages of multiplexing luminescence of UCNPs converted from NIR laser illumination, two specific ROS and RNS-sensitive cyanine fluorophores with their absorbance overlapping the upconversion emissions were individually incorporated onto nanoprobe surface. Such unique chromogenic modification guaranteed UCNPs with a broad absorbance in NIR region and allowed to monitor the MSOT signal variations. Under oxidative or nitrosative stimulation, the reactions triggered the structural rearrangement or degradation in fluorophores, which thus contributed spectrally opposite MSOT response for reversed ratiometric tracking of multiple radicals with a longitudinal manner, and meanwhile, for dynamic profiling of their correlations with the pathogenesis in living mice.

2.2 Results and Discussion

2.2.1 Rationale of nanoprobes design

As shown in Figure 1, a rational design by integration of NIR light-mediated nanoprobe with a unified MSOT imaging was demonstrated to simultaneously identify disparate oxidative or nitrosative radicals (*e.g.*, $O_2^{\bullet-}$ or $ONOO^{\bullet-}$). Different from conventional probing systems *via* “on or off” signal changes for radicals sensing, the rationale of current imaging probes mainly relies on the unique spectral characteristics of two different NIR fluorophores on UCNPs surface, which can concomitantly respond ROS and RNS stimulation to produce optoacoustic (OA) signals in opposite directions at different wavelengths. Such redox-triggered spectral

changes could be interlocked in a ratiometric manner, providing great benefits for simultaneous and dynamic MSOT imaging of ROS and RNS under two independent optical channels.

Figure 1. Illustration of the design of multiple radicals-sensitive upconversion nanoprobe (UCNs) and their application in dynamic profiling of pathophysiology implications *in vivo*.

Typically, the nanoprobe was fabricated by embedding two ROS/RNS responsive substrates in a branched polymer layer on the surface of lanthanide-doped UCNPs *via* hydrophobic interaction.²⁸ Upon ROS treatment, the hydro-cyanine substrate (HCy5) regenerated the π -conjugation structure of Cy5 with a maximum absorbance at ~ 640 nm,²⁹ leading to the OA signal enhancement. While upon addition of RNS, the cyanine substrate (Cy7) with a maximum absorbance at 780 nm underwent the structural degradation, causing a reduction of OA signal at 800 nm.³⁰ Moreover, considering the multicolor emissions of UCNPs under 980 nm laser irradiation, the UCL decreased at 660 nm with ROS treatment, and increased at 800 nm with RNS incubation *via* luminescence resonance energy transfer (LRET).³¹ Such promising imaging strategies through MSOT and UCL provide a multiplex signal readout for different radicals at NIR spectral window, which facilitate the non-invasive multiple radicals monitoring in complex living conditions.

2.2.2 Characterization of nanoprobe

The upconversion nanoplateforms with multiple emissions at 660 and 800 nm, were established by doping core-shell nanoparticles with Er^{3+} and Tm^{3+} ions.³² These core-shell nanostructures were further modified with branched polyethylenimine (PEI_{25000}) and polyethylene glycol (PEG_{5000}) to enhance biocompatibility and to facilitate the incorporation of radical responsive HCy5/Cy7 fluorophores into the particles surface layer. Transmission electron microscopy (TEM) image indicated a spherical morphology of well-defined UCNs with a size distribution at ~ 20 nm, while the dynamic light scattering (DLS), and zeta potential analysis indicated a uniform hydrodynamic diameter (100 ± 27 nm) and positive charge surface (21.8 ± 2.8 mV) (Figure 2a-b). Laser irradiation (*e.g.*, at 980 nm) of the polymer-modified UCNPs induced strong UCL signals at both 660 nm and 800 nm, which were dramatically reduced after HCy5 and Cy7 coating on particle surface due to LRET effect (Figure 2c), as demonstrated by the luminescence lifetime decays of UCNs upon 980 nm excitation (Figure 2d). The obvious absorbances of cyanine fluorophores were used to determine the loading efficacy, and there were 8.9 % of HCy5 and 12.2 % Cy7 found on UCNs surface, respectively (Figure 2e).

Figure 2. Characterization of as-prepared nanoprobes. (a) TEM and DLS analysis of UCNs, Scale bar: 50 nm. (b) Zeta potential analysis. (c, d) The UCL spectra and lifetime decay curves of UCNs at 800 nm with (red) or without (gray) surface encapsulation of Cy7 (Ex: 980 nm). (e) HPLC quantification of Cy7 and HCy5 loading efficacies. The standard curves for Cy7 and HCy5 based on their absorbance at 780 and 385 nm, respectively.

2.2.3 ROS/RNS response of nanoprobes

We first evaluated the capabilities of UCNs to simultaneously differentiate multiple radicals in buffers (pH 7.4). Prior to radical treatment, UCNs alone presented a strong absorbance of Cy7 at 780 nm (Figure 3a). In the presence of typical ROS, *e.g.*, superoxide anion ($O_2^{\bullet-}$, 100 μM), a significant absorbance enhancement at 640 nm was observed, due to the HCy5 structure conversion induced by rapid $O_2^{\bullet-}$ oxidation, while similar ROS treatment will not alter Cy7 structure and there was no obvious spectral change at 780 nm, suggesting the selective $O_2^{\bullet-}$ recognition between HCy5 and Cy7. Conversely, in the presence of RNS, *e.g.*, peroxynitrite ($ONOO^-$, 100 μM), almost no absorbance change was detected at 640 nm. However, RNS

treatment of UCNs easily caused the absorbance decrease at 780 nm due to the irreversible oxidative degradation of Cy7 structure. Moreover, the combined stimulation of ROS ($O_2^{\cdot-}$, 20 μM) and RNS ($ONOO^-$, 20 μM) for UCNs led to the selective absorbance increase at 640 nm and decrease at 780 nm simultaneously, which could be further differentiated by utilizing the quantitative mathematical deconvolution technique,³³ suggesting such specific reaction-mediated absorbance change provided unique advantages for selective ROS and RNS status in living settings. Moreover, apart from a certain similarity between $O_2^{\cdot-}$ and $\cdot OH$, as well as the oxidative Cy7 degradation between $ONOO^-$ and ClO^- , no obvious response was observed upon other radical treatment including $ROO^\cdot$, NO and H_2O_2 *etc.* (Figure 3b), clearly showing the beneficial specificity of UCNs for simultaneously differentiation of ROS (*e.g.*, $O_2^{\cdot-}$) and RNS (*e.g.*, $ONOO^-$).

Figure 3. OA and UCL responses of UCNs upon ROS/RNS treatment in buffers. (a) UV-vis absorption spectra of UCNs (1 mg mL⁻¹) upon $O_2^{\cdot-}$ and $ONOO^-$ treatment. (b) Specificity of UCNs with the normalized absorbance at 640 and 780 nm upon various radical species (100 μM) stimulation. (c, d) OA response upon different concentration of $O_2^{\cdot-}$ and $ONOO^-$ treatment. (e) UCL spectra of UCNs upon $O_2^{\cdot-}$ and $ONOO^-$ treatment (E_x : 980 nm).

We also examined the capability of UCNs as optoacoustic contrast agent to differentiate ROS and RNS. Typically, we incubated UCNs with $O_2^{\bullet-}$ and $ONOO^-$ in PBS (pH 7.4), and the radicals triggered OA changes were measured by a unique MSOT setup with NIR excitation at the range of 680-980 nm. UCNs treated with $O_2^{\bullet-}$ demonstrated a significant OA signal enhancement at 680 nm but negligible change at 800 nm, leading to a ratiometric value ($\Delta OA_{680}/OA_{800}$) of 0.64 ± 0.06 (Figure 3c). Whereas addition of $ONOO^-$ obviously decreased the maximum OA signal at 800 nm rather than that at 680 nm, thus contributing a dramatic reverse ratiometric change (Figure 3d). The optoacoustic response was also linearly correspondent to different radicals in PBS (pH 7.4) with the sensing limit down to 65 nM and 118 nM for $O_2^{\bullet-}$ and $ONOO^-$, respectively. Such opposite OA signal variations supplied an ideal amenability to dual-channel sensing of various ROS and RNS at 680 and 800 nm, respectively.³⁴

Moreover, the simultaneous multiple radical responses were also evaluated by the multiplexing UCL signals upon a single NIR photonic irradiation of UCNs at 980 nm. As shown in Figure 3e, stimulation of UCNs with $O_2^{\bullet-}$ led to a reduced UCL emission at 660 nm due to the LRET between UCNs and regenerated Cy5 structures triggered by ROS oxidation. While $ONOO^-$ treatment degraded Cy7 structure on UCNs surface, which directly abated LRET and therefore caused an obvious recovery of UCL emissions at 660 nm and 800 nm, respectively.

2.2.4 *In vitro* UCNs response to endogenous redox species

We further investigated the capability of UCNs to monitor the endogenously generated radicals in murine RAW264.7 macrophage cells through both MSOT and optical UCL imaging. Briefly, the excessive $O_2^{\bullet-}$ and $ONOO^-$ generation in cells were achieved by pretreating with phorbol 12-myristate 13-acetate (PMA) and mixture of lipopolysaccharide (LPS)/interferon- γ (INF- γ)/PMA, respectively, which were confirmed by confocal microscopy based on the

standard fluorimetric intracellular peroxynitrite (green) and superoxide (red) assay trackers (Figure 4a).^{6,35}

Figure 4. OA and UCL responses of UCNs toward endogenous radical species in living cells. (a) Confocal imaging of ROS/RNS generation in RAW264.7 cells upon the treatment of PMA and LPS/INF- γ /PMA followed by MnTBAP and MEG as specific scavenger for superoxide ($O_2^{\bullet-}$) peroxynitrite ($ONOO^-$) species, respectively. Blue: Hoechst 33342 (E_x : 405 nm, E_m : 460/50 nm), green: peroxynitrite indicator (E_x : 480 nm, E_m : 520/50 nm), red: superoxide indicator (E_x : 565 nm, E_m : 590/40 nm). (b) OA signals at 680 and 800 nm of macrophage cells in different groups: (1) cells only; (2) cells incubate with UCNs; (3) PMA-stimulated cells ($O_2^{\bullet-}$ generation) with UCNs; (4) PMA-stimulated cells with $O_2^{\bullet-}$ inhibitor (MnTBAP) and UCNs; (5) LPS/INF- γ /PMA-stimulated cells ($ONOO^-$ generation) with UCNs; (6) LPS/INF- γ /PMA-stimulated cells with $ONOO^-$ inhibitor (MEG) and UCNs, respectively. Scale bar, 1 mm. (c) UCL fluorescence imaging of RAW264.7 cells incubated with UCNs ($100 \mu\text{g mL}^{-1}$) in different groups. Green: UCL540 (E_x : 980 nm, E_m : 540/50 nm), red: UCL660 (E_x : 980 nm, E_m : 640/50 nm), violet: UCL800 (E_x : 980 nm, E_m : 790/30 nm). Scale bar, 20 μm .

Then, the cell samples were collected for MSOT measurement after 4 h incubation with UCNs. As shown in Figure 4b, cellular inflammation triggered by PMA produced $O_2^{\bullet-}$ species (in group 3), which presented an obvious OA enhancement at 680 nm but less change at 800 nm. Whereas the cell stimulation with LPS/INF- γ /PMA for $ONOO^-$ generation (group 5) displayed an obvious attenuation at 800 nm but less change at 680 nm. Additionally, cellular treatment with scavenger (*e.g.*, Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) for $O_2^{\bullet-}$

or mercaptoethyl guanidine (MEG) for ONOO^-) caused a less OA change similar to the cells under resting states.

The potential UCL signal changes corresponding to multiple ROS/RNS treatment were also examined by confocal microscopy upon 980 nm laser excitation (Figure 4c). The decrease of UCL emissions was observed at 660 nm with PMA stimulation, indicating the LRET between UCNs and regenerated Cy5 mediated by ROS oxidation, while the cellular treatment with LPS/IFN- γ /PMA led to the ONOO^- oxidation of RNS-responsive Cy7 degradation, thus enhanced the UCL emission at 800 nm due to the split LRET. Both the radical responsive UCL changes could be inhibited with the treatment of $\text{O}_2^{\cdot-}$ and ONOO^- scavengers.

2.2.5 Orthogonally screening the dynamics of multiple redox species *in vivo*

The *in vivo* MSOT and UCL fluorescent imaging were performed for simultaneously screening of ROS and RNS dynamics *via* intravenous (*i.v.*) injection of UCNs (5 mg/mL in 100 μL saline) into living Balb/c nude mice ($n = 5$). The OA and UCL imaging were captured to monitor the distribution of UCNs in living mice. Both OA and UCL analysis consistently showed the obvious particles accumulation in the liver (Figure 5), suggesting the feasibility of UCNs as a potent nanoprobe to real-time correlate the dynamics of redox species in hepatocytes with various stages of inflammatory progression.

Figure 5. *In vivo* dynamic biodistribution of UCNs. (a) UCL800 signals in various organs post tail-vein UCNs injection in different time. (E_x : 980 nm, E_m : 790/30 nm). The organs from 1-7:

1, Heart; 2, Liver; 3, Spleen; 4, Lung; 5, Kidney; 6, Stomach; 7, Intestine. Scale bar: 1 cm. Statistical significance assessed by a Student's t-test (heteroscedastic, two-sided). *** $P < 0.001$; ** $P < 0.01$. (b) Scheme of liver cross-section from MSOT and time-resolved MSOT images at 680 and 800 nm in liver post UCNs injection with pseudo-color ($n = 5$).

As proof-of-concept, in order to mimic early stage inflammation, LPS, a bacterial endotoxin from Gram-negative pathogens, which can induce substantial over-production of ROS *in vivo* (e.g. $O_2^{\bullet-}$), and acetaminophenol (APAP), a typical anti-pain/fever drug, which can stimulate excessive RNS (e.g., $ONOO^-$) were intraperitoneally (*i.p.*) injected into the nude mice.³⁸ Upon subsequent tail-vein administration of UCNs, the MSOT imaging was performed at different time intervals, and the anatomy OA signals at the region of interest (ROI) in liver cross-section were evaluated at 680 and 800 nm by means of pseudo-colour processing, and further analyzed based on the established mathematical deconvolution processing.³³ As shown in Figure 6a, the radical stimulation showed negligible variations at both 680 and 800 nm over the course of imaging at initial 120 min post-injection of UCNs alone, showing the reliable baseline and great stability of UCNs in liver tissues.

The sequential MSOT and UCL optical imaging were acquired immediately after tail-vein injection of UCNs (5 mg/mL in 100 μ L saline). Typically, LPS stimulation resulted in a gradual increasing of ROS generation in mouse liver over the imaging process (Figure 6b). As compared to the signal variations in a control group, APAP treatment displayed a slightly enhancement of deconvoluted OA signal at 680 nm related to ROS production (e.g. $O_2^{\bullet-}$) at initial injection of UCNs. However, a slightly decreasing trend toward ROS response occurred and continuous increment of *in vivo* $ONOO^-$ production were easily observed within 3 h of APAP administration (Figure 6c), suggesting the dynamic correlation between ROS and RNS, and the process of RNS generation was later than that of ROS. When N-acetylcysteine (NAC), a glutathione precursor well-known to scavenge reactive metabolites in hepatocyte cells,³⁹ was used to treat living mice together with LPS or APAP stimulation. Both the deconvoluted OA

signals at 680 and 800 nm returned to the levels close to the control group done by UCNs itself, suggesting the over-production of radicals could be effectively inhibited by NAC.

Figure 6. Simultaneous OA/UCL imaging of multiple radicals *in vivo*. (a) MSOT images at 680 and 800 nm in ROI of liver upon UCNs, LPS and APAP administration for 90 min ($n = 5$). Scale bar: 5 mm. (b, c) Dynamic OA signal variations in hepatic inflammation models. (d) Representative *in vivo* UCL imaging at 660 and 800 nm upon saline, UCNs, LPS and APAP treatment for 90 min (E_x : 980 nm, $n = 5$). Scale bar: 1 cm.

During the same time period, the UCL imaging also showed an obvious signal drop (~ 4.4 folds) at 660 nm in LPS-treated mice and a significant increment (~ 4.7 folds) at 800 nm in APAP-stimulated mice (Figure 6d), which was due to the LRET process between the radical responsive fluorophores and UCNs, further confirming the great potential of UCNs as a promising multimodality nanoprobe for the screening of dynamic oxidative and nitrosative stress *in vivo*.

2.2.6 *In vivo* monitoring of dynamic radical status under various hepaopathological progression

Inspired by the promising results in standard inflammation animal models for orthogonally screening of redox species *in vivo*, it was highly essential to investigate the heterogeneity of multiple radicals to correlate their dynamic changes with the pathological progression in living subjects. To this end, isoniazid, also known as isonicotinylhydrazide (INH), a most commonly used anti-tuberculosis drugs,⁴⁰ and tacrine (THA), a FDA approved agent for Alzheimer's

disease treatment,⁴¹ have been chosen to mimic the different hepatotoxic pathology. INH and THA are capable of inducing oxidative or nitrosative stress, which are associated with severe liver damage in clinics while the detailed mechanisms have not been fully elucidated.⁴²

Figure 7. Dynamic profiling of multiple radical variations in drug-induced hepatotoxic models. (a, b) Time-resolved MSOT signals of UCNs at 680 and 800 nm in mice liver upon overdose INH (200 mg Kg⁻¹), or THA (30 mg Kg⁻¹) treatment ($n = 5$). Scale bar: 5 mm. (c) *In vivo* UCL imaging at 660 and 800 nm upon UCN, INH and THA treatment (E_x : 980 nm, $n = 5$). Scale bar: 1 cm. (d, e) Dynamic profiling of OA signals variations based on UCN in mouse liver at 680 and 800 nm upon INH, THA and their reactive metabolites scavenger (NAC) treatment ($n = 5$). Statistical significance assessed by a Student's t-test (heteroscedastic, two-sided). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

By using radical-responsive NIR fluorophores conjugated UCNs as contrast agents, we performed a dynamic MSOT imaging of ROS/RNS in liver upon overdose INH and THA administration. As shown in Figure 7a, INH treatment presented a short-lived oxidative burst at 680 nm within 30 min and an obvious nitrosative stress-induced signal attenuation at 800 nm within 120 min after UCNs injection. Similarly, the UCL imaging in INH-treated mice also displayed a noticeable emission reduction at 660 nm and an enhancement (~ 2.9-folds) at 800 nm upon NIR light excitation at 980 nm (Figure 7c). The consistent imaging results (Figure 7d-e) suggested a threshold dose-type generation of ROS (*e.g.*, O₂^{•-}) at early stage but a dose-

dependent excessive RNS (*e.g.*, ONOO⁻) generation at prolong stage. Unlike INH, THA administration (30 mg Kg⁻¹) exhibited a sustained OA increment at 680 nm within 60 min while a slight reverse variation at 800 nm up to MSOT imaging at 120 min (Figure 7b and 7e). Furthermore, the UCL imaging displayed considerable emission drop (~ 4.2-folds) at 660 nm but less change at 800 nm (Figure 7c), demonstrating more predominant ROS production and accumulation than that of RNS in the process of MSOT imaging upon overdose THA treatment.

2.2.7 Exploration the RO/NS correlations underlying hepatopathological implications

It has been established that numerous redox species in living systems closely link to a wide variety of pathophysiological events from intrinsic metabolism to acute inflammation, and they can also directly reflect the etiological processing of many chronic diseases.³ In line with the dynamic changes of ROS and RNS levels *in vivo*, the ratiometric MSOT screening of ROS/RNS at 680 and 800 nm could serve as an ideal strategy to real-time track the changes of multiple radicals in a highly complicated living condition (Figure 8a). Therefore, such unique redox-responsive ratiometric MSOT imaging was also used to investigate the correlations between the ROS/RNS variations and inflammation development in living animals.

We examined the liver damage procession by monitoring the activities of two typical enzymes: cytochrome P450 (CYP450), one major redox source for most phase I drug metabolism, and uridine 5'-diphospho-glucuronosyltransferases (UGTs), a key role for phase II glucuronidation to facilitate the drugs biotransformation *in vivo*.^{43, 44} As shown in Figure 8b-c, a gradual production of CYP450 and rapid consumption of UGTs activities were observed at the incipient 60 min after individual administration of APAP, INH and TNH, indicating the intensive drug metabolism occurred in liver. As a typical hepatotoxin, APAP is known to mainly undergo phase II metabolism pathway before its excretion *via* glucuronidation and sulfation. And only a small proportion of CYP450 dependent phase I metabolism was presented to produce a metabolic iminoquinone, N-acetyl-p-benzoquinone imine (NAPQI), which

induced the mitochondrial dysfunction and subsequently initiated nitrosative stress in over-dosage.⁴²

Figure 8. Exploration the RO/NS correlations underlying hepatopathological implications. (a) Normalized ratiometric OA values ($\Delta OA_{680}/OA_{800}$) upon different drugs treatment in living mice. (b, c) Assessment of metabolism-related enzymes including CYP450 and UGTs at different time in liver upon drugs treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (d-f) Variations of serum transaminase (AST, ALT) and inflammation-associated cytokine (IL-10) variations at different time upon different drugs administration ($n = 5$). Data were represented as mean $\pm$ SD. (g) H&E staining in liver tissues at 180 min after INH and THA treatment ($n = 5$). Arrowheads mark centrilobular vein fibrosis (blue), swollen hepatocytes (green) and inflammatory infiltration (red) respectively. CV: central vein. Scale bars, 50 μm . (h, i) Immunohistochemical analysis of 4-HNE and 3-nitrotyrosine in liver sections at 60 and 180 min after UCN, APAP, INH and THA treatment ($n = 5$). Arrowheads mark 4-HNE-positive lesions (black) and nitrotyrosine-positive foci (white) respectively. Scale bars, 100 μm .

Compared to APAP, a similar CYP450 level along with a dramatic depletion of UGTs activities was determined upon INH treatment for 60 min, suggesting that the metabolism of INH *in vivo* would mainly go through phase II pathway, while a fraction of INH oxidation *via* phase I metabolism led to initial radicals generation including ROS/RNS, as indicated in

radiometric MSOT analysis. Additionally, different from APAP, more significant CYP450 enhancements and UGTs suppression were observed upon THA stimulation of living mice for 60 min, clearly indicating the possibility of both phase I and II pathways occurred in THA metabolism, and the higher phase I metabolism presented to induce more oxidation of THA for free radicals generation (*e.g.*, ROS). This data corroborated the controversial metabolic mechanism that THA would be mainly excreted through the glucuronidation process in phase II pathway, and the relevant THA bioactivation involved the CYP450-mediated oxidation to form 1-hydroxytacrine, a reactive metabolite that could cause obvious hepatotoxicity with an increasing radical production.^{45, 46} With regard to the correlations with conventional liver function tests, the serum AST, ALT and inflammation-associated cytokine (IL-10) levels in Figure 8d-f showed significant increase at the 60 min time point after drugs injection, indicating that drug-induced liver damages were gradually appeared.

Moreover, we also performed the liver tissue histological and immunohistochemical analysis at 60 min and 180 min to evaluate the inflammatory response after APAP, INH and THA treatment, respectively. H&E staining indicated a minimum hepatotoxicity within 60 min upon treatment with APAP, INH and THA, however, the typical lobular hepatocyte structures were dramatically destroyed and the disparate histologic changes, such as centrilobular vein fibrosis, swollen hepatocytes, sinusoidal congestion, and inflammatory infiltration were readily observed up to 180 min after drug injection (Figure 8g). These studies clearly suggested the definite hepatotoxicity occurred later than the systematic drug metabolism and initial inflammation reaction, which is the process well-accepted in drug induced liver injury (DILI).

In order to validate the correlations between the dynamic RO/NS changes and the inflammation procession in DILI, the 4-hydroxynonenal (4-HNE) assay, known as a specific hallmark of ROS overproduction *in vivo*, was used to examine the ROS-mediated cell damage. Moreover, as a product of tyrosine nitration in protein, 3-nitrotyrosine has been considered as

a specific biomarker of nitrosative stress in living system. As shown in Figure 8h-i, negligible histological changes in 4-HNE and 3-nitrotyrosine were observed in liver tissue before and after the different drug overdose APAP or INH treatment within 60 min. However, slightly production of 4-HNE-positive foci and obvious generation of nitrotyrosine-positive lesion were detected at 180 min, indicating the occurrence of both oxidative and nitrosative stress after overdose APAP or INH treatment. Furthermore, both APAP and INH administration caused more significant 3-nitrotyrosine staining in live mice, demonstrating the major roles of RNS (*e.g.* ONOO⁻) in the observed inflammation.

