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**NANYANG
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SINGAPORE

DEVELOPMENT OF SMART OPTICAL IMAGING

AGENTS FOR DISEASE DIAGNOSIS

CHENG PENGHUI

School of Chemical and Biomedical Engineering

Interdisciplinary Graduate Programme

NTU Institute for Health Technologies (HealthTech NTU)

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AGENTS FOR DISEASE DIAGNOSIS

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School of Chemical and Biomedical Engineering

**Interdisciplinary Graduate Programme
NTU Institute for Health Technologies (HealthTech NTU)**

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

2022

Statement of Originality

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

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Supervisor Declaration Statement

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

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A handwritten signature in black ink, appearing to be 'Pu Kanyi', is written over a rectangular stamp. The stamp contains the text 'TU NTU NTU NTU NTU NTU NTU' repeated in a grid pattern.

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Prof. Pu Kanyi

Authorship Attribution Statement

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

Chapter 3 is published as Penghui Cheng, Jianjian Zhang, Jiaguo Huang, Qingqing Miao, Chenjie Xu, Kanyi Pu. Near-infrared fluorescence probes detect reactive oxygen species for keloid diagnosis. *Chemical Science* **9**, 6340-6347 (2018). DOI: 10.1039/c8sc01865k.

The contributions of the co-authors are as follows:

- A/Prof Kanyi Pu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Prof. Chenjie Xu, Dr Jiaguo Huang and Dr. Qingqing Miao.
- I co-designed the study with A/Prof Kanyi Pu and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- Dr. Jianjian Zhang provided guidance in the synthetic work and data process.
- Dr Qingqing Miao, Dr. Jiaguo Huang provided guidance in the experiment operation and data analysis.

Chapter 4 is published as Penghui Cheng, Qingqing Miao, Jingchao Li, Jiaguo Huang, Chen Xie, Kanyi Pu. Unimolecular chemo-fluoro-luminescent reporter for crosstalk-free duplex imaging of hepatotoxicity. *Journal of the American Chemical Society*, 2019, 141, 10581-10584. DOI: 10.1021/jacs.9b02580.

The contributions of the co-authors are as follows:

- A/Prof Kanyi Pu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr Qingqing Miao.
- I co-designed the study with A/Prof Kanyi Pu and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- Dr. Jiaguo Huang and Dr. Chen Xie provided guidance in the synthetic work and data analysis.
- Dr. Jingchao Li assisted in the paraffin-embedded sectioning, cryo-sectioning, and provided guidance in the data analysis.
- Dr. Qingqing Miao provided guidance in the experiment operation and data process.

Chapter 5 is published as Penghui Cheng, Wan Chen, Shenhua Li, Shasha He, Qingqing Miao, Kanyi Pu. Fluoro-photoacoustic polymeric renal reporter for real-time dual imaging of acute kidney injury. *Advanced Materials*, 2020, 32, 1908530. DOI: 10.1002/adma.201908530.

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- A/Prof Kanyi Pu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr Qingqing Miao.
- I co-designed the study with A/Prof Kanyi Pu and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- Prof. Qingqing Miao, Wan Chen, and Shenhua Li assisted in the photoacoustic imaging work at State Key Laboratory of Radiation Medicine and Protection, School for Radiological and Interdisciplinary Sciences (RAD-X), Collaborative Innovation Center of Radiation Medicine of Jiangsu Higher Education Institutions, Soochow University.
- Prof. Qingqing Miao and Wan Chen analyzed the data of in vivo photoacoustic imaging.
- Prof. Qingqing Miao provided guidance in the experiment operation and data process.

Chapter 6 is published as Penghui Cheng, Qingqing Miao, Jiaguo Huang, Jingchao Li, Kanyi Pu. Multiplex optical urinalysis for early detection of drug-induced kidney injury. *Analytical Chemistry*, 2020, 92, 6166-6172. DOI: 10.1021/acs.analchem.0c00989.

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List of Abbreviations

AAP: Alanine Aminopeptidase

AKI: Acute Kidney Injury

AML-12: Alpha Mouse Liver-12

BUN: Blood Urea Nitrogen

Casp3: Caspase-3

CHL: chemiluminescence

CFR: Chemo-fluorescent Reporter

CKD: Chronic Kidney Injury

CyBA: Hemicyanine dye with boronic acid as the responsive group

CyTF: Hemicyanine dye with trifluoromethyl ketone as the responsive group

CYP: Cytochrome P450

DAPI: 4',6-diamidino-2-phenylindole

DIH: Drug-induced Hepatotoxicity

DIKI: Drug-induced Kidney Injury

DIPEA: *N,N*-Diisopropylethylamine

DMF: Dimethylformamide

DNA: Deoxyribonucleic Acid

EEDQ: *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline

FAP- α : Fibroblast Activation Protein- α

FBS: Fetal Bovine Serum

FDA: Food & Drug Administration

FPRR: Fluro-Photoacoustic Renal Reporter

GFR: Glomerular Filtration Rate

GGT: γ -Glutamyl Transpeptidase

HBTU: 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate

H&E: Hematoxylin & Eosin

HoBT: Hydroxybenzotriazole

HK-2: Human Kidney-2

KF: Keloid Fibroblast

HP β CD: hydroxylpropyl- β -cyclodextrin

NAC: *N*-acetyl-L-cysteine

NDF: Normal Dermal Fibroblast

HPLC: High-performance liquid chromatography

ID: Injected Dosage

IVIS: In Vivo Imaging System

i.p.: intraperitoneally

i.v.: intravenously

MeOH: Methanol

MSOT: Multi-spectral Optoacoustic Tomography

MTS: CellTiter 96[®] AQueous One Solution Cell Proliferation Assay

MUR: Multiplex Urinalysis Reporter

NAG: N-acetyl glycosaminidase

NIR: Near-infrared

NIRF: Near-infrared fluorescence

NMR: Nuclear Magnetic Resonance

n.s.: not significant

$O_2^{\bullet-}$: Superoxide anion

PA: Photoacoustic

PABA: *p*-aminobenzyl alcohol

PanIN: Pancreatic Intraepithelial Neoplasia

PBS: Phosphate Buffered Solution

RCOA: Renal-clearable optical agents

PEG: Polyethylene Glycol

ROI: Region-of-interest

RONS: Reactive Oxygen and Nitrogen Species

ROS: Reactive Oxygen Species

rt: room temperature

SBR: Signal-to-Background Ratio

sCr: serum creatinine

SD: Standard Deviation

t_{dpt} : drug post-treatment time

TFA: Trifluoroacetic Acid

THF: Tetrahydrofuran

uPA: Urokinase-type Plasminogen Activator

VPA: Valproic acid

List of Publications

1. **Penghui Cheng**, Kanyi Pu*. Molecular imaging and disease theranostics with renal-clearable optical agents. *Nature Reviews Materials*, 2021, 6, DOI: 10.1038/s41578-021-00328-6.
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3. **Penghui Cheng**, Wan Chen, Shenhua Li, Shasha He, Qingqing Miao, Kanyi Pu. Fluoro-photoacoustic polymeric renal reporter for real-time dual imaging of acute kidney injury. *Advanced Materials*, 2020, 32, 1908530.
4. **Penghui Cheng**, Kanyi Pu. Activatable phototheranostic materials for imaging-guided cancer therapy. *ACS Applied Materials & Interfaces*, 2020, 12, 5286–5299. [Review]
5. **Penghui Cheng**, Qingqing Miao, Jingchao Li, Jiaguo Huang, Chen Xie, Kanyi Pu. Unimolecular chemo-fluoro-luminescent reporter for crosstalk-free duplex imaging of hepatotoxicity. *Journal of the American Chemical Society*, 2019, 141, 10581-10584.
6. **Penghui Cheng**, Jianjian Zhang, Jiaguo Huang, Qingqing Miao, Chenjie Xu, Kanyi Pu. Near-infrared fluorescence probes detect reactive oxygen species for keloid diagnosis. *Chemical Science*, 2018, 9, 6340-6347. [2018 Chemical Science HOT Article Collection][Cover]

7. Jianjian Zhang, **Penghui Cheng**, Kanyi Pu. Recent advances of molecular optical probes in imaging of β -galactosidase. *Bioconjugate Chemistry*, 2019, 30, 2089-2101. [Invited Review]
8. Xu Zhen, **Penghui Cheng**, Kanyi Pu. Recent advances in cell membrane-camouflaged nanoparticles for cancer phototherapy. *Small*, 2019, 15, 1804105. [Invited Review]
9. Chen Xie, **Penghui Cheng**, Kanyi Pu. Synthesis of PEGylated semiconducting polymer amphiphiles for molecular photoacoustic imaging and guided therapy. *Chemistry – A European Journal*, 2018, 24, 12121-12130. (Invited Concept)
10. Houjuan Zhu, **Penghui Cheng**, Peng Chen, Kanyi Pu. Recent progress on the development of near-infrared organic photothermal and photodynamic nanotherapeutics. *Biomaterials Science*, 2018, 6, 746–765. [Review]
11. Shasha He, Jingchao Li, **Penghui Cheng**, Ziling Zeng, Chi Zhang, Hongwei Duan, Kanyi Pu. Charge-reversal polymer nano-modulators for photodynamic immunotherapy of cancer. *Angewandte Chemie International Edition*, 2021, 60, DOI: 10.1002/ange.202106392.
12. Jingsheng Huang, Jiaguo Huang, **Penghui Cheng**, Yuyan Jiang, Kanyi Pu. Near-infrared chemiluminescent reporters for in vivo imaging of reactive oxygen and nitrogen species in kidneys. *Advanced Functional Materials*, 2020, 30, 2003628 .
13. Jiaguo Huang, Yan Lyu, Jingchao Li, **Penghui Cheng**, Yuyan Jiang, Kanyi Pu. Renal-clearable duplex optical reporter for real-time imaging of contrast-induced acute kidney injury. *Angewandte Chemie International Edition*, 2019, 58, 17796-17804.

14. Qing Li, Shenhua Li, Shasha He, Wan Chen, **Penghui Cheng**, Yan Zhang, Qingqing Miao, and Kanyi Pu. Activatable polymeric reporter for near-infrared fluorescent and photoacoustic imaging of invasive cancer. *Angewandte Chemie International Edition*, 2020, 59, 7018-7023. [\[Cover\]](#)
15. Jingchao Li, Dong Cui, Yuyan Jiang, Jiaguo Huang, **Penghui Cheng**, Kanyi Pu. Near-infrared photoactivatable semiconducting polymer nanoblockaders for metastasis-inhibited combination cancer therapy. *Advanced Materials*, 2019, 31, 1905091.

Abstract

Optical imaging has become an indispensable tool in disease diagnosis and treatment. As compared to other imaging modalities, it exhibits high-throughput capacity, high sensitivity with detection at molecular and cellular levels, avoidance of radiative toxicity, and relatively low instrumentation costs. In the clinical settings, optical instrumentations such as endoscopy, microscopy and photoacoustic tomography, has facilitated both pre-clinical and clinical optical imaging applications.

Encouraged by preclinical results and the translational potential of optical imaging, a variety of optical imaging agents have been developed, which could be classified into inorganic nanoparticles and organic fluorophores, based on the material of construction. Organic fluorophores show high structural versatility with clear structure-property relationships. These optical agents feature modifiable groups, which enables the combination of different functional moieties to achieve desired biochemical properties in biomarker detection, targeting and even therapy. Particularly, biomarker-activatable optical imaging agents that specifically turn on the optical signals in the presence of disease biomarkers at the molecular and cellular level have the potential for disease detection at an incipient stage.

This thesis aims to discuss the application of biomarker-activatable molecular fluorophore for disease diagnosis via both real-time in vivo imaging and in vitro urinalysis. In the real-time imaging approach, both attempts to improve probe design for clinical translation and explore different diseases will be reported. The probe design could be improved from two different aspects: i) tuning optical properties to

improve penetration depth and signal-to-noise ratio; ii) tuning pharmacokinetic properties to accelerate body excretion and minimize organ toxicity. Such activatable fluorophores are utilized to detect malignant skin diseases, drug-induced liver injury, drug-induced kidney injury in living mice models.

Last but not least, the application of activatable optical probes for in vitro urinalysis is introduced. The in vitro application liberates the stringent requirement of biocompatibility and pharmacokinetic properties on molecular fluorophores. Therefore, the emphasize is on finding appropriate disease biomarkers that are both urinary-secreted and disease-specific. Multiplex urinalysis reporters that specifically emit optical signals in the presence of three different biomarkers are developed and they are applied for disease detection in drug-induced kidney injury model.

Chapter 1 Introduction

1.1. Background

Optical imaging has become an indispensable tool in disease diagnosis and treatment. As compared to other imaging modalities, it exhibits high-throughput capacity, high sensitivity with detection at molecular and cellular levels, avoidance of radiative toxicity, and relatively low instrumentation costs.¹ The development of optical instrumentations, such as endoscopy, microscopy and photoacoustic tomography, has facilitated pre-clinical and clinical optical imaging applications, including the detection of molecular and cellular pathways in living cells and animals, preclinical drug screening, exploitation of new disease biomarkers, image-guided surgery and therapy.^{2,3} Additionally, deep-tissue imaging is made possible with photoacoustic imaging, which integrates optical and ultrasound imaging, thereby enabling a tissue penetration depth of up to 5 cm.⁴

Encouraged by preclinical results and the translational potential of optical imaging, a variety of optical imaging agents have been developed, mainly based on inorganic nanoparticles and organic fluorophores, each with their own merits and demerits. Inorganic nanoparticles, such as metal nanoparticles and nanoclusters,⁵ quantum dots,⁶ carbon-based⁷ and silica-based nanomaterials⁸, have a size- and composition-tunable signal wavelength, high photostability and large Stokes shift owing to quantum-confined properties. In comparison, organic materials, such as macromolecular and small-molecular fluorophores, show high structural versatility

with clear structure-property relationships.⁹ These optical agents feature modifiable groups, which enables the combination of different functional moieties to achieve desired biochemical properties in detection, targeting and even therapy.^{10,11}

1.2. Motivation

Biomarker-activatable small-molecular fluorophores that specifically turn on the optical signals in the presence of disease biomarkers achieve disease diagnosis at the molecular and cellular level. Compared to anatomical imaging to detect physiological damages, molecular imaging provides higher sensitivity and accuracy, is capable to detect the onset of disease at an earlier time point.¹² However, most of the biomarker-activatable fluorophores emit fluorescence signals in the first NIR range that exhibit poor tissue penetration depth (1 cm). The poor penetration is limited by the interface reflection and scattering of excitation light, the non-specific tissue absorption and autofluorescence.¹³ To solve this problem, self-luminescent and photoacoustic imaging have emerged, which drastically increased imaging sensitivity and signal-to-noise ratio due to reduced light scattering and tissue autofluorescence.¹⁴ However, biomarker-activatable fluorophores that emit self-luminescent or photoacoustic signals have been limited.

Furthermore, the pharmacokinetic properties of optical agents are crucial for clinical translation. After intravenous administration into the body, optical agents enter the blood circulation and can be distributed into various organs and tissues, followed by in vivo metabolism and clearance by the hepatobiliary or renal clearance pathways. The clearance pathways are determined by the inherent properties of the agents, such

as size, shape, surface charges and other factors. In the hepatobiliary clearance pathway, organic optical agents are slowly metabolized and degraded by macrophages in the mononuclear phagocyte system (that is, liver and spleen). The metabolites are then secreted into the duodenum via the bile duct.¹⁵ Non-biodegradable nanoparticles can accumulate in the body and cause acute or chronic toxicity.

Most of the optical agents are excreted via the hepatobiliary pathway and cleared by the mononuclear phagocyte system (MPS). The non-specific protein adsorption on optical agents in the blood first triggers their sequestration by the MPS system, eventually leading to intracellular degradation in hepatocytes, followed by excretion into the bile and into feces in days to months post injection.¹⁶ Accumulation in hepatocytes can last for > 6 months, which is associated with chronic organ toxicity¹⁷.

By contrast, renal-clearable optical agents are rapidly filtrated by the glomerular filtration membrane (GFM), transported through the renal tubules, and eventually excreted into the bladder through the ureter. In this process, the optical agents are minimally metabolized and rapidly cleared. Thus, renal-clearable optical agents minimize toxicity issues and side effects in the time course of imaging, which is a key criterion for clinical translation. Therefore, it is of great importance to fine-tune the pharmacokinetics and excretion pathways of optical agents for successful clinical translation.

1.3. Objectives of This Work

There are 4 objectives in this work.

The 1st objective is to enhance the sensitivity of optical imaging agents for disease diagnosis. To achieve this purpose, an activatable fluorescent probe that specifically activates fluorescent signals in the presence of reactive oxygen and nitrogen species was applied to detect a malignant proliferative skin scarring, keloid.

The 2nd objective is to improve the accuracy of optical imaging agents for disease diagnosis. To achieve this goal, in Chapter 4, the design of a unimolecular optical agent that integrates fluorescence and chemiluminescence imaging for detection of two interlinked molecular events, oxidative stress (superoxide anion, $O_2^{\cdot-}$) and cell apoptosis (caspase-3) was reported. Such a crosstalk-free dual channel probe was applied for duplex imaging in the detection of drug-induced liver injury by longitudinal monitoring of two sequential biomolecular events and achieved disease diagnosis with high accuracy.

The 3rd objective is to accelerate the body clearance of optical imaging agents for biosafety purposes. To achieve this goal, in Chapter 5, a biomarker-activatable probe that emit both fluorescence and photoacoustic signals was reported; and applied for detection of drug-induced kidney injury in living mice. The probe had high renal clearance efficiency and demonstrated high biocompatibility. Such probe demonstrated both high signal-to-noise ratio and deep tissue penetration depth; and showed high translational potential.

The 4th objective is to explore the application of biomarker-activatable optical agents towards clinical translation. In Chapter 6, the application of biomarker-activatable optical probe for in vitro disease diagnosis via optical urinalysis was illustrated. A cocktail of three molecular urinary reporters (MURs) were applied for detection of DIKI in mouse models, and achieved better diagnostic accuracy as compared to traditional diagnostic methods.

1.4. Organization of This Thesis

This thesis aims to discuss the application of biomarker-activatable molecular fluorophore for disease diagnosis via both real-time in vivo imaging and in vitro urinalysis. In the real-time imaging approach, both attempts to improve probe design for clinical translation and explore different diseases will be reported. The probe design could be improved from two different aspects: tuning optical properties to improve penetration depth and signal-to-noise ratio, and tuning pharmacokinetic properties to accelerate body excretion and minimize organ toxicity. Secondly, the application of activatable optical probes for in vitro urinalysis will be introduced. The in vitro application liberates the stringent requirement of biocompatibility and pharmacokinetic properties on molecular fluorophores. Therefore, the emphasize is on finding appropriate disease biomarkers that are both urinary-secreted and disease-specific. Thereafter, sensitive probes could be designed and developed for biomarker detection.

Figure 1-1. The research directions of optical agents for disease diagnosis via both in vivo real-time imaging and in vitro urinalysis.

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

Chapter 2 reviews the basic concepts of medical imaging, the advantages and disadvantages of different imaging modalities, the current development status, and applications of biomarker-activatable optical probes. The following chapters report the individual work to achieve the objectives of this thesis. In **Chapter 3**, an ONOO⁻-activatable NIR probe (CyTF) is reported for sensitive detection of a malignant skin condition, keloid scar. CyTF specifically distinguishes malignant keloid from non-malignant hypertrophic scars and normal skin tissues. In **Chapter 4**, a chemo-fluoro-luminescent reporter (CFR) that independently activates NIRF and chemiluminescent channels in the presence of caspase-3 and O₂^{•-}, respectively. CFR is then applied to detect drug-induced hepatotoxicity, and enables longitudinal monitoring of sequential biomolecular events, oxidative stress and cell apoptosis. In **Chapter 5**, a polymeric optical reporter (FPRR) that activates both fluorescence and photoacoustic signals in the presence of disease biomarker (γ-glutamyl transpeptidase) is developed to detect drug-induced acute kidney injury. FPRR demonstrates superior signal-to-noise ratio

and higher penetration compared to fluorescent probes, showing its great potential for clinical translation. Besides in vivo real-time imaging, the application of optical probes for in vitro disease diagnosis via urinalysis will be illustrated. In **Chapter 6**, molecular urinary reporters (MURs) that release fluorescence signals in the presence of kidney injury biomarkers (γ -glutamyl transpeptidase, alanine aminopeptidase, and N-acetyl- β -D-glucosaminidase) are reported for multiplex optical urinalysis in early detection of acute or chronic kidney injury.

Last but not least, in **Chapter 7**, a conclusion is given to summarize all the finished works. Some possible research directions in the future is also suggested.

Chapter 2 Literature Review

2.1. Medical Imaging for Disease Diagnosis

Medical imaging is a powerful tool for disease diagnosis in both clinical applications and preclinical studies.¹⁸ Currently, a broad range of imaging modalities are available, such as positron emission tomography (PET), magnetic resonance imaging (MRI), ultrasound, computed tomography (CT), single photon emission tomography (SPECT), planar X-ray imaging, and optical imaging.¹⁹⁻²⁵ Based on the level of imaging, medical imaging can be categorized into both anatomical imaging and molecular imaging.

2.1.1. Anatomical Imaging

Anatomical imaging refers to the accurate visualization of inner body structures without trauma or risk of exploratory surgery. Conventional high resolution anatomic imaging modalities include CT, MRI, PET, SPECT.²⁶ Clinicians make disease diagnosis by finding abnormal structural changes, and common diseases such as carcinoma, cardiovascular diseases, and other diseases with physiological changes could be detected.²⁷⁻²⁹ Besides, the combination of these imaging modalities improves the ability of clinicians to noninvasively improve diagnostic accuracy and monitor the disease progression.³⁰ However, such imaging methods only detect disease at a stage of morphological injuries and functional damages when the disease progression has become irreversible. On the contrary, molecular imaging that sensitively detect disease biomarkers is attracting more and more attentions.^{12,31}

2.1.2. Molecular Imaging

Molecular imaging, the non-invasive, real-time visualization of biochemical events at the cellular and molecular level within living cells, tissues, and/or intact subjects, has become a rapidly emerging biomedical research discipline in the 20th century.²⁰ As an emerging multidisciplinary field, it integrates biology, pathophysiology, and chemistry, with research studies from cellular and molecular pathways, disease progression mechanisms, and biomarker-activatable detection probes.^{32,33} In essence, it represents a paradigm shift from anatomical imaging to assess disease progression at an early stage, before any morphological damages.³⁴⁻³⁶ By developing contrast agents with molecular targets, traditional imaging modalities such as optical imaging, PET, SPECT, US, MRI, and Raman imaging have been explored for molecular imaging.³⁷⁻⁴¹ The properties and features of these imaging modalities for molecular imaging are listed and compared in Table 2.1.

Table 2-1. Features of common imaging modalities for molecular imaging. Adapted from ref. 20

Modality	Temporal resolution	Spatial resolution	Penetration depth	Sensitivity	Safety Profile
CT	Minutes	50-200 μ m	Limitless	ND	Ionizing radiation
MRI	Minutes-hours	25-100 μ m	Limitless	10^{-3} to 10^{-5} M	No ionizing radiation
PET	Seconds-minutes	1-2mm	Limitless	10^{-11} to 10^{-12} M	Ionizing radiation
SPECT	Minutes	1-2mm	Limitless	10^{-10} to 10^{-11} M	Ionizing radiation
US	Seconds-minutes	0.01-0.1mm	mm-cm	10^{-12} M	Good safety profile
Optical imaging	Seconds-minutes	2-3mm	<1cm	10^{-9} to 10^{-12} M	Good safety profile
Raman imaging	Minutes-days	mm	~5mm	10^{-12} to 10^{-15} M	-

2.1.3. Optical Imaging

Optical imaging has played a vital role in biology and medicine due to its non-invasiveness, high temporospatial resolution, and high sensitivity. In the clinical settings, optical imaging has a wide range of applications, ranging from drug screening and disease diagnosis to intraoperative imaging-guided surgery and disease prognosis.⁴²⁻⁴⁵ The widely available imaging instrumentations have facilitated the broad applications from bench to bedside, such as optical microscopy, spectroscopy, endoscopy, scanning laser ophthalmoscopy, laser Doppler imaging, and optical coherence tomography.^{46,47} Besides, a wide variety of optical imaging agents are available, which will be discussed in detail in the next section.

2.2. Optical Imaging Agents

Optical imaging agents encompass a variety of materials from inorganic nanoparticles to organic fluorophores. A range of optical agents are available, such as organic dyes,⁴⁸ quantum dots (QDs),⁴⁹ up-conversion nanoparticles,⁵⁰ metal nanoclusters,⁵¹ carbon-based,⁷ silica-based nanomaterials⁵², and organic semiconducting polymer nanoparticles⁵³ and their applications in molecular imaging for disease diagnosis in preclinical studies have been reported. However, with intrinsic metal – induced toxicity and prolonged body retention, inorganic nanoparticles sometimes suffer from critical safety concerns. Therefore, organic systems including organic fluorophores, semiconducting polymer nanoparticles (SPNs) and semiconducting molecule nanoparticles (SMNs) are preferred for clinical translation purposes. In particular, organic fluorophores will be the focus of this thesis, considering the ease of structural

modification to design optical probes with active-targeting or biomarker-activatable properties.⁵⁴⁻⁵⁶

Based on optical and pharmacological properties, organic fluorophores can be classified into “always-on” agents, actively-targeting optical agents, and biomarker-activatable optical agents. Briefly, “always-on” optical agents constantly emit optical signals and passively accumulate at the disease site; active targeting optical agents is constructed by conjugating “always-on” fluorophore to a small protein or antibody that is preferentially taken up by cancer cells through endocytosis; biomarker-activatable optical agents could specifically switch optical signals from “off” to “on” in the presence of disease biomarkers.

Figure 2-1. Schematic illustration of “always-on” fluorophores, targeting fluorophores, and biomarker-activatable optical agents for tumour imaging.

By virtue of biocompatibility and ease of structure modification, small molecular or macromolecular fluorophores have become a valuable tool for developing optical agents with active disease targeting capability or biomarker-activatable optical signals. Additionally, the development of fluorophores with emission in the first or second

near-infrared (NIR) regions have enabled deeper tissue penetration, minimized tissue auto-fluorescence and enhanced signal-to-noise ratio.^{13,57}

2.2.1. Biomarker-activatable Optical Agents

By definition, a biomarker is a measurable indicator of the severity or presence of some disease state.⁵⁸ From a functional point of view, disease biomarkers can be categorized into susceptibility, predictive, diagnostic, monitoring, prognostic, and safety biomarkers.⁵⁹ A variety of compounds ranging from DNA, RNA, small molecules, proteins can serve as biomarkers.^{60,61}

Molecular imaging is a field of medical imaging that focuses on imaging molecules of medical interest within living cells, tissue, and patients.⁶² Compared to anatomical imaging to detect functional damage, molecular imaging to detect disease biomarkers allow for sensitive detection of biomolecular events in a pathophysiological pathway with high sensitivity and signal-to-background ratio; is capable of disease diagnosis at relatively early stages.³⁴⁻³⁶ Therefore, it is important to design biomarker-activatable optical probes that specifically switch on the optical signals in response to disease-related biomarkers. Besides, optical probes with emission signals in the near-infrared region (NIR I: 650 – 1000 nm) have drastically reduced the noise from tissue auto-fluorescence and increases tissue penetration up-to 1 cm, which is sufficient to penetrate the dermis layer (~ 2 mm) for detection of skin diseases.⁶³ By selecting disease-specific biomarkers and tuning the in vivo biodistribution and pharmacokinetic pathway, such biomarker-activatable probes have been used to

diagnose a variety of diseases including skin diseases,^{64,65} acute and chronic kidney injury,^{66,67} drug-induced liver injury,⁶⁸ and carcinogenesis.^{69,70}

2.2.2. Tuning the Optical Properties of Biomarker-activatable Probes

The biomarker-activatable probes could specifically emit optical (fluorescence/chemiluminescence/photoacoustic) signals in the presence of biomarkers. The optical properties, penetration depth, and depth-to-resolution ratio have been summarized in Table 3.

Table 2-2. Comparison of the tissue-penetration depth for fluorescence, self-luminescence, and PA imaging. Adapted from Ref. 76.

Imaging Modality	Fluorescence		Bioluminescence	Chemiluminescence	Afterglow	Photoacoustic	
	NIR-I	NIR-II				NIR-I	NIR-II
Penetration depth (cm)	≈1	≈3-5	≈2	≈2.5	4	5.2	11.6
Depth-to-resolution ratio	≈15	≈400	≈1	N.A.	N.A.	≈200	N.A.

2.2.2.1. Fluorescence Imaging

As compared to fluorescence signals in the visible range, NIR fluorescence signals have higher penetration depth and depth-to-resolution ratio. Besides, due to reduced photon scattering, absorption and tissue autofluorescence, NIR fluorescence imaging offers higher signal-to-noise ratio at deeper tissue depths as compared to fluorescence imaging at visible range.⁷¹

2.2.2.2. Self-luminescence Imaging

In contrast to fluorescence imaging, self-luminescence imaging detects photons without the requirement of real-time light excitation. Therefore, such imaging modalities have drastically increased imaging sensitivity and signal-to-noise ratio due to reduced light scattering and tissue autofluorescence.⁷² There are three approaches in self-luminescence imaging: bioluminescence relies on enzymatic reaction to generate photons; chemiluminescence relies on chemical reaction to generate photons; afterglow requires pre-irradiation of light to generate chemical or energy defects which release photons after cessation of light.⁷³

2.2.2.3. Photoacoustic Imaging

Photoacoustic (PA) imaging is an emerging hybrid imaging modality that combines the advantages of fluorescence and acoustic imaging. In essence, photoacoustic imaging allows the delivery of light energy that is absorbed by tissues causing a thermoelastic expansion, which subsequently generates ultrasound waves that are detected by the transducer.⁴ As ultrasonic signals possess minimal attenuation due to negligible sound scattering in biological tissues, the imaging depth of PA imaging is mainly limited by the penetration capability of excitation photons. Thereafter, PA imaging breaks through the imaging depth limit by photons and affords deep tissue penetration depth of 5.2, 11.6 cm for PA imaging in the first and second NIR region, respectively.^{4,74-76}

2.3. Tuning the Pharmacokinetic Properties

The pharmacokinetic properties of optical agents are crucial for clinical translation.⁷⁷ After intravenous administration into the body, optical agents enter the blood

circulation and can be distributed into various organs and tissues, followed by *in vivo* metabolism and clearance by the hepatobiliary or renal clearance pathways. The clearance pathways are determined by the inherent properties of the agents, such as size, shape, surface charges and other factors. In the hepatobiliary clearance pathway, organic optical agents are slowly metabolized and degraded by macrophages in the mononuclear phagocyte system (that is, liver and spleen). The metabolites are then secreted into the duodenum via the bile duct.¹⁵ Non-biodegradable nanoparticles can accumulate in the body and cause acute or chronic toxicity. By contrast, renal-clearable optical agents (RCOAs) are rapidly filtrated by the glomerular filtration membrane (GFM), transported through the renal tubules, and eventually excreted into the bladder through the ureter. In this process, RCOAs are minimally metabolized and rapidly cleared. Thus, RCOAs minimize toxicity issues and side effects in the course of imaging, which is a key criterion for clinical translation. Indeed, RCOAs have been developed for glomerular filtration rate (GFR) measurement, real-time imaging and fluorescence-guided surgery (FGS) (Figure 2-2).

Figure 2-2. a) The hepatobiliary clearance pathway typically lasts for days to months. Optical agents are taken up by hepatocytes, followed by bile duct transportation, elimination into the small intestines and eventually excretion into faeces. b) The renal clearance pathway lasts hours to days. Optical agents undergo glomerular filtration, tubular secretion or reabsorption and urinary excretion. Adapted from Ref. 21

2.3.1. Hepatobiliary-clearance Pathway

Non-RCOAs are mainly cleared by the mononuclear phagocyte system (MPS), whereas RCOAs are cleared by the kidneys⁷⁸. Protein adsorption on non-RCOAs in the blood first triggers their sequestration by the MPS system, eventually leading to intracellular degradation in hepatocytes, followed by excretion into the bile and into feces in days to months post injection¹⁶ (Figure 2-2a). Accumulation in hepatocytes can last for > 6 months, which is associated with chronic organ toxicity¹⁷. By contrast, renal clearance leads to rapid elimination of RCOAs (hours to days) with little metabolic degradation (Figure 2-2b 2b). In the kidneys, RCOAs are filtrated at the glomerulus through the GFM, and then transported into the renal tubules, where they can be secreted or reabsorbed. Eventually, RCOAs are secreted into urine via the bladder.⁷⁹ This rapid elimination process results in less long-term organ toxicity and side effects, as compared to agents cleared by the MPS. For example, liver accumulation of non-RCOAs in living mice remains at 10-100 % injected dose (ID) g^{-1} at 4–48h post injection (p.i.), which is 10-1000 times higher than for RCOAs ($0.1\text{-}1\text{ \%ID g}^{-1}$).⁸⁰ In addition, more than 90% ID of CdSe/CdS/ZnS quantum dots (QDs) (hydrodynamic diameter (HD) = 52 nm) remain in the major organs (kidneys, liver and spleen) in non-human primates at

90 days p.i.,⁸¹ whereas more than 60% ID of renal-clearable CdSe/ZnS QDs (HD < 5.5 nm) are urine-excreted in living mice within 4h p.i.⁸²

2.3.2. Renal-clearance Pathway

To achieve renal clearance, optical agents have to be smaller than the GFM clearance threshold (6-8 nm) and protein fouling-resistant to avoid MPS sequestration.^{82,83} Properties, such as charge, shape and chemical composition also affect the clearance rate and efficiency.^{84,85} Certain molecular design strategies have been explored for inorganic nanoparticles to make them clearable by the kidneys.^{80,86} Inorganic nanoparticles with a HD < 6 nm are filtered by the glomerulus; the clearance efficiency increases with decreasing size in the size range of 2-6 nm and reaches maxima in the size range of 1 – 2nm. By contrast, in the sub-nanometer regime (< 1 nm), an inverse size-scaling law (decreasing clearance efficiency with decreasing size) is observed owing to physical interactions with the endothelial glycocalyx.⁸⁷

2.4. Conclusions, Challenges and Perspectives

This chapter gives a systematic summary of the role of medical imaging in disease diagnosis, introduces optical agents with different imaging modalities (fluorescence, chemiluminescence, or bioluminescence signals), compares the biodistribution and clearance pathways of hepatobiliary-clearable and renal-clearable optical agents. As compared to always-on, disease-targeting optical agents, biomarker-activatable optical agents that specifically emit signals in the presence of disease biomarkers, improve imaging quality with higher signal-to-background ratio and achieve disease diagnosis with better accuracy. Although biomarker-activatable optical agents have

been vastly applied in preclinical studies, the clinical applications have been limited due to the intrinsic poor penetration depth of fluorescence signals. To address this problem, biomarker-activatable optical agents that emit excitation-free chemiluminescence signals or photoacoustic signals should be developed. In Chapter 4 and 5, we demonstrated feasible methods to develop optical agents with multiplex imaging capabilities: fluorescence/ chemiluminescence signals or fluorescence/ photoacoustic signals.

Furthermore, chronic organ toxicity and long-term body retention remain the major hurdle for the translation of optical agents that are cleared via the hepatobiliary pathway. In Chapter 5, we presented a proof-of-concept approach to design renal-clearable probes with up-to 80% clearance efficiency.

Chapter 3. Near-infrared Fluorescence Probes Detect Reactive Oxygen Species for Keloid Diagnosis

3.1. Introduction

Reactive oxygen and nitrogen species (RONS) are essential to regulate normal physiological processes, while abnormal high levels of RONS are associated with pathogenesis. In addition to cancer, inflammation, cardiovascular diseases and organ fibrosis,⁸⁸ an abnormally high cellular level of RONS production has been reported in chronic wound healing and keloid scars.^{89,90} Keloid is a type of tissue fibrosis after initial lesion with characteristics of excess collagen neoplasm and invasiveness to surrounding tissues, causing severe cosmetic problems, pruritus, and pain. The current treatments of keloid including surgical incision, radiation, pressure and laser therapy suffer from high recurrence rate, not to mention that radiation therapy has the risk of carcinogenesis.⁹¹ However, only a few diagnostic probes have been developed for keloids based on the overexpression of FAP α (fibroblast activation protein-alpha)⁹² and CTGF (connective tissue growth factor).⁹³ In scar formation process, an abnormal high RONS level could affect the processes of fibroblasts epithelial to mesenchymal transition (EMT) required for ECM deposition,⁹⁴ fibroblasts migration and adhesion,⁹⁵ and extra collagen deposition.^{96,97} Abnormal expressions of nuclear factor erythroid 2-related factor 2 (Nrf2),⁹⁸ and fibrogenic cytokine transforming growth factor β (TGF- β)^{99,100} could account for this high RONS level. It yet still remains unclear which specific RONS plays a part in developing keloid pathogenesis. Thereby, understanding the molecular mechanism of RONS in keloid

formation not only can provide useful information for early diagnosis of keloid but also may bring in new therapeutic interventions.

As compared to other imaging modalities, optical imaging has the advantages of rapid detection with high-throughput capacity, high sensitivity with detection at molecular and cellular levels, and relatively low instrumentation costs.¹ Molecular probes with high detection specificity and sensitivity have been readily developed for RONS such as $O_2^{\bullet-}$,¹⁰¹ H_2O_2 ,¹⁰² $ONOO^-$,¹⁰³⁻¹⁰⁶ $NO^{\bullet}$,¹⁰⁷ $HO^{\bullet}$,¹⁰⁸ OCI^- ¹⁰⁹ and O_3 .¹¹⁰ A natural extension of such efforts is the detection of RONS in living cells and living organisms with different disease models. Particularly, the development of near-infrared (NIR) probes have enabled deeper tissue penetration, minimized tissue auto-fluorescence and enhanced signal-to-noise ratio. These RONS probes have been successfully applied in evaluation of drug-induced toxicity,¹¹¹ ischemia-reperfusion,¹¹² acute peritonitis inflammation¹¹³ and neuroinflammation¹¹⁴ models. However, optical molecular probes have not yet been used to study the role of RONS in keloids and early diagnosis of keloids.

Here, the design and syntheses of two near-infrared (NIR) fluorescent activatable molecular probes for detection of RONS in keloids is reported. These two probes had different responses towards peroxynitrite ($ONOO^-$) in terms of specificity (**Scheme 3-1a**). CyTF was designed by linking a Trifluoromethyl ketone group to a NIR hemicyanine dye (CyOH), and it could undergo $ONOO^-$ -mediated oxidation via a dioxirane intermediate in a highly-selective manner.^{103,104} Meanwhile, CyBA was designed by conjugating a boronic acid-based self-immolative group to CyOH. Its responsiveness to both $ONOO^-$ and H_2O_2 were reported, after oxidation the boronate

ester group was released first, followed by the self-immolative linker.^{105,106,115} Both CyTF and CyBA were non-fluorescent initially as the electron-donating oxygen atom was caged by the ROS responsive group. After reaction with RONS, the caging groups were released, leading to free fluorescent CyOH. This characteristic fluorescence turn-on mechanism could be used to specifically distinguish keloid scar cells from normal dermal fibroblasts, where an abnormally high oxidative stress was reported (**Scheme 3-1b**).¹⁶

Scheme 3-1 (a) Design and mechanism of CyTF and CyBA for ROS imaging. (b)

Illustration of CyTF and CyBA activation in KF cells due to higher ROS level.

3.2. Materials and Methods

Chemicals. All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated. Commercially available reagents were used without further purification unless noted otherwise. Corning Matrigel Matrix was purchased from Corning Life Sciences.

Materials Characterization. UV-vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were carried out on a Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). HPLC analyses were performed on an Agilent 1260 system equipped with a G1311B pump, UV detector and an Agilent Zorbax SB-C18 RP (9.4 × 250 mm) column, with CH₃CN (0.1% of TFA) and water (0.1% of TFA) as the eluent. NMR spectra were measured using a Bruker 300 MHz instruments (Germany). All ¹H chemical shifts (δ) are relative to residual protic solvent (CHCl₃: δ 7.26 ppm). Mass spectra were measured using Thermo LCQ Fleet LC-MS with ESI mode (America). Fluorescence images of cells were acquired on Laser Scanning Microscope LSM800 (Zeiss).