Unlike APAP or INH, as an acute hepatotoxic drug withdrawn by FDA,⁴¹ THA stimulation led to a minor extent of nitrosative-positive foci, while more 4-HNE-positive lesions observed at 60 min and 180 min, unequivocally indicating that the oxidative stress may predominantly contribute to the THA-induced liver injury. This histological analysis was in good accordance with the ratiometric MSOT analysis acquired at the initial 180 min, suggesting the close correlations of RO/NS during the DILI processes of each drug, that is, the APAP or INH treatment in living mice may induce the quick burst of ROS and high conversion to RNS, while THA-treated mice will undergo more ROS but a minor RNS generation. These results demonstrated the great potential of UCNs as multispectral responsive nanoprobe to real-time and orthogonal monitor the dynamic changes and correlations of RO/NS biomarkers, and importantly, to precisely sense initial hepatotoxic conditions.⁴⁷⁻⁴⁹

2.3 Conclusion

In summary, an innovative strategy has been successfully presented by integration of unique NIR light-mediated upconverting nanoprobe with multispectral MSOT imaging for reversed ratiometric tracking of disparate oxidative and nitrosative stresses *in vitro* and *in vivo*. Such a promising strategy realizes the multiplex screening of diverse endogenous biomarkers and precise interrelation their dynamic correlations in complex living settings through one

unified imaging modality, which are particularly meaningful to systematically exploit the underlying metabolism pathways and to real-time map out pathological implications towards the diseases at the different stages. This study not only facilitates the better understanding of the pathophysiological roles of various redox species in living animals with non-invasive manner, but more importantly, it also promotes the development of MSOT technology towards the exploration of undefined mechanism of potential pathogenesis in clinics, which may thus boost the pharmaceutical industry for high-throughput drug screening and pathological profiling of dynamic inflammation processing to benefit the healthcare communities in the future.

2.4 Experimental Methods

2.4.1 Materials and instruments

Chemicals and reagents. $\text{Gd}(\text{CH}_3\text{CO}_2)_3$, $\text{Yb}(\text{CH}_3\text{CO}_2)_3$, $\text{Er}(\text{CH}_3\text{CO}_2)_3$, $\text{Tm}(\text{CH}_3\text{CO}_2)_3$, oleic acid, 1-octadecene, NH_4F , NaOH , polyacrylic acid (PAA, Mw 1800), diethylene glycol (DEG), polyethylenimine (PEI, Mw 25000, branched), cytotoxicology assay kit (TOX8, resazurin based), and Hoechst33342 were purchased from Sigma-Aldrich. The MitoSOX mitochondrial superoxide indicator (red fluorescence) and Cell MeterTM fluorimetric intracellular peroxynitrite assay kit (green fluorescence), anti-3-nitrotyrosine anti-4-hydroxynonenal antibody were bought from Abcam (China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Invitrogen (USA). All the commercially reagents were used as received unless otherwise noted.

Instruments. ^1H -NMR and ^{13}C -NMR spectra were measured on a 300 MHz Bruker spectrometer. Mass spectra were measured on a Thermo Polaris Q instrument for EI measurements and a Thermo LCQ Deca XP MAX instrument for ESI measurements. High performance liquid chromatography (HPLC, Shimadzu) system was performed on an Alltima C-18 column of 250×10 mm at a flow rate of 3 mL min⁻¹. UV-vis absorption spectra were

measured using a Beckman coulter DU800 spectrometer. Fluorescence emission spectra were captured with an Agilent Varian Cary eclipse fluorescence spectrophotometer. The fluorescence emission spectra of UCNs were recorded at an angle of 90 °to the excitation laser (980 nm, 2 W cm⁻²) and an optical SEC-2000 spectrometer coupled 2048 pixels CCD assay (ALS Co., Ltd, Japan). Transmission electron microscope (TEM) images were recorded using a FEI EM208S TEM (Philips) operated at 100 kV. Dynamic light scattering (DLS) and zeta potential measurements were performed by Brookhaven 90 Plus nanoparticle size analyzer. The cell viabilities were measured by a Bio-Tek EL-311 microplate reader. The confocal imaging of cells was carried out on Carl Zeiss LSM 800 confocal laser microscope (Germany). The upconversion luminescence (UCL) and Cy7 emission in living cells were recorded separately on a Nikon confocal fluorescence microscope (Nikon, Eclips TE2000-E, Japan) equipped with 980 nm and 786 nm laser wide-field fluorescence add-on (EINST Technology Pte Ltd, Singapore) in our lab. For UCL lifetime measurements, the UCL emission decays of samples were collected on an Edinburgh FLS920 fluorescence spectrometer with an external continuous 980 nm NIR LED laser diode, which was coupled with a chopper to modulate the excitation into pulse mode. All the optoacoustic (OA) imaging experiments *in vitro* and *in vivo* were performed by using a real-time multispectral optoacoustic tomographic (MSOT) imaging system from iThera Medical GmbH (Neuherberg, Germany). The UCL imaging in living mice was performed with an IVIS Lumina II imaging system (Caliper Life Sciences, France) with 980 nm NIR laser irradiation.

2.4.2 Preparation of nanoprobe

2.4.2.1 Synthesis of small-molecule fluorophores⁵⁰

a) Synthesis of Cy7-S

Typically, the starting material **IR780** (183 mg, 0.28 mmol), 3-mercaptopropionic acid (28 μL , 0.32 mmol) and triethylamine (TEA, 44 μL , 0.32 mmol) were mixed in 6.0 mL dimethylformamide (DMF) and stirred at room temperature for 24 h. Then the mixture solution was added drop-wisely into ice-cold methyl ether and the crude product as green precipitate was filtrated. The final product was purified by flash chromatography with the gradient of $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (changed from 100: 1 to 10: 1) to afford **Cy7-S** as a powder (169.3 mg, yield 75%). ^1H NMR (300 MHz, MeOD): δ 8.90 (d, J = 14.2 Hz, 2H), 7.53 (d, J = 7.4 Hz, 2H), 7.47-7.39 (m, 2H), 7.30 (dd, J = 15.6, 7.8 Hz, 4H), 6.32 (d, J = 14.2 Hz, 2H), 4.15 (t, J = 7.2 Hz, 4H), 3.33 (dt, J = 3.3, 1.6 Hz, 4H), 3.09 (t, J = 6.9 Hz, 2H), 2.70 (s, 2H), 2.61 (t, J = 6.9 Hz, 2H), 1.91 (dd, J = 14.7, 7.4 Hz, 4H), 1.78 (s, 12H), 1.08 (t, J = 7.4 Hz, 6H). ^{13}C NMR (75 MHz, MeOD) δ 173.80, 172.73, 145.79, 142.48, 141.07, 134.81, 128.39, 124.83, 122.07, 110.67, 100.69, 49.13, 45.17, 34.70, 32.75, 26.99, 25.84, 20.79, 20.43, 14.00, 10.28. ESI-MS: $[\text{M}-\text{I}]^+$ m/z : 609.59.

b) Synthesis of **HCy5**

Synthesis of compound 3. The compounds 2,3,3-trimethylindolenine (**1**, 1 ml, 6.2 mmol) and methyl iodide (**2**, 0.9 ml, 12.5 mmol) were dissolved in 20 ml acetonitrile (ACN). The mixture solution was refluxed for 10 h, then allowed to cool down to room temperature, and the precipitate was collected by filtration. The solid was washed with *n*-hexane and dried under reduced pressure to afford compound **3** as a brown solid. Yield: 1.7 g (85%). ¹H NMR (300 MHz, CDCl₃): δ 7.61 (d, J = 13.5, 8.0, 3.6 Hz, 4H), 4.29 (s, 3H), 3.13 (s, 3H), 1.59 (s, 6H). ESI-MS: [M-I]⁺ m/z: 232.30.

Synthesis of compound 5. Malonaldehyde dianilide hydrochloride **4** (400 mg, 1.55 mmol) was dissolved in a mixture of dichloromethane (10 mL) and DIEA (0.6 mL). To the mixture was added dropwise a mixture of acetic anhydride (0.4 mL) and dichloromethane (3 mL), followed by stirring at room temperature for 6 hours. Then the mixture was evaporated to obtain compound **5** without further purification.

Synthesis of compound Cy5. Acetonitrile solution of compound **4** (130 mg, 0.5 mmol) was added dropwise to a refluxing acetonitrile solution (10 mL) containing **3** (300 mg, 0.1 mmol) and sodium acetate (50 mg, 0.78 mmol). Reflux was continued for 12 hours, then the reaction mixture was allowed to cool to room temperature, and the resulting solid was removed by filtration. The crude product was further purified with silica gel column chromatography using

DCM/0-2% MeOH as the eluent to afford target product **Cy5** (45%, 114 mg). ^1H NMR (300 MHz, CDCl_3) δ 7.95 (t, $J = 13.1$ Hz, 2H), 7.50 – 7.32 (m, 4H), 7.25 (d, $J = 7.6$ Hz, 2H), 7.13 (d, $J = 7.8$ Hz, 2H), 6.89 (t, $J = 12.7$ Hz, 1H), 6.35 (d, $J = 13.6$ Hz, 2H), 3.70 (s, 6H), 1.76 (s, 12H). ^{13}C NMR (75 MHz, CDCl_3) δ 173.37, 152.81, 142.40, 140.73, 128.42, 126.52, 125.40, 122.13, 110.33, 104.04, 54.61, 31.97, 28.14. ESI-MS $[\text{M}-\text{I}]^+$ m/z 383.43. HRMS (ESI) m/z 383.2491 $[\text{M}-\text{I}]^+$ (calcd for 383.2482, $\text{C}_{27}\text{H}_{31}\text{N}_2$).

*Synthesis of compound **HCy5**.* The prepared **Cy5** (52 mg, 0.1 mmol) was dissolved in 5 mL methanol, then 0.5 mL methanol solution containing NaBH_4 (3.0 mg, 0.08 mmol) was added drop-wisely to the **Cy5** solution and stirred for 10 min until the solution changed to colorless. The reaction mixture was further magnetically stirred for 20 min and removed the solvent under reduced pressure. The residue was dissolved in 10 mL DCM and extracted by 5 mL water with vigorously shaken. The collected organic layer was dried by anhydrous Na_2SO_4 , and the solvent was finally removed under reduced pressure. The product **HCy5** thus was obtained without further purification. ^1H NMR (300 MHz, CDCl_3) δ 7.17 (dd, $J = 15.7, 7.5$ Hz, 3H), 7.04 (t, $J = 11.8$ Hz, 1H), 6.92-6.73 (m, 3H), 6.59 (dd, $J = 12.3, 7.9$ Hz, 2H), 6.46 (dd, $J = 14.8, 10.8$ Hz, 1H), 6.17 (dd, $J = 14.0, 11.1$ Hz, 1H), 5.62 (dd, $J = 14.9, 9.1$ Hz, 1H), 5.34 (d, $J = 11.4$ Hz, 1H), 3.33 (d, $J = 9.1$ Hz, 1H), 3.13 (s, 3H), 2.71 (s, 3H), 1.63 (s, 6H), 1.34 (s, 3H), 1.10 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 156.58, 151.64, 145.38, 139.40, 138.74, 136.15, 129.29, 127.69, 127.45, 125.66, 124.44, 121.67, 121.50, 119.10, 118.38, 107.88, 105.57, 95.83, 81.07, 45.26, 44.29, 34.36, 28.99, 28.34, 28.31, 25.62, 24.12. ESI-MS: $[\text{M}+\text{H}]^+$ m/z : 385.47. HRMS (ESI): $[\text{M}+\text{H}]^+$ m/z : 385.2647 (calcd for 385.2644, $\text{C}_{27}\text{H}_{33}\text{N}_2$).

2.4.2.2 Preparation and modification of UCNs

a) Preparation of NaGdF₄:Yb/Er/Tm@NaGdF₄ (20/2/1%) core-shell UCNPs⁵¹

2 mL methanol containing RE(CH₃CO₂)₃ (RE = Gd, Yb, Er and Tm), 3 mL oleic acid and 7 mL 1-octadecene were added in a 50 mL three-neck flask. The ratio of Yb/Er/Tm is 20/2/1% and the total lanthanide amount is 0.4 mmol. The mixture was heated to 150 °C for 60 min before cooling down to room temperature. Subsequently, a methanol solution (6 mL) containing NH₄F (59.3 mg) and NaOH (40.0 mg) was added and stirred for 30 min at 50 °C. Then methanol was evaporated, and the solution was kept at 290 °C for 1.5 h under nitrogen atmosphere. The core particles were then precipitated by ethanol after cooling down to room temperature, collected through centrifugation several times after ethanol washing, and re-dispersed in 4 mL hexane for next step.

For the core-shell UCNPs synthesis, 3 mL oleic acid and 7 mL 1-octadecene were added in a 50 mL three-neck flask, then 2 mL methanol containing 0.4 mmol Gd(CH₃CO₂)₃ was also added in the solution. The mixture was heated to 150 °C for 60 min before cooling down to room temperature. The as-synthesized NaGdF₄:Yb/Er/Tm core particles in 4 mL hexane were added along with a methanol solution (6 mL) containing NH₄F (59.3 mg) and NaOH (40.0 mg). The resulting mixture was stirred for 30 min at 50 °C. Then methanol was evaporated and the solution was kept at 290 °C for 1.5 h under nitrogen atmosphere. After cooling down to room temperature, the core-shell nanoparticles were precipitated using ethanol and collected through centrifugation several times after ethanol washing. Finally, the core-shell UCNPs were re-dispersed in 4 mL cyclohexane and conserved in 4 °C fridge.

b) UCNPs modification and fluorophores loading

Synthesis of PEI and PEG modified UCNPs. Briefly, the as-prepared oleate-capped UCNPs were precipitated using ethanol and re-dispersed in a 10 mL acid aqueous solution (pH = 4) adjusted by HCl (0.1 M). Then the solution was sonicated for 30 min following with vigorous stirring for 2 h. During this reaction, the carboxylate groups of the oleate ligands were

protonated (to yield oleic acid). Then the aqueous solution was extracted with 30 mL diethyl ether to remove the oleic acid for three times. The combined ether layers were re-extracted with 10 mL water. Finally, the dispersible ligand-free UCNPs in the water were recuperated by centrifugation (20,000 rpm, 10 min) after precipitation with 20 mL acetone. The ligand-free UCNPs were re-dispersed in 10 mg/mL branched PEI solution ($M_w = 25,000$) by sonication. After stirring for 24 h at room temperature, the products (PEI-UCNs) were collected by centrifugation (20,000 rpm for 10 min), washed with water for several times, and re-dispersed in water.

Then PEI-UCNPs were further functionalized with PEG-COOH by the coupling reaction. Briefly, 100 mg of PEG₃₄₀₀-COOH (Merck) were dissolved in water (5 mL) containing EDC (47.9 mg, 0.25 mmol) and NHS (57.5 mg, 0.5 mmol) to form the active succinimidyl ester for 30 min. Then the mixture was added slowly to 5 mL PEI-UCNPs (10 mg mL^{-1}) water solution with 30 min sonication. After vigorous stirring overnight, the UCNPs@PEI-PEG was washed with DMF and water for several times respectively by centrifugation (18,000 rpm for 10 min) and re-dispersed in 5 mL PBS with the concentration of 10 mg mL^{-1} .

The cyanine fluorophores HCy5 and Cy7 were loaded on the surface of PEG/PEI-UCNPs by hydrophobic interaction. The mixture of Cy7 (10 mg) and HCy5 (2 mg) in dimethyl sulfoxide (DMSO) were added drop-wisely to the PEG/PEI-UCNPs solutions (5 mg mL^{-1}) in a 10 mL vial under sonication for 30 min. Finally, the mixture was stirred vigorously for overnight at room temperature, and the HCy5/Cy7-UCNPs were collected by centrifugation (18,000 rpm, 10 min) followed by several times washing to remove excess reactant. The loading amounts of Cy7 was determined based on its specific absorption peak at 780 nm, and the embedded HCy5 molecule was extracted by DMSO for further HPLC analysis based on its specific absorbance at 385 nm.

2.4.3 Preparation of various radical species

The different stock solutions of these radical species were prepared by following the reported methods.⁵²

OONO⁻: The peroxyxynitrite (ONOO⁻) stock solution was prepared by adding three kinds of precursor solutions, including the mixture of hydrogen peroxide (0.7 M, 1.5 mL) in hydrochloric acid (0.6 M, 1.5 mL), sodium nitrite solution (0.6 M, 3 mL) and sodium hydroxide solution (1.5 M, 3 mL) simultaneously. The resulting solution was stored at -20 °C for further use. The concentration of the OONO⁻ solution was determined by measuring the absorbance at 302 nm with a molar extinction coefficient of 1670 M⁻¹ cm⁻¹ in 0.1 M NaOH.

$$C_{\text{ONOO}^-} = \text{Abs}_{302 \text{ nm}} / 1.67 \text{ (mM)}$$

ClO⁻: The hypochlorite (ClO⁻) stock solution was prepared by dissolving appropriate commercial NaClO solution (4-5%) in 10 mL deionized water and the concentration of the ClO⁻ was determined by measuring the absorbance at 209 nm with a molar extinction coefficient of 350 M⁻¹ cm⁻¹.

$$C_{\text{ClO}^-} = \text{Abs}_{209 \text{ nm}} / 0.35 \text{ (mM)}$$

H₂O₂: The 3% H₂O₂ stock solution was purchased from Sigma-Aldrich, which can be diluted accordingly to the desired concentration before using.

O₂^{•-}: The superoxide radical anion (O₂^{•-}) was freshly prepared before using, based on reported methods by dissolving commercial available solid potassium superoxide (KO₂) in the buffer.

•OH: The •OH stock solution (1 mM) was generated in the Fenton reaction from iron (II) chloride (FeCl₂) and hydrogen peroxide (H₂O₂) under the following equation. Briefly, the FeCl₂ was dissolved in water (2 mM), and added quickly to the equivalent H₂O₂ solution (1 mM) for further use. $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{•OH} + \text{OH}^-$

ROO[•]: The peroxy radicals (ROO[•]) stock solution (0.1 mM) was prepared by diluting 0.137 mL commercially available tert-butyl hydroperoxide (t-BuOOH, 70%) in 10 mL water.

NO: The nitric oxide (NO, 1 mM) was generated from sodium nitroferricyanide (III) dehydrate ($\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}] \cdot 2\text{H}_2\text{O}$, 3.0 mg) in 10 mL water.

2.4.4 Multiple radical species detection in buffers

Different concentration of RO/NS including OONO^- , ClO^- , $\text{O}_2^{\cdot-}$, $\cdot\text{OH}$, $\text{ROO}^\bullet$, NO and H_2O_2 were added to 1 mL UCNs nanoprobe solution (1 mg mL^{-1}) in PBS (pH = 7.4), respectively. The mixture solutions were kept at ambient temperature for 5 min followed by UV-vis absorption and fluorescence emission spectra tests in 1 cm cuvette, and their UCL spectra were further recorded upon continues 980 nm NIR laser irradiation (2 W cm^{-2}). Moreover, the OA signals from 680 nm to 980 nm were recorded by iThera MSOT imaging system. In addition, the UCL signals at 660 nm and 800 nm were separately determined based on IVIS Lumina II imaging system with specific filters (*e.g.*, 640/50 nm and 790/30 nm) upon 980 nm NIR laser irradiation (10 W cm^{-2}).

2.4.5 *In vitro* endogenous radical species sensing

Murine macrophages RAW264.7 cells were purchased from American-type culture collection (ATCC) and cultured in high-glucose DMEM containing 10% fetal bovine serum (FBS), penicillin ($100 \text{ I. U. mL}^{-1}$) and streptomycin ($100 \mu\text{g mL}^{-1}$) in a humidified atmosphere with 5% CO_2 at 37°C .

For cell imaging, RAW264.7 cells were seeded in confocal dish overnight at a density of 1×10^5 cells per milliliter DMEM. For the endogenous radical species generation and imaging studies, the excessive $\text{O}_2^{\cdot-}$ in macrophage cells was activated by pretreating with phorbol 12-myristate 13-acetate (PMA) (200 ng mL^{-1}) for 1 h to generate oxidative stress, and the excessive ONOO^- in RAW264.7 cells was produced by stimulating with lipopolysaccharide (LPS, $1 \mu\text{g mL}^{-1}$) and interferon- γ (INF- γ , 50 ng mL^{-1}) for 4 h followed by PMA (10 nM) treatment for 0.5 h to induce nitrosative stress.⁵² The generation of ROS (*e.g.*, $\text{O}_2^{\cdot-}$) and RNS (*e.g.*, ONOO^-) were further confirmed by the standard MitoSOX mitochondrial superoxide indicator (red

fluorescence) and Cell Meter™ fluorimetric intracellular peroxynitrite indicator (green fluorescence), respectively. To identify the specificity of these two radicals monitoring, the cells were pre-treated with Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP, 100 μ M) as $O_2^{\bullet-}$ scavenger and mercaptoethyl guanidine (MEG, 100 μ M) as ONOO $^-$ scavenger for 1 h before the oxidative or nitrosative stress generation upon PMA and LPS/IFN- γ /PMA stimulation, respectively. After refreshing the medium, the RAW264.7 cells were further incubated with UCN (100 μ g mL $^{-1}$) for 4 h in confocal dish with 1 mL DMEM, and the UCL cellular imaging was performed in Nikon fluorescence microcopy upon 980 nm NIR light excitation (5 W cm $^{-2}$).

Moreover, the MSOT images in living cells were also collected. Briefly, the UCNs probe was incubated with the RAW264.7 macrophage cells after PMA ($O_2^{\bullet-}$ generation) and LPS/IFN- γ /PMA (ONOO $^-$ production) stimulation and collected in 1 mL DMEM (1 $\times$ 10 6 cells mL $^{-1}$). Then these cell samples in different groups were encapsulated into an OA phantom containing two-channel polyurethane cylindrical, one for holding the control medium (DMEM) and the other for holding cell samples with UCNs probe treatment in different groups. The MSOT images and OA signals were finally recorded using a 128-element concave transducer array spanning a circular arc of 270 $^\circ$ with the optimal excitation wavelength at 680 nm to 980 nm.

2.4.6 MSOT and UCL imaging of multiple radical species *in vivo*

All animal experimental procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Soochow University. The Balb/c nude mice (~6-8 weeks old) were purchased from Shanghai Laboratories Animal Center in China.

To study the biodistribution of UCNs, the nude mice were intravenously injected with UCN (5 mg/mL in 100 μ L saline) and were further sacrificed at different time points after UCNs administration. Different organs including heart, liver, spleen, lung, kidney, stomach and

intestines were collected and washed with PBS (10 mM, pH = 7.4) for three times, and the UCL signals were recorded based on the IVIS Lumina II imaging system upon NIR light irradiation (E_x : 980 nm, E_m : 790/30 nm).

To further monitor the dynamic radical oxidative or nitrosative stress *in vivo*, the mice were fasted overnight and intraperitoneal (*i.p.*) treated with sterilized saline solutions containing different modeling drugs including LPS (20 mg Kg⁻¹), acetaminophenol (APAP, 300 mg Kg⁻¹), isoniazid (INH, 200 mg Kg⁻¹) and tacrine (THA, 30 mg Kg⁻¹), respectively ($n = 5$). For reactive metabolites scavenger studies, the animals were pre-treated with N-acetyl-cysteine (NAC, 200 mg Kg⁻¹) by *i.p.* injection at five minutes before drugs stimulation as indicated in previous reports. Fifteen minutes after the drugs treatment, the UCN (5 mg/mL in 100 μ L saline) were injected intravenously and the mice were then anesthetized with 3% isoflurane for UCL imaging at 660 and 800 nm on the IVIS Lumina II animal imaging system with specific filters (640/50 nm and 790/30 nm) upon 980 nm NIR light irradiation (10 W cm⁻²). The real-time MSOT imaging was also performed by whole body screening from 680 to 980 nm after drugs and UCN administration at different time points with 10 min interval. To dynamic profile the variations of oxidative and nitrosative stress *in vivo*, the time-resolved MSOT signals at 680 and 800 nm in mouse liver with pseudo-color processing were collected upon diverse hepatotoxins injection, and the ROI intensity was recorded on the tomographic anatomical image of liver cross section in iThera software.

2.4.7 Histological and immunohistochemical analysis

To evaluate the tissue damage and inflammatory response, the animals were euthanized at 60 min and 180 min post UCN, APAP, INH and THA treatment. The various organs including liver, heart, spleen, lung, and kidney were resected and placed into 4% formalin solutions overnight at 4 °C, and all tissues were embedded in paraffin followed by 10 μ m sectioning. For the histological studies, these organ tissues were stained by hematoxylin and eosin (H&E) under

standard protocols. For the immunohistochemical staining, the liver tissues were processed for 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine staining based on the anti-4-HNE primary antibody and anti-3-nitrotyrosine primary antibody (Abcam) at 1:500 dilution by following the previous reported methods.^{53,54} All images were acquired using an Olympus IX53 inverted fluorescence microscope equipped with a Nuance (CRi Inc.) hyperspectral camera capable of bright field full-color imaging.

2.4.8 Liver metabolic enzymes activity test

To examine the metabolic mechanism and inflammation procession during the drug induced liver injury (DILI), the mice were sacrificed to collect the livers at different time points. Then frozen liver tissues were thawed and homogenized in ice-cold PBS using a tissue homogenizer (Omni) for 10 min (3,000 rpm, 4 °C) and the supernatants were collected for the UGTs assay ($n = 5$). Liver microsomes in the endoplasmic reticulum of hepatic cells were also isolated from liver tissues by utilizing the classical methods reported previously to determine the amounts of the cytochrome P450 (CYP450).⁵⁵ Both of these specific biomarkers were determined by enzyme-linked immunosorbent assay (ELISA) kit according to the standard procedure described by the manufacturer, and the protein content in liver tissues was determined by bicinchoninic acid (BCA) method based on the standard protocols.

2.5 References

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2.6 Annex

¹H NMR spectra of Cy7-S in MeOD

¹³C NMR spectra of Cy7-S in MeOD

cy7-s #2-14 RT: 0.02-0.17 AV: 13 SB: 115 0.27-1.96 NL: 2.72E6
T: ITMS + c ESI Full ms [50.00-2000.00]

ESI-MS spectra of Cy7-S

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -3.0, max = 70.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
1166 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 10-39 H: 10-49 N: 0-2 O: 0-2 Si: 0-14 S: 0-2
C₃₉H₄₉N₂O₂S
Cy7-S 15 (0.314)

1: TOF MS ES+
1.14e+003

HR-MS spectra of Cy7-S

¹H NMR spectra of Cy5 in CDCl₃

¹³C NMR spectra of Cy5 in CDCl₃

C:\Xcalibur\...Wang Zhimin\cy5-me
MeOH

07-Mar-17 9:58:51 PM

cy5-me #2-11 RT: 0.02-0.13 AV: 10 NL: 1.04E6
T: ITMS + c ESI Full ms [50.00-2000.00]

ESI-MS spectra of Cy5

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -3.0, max = 70.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

97 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 10-28 H: 10-31 N: 0-4 Si: 0-14

C₂₇H₃₁N₂

Cy5-Me 6 (0.167) Cm (8)

1: TOF MS ES+
9.71e+003

Minimum:
Maximum:

5.0 10.0 -3.0
70.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
383.2491	383.2487	0.4	1.0	13.5	159.9	0.0	C ₂₇ H ₃₁ N ₂

HR-MS spectra of Cy5

hcy5-me-b #8 RT: 0.10 AV: 1 NL: 5.51E4
T: ITMS + c ESI Full ms [50.00-2000.00]

ESI-MS spectra of Hcy5

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -3.0, max = 70.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

60 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 10-27 H: 10-33 N: 0-2 Si: 0-14

C₂₇H₃₂N₂

Hcy5-Me 32 (0.666)

1: TOF MS ES+
1.02e+002

Minimum:

Maximum:

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
385.2647	385.2644	0.3	0.8	12.5	19.8	0.0	C ₂₇ H ₃₃ N ₂

HR-MS spectra of Hcy5

Chapter 3 NIR nanoprobe-facilitated cross-referencing manifestation of local disease biology for dynamic therapeutic response assessment

This chapter has been published in Z. Wang[†], X. Ai[†](Co-first author), Z. Zhang, Y. Wang, X. Wu, R. Haindl, E. K. L. Yeow, W. Drexler, M. Gao, B. Xing*, *Chem. Sci.*, 2020, 11, 803-811.
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3.1 Introduction

The growing prevalence of various diseases such as tumor, infection, cardiovascular and other metabolic illnesses engenders one of leading global burdens owing to its substantial impacts on public health.¹⁻⁴ Due to the symptoms occult and pathological complexity, effective treatment outcomes require advances not only in the pharmaceutical discovery, but also in urgent demanding innovative diagnostics for dynamic and accurate identification of biological indicators response in disease progression and/or opportunely therapeutic assessment.⁵ Although tissue biopsy and blood biomarkers test can serve as gold standards for disease diagnosis and pathological profiling (*e.g.*, in major organs like liver, kidney *etc.*),⁶ the high invasiveness, non-real-time reporting, unsatisfactory precision and risk of complications are considerable defects in clinical practice.⁷ Alternatively, molecular imaging approaches such as ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI) and optical imaging enable non-invasive and spatiotemporal mapping of pathophysiological changes in intact organisms,⁸⁻¹³ however, lack of valid means utilized in real-time monitoring of diseases dynamics, and importantly, punctually feeding back the therapeutic efficacy remain a huge technical barrier, which is vital for personalized and precision medicine.¹⁴

As essential biological messengers, reactive oxygen species (ROS) and their induced oxidative stress play significant roles in both biological signal transduction and the progression of pathologies ranging from cancers,^{15,16} metabolic diseases,^{17,18} cardiovascular,¹⁹ and even neurodegenerative disorders.^{20,21} Moreover, extensive investigations witnessed the great potential of ROS as valuable biomarkers for effective disease diagnosis and feedback-informed therapy. In spite of the remarkable advances of ROS sensing techniques, accurate monitoring various radical species dynamics, and more importantly, real-time correlating these pervasively changing biological indicators with disease progression and therapeutic response remain a bottleneck. Recent studies have attempted to monitor dynamic ROS metabolisms for the

measurement of pathophysiological evolution by utilizing multispectral optoacoustic tomography (MSOT) and near-infrared (NIR) light-mediated nanotechnology,²²⁻²⁵ particularly, some oxidative stress-responsive photoacoustic or luminescent imaging platforms for oxidative pathological diagnosis have received tremendous attention.²⁶⁻³⁴ However, despite the initial success in disease sensing, relevant research focusing on dynamic manifestation of oxidative stress status for non-invasive monitoring of therapeutic response and beyond, especially in the precise pharmaceutical intervention guidance in complex living conditions remains a pressing and challenging goal, relevant research has been rarely reported.

Here, an innovative nanoprobe-facilitated dual-modal imaging based on specific ROS activation is presented for precise evaluation of liver disease biology and dynamic therapeutic response assessment in living mice. Typically, we innovate such a cross-referencing imaging strategy by integration of MSOT with upconversion nanoparticles (UCNPs) mediated luminescence imaging through the process of optical energy transfer (OET), thereby taking unique advantages of the deep-tissue penetration, high spatiotemporal resolution and mutual corrective accuracy from both MSOT and upconversion luminescence (UCL) imaging modalities.³⁵⁻³⁶ By tailoring spectral overlap between the multiplexing UCL response and the absorption shift of the H₂O₂-sensitive cyanine dye, this UCNPs/cyanine-based nanoprobe (UCN) offers a convertible absorption blue-shift due to the chemo-specific reaction between ROS with the probe molecule that can be captured by MSOT. Meanwhile, the ROS-triggered response simultaneously induces multispectral luminescence changes, which exhibits an opposite trend of the UCL signal variation in comparison to the one observed in MSOT. Such spectrally opposite dual-modal imaging outcomes supply an ideal and ratiometric cross-referencing system that enables a full-package solution *via* the combination of the advantages of both the optical full-field implementation and high-resolution photoacoustic (PA) technology, thus allows more sensitive and accurate evaluation of local disease biology and drug treatment

response, due to the self-calibrating feature of such a reverse-ratiometric PA and UCL imaging platform (Scheme 1).

Scheme 1. The principle of UCN design. (a) NIR nanoprobe-facilitated PA and UCL cross-referencing technique for simultaneous hepatopathological profiling and liver disease therapeutic assessment. (b) ROS responsive mechanism of the small-molecule probe, CyB. (c) Optical energy-transfer processes of the nanoprobe UCN before and after H₂O₂ stimulation.

Comparing with conventional therapeutic evaluation techniques like tissue biopsy and blood tests, our works not only effectively overcome the limitations of invasiveness, discontinuity and hysteresis, but also exhibits superior sensitivity and spatiotemporal precision based on PA and UCL cross-referencing imaging, which greatly promotes the feasibility for opportune guidance of the pharmaceutical intervention and new drugs development.

3.2 Results and Discussion

3.2.1 Design of the PA and UCL cross-referencing nanoprobe

Scheme 1a illustrates the integration of MSOT with UCL imaging modality by a cross-referencing ROS-sensitive nanoprobe, which offers a distinct paradigm for non-invasive and

precise evaluation of disease pathological progression and assessment of the therapeutic response. Given that liver disease is of great harm and known as a major killer to the population under different ages,^{4, 37} we chose liver failure as the disease model to prove our concept. So far, the extensive studies clearly indicate the correlation of cumulative ROS production with its induced cellular redox imbalance, oxidative damage and liver dysfunction.^{17, 18} Therefore, real-time monitoring of oxidative stress status in liver damage progression could contribute to the early disease identification, pathological profiling as well as effective diagnostics and treatment evaluation in clinical settings.

The rationale for our nanoprobe design is mainly based on the principle that the ROS-sensitive chromogenic-convertible cyanine molecule acts as a specific PA reporter; meanwhile, ROS triggered reaction can also lead to UCL response due to the optical energy-transfer (OET) between robust multi-emissive UCNP and cyanine chromophores. Typically, the NIR-responsive UCNP with maximum emission (λ_{UCL}) at 800, 660 nm and cyanine dye molecule (CyB) are combined into one unified nanoplatfrom. Upon H_2O_2 stimulation, the electron-withdrawing boronate-substituted CyB can be specifically converted to its precursor structure, CyA, which triggers a hypsochromic shift from 800 to 650 nm (Scheme 1b and c).³⁸ More importantly, the desirable spectral overlaps between UCL and the absorption band of either CyB or CyA (Scheme 1c),³⁹ enabling a decreased ratio of UCL_{660}/UCL_{800} but an opposite signal change of PA_{680}/PA_{800} . Such an interlocking strategy can internally cross-reference two individual modalities, and permits high spatiotemporal and quantitative precision for H_2O_2 analysis, which therefore provides an omnidirectional solution for systemic manifestation of dynamic oxidation variation in the disease progression and drugs treatment by taking advantages of high-resolution PAT and full-field luminescent imaging.

3.2.2 Fabrication and characterization of UCN

The cyanine molecule CyB was synthesized through a starting framework (IR780) with the absorption at ~ 800 nm. To achieve an effective photon upconversion, the core-shell UCNPs ($\text{NaGdF}_4\text{:Yb/Tm/Er@NaGdF}_4$) were prepared by the solvothermal method (Figure 1a-b),^{39, 40} and the modulation of Tm^{3+} and Er^{3+} lanthanide elements enables multicolor emissions ($\lambda_{\text{UCL}} = 800, 660$ nm *etc.*) that well match the absorption of either CyB or CyA. The oleic acid (OA)-coated UCNPs were further functionalized with branched polyethylenimine (PEI) and polyethylene glycol (PEG) to improve the biocompatibility, which also facilitates the physical encapsulation of hydrophobic chromophore CyB into the inner hydrophobic spaces on UCNPs surface.⁴¹ The CyB loading efficacy in UCN was determined to be 4.84 % by the absorbance at 800 nm, and the persistent absorbance indicates a good stability where UCN was kept in the HEPES buffer with or without 10 % FBS from 0 to 72 h (Figure 1c-d). The typical transmission electron microscopic (TEM) images in Figure 2a show a spherical morphology of bare UCNPs and the nanoprobe, respectively. The average hydrodynamic diameter of the nanoprobe was further determined to be ~ 78 nm.

Figure 1. Preparation and characterization of UCN. (a) Synthesis route of UCN. (b) Upconversion luminescence (UCL) spectra of the core-shell UCNPs under 980 nm laser excitation. (c) Absorbance at 800 nm as a function of CyB with different concentrations (0.0156

- 10 $\mu\text{g/mL}$). The CyB loading content (red symbol) in UCN (0.1 mg/mL) was determined to be 4.84 %. (d) Absorbance at 800 nm as a function of different incubation time (0 - 72 h) of 0.5 mg/mL UCN in HEPES buffer (10 mM HEPES, 50 mM NaCl, pH = 7.4) with or without 10% FBS.

3.2.3 ROS response of UCN

Firstly, the sensing specificity was evaluated by incubation of UCN with various radical species. As shown in Figure 2b, only H_2O_2 incubated group shows the highest $\text{Abs}_{650}/\text{Abs}_{800}$ ratio (~ 3.8) due to the absorption blue-shift triggered by a chemo-specific cleavage of the caged boronate group; whereas no significant ratio change (< 0.2) could be found in the groups incubated with any other types of ROS, indicating the superior selectivity of the nanoprobe to H_2O_2 . Moreover, the absorption spectra of UCN towards different concentrations of H_2O_2 were observed in Figure 2c with a good linear correlation. Remarkably, the PA signals change, upon response to H_2O_2 , exhibits the similar trends that the PA_{800} intensity is significantly decreased, while only a slight enhancement of PA_{680} can be found (Figure 2d). This dual-wavelength PA response can thus provide the possibility for ratiometric PA imaging of H_2O_2 . Interestingly, comparing with PA results, the radical triggered UCL spectra changes (Figure 2e) present an opposite trend that UCL_{800} is greatly increased, whereas UCL_{660} is quenched slightly, due to the OET process between UCNPs and the convertible small-molecule dyes (CyB or CyA), further ensuring that our nanoprobe could also work for ratiometric UCL detection of H_2O_2 .

Both steady-state UCN luminescence and dynamic luminescence lifetime measurements validate the UCL energy transfer conversion for H_2O_2 sensing. As shown in Figure 2f, the UCL intensity at 800 nm (blue curve) is significantly quenched as compared to the bare UCNPs (black dashed curve) with the OET efficiency of $\sim 81\%$.³⁹ However, upon H_2O_2 reaction (red curve), the OET efficiency of UCL_{800} decreases approximately to 40% while the OET value of UCL_{660} is found to enhance from 46% to 68%. Likewise, the luminescence lifetime at 800 and 660 nm show the similar trends. Besides, the H_2O_2 induced UCN lifetime variations demonstrate the significant enhancement of UCL_{800} (from 74 to 169 μs), while only the slight

change of UCL₆₀₀ is observed (140 and 145 μ s). Compare with free UCNPs, the UCL₈₀₀ lifetime of the CyB-encapsulated nanoprobe decreases from 202 to 74 μ s (Figure 2g), mostly attributed to the process of energy transfer between UCNPs and the absorption convertible dyes. Such dual-wavelength luminescence changes provide a ratiometric analysis to facilitate precise manifestation of dynamic oxidative status through the as-prepared NIR nanoprobe.

Figure 2. The characterization and ROS response of UCN in buffers. (a) TEM image and hydrodynamic diameter distribution of the as-prepared UCN. (b) The specificity of UCN response towards different ROS: H₂O₂, peroxyxynitrite (ONOO⁻), hypochlorite (OCl⁻), tert-butyl hydroperoxide (TBHP), tert-butoxy radical (tBuO[•]), hydroxyl radical (•OH), nitric oxide (NO) and superoxide radical (O₂^{•-}). (c) Absorption spectral changes of UCN (0.1 mg/mL) upon incubation with H₂O₂ from 0 to 100 μ M. (d) The PA response of UCN (0.5 mg/mL) with 100 μ M H₂O₂. (e) UCL responses of 0.5 mg/mL UCNP5, UCN and 100 μ M H₂O₂-treated UCN, respectively (Ex. 980 nm). (f, g) UCL₈₀₀ and UCL₆₆₀ lifetime spectra of bare UCNP5, nanoprobe UCN and H₂O₂-treated UCN, respectively (Ex. 980 nm). (h) PA (red) and UCL (blue) ratiometric cross-referencing for H₂O₂ detection.

Then, the capability of the nanoprobe for quantitative H₂O₂ detection was investigated by PA and UCL modalities. The results in Figure 2h reveal good linear correlations but opposite ratio slopes in both PA₆₈₀/PA₈₀₀ and UCL₆₆₀/UCL₈₀₀, as a function of the H₂O₂ concentration from 0 to 500 μ M. Notably, the limits of detection (LODs) of PA and UCL were determined to be $\sim$ 0.1 and 0.07 μ M, respectively, suggesting the nanoprobe is sensitive enough to detect biological H₂O₂ as low as the concentration at submicromolar level. In line with the good sensitivity and quantitative capability for H₂O₂ sensing, such nanoprobe demonstrated opposite

signal changes between PA₆₈₀/PA₈₀₀ and UCL₆₆₀/UCL₈₀₀, and therefore offers a novel cross-referencing strategy to orthogonally well correlate PA and UCL, thus greatly minimizing the pseudo signal response to guarantee the precise validation of abnormal oxidation under highly complex and dynamic living conditions.

3.2.4 Cross-referencing detection of endogenous H₂O₂ by PA and UCL

The potential of UCN for endogenous H₂O₂ sensing was further investigated in murine RAW264.7 macrophage cells by both PA and UCL imaging modalities. Briefly, the excessive cellular ROS production was achieved by treatment of cells with lipopolysaccharide (LPS), a commonly used bacterial endotoxin. Upon 4 h LPS stimulation and the nanoprobe incubation (0.1 mg/mL), both living cells and cell lysates were collected for confocal imaging and MSOT analysis.

As shown in Figure 3a, the PA signal at 680 nm for LPS-treated cell lysates is much higher than the control without LPS as well as the group pretreated with N-acetylcysteine (NAC, a ROS scavenger), while the PA₈₀₀ intensity in LPS-stimulated group exhibits an obvious attenuation as compared to other normal cells. Furthermore, the ratio of PA₆₈₀/PA₈₀₀ in LPS-treated group is determined to be ~ 0.96, whereas a ratio of only ~ 0.70 can be found in the control group (Figure 3b), suggesting a positive PA ratiometric change triggered by the endogenous H₂O₂. In contrast, the UCL imaging shows different trends of luminescence ratio changes (Figure 3c). Without LPS, intracellular red emission (UCL₆₆₀) is clearly detected, with the UCL₈₀₀ emission (cyan) particularly weak due to the OET process. As expected, the obvious UCL₈₀₀ signal can be found upon LPS stimulation, indicating a reduced energy transfer between UCNPs and CyB, probably owing to the absorption band blue-shift induced by H₂O₂ reaction. Although similar UCL₆₆₀ signals are observed in different groups, the ratiometric UCL₆₆₀/UCL₈₀₀ values in Figure 3d reflect the obvious difference, in which the ratio of LPS-

treated group (~ 1.7) is significantly lower than those observed in the control (~ 3.1) and NAC-pretreated cells (~ 2.5).

Figure 3. Endogenous H_2O_2 detection in RAW264.7 cells by UCN. (a) PA images of cell lysates incubated with 0.1 mg/mL UCN in the absence or presence of LPS (2 μ g/mL) or the ROS scavenger NAC (0.3 mM), respectively. Scale bar: 1 mm. (b) The plot of PA₆₈₀/PA₈₀₀ ratios in different groups. Data were represented as mean $\pm$ SD. (c) UCL imaging of RAW264.7 cells treated with UCN in different groups as above. Hoechst 33342 (Blue, Ex: 405 nm, Em: 460/50 nm), UCL₆₆₀ (Red, Ex: 980 nm, Em: 640/50 nm), UCL₈₀₀ (Cyan, Ex: 980 nm, Em: 790/30 nm). Scale bar: 50 μ m. (d) The plot of UCL₆₆₀/UCL₈₀₀ ratios in different groups. Data were analyzed by ImageJ and represented as mean $\pm$ SD.

These results demonstratively prove that our nanoprobe is feasible for cellular H_2O_2 sensing by both PA and UCL imaging, more importantly, such a cross-referencing strategy based on reversed ratiometric PA₆₈₀/PA₈₀₀ and UCL₆₆₀/UCL₈₀₀ holds superior sensitivity and accuracy for endogenous H_2O_2 detection, which may provide robust applicability in complex living conditions.

3.2.5 *In vivo* dynamic biodistribution and biosafety of UCN

As a major metabolic and immunologic organ, liver serves various important functions including energy metabolism, storage and poisons excretion.⁴² However, a variety of genetic or acquired factors such as viruses, bacteria, drugs and alcohol usage that can disturb liver functions and lead to different types of disorders,⁴³⁻⁴⁵ which engender substantial impacts on

public health.⁴⁶ In light with the close-knit interplay between dynamic redox imbalance and hepatic diseases,^{47, 48} it is highly essential to investigate the detailed mechanisms in the hepatopathological progression by using our nanoprobe.

Figure 4. *In vivo* dynamic biodistribution and toxicity of UCN. (a) *Ex vivo* UCL_{800} imaging (Ex: 980 nm, Em: 790/30 nm) of different organs after tail-vein injection of the nanoprobe (5 mg/mL, 100 μL) at different time points. Scale bar: 1 cm. Organs from 1-7: 1, Heart; 2, Liver; 3, Spleen; 4, Lung; 5, Kidney; 6, Stomach; 7, Intestine. (b) Quantification of UCL_{800} signals in different organs at different times. Data were represented as mean $\pm$ SD. (c) MSOT imaging of the mouse liver at 680 and 800 nm post UCN injection. Scale bar: 1 cm. (d) Serum ALT and AST activities after the administration of saline (control) and UCN (5 mg/mL, 100 μL). Values are expressed as mean $\pm$ SD (n = 5). (e) Histomorphological appearance of the major organs of mice (n = 3) after treatment with the saline only (control) or 24 h after administration of UCN (5 mg/mL, 100 μL). HE, scale bar: 100 μm .

We firstly evaluated the *in vivo* biodistribution and biosafety of our nanoprobe *via* intravenous (*i.v.*) injection of UCN into the mice and monitored the signals with MSOT and UCL imaging. As shown in Figure 4a and 4b, *ex vivo* UCL imaging confirm the higher accumulation of UCN in the liver than other main organs, and the nanoprobe could be cleared out after 24 h. The obvious liver localization of UCN was also validated *via* PA₈₀₀ imaging in Figure 4c, the consistent PA₈₀₀ signals further indicate the good biostability of the loaded dye in the UCNs surface. Moreover, the potential biosafety issue of UCN was studied by biochemical serum assay and histological analysis. The results reveal negligible toxicity towards mice liver and other organs (*e.g.*, heart, lung, liver, spleen and kidney *etc*) after UCN administration (Figure 4d and 4e), further demonstrating the good biocompatibility and feasibility of our nanoprobe for *in vivo* liver sensing applications.

3.2.6 *In vivo* cross-referencing manifestation of oxidative stress induced hepatopathology

So far, the first-line antituberculosis drug, isoniazid (INH)-induced hepatotoxicity has been proposed to closely link with oxidative stress, however, exact mechanisms are still needed to be identified.⁴⁹ In this study, we established the acute liver failure model in Balb/c nude mice (*n* = 5) *via* intraperitoneal injection (*i.p.*) of toxic dose INH (100 mg/kg). Inspired by the reliable biodistribution results aforementioned, the liver accumulation of UCN reaches a plateau from 1 to 3 h after *i.v.* injection, suggesting a good dynamic imaging window with less interference caused by the nanoprobe metabolism *in vivo*. Thereby, time-resolved PA and UCL imaging of INH-pretreated or normal mice were performed 1 h post UCN (5 mg/mL, 100 μ L) administration to explore the complications of INH-induced oxidative stress in liver pathological progression.

The dynamic luminescence signal changes of UCL₆₆₀ and UCL₈₀₀ were determined in the liver with different time intervals. The intensities of UCL₈₀₀ in INH-treated mice are gradually

enhanced in comparison to the control mice without INH treatment, while the similar UCL₆₆₀ signals can be detected in both groups within the imaging period. The ratiometric UCL₆₆₀/UCL₈₀₀ values in Figure 5a clearly show a remarkable downward trend in the mice with INH stimulation. Moreover, the dynamic ratiometric imaging clearly indicates the oxidative burst induced by INH within 30 min with a subsequent trend of UCL₆₆₀/UCL₈₀₀ declined onwards. As indicated in Figure 5b, there is a much higher UCL₈₀₀ intensity observed at 30 min, and only a slight decrease of UCL₆₆₀ is detected in INH-treated mice when compared to the mice in control group. These results demonstrate the great potential of our nanoprobe for ratiometric screening of oxidation dynamics in living animals.

Figure 5. *In vivo* ratiometric UCL and PA imaging. (a, c) Dynamic ratios of UCL₆₆₀/UCL₈₀₀ and PA₆₈₀/PA₈₀₀ at varied time points in UCN-pretreated (5 mg/mL, 100 μ L) mice upon INH (100 mg/kg) stimulation with or without antioxidant therapeutic reagents (*e.g.*, GSH, NAC and SIL, 200 mg/kg, respectively). Ratiometric values represent means $\pm$ SD ($n = 5$). (b, d) UCL and PA images at 30 min post INH-treated or hepatoprotective drugs-pretreated mice upon UCN injection.

To validate the applicability of oxidative stress photoacoustic imaging *in vivo*, the cross-sectional MSOT images covering the whole liver were captured at a 10 min interval in normal mice and animals treated with hepatotoxic INH after 1 h UCN administration. The PA signal changes at 680 and 800 nm were monitored and quantified. Interestingly, the dynamic changes of both PA₆₈₀ and PA₈₀₀ signals show opposite trends in comparison to UCL imaging. Typically, the PA₈₀₀ intensity in INH-treated mice displays a noticeable reduction at initial 30 min and then subsequently rises over the time duration, most likely due to the ROS response and dynamic metabolism of UCN in liver. Consequently, the ratiometric variation of PA₆₈₀/PA₈₀₀ in Figure 5c exhibits an upward trend with the peak value at ~30 min in INH-treated group, such a ratiometric change is exactly opposite to UCL₆₆₀/UCL₈₀₀, strongly suggesting the cross-referencing feature of PA and UCL capable for *in vivo* ROS imaging. Additionally, we also constructed the pseudo-color PA images and analyzed the cross-section anatomy of liver functions (Figure 5d). The increased PA₆₈₀/PA₈₀₀ (blue to red) can be visualized between normal mice and liver injured animals treated with INH, clearly demonstrating the great feasibility of UCN for dynamic and precise radical stress screening *in vivo*.

In line with the great potential of UCNs for radical screening, our nanoprobe successfully builds a bridge between MSOT and luminescence imaging for spatiotemporal sensing of ROS dynamics *in vivo*. More importantly, the internal ratiometric cross-referencing of PA and UCL enables much higher accuracy for real-time manifestation of oxidative stress in drug-induced liver pathological progression, which can greatly facilitate further investigations into the non-invasive pharmacological assessment in the therapeutic process.