Scheme 3-2 Synthetic route of CyTF and CyBA. (a) H₂SO₄ (con.), reflux, 4 h, EtOH, 97%; (b) TMSCF₃, CsF, THF, 18 h, 86%; (c) TBAF, THF, 5 h, then 4 M HCl, 3 h, 97%; (d) BBr₃, –78 °C, room temperature, 12 h, CH₂Cl₂, 74%; (e) K₂CO₃, Resorcinol, CH₃CN, 2h; (f) 5, BTC, Et₃N, 0 °C, 2 h, CH₂Cl₂, 28%; (g) 4-methylphenylboronic acid pinacol ester, K₂CO₃, CH₃CN.

Synthesis of compound 2: To a solution of 3-(4-methoxyphenyl)propanoic acid (2.7 g, 15.0 mmol) in ethanol (20 mL) was added H₂SO₄ (0.7 mL, con.) at room temperature under nitrogen atmosphere. The resulting mixture was refluxed for 4 h. Then ethanol was evaporated under reduced pressure, and the residue was added ethyl acetate (60 mL). The resulting solution was washed with H₂O (3 × 50 mL), saturated sodium bicarbonate (30 mL) and saturated sodium chloride (30 mL), respectively. The organic phase was dried with anhydrous Na₂SO₄, and then concentrated under reduced pressure, which led to ethyl 3-(4-methoxyphenyl)propanoate **2** (2.99 g, yield 96%) without purification. ¹H NMR (300 MHz, CDCl₃): δ = 7.12 (d, *J* = 8.6 Hz, 2H), 6.83 (d, *J* =

8.7 Hz, 2H), 4.12 (q, $J = 7.1$ Hz, 2H), 3.78 (s, 3H), 2.89 (d, $J = 7.8$ Hz, 2H), 2.58 (d, $J = 7.8$ Hz, 2H), 1.23 (t, $J = 7.2$ Hz, 3H) (**Figure 3-9**). MS (ESI): $m/z = 209.13$ $[M+H]^+$.

Synthesis of compound 3: To a solution of ethyl 3-(4-methoxyphenyl) propanoate **2** (2.91 g, 14.0 mmol) and TMSCF_3 (3.1 mL, 21.0 mmol) was added CsF (21.2 mg, 0.14 mmol). The mixture was stirred at room temperature under nitrogen atmosphere for 18 h and then CH_2Cl_2 (30 mL) was added to the resulting solution, the mixture was treated with water (3×10 mL) and saturated sodium chloride (10 mL). The organic phase was dried with anhydrous Na_2SO_4 , and then was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 5%) to afford (2-ethoxy-1,1,1-trifluoro-4-(4-methoxyphenyl) butan-2-yl)trimethylsilane **3**. (4.02 g, yield 86%). ^1H NMR (300 MHz, CDCl_3): $\delta = 7.14$ (d, $J = 8.2$ Hz, 2H), 6.86 (d, $J = 8.0$ Hz, 2H), 3.81 (s, 3H), 3.81-3.56 (m, 2H), 2.78-2.63 (m, 2H), 2.18-1.96 (m, 2H), 1.26 (td, $J = 7.0, 0.9$ Hz, 3H), 0.24 (d, $J = 0.9$ Hz, 9H) (**Figure 3-10**). MS (ESI): $m/z = 335.19$ $[M+H]^+$.

Synthesis of compound 4: To a solution of compound **3** (4.0 g, 12 mmol) in THF (2 mL) was added TBAF (1 M in THF, 13.0 mL) dropwise under N_2 atmosphere. The mixture was stirred at room temperature for 5 h and then was treated with hydrochloric acid (4.0 M, 4.0 mL) for 3 h. CH_2Cl_2 (30 mL) was added to the resulting solution, and then washed with water and saturated sodium chloride, respectively. The organic phase was dried with anhydrous Na_2SO_4 , and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane, 5%) to obtain trifluoro-4-(4-methoxyphenyl)butan-2-one (2.45 g, yield: 88%); ^1H NMR

(300 MHz, CDCl₃): δ = 7.11 (d, J = 8.7 Hz, 2H), 6.84 (d, J = 8.7 Hz, 2H), 3.78 (s, 3H), 3.05-2.98 (m, 2H), 2.96-2.89 (m, 2H) (**Figure 3-11**). MS (ESI): m/z = 233.09 [M+H]⁺.

Synthesis of compound 5: To a solution of 1,1,1-trifluoro-4-(4-methoxyphenyl) butan-2-one **4** (2.4 g, 10.3 mmol) in CH₂Cl₂ (20 mL) was added BBr₃ (5.2 mL, 13.4 mmol) at –78 °C under nitrogen atmosphere. The resulting mixture was then warmed to room temperature and stirred 10 h. Then ice-water was added slowly, and the mixture was added ethyl acetate (50 mL). The resulting solution was washed with H₂O (3 × 20 mL), saturated sodium bicarbonate (30 mL) and saturated sodium chloride (30 mL), respectively. The organic phase was dried with anhydrous Na₂SO₄, and then concentrated under reduced pressure, which was purified by silica gel column chromatography (ethyl acetate/hexane, 25%) to obtain 1,1,1-trifluoro-4-(4-hydroxyphenyl)butan-2-one **5** (1.84 g, yield 82%); ¹H NMR (300 MHz, CDCl₃): δ = 7.06 (d, J = 8.6 Hz, 2H), 6.77 (d, J = 8.6 Hz, 2H), 3.05-2.97 (m, 2H), 2.95-2.80 (m, 2H) (**Figure 3-12**). MS (ESI): m/z = 219.16 [M+H]⁺.

Synthesis of CyOH: CyOH was synthesized according to procedure reported in the literature.²⁵

Synthesis of CyTF: To a stirred solution of BTC (17.8 mg, 0.06 mmol) dissolved in dichloromethane (1 mL) and cooled to 0 °C, was added drop-wise to a solution of the compound **5** (17.4 mg, 0.08 mol) in dichloromethane (2 mL) and then triethylamine (8.0 mg, 0.08 mol) was added. During addition, the temperature was kept at 0 °C. After the addition was completed, the mixture was allowed slowly to rise to room temperature and stirring was continued for 2h. Then a solution of CyOH (30 mg, 0.06

mmol) in dichloromethane (2 ml) was added to the above mixture and stirred 30 min. The resulting mixture was concentrated under vacuum, which was purified by silica gel column chromatography (ethyl acetate/hexane, 10%) to obtain CyTF (12.7 mg, yield 28%). ^1H NMR (400 MHz, CDCl_3): δ = 8.63 (d, J = 14.8 Hz, 1H), 7.54-7.48 (m, 3H), 7.47-7.42 (m, 2H), 7.39 (d, J = 8.4, 1H), 7.22 (q, J = 19.2, 7.2 Hz, 4H), 7.10 (d, J = 6.8 Hz, 2H), 7.01 (d, J = 8 Hz, 1H), 6.84 (d, J = 15.2 Hz, 1H), 4.22 (s, 3H), 3.12 (m, 4H), 2.88 (t, J = 5.6 Hz, 2H), 2.74 (t, J = 6.0 Hz, 2H), 1.97-1.93 (m, 2H), 1.81 (s, 6H) (**Figure 3-13**). MS (ESI): m/z = 628.35 $[\text{M}+\text{H}]^+$. (**Figure 3-14**)

Synthesis of CyBA: A mixture of compound CyOH (19.2 mg, 0.05 mmol) and Cs_2CO_3 (70 mg, 0.20 mmol) in dry CH_2Cl_2 (3 mL) in a 15 mL round bottom flask was stirred at room temperature for 15 min. 4-Bromomethylphenylboronic acid pinacol ester (30 mg, 0.10 mmol) was then added and the reaction mixture was further stirred at room temperature overnight. After that, the mixture was washed with water (15 mL) and extracted by using dichloromethane. The dichloromethane layer was dried with anhydrous magnesium sulphate and concentrated under reduced vacuum to yield crude product. The resulting crude product was purified by a silica gel column ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ = 10:1) to afford compound CyBA as a blue solid (75%). ^1H NMR (300 MHz, CDCl_3): δ = 8.61 (d, J =15, 1H), 7.86 (d, J =9, 2H), 7.48 (m, 3H), 7.40 (m, 1H), 7.32 (m, 1H), 7.16 (s, 1H), 6.86 (m, 2H), 6.60 (d, J =15, 1H), 5.22 (s, 2H), 4.04 (s, 2H), 3.67 (m, 2H), 3.12 (m, 2H), 1.75 (s, 3H), 1.60 (m, 2H), 1.46 (s, 6H), 1.32 (s, 12H) (**Figure 3-15**). MS (ESI): m/z = 600.3 $[\text{M}+\text{H}]^+$. (**Figure 3-16**).

Cell Culture and Fluorescence Imaging. Normal dermal fibroblasts (NDF) and keloid-derived fibroblasts (KF) were cultured in DMEM (Dulbecco's Modified Eagle Medium)

supplemented with heat-inactivated fetal bovine serum (10%) in a humidified environment containing 5% CO₂ and 95% air at 37 °C, respectively. Cell media was replaced every three days. For cell fluorescence imaging, NDFs and KFs (6×10^4 cells/well in 1mL) were seeded in the dishes (Dia. 35mm) and incubated overnight to reach 70% confluency. Cells in the negative control groups were treated with NAC alone or NAC followed by TGF- β 1 and NAC. Cells in the positive control were treated with TGF- β 1. Cells in the control group received the same treatment procedure with DMEM medium (10% FBS, 1%PE). After incubation, the medium was removed, and the cells were washed three times with PBS buffer. Then the cells were stained with Hoechst 33342 (NucBlue Live ReadyProbesReagent, Thermo Fisher) for nuclei and fixed with 4% polyformaldehyde solution. Fluorescence images of the live cells were acquired on Laser Scanning Microscope LSM800 (Zeiss). The excitation and emission wavelength for cell imaging were 640/655-710 nm for CyTF/CyBA and 405/410-470 nm for Hoechst. ImageJ software was utilized to remove signal background and quantify cellular fluorescence intensity.

Cytotoxicity Assay. Normal dermal fibroblasts (NDF) and keloid-derived fibroblasts (KF) were seeded in 96 well plates (2×10^4 cells/well) for 24 h, and then CyTF or CyBA (final concentration 5, 10, 15, 20 μ M) was added to the cell culture medium. Cells were incubated with or without (control) CyTF or CyBA, followed by the addition of MTS assay (Promega Cat. No. G3581, 100 μ L, 0.1 mg/mL) for 4 h. The absorbance of MTS at 490 nm was measured by using a microplate reader. The cytotoxic effects (VR) of CyTF or CyBA were assessed using the following equation: $VR = A/A_0 \times 100\%$, where A

and A_0 are the absorbance of the experimental group and control group, respectively. The assays were performed in five sets for each concentration.

In Vivo Imaging. All animal studies were performed in compliance with the Guidelines established by the Institutional Animal Care and Use Committee (IACUC), Sing Health. NDF, KF cell suspensions in DMEM-diluted Matrigel were freshly prepared. Then the mixtures were injected at three positions on the back under the skin of nude 8-week-old female mice with equal volume. CyTF was intravenously injected. Fluorescence imaging was acquired using IVIS imaging system (exposure time: 60 s, IVIS-CT machine, PerkinElmer).

Statistics Analysis. The fluorescence images were quantified with ROI analysis using Living Image 4.0 Software. Results are expressed as the mean SD deviation unless otherwise stated. Statistical comparisons between two groups were determined by student's t-test. For all tests, $p < 0.05$ was considered as statistically significant. All statistical calculations were performed using GraphPad Prism v.6 (GraphPad Software Inc., CA, USA).

3.3. Results and Discussions

To validate and compare the RONS sensing ability between CyTF and CyBA, their absorption and fluorescence spectra in the absence and presence of different RONS were measured. Both CyTF and CyBA had two absorption peaks at 605 and 654 nm and were initially non-fluorescent; upon adding ONOO^- , the absorption peak at 605 nm disappeared and a new peak at 690 nm – the fingerprint spectrum of free CyOH

appeared.¹¹⁶ Such a cleavage led to fluorescence enhancement up to 15-fold at 717 nm (**Figure 3-1a, b, Figure 3-2**).

High performance liquid chromatography (HPLC) and liquid chromatography mass spectroscopy (LCMS) further confirmed that CyTF (HPLC retention time, T_R = 24 min) and CyBA (T_R = 24.1 min) were totally converted into free CyOH (T_R = 20.8 min) after incubation with ONOO^- (**Figure 3-1c, d**) and H_2O_2 (**Figure 3-4**). In addition, a gradual increase in fluorescence intensity with ONOO^- concentration could be observed, and linearity existed at concentration ranging from 0 - 8 μM (**Figure 3-1e**). Under such conditions, the limits of detection for CyTF and CyBA were determined to be 89.4 and 71.7 nM, respectively.

The selectivity of CyTF and CyBA was tested against other RONS including $\text{O}_2^{\bullet-}$, OCl^- , $^{\bullet}\text{OH}$, and H_2O_2 . As expected, CyTF was highly specific towards ONOO^- . However, CyBA showed responses to both ONOO^- and H_2O_2 and the fluorescence enhancement for H_2O_2 was 5-fold (**Figure 3-1f**). Moreover, the response rates of both CyTF and CyBA were tested by time-course fluorescence measurements (**Figure 3-3**). Upon addition of ONOO^- , the fluorescence intensity of both two probes at 717 nm increased drastically and reached plateau in less than two minutes, indicating rapid response of both two probes to ONOO^- . Thus, CyTF and CyBA showed similar fluorescence enhancement and kinetics upon adding ONOO^- , yet CyBA showed poorer selectivity as it was responsive towards both ONOO^- and H_2O_2 .

Figure 3-1 UV-Vis absorption spectra (a) and fluorescence (b) of CyTF or CyBA (20 μM) in the absence or presence of ONOO^- (25 μM) at 37 $^\circ\text{C}$ in PBS (1 $\times$, pH = 7.4) containing 20% DMSO. Excitation: 640nm. (c) High performance liquid chromatography (HPLC) traces of the incubation mixture of CyTF (c) and CyBA (d) in the absence (upper panel) or presence (middle panel) of ONOO^- (25 μM), and HPLC traces of CyOH in water (lower panel). Wavelength: 600 nm. (e) Fluorescence titration of CyTF or CyBA (20 μM) as a function of ONOO^- concentration. Each spectrum was recorded at 5 min after addition of ONOO^- . Regression equation for CyTF: $\text{FL} = 1.07 \times 10^6 + 1.16 \times 10^6 [\text{ONOO}^-]$ μM ($R^2 = 0.99$); regression equation for CyBA: $\text{FL} = 1.14 \times 10^6 + 1.27 \times 10^6 [\text{ONOO}^-]$ μM ($R^2 = 0.99$). (f) Fluorescence intensity of CyTF and CyBA in the presence of various

RONS (50 μM) for 5 min in PBS ($1\times$, pH = 7.4) containing 20% DMSO. Control represents assay buffer only. ONOO^- was 25 μM . The incubation time for H_2O_2 was 2 hours. Error bars represented standard deviations of three separate measurements.

Figure 3-2 (a) UV-Vis absorption spectra and (b) fluorescence of CyTF (20 μM) upon titrating different concentrations of ONOO^- . (c) UV-Vis absorption and (d) fluorescence of CyBA (20 μM) upon titrating different concentrations of ONOO^- . (e) UV-Vis absorption and (f) fluorescence of CyBA (20 μM) upon titrating different

concentrations of H_2O_2 . Experiments were performed at 37°C in PBS ($1\times$, $\text{pH} = 7.4$) containing 20% DMSO. Excitation: 640nm.

Figure 3-3 Time-dependent fluorescence changes of CyTF ($20\mu\text{M}$) and CyBA ($20\mu\text{M}$) upon addition of $25\mu\text{M}$ ONOO^- .

Figure 3-4 High performance liquid chromatography (HPLC) traces of the incubation mixture of CyBA in the absence (upper panel) or presence (middle panel) of H_2O_2 (500 μM), and HPLC traces of CyOH in water (lower panel). Wavelength: 600nm.

Imaging of RONS in Cells. After confirming the low cytotoxicity of CyTF and CyBA against living cells by MTS assay (**Figure 3-5**), these probes were used to detect endogenously generated RONS in cultured normal dermal fibroblasts (NDFs). NDFs without treatment served as control group. NDFs treated with Transforming Growth Factor (TGF)- β 1 alone were used as the positive control, whereas the cells treated with TGF- β 1 along with a RONS scavenger (NAC: N-acetyl-L-cysteine) and the cells treated with NAC only were used as the negative control groups. All groups were then incubated with CyTF or CyBA for fluorescence imaging.

Figure 3-5 MTS assay for the relative viability of Normal Dermal Fibroblasts (NDFs) and Keloid-derived fibroblasts (KFs) treated with various concentrations of (a) CyTF and (b) CyBA. Error bars represent the standard deviation of 5 trials.

As a whole, for control and negative control groups, weak fluorescence signals were observed for both CyTF and CyBA (**Figure 3-6a, b**), indicating low RONS levels in NDFs.

However, in the control group, CyBA had a slightly higher background signal than CyTF in the untreated cells (**Figure 3-6a**). As CyBA could react with both H_2O_2 and ONOO^- , this implied a higher basal level of H_2O_2 than ONOO^- in untreated cells. This is reasonable because H_2O_2 is a less reactive and hence more stable oxidative species than ONOO^- .^{117,118} After TGF- β 1 stimulation, the fluorescence of both CyTF and CyBA dramatically increased, and the fluorescence enhancement for CyTF (3.76 fold) was 2.6-times higher than CyBA (1.43 folds) (**Figure 3-6a, b**). The overall increased fluorescence signals after TGF- β 1 stimulation were caused by the fact that high concentration of cytokine TGF- β 1 could induce inflammatory response and in turn oxidative stress. Furthermore, upon NAC addition into these stimulated cells, the fluorescence level decreased to basal levels similar to those untreated cells (**Figure 3-6a, b**), confirming the role of TGF- β 1 in inducing oxidative stress.

Figure 3-6 (a) Fluorescence microscopy of NDF cells incubated with CyTF or CyBA (10 μ M, 30 min) before imaging; first panel: untreated cells; second panel: cells treated with TGF- β 1 (36 h); third panel: cells pre-treated with NAC (2 h) before treated with TGF- β 1 (0.5 h), followed with NAC for 1 h; fourth panel: cells treated with NAC for 20 min. [TGF- β 1] = 2.5 ng/ml, [NAC] = 5 mM. Scale bars: 50 μ m (b) Quantification of fluorescence intensities of NDF cells after incubation with CyTF or CyBA in Figure 2a, the fluorescence intensities were normalized by total cell nuclei signal, and values presented were relative to the control groups. Error bars represent standard deviations of three separate measurements. (c) Schematic illustration of TGF- β 1 signalling and RONS generation in NDF cells. *: $p < 0.05$, **: n.s.

The great fluorescence enhancement for CyTF and CyBA in TGF- β 1 stimulated NDFs further implies that TGF- β 1 could especially increase intracellular ONOO $^-$ level. In TGF- β 1 signalling cascade (**Figure 3-6c**), it first binds to and phosphorylates transmembrane TGF- β type II receptor (T β RII), and then trans-phosphorylates TGF- β type I receptor (T β RI). This subsequently activates Smad signalling proteins, which then translocate into nucleus to interact with other transcription factors to regulate transcription pathways.¹¹⁹ In particular, TGF- β 1 could increase the expressions of transmembrane proteins NADPH oxidase 4 (NOX4)¹²⁰ and endothelial nitric oxide synthase (eNOS).¹²¹ eNOS generates nitric oxide, NO $^{\bullet}$ from L-Arginine and oxygen molecule; and NOX4 produces superoxide anion, O $_2^{\bullet-}$ from oxygen molecule. The generated O $_2^{\bullet-}$ could subsequently react with NO $^{\bullet}$ to produce ONOO $^-$ ($k \approx 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) or convert to H $_2$ O $_2$ by enzyme superoxide dismutase ($k \approx 10^9 \text{ M}^{-1} \text{ s}^{-1}$).¹¹⁸ As both reactants O $_2^{\bullet-}$ and NO $^{\bullet}$ have high intracellular levels, and the reaction producing

ONOO⁻ is more kinetically favoured, the level of ONOO⁻ should be higher than H₂O₂ in TGF-β1 stimulated NDFs (**Figure 3-6c**).