3.2.7 *In vivo* real-time cross-referencing assessment of treatment responses

Inspired by the promising results in real-time monitoring of ROS-induced hepatotoxicity *in vivo*, we further investigate the possibility of UCN for dynamic assessments of the liver damage treatment. To this end, three clinically used drugs including glutathione (GSH), N-

acetylcysteine (NAC) and silibinin (SIL) that can effectively trigger the antioxidant defense and cure various types of drug-induced liver injury (DILI) are selected to evaluate the applicability of UCN. Generally, MSOT and UCL imaging were performed in INH-treated mice administrated with UCN. The DILI treatment effect based on drug molecules of GSH (200 mg/kg, *i.v.*), NAC (200 mg/kg, *i.p.*) and SIL (200 mg/kg, *i.g.*) were systematically evaluated through MSOT and UCL analysis.⁵⁰⁻⁵² As expected, all these drugs definitely play effective roles in the inhibition of liver damage by suppressing the ROS overproduction induced from hepatotoxic INH treatment, which can be non-invasively and sensitively reflected by integrated PA and UCL imaging.

As shown in Figure 5a and 5b, the significant signal enhancement in UCL₆₆₀/UCL₈₀₀ can be observed, whereas, an obvious ratio decrease for PA₆₈₀/PA₈₀₀ (Figure 5c and 5d) in comparison to INH-stimulated hepatotoxic mice. Moreover, representative PA and UCL images of drugs treated mice show the similar signals within short time period therapy (~30min) as observed in normal mice, suggesting relatively low oxidative stress in the liver after treatment. Notably, there are still slightly progressive oxidation observed after INH injection within ~1 h in the mice treated with different liver protective drugs. Among the therapeutic assessment, it is easy to find that SIL drug molecule exhibits superior efficacy to curb liver ROS generation caused by INH metabolism, the antioxidant GSH performs slightly better action than that of NAC against the early stage of liver injury (<1 h), while almost the identical hepatoprotection can be detected after prolong INH stimulation (1 - 3 h). Such manifestations enable the horizontal comparison of different therapeutic effects, which is of great vitality in practical high-through drug screening.

3.2.8 Comparison with conventional liver function tests (LFTs)

To further validate the reliability of UCN based therapeutic response assessments, the commonly used biological assay including serum transaminase tests and liver histological

studies were carried out to correlate the dynamic oxidative implications with the different disease stages, and the dynamic treatment responses were systematically verified by our PA and UCL cross-referencing nanoprobe. As shown in Figure 5 above, both photoacoustic and luminescence imaging exhibit obvious signals variations in INH-treated hepatotoxic mice pretreated with antioxidant drugs as compared to those in control mice. Particularly, the most significant upregulation of PA₆₈₀/PA₈₀₀ can be easily observed, while, the simultaneous downregulation response in UCL₆₆₀/UCL₈₀₀ can be detected within 30 min (Figure 6a and b), clearly indicating that the early oxidative stress burst in liver injuring process could be distinctly manifested by UCN.

Although the gold-standard liver function tests (LFTs) through the amount of aspartate transaminase (AST) and alanine aminotransferase (ALT) (Figure 6c and d) demonstrate the tangible evidence of time-dependent liver damage triggered by INH, while the administration of GSH, NAC and SIL can remarkably suppress or even reverse the pathological progression. It should be noted that such serum biochemical assays have difficulty in differentiating hepatotoxic responses at initial stage (*e.g.* < 30 min). Furthermore, liver histological studies with hematoxylin & eosin (H&E) staining indicate no morphological changes observed in liver tissues at an earlier stage (*e.g.* 30 min) (Figure 6e), mainly due to a low degree of substantial damage. The obvious swollen hepatocytes and inflammatory infiltration can only be found earliest 3 h post INH stimulation. Furthermore, since oxidative stress plays crucial roles in overdosed INH that caused acute liver failure, an immunohistochemical study based on 4-hydroxynonenal (4-HNE) was utilized to investigate the possibility of lipid peroxidation (LPO) occurred in damaged liver. As shown in Figure 6f, prolong INH stimulation (*e.g.* ~ 3 hr) significantly induces 4-HNE positive foci; meanwhile, the liver tissues with GSH, NAC or SIL pretreatment demonstrate negligible LPO lesion, clearly indicating their promising antioxidant effects against liver damage. Similarly to the serum AST/ALT tests, LPO-associated

histochemical analysis could not effectively manifest the dynamic progression of liver pathology and related therapeutic responses, especially at the early stage.

Figure 6. Multiplexed profiling of liver pathological alternations and comparative assessments of therapeutic responses. (a, b) PA_{680}/PA_{800} and UCL_{660}/UCL_{800} values at 30 min in liver for the INH-treated or antioxidative drugs (GSH, NAC and SIL)-pretreated mice upon UCN injection. Columns represent means $\pm$ SD (n = 5). # indicates the control group. (c, d) Serum aspartate transaminase (AST) and alanine aminotransferase (ALT) levels for the mice upon treatment of INH or hepatoprotective drugs at different times. Data represent means $\pm$ SD (n = 5). (e) H&E staining of liver tissues at 30 and 180 min after INH treatment with or without drugs (n = 5). Arrowheads mark centrilobular vein fibrosis (blue), swollen hepatocytes (green), and inflammatory infiltration (red), respectively. CV: central vein. Scale bars: 100 μ m. (f) Immunohistochemical study of 4-hydroxynonenal (4-HNE) staining in liver sections at 30 and 180 min after INH treatment with or without drug molecules (n = 5). Black arrowheads mark 4-HNE-positive lesions. Scale bars: 100 μ m. (g) Illustration of INH-induced liver dysfunction and antioxidative drugs treatment effects. The p-values (**p < 0.01, ***p < 0.001) were determined by t-test.

Apparently, although the conventional serum tests and histological analysis are capable of diagnosing the substantive liver injury and overall pharmacological actions, these methods are heavily limited to characterize the incipient hepatotoxic response and dynamic monitoring the therapeutic effects, owing to the complicated sampling and testing processes. In contrast, UCN assisted PA and UCL imaging display higher sensitivity and real-time capability for precise detection of dynamic liver pathogenesis and early therapeutic effects without unpleasant operations, hysteric reporting and risk of complications usually suffered in clinical blood tests and liver biopsies. Most critically, such a unique technique offers a cross-referencing profile *via* reverse-ratiometric of PA_{680}/PA_{800} and UCL_{660}/UCL_{800} during the therapeutic assessments against liver dysfunction, which greatly enhance the sensing precision in complex conditions and further provide an archetypal research to boost its applications in broader oxidative stress or even antioxidant pharmacological profiling beyond the liver disease (Figure 6g). Other than great merits for the applicability of UCN, the concept of cross-referencing sensing also paves a new way to design highly accurate and versatile bioimaging probes.

3.3 Conclusion

In summary, we introduce an innovative cross-referencing molecular imaging technique based on environmental stimulation triggered photoacoustic and upconversion luminescence responses for reversed-ratiometric and noninvasive monitoring of oxidative dynamics *in vivo* *via* an optical energy transfer-facilitated nanoprobe, such a unique nanoprobe further promotes precise recognitions of the complex interplay between oxidative stress status and liver pathological evolution, as well as utilization in early identification of local pathological progression and dynamic therapeutic monitoring during the disease treatment. Owing to the better diagnostic performance compared to conventional blood tests and histological analysis, we envision that our study may serve as an archetypal system to encourage innovative diagnostics not only for precise assessment of therapeutic efficacy, but also boost its

applications in a brand-new field of real-time antioxidant pharmaceutical screening and beyond, especially in the feedback informed intervention guidance and new drugs development.

3.4 Experimental Methods

3.4.1 Materials and instruments

Except for some chemicals IR780, triphosgene, benzylamine, 4-(Hydroxymethyl) phenylboronic acid pinacol ester, pyridine that were purchased from Sigma-Aldrich, other commonly used materials and instruments are the same as aforementioned information in the section of 2.4.1.

3.4.2 Preparation of the nanoprobe UCN

3.4.2.1 Synthesis of the small-molecule ROS probe (CyB)

Synthesis of CyB

As shown the scheme in below, the small-molecule H_2O_2 probe (CyB) was synthesized as follows according to the previously reported method with some slight modifications.³⁸

Synthesis of intermediate 2. To flame dried two-neck round bottom flask containing triphosgene (254 mg, 0.85 mmol), a solution of (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-

yl)phenyl) methanol (**4**) (100 mg, 0.43 mmol) dissolved in dry DCM (10 mL) was drop-wisely added. The solution was cooled to 0 °C using an ice-water bath, then pyridine (52 µl, 0.65 mmol) was added *via* syringe. Then the reaction was slowly warmed to room temperature, stirred for 3 h, followed by dilution with ethyl acetate (25 mL). The resulting mixture was partitioned with 1M HCl (2 mL) and the biphasic mixture was placed into a separatory funnel. The organic layer was washed with brine and water, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was used for the subsequent reaction without further purification. ¹H NMR (CDCl₃, 300 MHz) δ 7.83 (d, *J* = 8.1 Hz, 2H) 7.41 (d, *J* = 8.1 Hz, 2H), 5.32 (s, 2H), 1.36 (s, 12H).

Synthesis of intermediate CyA. IR780 (**3**) (134 mg, 0.2 mmol) was dissolved in dry acetonitrile, then the solution was mixed with benzylamine (**4**) (44 µl, 0.4 mmol) and N, N-diisopropylethylamine (DIEA) (33 µl, 0.2 mmol). The reaction mixture was heated at 80 °C for 40 min under N₂ protection, then quenched with 0.1 M HCl. The mixture was evaporated and the residue was extracted with H₂O/DCM (3 × 10 mL), then combined the organic phase and dried over anhydrous Na₂SO₄. The solvent was removed to afford the crude product, which was then purified with silica gel column chromatography using DCM/0-5% MeOH as eluent to obtain the desired product **CyA**. Yield 62% (122 mg). ¹H NMR (500 MHz, DMSO-d₆) δ 7.65 (d, *J* = 12.7 Hz, 2H), 7.45 (d, *J* = 7.0 Hz, 2H), 7.42 – 7.38 (m, 2H), 7.35 (d, *J* = 7.1 Hz, 1H), 7.30 – 7.21 (m, 4H), 7.06 (t, *J* = 7.4 Hz, 2H), 6.88 (d, *J* = 7.8 Hz, 2H), 5.67 (d, *J* = 12.9 Hz, 2H), 5.03 (s, 2H), 3.94 – 3.78 (m, 4H), 1.86 – 1.80 (m, 4H), 1.48 (s, 12H), 1.03 (t, *J* = 7.4 Hz, 6H). ESI-MS *m/z*: 610.5 [M+H]⁺.

Synthesis of final compound CyB. The intermediate **2** (118 mg, 0.4 mmol) and **CyA** (73 mg, 0.1 mmol) were dissolved in dry DCM (10 mL), then DIEA (66 µl, 0.4 mmol) was dropwise added in the mixture. The solution was stirred at room temperature for 24 h under N₂ atmosphere. The reaction mixture was washed with saturated NH₄Cl solution and brine,

extracted with DCM then dried over anhydrous Na_2SO_4 . The organic solvent was evaporated to obtain the crude product and then purified with HPLC using $\text{H}_2\text{O}/50\text{-}100\%$ in ACN as eluent to afford the product CyB. Yield $\sim 10\%$ (20 mg). ^1H NMR (500 MHz, CDCl_3) δ 7.65 (d, $J = 12.7$ Hz, 2H), 7.45 (d, $J = 7.0$ Hz, 2H), 7.42 – 7.38 (m, 2H), 7.35 (d, $J = 7.1$ Hz, 1H), 7.30 – 7.21 (m, 4H), 7.06 (t, $J = 7.4$ Hz, 2H), 6.88 (d, $J = 7.8$ Hz, 2H), 5.67 (d, $J = 12.9$ Hz, 2H), 5.03 (s, 2H), 3.94 – 3.78 (m, 4H), 1.86 – 1.80 (m, 4H), 1.48 (s, 12H), 1.03 (t, $J = 7.4$ Hz, 6H). ESI-MS m/z : 870.87 $[\text{M}+\text{H}]^+$; HR-MS m/z : 870.5394 (Calc. 870.5381) $[\text{M}+\text{H}]^+$, $\text{C}_{57}\text{H}_{69}\text{BN}_3\text{O}_4$.

3.4.2.2 Preparation and modification of UCN

Typically, the preparation of $\text{NaGdF}_4\text{:Yb/Er/Tm@NaGdF}_4$ core-shell UCNPs, and their modification with PEI and PEG are the same as the section 2.4.2.2.

For the preparation of UCNP@PEI-PEG/CyB nanoconjugates, the ROS-response probe CyB was loaded on the polymeric surface of PEG-PEI-UCNPs by the hydrophobic interaction. The DMSO solution of CyB (5 mg) was drop-wise added to the UCNPs@PEI-PEG water solution (10 mg) under sonication for 30 min. The mixture was then stirred vigorously overnight at room temperature. Finally, the UCNP@PEI-PEG/CyB nanoconjugates were collected by centrifugation (18,000 rpm, 10 min) followed by two times washing to remove excess reactant. The loading amounts of CyB was determined by the UV-vis absorbance at 800 nm. Transmission electron microscope (TEM) and dynamic light scattering (DLS) measurements were performed to characterize the successful preparation of the nanoprobe.

3.4.3 Ratiometric detection of H_2O_2 *in vitro*

The specificity of H_2O_2 sensing was firstly tested by incubating UCN (0.5 mg mL^{-1}) in HEPES buffer with various reactive oxygen species (20 μM), including H_2O_2 , OONO^- , ClO^- , TBHP, $\text{BuO}^\bullet$, $^\bullet\text{OH}$, NO and $\text{O}_2^{\bullet-}$. After 10 min incubation at ambient temperature, the UV-vis absorption was determined for those solutions. The selectivity of various radicals were plotted by the ratio of $\text{Abs}_{650}/\text{Abs}_{800}$. For the upconversion luminescence (UCL) test of H_2O_2 response,

the samples were added in a 1 cm cuvette and irradiated with 980 nm continuous laser, then UCL spectra were recorded and the H₂O₂ response was further plotted by the ratio of UCL₆₆₀/UCL₈₀₀. The photoacoustic signals of UCN incubating with different concentrations of H₂O₂ were recorded by iThera MSOT imaging system from 680 nm to 980 nm.

The ratiometric PA plot (PA₆₈₀/PA₈₀₀) was also carried out to evaluate H₂O₂ response. The lifetime decay curves of free UCNPs and the nanoprobe before and after H₂O₂ reaction at 800 and 660 nm were determined on the lifetime spectrometer (FSP920). The optical energy transfer (OET) efficiency (E) from the donor (UCNP) to the acceptor (CyB) could be calculated based on the following formula: $E = 1 - UCL_{DA}/UCL_D$ Where UCL_{DA} and UCL_D are the luminescence intensity of UCNPs in the presence and absence of loaded dye (e.g., CyB), respectively.

3.4.4 Cell culture and imaging

RAW264.7 cells were cultured in high-glucose DMEM containing 10% fetal bovine serum (FBS), penicillin (100 I. U. mL⁻¹) and streptomycin (100 µg mL⁻¹) in a humidified atmosphere with 5% CO₂ at 37 °C.

For the cellular ROS imaging, RAW264.7 cells were cultured in the confocal dish and incubated with UCN (0.1 mg mL⁻¹), then lipopolysaccharide (LPS, 2 µg mL⁻¹) was treated to induce oxidative stress and H₂O₂ overgeneration. After 4 h co-incubation, both LPS-treated, NAC-treated and control groups in confocal dishes were washed, the cellular UCL imaging was performed in Nikon fluorescence microscopy upon 980 nm NIR light excitation (5 W cm⁻²). Moreover, MSOT imaging for these samples in different groups were also performed by encapsulation the cell lysates into PA phantom respectively, which contains two-channel polyurethane cylindrical, one for holding the control medium (DMEM) and the other for holding the cell sample with UCN treatment. The MSOT images and signals were finally

recorded using a 128-element concave transducer array spanning a circular arc of 270° with the optimal excitation wavelength from 680 to 980 nm.

3.4.5 Animal model and dynamic biodistribution study

All animal experimental procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Soochow University. The Balb/c nude mice (~6-8 weeks old) were purchased from Shanghai Laboratories Animal Center in China. To study the biodistribution of UCN, the nude mice were intravenously injected with UCN (5 mg mL⁻¹ in 100 μL saline) and were further sacrificed at different time points post administration. Major organs including heart, liver, spleen, lung, kidney, stomach and intestines were collected and washed with PBS (10 mM, pH = 7.4) for three times, and then UCL signals from UCN were recorded based on the IVIS Lumina II imaging system upon NIR light irradiation (E_x: 980 nm, E_m: 790/30 nm).

3.4.6 *In vivo* PA and UCL imaging

To monitor the dynamic ROS production in the antituberculosis drug (INH)-induced acute hepatic disease model, mice were fasted overnight and intraperitoneal (*i.p.*) treated with sterilized saline solution with or without isoniazid (INH, 100 mg Kg⁻¹), respectively (*n* = 5). For hepatoprotective pharmacological assessments, INH-stimulated mice were pre-treated with the clinically used drugs including glutathione (GSH, 200 mg Kg⁻¹) intravenously (*i.v.*) 5 min before INH treatment, with N-acetylcysteine (NAC, 200 mg Kg⁻¹) intraperitoneally (*i.p.*) 1 h before INH treatment, and Silymarin (SIL, 200 mg Kg⁻¹) intragastrically (*i.g.*) 12 h before INH treatment, respectively, thereby these hepatoprotective drugs could reach an effective plasma concentration.⁵⁰⁻⁵² Meanwhile, UCN (5 mg/mL in 100 μL saline) were injected intravenously before 1 h of INH stimulation to reach an effective liver accumulation, and the mice were then anesthetized with 3% isoflurane for UCL imaging at 660 nm and 800 nm on the IVIS Lumina

II animal imaging system with specific filters (*e.g.*, 640/50 nm and 790/30 nm) upon 980 nm NIR light irradiation (10 W cm⁻²).

The real-time MSOT imaging was also performed from 680 nm to 980 nm after hepatoprotective drugs and UCN administration at different time points with 10 min interval. To dynamic and ratiometric profile the ROS status in the liver, both the time-resolved MSOT and UCL signals with pseudo-color processing were collected upon INH injection, and the ROI intensity was recorded on the tomographic anatomical image of liver cross section in iThera software.

3.4.7 Histological and immunohistochemical analysis

The main organs including liver, heart, spleen, lung and kidney were resected and placed into 4% formalin solutions overnight at 4 °C, and all tissues were embedded in paraffin with 10 μm section. For the histological studies, these organ tissues were stained by hematoxylin and eosin (H&E). For the immunohistochemical staining, the samples were processed for 4-hydroxynonenal (4-HNE) staining based on the anti-4-HNE primary antibody primary antibody (Abcam) at 1:500 dilution by following the manufacturer's methods. All images were acquired using an Olympus IX53 inverted fluorescence microscope equipped with a Nuance (CRi Inc.) hyperspectral camera capable of bright field full-color imaging.

3.4.8 Serum aminotransferases test

The mice were anesthetized, and the blood was collected from the vena cava at different time points (n = 5). Then heparinized serum was separated immediately to measure aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities by following the standard protocols. The liver function was evaluated by analyzing the relative level of these two indicators.

3.5 References

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3.6 Annex

¹H NMR spectra of CyA (DMSO-d₆)

ESI-MS spectra of CyA

¹H NMR spectra of CyB (MeOD)

4P-2A #2-16 RT: 0.02-0.21 AV: 15 SB: 84 0.20-1.97 NL: 4.50E4
 f: ITMS + c ESI Full ms [50.00-2000.00]

ESI-MS spectra of CyB

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -3.0, max = 70.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

42 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 10-57 H: 10-69 B: 0-4 N: 0-3 O: 0-4

C57H69BN3O4

Cy7-H2O2-Et 9 (0.203)

HR-MS spectra of CyB

Chapter 4 pH-sensitive and biodegradable charge-transfer nanocomplex for NIR-II photoacoustic tumor imaging

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4.1 Introduction

As a hybrid technique emerging in biomedical analysis, photoacoustic imaging (PAI) has been well recognized to provide reliable sensing at penetration depth and spatial resolution close to practical requirements, which may overcome the limitations occurred in conventional optical imaging (*e.g.*, low penetration, light diffusion *etc*) and ultrasonic imaging (*e.g.*, low contrast), therefore enable its great possibility for clinical translation.¹⁻⁶ Despite such great potential, the conventional PAI for numerous biomedical applications based on intrinsic or extrinsic contrasts are still flawed due to the overwhelming light scattering of biological tissues and serious nonspecific PA signal readout.⁷ Remarkable efforts to technically improve the quality of PAI have been made in the engineering of more sensitive photoacoustic setups as well as the development of more efficient contrast agents (CAs).⁸⁻¹⁰ Of which the development of the commercially available Q-switched Nd:YAG pulse-laser (1064 nm) and new second NIR window (NIR-II, 960 - 1700 nm) light-absorbing PA contrast materials such as silver nanoplates, copper sulfide nanoparticles, phosphorus phthalocyanine and semiconducting polymer nanoplateforms, have allowed for the photoacoustic sensing in the NIR-II window. So far, NIR-II PA imaging has received worthy attention in deep tissue, brain vasculature and tumor detection due to its centimeter penetration and higher signal-to-noise ratio (SNR).¹¹⁻¹⁸

However, these NIR-II PA contrast agents for *in vivo* applications still faced challenges that the endogenous NIR-II absorption and non-targeted contrast extravasation would dramatically affect the imaging sensitivity and specificity. In addition, the biological incompatibility and long-term accumulation of these applied PA contrast agents further raised considerable concerns for their biosafety in practice.^{9, 11} Although some NIR-II contrast agents were designed for targeted PA imaging by conjugation of targeting-moieties (*e.g.*, antibodies), intelligent and environment-responsive NIR-II PA sensing still cannot be fully achieved yet.¹⁹⁻

²¹ Therefore, the development of sensitive and biodegradable NIR-II PA imaging probes that

can specifically respond to external stimulation for more reliable molecular sensing of various biological and pathological implications, and more importantly, can potentially realize successful clinical translations will be highly desired.^{22, 23}

Charge-transfer complex (CTC) materials are assemblies of charge-accepting (A) and charge-donating (D) molecules that have been intensely studied for several decades owing to their wide range of properties, such as the anisotropic conductivity, photoconductivity, paramagnetism, ferroelectricity and ambipolar transport.²⁴⁻³⁰ Extensive investigations toward the intramolecular charge-transfer (ICT) have shown that the excitation energy of the transitional state could contribute to the formation of typical charge-transfer (CT) bands in the visible and NIR regions in the electromagnetic spectrum, which therefore results in an intense light harvesting, and such kind of CTC materials are expected to have multi-field applications in solar cells, ferromagnets, superconductors, sensors and devices.³¹⁻³³ In particular, the colorimetric assay based on the formation of charge-transfer nanocomplex (CTN) by TMB (3, 3', 5, 5'-tetramethylbenzidine) and cationic TMB (TMB^{++}) has been well applied for free radicals detection, blood test and the enzyme-linked immunosorbent assay (ELISA) in preclinical research and clinical practice.³⁴⁻³⁶ Preliminary studies also disclosed that the TMB oxidation product (diiminium cation, TMB^{++}) can accept the electron (or charge) of TMB and produce charge-transfer nanocomplex, which is responsible for the blue colored CT bands with maximum absorbance at 370 and 652 nm, respectively. Further oxidation of the cationic TMB^{++} or moderate changes in pH yields a yellow diimine product (maximum absorbance at 450 nm), resulting in the degradation of CTN (Scheme 1).^{37, 38}

Scheme 1. Schematic illustration of CTN formation, pH sensitivity and *in vivo* NIR-II PA imaging applications.

Interestingly, we recently observed that this CTN shows one more broadband NIR absorption peak ranging from 750 nm to 1200 nm on the optical spectrum. Such strong absorption band could be greatly enhanced in the acidic environment (pH 5) compared to the neutral or slight basic condition (pH > 7), which provides the feasibility to enable CTN as a promising pH-sensitive NIR-II absorber for biological applications. Currently, extensive studies have demonstrated the acidic microenvironment within growing tumor, mostly attributed to the glycolytic metabolism, deficient blood perfusion and hypoxia, which plays significant roles in tumor cells proliferation, metastasis and chemoresistance.³⁹⁻⁴¹ Accordingly, focusing on low tumor pH has been widely regarded as an effective strategy for tumor diagnostic and therapeutic purposes.⁴²⁻⁴⁴

In terms of the unique pH-responsive feature of CTN, in this study, the broadband NIR-absorbing and environment-sensitive CTN could be a promising pH-activatable PA probe for specific and sensitive sensing of tumor acidic microenvironment. Considering the biomedical applicability of this TMB/TMB⁺⁺ based CTN, the potential cytotoxicity and clearance will be evaluated in combination with the biosafety studies of TMB.⁴⁵⁻⁴⁷

4.2 Results and Discussion

4.2.1 Preparation and characterization of CTN

Here, the charge-transfer nanocomplex material used was prepared as described in the experimental section.⁴⁸ The diameter optimization was achieved through alteration of the relative concentration of the substrates (TMB and AgNO_3), wherein the intermolecular charge-transfer degree of the π -conjugated push-pull system was controllable.