The NIR probes were then applied to detect keloid-derived fibroblasts (KFs) in culture, with NDFs as control group. Compared with low basal fluorescence levels in NDFs, both probes incubated in untreated KFs had shown enhanced fluorescence levels.

Figure 3-7 (a) Fluorescence microscopy of NDF, KF and KF cells treated with RepSox incubated with CyTF or CyBA (10 μM, 30 min) before imaging, [RepSox] = 25 μM. Scale bars: 50 μm (b) Quantification of fluorescence intensities of NDF cells after incubation with CyTF or CyBA in Figure 3a, the fluorescence intensities were normalized by total

cell nuclei signal, and values presented were relative to the control groups. Error bars represent standard deviations of three separate measurements. (c) Schematic illustration of RepSox inhibiting TGF- β Type 1 receptor signalling pathway in KF cells.

*: $p < 0.05$.

The fluorescence enhancement in CyTF (2.26-fold) was 1.5-times higher than CyBA (1.5-fold) due to a higher background level of H_2O_2 as explained earlier (**Figure 3-7a, b**). However, when KFs were further treated with a selective T β R1 inhibitor (RepSox),¹²² the fluorescence levels for both probes decreased to a large extent, suggesting the role of TGF- β 1 signalling in causing keloid oxidative stress. As a result, by targeting TGF- β 1 signalling pathway, keloids therapeutic drugs like RepSox could be developed.

An uncontrolled TGF- β 1 signalling was found in keloid pathogenesis, originating from both overproduction of the cytokine itself and higher expression levels of T β RI & T β RII in keloid cells.³ Specifically, the distinguishable fluorescence enhancement from CyTF had demonstrated a much higher ONOO⁻ level in KF cells. When RepSox was subsequently added to inhibit TGF- β 1 signalling by blocking T β RII transphosphorylation, intracellular ONOO⁻ level is reduced (**Figure 3-7c**). Hence, ONOO⁻ could become a specific biomarker for keloid disease, and CyTF has the potential to be applied in early diagnosis. Furthermore, identifying the significance of TGF- β 1 signalling in keloid pathogenesis has made developing TGF- β signalling pathway-targeting drug treatment towards keloid disease plausible. By targeting intracellular ONOO⁻ level, CyTF could also become a potent drug screener.

In Vivo Keloid Imaging. The ability of CyTF to distinguish keloid cells from normal skin cells was further tested in a xenograft mice model. The KF cells pellets (6×10^5 cells), NDF cells pellets (6×10^5 cells) separately dissolved in 200 μ L solution (Matrigel/DMEM = 1:1) and Matrigel (200 μ L) only as control group were injected into three points on the back of living mice, and 200 μ L CyTF (0.2mM in deionized water containing 20% ethanol) was intravenously injected (**Figure 3-8a**). By virtue of its NIR fluorescence with deep tissue penetration and minimized tissue auto-fluorescence, fluorescence signals from CyTF being turned on to CyOH could be readily observed in living mice (**Figure 3-8b**). The fluorescence signal of CyTF at the keloid site was found to gradually increase from $t = 1$ h and reached its maximum at $t = 3.5$ h. At this time point, the fluorescence intensity was 3-fold compared to NDF cells and control (**Figure 3-8d**). Afterwards, the fluorescence signal at the keloid site decreased, until reaching a similar level to the background, suggesting that CyTF could be readily cleared over time. After 6 h, the mice were sacrificed to study in vivo distribution, the organs were firstly incubated in ONOO⁻ solution to activate any remaining CyTF to the fluorescent CyOH, and most of the probes were found accumulated in the liver (**Figure 3-8c, e**). The injected cells pellets were also extracted, and KF cells pellets showed stronger fluorescence signal as compared to the NDF cells pellets and control group. The in vivo experiments confirmed that CyTF could be activated by ONOO⁻ and used to image implanted KF cells in living mice.

Figure 3-8 In vivo fluorescence imaging of keloid. (a) Schematic illustration of keloid fluorescence imaging in living nude mice. (b) Whole animal dorsal (upper panel) and ventral (lower panel) fluorescence imaging before intravenous injection (left panel) and 3.5 hours after intravenous injection (right panel). The upper left green circle indicated subcutaneous injected Matrigel as a control. The lower left blue circle indicated subcutaneous injected mixture of NDF cells and Matrigel. And the lower right red circle indicated subcutaneous injected mixture of KF cells and Matrigel. (c) Fluorescence imaging of different organs and injected Matrigel. The organs were imaged after incubating in 200 μ M ONOO⁻ solution for 30 minutes. (d) Quantification of fluorescence intensity as a function of time. (e) Fluorescence quantification of

major organs of mice. The fluorescence images were acquired at 720 nm upon excitation at 675 nm.

3.4. Conclusions

In summary, NIR molecular probes CyTF and CyBA ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 640/717$ nm) to detect ONOO^- were developed and applied to distinguish keloid fibroblasts from normal dermal fibroblasts. CyTF was highly specific towards ONOO^- , while CyBA was responsive to both ONOO^- and H_2O_2 . In living cells, CyTF showed higher fluorescence enhancement than CyBA, which were 3.76 and 2.26-fold in TGF- β 1 stimulated normal dermal fibroblasts and keloid fibroblasts, respectively. In keloid fibroblasts treated with RepSox, the fluorescence signals decreased to a large extent, proving CyTF as a potent drug screener. This cellular imaging results proved that keloid fibroblasts had a higher intracellular level of ONOO^- than H_2O_2 , which could be explained by the rapid reaction between elevated $\text{NO}^\bullet$ and $\text{O}_2^{\bullet-}$ from abnormal TGF- β signalling. In the xenograft mice model, obvious fluorescence signal from CyTF was detected, which was 3-fold higher than that from normal tissues. This study not only highlights the role of ONOO^- in keloid pathogenesis, but validates the feasibility of applying optical molecular probes in keloid diagnosis.

Even though CyTF has been proven for sensitive detection of keloid, situation gets complicated in real-life situations as ONOO^- is up-regulated in a range of medical conditions such as stroke, myocardial infarction, diabetes, inflammatory diseases, cancer, and neurodegenerative disorders. To increase diagnostic accuracy of a particular disease, usually multiple tests are conducted in the clinicals, which however,

is tedious and time-consuming. In the next chapter, we reported the synthesis and application of a unimolecular chemo-fluoro-luminescent reporter (CFR) for crosstalk-free duplex imaging of drug-induced hepatotoxicity (DIH). CFR had two independent optical channels that sensitively and specifically detected $O_2^{\bullet-}$ and casp3 *via* CHL and NIRF signals, respectively. Such a molecular design not only enabled longitudinal monitoring of two correlated sequential biomolecular events (oxidative stress, cell apoptosis), but also achieved diagnosis of DIH with high accuracy.

Figure 3-9 ¹H NMR spectrum of compound 2 in CDCl₃.

Figure 3-10 ¹H NMR spectrum of compound 3 in CDCl₃.

Figure 3-11 ¹H NMR spectrum of compound 4 in CDCl₃.

Figure 3-12 ^1H NMR spectrum of compound 5 in CDCl_3 .

Figure 3-13 ^1H NMR spectrum of CyTF in CD_3OD .

Figure 3-14 Mass spectrum of CyTF.

Figure 3-15 ¹H NMR spectrum of CyBA in CDCl₃.

Figure 3-16 Mass spectrum of CyBA.

Chapter 4. A Unimolecular Chemo-fluoro-luminescent Reporter for Crosstalk-free Duplex Imaging of Hepatotoxicity

4.1. Introduction

Real-time simultaneous imaging of multiple biomarkers not only can provide unique opportunities to study the fundamental correlation between different biomarkers within a certain pathological pathway in living organisms, but also can improve the accuracy of disease diagnosis.^{123,124} Activatable fluorescent probes have become valuable tools in medicine, because they have high sensitivity and emit specific signals in response to the biomarker of interest.^{11,125,126} However, only a few have been developed for duplex-biomarker imaging,¹²⁷⁻¹³⁰ not to mention multi-biomarker imaging. Since these probes generally rely on the respective activation of two fluorescent moieties, signal crosstalk often occurs due to the emission spectral overlap,¹³¹ potentially compromising biomarker resolution.

In contrast to fluorescence probes, chemiluminescence (CHL) probes have minimized background noise, because of the elimination of real-time light excitation.¹³² However, most CHL substrates have weak luminescence under physiological conditions.⁷² Recently, it was found that molecular engineering of Schaap's chemiluminescence dioxetanes through addition of an electron-withdrawing substituent on the phenol donor dramatically improved the luminescence efficiency by three orders of magnitude in aqueous medium.¹³³ Such a major advance has substantially facilitated the application of CHL probes for in vivo molecular imaging. Until now, a variety of

biomarkers including enzymes,¹³³⁻¹³⁵ reactive oxygen species (ROS),^{133,136} and other small molecules¹³⁷ have been detected using CHL probes. However, CHL imaging has been rarely exploited for multi-biomarker imaging.

In this study, the synthesis of a unimolecular chemo-fluoro-luminescent reporter (CFR) is reported for crosstalk-free duplex imaging of drug-induced hepatotoxicity (DIH). DIH is one of the most significant cause for US Food and Drug Administration (FDA) non-approval and drug withdrawal from the market.¹³⁸ Thereby, detection of DIH is essential for both drug screening and clinical care.¹³⁹ However, sensitive probes able to image DIH in living animals remain lacking.¹⁴⁰⁻¹⁴² Because oxidative stress and cellular apoptosis have been found to be upregulated at the early stage of DIH prior to inflammatory response and liver failure,¹⁴³ superoxide anion ($O_2^{\bullet-}$) and caspase-3 (casp3) were chosen as the early biomarkers for the design of CFR.^{144,145}

CFR represents the first kind of optical small-molecular probes having two independent activation channels (**Scheme 4-1**): near-infrared fluorescence (NIRF) for casp3 and CHL for $O_2^{\bullet-}$, and the synthetic route is shown in Scheme S1. The NIRF moiety is modified on a hemi-cyanine unit (CyU) with its hydroxyl group caged by a self-immolative linker connected to a casp3-cleavable peptide [Asp-Glu-Val-Asp] to diminish the electron-donating ability of the oxygen atom. The CHL moiety is derived from a CHL dioxetane whose activity is inhibited by caging the phenol with a $O_2^{\bullet-}$ -cleavable trifluoromethanesulfonate (Tf) group.¹⁴⁶ Thereby, the presence of casp3 leads to the cleavage of the amide linkage between peptide and self-immolative linker of CFR, followed by the spontaneous generation of CyU *via* 1,6-elimination. Due to the enhanced electron-donating ability from the oxygen atom of CyU, the NIRF signal of

resulted product is turned on. Differently, $O_2^{\bullet-}$ attacks the sulfonate ester of CFR and induces deprotection of Tf group, leading to a highly unstable phenolate-dioxetane derivative. This intermediate undergoes spontaneous decomposition and releases photons as one of its products. Such a duplex molecular design allows CFR to specifically activate its NIRF and CHL signals in response to casp3 and $O_2^{\bullet-}$, respectively.

Scheme 4-1. Duplex imaging mechanism of CFR.

4.2. Materials and Methods

Chemicals. All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Commercially available reagents were used without further purification unless otherwise noted. 2-Chlorotrityl chloride polystyrene resin, Fmoc-Asp(OtBu)-OH, Fmoc-Val-OH, Fmoc-Glu-OH, 1-hydroxybenzotriazole hydrate (HoBT), *N,N,N',N'*-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from Sangon Biotech for SPPS synthesis. Alanine aminopeptidase (AAP), fibroblast activation protein- α (FAP- α), furin, urokinase (uPA) and casp3 were purchased from R&D Systems.

Characterization. UV/Vis spectra were measured on a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were performed on a Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). HPLC analyses were done on an Agilent 1260 system equipped with a G1311B pump, UV detector and an Agilent Zorbax SB-C18 RP (9.4 × 250 mm) column, with methanol/acetonitrile and water as the eluent. Fluorescence images of living cells were acquired on Laser Scanning Microscope LSM800 (Zeiss). Chemiluminescence images of living cells were acquired on LX71 inverted microscope (Olympus) equipped with infinity 3-1 (Lumenera) CCD camera. Living mice imaging was acquired with IVIS Spectrum imaging system (PerkinElmer, Inc.). Proton-nuclear magnetic resonance (^1H NMR) spectra were recorded on a Bruker Advance II 300 MHz NMR. Electrospray ionization-mass spectrometry (ESI-MS) spectra were acquired on a Thermo Finnigan Polaris Q quadrupole ion trap mass spectrometer (ThermoFisher Corporation) equipped with a standard ESI source.

Synthesis for compound 2. The mixture of 1-bromo-4-chlorobutane (5.1 g, 30 mmol) and sodium azide (2.05 g, 31.5 mmol) was dissolved and stirred in DMF at 50 °C overnight. After reaction, the residue was diluted with ethyl acetate and washed with DI water for three times. The organic layer was dried over sodium sulfate and concentrated under reduced pressure. After silica gel chromatography (petroleum ether), pure compound 2 could be obtained (1.9 g, 48%).

Scheme 4-2. Synthetic route for CFR. Reagents and conditions: (i) NaN_3 , DMF, 50°C , 12h; (ii) NaI , Acetone, 50°C , 24h; (iii) compound 3, solvent free, N_2 , 90°C , reflux; (iv) Ac_2O , AcONa , N_2 , 90°C , reflux; (v) resorcinol, K_2CO_3 , ACN, N_2 , 55°C , 2h; (vi) PABA, EEDQ, DCM, rt, 6 h; (vii) PBr_3 , THF, 0°C , 2 h; (viii) CyU, DIPEA, CH_3CN , 55°C , 5 h; (ix) TFA, DCM, 0°C , 2 h; (x) Tf_2O , pyridine, DCM, -78°C to 25°C , 2 h; (xi) NaOH, THF, rt, 24 h;

(xii) propargylamine, HoBT, HBTU, DIPEA, DMF, rt, 2 h; (xiii) methylene blue, O₂, DCM/MeOH, 0 °C, 2 h; (xiv) CuSO₄•5H₂O, sodium ascorbate, DMSO/H₂O, rt, 1 h. Firstly, CyU was synthesized from CyCl and resorcinol via a retro-Knoevenagel reaction. Next, the peptide (5) was coupled to p-aminobenzyl alcohol (PABA) followed by bromination of 1° alcohol to yield a compound 7. Then, 7 was reacted to the hydroxyl group of CyU, from which the deprotection of OtBu groups on the peptide chain afforded the azide-modified casp3-responsive moiety (9). Compound 11 was synthesized from 10 by a one-step triflation, and hydrolysis of 11 led to 12. The reaction of 12 with propargylamine yielded 13, followed by [2+2] cycloaddition with ¹O₂ to afford the alkyne-functionalized CHL moiety (14). At last, CFR was obtained by coupling 9 and 14 via click reaction. The structures of these compounds were confirmed by NMR and mass spectra.

Synthesis for compound 3. The mixture of compound 2 (1 g, 7.5 mmol) and sodium iodide (1.2 g, 7.85 mmol) was dissolved and vigorously stirred in acetone at 50 °C for 24 h. The resulting residue was concentrated and purified with silica gel chromatography (petroleum ether) to obtain pure compound 3 (1g, 60%). ¹H NMR (300 MHz, CDCl₃) δ: 3.32 (t, 2H), 3.21 (t, 2H), 1.87-1.96 (m, 2H), 1.69-1.76 (m, 2H). MS (ESI): m/z = 224.98 [M + H]⁺.

Synthesis for compound 4. 2,3,3-trimethylindolenine (318 mg, 2 mmol) and compound 3 (450 mg, 2 mmol) were mixed and vigorously stirred under N₂ atmosphere at 90 °C for 2 h. The residue was then diluted with 3 mL CH₂Cl₂, precipitated into excess diethyl ether, and washed three times with excess diethyl

ether. The product was directly used for next step synthesis without further purification.

Synthesis for CyCl. Compound 4 (160 mg, 0.63 mmol), 2-chloro-3-(hydroxymethylidene)cyclodex-1-ene-1-carbaldehyde (53 mg, 0.3 mmol) and sodium acetate (25 mg, 0.3 mmol) were dissolved in 5 mL acetic anhydride. The mixture was vigorously stirred at 90 °C under N₂ atmosphere for 4 h. The residue was then diluted with CH₂Cl₂ and washed with saturated sodium bicarbonate for three times to remove residue acid. The product was purified after silica gel chromatography (CH₂Cl₂/CH₃OH = 50:1) to obtain pure CyCl (120 mg, 70%). ¹H NMR (300 MHz, CDCl₃) δ: 8.34 (d, J = 15 Hz, 2H), 7.39 (m, 4H), 7.28 (s, 1H), 7.24 (d, 2H), 7.20 (d, J = 6 Hz, 2H), 6.32 (d, J = 12 Hz, 2H), 4.31 (t, 4H), 3.46 (t, 4H), 2.80 (t, 4H), 1.98 (m, 6H), 1.84 (m, 4H), 1.73 (s, 12H). MS (ESI): m/z = 649.35 [M + H]⁺.

Synthesis for CyU. Grinded potassium carbonate (130 mg, 0.9 mmol) and resorcinol (100 mg, 0.9 mmol) were stirred in 5 mL CH₃CN at 55 °C under N₂ atmosphere for 15 minutes, before CyCl (200 mg, 0.3 mmol) dissolved in 5 mL CH₃CN was added dropwise into the mixture. The reaction mixture was then stirred for 2 h. The residue was then diluted with CH₂Cl₂ and washed with DI water for three times. The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The residue was then purified with silica gel chromatography (CH₂Cl₂/CH₃OH = 25:1) to obtain pure CyU (100 mg, 60%). ¹H NMR (300 MHz, MeOD) δ: 8.63 (d, J = 15 Hz, 1H), 7.61 (s, 1H), 7.45 (m, 3H), 7.40 (m, 2H), 7.10 (t, 2H), 6.00 (d, J = 13 Hz, 1H), 4.09 (t, 1H), 3.46 (m, 2H), 2.77 (t, 2H), 2.64 (t, 2H), 1.95 (m, 3H), 1.78 (s, 5H), 1.57 (s, 6H). MS (ESI): m/z = 467.24 [M + H]⁺.

Synthesis for compound 6. *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 592 mg, 2.4 mmol) was added to anhydrous CH₂Cl₂ dissolved Ac-Asp-Glu-Val-Asp-OH (compound 5, 400 mg, 0.6 mmol) under N₂ atmosphere and the reaction mixture was stirred for 20 min. Then 4-aminobenzyl alcohol (296 mg, 2.4 mmol) dissolved in anhydrous CH₂Cl₂ was added via a syringe and stirred for 6 h at room temperature. After completion, the mixture was concentrated under reduced pressure and purified by preparative HPLC to obtain pure product 6 (380 mg, 80%). ¹H NMR (300 MHz, CDCl₃) δ : 7.70 (d, *J* = 9Hz, 2H), 7.30 (d, *J* = 9Hz, 2H), 4.94 (s, 1H), 4.63 (m, 3H), 4.18 (t, 2H), 2.90-3.05 (m, 5H), 2.30-2.52 (m, 4H), 2.02 (s, 3H), 1.43 (m, 27H), 1.01 (t, 6H). MS (ESI): *m/z* = 853.35 [M + H]⁺.

Synthesis for compound 7. Pure product 6 (100 mg, 0.12 mmol) was dissolved in anhydrous tetrahydrofuran in ice bath under N₂ atmosphere, and PBr₃ (23 μ L, 0.24 mmol) in anhydrous tetrahydrofuran was added via a syringe. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure. The resulting mixture was diluted with ethyl acetate and washed with saturated sodium bicarbonate solution for three times to remove residual acid. The organic layer was dried with anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was then taken for next step synthesis without further purification.

Synthesis for compound 8. The mixture of the hemicyanine (CyU, 10 mg, 0.025 mmol) and *N,N*-diisopropylethylamine (DIPEA, 17.5 μ L, 0.10 mmol) dissolved in anhydrous acetonitrile was stirred at 55 °C under N₂ atmosphere. After stirring for 20 minutes, compound 7 (80 mg, 0.10 mmol) was added dropwise into the mixture and the reaction mixture continued to stir at 55 °C for 5 h under N₂ atmosphere. After

completion, the resulting mixture was concentrated under reduced pressure and purified by preparative HPLC to obtain pure product 8 (24 mg, 85%). ^1H NMR (300 MHz, MeOD) δ : 8.76 (d, J = 15 Hz, 1H), 8.31 (m, 2H), 7.95 (d, J = 6 Hz, 1H), 7.72 (t, 2H), 7.48 (m, 5H), 7.08 (m, 2H), 6.52 (d, J = 15 Hz, 1H), 5.26 (m, 3H), 4.67 (m, 1H), 4.38 (m, 2H), 4.04 (m, 1H), 3.45 (t, 2H), 2.91 (m, 2H), 2.82 (m, 5H), 2.64 (m, 2H), 2.28 (m, 2H), 1.99 (s, 6H), 1.82 (s, 4H), 1.61 (s, 2H), 1.41 (m, 27H), 1.29 (m, 6H), 0.96 (m, 6H). MS (ESI): m/z = 1240.88 $[\text{M} + \text{H}]^+$.

Synthesis for compound 9. Pure product 8 (24 mg, 0.02 mmol) was stirred in TFA/ CH_2Cl_2 (50% v/v) in ice bath for 2 h. The reaction was monitored by analytical HPLC. Upon completion, saturated NaHCO_3 solution was added dropwise into the reaction mixture until pH was adjusted to 7.4. Pure product 9 (14 mg, 70%) was then obtained after preparative HPLC purification. ^1H NMR (300 MHz, MeOD) δ : 8.76 (d, J = 15 Hz, 1H), 7.74 (m, 4H), 7.50 (m, 6H), 7.04 (m, 2H), 6.50 (d, J = 15 Hz, 1H), 5.25 (s, 3H), 4.67 (m, 1H), 4.37 (m, 2H), 4.02 (m, 1H), 3.45 (t, 2H), 2.99 (m, 1H), 2.86 (m, 1H), 2.79 (m, 5H), 2.42 (m, 2H), 2.13 (m, 2H), 1.99 (m, 7H), 1.82 (m, 7H), 1.30 (m, 4H), 0.96 (t, 6H). MS (ESI): m/z = 1072.45 $[\text{M} + \text{H}]^+$.

Synthesis for compound 10 was carried out according to literature report (ACS Cent Sci, 2017, 3, 349-358). ^1H NMR (300 MHz, CDCl_3) δ : 7.88 (d, J = 15 Hz, 1H), 7.69 (d, J = 8 Hz, 1H), 7.36 (m, 2H), 6.58 (d, J = 15 Hz, 1H), 3.85 (s, 1H), 3.35 (s, 1H), 3.27 (s, 1H), 2.71 (s, 1H), 1.72-2.01 (m, 12H). MS (ESI): m/z = 354.18 $[\text{M} + \text{H}]^+$.

Synthesis for compound 11. Mixture of compound 10 (30 mg, 0.08 mmol) and pyridine (430 μL , 5 mmol) dissolved in anhydrous CH_2Cl_2 was added into the reaction

tube, and the reaction vessel degassed by freeze-pump-thaw cycles for three times. The mixture was stirred at room temperature under N₂ environment, before trifluoromethanesulfonic anhydride (29 μ L, 0.16 mmol) was added via a syringe. The reaction mixture was stirred for 2 h, and then washed with saturated ammonia chloride solution for three times to remove residue acidic anhydride. The organic layer was dried with anhydrous sodium sulfate and concentrated under reduced pressure. Pure compound 11 (35 mg, 90%) was obtained after HPLC purification. ¹H NMR (300 MHz, CDCl₃) δ : 7.88 (d, J = 15 Hz, 1H), 7.69 (d, J = 9 Hz, 1H), 7.35 (m, 2H), 6.55 (d, J = 15 Hz, 1H), 3.85 (s, 3H), 3.34 (s, 3H), 3.27 (s, 1H), 2.71 (s, 1H), 1.78-2.01 (m, 12H). MS (ESI): m/z = 486.13 [M + H]⁺.

Synthesis for compound 12. Pure compound 11 was stirred in 1 M NaOH/THF (50% v/v) for 24 h to obtain compound 12. Saturated ammonia chloride solution was added dropwise into the reaction mixture until pH was adjusted to 8; the organic layer was then washed with brine, dried over sodium sulfate, and evaporated under reduced pressure. Crude product was directly used for next step synthesis without further purification.

Synthesis for compound 13. Crude product 12 (6 mg, 0.012 mmol), HoBT (1.94 mg, 0.014 mmol), HBTU (9.1 mg, 0.024 mmol) and DIPEA (5.1 μ L, 0.03 mmol) dissolved in anhydrous N, N-dimethylformamide was stirred at room temperature for 30 minutes, before propargylamine (2.3 μ L, 0.036 mmol) was added. The reaction mixture was then stirred for 2 hours, and pure product compound 13 was obtained after HPLC purification. ¹H NMR (300 MHz, MeOD) δ : 7.84 (d, J = 15 Hz, 1H), 7.45 (d, J = 9 Hz, 1H),

6.81 (m, 2H), 6.72 (d, $J = 15$ Hz, 1H), 4.08 (m, 2H), 3.30 (s, 3H), 3.22 (s, 1H), 2.68 (s, 1H), 2.60 (t, 1H), 1.82-2.01 (m, 12H). MS (ESI): $m/z = 509.15$ $[M + H]^+$.

Synthesis for compound 14. The pure compound 13 (10 mg, 0.02 mmol) and catalytical amount of methylene blue were dissolved in mixture of CH_2Cl_2 and CH_3OH ($v/v = 1$). Oxygen was bubbled through the solution while irradiated with white light. The reaction was monitored by TLC. Upon completion, the reaction mixture was concentrated under reduced pressure and washed with H_2O to remove most of the methylene blue. Crude compound 14 was used to next step without further purification.

Synthesis for CFR. Compound 9 (15 mg, 0.015 mmol) and compound 14 (7.5 mg, 0.015 mmol), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (4 mg, 0.015 mmol) and sodium ascorbate (1.5 mg, 0.007 mmol) was dissolved and stirred in $\text{DMSO}/\text{H}_2\text{O}$ (50% v/v) under N_2 environment at room temperature for 1 h. The reaction mixture was then purified with preparative HPLC to obtain pure CFR (16 mg, 75%). ^1H NMR (300 MHz, MeOD) δ : 8.65 (m, 1H), 7.80 (m, 2H), 7.70 (m, 3H), 7.30-7.55 (m, 7H), 7.10 (m, 2H), 6.80 (m, 3H), 6.35 (m, 1H), 5.22 (d, $J = 9$ Hz, 2H), 4.51-4.69 (m, 5H), 4.30 (m, 3H), 4.03 (m, 1H), 3.73 (m, 1H), 3.53 (m, 1H), 3.21 (s, 1H), 2.99 (m, 1H), 2.80 (m, 4H), 2.42 (m, 2H), 2.10 (m, 4H), 1.99 (m, 9H), 1.85 (m, 14H), 1.45 (m, 4H), 1.29 (s, 5H), 0.90 (m, 7H). MS (ESI): $m/z = 1613.62$ $[M + H]^+$.

In vitro enzyme incubation and selectivity studies. Casp3 was incubated in freshly prepared incubation medium (HEPES (25 mM HEPES, $\text{pH} = 7.4$ containing 0.1% CHAPS and 10 mM DTT) at 37°C for 3 minutes before CFR dissolved in DMSO (5% v/v HEPES) was added. $^1\text{O}_2$ was prepared from equal molar concentrations of NaOCl and H_2O_2 ,

OCI⁻ was prepared from NaOCl and H₂SO₄, •OH was prepared from H₂O₂ and Fe(ClO₄)₂. ONOO⁻ was prepared by the procedure described by Hughes and Nicklin (J. Chem. Soc. A, 1970, 925-928).

Enzyme kinetic studies. Various concentrations of CFR (3, 5, 13, 26, 39, 80 μM) were incubated with casp3 (1.78*10⁻⁶ μmol) at 37 °C for 5 min in a 100μL system of HEPES buffer (25 mM, pH = 7.4) containing 0.1% CHAPS and 10 mM DTT. After incubation, the mixture was injected into HPLC (eluent: acetonitrile/water) for quantification analyses. The initial reaction velocity (μmol/s) was calculated, plotted against CFR concentration, and fitted to a Michaelis-Menten curve. The kinetic parameters were calculated using Michaelis-Menten equation: $V = V_{\max} * [S] / (K_M + [S])$, where V is initial velocity, and [S] is substrate concentration. The calculated parameters are as follows: $V_{\max} = 0.14 \mu\text{M/s}$, $K_M = 19.9 \mu\text{M}$, $k_{\text{cat}} = 7.88 \text{ s}^{-1}$, $k_{\text{cat}}/K_M = 4.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$.

Cell culture and drug treatment. AML-12 cells were cultured in DMEM:F12 (1:1) medium supplemented with heat-inactivated fetal bovine serum (10%), 10μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium, 40 ng/mL dexamethasone and antibiotics penicillin/streptavidin (1%) in a humidified environment containing 5% CO₂ and 95% air at 37 °C. Cell media were replaced every two to three days. For VPA treatment experiment, cells were treated with VPA (100 mM) in DMEM medium (10% FBS) for indicated duration. For control groups, cells were treated with NAC (100 mM) first for 1 h, before co-incubation with mixture of VPA (100 mM) and NAC (100 mM) for indicated duration.

Cell fluorescence imaging. For cell fluorescence imaging, AML-12 cells (10^5 cells per well in 1 mL) were seeded in the dishes (dia. 35 mm) and incubated overnight to reach 80% confluency. After incubation with different drugs as indicated in the captions under Figure 2, the medium was removed, and the cells were washed three times with PBS buffer. Then the cells were incubated with CFR (10 μ M) in DMEM medium for 20 minutes. Then the medium was removed, and the cells were washed with PBS buffer for three times. Then the cells were stained with Hoechst 33342 (NucBlue Live ReadyProbes Reagent, Thermo Fisher) for nuclei and fixed with 4% polyformaldehyde solution. Fluorescence images of the live cells were acquired on a Laser Scanning Microscope LSM800 (Zeiss). The excitation and emission wavelengths for cell imaging were 640/655–710 nm for CFR and 405/410–470 nm for Hoechst. ImageJ software was utilized to remove signal background and quantify cellular fluorescence intensity.

Cell chemiluminescence imaging. For cell chemiluminescence imaging, AML-12 cells (10^5 cells per well in 1 mL) were seeded in the dishes (dia. 35 mm) and incubated overnight to reach 80% confluency. After incubation with different drugs as indicated in the captions under Figure 2, the medium was removed, and the cells were washed three times with PBS buffer. Then the cells were incubated with CFR (10 μ M) in DMEM medium for 20 minutes. Then the medium was removed, and the cells were washed with PBS buffer for three times. The cells were immediately taken for chemiluminescence imaging, and imaging was acquired within 20 minutes. Chemiluminescence images of living cells were acquired on LX71 inverted microscope (Olympus) equipped with infinity 3-1 (Lumenera) CCD camera. During imaging, the shutter for excitation light was closed, and exposure time was adjusted to 3000 ms.

Cytotoxicity assay. AML-12 cells were seeded in 96-well plates (10^4 cells per well) for 24 h, and then different concentrations of CFR (3, 5, 10, 15, and 20 μ M) were added to the cell culture medium. Cells were incubated with or without (control) CFR, followed by addition of MTS assay (Promega Cat. no. G3581, 100 μ L, 0.1 mg/mL) for 4 h. The absorbance of MTS at 490 nm was measured by using a microplate reader. The cytotoxic effects (VR) of CFR were assessed using the following equation: $VR = A/A_0 \times 100\%$, where A and A_0 are the absorbance of the experimental group and control group, respectively. The assays were performed in three sets for each concentration.

Live mice imaging. All animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Nanyang Technological University-Institutional Animal Care and Use Committee (NTU-IACUC) and approved by the Institutional Animal Care and Use Committee (IACUC) for Animal Experiment, Singapore. Adult female Ncr nude mice (18 – 20 g) were housed in a temperature controlled (22 °C) room with 12 h dark light cycles (0700 h on and 1900 h off). The animals were provided ad libitum with food (Tecklad T.2918.CS Irradiated Rodent Diet purchased from Acre Engineering Pte Ltd) and water. Mice were first i.p. injected with hepatotoxic dosage (750 mg/kg bw) of VPA (10% dissolved in 9% NaCl solution) without anesthesia. After different drug post-treatment time ($T = 0.5, 12, 18$ and 24 h), mice were i.v. injected with CFR (2 μ mol/kg bw, dissolved in 8% v/v DMSO/PBS) and were anesthetized with inhaled isoflurane or received i.p. injection of ketamine/xylazine (0.1 mL/10g) for anaesthesia throughout real-time imaging (2 h). NIRF imaging was acquired using IVIS system fluorescence mode ($Ex/Em = 640/720$ nm), and CHL

imaging was acquired using IVIS system bioluminescence open filter mode with exposure time of 180 s.

Statistics analysis. The fluorescence images were processed with ROI analysis using Living Image 4.0 Software. The results are expressed as the mean $\pm$ standard deviation unless otherwise stated. Statistical comparisons between the two groups were determined by Student's t-test (1-tailed, unpaired). For all tests, $p < 0.05$ was considered as statistically significant. All statistical calculations were performed using GraphPad Prism v.6 (GraphPad Software Inc., CA, USA).

4.3. Results and Discussions

To investigate the optical properties and sensing ability of CFR, its absorption, NIRF and CHL spectra were measured in the absence or presence of both biomarkers. CFR intrinsically had absorption maxima at 610 and 660 nm, and was barely fluorescent or chemiluminescent. After incubation of CFR with casp3, a new absorption peak at 690 nm appeared, due to the cleavage of peptide segment and generation of the CyU unit (**Figure 4-1a**). This was further confirmed by emergence of a new high-performance liquid chromatography (HPLC) peak corresponding to PABA (**Figure 4-1d**). Such a casp3-induced cleavage resulted in 12-fold NIRF enhancement at 710 nm (**Figure 4-1b**), and the catalytic efficiency (K_m/k_{cat}) of casp3 towards CFR was $4.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (**Figure 4-2**). These properties were similar to the precursor (compound 9 in Scheme 4-1, **Figure 4-3**), showing the presence of the CHL moiety had little influence on the enzymatic response. In the presence of $\text{O}_2^{\bullet-}$, the absorption and NIRF spectra of CFR remained nearly unchanged, while its CHL signal was activated at 540 nm (**Figure 4-**

1c). The structural change and release of 2-admantanone were confirmed by HPLC analysis (**Figure 4-1d**). The $O_2^{\bullet-}$ -induced CHL of CFR was ~40000-fold stronger than background (**Figure 4-1c**), whose half-life was 3.5 min (**Figure 4-2**) and the limit of detection (LOD) was as low as ~10 nM (**Figure 4-1e**). The reaction kinetic coefficient of CFR towards $O_2^{\bullet-}$ was determined to be $5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (**Figure 4-4**). Moreover, the presence of both $O_2^{\bullet-}$ and casp3 still led to similar CHL emission at 540 nm, indicating no energy transfer from dioxetane to free CyU probably due to the large donor-acceptor distance. Nevertheless, CFR showed negligible NIRF and CHL responses towards other interfering enzymes and ROS (**Figure 4-1f**). These data suggested that CFR sensitively and specifically detect casp3 and $O_2^{\bullet-}$ with NIRF and CHL signals, respectively.

Figure 4-1. In vitro detection. (a) UV/Vis absorption spectra, (b) NIRF and (c) CHL spectra of CFR (10 μ M) in the absence or presence of casp3 (0.1 U/mL) or $O_2^{\bullet-}$ (50 μ M) in PBS (10 mM, pH = 7.4) at 37 °C. Inset: (a) White light, (b) NIRF and (c) CHL images of CFR (10 μ M) in the absence or presence of casp3 or $O_2^{\bullet-}$. (d) HPLC traces of CFR in the absence or presence of $O_2^{\bullet-}$ or casp3, and the traces of pure 2-admantanone, PABA. (e) CHL intensities of CFR (10 μ M) as a function of $[O_2^{\bullet-}]$ (0 – 15 μ M). (f) NIRF (710 nm) and CHL changes (540 nm) of CFR (10 μ M) after incubation with indicated ROS (50 μ M) in PBS buffer (10 mM, pH = 7.4) and enzymes (0.1 U/mL) at 37 °C. NIRF excitation at

640 nm and CHL acquired under bioluminescence mode with the exposure time of 5 s. Alanine aminopeptidase (AAP), fibroblast activation protein- α (FAP- α), urokinase (uPA). Error bars: standard deviation from three separate measurements.

Figure 4-2. (a) CHL signals of CFR (10 μM) change with time after addition of KO_2 (50 μM) in PBS (10 mM, pH = 7.4). (b) Enzyme kinetics of casp3 (0.5 U/mL) towards CFR, [CFR] = 3 to 80 μM . $K_M = 2.0 \times 10^{-5} \text{ M}$, $k_{\text{cat}} = 7.9 \text{ s}^{-1}$, $k_{\text{cat}}/K_M = 4.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$.

Figure 4-3. In vitro detection of CyDEVD (Compound 9). (a) UV/Vis absorption and (b) fluorescence of CyDEVD in the absence or presence of casp3 (0.1 U/mL) in HEPES (25 mM, pH = 7.4) containing 0.1% CHAPS and 10 mM DTT at 37 °C. Inset: white light (a) and fluorescence images (b) of CyDEVD (10 μ M) in the absence or presence of casp3. (c) Enzyme kinetics study of 0.1U/mL casp3 with [CyDEVD] 3 – 80 μ M. $K_M = 6.4 \times 10^{-6}$ M, $k_{cat} = 1.9 \text{ s}^{-1}$, $k_{cat}/K_M = 2.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. NIRF (710 nm) changes (d), UV/Vis absorption (e) and fluorescence spectra (f) of CyDEVD (10 μ M) after incubation with indicated enzymes (0.1 U/mL) in respective buffers at 37 °C. Alanine aminopeptidase (AAP), fibroblast activation protein-alpha (FAP- α), urokinase (uPA). (g) CyDEVD sensing capability evaluation through HPLC analysis of incubation mixture of CyDEVD (10 μ M) in the presence of casp3 (0.1 U/mL) in HEPES (25 mM, pH = 7.4) containing 0.1% CHAPS and 10 mM DTT at 37 °C. (h) HPLC traces of CyDEVD (10 μ M) in the absence or presence of casp3 (0.1 U/mL) in HEPES (25 mM, pH = 7.4) containing 5% DMSO, 0.1% CHAPS and 10 mM DTT at 37 °C. Error bars: standard deviation from three separate measurements.

Figure 4-4. (a) Time course of CHL signals ($\lambda = 540 \text{ nm}$) of CFR (10 μ M) upon adding $\text{O}_2^{\bullet -}$ (100 μ M, 10 equivalents); (b) Plot of V_0/V with [GSH] (from 1 to 5 μ M) for

estimation of k_{CFR} ($5.0 \pm 0.7 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$. $V_0/V = 1 + k_{\text{GSH}}[\text{GSH}]/k_{\text{CFR}}[\text{CFR}]$, $k_{\text{GSH}} = 200 \text{ M}^{-1}\text{s}^{-1}$.