Figure 1. Preparation and characterization of CTN. (a) SEM images and diameter distribution of CTN. (b) XRD spectrum of CTN. (c) XPS survey spectra of CTN. (d) High resolution XPS spectrum of CTN (N1s). (e) FTIR spectrum of TMB and CTN. (f) Frontier molecular orbitals and relative energy level of TMB and TMB⁺⁺.

The diameters of the prepared CTN were varied from 418.4 ± 15.4 to 79.6 ± 2.2 nm and the optimal sized-CTN (< 100 nm, Figure 1a) was chosen as the candidate for further investigation. The XRD analysis (Figure 1b) indicated the peaks at 10.5, 17.4 and the broad peak can be ascribed to the formation of organic CTN by charge-transfer process between TMB and TMB⁺⁺. The XPS studies in Figure 1c show the occurrence of carbon, oxygen and nitrogen signals. The peaks at 401.9 and 399.8 eV in the N1s high-resolution XPS spectrum (Figure 1d) can be assigned to the protonated imine TMB⁺⁺ and the neutral amine TMB of CTN. Differences in the FTIR spectra (Figure 1e) between TMB and CTN could also be direct

evidence for the formation of CTN containing the typical phenyl ring of TMB and C=N bond of quinonediimine like cation (TMB⁺⁺). To further validate the charge-transfer of CTN, we carried out computational studies using Gaussian 09 under HF/6-31+G(d,p) scheme.⁴⁹ The simulated molecular orbitals in Figure 1f showed that the energy level of LUMO of TMB⁺⁺ (-7.0341 eV) was lower than that of TMB with an energy-gap of 0.0468 eV, which demonstrated the possibility of the CT process from TMB to TMB⁺⁺. All these results could be deduced the structure and charge-transfer mechanism of as-prepared CTN.

4.2.2 *In vitro* photoacoustic performance of CTN

As shown in Figure 2a, the optical spectrum of the CTN aqueous solution exhibited the broadband absorption ranging from NIR-I to NIR-II window, which can be attributed to the CT bands of TMB/TMB⁺⁺-based CTN. Most importantly, this pH-sensitive CTN was highly stable under an acidic environment (PBS, pH 5.0) for 72 h, while it was gradually degraded in normal physiological conditions (pH 7.4) (Figure 2b). Such observed pH-responsive phenomenon of CTN could be likely attributed to the different charge-transfer efficiency between TMB and TMB⁺⁺ when upon their exposure to varying pH conditions.^{50, 51} In addition, the potential changes of CTN structure under different pH environment were monitored by SEM after incubation with different buffers after 24 hours (Figure 2b), the images clearly showed the CTN nanofibers were almost degraded in pH 7.4 PBS buffer whereas no morphological changes were observed in the acidic condition.

Therefore, the pH-sensitivity and biodegradability of such CTN could not only act as valuable feature for specifically responding to the critical parameter, pH, in physiological and pathological processes (*e.g.*, tumor acidity), but also as a property that will alleviate the health risk caused by nanoparticles enrichment *in vivo*.^{52, 53} Moreover, CTN was incubated with different cell lines, HEK293 (human embryonic kidney cells) and RAW264.7 (mouse macrophage cells) for 24 h. No statistical difference in the cell viability was found with either

of the two cell lines even at concentrations of up to 500 $\mu\text{g/mL}$ (Figure 2c), which indicated the good biosafety for potential biological application.

Figure 2. *In vitro* studies of CTN. (a) UV-Vis-NIR absorption spectrum of CTN (0.05 mg/mL) in different PBS buffers (pH 5 and pH 7.4). (b) Sensitivity evaluation of CTN in different PBS buffers within 72 hours, scale bar, 1 μm . (c) Cell viability of NIH/3T3 and RAW264.7 cells after 24 h incubation with CTN at different concentrations. (d) Normalized PA spectra of CTN and PBS. (e) PA signal of CTN (0.1 mg/mL) and fresh mouse blood at 1064 nm. (f) Plot of the PA amplitude at 1064 nm as a function of CTN concentration.

We further evaluated the feasibility of CTN as a NIR-II PA contrast agent. Considering the lower native absorption from the tissue components in NIR-II region, as a proof of concept, we mainly monitored the PA signal of CTN with a 1064 nm laser excitation. The photoacoustic spectra in an aqueous solution was collected and normalized by a tunable pulse-laser illumination from 700 to 1200 nm (Figure 2d), which suggested that CTN is suitable for NIR-II PA imaging due to the excellent photon-caustic converting efficacy.⁵⁴ In addition, to reasonably optimize the CTN usage amount for efficient PA imaging contrast at 1064 nm, we determined through comparison with the PA signal of fresh mouse blood and found that the critical concentration of CTN was 0.1 mg/mL (Figure 2e). Importantly, the linear correlation between the concentration of CTN and PA intensity at 1064 nm was observed in Figure 2f with

$R^2 = 0.995$, indicating the applicability for the potential of PAI quantification. These results suggest that CTN could act as an attractive contrast candidate for NIR-II PA bioimaging and sensing.

4.2.3 Deep tissue photoacoustic imaging

To validate the availability of CTN for *in vivo* application, we firstly tested the penetrating ability of 1064 nm PAI with a tissue mimicking phantom made of agar gel embedding CTN, and the chicken breast muscles were covered to achieve different thicknesses (Figure 3a). The PA images were obtained by increasing tissue depths from 0 to 5 cm.

Figure 3. Deep tissue NIR-II PA imaging. (a) Agar gel phantom with different concentrations of CTN (0.1, 0.5 and 1 mg/mL) for deep tissue penetration test of NIR-II PAI. (b) Two-dimensional PA images at different depths (0 to 5 cm) with stacked chicken breast muscle blocks, laser energy density is 20 mJ/cm². (c) Plot of SNR with different CTN concentrations at 1064 nm as a function of the tissue depth.

As shown in Figure 3b, all the agar gel cylinders with different concentrations (0.1, 0.5 and 1 mg/mL) of CTN were clearly visualized, indicating the promising PA contrast of CTN

and the superior feature of NIR-II imaging. However, the signal-to-noise ratio (SNR) of the reconstructed images gradually decreased along with the increased depths of the chicken breast tissues (Figure 3c). It is noteworthy that the PA penetration of 0.1 mg/mL CTN was able to reach ~3 cm tissue depth while the signal of agar gel objects with higher concentrations (CTN, 0.5, 1 mg/mL) remained detectable even up to 5 cm tissue depth, which could recognize the advantages of NIR-II PAI for deep penetration and high-contrast in the particle bioimaging trials.

4.2.4 *In vivo* photoacoustic tumor imaging

To further examine the imaging capability of CTN for tumor sensing *in vivo*, PA scanning of 4T1 tumor-bearing mouse was conducted on a homemade PA setup under 1064 nm pulsed laser excitation. Then 200 μ L of CTN (1 mg/mL) was intratumorally injected into the tumor-bearing mice, and the control mice were treated with the same amount of CTN by subcutaneous injection. As shown in Figure 4a, prior to the injection of CTN, only PA signals of the background noise were observed in both tumor-bearing and control mice, which should be derived from the tissues' absorption in NIR-II region. 5 min after CTN injection, obvious PA signals appeared in the tumor area and normal tissue were observed, and no significant difference was found at this stage. Then the PA amplitudes in the tumor were markedly enhanced over time, which should be attributed to the CTN response under the acidic tumor microenvironment. In contrast, the mean PA intensity at the focused region of the control mice was gradually weakened mainly due to the degradation of CTN under normal physiological condition. At 4 hours post injection, the extremely significant difference ($p < 0.001$) of PA intensity was observed between tumor and normal tissue. After 24 hours, there were no detectable PA signals in control mice whereas the tumor-bearing mice still showed significant PA signals, which was corresponded with the pH-sensitivity investigations of CTN *in vitro* (Figure 4b). Additionally, we have performed the PA sensing in tumor models after 4T1 cells

seeding at different stages (Figure 4c). From the results, PA signals were gradually increased in both tumor models after CTN injection. However, there was significant difference of the PA intensity between 7 days tumor (small size) and 14-day tumor (large size). The results above demonstrated the capability of CTN being sensitive NIR-II PA probe for tumor imaging.

Figure 4. *In vivo* NIR-II PA imaging of tumor. (a) Representative PA images of the 4T1 tumor bearing mice and normal mice before and postinjection of CTN. (b) Normalized mean PA amplitudes as a function of time at 1064 nm. Energy density, 5.5 mJ/cm². Statistically significant difference in PA amplitude (a.u.) between tumor and normal tissue. (c) Photos for different tumor sizes (up: 4T1 cells seeding for 7 days; down: 4T1 cells seeding for 14 days), and the differences of PA signals after CTN injection (50 μ L, 1mg/ml). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$)

4.3 Conclusion

In conclusion, we have successfully developed a novel, organic self-assembled, pH-sensitive and biodegradable photoacoustic probe, CTN, for NIR-II PA tumor imaging in mice. The results of these studies demonstrated that CTN is suitable for *in vivo* NIR-II PA imaging with good contrast and deep penetration up to 5 cm due to the strong CT band in the NIR-II

region where the tissue background is minimal; Such self-assembled CTN is pH-responsive and the PA signal can be significantly enhanced in acidic conditions compared to in neutral conditions, which allows specific sensing of the tumor acidic microenvironment. More important, the biodegradable feature of CTN in physiological condition (pH 7.4) and the preliminary cell viability evaluation indicated negligible biosafety concern. This is particularly vital for *in vivo* imaging applications. Furthermore, refinements of this CTN to improve the sensitivity or specific targeting, as well as functional diversification will allow for better biocompatibility and utility of *in vivo*. Overall, this TMB/TMB⁺⁺-based organic CTN can serve not only a new class of NIR-II PA contrast agent but also as an pH-activatable probe for tumor imaging in mice. Given the many merits of CTN, we believe that such kind of NIR-II light absorbing charge-transfer nanocomplexes hold great potentials for further advancing PA imaging in biology and medicine.

4.4 Experimental Methods

4.4.1 Preparation, optimization, and characterization of CTNs

3, 3', 5, 5'-tetramethylbenzidine (TMB) and silver nitrate were purchased from Sigma-Aldrich. Unless otherwise stated, all commercial available reagents for synthesis and assay were used without further purification.

The CTN nanofibers were prepared and characterized according to a previously reported method with slight modifications.⁴⁸ Typically, 1 mL of AgNO₃ aqueous solutions with different concentrations (1 - 20 mM) were added into 2 mL of TMB (10 mM) ethanol solution respectively under vigorous stirring at room temperature. The color of the reaction mixture started to change from green to dark purple after the reaction had proceeded for 2 hours. The top purple CTN nanofibers were collected by centrifugation and then washed two times with DI water for the following experiments, the bottom gray precipitated Ag nanoplates were

removed. The morphology and diameters of prepared CTNs were characterized by SEM (Figure 5).

Figure 5. SEM images and the optimized diameter distributions of prepared CTNs.

UV-Vis-NIR absorption spectra were measured using a Perkin Elmer Lambda 950 spectrometer. The material morphology of CTN was investigated by a JEOL JSM-6700F field emission SEM. A Bruker D8 X-ray diffraction (XRD) system, a Perkin-Elmer model PHI 5600 X-ray Photoelectron Spectroscopy (XPS) system and a Bruker Vertex 80v vacuum FTIR spectrometer were used to study the structure.

4.4.2 *In vitro* PA test of CTNs

The laser source used is from OPO tunable (700 - 1200 nm) wavelength and fiber coupling. The output light from the fiber is then collimated and focused by condenser lens on the sample with a spot size of $\sim 500 \mu\text{m}$ diameter. Meanwhile, a beam splitter and photodiode are utilized to monitor the laser intensity variation. During the experiment, the MPE were well kept within the ANSI standard. An ultrasound transducer (V303SU, Olympus) with 2 MHz central frequency is placed close to the sample to detect the PA resonance signals. The transducer is immersed in water tank for optimum optical and acoustic coupling. The PA resonance signals

are firstly amplified by a low-noise amplifier (5662, Olympus) with 54 dB gain, and then recorded by an oscilloscope (LeCroy) with 500 MS/s sampling rate. Peak-to-peak voltage of the PA signals were then normalized with the laser energy at each wavelength and plotted against the wavelength to generate the PA spectrum of CTN.

For the concentration-dependent 1064 nm PA testing of CTN, a Q-switched Nd:YAG pump laser was used as the laser source of PA instrumentation. CTN solutions or fresh mouse blood were placed inside a low-density polyethylene (LDPE) tube with an inner diameter (i.d.) of 0.59 mm and outer diameter (o.d.) of 0.78 mm. LDPE tube containing samples and a single-element ultrasound transducer (UST, V323-SU/2.25 MHz, 13 mm active area, and 70% nominal bandwidth, Panametrics) were both immersed in water for coupling of PA signals to UST. The LDPE tube was irradiated with laser pulses at wavelength 1064 nm. PA signals were collected by UST, amplified with a gain of 50 dB and subsequently band pass filtered (1-10 MHz) by a pulse/receiver unit (Olympus-NDT, 5072PR). Finally, the output signals from the pulser/receiver unit were digitized with a data acquisition card (DAQ, GaGe, compuscope 4227) operated at 25 MHz and the acquired signals were stored in the computer. PA signals of with different concentrations of CTN at 1064 nm were measured and plotted.

4.4.3 NIR-II PA imaging in deep tissue

CTN spots of three different concentrations were embedded in an agar gel phantom (0.1, 0.5 and 1 mg/mL, respectively). Chicken breast tissues were stacked layer by layer above agar gel phantom to obtain various imaging depths. A home-made photoacoustic tomography system was used for deep tissue and tumor imaging. The system uses a high energy Q-switched 1064 nm Nd:YAG laser (Continuum, Surelite Ex) as a PA excitation source. The laser can deliver 5 ns pulses at 10 Hz repetition rate. The near-infrared 1064 nm beam was delivered to the ultrasound scanner through right angle prisms. The narrow laser beam was expanded and homogenized using an optical diffused (OD). The ultrasound transducer (UST) was immersed

in water for coupling the PA signal from the sample to the transducer. The PA signals generated by the tumor were received by a single-element transducer (V323-SU/2.25 MHz, Olympus NDT). The UST has 13 mm active area and 70% nominal bandwidth. The UST was continuously rotated in a circular geometry by a stepper motor (SM) to acquire PA signals from all angles of the tissue and tumor. The signals collected were subsequently amplified, band-pass filtered (1-10MHz) by an ultrasound amplifier and filter unit (A/F), (Olympus NDT, 5072PR) and then digitized. The signals were recorded by the PC with a 25 Ms/s data acquisition (DAQ) card from GaGe (compuscope 4227). Finally, the acquired PA signals were used to reconstruct the tumor image using a delay-and-sum back projection algorithm.

4.4.4 *In vivo* NIR-II PA tumor imaging

In vivo PA imaging of tumor-bearing mouse was acquired by the homemade 1064 nm PA imaging system mentioned above. Animal experiments were performed in accordance with the approved guidelines and regulations, and were approved by the institutional Animal Care and Use committee of Nanyang Technological University, Singapore (Animal Protocol Number ARF-SBS/NIE-A0263). Subcutaneous injection of two million 4T1 xenograft tumor cells suspended in 0.2 mL of DMEM (10% FBS, 1% antibiotics) into one side of the backs of female NCr nude was performed. After two weeks, the tumor-bearing mice were used for 1064 nm PA imaging. A layer of ultrasonic coupling gel was applied on the mouse's skin at tumor area. The mouse was subsequently anesthetized and placed into PA setup with its tumor protruding above to the water tank through a hole that had been sealed by transparent polyethylene film. The tumor-bearing mouse and the control mouse without tumor were monitored and imaged before and after intratumoral or subcutaneous injection of CTN (1 mg/mL, 200 μ L per mouse, $n = 3$) under 1064 nm laser with the power intensity of 5.5 mJ/cm². At different post injection times, PA images were acquired and reconstructed for the tumor and the normal tissue.

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Chapter 5 Redox-activatable and acid-enhanced nanotheranostics for NIR-II photoacoustic tomography and combined photothermal tumor therapy

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5.1 Introduction

In the past few years, significant advances in near-infrared (NIR) light-mediated nanoplatforms ranging from organic dye assemblies, semiconducting polymers, carbon nanotubes, to inorganic metallic gold nanoparticles, quantum dots and upconversion nanoparticles, have remarkably promoted the developments of phototheranostics for the healthcare and life sciences.¹⁻³ These NIR nanotheranostic agents hold great promise in the personalized medicine due to their simultaneous disease diagnosis and therapy with specific targeting, deep-tissue penetration, high spatiotemporal precision and minimal invasiveness.⁴⁻⁷ As an emerging phototheranostic modality, NIR photoacoustic tomography (PAT) and its' combined photothermal therapy (PTT) gain considerable interests in recent tumor treatment research.⁸⁻¹² Compare with most of NIR-I (650-1000 nm) phototheranostics, some NIR-II widow (1000-1700 nm) theranostic studies have been validated that both the PAT and PTT in this wavelength range exhibit superior signal-to-noise ratio (SNR) and enhanced treatment efficacy.¹³⁻²⁶ However, despite these great promises, current “always-on” NIR-II phototheranostic agents remain restricted by the inherent nonspecificity from the pseudo signal readout and undesirable treatment-related side effects.

To address these challenges, exploring environment-activatable “turn-on” NIR-II phototheranostics that generates diagnostic and therapeutic effects only after specific activation in tumorous area while keeping silent at normal tissues could be an effective strategy.²⁷⁻²⁹ In view of the intrinsic complexity of tumor physiology, tumor microenvironment (TME) characteristics (*e.g.*, high redox state, low pH, hypoxia, and so forth) have attracted tremendous attention for rational design of stimuli-responsive, on-demand and effective tumor theranostic agents.³⁰⁻³⁴ Although several microenvironmental stimuli-activatable NIR-II fluorescent nanoplatforms have been successfully constructed and applied in tumor detection and guided phototherapy,³⁵⁻³⁷ it should be noted that these NIR-II emissive systems were actually excited

by the NIR-I (*e.g.*, 800 nm) laser for imaging and therapeutic purposes in practice. Particularly, the activatable phototheranostics for higher efficacy and deeper tissue PA sensing as well as combined PTT both in the NIR-II window has not been fully developed to date.

In view of our previous results, the broadband absorbing and biodegradable charge-transfer complex (CTC) by TMB oxidation not only possesses strong NIR-II (1064 nm) PA signal but also can specifically respond to the environmental pH change.³⁸ Although such a promising pH-sensitive NIR-II PA probe allows tumor acidity imaging, the sensitivity, targeting specificity and the administration mode are worthy for further refinements.

Scheme 1. Illustration of SHT formation and its H₂O₂-activation and acid-enhancement for tumor-specific NIR-II photo-nanotheranostics.

In this study, we report an evolutionary version of tumor microenvironment-activated nanotheranostics (SHT, SiO₂@HRP/TMB) for precise PAT and combined PTT both in the NIR-II window. This intelligent chromogenic nanotheranostics is fabricated by tumor folate (folic acid, FA)-specific modified mesoporous silica nanocarrier that are simultaneously loaded with the enzyme horseradish peroxidase (HRP) and its substrate, 3, 3', 5, 5'-tetramethylbenzidine (TMB), enabled specific turn-on response by the key tumor microenvironmental redox parameter, H₂O₂.³⁹⁻⁴¹ Once reaching the tumor area, the colorless SHT nanoprobe could be converted to the strong broadband absorbing form with the wavelength ranging from visible to the NIR-II window; meanwhile the chromogenic response would be significantly amplified in the acidic condition owing to the relatively high HRP

activity and better stability of CTC than that at neutral physiological pH.^{42,43} Such intelligent H₂O₂-activable and acid-enhanced nanotheranostics allow *in vivo* nearly zero-background PA tumor tomography and combined effective PTT with NIR-II light irradiation (**Scheme 1**). Considering the high sensitivity, superior penetration depth and excellent biocompatibility, this NIR-II photo-nanotheranostics holds broad prospects for the investigation and intervention of deep tissue H₂O₂ production and acid-associated physiological and pathological events.

5.2 Results and Discussion

5.2.1 Preparation and characterization of SHT

Figure 1. Preparation and characterization of SHT. (a) Synthesis route of SHT. (b) TEM image of dendritic mesoporous silica nanospheres. (c) Size distribution of SHT nanospheres in water determined by dynamic light scattering. (d) The TEM image of prepared SHT. (e) FTIR characterization of SiO₂, SiO₂-FA and SiO₂-FA-TMB. (f) UV-vis absorption spectrum of SiO₂-

FA, folic acid (FA) and SiO₂. (g) Absorbance at 320 nm as a function of FA with different concentrations (3.125-100 μ g/mL). The FA content (blue symbol) of SiO₂-FA (0.5 mg/mL) was determined to be 12.7%. (h) Absorbance at 650 nm as a function of TMB with different concentrations in presence of 2 μ g/mL HRP and 20 μ M H₂O₂. The TMB content (blue symbol) of SiO₂-FA-TMB (0.1 mg/mL) was determined to be 19.9%. (i) Absorbance at 650 nm as a function of HRP with different concentrations in presence of SiO₂-FA-TMB (0.1 mg/mL) and 20 μ M H₂O₂. The HRP content (blue symbol) of SiO₂-FA-TMB-HRP was determined to be 2.47%.

As indicated in Figure 1a, to construct the activatable SHT nanotheranostics, we firstly prepared 3D dendritic biodegradable and amino-functionalized mesoporous silica nanospheres according to the heterogeneous oil-water biphasic stratification reaction strategy.⁴⁴ Transmission electron microscopy (TEM) observation clearly revealed that the as-prepared SiO₂ nanospheres feature a dendritic hierarchical mesostructure (Figure 1b).

To further fabricate the tumor-specific nanotheranostics, folic acid (FA) as a targeting ligand was successfully modified on the surface of SiO₂ (-NH₂) by the coupling reaction between -COOH and -NH₂ groups, the successful conjugation was confirmed by FTIR (Figure 1g).⁴⁵ Then the HRP and TMB were encapsulated into the pores of mesoporous silica-based nanocarriers through the processes of physical diffusion and adsorption.^{46, 47} The TEM image in Figure 1c and 1d indicated the formation of nanocompositions for the obtained SiO₂@HRP/TMB (SHT), which showed monodispersed, uniform spherical framework with the average diameter verified to be 145.5 nm by dynamic light scattering (DLS) measurement. The loading efficacy of FA, TMB and HRP was determined to be 12.7%, 19.9% and 2.47% by using the absorption spectroscopy, respectively (Figure 1f-i).

5.2.2 ROS and pH responses of SHT

The HRP/TMB based colorimetric assay has been extensively applied for reactive oxygen species (ROS, *e.g.*, H₂O₂ etc.) sensing as well as the enzyme-linked immunosorbent assay (ELISA) in biochemical research.^{43, 48} In view of our turn-on activatable nanotheranostics, the chromogenic substrate TMB and the catalase HRP were encapsulated into SHT as an integrated colorimetric assay system, which enabled the capability of sensitive detection of environmental

H₂O₂. The sensing specificity of SHT was evaluated by incubation with various radical species, including H₂O₂, peroxyxynitrite (ONOO⁻), hypochlorite (OCl⁻), tert-butyl hydroperoxide (TBHP), tert-butoxy radical (•OtBu), hydroxyl radical (•OH) and nitric oxide (NO). Notably, only SHT and H₂O₂ co-incubated group showed strong NIR-II absorbance at 1064 nm, whereas no significant color response was found in the groups with any other types of free radicals' incubation (Figure 2a). Moreover, the absorbance change of SHT towards H₂O₂ in the slight acidic condition (pH 6.0) was more obvious than those in the neutral condition (pH 7.4). Additionally, the strong NIR-II absorbance of the H₂O₂/SHT solution in acidic condition (pH 6.0) remained no change even after 24 h, however, the absorption of the same sample at pH 7.4 was almost entirely disappeared due to the biodegradability of the generated charge-transfer complex (Figure 2b). To further validate the sensitivity of SHT activation in various pH environments, the pH-dependent absorption responses of SHT toward H₂O₂ were measured in varying pH buffers ranging from 5.0 to 9.0. The results indicated that SHT exhibited high sensitivity in an acidic buffers in the presence of H₂O₂, while the response in the basic condition was extremely slow (Figure 2c and 2d).

Figure 2. Preparation and characterization of the activatable nanotheranostics SHT. (a, b) Absorbance of SHT at 1064 nm after incubation with various types of free radical species (25 μ M) for 10 min. (b) Relative sensitivity and specificity of SHT incubated with different radical species under different pH conditions for 24 h. (c, d) Determination of SHT sensitivity for H₂O₂ detection in different pH buffers. (e) UV-vis–NIR absorbance spectra of SHT (0.1 mg/mL) with different H₂O₂ concentrations in PBS buffer. (f) PA imaging and signal plot at 1064 nm as a function of the concentration of H₂O₂.

To determine the feasibility of H₂O₂ sensing by using PAT modality, SHT solutions with different concentrations of H₂O₂ were tested for both absorbance and PA signal changes. As shown in Figure 2e, the absorbance increase was found to be H₂O₂-concentration-dependent. Moreover, the linear correlation between the concentration of H₂O₂ and the NIR-II PA amplitude were observed in Figure 2f, indicating the potential applicability for quantitative H₂O₂ detection. Considering a high-redox and acidic microenvironment in solid tumors due to the increased glycolytic metabolism, deficient blood perfusion and the processes of hypoxia.^{49, 50} Such a H₂O₂-activated and acidity-enhanced nanotheranostics holds promising potentials for further applications of tumor-specific activatable diagnostics and therapeutics.