CFR was used for duplex imaging of endogenous $\text{O}_2^{\bullet-}$ and casp3 in mouse hepatocytes (AML-12) after confirming its low cytotoxicity (**Figure 4-5**). The cells were treated with a widely prescribed antiepileptic drug – valproic acid (VPA), and CHL and NIRF imaging were conducted at two drug treatment times (t_{dt}) (0.5 and 6 h). The VPA dosage was chosen at 100 mM, known to be cytotoxic.¹⁴⁷ At $t_{\text{dt}} = 0.5 \text{ h}$, the CHL intensity of VPA-treated cells was ~710 – fold higher than the control cells; however, it returned to the basal level at $t_{\text{dt}} = 6 \text{ h}$ (**Figure 4-6a,b**). In contrast, the NIRF intensity of VPA-treated cells remained nearly the same as the control at $t_{\text{dt}} = 0.5 \text{ h}$, but increased by 3.6 – fold at $t_{\text{dt}} = 6 \text{ h}$ (**Figure 4-6a,c**). When the cells were pre-treated with an antioxidant, *N*-acetyl L-cysteine (NAC), both CHL and NIRF signals decreased to the basal levels (**Figure 4-6**). This was because NAC scavenged cellular ROS, preventing oxidative stress and subsequently inhibiting apoptosis.¹⁴⁸ These results not only confirmed the feasibility of CFR to image $\text{O}_2^{\bullet-}$ and casp3 in living cells, but also revealed that $\text{O}_2^{\bullet-}$ was upregulated prior to the activation of casp3 after VPA challenge.

Figure 4-5. CFR cytotoxicity test by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) assay in AML-12 living cells. Error bars represent the standard deviation of 3 trials.

Figure 4-6. In vitro duplex imaging in mouse hepatocytes (AML-12). (a) CHL and NIRF cell images of cells with different pre-treatments before incubation with CFR (10 μ M, 20 min). From left to right: untreated cells, cells treated with VPA (100 mM) (0.5 h and 6 h), and cells pre-treated with NAC (1 h) before co-incubation with VPA and NAC (both 100 mM) for 6 h. Mean CHL (b) and NIRF (c) intensity enhancement of single hepatocyte in Figure 2a. The values relative to the control groups. ** $p < 0.01$ ($n=3$), n.s.: not significant.

The duplex imaging capability of CFR was further validated in a DIH mouse model after confirming its blood elimination half-life (10.4 min) (**Figure 4-7**) and the in vivo sensitivity (at least 15 nM) (**Figure 4-8**). The mice were treated with VPA at a hepatotoxic dosage (750 mg/kg bw),¹⁴⁹ and CFR was administered *via* intravenous (i.v.) injection at different drug post-treatment times (t_{dpt}) (0.5, 12, 18 and 24 h). The control mice were injected with saline, or protected with NAC. Longitudinal NIRF and CHL imaging were conducted for 2 h for different treatment groups (**Figure 4-9a**). The signals in the liver were quantified for analysis. At $t_{dpt} = 0.5$ h, the maximal CHL intensity of VPA-treated mice was observed at 2 min after i.v. injection of CFR, 3-times higher than saline-treated mice (**Figure 4-9 b-d**); in contrast, the NIRF intensities were similar to those of the control. The CHL intensity dropped to the basal level at later t_{dpt} , while the NIRF intensities increased by 1.5 and 1.4 – fold at $t_{dpt} = 18$ and 24 h, respectively (**Figure 4-9 b-d**). After imaging, the mice were dissected and organs were collected for biodistribution (**Figure 4-10**) and histological analysis (**Figure 4-9 e** and **Figure 4-11,12**). The results showed that only at the late t_{dpt} (18&24 h) could the tissue damage (dilated sinusoids and hepatocyte debris) be observed (**Figure 4-9e**). Moreover, the green signals from the casp3 immunostaining superimposed well with the NIRF signal of CFR (**Figure 4-9e**). The fact that the CHL signal of CFR detected the upregulation of $O_2^{\bullet-}$ at a much earlier t_{dpt} (0.5 h) highlighted that CFR acted as an early reporter to detect DIH prior to histological changes.

Figure 4-7. Pharmacokinetics of CFR in saline-treated healthy mice. Blood (20 μ L) was collected at different time points ($t = 3, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105$ and 120 min) post i.v. injection of CFR (2 μ mol/kg). The blood elimination half-life ($t_{1/2}$) is determined to be 10.4 min.

Figure 4-8. In vivo sensitivity of CFR towards $O_2^{\bullet-}$. (a) CFR (1 μ M) in the presence or absence of 15 nM $O_2^{\bullet-}$ were subcutaneously injected into living mice. (b) Quantification of CHL signals in (a), error bars are standard deviation from three separate measurements. *** $p < 0.001$.

The trend of in vivo duplex signal evolution for CFR was similar to that of in vitro (Figure 4-6), which can be rationalized by the cytotoxic mechanism of VPA as summarized in Figure 4-9 f. VPA was first metabolized by CYP enzymes into E-2,4-

diene VPA. This metabolite is a potent inhibitor of mitochondria β -oxidation and normal functions, and thus can cause mitochondrial dysfunction and subsequently induce the overproduction of $O_2^{\bullet-}$.¹⁴⁴ The elevated ROS level then results in both DNA damages and mitochondria permeability transition, activating casp3.¹⁴⁵ At last, cellular apoptosis occurs, leading to cell death and tissue damage. Thus, the duplex signal evolution of CFR is consistent with the mechanism of DIH, further confirming that CFR longitudinally and differentially detected the sequential upregulation of $O_2^{\bullet-}$ and casp3 during the progression of DIH.

Figure 4-9. Real-time duplex imaging of DIH in living mice. (a) Timeline for development of VPA-induced DIH and duplex imaging. (b) Representative CHL images of living mice at 2 min and NIRF images at 15 min after i.v. injection of CFR in different treatment groups. The white circles indicate the site of livers. The dynamic CHL (c) and

NIRF (d) signals as a function of time post-injection of CFR in living mice. (e) Representative haematoxylin&eosin (H&E) staining and fluorescence images of liver tissues. Blue: DAPI; green: caspase-3 antibody staining; red: CFR. (f) Schematic illustration of how CFR sequentially detects the upregulation of $O_2^{\bullet-}$ and casp3 during the progression of DIH. **: $p < 0.01$ (n=3), n.s.: not significant.

Figure 4-10. Biodistribution studies. (a) Organs dissected from different experimental groups were imaged under IVIS fluorescence mode. Ex = 640 nm, Em = 720 nm. (b) HPLC analysis of area under curve (AUC) for major organs from control group, and experimental groups $t_{dpt} = 0.5, 18$ h. Major organs were homogenized in methanol and centrifuged to remove insoluble components. The supernatant containing extracted molecules were taken for HPLC analysis. The calculated AUC refers to both enzyme-cleaved and noncleaved CFR in major organs and showed similar liver accumulation in both control and $t_{dpt} = 0.5, 18$ h groups. (c) The body weight of mice as a function of VPA treatment time. No obvious mice weight loss was observed within $t_{dpt} = 24$ h.

Figure 4-11. Histological and Immunohistochemistry studies of organs from healthy mice after i.v. injection of CFR ($2 \mu\text{mol/kg bw}$, dissolved in 8% v/v DMSO/PBS). Mice were dissected 24 h after injection and organs were taken out for sectioning. Green

signals after staining with caspase-3 antibodies were not observed, suggesting no major organs are damaged from CFR injection. Scale bar: 50 μm .

Figure 4-12. Histology studies for NAC/VPA and $t_{\text{dpt}} = 1, 2$ and 6 h groups. Upper panel: representative photomicrographs of H&E staining in paraffin-embedded liver sections from different experimental groups.). Lower panel: representative confocal fluorescence microscopy images of liver cryo-sections. Blue channel indicates signals from DAPI staining; green channel indicates signals from cleaved casp3 antibody staining; and red channel indicates signals from enzyme cleaved CFRs. Scale bar: 50 μm .

4.4. Conclusions

In conclusion, a duplex optical probe (CFR) was reported and its proof-of-concept application in early detection of DIH was demonstrated. CFR had two independent optical channels that sensitively and specifically detected $\text{O}_2^{\bullet-}$ and casp3 *via* CHL and NIRF signals, respectively. The crosstalk-free duplex imaging ability of CFR enabled longitudinal monitoring of two correlated sequential biomolecular events, confirming that $\text{O}_2^{\bullet-}$ is upregulated before casp3 in drug-challenged cells and mice. Moreover, the

CHL channel of CFR detected DIH 17.5 h earlier than histological analysis. Thus, CFR holds great promise for both pre-clinical drug screening and clinical diagnosis of DIH. Therefore, CFR represents the first small molecule-based duplex optical probe with both light excitation and excitation-free channels. Such a molecular design of CFR can be generalized for duplex imaging of other biomarkers simply by changing the caging groups on both CHL and NIRF signalling moieties.

Despite of the high sensitivity and time resolution of CFR, its clinical translation could be hindered by the shallow penetration depth of fluorescence signals. Near-infrared fluorescence signals has a tissue penetration depth of ≈ 1 cm, which is not satisfactory for real-time imaging in human patients. To resolve this issue, in Chapter 5, we reported the synthesis and application of a fluoro-photoacoustic polymeric renal reporter (FPRR) for real-time non-invasive near-infrared fluorescence (NIRF) and photoacoustic (PA) dual imaging of drug-induced acute kidney injury (AKI) in murine mouse model.

Figure 4-13 ^1H NMR spectrum of compound 6 in CDCl_3 .

Figure 4-14 ^1H NMR spectrum of compound 6 in MeOD.

Figure 4-15 Mass spectrum of compound 8.

Figure 4-16 ^1H NMR spectrum of compound 9 in MeOD.

Figure 4-17 Mass spectrum of compound 9.

Figure 4-18 ^1H NMR spectrum of compound 10 in CDCl_3 .

Figure 4-19 ^1H NMR spectrum of compound 11 in MeOD. Figure 4-20 ^1H NMR spectrum of compound 13 in MeOD.

Figure 4-21 ¹H NMR spectrum of CFR in MeOD.

Chapter 5. Fluoro-photoacoustic Polymeric Renal Reporter for Real-time Dual Imaging of Acute Kidney Injury

5.1. Introduction

Photoacoustic (PA) imaging that combines the advantages of optical and ultrasound imaging has become an increasingly important tool in preclinical studies and even clinical applications.¹⁵⁰⁻¹⁵³ By detecting the acoustic signals generated by thermoelastic expansion followed by photo absorption, PA imaging substantially overcomes tissue scattering, and thus possesses better spatial resolution and increased penetration depth as compared to optical imaging.¹⁵⁴ Despite the fact that many materials have been exploited as contrast agents for PA imaging such as small-molecular dyes,^{155,156} gold nanoparticles,¹⁵⁷ carbon nanotubes,¹⁵⁸ semiconducting polymer nanoparticles,^{132,159-162} most of them rely on passive accumulation at the disease region to cause signal enhancement relative to healthy tissue. These accumulation agents have been used to detect primary and metastatic tumors,^{159,161} vascular,¹⁶³ ocular and lymphatic diseases.^{164,165} In contrast, activatable PA probes that only emit signal responding to disease biomarkers provide specific and accurate molecular information for disease diagnosis.¹⁶⁶ Until now, a variety of biomarkers including pH,¹⁶⁷ antioxidants,¹⁶⁸ reactive oxygen species,^{169,170} enzymes^{171,172} and metal ions¹⁷³ have been detected by activatable PA probes. However, most of the PA agents have high uptake by the mononuclear phagocytic system (MPS) and undergo slow excretion via hepatobiliary pathway.¹⁷⁴ This prolonged exposure can potentially result in the acute or chronic toxicity to major organs, hindering these agents from

clinical translation.¹⁷⁵ Thus, development of activatable PA agents with high renal clearance is a promising approach towards future clinical practices.^{80,176}

Kidney receives up to 25% cardiac output and serves as the primary site for metabolism and excretion of body wastes. Due to the local high concentration of drugs and metabolites, kidney is vulnerable to toxic exposure and easy to be injured.¹⁷⁷ Development of renal-clearable optical imaging agents is thus important for imaging and diagnosis of kidney diseases.⁸⁶ By fine-tuning the size, surface charges, and shape, quantum dots,¹⁷⁸ gold nanoparticles,^{5,87,179} silica nanoparticles^{180,181} and carbon nanotubes¹⁸² have been developed to be renal-clearable, which however were rarely validated for PA imaging as well as imaging of kidney function. A series of molecular fluorescence/chemiluminescence probes have been recently developed by Pu's group that are both highly renal clearable and capable of detecting disease biomarkers in kidney injury.^{183,184} Additionally, Zheng's group have reported gold nanoclusters (size < 3.5 nm) for real-time PA imaging in a unilateral ureter obstruction (UUO) mouse model. However, such gold nanoclusters exhibit always-on PA signals and the signal difference relies on the different accumulation in the obstructed kidney due to decreased glomerular filtration rate (GFR).¹⁸⁵ Therefore, development of activatable PA probes for real-time detection and diagnosis of kidney injury remains challenging.

Herein, the synthesis of a fluoro-photoacoustic polymeric renal reporter (FPRR) is reported for real-time non-invasive near-infrared fluorescence (NIRF) and PA dual imaging of drug-induced acute kidney injury (AKI) in murine mouse model (**Figure 5-1a**). AKI is characterized by the rapid decline in kidney function associated with high morbidity and mortality.¹⁸⁶ Among major AKI etiologies such as ischemia/reperfusion,

sepsis, and nephrotoxin exposure, nephrotoxin induced AKI accounts for 25% of all AKI cases.¹⁸⁷ The sequential pathophysiological events induced by nephrotoxic drug (cisplatin) include renal epithelial cell damage and death, vigorous inflammatory reactions, GFR decline, and ultimately kidney dysfunction.¹⁸⁸ After proximal tubular epithelial cell damage, γ -glutamyl transferase (GGT), a brush border enzyme highly expressed in proximal tubular epithelial cells, is released by exocytosis or leakage and can be readily detected in the urine.^{189,190} Thus, GGT was chosen as the targeted biomarker for the design of FPRR.

5.2. Materials and Methods

Chemicals. All chemicals used in the experiments were purchased from Sigma-Aldrich unless otherwise stated. Commercially available reagents were used without further purification unless noted otherwise. Furin, urokinase, caspase-3, alanine aminopeptidase were purchased from RnD Systems. Dulbecco's modified eagle medium (DMEM) with L-glutamine was purchased from Lonza. Trypsin–EDTA (0.05%), penicillin–streptomycin (10,000 U mL⁻¹) and fetal bovine serum (FBS) were purchased from Gibco. NucBlue Live Ready Probes Reagent were obtained from Thermo Fisher Scientific. CellTiter 96(R) AQueous One Solution Assay (MTS Assay) was purchased from Promega.

Characterization. Proton nuclear magnetic resonance (¹H NMR) spectra were recorded by using a Bruker Avance II 300 MHz NMR, MeOD was used as the solvent. UV/vis absorption spectra were recorded on a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were conducted on a Fluorolog 3-TCSPC

spectrofluorometer (Horiba Jobin Yvon). Photoacoustic measurements were conducted by using a real-time multispectral optoacoustic tomographic (MSOT) imaging system from iThera Medical GmbH (Neuherberg, Germany). High performance liquid chromatography (HPLC) analyses were done on an Agilent 1260 system equipped with a G1311B pump, UV detector and an Agilent Zorbax SB-C18 RP (9.4 × 250 mm) column, with methanol (0.1% of TFA) and water (0.1% of TFA) as the eluent. HPLC purification were performed on an Agilent 1260 gradient preparative system equipped with a G1361A pump, UV detector and an Agilent Zorbax SB-C18 RP (21.2 × 250 mm) column, with methanol (0.1% of TFA) and water (0.1% of TFA) as the eluent. Fluorescence images of living cells were acquired on Laser Scanning Microscope LSM800 (Zeiss). Living mice imaging was acquired with IVIS Spectrum imaging system (PerkinElmer, Inc.).

Synthesis of CyOH. Synthesis was carried out according to previous literature report.¹⁸³ ¹H NMR (300 MHz, CDCl₃) δ: 8.64 (d, J = 15 Hz, 2H), 7.44 (m, 5H), 7.12 (t, 3H), 6.00 (d, J = 15 Hz, 2H), 4.10 (t, 1H), 3.47 (t, 2H), 2.78 (t, 2H), 2.65 (t, 2H), 1.96 (d, 4H), 1.79 (s, 7H).

Synthesis of compound 1. Fmoc-Glu-OtBu (CAS 84793-07-7, 300 mg, 0.7 mmol) was dissolved and stirred in anhydrous CH₂Cl₂ under N₂ atmosphere and then the mixture of N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 691 mg, 2.8 mmol) and *p*-aminobenzyl alcohol (PABA, 344 mg, 2.8 mmol) dissolved in anhydrous CH₂Cl₂ was added via a syringe. The mixture was then stirred for 3 h at room temperature. After completion, the mixture was concentrated under reduced pressure and purified by preparative HPLC to obtain pure product 1 (326 mg, 85%). ¹H NMR (300 MHz, CDCl₃)

δ : 7.75 (d, J = 6 Hz, 2H), 7.56 (d, J = 9 Hz, 4H), 7.38 (m, 2H), 7.28 (m, 4H), 4.60 (s, 2H), 4.42 (m, 2H), 4.14 (m, 2H), 2.33 (m, 2H), 1.90 (m, 2H), 1.45 (s, 9H) (**Figure 5-13**).

Synthesis of compound 2. Pure product 1 (150 mg, 0.28 mmol) was dissolved in anhydrous tetrahydrofuran (THF) in ice bath under N_2 atmosphere, and PBr_3 (53 μ L, 0.56 mmol) dissolved in anhydrous THF was added via a syringe. The reaction mixture was stirred in ice bath for 2 h and the reaction was monitored by thin layer chromatography (TLC). The reaction mixture was then concentrated under reduced pressure and diluted with ethyl acetate (30 mL). The organic phase was washed sequentially with saturated sodium bicarbonate (30 mL $\times$ 3) and brine (30 mL) and dried over anhydrous sodium sulfate before concentrated under reduced pressure. The crude compound 2 was then taken for next step synthesis without further purification.

Synthesis of compound 3. The mixture of pure hemi-cyanine (CyOH, 32 mg, 0.07 mmol), and N,N -Diisopropylethylamine (DIEA, 47 μ L, 0.28 mmol) was dissolved and stirred in anhydrous CH_3CN at 55 $^{\circ}C$ under N_2 atmosphere. After stirring for 30 min, compound 2 (165 mg, 0.28 mmol) was added into the mixture and the reaction mixture was continued to stir at 55 $^{\circ}C$ for 8 h under N_2 atmosphere. The reaction was monitored by analytical HPLC. After completion, and resulting mixture was concentrated under reduced pressure and purified by preparative HPLC to obtain pure product 3 (48 mg, 70%). 1H NMR (300 MHz, CD_3OD) δ : 8.64 (d, J = 15 Hz, 1H), 7.70 (m, 4H), 7.40 (m, 14H), 6.98 (s, 2H), 6.42 (d, J = 15 Hz, 1H), 5.23 (s, 2H), 4.33 (m, 6H), 3.44 (t, 2H), 2.48-2.71 (m, 5H), 2.18 (m, 2H), 1.80 (m, 11H), 1.42 (m, 11H) (**Figure 5-14**).

Synthesis of CyG. Pure product 3 (30 mg, 0.03 mmol) was stirred in Trifluoroacetic acid (TFA)/CH₂Cl₂ (4 mL, 50% v/v) in ice bath for 1.5 h. The reaction was continuously monitored by analytical HPLC. Upon completion, the reaction mixture was diluted with CH₂Cl₂ (20 mL) and washed with saturated sodium bicarbonate (30 mL × 3). The organic phase was then dried over sodium sulfate and concentrated under reduced pressure to yield crude product. To a solution of the above crude product dissolved in dimethylformamide (DMF, 4 mL), piperidine (200 µL, 2.7 mmol, 5% v/v in DMF) was added. The reaction mixture was stirred at room temperature for 20 min and purified by preparative HPLC to obtain pure CyG (9 mg, 42%). ¹H NMR (300 MHz, CD₃OD) δ: 8.76 (d, J = 15Hz, 1H), 7.66 (m, 3H), 7.48 (m, 7H), 7.07 (m, 2H), 6.53 (d, J = 15Hz, 1H), 5.23 (s, 2H), 4.38 (t, 2H), 4.06 (t, 1H), 3.45 (t, 2H), 2.69 (m, 5H), 2.25 (m, 2H), 1.97 (m, 4H), 1.80 (m, 7H), 1.28 (m, 2H) (**Figure 5-15**).