5.2.3 ROS and acid-activatable photothermal and photoacoustic studies

The broadband NIR absorbing feature of SHT upon H₂O₂ action that not only enables a good NIR-II absorber for PAT, but also it may serve as a photothermal agent for *in vivo* tumor PTT. Notably, very recent research has demonstrated that TMB oxidation based CTC showed strong NIR laser-driven photothermal effect and the TMB-H₂O₂ colorimetric system could be potentially used for photothermal immunoassay.⁵¹

Figure 3. *In vitro* photothermal and photoacoustic studies of SHT with or without H₂O₂ in different pH conditions. (a, b) Temperature increase and IR thermal images of SHT (0.1 mg/mL) in different pH PBS buffers with or without H₂O₂ (50 μM) incubation under 1064 nm laser irradiation (1.0 W/cm²). (c) Photostability study of SHT after H₂O₂ response in pH 6.0 PBS buffer by reversibly photothermal heating and natural cooling cycles. (d) Schematic illustration of deep tissue NIR-II PT and PA studies of SHT after H₂O₂ response. (e) Temperature changes of H₂O₂-incubated SHT at different tissue depths under 1064 nm laser irradiation (1.0 W/cm²). (f) Plot of photoacoustic SNR with H₂O₂-incubated SHT at 1064 nm as a function of the tissue depth. Laser energy density was 20 mJ/cm².

We examined the photothermal property of SHT upon H₂O₂ activation by the NIR-II laser (1064 nm) irradiation at a power density of 1.0 W/cm². As shown in Figure 3a and 3b, the IR thermal camera images clearly showed that dramatic temperature increases with the blue colored H₂O₂-incubated SHT solutions after 5 min irradiation. However, no obvious temperature change was found with the colorless SHT solutions in the absence of H₂O₂. Furthermore, there was remarkable enhanced-photothermal heating effect observed in the acidic SHT/H₂O₂ reaction solution (pH 6.0) than that in the neutral condition (pH 7.4) with the same NIR-II irradiation, indicating the excellent photothermal specificity for potential tumor-selective PTT without normal tissue damage caused by nonspecific overheating. Moreover, the

H₂O₂-activated SHT solution exhibited superior photothermal stability (Figure 3c) that reveals the possibility for potential *in vivo* PTT studies.

5.2.4 Deep-tissue NIR-II photoacoustic and photothermal effects

To validate the deep-tissue penetration performances of NIR-II PAT and photothermal effects, a home-made PAT system and a tissue mimicking phantom covered with different thicknesses of chicken breast muscles were used to test the penetrating features (Figure 3d).¹⁶ The results in Figure 3e demonstrated tissue-depth-dependent temperature decreases of H₂O₂-activated SHT solutions under 10 min of 1064 nm laser irradiation (1.0 W/cm²). It should be noted that a 14.7 °C extra temperature increase was observed with 5 mm-tissue-depth covered SHT/H₂O₂ sample, such heating enhancement was sufficient to ablate tumor cells according to the threshold temperature increase (10-13 °C) for *in vivo* PTT.⁵² Additionally, the deep-tissue 1064 nm PA signals of H₂O₂-activated SHT solutions were examined and plotted in Figure 3f. Although the SNR of the irradiated samples was gradually decreased along with the tissue depths increasing from 0 to 4 cm, the clear PA contrast could be still detectable (SNR, ~10 dB) even up to 2 cm tissue depth. These results demonstratively proved the excellent tissue-penetrating capabilities of both PAT and PT effects in the NIR-II window.

5.2.5 *In vitro* NIR-II photothermal tumor cells ablation

To further investigate the specificity and capability of NIR-II photothermal tumor cells ablation by H₂O₂-activated SHT nanotheranostics, 4T1 cells were incubated with 0.1 mg/mL of SHT for 4 hours, removed the culture medium, then replenished with fresh one without or with 50 μM H₂O₂ to mimic the normal or tumor redox microenvironment. After 1 hour incubation, the cells were irradiated for 6 min with a 1064 nm laser (1.0 W/cm²). The PTT efficacy was evaluated by live/dead cells staining (calcein AM and propidium iodide) and quantitatively measured through using resazurin-based cell viability assays.

Figure 4. NIR-II PT study in cells. (a) Confocal fluorescence images of SHT incubated 4T1 cells after laser irradiation at 1064 nm (1 W cm^{-2}) for 6 min. Live/dead stain shows dead cells as red while viable cells as green; Scale bars, $100 \mu\text{m}$. (b) Viability test of photothermal destruction 4T1 cells. (c) Hsp70-expression analysis of 4T1 cells treated with SHT and activated by H_2O_2 upon 1064 nm irradiation (1 W cm^{-2} , 6 min). Treated 4T1 cells were stained with Alexa Fluor 488 labeled anti-Hsp70 antibody; Scale bars, $100 \mu\text{m}$. (d) Cytotoxicity of different concentrations SHT against 4T1 cells.

As shown in Figure 4a, b and d, there was no obvious cytotoxicity in both SHT and H_2O_2 treatment groups without laser irradiation, while remarkable dead cells staining and much lower viability (29.7%) were observed in the SHT/ H_2O_2 coincubated group after photoirradiation. Moreover, the significant difference of cell viability was found between H_2O_2 -treated and control (no H_2O_2 treatment) groups with combined SHT/NIR-II laser operations, indicating the effective photothermal damage specifically activated by H_2O_2 . These results clearly demonstrated the selective photothermal tumor cell-killing ability of our activatable nanotheranostics. To clarify the potential mechanism of such H_2O_2 -activated SHT in PTT, we analyzed the expression level of thermal-stress-associated heat shock protein 70 (Hsp70) in 1064 nm laser irradiated 4T1 cells.⁵³ After the immunofluorescent staining by Alexa Fluor 488 anti-Hsp70 antibody, an obvious green fluorescence was shown in SHT/ H_2O_2 treated cells

(Figure 4c), which confirmed the heat-induced Hsp70 over expression. In contrast, 4T1 cells with SHT or H₂O₂ treatment only showed relatively low Hsp70 expression after the same photoirradiation. The results further validated the NIR-II PTT triggered tumor cells response specifically derived from the external heat stimuli that generated by H₂O₂-activated SHT.

5.2.6 *In vivo* NIR-II tumor PAT

In order to evaluate the capability of SHT for NIR-II PA H₂O₂ sensing in the tumor microenvironment, the 4T1 tumor-bearing mouse model was established by following the standard protocol⁵⁴, then mice administrated with SHT (200 μ L, 2 mg/mL) via tail vein injection were scanned on a homemade PAT setup with 1064 nm pulsed laser excitation (Figure 5a). The PA signals of tumor area at one back side were gradually increased over time, which indicated the formation of the broadband absorbing charge-transfer complex derived from the SHT response towards tumorous H₂O₂ (Figure 5b and 5c). However, no obvious enhanced PA contrast except for the background noise was appeared in the normal tissue at different time periods post SHT injection. It was noted that the 1064 nm PA signal reached plateau after SHT injection for 4-6 h, the significant difference ($p < 0.001$) of PA intensity could be observed between tumor and normal tissue images.

Figure 5. *In vivo* NIR-II PA tumor detection. (a) Schematic diagram of 1064 nm Nd:YAG laser based PA tumor imaging system. Here, RP - Right angle prism, CRD - Circular rotating disc, SM - Stepper Motor, DAQ - Data Acquisition card, RFA - Ultrasound receiver/filter/amplifier unit, UST - Ultrasound transducer, OD - Optical Diffuser, PF - 100 μm transparent polythene membrane. (b) PA images of tumor and normal tissues in the 4T1 tumor bearing mice post injection of SHT (2 mg/mL, 200 μL). (c) Normalized mean PA amplitudes as a function of SHT post injection time. Energy density of 1064 nm pulse laser irradiation, 5.5 mJ/cm². Statistically significant difference in PA amplitude (a.u.) between tumor and normal tissue (*p < 0.05, **p < 0.01, ***p < 0.001, n = 3).

Besides, *in vivo* and *ex vivo* biodistribution analysis (Figure 6) by systematic administration of fluorescent dye (Cy7) labeled SHT further validated the superior tumor accumulation efficacy of this nanotheronastics due to the effects of both the enhanced permeability and retention (EPR) as well as the feature of folate-mediated tumor targeting.^{55, 56} Although the PA intensity in tumor was decreased over time attributed to the metabolism and clearance, there are still detectable PA signal after 24 h. This phenomenon was consistent with

our previous results that tumorous acidity significantly enhances the stability of CTC generated by H_2O_2 /SHT response. In contrast, the CTC would be degraded under the physiological condition (pH 7.4).³⁸ These data demonstratively proved that SHT could be used for NIR-II PA tumor detection based on the specific microenvironmental- H_2O_2 -activated strategy.

Figure 6. Biodistribution study of SHT in tumor-bearing mice. (Left) *In vivo* optical imaging of SHT-Cy after i.v. injection in 4T1 tumor-bearing mice; *Ex vivo* imaging of tumor and major organs post SHT-Cy injection (T, Li, Sk, H, Ki, Sp and SI stand for tumor, liver, skin, heart, kidney, spleen, and small intestine, respectively). (Right) Quantification of *ex vivo* average fluorescence intensity of different organs.

5.2.7 *In vivo* NIR-II tumor PTT

In light with the tumor PAT results, the PTT capability and efficacy were investigated in the 4T1 xenograft tumor model. The maximum NIR-II PA signals in the tumor region could be detected at 4 h post SHT injection, PTT was conducted accordingly with a 1064 nm laser irradiation (1.0 W/cm^2) for 6 min and the heating process was monitored every minute by an IR camera. The tumor temperature with SHT-treated mice gradually increased from the lowest 31.2°C to the maximum 52.9°C during the irradiation, which was significantly higher than that of saline-treated mice with a plateau temperature of 37.4°C (Figure 7a and 7b). Considering the threshold temperature (43°C) of PTT, such temperature change generated by H_2O_2 -activated SHT was high enough to induce tumor ablation.

To evaluate the antitumor effect for the NIR-II PTT using SHT, tumor volumes were continuously monitored for two weeks. As indicated in Figure 7c and 7d, the growth of tumors for SHT-treated mice was significantly suppressed without reoccurrence observed after 1064

nm photoirradiation. In contrast, saline-treated mice with the same irradiation failed to show any antitumor capability. Besides, no notable therapeutic effect was found for both SHT and saline-treated mice without NIR-II laser irradiation. These results showed that SHT offers excellent activatable photothermal therapeutic ability against tumor.

Figure 7. *In vivo* NIR-II PTT against tumor. (a) IR thermal images of 4T1 tumor-bearing mice with intravenous injection of SHT under laser irradiation at 1064 nm (1 W cm⁻²) for different times. (b) Temperature changes during laser irradiation after intravenous injection of saline and SHT into 4T1 tumor-bearing mice (***p* < 0.01, *n*=4). (c) Representative photographs of mice taken from different group after 1064 nm PTT treatment. White dotted circle indicates the region of tumor tissue (ROI). (d) Tumor growth curves and (e) body weight data of mice after NIR-II PTT operations (***p* < 0.01, *n*=4).

Moreover, there was no obvious body weight loss observed for all the mice throughout the experimental period (Figure 7e). Additionally, the histological studies by using TUNEL and H&E staining methods were carried out to further evaluate the PTT outcomes and potential side

effects.^{57, 58} As expected, no appreciable histopathological damage or inflammation could be observed in hearts, livers, spleens, and kidneys of both SHT and saline-treated mice with or without NIR-II laser irradiation (Figure 8). In contrast, compared to saline-treated tumor tissues or free NIR-II laser irradiated groups, tumor tissues in the SHT-treated group after PTT indicated obviously damaged effects (Figure 9a). Most importantly, the TUNEL staining of tumor tissues also indicated the apoptotic tumor cells for SHT-treated mice after PTT, while no noticeable apoptosis could be observed for saline-treated mice after laser irradiation. Further serum biochemical tests of SHT-administrated mice indicated that the pathological indicators of both liver (*e.g.* ALT, AST, ALP) and renal (*e.g.* CREA, BUN, LDH) were all in the normal range in comparison to the control (Figure 9b). These data validated that the SHT-mediated microenvironmental activatable NIR-II PTT exhibited a highly specific and effective tumor therapeutic action along with the good biosafety.

Figure 8. H&E staining of various organs upon saline or SHT (n = 4) with or without 1064 nm laser irradiation. The organs including heart, liver, spleen and kidney were harvested at 15 days after PTT experiments. Scale bars: 100 μ m.

Figure 9. (a) H&E and TUNEL staining of tumor upon saline or SHT treatment (n = 4) with or without 1064 nm laser irradiation. The tumor tissues were harvested at 15 days after PTT experiments. Scale bars: 100 μ m. (b) Serum biochemistry test of the mice after treatment with SHT (2 mg/mL, 200 μ L) or saline (n=3). Data are expressed as means $\pm$ SD. Alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (CRE) and lactate dehydrogenase (LDH).

5.3 Conclusion

In conclusion, we have successfully developed a microenvironmental stimuli-activatable nanotheranostics (SHT) for PA tumor detection and combined PTT both in the NIR-II optical window. Such a smart phototheranostics showed highly sensitive H_2O_2 -activated/acid-enhanced PA signal changes as well as precision PTT *in vitro* and *in vivo*, which significantly

minimized the nonspecific signal readout and therapeutic side effects encountered in the conventional “always-on” theranostic agents. Most importantly, the NIR-II light-mediated tumor PAT and PTT eventually led to superior deep-tissue photoacoustic contrast and photothermal heating capability in living mice. To the best of our knowledge, our work represents the first activatable PAT and PTT nanotheranostics both in the NIR-II range for simultaneous tumor diagnosis and phototherapy. In view of the numerous merits of stimuli-activatable NIR-II photo-nanotheranostics, this study provides great opportunities for the investigation and management of redox/acid-associated (patho)physiological alterations, as well as contributing to the development of future nanomedicine.⁵⁹

5.4 Experimental Methods

5.4.1 Materials and instruments

3, 3', 5, 5'-tetramethylbenzidine (TMB), Horseradish peroxidase (HRP), folic acid, tetraethyl orthosilicate (TEOS), ammonium nitrate (NH₄NO₃) and cetyltrimethylammonium chloride (CTAC) were purchased from Sigma-Aldrich. Unless otherwise stated, all commercially available reagents for synthesis and assay were used without further purification. *In vitro* cytotoxicity resazurin-based TOX-8 assay kit, Live/dead cell double staining kit and the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit were purchased from Sigma-Aldrich (Merck).

5.4.2 Preparation of mesoporous SiO₂ NPs (MSNs)

The dendritic MSNs were prepared following the previous reported one-pot biphasic stratification approach.⁴⁴ Typically, 12 mL of (25 wt %) CTAC solution and 45 μ L TEA were added to 18 mL of DI water and stirred gently at 60 °C for 1 h in a 100 mL round bottom flask, followed by 5 mL of (5 v/v %) TEOS in cyclohexane was carefully added to the water-CTAC-TEA solution and kept at 60 °C in an oil bath under slow stirring (~ 100 rpm) for 30 min. Then 1 mL of (10 v/v %) APTES in cyclohexane was drop-wisely added and kept at a constant

temperature with continuous stirring for another 6 hours. The products were collected by centrifugation (5000 rpm, 5 min) and washed for three times with ethanol to remove the residual reactants. Finally, the collected products were extracted with a 3 mg/mL ammonium nitrate (NH_4NO_3) ethanol solution at 60 °C for 6 h twice to remove the template. The obtained dendritic MSNs can be stored in ethanol solution in 4 °C for the subsequent experiments.

5.4.3 MSNs modification and TMB/HRP loading

The folic acid functionalized MSNs were prepared following a reported method.⁴⁵ Briefly, the folic acid (10 mg) was firstly activated by N-hydroxysuccinimide esterification of FA (22 mg, 0.05 mmol) with NHS (5.8 mg, 0.05 mmol) in 2 mL dry DMSO solution of EDC (19 mg, 0.1 mmol), HOBT (9 mg, 0.07 mmol) and 20 μL DIEA. The mixture was stirred for 30 minutes at room temperature. Then, the MSN- NH_2 suspension (20 mg in 3 mL DMSO) was dropped into the NHS-FA solution and stirred for 24 hours. Finally, the reaction mixture was collected by centrifugation (5000 rpm, 5 min) and washed with DMSO and DI water several times respectively to afford FA-MSNs.

For the TMB loading, 1 mL of FA-MSNs suspension (10 mg/mL) was added in 1 mL of 0.01 M TMB water solution with 10% DMSO, which was sonicated for 10 min and then stirred for 12 hours under N_2 gas atmosphere. The mixture was washed with 50% ethanol and DI water two times to produce TMB-loaded MSNs. For the HRP (Horseradish peroxidase, Sigma-Aldrich) loading, 10 mg of as-prepared TMB-MSNs were redispersed in 2 mL acetic acid-sodium acetate buffer (pH = 5.0) containing 1 mg of HRP. The mixed solution was then kept at 4 °C with gentle stirring for 12 hours. The TMB/HRP loaded MSNs (SHT) were obtained by centrifugation (5000 rpm, 5 min) after washing with DI water. The products were stored at 4 °C for further use. In all modification and loading processes, the loading efficacy was quantified by TMB/HRP/ H_2O_2 absorbance response with UV-vis spectroscopy measurements.

5.4.4 Specific and quantitative detection of H₂O₂

To study the specificity of H₂O₂ detection, 0.1 mg/mL SHT was incubated with various reactive oxygen species (ROS, 25 μ M) for 10 min with gentle shaking. Then, the UV-Vis-NIR absorption spectra of the mixtures after incubation were measured. To study the pH-sensitivity, SHT and H₂O₂ were added in PBS buffers with different pH values, the colorimetric test was subsequently performed following the same procedure. For the quantitative analysis, different concentrations of H₂O₂ were incubated with SHT for 10 min and then measure the absorbance and photoacoustic signals at 1064 nm.

5.4.5 *In vitro* NIR-II PAT and PT studies

The NIR-II PAT experiments were performed following the same protocol as described in the section 4.4.2.

For the NIR-II photothermal test, SHT solutions (200 μ L, 0.1 mg/mL) with H₂O₂ in different pH PBS buffers were irradiated by 1064 nm laser with a power density of 1 W/cm². The temperature changes were monitored every 20 s by an IR thermal camera (FLIR) during both the photothermal heating (0-3 min) and the cooling period (3-8 min). The photostability study of SHT with 50 μ M H₂O₂ was also evaluated by 1064 nm laser irradiation (1 W cm⁻²) for 5 repetitions.

For deep-tissue PAT and PT studies, 100 μ L of SHT (1 mg/mL) after 50 μ M H₂O₂ activation was firstly embedded in a sample phantom. Then chicken breast tissues were stacked layer by layer above the phantom to obtain various imaging depths (0-4 cm). The homemade PAT set up was used for deep-tissue imaging with both NIR-I (800 nm) and NIR-II (1064 nm) laser sources. Finally, the acquired PA signals were used to reconstruct the image using a delay-and-sum back projection algorithm. The deep-tissue photothermal studies with both 808 nm and 1064 nm laser irradiations were performed following a previously reported method.¹⁶ Typically, 200 μ L SHT solution with 50 μ M H₂O₂ was filled in a 96-well plate and covered by

chicken breast tissue of various thicknesses (0, 2.5, 5 and 10 mm) on top and then exposed to 808 nm or 1064 nm laser (1 W cm^{-2}) for 10 min. The temperature changes of the sample under chicken breast tissue were monitored by an IR thermal camera.

5.4.6 Cell culture and viability test

4T1 cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and it was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) containing penicillin ($100 \text{ I. U. mL}^{-1}$) and streptomycin ($100 \mu\text{g mL}^{-1}$) and maintained in an atmosphere containing 5% CO_2 and 95% humidified air at 37°C . For the cytotoxicity assay, cells were seeded in 96-well plates (8000 cells/well in $100 \mu\text{L}$) and incubated overnight. Then different concentrations of SHT were added to the cell culture medium, respectively, the cells were incubated for 24 h. The medium was then removed and washed with PBS. Fresh cell culture medium containing TOX8 was added to wells and the plate was incubated for another 3 h. The fluorescence at 590 nm was measured by a Tecan's Infinite M200 microplate reader with 560 nm excitation. Cell viability was expressed by the ratio of the fluorescence of cells incubated with SHT to that of control cells.

5.4.7 Cells staining and Hsp70-expression study

For live/dead cells staining, 4T1 cells were seeded in 8-well dish (ibidi) at a concentration of $2 \times 10^4 \text{ cells mL}^{-1}$ (0.3 mL) and incubated for 24 h. Then 0.1 mg/mL of SHT was added in the wells and incubated for another 4 h, then washed and treated with $50 \mu\text{M H}_2\text{O}_2$ containing or H_2O_2 -free medium. After 1 h incubation, the cells were irradiated with 1064 nm laser (1.0 W/cm^2) for 6 min. The control groups without NIR-II irradiation were performed. The cells were incubated overnight and then stained with calcein AM and propidium iodide (PI) for 30 min, washed with PBS, and then imaged by Carl Zeiss LSM 800 confocal laser microscope (Germany). Additionally, cell viability was also measured by using a TOX-8 assay kit.

For Hsp70-expression study, the irradiated 4T1 cells by 1064 nm laser (1.0 W cm^{-2} , 6 min) were incubated for another 3 h, then the medium was removed and washed with PBS. Subsequently, 4T1 cells were fixed with 4% paraformaldehyde solution for 20 min, after three times PBS washing, the cells were stained with anti-Hsp70 antibody conjugated with Alexa Fluor 488 (Biolegend Alexa Fluor 488 anti-Hsp70) and Hoechst 33258 at room temperature for 30 min. Finally, stained cells were washed with PBS again and imaged with the confocal laser microscope.

5.4.8 *In vivo* tumor PAT and PTT studies

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 model in female NC/nude mice, two million 4T1 cells suspended in 0.2 mL DMEM (10% FBS, 1% antibiotics) were subcutaneously injected into one side of the back of mice. *In vivo* NIR-II PTT was performed when the subcutaneous tumor volume reached approximately 100 mm^3 , while tumor-bearing mice with an average tumor size around 1000 mm^3 were used for NIR-II PAI.

In vivo tumor PAT was acquired by the homemade 1064 nm PAT system mentioned above. A layer of ultrasonic coupling gel was applied on the mouse's skin at the tumor area. The mouse was subsequently anesthetized and placed into the PA setup with the tumor protruding above to the water tank through a hole that was sealed by transparent polyethylene film. The tumor-bearing mice were monitored and imaged before and after tail vein injection of SHT (2 mg/mL, 200 μL per mouse, $n = 3$) using the 1064 nm laser with a power intensity of 5.5 mJ/cm^2 . At the different time post injection, PA images were acquired and reconstructed for the tumor and the normal tissue.

The mice bearing 4T1 xenograft tumors were administered with SHT (2 mg/mL, 200 μL), or saline (200 μL) via tail vein injection ($n = 4$). According to the PAT results, at 4 h post

injection, tumor regions of living mice were irradiated with 1064 nm laser at 1 W/cm² for 6 min. IR thermal camera was used to monitor the temperature changes of tumor region every 1 min during laser irradiation. After photothermal treatment, sizes of tumors and mice body weights were measured by a caliper every other day for two weeks.

5.4.9 *In vivo* biodistribution study of SHT

Female NCr nude mice bearing xenograft 4T1 tumors were administered with SHT-Cy (200 μ L, 2 mg/mL) through tail vein injection (n = 3). Fluorescence imaging of mice was performed by an IVIS imaging system (PerkinElmer, Inc.). Fluorescence data were acquired with 740 nm laser excitation and emission at 800 nm. Fluorescence intensity of mice were subsequently monitored at different time points post SHT-Cy (Figure 10) injection. After noninvasive fluorescence monitoring, mice were sacrificed by CO₂ asphyxiation. Tumors, livers, hearts, spleens, lungs, kidneys, and intestines were harvested and measure by IVIS imaging system for ex vivo fluorescence imaging to quantify the biodistribution. Quantification of fluorescence was conducted by Living Image software.

Figure 10. Scheme of SHT-Cy preparation. Absorbance at 780 nm as a function of Cy7 with different concentrations (1.6-25 μ g/mL), the red symbol shows that Cy7 loading efficacy in SHT was determined to be 6.13%.

5.4.10 Histological analysis

After NIR-II PTT and observations, mice were sacrificed (n = 4) and organs (livers, spleens, kidneys, lungs, and tumors) were fixed in 4% paraformaldehyde. Then paraffin-

embedded sectioning was performed, and hematoxylin and eosin (H&E) staining was carried out according to standard protocols.⁵⁸ As for the terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) assay, the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Merck) was used according to the manufacturer's instructions. Images of slices were captured by a Nikon ECLIPSE 80i microscope.

5.4.11 Serum biochemistry test

Animal care, handling procedures and study protocols were in agreement with the guidelines evaluated and approved by the ethics committee of Shanghai Model Organisms Center Inc. with AAALAC accreditation. Typically, SHT (2 mg/mL, 200 μ L), was *i.v.* injected into the healthy C57BL/6 mice and control mice ($n = 3$) that were treated with the same amount of saline. Blood was collected from mice eyes at 24 h postinjection and kept in heparinized tubes. Then the serum biochemical indicators, such as ALT, AST, ALP, CREA, BUN and LDH were systematically tested following the standard protocols and the assay kits from Shanghai Model Organisms Center Inc.