Synthesis of dextran-alkyne. To a solution of dextran (CAS 9004-54-0, 2 g, 0.34 mmol) and tetra-tert-butylammonium iodide (27 mg, 0.08 mmol) in anhydrous DMF (30 mL) was added a suspension of sodium hydride (120 mg, 5 mmol). The reaction mixture was continuously stirred in ice bath under N₂ atmosphere for 0.5 h. Then a solution of propargyl bromide (52 µL, 0.68 mmol) was added via a syringe and the mixture was stirred at room temperature for additional 24 h. Upon completion, 10-times diluted glacial acetic acid was added dropwise to adjust pH to 7.4. Water was then added into the mixture and the resulting solution was dialysis against pure water to remove any impurities. After dialysis, the resulting inorganic solution was washed three times with CH₂Cl₂, followed by lyophilization to obtain pure Dextran-alkyne as white solid (1.8 g,

85%): ^1H NMR (300 MHz, D_2O) δ : 4.95 (d, $J = 3\text{Hz}$, 37H), 3.53-3.88 (m, 295H), 2.69 (s, 1H) (**Figure 5-16**).

Synthesis of FPRR. CyG (15 mg, 0.02 mmol), dextran-alkyne (150 mg, 0.025 mmol), sodium ascorbate (1 mg, 0.004 mmol) and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.25 mg, 0.005 mmol) was dissolved in $\text{DMSO}/\text{H}_2\text{O}$ (3 mL/3 mL) and stirred at room temperature under N_2 atmosphere in the dark overnight. Then the reaction was precipitated in acetone. Pure FPRR could be obtained as blue solid by further purification with preparative HPLC (113 mg, 85%): ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ : 6.0-8.0 (m, 14H), 4.67 (s, 37H), 3.44 (t, 298H). 1.0-2.5 (m, 20H).

Synthesis of dextran-substituted CyOH (Dex-CD). The mixture of CyOH (20 mg, 0.04 mmol), dextran-alkyne (300 mg, 0.05 mmol), sodium ascorbate (2 mg, 0.008 mmol) and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (2.5 mg, 0.01 mmol) was dissolved and stirred in $\text{DMSO}/\text{H}_2\text{O}$ (3 mL/3 mL) at room temperature under N_2 atmosphere in the dark overnight. Then the reaction was precipitated in acetone. Pure Dex-CD could be obtained as blue solid by further purification with preparative HPLC (220 mg, 85%): ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ : 6.0-8.0 (m, 12H), 4.67 (s, 37H), 3.44 (t, 298H). 1.0-2.5 (m, 13H).

In vitro enzyme incubation and enzyme kinetic studies. GGT (Sigma G9270) was freshly prepared and suspended in biological H_2O to a concentration of $5\text{ }\mu\text{g}/\mu\text{L}$. FPRR was dissolved in PBS (10 mM, $\text{pH} = 7.4$) and GGT (20 μg) was added. The mixture was then incubated at $37\text{ }^\circ\text{C}$. For enzyme kinetic studies, various concentrations of FPRR (6, 15, 40, 65, 80, 160 μM) were incubated with GGT (12.5 μg) at $37\text{ }^\circ\text{C}$ for 5 min in PBS (10 mM, $\text{pH} = 7.4$). After incubation, the mixture was taken for NIRF measurement.

A calibration curve of NIRF signals was plotted against different CyOH concentrations, and the percentage of CyOH conversion was subsequently calculated. The initial reaction velocity ($\mu\text{mol/s}$) was calculated, plotted against FPRR concentration, and fitted to a Michaelis-Menten curve. The kinetic parameters were calculated using Michaelis-Menter equation: $V = V_{\text{max}} * [S] / (K_M + [S])$, where V is initial velocity, and $[S]$ is substrate concentration. The calculated parameters are as follows: $V_{\text{max}} = 0.03 \mu\text{M/s}$, $K_M = 10.21 \mu\text{M}$, $k_{\text{cat}} = 158.74 \text{ s}^{-1}$, $k_{\text{cat}}/K_M = 15.6 \mu\text{M}^{-1}\text{s}^{-1}$.

Cell culture and fluorescence imaging. NDF and HK-2 cells were cultured in DMEM medium supplemented with heat-inactivated fetal bovine serum (10%) and antibiotics penicillin/streptavidin (1%) in a humidified environment containing 5% CO_2 and 95% air at 37 °C. Cell media were replaced every two to three days. For cell fluorescence imaging, NDF and HK-2 cells (6×10^4 cells per well in 1 mL) were seeded in the dishes (dia. 35 mm) and incubated overnight to reach 80% confluency. Cells were randomly divided into three groups: 1) untreated cells, 2) cells treated with FPRR, and 3) cells treated with GGsTop before incubation with FPRR. For group 3, cells were incubated with GGsTop (100 μM) for 30 minutes. Then the medium was removed, and the cells were then washed with PBS buffer for three times. Afterwards, cells from group 2 and 3 were incubated with FPRR (25 μM) in DMEM medium for 1 h. Then the medium was removed, and the cells were washed with PBS buffer for three times. Then the cells from all three groups were stained with Hoechst 33342 (NucBlue Live ReadyProbes Reagent, Thermo Fisher) for nuclei and fixed with 4% polyformaldehyde solution. Cell fluorescence images were acquired on a Laser Scanning Microscope LSM800 (Zeiss). The excitation and emission wavelengths for cell imaging were 640/655–710 nm for

FPRR and 405/410–470 nm for Hoechst. ImageJ software was utilized to remove signal background and quantify cellular fluorescence intensity.

Cell cytotoxicity assay. NDF and HK-2 cells were seeded in 96-well plates (6×10^3 cells per well) for 24 h, and then different concentrations of FPRR (5, 10, 20, 40, 80 μM) were added to the cell culture medium. Cells were incubated with or without (control) FPRR, followed by addition of MTS assay (Promega Cat. no. G3581, 100 μL , 0.1 mg/mL) for 4 h. The absorbance of MTS at 490 nm was measured by using a microplate reader. The cytotoxic effects (VR) of FPRR were assessed using the following equation: $\text{VR} = A/A_0 \times 100\%$, where A and A_0 are the absorbance of the experimental group and control group, respectively. The assays were performed in five sets for each concentration.

In vivo biodistribution and renal clearance efficiency studies. All animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Nanyang Technological University-Institutional Animal Care and Use Committee (NTU-IACUC) and approved by the Institutional Animal Care and Use Committee (IACUC) for Animal Experiment, Singapore. Female nude mice (Tac:Cr:(NCR)-Fox1nu, 16 – 18 g) were housed in a temperature controlled (22 °C) room with 12 h dark light cycles (0700 h on and 1900 h off). The animals were provided ad libitum with food and water. Mice were i.v. injected with 0.2 mL FPRR (6.5 $\mu\text{mol/kg}$ bw) dissolved in PBS buffer and immediately placed in metabolic cages. Urine was collected at 3, 6 and 24 h post-injection, centrifuged at 4,500 r.p.m. for 10 min and filtered by a 0.22 μm syringe filter. After 24 h urine collection, the mice were dissected to collect major organs. The collected organs were homogenized in PBS buffer and

centrifuged at 4,500 r.p.m. for 15 min to remove insoluble components. Quantifications of FPRR in the urine excretion and major organs was done using analytical HPLC.

Pharmacokinetic studies. Female nude mice were i.v. injected with 0.2 mL FPRR (6.5 $\mu\text{mol/kg}$ bw) dissolved in PBS buffer, and blood sample (20 μL) was collected from tail vein at 3, 5, 8, 15, 30, 60, 90, 120 min post-injection. Blood was sampled in heparinized capillary tubes and stored in an ice box to prevent clotting before centrifugation at 3,500 r.p.m. for 10 min. The amount of FPRR in blood serum was quantified using HPLC and the % ID g^{-1} was plotted against post-injection time. The points were then fitted by a bi-exponential decay curve to estimate elimination half-life ($t_{1/2}$).

Hemolytic studies. Hemolytic studies were carried out according to literature report.¹⁹¹ Fresh mice blood was diluted by saline, and the red blood cells (RBC) were isolated from serum by centrifugation. The RBC were further washed with saline for three times, before a serial concentration of FPRR solution was added into the RBC suspension. PBS and Triton X-100 (a common surfactant for cell lysis, 10 g/L) were used as negative or positive controls, respectively. The solution was mixed by vortex, and then incubated at 37°C for 2 h. Afterwards, RBCs were centrifuged at 3000 rpm for 10 min and the supernatant of each sample was transferred into a 96-well plate. Free hemoglobin in the supernatant was measured with a Microplate reader at 540 nm. The hemolysis ratio of RBCs was calculated using the following formula: hemolysis (%) = $(A_{\text{sample}} - A_{\text{negative control}}) / (A_{\text{positive control}} - A_{\text{negative control}})$.

Establishment of cisplatin-induced AKI and real-time imaging in living mice. Female nude mice (16 – 18 g) were randomly selected and treated with cisplatin at reported nephrotoxic dosage (intraperitoneal injection, 20 mg/kg bw) without anesthesia. The control groups were treated with saline (0.2 mL) or NAC (intravenous injection, 400 mg/kg bw) 30 min before cisplatin administration. Real-time NIRF and PA imaging were conducted at different post-drug treatment times ($t = 16, 24, 48$ h). NIRF imaging was acquired using IVIS system fluorescence mode ($Ex/Em = 675/720$ nm, exposure time: 0.1s), images were acquired every 30 minutes for a duration of 2 h after i.v. injection of FPRR (6.5 $\mu\text{mol/kg bw}$). Mice were anesthetized with inhaled isoflurane throughout real-time imaging (2 h). In vivo PA imaging was conducted in the MSOT photoacoustic imaging system. Female nude mice (16 - 18g) received different drug treatment before imaging. Mice were positioned perpendicular to the scanning plane and remained anesthetized by 2% isoflurane during the PA imaging experiment. The real-time MSOT imaging was performed from 680 nm to 850 nm. 2 min after the start of sequential image acquisition, a bolus of FPRR in PBS solution was injected through the tail vein catheter and images were acquired every 30 min. The ROI intensities were recorded on the tomographic anatomical image of liver cross section in iThera software.

Online urinalysis. Urine samples were collected from living mice after i.v. injection of FPRR (6.5 $\mu\text{mol/kg bw}$) at $t = 16, 24$, and 48 h post-treatment with cisplatin (20 mg/kg bw) or saline as a control. The collected urine samples were centrifuged at 4,500 r.p.m. for 10 min, filtered by a 0.22 μm syringe filter, and taken for UV/vis absorption and NIRF measurement.

Histology. All tissues were fixed with 4% PFA, dehydrated in ethanol solution, embedded in paraffin and cut into sections with a thickness of 10 μm for H&E staining. The paraffin was removed by xylene washing and then the sections were incubated with haematoxylin for 4 min and eosin for 2 min, followed by washing with distilled water. The stained sections were examined using a Nikon ECLIPSE 80i microscope.

5.3. Results and Discussions

FPRR consists of three moieties, a renal clearance enabler polymeric moiety (dextran),^{192,193} a NIRF/PA signaling moiety (hemi-cyanine CyOH) and a biomarker responsive moiety (γ -glutamate). The hydroxyl group of CyOH was caged by a self-immolative linker connected to GGT-cleavable γ -glutamate moiety to diminish the electron-donating ability of the oxygen atom. In the presence of GGT, the amide bond next to γ -glutamate of FPRR can be specifically cleaved, followed by 1,6-immolation and spontaneous release of the linker *p*-aminobenzyl alcohol (PABA). Subsequently dextran substituted-CyOH (Dex-CD) was obtained with simultaneously enhanced NIRF and PA signals. The synthetic route of FPRR was shown in **Figure 5-1b**. First, Fmoc-Glu-OtBu was conjugated to PABA, from which the primary alcohol was brominated to obtain compound 2. Next, 2 was reacted to the hydroxyl group of CyOH, followed by deprotection of Fmoc and OtBu protecting groups on the γ -glutamate to afford CyG. Finally, CyG was coupled to alkyne-functionalized dextran (dextran-alkyne, molar ratio = 1 from ^1H NMR) molecule by click reaction to yield FPRR. There is one CyG moiety conjugated to one dextran molecule, as confirmed by ^1H NMR (Supporting Information).

Figure 5-1. a) Schematic illustration and molecular mechanism of FPRR for real-time NIRF and PA imaging of AKI. b) Synthesis of FPRR. i) *p*-aminobenzyl alcohol, N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), dichloromethane (DCM) anhydrous, 25°C, 3 h. ii) Phosphorus tribromide (PBr₃), tetrahydrofuran anhydrous, 0°C, 2 h. iii) Hemi-cyanine (CyOH), N,N-Diisopropylethylamine (DIEA), acetonitrile anhydrous, 55°C, 8 h. iv) Trifluoroacetic acid (TFA)/DCM (50% v/v), 0°C, 1.5 h; Piperidine, dimethylformamide (DMF), 25°C, 15 min. v) Dextran-alkyne, copper(II) sulfate pentahydrate (CuSO₄·5H₂O), sodium-L-ascorbate, dimethyl sulfoxide/H₂O, 25°C, 12 h.

The optical properties and detection capability of FPRR were studied by measurement of its UV/vis absorption, NIRF and PA spectra in the absence or presence of GGT under physiological conditions. Intrinsically, FPRR had two absorption peaks at 610 and 660 nm (**Figure 5-2a**). After incubation with GGT, both two absorption peaks diminished with the emergence of a new peak at 692 nm, corresponding to the generation of Dex-CD. Meanwhile, the NIRF signals at 710 nm was increased by 33-fold, and time-

dependent NIRF measurement showed a gradual signal increase until plateau within 2 h (**Figure 5-2b; Figure 5-3**). Furthermore, due to the elevated absorption signal at the NIR region, PA signal was enhanced by 6.5-fold at 700 nm (**Figure 5-2c**). High-performance liquid chromatography (HPLC) studies further confirmed that FPRR (retention time, $T_R = 12.8$ min) was completely converted into Dex-CD ($T_R = 22.2$ min) after incubation with GGT for 2 hours (**Figure 5-2d**). The catalytic coefficient (K_m/k_{cat}) of GGT towards FPRR was $15.6 \mu\text{M}^{-1}\text{s}^{-1}$, which was 25-fold lower than CyG (**Figure 5-2e; Figure 5-4**). This is due to the steric hindrance from the dextran polymer. In contrast, FPRR showed minimum NIRF enhancement in the presence of other enzymes such as renal intracellular enzymes (Furin, urokinase), apoptosis-related enzyme (caspase-3), proximal tubular enzymes such as lysosomal enzyme (N-acetyl- β -D-glucosaminidase) and brush border enzymes (alanine aminopeptidase) (**Figure 5-2f**). Thus, FPRR was able to activate both NIRF and PA signals towards GGT with high sensitivity and specificity.

Figure 5-2. In vitro characterization of FPRR. a) UV/vis absorption, b) NIRF and c) PA spectra of FPRR (25 μM) in the absence or presence of GGT (20 μg) in PBS (10 mM, pH = 7.4) at 37 $^{\circ}\text{C}$ for 2 h. d) HPLC traces of FPRR in the absence or presence of GGT, and the traces of pure Dex-CD. e) Enzyme kinetics of GGT (20 μg) towards FPRR (6, 15, 40, 65, 80, 160 μM). $K_M = 10.2 \mu\text{M}$, $k_{cat} = 158.7 \text{ s}^{-1}$, $k_{cat}/K_M = 15.6 \mu\text{M}^{-1}\text{s}^{-1}$. f) Relative NIRF (710 nm) enhancement after incubation with indicated enzymes in PBS (10 mM, pH = 7.4) at 37 $^{\circ}\text{C}$ for 2 h. Alanine aminopeptidase (AAP), caspase-3 (casp3), N-acetyl- β -D-glucosaminidase (NAG), urokinase (uPA). g) NIRF cell images of normal dermal fibroblasts (NDF) and proximal tubule epithelial cells (HK-2), and mean NIRF intensity of single cells after different treatments. From top to bottom: untreated cells, cells treated with FPRR, and cells treated with GGSTop before incubation with FPRR. GGSTop (100 μM , 30 min), FPRR (25 μM , 1 h). The cell nucleus stained with 4'-diamidino-2-phenylindole (DAPI) showed a blue fluorescence signal and FPRR showed

a pseudo-red fluorescence signal. h) Pharmacokinetics of FPRR in healthy mice after i.v. injection of FPRR (6.5 $\mu\text{mol/kg}$ bw). The blood elimination half-life ($t_{1/2}$) is determined to be 23 min. i) HPLC quantitative analysis of residual FPRR in major organs (gray bar) and FPRR excreted from kidney into urine (red bar) 24 h after i.v. injection. Data in (e-i) are presented as the mean $\pm$ SD, $n = 3$ independent experiments.

Figure 5-3. Time-dependent NIRF changes of FPRR (25 μM) in the presence of GGT (20 μg) in PBS (10 mM, pH = 7.4) at 37°C for 2 h.

After confirming its low cytotoxicity (**Figure 5-5**), FPRR was further tested in human-origin kidney proximal tubule epithelial cells (HK-2) with normal dermal fibroblast (NDF) as the control. After incubation with FPRR, HK-2 displayed 25-fold higher NIRF signals as compared to NDF (**Figure 5-2g**). However, if HK-2 received pre-treatment of GGsTop (GGT inhibitor) before incubation with FPRR, their NIRF signal decreased significantly by 91%. In comparison, NDF showed minimum NIRF signals after FPRR incubation in the presence or absence of GGsTop. These data confirmed that FPRR was specifically cleaved by GGT which was abundant in HK-2 cells.

Figure 5-4. In vitro characterization of CyG. a) UV/vis absorption, b) NIRF spectra of CyG (25 μM) in the absence or presence of GGT (20 μg) in PBS (10 mM, pH = 7.4) at 37 $^{\circ}\text{C}$ for 2 h. c) HPLC traces of CyG in the absence or presence of GGT, and the traces of pure CyOH. d) Enzyme kinetics of GGT (20 μg) towards CyG (6, 15, 40, 65, 80, 160 μM). $K_M = 29.1 \mu\text{M}$, $k_{\text{cat}} = 11087 \text{ s}^{-1}$, $k_{\text{cat}}/K_M = 380 \mu\text{M}^{-1}\text{s}^{-1}$. e) Time-dependent NIRF changes of CyG (25 μM) in the presence of GGT (20 μg) in PBS (10 mM, pH = 7.4) at 37 $^{\circ}\text{C}$ for 2 h. f) Relative NIRF (710 nm) enhancement after incubation with indicated enzymes in PBS (10 mM, pH = 7.4) at 37 $^{\circ}\text{C}$ for 2 h. Alanine aminopeptidase (AAP), caspase-3 (casp3), N-acetyl- β -D-glucosaminidase (NAG), urokinase (uPA).

Figure 5-5. FPRR cytotoxicity test by MTS assay in HK-2 and NDF living cells. Data are presented as mean $\pm$ SD, $n = 5$ independent measurement.

After confirming its low hemolysis toxicity to red blood cells (**Figure 5-6**), FPRR was then tested in living mice for pharmacokinetics and biodistribution studies, and the blood elimination half-life ($t_{1/2}$) was determined to be 23 min from a two-compartment fitting of plasma clearance kinetics (**Figure 5-2h**). At 24 h after intravenous administration of FPRR (6.5 $\mu\text{mol/kg}$ bw), the mice were dissected, and all major organs were taken out for biodistribution studies. The organs were homogenized in phosphate-buffered saline (PBS) and centrifuged to remove any insoluble particles, the residual FPRR in organs were analyzed and quantified by HPLC studies. As shown in **Figure 5-2i**, FPRR showed limited tissue accumulation and high renal excretion. Major organs such as liver, lung and kidney contained approximately 3% injected dose (ID), while heart, spleen, skin and muscle contained approximately 1% ID. Meanwhile, mice urine was collected and the renal clearance efficiency of FPRR at 24 h was quantified by HPLC and calculated to be 78%, which is higher than gold nanoclusters (52% ID)⁸⁷ and silica Cornell dots (73% ID),¹⁸⁰ and similar to porphyrin-polyethylene glycol (PEG) (80% ID).¹⁹⁴ In contrast, systematic administration of CyG,

the small molecule without polymeric dextran moiety showed major accumulation in liver and minimal renal clearance efficiency (~5%) (**Figure 5-7**). Thus, these data suggested that FPRR had high renal clearance efficiency, making it an excellent candidate for real-time imaging of kidney dysfunction.