5.4.12 Statistical analysis

Mean PA signals of *in vitro* sample test and *in vivo* tumor detection were measured by region of interest (ROI) analysis using Matlab. Results are expressed as the mean $\pm$ SD deviation unless otherwise stated. The results were plotted by OriginPro 8.5 software and statistical analysis were performed using t-test.

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Chapter 6 Cyanines-dyad molecular probe for simultaneous profiling of multiple free-radical evolutions in bacterial pathogenesis (Ongoing project)

6.1 Introduction

Bacterial infections remain the worldwide challenge in recent decades, mainly due to the evolving pathogenicity, emergence of foreign pathogens and development of antibiotic resistance.¹ Comprehensive efforts have been devoted to identify the infections pathogenesis, host defense and antibacterial mechanisms, which comprise the dynamic inflammatory response, immune activation as well as lethal actions.² Among the various bioactive factors in response to infectious diseases, phagocyte-derived free radical species are widely implicated to play essential roles in the regulation of bacteria-induced inflammatory cascade and triggering intrinsic bactericidal effects.³ Furthermore, several investigations have demonstrated the varied contributions of multiple radical mediators including reactive oxygen and nitrogen species (RONS, *e.g.*, $\bullet\text{O}_2^-$, $\bullet\text{OH}$, H_2O_2 , NO , ONOO^- , ClO^- *etc.*) to the control of infection pathological changes.⁴ However, these studies primarily relying on end-point analysis (EPA) such as histology and biochemical assays could not real-time monitor and profile the complex interplay among radical dynamics and pathological evolution of infections, which also been restricted to the invasiveness, time consumption and risk of complications.⁵ Therefore, simple, safe and effective methods are highly desired that would provide novel perspectives on precise diagnosis and therapeutic interventions.

As compare to conventional EPA analyses, optical imaging techniques have the advantages of highly sensitive, noninvasive and time/cost-effective capacities for broad biomedical detections from the cellular (or molecular) level up to whole-body sections in living systems.⁶ Furthermore, the diverse fluorescence molecular sensors are emerging as powerful tools to study bacterial infections through specific targeting or responses of infection-associated bioindicators such as toxins, enzymes, inflammatory cytokines and so forth.⁷ Particularly, some

free radical-responsive small molecular probes towards $\bullet\text{O}_2^-$, $\bullet\text{OH}$, NO , ClO^- , ONOO^- etc. have been readily developed and utilized in fluorescence imaging of bacterial infection with superb sensitivity and specificity.⁸ In spite of the great success for understanding the substantive relevance between those radical species and infections, detailed mechanisms of multi-radical dynamic variations in bacterial pathogenesis have not yet been exploited mainly due to lack of multi-responsive imaging platforms. Recent studies have attempted to directly measure infection-associated oxidative and nitrosative stress status through engineered redox-sensitive GFPs or the combination of two different fluorescent probes,^{9,10} however, these approaches are still limited by the multiplexing capacity, tissue penetration and unified sensing outcomes for real-time and simultaneous recognize native RONS dynamics during the infection pathological progression *in vivo*.

Scheme 1. Design and principle of HCy5-Cy7 for multiple free radicals imaging in the pathogenesis and host defense of bacterial infections.

Here, we report a NIR small-molecule probe based HCy5-Cy7 dyad to simultaneously and sensitively monitor multiple free radicals variations in the infectious disease. As illustrated in Scheme 1, upon the bacterial stimulus, HCy5-Cy7 specifically turns on its fluorescence at 660 nm due to the local overproduction of inflammation-associated $\bullet\text{O}_2^-$ or $\bullet\text{OH}$ triggers oxidation of reduced HCy5 moiety to conjugated Cy5, while the burst of ONOO^- or ClO^- specie induces Cy7 structural degradation thereby leading to the emission turn-off at 800 nm.¹¹ Such unique

multispectral signal responses allow HCy5-Cy7 to multiplexly investigate the dynamic oxidative/nitrosative stress events with high spatiotemporal precision. Most significantly, this molecular probe provides a great opportunity for the real-time manifestation of different redox alterations in bacterial pathogenesis, which may contribute to the development of effective antibacterial therapeutics.

6.2 Results and Discussion

6.2.1 Synthesis of HCy5-Cy7

Scheme 2. Synthesis of HCy5-Cy7. Chemicals and conditions: (a) 1,2-Dichlorobenzene, 90 °C, 12 h; (b) DCM, DIEA, r.t., 6 h; (c) ACN, AcONa, reflux, 16 h; (d) MeOH, NaBH₄, r.t., 10 min; (e) DMF, DIEA, r.t., 24 h; (f) DMF, HBTU, DIEA, r.t., 4 h; (g) DCM, TFA, r.t., 1 h; (h) DMF, EDC, HOBT, DIEA, r.t., 12 h.

We prepared the probe molecule HCy5-Cy7 by coupling of hydro-Cy5 (HCy5) and Cy7 according to Scheme 2. Briefly, the dual carboxylic-substituted Cy5 was firstly synthesized, which was then reduced by NaBH₄ to obtain HCy5. In parallel, Cy7 dye was modified with a cyclohexanediamine linker thereby enabling covalent link with as-synthesized HCy5. The final

molecule HCy5-Cy7 was purified with HPLC and characterized by NMR and mass spectroscopy (see the experimental section for details). Although the similar linked Cy5-Cy7 dye (FOSCY-1) has been previously described by Nagano *et al.* for the sensitive detection of ONOO⁻,¹¹ our design judiciously replaces the less susceptible Cy5 dye to the •O₂⁻ or •OH responsive HCy5 that introduces the multiplexing capacity towards both RNS and ROS. We envision that such a smart and multi-sensitive NIR fluorescent probe, HC5-Cy7 could be particularly suitable for the arduous task of real-time multiple free-radical dynamics profiling.

6.2.2 Multiple RONS responses of HCy5-Cy7

To validate the multiple radical sensing ability, the absorption and fluorescence spectra of HCy5-Cy7 were measured in the buffer with various RONS treatments. As shown in Figure 1a, the probe molecule only shows the major absorption peak at ~780 nm attributing to the Cy7 chromophore. Upon •O₂⁻ reaction, one more peak is appeared at ~640 nm, suggesting the structural conversion from HCy5-Cy7 to π -conjugation regenerated Cy5-Cy7 (Scheme 1). Differently, the typical ONOO⁻ treatment leads to a remarkable decrease of the absorption at ~780 nm, which could be ascribed to the HCy5-Cy7 framework disruption and the formation of π conjugation truncated HCy5-Cy7'. Furthermore, fluorescence responses of this probe with or without above representative radical species (•O₂⁻ and ONOO⁻) were tested with 620 and 740 nm excitations, respectively (Figure 1b). In comparison to HCy5-Cy7 along, •O₂⁻ adding induces the emission occurrence at ~660 nm while ONOO⁻ incubation significantly reduces the fluorescence peak at ~800 nm, which are consistent with the absorption changes. Although •O₂⁻ could cause a slight decrease of the fluorescence at 800 nm, the clearly dual-channel spectral responses are still able to identify these two different types of radicals. As expected, HCy5-Cy7 demonstrates superb sensing specificity and multiplexed capability towards various radical species (Figure 1c). The fluorescence intensity changes exhibit opposite manners that HCy5-Cy7 treated with ROS (•O₂⁻ or •OH) results in a positive enhancement of the emission at 660

nm, whereas no obvious increase is shown for any other radical species treatments. Meanwhile, fluorescence variations at 800 nm exhibit a negative trend only towards RNS (ONOO^-) or ClO^- action. In addition, both the fluorescence intensities at 660, 800 nm are linearly dependent with the concentrations of $\cdot\text{O}_2^-$ and ONOO^- (Figure 1d). The limits of detection (LODs) were accordingly determined to be 0.34 and 0.082 μM , respectively. Such dual-channel reversed fluorescent signals readout enables HCy5-Cy7 as a multi-responsive molecular reporter for the simultaneous multiple radicals detection.

Figure 1. Multiple free radicals detection *via* HCy5-Cy7. (a, b) UV-vis absorption and fluorescence spectra of HCy5-Cy7 (20 μM) in the absence or presence of $\cdot\text{O}_2^-$ (100 μM) or ONOO^- (50 μM). Excitation: 620 and 740 nm, respectively. (c) The sensing specificity of HCy5-Cy7 towards different radical species, including hypochlorite (OCl^-), peroxyxynitrite (ONOO^-), nitric oxide (NO), tert-butoxy radical ($\cdot\text{OtBu}$), tert-butyl hydroperoxide (TBHP), H_2O_2 , hydroxyl radical ($\cdot\text{OH}$), and superoxide radical ($\cdot\text{O}_2^-$); $\Delta\text{F} = \text{F}_1 - \text{F}_0$, where F_1 indicates the fluorescence intensity of HCy5-Cy7 after RONS reaction, F_0 indicates the fluorescence intensity of Hcy5-Cy7 alone. (d) The fluorescence intensity fitting of HCy5-Cy7 as a function of ONOO^- (or $\cdot\text{O}_2^-$, inset) concentration. $y = -5.83x + 278$ (blue); $y = 0.378x + 2.88$ (red).

6.2.3 Endogenous RONS imaging with HCy5-Cy7

We then validated the feasibility of HCy5-Cy7 to monitor the endogenous multiple radical correlations in murine RAW264.7 macrophage cells. The specifically cellular overproduction of $\bullet\text{O}_2^-$ and ONOO^- was performed by chemical stimulations using phorbol 12-myristate 13-acetate (PMA) and LPS/interferon- γ (IFN- γ)/PMA, respectively.^{11,12} Thereafter, RAW264.7 microphage cells were treated with the probe molecule for fluorescence confocal imaging. As indicated in Figure 2a, the control cells loaded with HCy5-Cy7 alone show very weak fluorescence in the Cy5 channel (red) but strong fluorescence in the Cy7 channel (magenta), suggesting a low level RONS under the normal condition. However, a dramatic increase of the red fluorescence can be observed in the PMA treated group and the magenta fluorescence signal remains unchanged. This result demonstrated that HCy5-Cy7 can specifically detect cellular $\bullet\text{O}_2^-$ production, which was further confirmed by a negative control study with ROS scavenger pretreatment. Moreover, LPS/IFN- γ /PMA costimulated cells with HCy5-Cy7 addition exhibit a significant Cy5 fluorescence enhancement while the Cy7 fluorescence intensity is largely decreased to the background level, implying both $\bullet\text{O}_2^-$ and ONOO^- could be endogenously generated. Although several studies proposed that LPS/IFN- γ /PMA stimulated macrophages induce ONOO^- upregulation through the activation of NADPH oxidase and nitric oxide synthase (iNOS),¹³ our results beforehand clearly evidence the coexistence and correlation of $\bullet\text{O}_2^-$ and ONOO^- in the typical inflammatory process.

Besides, HCy5-Cy7 was further proved to be a mitochondria targeted molecule that is able to in situ monitoring of oxidative stress caused oxidation, nitration as well as damages (Figure 2b). In light with the negligible cytotoxicity (Figure 3), such unique multi-responsive and dual channel sensing capabilities make HCy5-Cy7 more conducive to simultaneously manifest biological multi-radical variations in comparison to previously reported single channel peroxynitrite- or superoxide-selective probes.¹²

Figure 2. (a) Fluorescence imaging of endogenous multiple radical variations with HCy5-Cy7 ($10 \mu\text{M}$) in RAW264.7 cells stimulated by PMA ($0.2 \mu\text{g ml}^{-1}$) or LPS/ IFN- γ /PMA ($2/0.05/0.01 \mu\text{g ml}^{-1}$) accordingly. Blue: Hoechst 33342 (Ex: 405 nm, Em: 460/50 nm); Red: Cy5 channel (Ex: 640 nm, Em: 630/50 nm); magenta: Cy7 channel (Ex: 784 nm, Em: 800/30 nm). Scale bars: $50 \mu\text{m}$. (b) Confocal analysis of HCy5-Cy7 cellular localization in PMA-stimulated cells. Green: Mitochondria tracker (Ex: 488 nm, Em: 520/50 nm). Scale bar: $50 \mu\text{m}$.

Figure 3. The cell viability of RAW264.1 after 24 h incubation with different concentration of HCy5-Cy7.

6.3 Conclusion

In this ongoing project, I introduced a NIR small-molecule probe based on HCy5-Cy7 dyad for simultaneously and sensitively monitoring of multiple RONS variations. HCy5-Cy7 specifically turned on its fluorescence at 660 nm *via* superoxide or hydroxyl radical ($\bullet\text{O}_2^-$, $\bullet\text{OH}$)-mediated oxidation of reduced HCy5 moiety to Cy5, while peroxynitrite or hypochlorous specie (ONOO^- , ClO^-)-induced Cy7 structural degradation leads to the emission turn-off at 800 nm. Such multispectral signal responses allow HCy5-Cy7 to multiplexly investigate the dynamic oxidative/nitrosative stress events with high spatiotemporal precision. Most importantly, this multifunctional probe molecule further reveals the multiple radical correlations and implications during the pathogenic and defensive processes of bacteria-infected mice, which may provide new perspectives for profiling the pathology and advancing the precision medicine against infectious diseases.

6.4 Experimental Methods

6.4.1 Reagents and instruments

Unless otherwise stated, all reagents and chemicals for synthesis and assay were purchased from Sigma-Aldrich or Invitrogen (Carlsbad, CA, USA) and were used without further purification. The instruments for molecules characterization and cell imaging are the same as that described in chapter 2 (Section 2.4.1).

6.4.2 Synthesis and characterization of the intermediates and HCy5-Cy7

Synthesis of intermediate 1. 2,3,3-trimethyl-3H-indole (1.6 g, 10 mmol) was added to 1, 2-dichlorobenzene (5 mL) in a round-bottom flask, 3-iodopropionic acid (2.4 g, 12 mmol) was added to the mixture and refluxed overnight under the nitrogen gas atmosphere. The reaction mixture was then cooled to room temperature. The mixture was added dropwise into 100 ml cold diethyl ether and precipitation was observed. Finally, the precipitate was washed with cold n-hexane twice and dried to obtain the product as an off-white solid. ^1H NMR (300 MHz,

CDCB) δ 8.04 – 7.91 (m, 1H), 7.84 (dd, J = 5.4, 3.3 Hz, 1H), 7.71 – 7.51 (m, 2H), 4.65 (t, J = 7.0 Hz, 2H), 2.98 (t, J = 7.0 Hz, 2H), 2.86 (s, 3H), 1.53 (s, 6H). ^{13}C NMR (75 MHz, CDCB) δ 176.74, 146.98, 146.05, 134.57, 134.14, 128.70, 120.78, 59.48, 48.75, 36.32, 27.09, 19.57. ESI-MS: Calcd. For m/z , $\text{C}_{14}\text{H}_{18}\text{NO}_2\text{I}$ $[\text{M-I}]^+$ 232.13; Found, 232.25.

Synthesis of Cy5. Malonaldehyde dianilide hydrochloride (400 mg, 1.55 mmol) was dissolved in a mixture of dichloromethane (10 mL) and DIEA (0.6 mL), and then added dropwise to the solution of acetic anhydride (0.4 mL) and dichloromethane (3 mL) followed by vigorous stirring at room temperature for 6 h. The intermediate 2 was obtained by evaporating the solvent without further purification. Then compound 2 (130 mg, 0.5 mmol) was dissolved in 1 mL acetonitrile solution and added dropwise to a refluxing acetonitrile solution (9 mL) containing compound 1 (300 mg, 0.1 mmol) and sodium acetate (50 mg, 0.78 mmol). After 16 h refluxing, the reaction mixture was allowed to cool at room temperature, and the precipitation was removed by filtration. The crude product was further purified with silica gel column chromatography using DCM/0-20% MeOH as the eluent to afford the target product Cy5 as dark-blue solid. ^1H NMR (300 MHz, MeOD) δ 8.23 (t, J = 13.0 Hz, 2H), 7.48 (t, J = 7.1 Hz, 2H), 7.44 – 7.31 (m, 4H), 7.31 – 7.19 (m, 2H), 6.65 (t, J = 12.4 Hz, 1H), 6.53 – 6.21 (m, 2H), 4.60 – 4.19 (m, 4H), 2.65 (t, J = 7.4 Hz, 4H), 1.73 (d, J = 6.8 Hz, 12H). ^{13}C NMR (75 MHz, MeOD) δ 175.83, 173.12, 154.16, 141.99, 141.23, 128.30, 125.52, 124.76, 121.88, 110.80, 103.13, 49.13, 46.07, 41.14, 34.40, 26.52, 7.72. ESI-MS: Calcd. For m/z , $\text{C}_{31}\text{H}_{35}\text{N}_2\text{O}_4\text{I}$ $[\text{M-I}]^+$ 499.62; Found, 499.45.

Synthesis of HCy5. The Cy5 (125 mg, 0.2 mmol) was dissolved in 5 mL methanol, then 1 mL methanol solution containing NaBH_4 (5.0 mg, 0.11 mmol) was added dropwise to the blue color Cy5 solution, stirred for 10 min until the solution changed to colorless. The reaction mixture was magnetically stirred for another 20 min and removed the solvent under reduced pressure. The crude product was further purified with silica gel column chromatography using

DCM/0-10% MeOH as the eluent to afford the target product HCy5 as gray solid. ^1H NMR (300 MHz, MeOD) δ 6.85 (t, $J = 5.9$ Hz, 2H), 6.80 – 6.68 (m, 2H), 6.67 – 6.56 (m, 1H), 6.57 – 6.45 (m, 2H), 6.37 (dd, $J = 13.8, 7.4$ Hz, 2H), 6.21 (dd, $J = 14.9, 10.7$ Hz, 1H), 5.84 (dd, $J = 14.3, 10.8$ Hz, 1H), 5.36 – 5.15 (m, 2H), 3.71 – 3.54 (m, 2H), 3.40 – 3.28 (m, 2H), 2.27 – 2.08 (m, 4H), 1.66 (s, 1H), 1.30 (d, $J = 1.7$ Hz, 6H), 1.02 (s, 3H), 0.77 (s, 3H). ESI-MS: Calcd. For m/z , $\text{C}_{31}\text{H}_{36}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 501.27; Found, 501.16.

Synthesis of Cy7-S. The starting material Cy7 (IR775, 183 mg, 0.3 mmol), 3-mercaptopropionic acid (28 μL , 0.32 mmol) and triethylamine (44 μL , 0.32 mmol) were mixed in 6.0 mL DMF and stirred at room temperature for 24 h. Then the mixture solution was added dropwise into ice-cold methyl ether and the crude product as green precipitate was filtrated. Through the silica gel-based column chromatography with an eluting solvent system in which the gradient of DCM/0-10%MeOH, the purified product Cy7-S as a dark green powder was obtained. ^1H NMR (300 MHz, MeOD) δ 8.91 (d, $J = 14.3$ Hz, 2H), 7.50 (d, $J = 7.4$ Hz, 2H), 7.40 (dd, $J = 11.2, 4.2$ Hz, 2H), 7.33 – 7.17 (m, 4H), 6.25 (d, $J = 14.2$ Hz, 2H), 3.64 (s, 6H), 3.05 (t, $J = 7.5$ Hz, 2H), 2.50 (t, $J = 7.5$ Hz, 2H), 1.76 (s, 12H). ESI-MS: Calcd. For m/z , $\text{C}_{35}\text{H}_{41}\text{N}_2\text{O}_2\text{SI}$ $[\text{M}-\text{I}]^+$ 553.29, Found 553.21.

Synthesis of Cy7-S-NH₂. Cy7-S (220 mg, 0.33 mmol) and HBTU (173 mg, 0.46 mmol) were dissolved in dichloromethane (10 mL). To this solution were added tert-butyl-4-aminocyclohexyl)carbamate (98 mg, 0.46 mmol) and DIEA (75 μL), followed by stirring at room temperature for 4 hours. Then dichloromethane was added, and the whole mixture was washed with saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na_2SO_4 , filtered, and evaporated. The crude Cy7-S-Boc was used without further purification in the next reaction. Cy7-S-Boc was subsequently dissolved in a mixture of dichloromethane (10 mL) and trifluoroacetic acid (10 mL). The reaction mixture was stirred at room temperature for 1 hours, then evaporated, and the crude Cy7-NH₂ was precipitated from

1 drop of methanol and cold diethylether (about 150 mL). The precipitate was collected by filtration and purified by recrystallization from 2-propanol to afford desired product as dark green crystals. ^1H NMR (300 MHz, MeOD) δ 8.89 (d, J = 13.9 Hz, 2H), 7.52 (d, J = 6.9 Hz, 2H), 7.43 (d, J = 6.8 Hz, 2H), 7.38 – 7.09 (m, 4H), 6.29 (d, J = 14.0 Hz, 2H), 4.55 (s, 2H), 3.68 (s, 6H), 3.08 (s, 4H), 2.71 (s, 4H), 2.52 (s, 2H), 2.04 (s, 2H), 1.98 (s, 2H), 1.77 (s, 12H), 1.54 – 1.23 (m, 6H). ^{13}C NMR (75 MHz, MeOD) δ 208.67, 173.15, 170.89, 156.46, 145.68, 143.03, 140.91, 133.38, 128.42, 127.88, 127.21, 124.83, 121.90, 110.38, 100.81, 35.79, 32.97, 30.21, 29.78, 29.25, 29.12, 26.88, 25.82, 22.80, 20.76. ESI-MS: Calcd. For m/z , $\text{C}_{41}\text{H}_{53}\text{N}_4\text{O}_2\text{SI} [\text{M-I}]^+$ 649.39, Found 649.70.

Synthesis of HCy5-Cy7. HCy5 (75 mg, 0.15 mmol) was dissolved in DMF (2 mL). To the mixture was added dropwise a solution of EDC (33 mg, 0.18 mmol) and HOBT (25 mg, 0.18 mmol) in DMF (2 mL), followed by stirring for 30 mins. Then a solution of Cy7-S-NH₂ (80 mg, 0.11 mmol) and DIEA (15 μL) in DMF (3 mL) was dropwise added. The reaction mixture was stirred overnight under N₂ atmosphere at room temperature, and evaporated. The resulting solid was purified by preparative HPLC, affording HCy5-Cy7 as a dark green powder. ^1H NMR (300 MHz, MeOD) δ 8.87 (d, J = 14.2 Hz, 2H), 7.91 (s, 1H), 7.50 (d, J = 7.2 Hz, 2H), 7.46 – 7.38 (m, 2H), 7.35 – 7.22 (m, 4H), 7.15 – 6.91 (m, 8H), 6.82 – 6.76 (m, 1H), 6.68 (dd, J = 17.8, 10.4 Hz, 3H), 6.54 (d, J = 7.2 Hz, 1H), 6.27 (d, J = 14.2 Hz, 2H), 6.10 (dd, J = 14.4, 10.4 Hz, 1H), 3.93 (d, J = 6.0 Hz, 2H), 3.65 (s, 6H), 3.56 (d, J = 9.2 Hz, 2H), 3.07 (t, J = 7.1 Hz, 2H), 2.74 – 2.64 (m, 4H), 2.57 – 2.45 (m, 4H), 2.03 (s, 1H), 1.93 (d, J = 12.1 Hz, 2H), 1.83 (s, 2H), 1.76 (s, 12H), 1.56 (d, J = 5.9 Hz, 6H), 1.36 – 1.29 (m, 4H), 1.28 – 1.17 (m, 8H), 1.06 – 0.95 (m, 4H). ^{13}C NMR (75 MHz, MeOD) δ 201.35, 173.12, 149.42, 145.66, 143.02, 140.92, 138.87, 133.36, 128.42, 127.00, 124.84, 121.92, 121.36, 110.36, 107.03, 100.78, 86.00, 70.99, 60.13, 43.84, 33.05, 30.81, 30.21, 27.26, 26.90, 25.82, 24.96, 20.74, 13.05. ESI-MS: Calcd. For m/z , $\text{C}_{72}\text{H}_{87}\text{N}_6\text{O}_4\text{SI} [\text{M-I}]^+$ 1131.82, Found 1131.65.

6.4.3 Free radical species sensing in the solution

To a 1 mL solution of HCy5-Cy7 (50 μ M) in PBS (50 mM, pH 7.2), various concentration of free radical stock solutions including $\bullet\text{O}_2^-$, $\bullet\text{OH}$, $\text{ROO}\bullet$, OONO^- , ClO^- , NO and H_2O_2 were added, respectively. The resulting solution was kept at ambient temperature for 5-10 min in 1 cm quartz cuvette for UV-vis absorption and fluorescence emission tests.

6.4.4 Cell culture and fluorescence imaging

Generally, the experimental methods for RAW264.5 cells culture, the induction of endogenous RONS and confocal imaging were followed the similar protocols as described in the section 2.4.5, chapter 2. The concentration of HCy5-Cy7 was 10 μ M for cell treatment and incubated for 30 min.

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Chapter 7 Near-infrared upconversion optogenetic activation of Akt2 signaling for insulin resistance management (Ongoing project)

7.1 Introduction

The worldwide prevalence of diabetes and its metabolic complication have substantially increased in recent decades.¹ Such serious demographic change may pose significant pressures in human healthcare, which thus demand advanced techniques for in-depth understanding of molecular basis of disease evolutions and enabling the efficient combating diabetes-related metabolic dysfunctions. Generally, both the type 1 and type 2 diabetes (T2D) are the predominantly underlying risk factors for metabolic syndromes, which increases the development of various pathological consequences, including damages of the liver, kidneys, eyes, as well as cardiovascular and nerve systems.² It has been widely recognized that insulin resistance is the fundamental aspect of the etiology of T2D and associated complications.³ Physiologically, the pathology of insulin resistance vary according to the metabolic function of the organs and tissues concerned, including down-regulation or deficiencies of the insulin receptor (IR), insulin receptor substrate (IRS) proteins or phosphoinositide 3-kinases (PIP3Ks), or even the abnormalities of glucose transporters (*e.g.*, GLUT4) function.⁴ Considering the inherent physiological complexity and individual differences in metabolism, the direct pathological mechanisms of insulin resistance, especially in human remain to be understood.

So far, the typical approaches in clinic for the prevention of insulin resistance and relevant metabolic syndromes mainly rely on pharmaceutical interventions and diet therapy.⁵ However, these strategies can only alleviate the symptoms by controlling the glucose homeostasis, inflammation or delaying the complications. Moreover, the nonspecific accumulation of chemical drugs through the blood circulation would be inevitable and commonly causing off-target effects. The specifically targeted gene perturbations would also raise the concern of

chronic effects due to its inherent irreversibility. Therefore, it is highly essential to develop an effective yet straightforward technology for insulin resistance management.

Currently, the emerging optogenetics based on the light stimulation permits spatial and temporal manipulation of intracellular signaling pathways through either photo-sensitive channelrhodopsins (ChRs) or photosensitive protein-protein interaction modules,^{6,7} providing potential opportunities to dissect the complex insulin network function. In view of mounting evidence, it was suggested that defects of PI3K/Akt2 (there are three Akt isoforms, Akt1 is essential for cells survival and Akt2 play a role in the metabolic process, while Akt3 is involved in the neuronal development⁸⁻¹⁰) signaling pathway is closely related to insulin resistance¹⁷, recent studies have attempted to use optogenetic systems for remote activation of PI3K/Akt signaling and demonstrating the promising effect to mimic insulin action.^{11, 12}

However, these optogenetic photo-actuators for light-mediated cellular signaling regulation mainly rely on UV or visible light¹³, which may raise the issues of limited tissue penetration and unpleasant phototoxicity owing to the inherent absorption or scattering from endogenous biomolecules. Notably, a classical of near-infrared (NIR) luminescent nanomaterial, upconversion nanoparticles (UCNPs) can emit short-wavelength anti-Stokes shift luminescence upon NIR excitation (*e.g.*, 808 or 980 nm), which has been utilized as promising biophotonic nanotransducers for a wide range of applications, particularly in deep-tissue photoactivation, bioimaging and phototherapy.¹⁴ Taking advantages of the unique photon upconverting feature, a novel concept termed “upconversion optogenetics” has been developed by flexibly incorporating multiple emissive UCNPs into various photoreceptors, such technical integrations facilitate the realization of NIR optogenetic stimulation in living conditions.¹⁵

Herein we present an innovative NIR optogenetic system for precise photoactivation of Akt2 signaling in insulin resistance management (Scheme 1). Principally, the blue light-inducible protein interaction modules (Opto-Akt2) with CRY2-Akt2 and Myr-CIBN were

expressed in adipocytes; meanwhile upconversion nanotransducers (UCNs) emitting blue anti-Stokes luminescence (*e.g.*, at ~450, 480 nm) upon 980 nm laser irradiation is capable of triggering the CRY2/CIBN dimerization as well as is recruitment of inactive Akt2 to the cell membrane, which will subsequently be activated by upstream kinases (*e.g.*, PI3K).^{11, 16} Such a NIR optogenetic platform not only enables effective manipulation of insulin/Akt2 signaling pathways, but also permits the induction of GLUT4 vesicles trafficking and glucose uptake in insulin resistant cells.

Scheme 1. UCNs-mediated NIR optogenetic system for photoactivation of Akt2 signalling against insulin resistance. The photo-inducible protein interaction module CRY2-Akt2 and Myr-CIBN (Opto-Akt2) were expressed in 3T3-L1 adipocytes for the optogenetic Akt2 regulation. Upon NIR light (*e.g.*, at 980 nm) irradiation, UCNs can emit anti-Stokes blue emissions (*e.g.*, at ~ 450, 480 nm) that trigger the CRY2-CIBN dimerization; meanwhile, inactive Akt2 is recruited to the membrane and activated by upstream kinases (*e.g.*, PI3K).

7.2 Results and Discussion

7.2.1 Preparation and characterization of UCNs

The core-shell UCNP with the capability to convert deep-tissue penetrable NIR light to local blue emission for optogenetic regulation were prepared based on the synthetic route shown in Figure 1a. The physicochemical and optical properties were tested by TEM, DLS and

photospectrometer. As indicated in Figure 1b and c, as-prepared UCNPs showed a spherical morphology with the average size distribution of ~ 30 nm. Meanwhile, the UCNPs displayed a strong blue emission band with the peaks at 450 and 475 nm, respectively, upon 980 nm NIR laser excitation (Figure 1d). To improve the biocompatibility of UCNPs for cellular applications, we used pegylated phospholipid (DSPE-PEG-NH₂) to convert the hydrophobic surface to hydrophilic phase (denoted as UCN). In addition, UCNs were covalently labeled with a fluorescence molecule (Cy5) for further cellular tracking studies.

Figure 1. Preparation and characterization of UCNs. (a) Schematic illustration of UCNs preparation and modification. (b, c) The TEM image and DLS analysis of core-shell UCNPs. (d) Upconversion luminescence (UCL) spectra and the photograph of UCNs under 980 nm laser excitation.

7.2.2 Optogenetic activation of CRY2-Akt2

The opto-Akt2 system was designed following a previously reported study by Ozawa et al. using the similar light-sensitive protein dimerization module (CRY2/CIBN).¹⁸ As Akt2 phosphorylation is regulated by both its membrane tracking and upstream kinases. In our study, the kinase domain of Akt2 was fused with CRY2phr (named CRY2-Akt2), and the membrane-targeting myristoylation sequence (named Myr-CIBN) was fused with the N-terminal portion

of CIB1 (CIBN) (Scheme 1), the plasmids were successfully constructed and infected in 3T3-L1 cells.

Figure 2. Optogenetic activation of Akt2 in 3T3-L1 cells. (a) Blue light induced CRY2-Akt2 membrane translocation and recovery. (Green channel, Ex: 488 nm, Em: 520/20 nm) Scale bar: 20 μ m. The Venus fluorescence distribution change shows the light-induced Akt2 translocation. (b) Photoactivation of Akt2 through NIR light (980 nm, 1 W/cm², 1 min). (Cyan channel, Ex: 640 nm, Em: 670/20 nm) Scale bar: 20 μ m. (c) The plot of fluorescence intensity ratio (membrane/cytosol) under NIR light irradiation, showing the opto-Akt2 recruitment from cytosol to the cell surface. (Mean $\pm$ SD, n = 10). (d) Western blot analysis of NIR optogenetic activation of CRY2-Akt2.

To evaluate the capability of optogenetic Akt2 regulation, the blue light (*e.g.*, 480 nm) excitation was firstly performed and monitored by confocal microscope. As shown in Figure 2a, the Venus fluorescence of CRY2-Akt2 was clearly accumulated on the membrane after blue light stimulation, and the membrane localized CRY2-Akt2 was redistributed in the cytosol under the dark condition. This demonstrated that such an optogenetic platform can reversibly regulate Akt2 function. Next, the cells were pretreated with UCNs and irradiated with a 980 nm laser, it was found that CRY2-Akt2 could also translocate to the cell membrane (Figure 2b and c), indicating that upconversion luminescence (UCL) was also capable of triggering Akt2

activation. The biochemical studies proposed that membrane-localized Akt2 is activated by phosphorylation at two sites, Thr308 and Ser473 by PDK1 and TORC2, respectively.²⁷ The phosphorylation level of CRY2-Akt2 after optogenetic regulation was investigated using western blot, like insulin stimulation, the phosphorylated Akt2 expression was clearly observed in either blue light or NIR-excited UCL stimulated groups (Figure 2d). Notably, the phosphorylation of CRY2-Akt2 showed negligible effect towards the Akt substrate, GSK3, the detailed mechanism need to be further explored.

7.2.3 Optimization of NIR stimulation for effective opto-Akt2 activation

Despite the initial success to optogenetic activation of CRY2-Akt2, the experimental conditions of NIR stimulation such as laser power density, irradiation time and potential phototoxic effect are essential to be rationally optimized. As shown in Figure 3 below, the live/dead staining results indicated no significant photodamage of the NIR laser irradiated cells with the laser power density lower than 1.5 W/cm² for 30 min (3 min break after 3 min irradiation). However, the phototoxicity was appeared once the laser power density reaching up to 2 W/cm². Therefore, the optogenetic activation of CRY2-Akt2 was performed with a power density at 1.0 W/cm² in practice. In addition, the NIR irradiation time was further optimized for effective optogenetic activation. The results in Figure 4 demonstrated the NIR-triggered membrane translocation of CRY2-Akt2 in a time- and power-density-dependent manner. The obvious recruitment of CRY2-Akt2 to the plasma membrane was detected once the NIR photo-irradiation reaching 0.5 W/cm² (3 min) or 1.0 W/cm² (1 min). However, very minimal CRY2-Akt2 membrane translocation could be observed with the NIR power density lower than 0.25 W/cm² and even for 3 min continuous irradiation.

Figure 3. Live/dead cells staining after 980 nm laser irradiation for phototoxicity evaluation. Scale bar: 50 μm .

Figure 4. Optimization of the laser power density and irradiation time for NIR optogenetic Akt2 activation. The CRY2-Akt2 membrane traslocation was monitored with a confocal microscope.

7.3 Conclusion

In summary, this work presents an innovative technology by tailoring the emerging optogenetics with near-infrared (NIR) nanophotonics for precise photoactivation of Akt2 signaling and mimicking insulin stimulation. Such a NIR optogenetic platform further enables effective manipulation of insulin/Akt2 signaling pathways with deep penetration, minimum

photodamage and high spatiotemporal resolution. Although further investigations focus on the optogenetic induction of GLUT4 vesicles trafficking and glucose uptake enhancement in insulin resistant models are currently still ongoing, I can envision this creative NIR optogenetic system will provide experimental evidence for effective insulin resistance or T2D management *in vitro* and *in vivo*.

7.4 Experimental Methods

7.4.1 Reagents and instruments

Lanthanide ions, 1-octadecene, oleic acid, NaOH, NH_4F , insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, glucose uptake fluorometric assay kit and TOX8 (resazurin based) were ordered from Sigma-Aldrich. Antibodies such as Myc-tag antibody (Alexa Fluor® 647), Akt/PKB, and phospho-Akt antibodies were obtained from Cell Signaling Technology; Glycogen synthase kinase 3β (GSK3 β) and P-GSK3 β antibodies were obtained from Transduction Laboratories; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from BD Biosciences. All the commercially chemicals and reagents were directly used as received unless otherwise mentioned. The instruments for materials characterization and cell imaging are the same as that described in chapter 2 (Section 2.4.1).

7.4.2 Preparation of core-shell UCNPs

Typically, 2 mL $\text{RE}(\text{CH}_3\text{CO}_2)_3$ (RE = Y, Yb and Tm) methanol solution, 3 mL oleic acid (OA) and 7 mL 1-octadecene were successively added in a 50 mL three-neck flask, and the total lanthanides amount was 0.4 mmol. The reaction mixture was heated up to 150 °C for 1 h before cooling down to room temperature. Then 6 mL methanol solution containing NaOH (40.0 mg) and NH_4F (59.3 mg) was dropwise added and stirred for half hour at ~50 °C. The methanol was evaporated and removed by a pump, and the reaction mixture was then gradually heated and kept at 290 °C for 2 h under N_2 atmosphere. After cooling down to room temperature,

the core lanthanide nanoparticles were precipitated using ethanol for three times by centrifugation and re-dispersed in 5 mL hexane for the next step reaction.

Similarly, 7 mL 1-octadecene, 3 mL OA and 2 mL $\text{Y}(\text{CH}_3\text{CO}_2)_3$ methanol solution (0.4 mmol) were added in a 50 mL three-neck flask. The reaction mixture was heated up to 150 °C for 1 h and then cooling down to room temperature. The as-prepared core lanthanide nanoparticles in hexane were added along with 6 mL methanol solution containing NaOH (40.0 mg) and NH_4F (59.3 mg). The mixture was stirred for 30 min at 50 °C and then removed MeOH. The solution was gradually heated and kept at 290 °C for 2 h under N_2 atmosphere. After cooling down to room temperature, the core-shell particles were precipitated using EtOH and collected through centrifugation after three times washing. Finally, the core-shell UCNPs were re-dispersed in hexane and kept in 4 °C fridge.

7.4.3 Surface modification of UCNPs

(a) *Pegylation*. Briefly, 5 mL as-prepared OA-caped UCNPs chloroform solution (10 mg/mL) was mixed with a 5 mL DSPE-PEG₂₀₀₀-NH₂ solution in chloroform (10 mg/mL), and stirred at room temperature overnight. The chloroform was then evaporated, and the residue was dissolved in 5 mL DI water. After sonication, the pegylated UCNPs were collected and dispersed in water and further filtered through a 0.22 μm membrane filter to remove the large aggregates, the resulting solution was then kept at 4 °C for later biological applications.

(b) *Cy5 labeling*. 5 mg of as-prepared UCNPs@PEG-NH₂ was dispersed in 2.5 mL PBS (pH 7.4), then 1 mg of Cy5-NHS (Lumiprobe Corporation) in 100 μL DMSO was added. After 2 min sonication, 5 μL DIEA was added in the reaction solution and followed by 24 h stirring in the dark at room temperature. Finally, the nanoparticles were collected by centrifugation (12,000 rpm, 10 min) and washed by ethanol and PBS to remove the free reactants. The Cy5-conjugated UCNPs (UCN-Cy5) were re-dispersed in PBS and stored in -20 °C fridge before use.

7.4.4 Construction of Opto-Akt2 plasmids and retrovirus infection

This section was finished in Professor Takeaki Ozawa's lab by Dr Genki Kawamura and Dr Mizuki Endo, so I will not draw up the experimental details in the thesis.

7.4.5 Cell imaging and optogenetic regulation

To evaluate the light-mediated translocation of CRY2-Akt, the plasmid DNAs transfected 3T3-L1 cells were seeded in the glass-bottom confocal dish (35-mm) in phenol red-free DMEM medium containing 10% FBS. After 24 h incubation, the cells were observed using a confocal microscope (Carl Zeiss LSM 800, Germany), 488 nm laser for the Venus excitation and 640 nm laser for the membrane tracker excitation (CellMask™ Deep Red, Thermo fisher).

a) Due to the absorption band of the photoreceptor CRY2 ranging from 400–500 nm, the 488 laser was used as the illumination power (~10 mW, 2 s intervals) for both the photo-activation of CRY2-Akt and real-time imaging. The fluorescence intensity changes of the cytosol and membrane were quantified using ZEN imaging software. The M/C (Membrane/Cytosol) ratio change was calculated by dividing an average M/C ratio after light stimulation to reach a stable state of CRY2-Akt translocation.

b) For the CRY2-Akt activation by NIR light, transfected 3T3-L1 cells were incubated in the confocal dish for 24 hours, then pegylated UCNs (0.1 mg mL⁻¹) was treated and incubated for another 4 h, washed the cells and replaced with phenol red-free DMEM medium before NIR optogenetic regulation and confocal imaging. Moreover, 3T3-L1 cells were treated with Cy5-labeled UCNs as a control to investigate the cellular uptake and localization of nanoparticles.

c) The live/dead cell staining was performed to evaluate the potential photodamage effect. Typically, 3T3-L1 cells were seeded in the confocal dish (Ibidi, 8-well) at a cell density of 2×10^4 cells/mL (0.3 mL) and incubated overnight. Then 0.1 mg/mL of UCNs were added in the wells for 4 h, then cells were washed with fresh culture medium and irradiated with a 980 nm

laser (1.0 W/cm²) for 30 min (3 min break after 3 min irradiation). The cells were incubated overnight and stained with propidium iodide (PI) and calcein AM for 30 min, washed with PBS and then imaged by confocal laser microscope.

7.4.6 Western blot study

Prior to the assay, the culture medium of 3T3-L1 cells with or without UCNPs treatment in confocal dishes (35-mm) was replaced with the phenol red-free DMEM medium with 0.1% FBS in the dark overnight. Then the serum-starved cells were irradiated with blue light (480 nm, 10 mW cm⁻²) or 980 nm laser (1 W cm⁻²) for 30 min (3 min break, after 3 min irradiation) towards UCNs treated cells. Cells were washed with cold PBS and lysed with 100 μ L ice cold RIPA buffer (Thermo Fisher) containing phosphatase and protease inhibitors, then treated with 100 μ L of sampling buffer (125 mM Tris-HCl, pH 6.8, 10% glycerol, 5% SDS and 10% 2-mercaptoethanol). The mixture was collected and gently sonicated. The protein samples were boiled at 95 °C for 5 min, running on SDS-polyacrylamide gels for the separation. Then the electrophoretical proteins gels were transferred onto PVDF (GE Healthcare, UK) membranes, and blocked with 1% skimmed-milk in Tris-buffer, containing Tween-20 (TBS-T: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1% Tween-20) for 2 h, subsequently immunoblotted with specific antibodies incubation overnight. Finally, the immunostained protein bands were visualized using ECL detection reagents with a gel imaging system.

7.5 References

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Chapter 8 Summary and Future Works

8.1 Summary and Perspective

Recent advances in NIR biophotonics-based theranostic platforms have witnessed the great success in the field of biomedicine: bioimaging, phototherapy and photoactivation. Various NIR photon-converting systems with their unique photophysical processes, *e.g.* photon upconversion, photon downshifting, photothermal and photoacoustic conversions enable miscellaneous applications in the biological regulation and diseases treatment (chapter 1). In this thesis, I have successfully developed two series of stimuli-responsive optical nanoprobes and nanotheranostics by using UCNPs and CTNs, respectively. These studies not only diversify the molecular design of sensitive photoacoustic and luminescence nanoprobes towards pathophysiological biomarkers in the NIR-I and NIR-II windows, but also highlight their potentials in the clinical translation for early liver failure diagnosis (chapter 2), precise pharmaceutical screening (chapter 3), effective tumor phototheranostics (chapter 4 and 5), bacterial infection sensing (chapter 6) as well as type-2 diabetes management (chapter 7). I draw the conclusions of above works as following:

Firstly, a multi-responsive PA/UCL nanoprobe has been prepared through the combination of multispectral UCNPs and RONS sensitive cyanine dyes, which allowed simultaneous MSOT and luminescence imaging for reversed ratiometric tracking of disparate oxidative and nitrosative stresses *in vitro* and *in vivo*. Most importantly, this study provides a promising strategy to screen the multiple biomarkers and dynamically correlate their interplays in complex living settings, which are particularly meaningful to exploit the pathological progression underlying drug-induced liver failures. Moreover, this hybrid imaging technology demonstrates the opportunities for potential clinical applications owing to the noninvasiveness, high sensitivity and imaging resolution.

Secondly, a new type of PA/UCL cross-referencing nanoprobe was designed for noninvasive and reversed-ratiometric imaging of oxidative dynamics *in vitro* and *in vivo*. Such a nanoprobe not only enables the precise recognition of the complex interplay between oxidative stress and hepatopathological evolution, but also facilitates the real-time therapeutic response monitoring, hepatoprotective intervention guidance and pharmaceutical screening for new drugs development. In addition, this study introduces a new concept of cross-referencing optical sensing that paves a way to design highly sensitive and versatile bioimaging probes.

Thirdly, I have successfully developed two organic CTNs-based phototheranostics working in the NIR-II optical window, in which the pH-sensitive and biodegradable CTN applied for NIR-II PA tumor imaging shows good contrast, deep penetration and negligible toxicity; another redox-activatable and acid-enhanced nanoprobe also enables sensitive tumor NIR-II PA imaging and combined PTT. These studies significantly minimize the nonspecific signal interference and therapeutic side effects in the comparison of currently used “always-on” phototheranostic agents. Furthermore, this study provides great potential for the investigation of other redox/acid-associated pathophysiological alterations in living settings.

In addition, I have designed and synthesized a NIR small-molecule probe based on HCy5-Cy7 dyad for simultaneous and sensitive monitoring of multiple RONS variations. The RONS responses in the solution and in cells revealed that the multispectral signal changes allowed HCy5-Cy7 to multiplexly investigate the dynamic oxidative/nitrosative stress events with high spatiotemporal precision. Although further *in vivo* studies are still ongoing, it can be proposed that the multifunctional probe molecule may be useful in profiling the multiple RONS correlations and implications during the pathogenic and defensive processes of bacteria-infected models.

Finally, I have presented a novel NIR optogenetic technology by tailoring UCNP-photo-transducers and the photosensitive protein interaction module (CRY2-CIBN) for remote control

of Akt2 activity, further facilitating the potential of NIR photoactivation of insulin/Akt2 signaling for insulin resistance management.

8.2 Future works

In chapter 6, I have successfully synthesized the HCy5-Cy7 dyad molecular probe and tested its capability for multiple free radical species sensing in both the solution and cellular models. The results demonstrated good specificity and sensitivity for ROS and RNS variations analysis and optical imaging. Further studies are currently ongoing and focused on the fluorescence manifestation of oxidative and nitrosative status in bacterial infection pathogenesis and antibiotics defense. The next plans include two parts, firstly the cell infection model will be established and treated with the probe molecule for RONS imaging using confocal microscope; the second part will focus on the *in vivo* imaging study, and we will performed IVIS imaging to monitor the dynamic RONS changes once the mice infection models are ready.

In chapter 7, I have verified that UCNPs are capable of manipulating the optogenetic module (CRY2-Akt2/CIBN) and can effectively induce Akt2 phosphorylation in the cellular level. Further studies in the insulin resistant model prove that the NIR optogenetic Akt2 signaling activation shows experimental evidence of both glucose uptake and GLUT4 vesicles upregulations. However, it is necessary to investigate whether this NIR optogenetic technique is applicable for the regulation of type-2 diabetes in living animals. Therefore, the future work can be conducted as following procedures: (1) the establishment of insulin resistance animal models; (2) the infection of opto-Akt2 system in insulin resistant mice; (3) UCNPs administration and NIR laser irradiation for optogenetic manipulation; (4) the evaluation of photoregulation effects and pharmacological implications *in vivo*.

In conclusion, this thesis presents the development of novel NIR biophotonic systems and their potential utilizations in photo-mediated diseases diagnosis and therapy. Despite the great

promise of above results, these NIR platforms still need further refinements to fulfil the requirements of clinical translations. For example, the photon upconversion efficiency of UCNPs should be improved for more effective photoregulation of biological functions; the targeting capability and response-specificity of the nanoprobe need to be optimized for *in vivo* applications; the potential toxicity or biocompatibility are also major issues to advance the current nanomedicine into clinical research. Overall, along with the continuous efforts in improving the optical energy transfer efficacy, minimizing the toxicity and extending the applicability in this field, the translational studies of NIR photon-converting systems will have a bright prospect.

List of Publications

First and co-first author publications:

1. Xiangzhao Ai[†], **Zhimin Wang**[†](Co-first author), Haolun Cheong, Yong Wang, Ruochong Zhang, Jun Lin, Yuanjin Zheng, Mingyuan Gao*, Bengang Xing*. *Nat. Commun.*, 2019, 10, 1087.
2. **Zhimin Wang**, Xu Zhen, Paul Kumar Upputuri, Yuyan Jiang, Junwei Lau, Manojit Pramanik, Kanyi Pu*, Bengang Xing*. *ACS Nano*, 2019, 13, 5816.
3. **Zhimin Wang**[†], Xiangzhao Ai[†](Co-first author), Zhijun Zhang, Yong Wang, Xiangyang Wu, Richard Haindl, Edwin K. L. Yeow, Wolfgang Drexler, Mingyuan Gao, Bengang Xing*. *Chem. Sci.*, 2020, 11, 803-811.
4. **Zhimin Wang**, Paul Kumar Upputuri, Xu Zhen, Ruochong Zhang, Yuyan Jiang, Xiangzhao Ai, Zhijun Zhang, Ming Hu, Zhenyu Meng, Yunpeng Lu, Yuanjin Zheng, Kanyi Pu, Manojit Pramanik*, Bengang Xing*. *Nano Res.*, 2019, 12, 49.
5. **Zhimin Wang**, Ming Hu, Xiangzhao Ai, Zhijun Zhang, Bengang Xing*. *Adv. Biosys.*, 2019, 3, 1800233. (Review Article)
6. **Zhimin Wang**, Do Cong Thang, Qingyu Han, Xuan Zhao, Xilei Xie, Zhiyong Wang, Jun Lin, Bengang Xing*. *J. Control. Release*, 2020, 324, 104-123. (Review Article)
7. **Zhimin Wang**, and Bengang Xing*. *Chem Asian J.* 2020, 15, 2076-2091. (Review Article)

Other co-author publications:

1. Thang Do Cong., **Zhimin Wang**, Xiaoling Lu, Bengang Xing*. *Theranostics*, 2019, 9, 3308-3340. (Review Article)
2. Thang Do Cong., **Zhimin Wang**, Ming Hu, Qingyu Han, Bengang Xing*. *ACS Nano*, 2020, 14, 5836–5844.