Figure 5-6. Hemolysis test of FPRR in fresh mice blood. Red blood cells (RBCs) were prepared from fresh mice blood after repeated wash with saline and centrifugation. RBCs suspension was then added into FPRR solution (1.88 - 60 μM, dissolved in 1 × PBS) for experimental groups, 1 × PBS and Triton X-100 (10 g/L) as negative and positive control groups, respectively. After incubation at 37°C for 2 h, the RBCs were centrifuged at 3000 rpm for 10 min, and the supernatant was transferred into 96-well plate for absorption measurement (A_{540}). The hemolysis ratio was calculated by the following equation: $\text{hemolysis (\%)} = (A_{\text{sample}} - A_{\text{negative control}}) / (A_{\text{positive control}} - A_{\text{negative control}})$. Data are presented as mean $\pm$ SD, $n = 3$ independent measurement.

Figure 5-7. a) Representative NIRF images of living mice after i.v. injection of CyG (6.5 $\mu\text{mol/kg}$ bw, 200 μL in saline) at post-injection time ($t = 30, 60$ min), ($\text{Ex/Em} = 675/720$ nm). b) Renal clearance efficiency as a function of time post-injection of CyG in living mice, two lines represent the measurements in two independent mice. c) Organs dissected from living mice after real-time imaging were imaged under IVIS fluorescence mode ($\text{Ex} = 675$ nm, $\text{Em} = 720$ nm).

FPRR was then evaluated for real-time imaging of AKI in a cisplatin mouse model (**Figure 5-8a**). The mice were challenged with cisplatin (an antineoplastic drug) at a reported nephrotoxic dosage (intraperitoneal injection, 20 mg/kg bw).¹⁹⁵ The control mice were treated with saline or protected with N-acetyl-L-cysteine (NAC, intravenous injection, 400 mg/kg bw) 0.5 h prior to cisplatin challenge, and real-time imaging was conducted at $t_{\text{dpt}} = 48$ h.¹⁹⁶ NAC was reported to be able to attenuate kidney damage by reducing oxidative stress and alleviating kidney inflammation in mouse model.¹⁹⁷ Subsequently, FPRR was intravenously administered at different drug post-treatment time ($t_{\text{dpt}} = 16, 24, 48$ h) at dosage of 6.5 $\mu\text{mol/kg}$ bw (**Figure 5-8b**). Longitudinal real-time NIRF and PA imaging were then carried out on in vivo imaging system (IVIS) and

multispectral optoacoustic tomography (MSOT) for 2 and 3 h, respectively. The signals were then quantified by placing the region of interest (ROI) at the two kidneys on the dorsal side or transverse sections. At $t_{\text{dpt}} = 16$ h, both NIRF and PA signals from kidneys showed no statistically significant difference from the control groups throughout the course of imaging. However, at $t_{\text{dpt}} = 24$ and 48 h, gradual NIRF and PA signals increase were observed in the first 60 min until plateau followed by minimum decrease in the rest of imaging course (**Figure 5-9,10**). This could be explained by the combinational effects of gradual enzyme cleavage and renal clearance of FPRR, which completed with each other to increase and decrease the kidney signal, respectively. At 60 min post-injection of FPRR, the NIRF signals from activated FPRR clearly delineated the shapes of two kidneys on the dorsal side (**Figure 5-8c**); while the PA signals from activated FPRR could be observed at the sites of renal parenchyma and pelvis on the two sides of aorta in the cross-sectional imaging (**Figure 5-8d**). In addition, the in vivo PA spectrum of the kidney resembled the in vitro solution spectrum (**Figure 5-8e**), confirming that the increased PA signals in the kidney region was due to GGT-activated FPRR. Quantification showed that the kidney NIRF (PA) signals at $t_{\text{dpt}} = 24$ and 48 h were 1.36 (1.97) and 1.54 (1.93)-fold higher as compared to control group (**Figure 5-8f**). For mice pre-treated with nephroprotective NAC, both NIRF and PA signals showed no statistically significant difference compared to the control group. Moreover, the signal-to-background ratio (SBR), defined as signals from kidney site relative to skin, was calculated and compared for NIRF and PA imaging (**Figure 5-8g**). For all the drug treated groups $t_{\text{dpt}} = 16, 24$ and 48 h, PA imaging showed an average of ~2.3-fold higher SBR as compared to NIRF imaging. As for the control groups, the SBR for PA imaging had no statistically significant difference from NIRF imaging.

Therefore, these data not only confirmed that FPRR-based real-time imaging detected AKI at 24 h post-cisplatin treatment, but also revealed that PA imaging had higher SBR relative to NIRF imaging due to its higher tissue penetration.

Figure 5-8. Real-time NIRF and PA imaging of cisplatin-induced AKI. a) Schematic illustration of using FPRR for bimodal imaging of AKI. b) Timeline for development of cisplatin-induced AKI and bimodal imaging. c) Representative NIRF images of living mice at 60 min after i.v. injection of FPRR in different treatment groups (Ex/Em = 675/720 nm). For NAC-protected mice, real-time imaging was conducted at 48 h post-cisplatin treatment. The white circles indicate the site of kidneys and bladder respectively in dorsal and ventral side. d) Representative PA images of mice transverse section at 120 min after i.v. injection of FPRR in different treatment groups (700 nm). The white circles indicate the site of kidneys. Right kidney (RK), left kidney (LK). e) In vivo PA spectra of kidney areas after intravenous administration of FPRR at 120 min.

f) Relative NIRF and PA signals enhancement at kidney site for different treatment groups as compared to control. g) Comparison of signal-to-background ratio between NIRF and PA imaging. Data in (f-g) are presented as the mean $\pm$ SD, $n = 3$ independent mice. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, n.s.: not significant.

Figure 5-9. The dynamic NIRF intensities of (a) kidneys and (b) bladder as a function of time post-injection of FPRR (6.5 μ mol/kg bw, 200 μ L in saline) in living mice after treatment of cisplatin for 16, 24 or 48 h, PBS or NAC prior to cisplatin administration. c) Relative NIRF signals enhancement at bladder site for different treatment groups as compared to control. Data are presented as mean $\pm$ SD. $n = 3$ independent mice. Two-tailed student's t-test. n.s: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5-10. a) Representative PA images of living mice with i.v injection of FPRR (6.5 $\mu\text{mol/kg}$ bw, 200 μL in saline) after treatment of cisplatin (20 mg/kg bw, i.p. injection) for 16, 24, 48 h. The control groups were treated with PBS or a nephroprotective antioxidant NAC (400 mg/kg bw, i.v. injection) 0.5 h prior to cisplatin administration. c) The dynamic PA intensities of kidneys as a function of time post-injection of FPRR in living mice after treatment of cisplatin for 16, 24 or 48 h, PBS or NAC prior to cisplatin administration. Data are presented as mean $\pm$ SD. n = 3 independent mice. Two-tailed student's t-test. n.s: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As a proof-of-concept demonstration for clinical translation, FPRR was tested for optical urinalysis. Mice urine was collected from both saline and drug-treated mice after systematic administration of FPRR (**Figure 5-11a**). At 24 h post-injection, FPRR showed a renal clearance efficiency of 78% (**Figure 5-11b**). The urine samples were then taken for UV/vis absorption and NIRF measurement (**Figure 5-11c-e**). Compared to the urine samples from control mice, urine from cisplatin-treated mice showed an elevated absorption at 690 nm along with 2.3, 2.7-fold NIRF enhancement at 710 nm respectively for $t_{\text{dpt}} = 24, 48$ h, which was attributed to the activation of FPRR to Dex-CD in kidney-injured mice. In comparison, the urine samples collected from untreated mice did not show obvious changes in the absorption or NIRF signals. Thus, optical urinalysis detected cisplatin-induced AKI at 24 h post-drug treatment, consistent with the results from in vivo imaging. After imaging experiments, the mice were dissected, and all major organs were collected for histological analysis (**Figure 5-11 f-g; Figure 5-12**). Histological hematoxylin & eosin staining showed normal tissue morphology, suggesting the minimal toxicity of FPRR. In addition, only at a late drug-treatment time point ($t_{\text{dpt}} = 72$ h) could kidney tissue damage such as renal cell debris and loss of brush border be observed. Thereafter, similar to real-time imaging, FPRR-based urinalysis noninvasively detected AKI at 24 h post-cisplatin treatment, which was 48 h earlier than reported detection time points for elevated serum creatinine and kidney tissue injury ($t_{\text{dpt}} = 72$ h).¹⁹⁵

Figure 5-11. a) Schematic illustration of online urinalysis. b) Renal clearance efficiency as a function of time post-injection of FPRR in living mice, three lines represent the measurements in three independent mice. c) UV/vis absorption and d) NIRF spectra of FPRR in urine samples excreted from both control and AKI mice after FPRR i.v. injection, and urine samples from untreated mice. e) Relative NIRF signals (710 nm) enhancement of urine samples excreted from drug treated mice as compared to control mice after FPRR i.v. injection. Data are presented as mean $\pm$ SD, $n = 3$ independent experiment. f) Representative hematoxylin&eosin staining of major organ tissues from healthy mice after i.v. injection of FPRR. Mice were dissected 24 h after injection and organs were taken out for sectioning. g) Representative hematoxylin&eosin staining of kidney tissues from different drug post-treatment

groups. Green arrows and blue triangle indicate renal cell debris and loss of brush border, respectively. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, n.s.: not significant.

Figure 5-12. a) Organs dissected from different experimental groups 2 h post FPRR-injection were imaged under IVIS fluorescence mode. Ex = 675 nm, Em = 720 nm. b) Quantifications of NIRF intensities of different major organs in (a).

5.4. Conclusions

In conclusion, a highly renal-clearable polymeric reporter (FPRR) that could simultaneously activate its NIRF and PA signals towards a AKI biomarker (GGT) was reported, and its application for real-time imaging of cisplatin-induced AKI in mouse model was demonstrated. FPRR not only exhibited high renal clearance efficiency (78% ID after 24 h injection) in living mice, but also specifically reacted with the biomarker in the kidney to turn on its NIRF and PA signals to report the onset of AKI. Proven as a sensitive optical AKI reporter, FPRR detected AKI detection at 24 h after cisplatin treatment through both real-time imaging and optical urinalysis, which was 48 h earlier than increase in serum creatinine and GFR decline. Additionally, as compared to NIRF imaging, PA imaging showed 2.3-fold higher SBR in kidney imaging and thus could potentially provide more sensitive and accurate disease information. Hence,

FPRR represents the first activatable PA probe that enables real-time molecular imaging of AKI. In addition, this study provides molecular guidelines for the design of polymeric optical agents with high renal clearance, which is of significance for clinical translation.

In Chapters 3 to 5, we have reported the applications of biomarker-activatable optical agents for real-time in vivo imaging and disease diagnosis of keloid, drug-induced liver injury, and acute kidney injury. In addition, in vitro diagnosis via non-invasive urinalysis plays an important role for preclinical and clinical disease diagnosis. In the next chapter, the synthesis and application of molecular urinary reporters (MURs) were demonstrated for multiplex optical urinalysis in early detection of drug-induced kidney injury (DIKI). MURs1-3 are devised to emit blue, orange and near-infrared (NIR) fluorescence signals upon activation by GGT, AAP, and NAG, respectively. Due to the minimal spectral overlap, MURs1-3 can form a cocktail sensor for multiplex optical urinalysis of DIKI.

Figure 5-13. ^1H spectrum of compound 1 in CDCl_3 . Figure 5-14. ^1H spectrum of compound 3 in CD_3OD .

Figure 5-15. ^1H spectrum of CyG in CD_3OD .

Figure 5-16. ^1H spectrum of compound dextran-alkyne in D_2O .

Chapter 6. Multiplex Optical Urinalysis for Early Detection of Drug-induced Kidney Injury

6.1. Introduction

Kidney is a vital organ in the body that plays important roles in regulating body fluid homeostasis and blood filtration as well as maintaining electrolyte balance. As it is the primary site for metabolism and excretion of drugs, imaging contrast agents and environmental hazards, it is vulnerable to the exposure of these toxicants.¹⁹⁸ In particular, drug-induced kidney injury (DIKI) is a frequent complication in intensive care unit (ICU) and has a high risk of morbidity and mortality.¹⁹⁹ Moreover, DIKI remains a significant contributor to acute kidney injury (AKI) and underlines 19-25% of all cases in critically ill patients.²⁰⁰ Therefore, development of sensitive detection methods for DIKI is of great importance for pharmaceutical companies to screen nephrotoxic drugs in drug development process, and for clinicians to closely monitor patient safety in clinical care.

Current clinical diagnosis often relies on measurement of serum creatinine (sCr) and blood urea nitrogen (BUN), which are factors reflecting changes in glomerular filtration rate (GFR).²⁰¹ However, sCr/BUN methods not only fail to sensitively detect kidney injury because substantial histological injuries could occur before measurable GFR change,²⁰² but also lack in accuracy because patients' age and muscle mass can cause fluctuations in the biomarker levels.²⁰³ In contrast, urinalysis using the biomarkers such as clusterin, Cystatin-C and β 2-microglobulin has recently shown to

be more sensitive and reliable than clinical diagnostic methods for detection of DIKI in animal studies.²⁰⁴ However, the detection methods for these urinary biomarkers are limited to commercial immunoassays and matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF-MS). Commercial immunoassays generally suffer from low sensitivity and tedious measurement procedures;²⁰⁵ MALDI-TOF-MS is a highly specific method in biomarker detection, but its application has been limited by number of reference MS spectra in the database.²⁰⁶

Activatable fluorescent probes that specifically emit signals in response to the biomarker of interest have become indispensable tools in fundamental biology,²⁰⁷⁻²⁰⁹ and medical applications.²¹⁰⁻²¹³ With a high signal-to-background ratio, fluorescent probes often have high sensitivity and strong ability for early detection of diseases such as cancer,^{69,214-216} skin diseases,^{65,217} acute liver injury^{68,218,219} and diabetes.²²⁰ Additionally, multiplex fluorescence imaging provides a unique approach to simultaneously study multiple interrelated biomarkers at the same time, potentially improving diagnostic accuracy.^{221,222} However, activatable fluorescent probes have been less exploited for detection of DIKI,²²³⁻²²⁵ not to mention multiplex fluorescence imaging.

In this study, three molecular urinary reporters (MURs) were developed for multiplex optical urinalysis in early detection of DIKI. Because elevated urinary excretion of proximal tubule brush border enzymes (γ -glutamyl transferase: GGT; alanine aminopeptidase: AAP) and lysosomal enzyme (N-acetyl-D-glucosamine: NAG) has been found at the early stage of both glomerulonephritis²²⁶ and acute tubular necrosis,²²⁷ they are chosen as the DIKI biomarkers for the design of MURs. Activatable

molecular fluorophores responsive towards GGT,²²⁸⁻²³⁰ AAP,²³¹ and NAG^{232,233} have been previously reported, the molecules are designed by caging the fluorophore optically-tunable atoms (typically oxygen or nitrogen) with an enzyme cleavable moiety. However, they were not applied for multiplex imaging. MURs1-3 are devised to emit blue, orange and near-infrared (NIR) fluorescence signals upon activation by GGT, AAP, and NAG, respectively. Due to the minimal spectral overlap, MURs1-3 can form a cocktail sensor for multiplex optical urinalysis of DIKI.

6.2. Materials and Methods

Materials. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Commercially available reagents were used without further purification unless otherwise noted. Fmoc-Ala-OH was purchased from Sangon Biotech. Alanine aminopeptidase (AAP), fibroblast activation protein- α (FAP- α), furin, matrix metalloproteinase-2 (MMP2), and dipeptidyl peptidase-4 (DPP4) were purchased from R&D Systems.

Instrumentation. UV/Vis spectra were measured on a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were performed on a Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). HPLC analyses were done on an Agilent 1260 system equipped with a G1311B pump, UV detector and an Agilent Zorbax SB-C18 RP (9.4 $\times$ 250 mm) column, with methanol (0.1% CF₃COOH) and water (0.1% CF₃COOH) as the eluent. Fluorescence images of living cells and immunofluorescent imaging of kidney slices were acquired on Laser Scanning Microscope LSM800 (Zeiss). Proton-nuclear magnetic resonance (¹H NMR) spectra

were recorded on a Bruker Advance II 300 MHz NMR. Electrospray ionization-mass spectrometry (ESI-MS) spectra were acquired on a Thermo Finnigan Polaris Q quadrupole ion trap mass spectrometer (ThermoFisher Corporation) equipped with a standard ESI source.

Synthesis of Compound 1. Fmoc-Ala-OH (310 mg, 1 mmol), p-aminobenzyl alcohol (250 mg, 2 mmol) and N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (300 mg, 2 mmol) were dissolved and mixed in anhydrous dichloromethane (DCM) and stirred overnight under N₂ atmosphere. The organic solvent was concentrated under reduced pressure. After HPLC purification, pure compound 1 could be obtained (330 mg, 0.8 mmol). ¹H NMR (300 MHz, CDCl₃) δ : 7.76 (d, J = 6 Hz, 2H), 7.57 (d, J = 6 Hz, 2H), 7.47 (d, J = 9 Hz, 2H), 7.39 (t, 2H), 7.30 (d, J = 9 Hz, 4H), 4.65 (s, 2H), 4.46 (d, J = 6 Hz, 2H), 4.36 (s, 1H), 4.21 (t, 1H), 1.46 (d, J = 6 Hz, 3H) (**Figure 6-11**). MS (ESI): m/z = 417.24 [M + H]⁺.

Synthesis of compound 2. Pure compound 1 (100 mg, 0.25 mmol) was dissolved in anhydrous tetrahydrofuran (THF) in ice bath under N₂ atmosphere, and PBr₃ (37 μ L, 0.38 mmol) in anhydrous THF was added via a syringe. The reaction mixture was stirred for 2 h before the solvent was concentrated under reduced pressure. The resulting mixture was diluted with ethyl acetate and washed with saturated sodium bicarbonate solution for three times to remove residual acid. The organic layer was dried with anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was then taken for next step synthesis without further purification.

Synthesis of compound 3. Resorufin (10 mg, 0.05 mmol) and grinded potassium carbonate (25 mg, 0.2 mmol) were vigorously stirred in anhydrous dimethylformamide (DMF) under N₂ atmosphere for 30 minutes, before compound 2 (95 mg, 0.2 mmol) dissolved in anhydrous DMF was added via a syringe. The mixture was continuously stirred for 5 h, and then pure mg, 0.2 mmol) dissolved in anhydrous DMF was added via a syringe. The mixture was continuously stirred for 5 h, and then pure compound 3 (29 mg, 95%) could be obtained after HPLC purification. ¹H NMR (300 MHz, DMSO-d₆) δ : 7.89 (d, J = 6 Hz, 2H), 7.63-7.79 (m, 6H), 7.53 (d, J = 12Hz, 1H), 7.42 (m, 3H), 7.32 (m, 2H), 7.18 (d, J = 3 Hz, 1H), 7.12 (m, 1H), 6.78 (m, 1H), 6.27 (d, J = 2.1 Hz, 1H), 5.22 (s, 2H), 4.24 (m, 4H), 1.30 (d, J = 7 Hz, 3H) (**Figure 6-12**). MS (ESI): m/z = 611.75 [M + H]⁺.

Synthesis of MUR2. Pure compound 3 (20 mg, 0.03 mmol) was stirred in 5% piperidine/DMF for 10 minutes at rt, before 10-times diluted acetic acid was added dropwise until pH was adjusted to 7. Pure MUR2 (10 mg, 90%) could be obtained after HPLC purification. ¹H NMR (300 MHz, CD₃CN) δ : 7.64 (d, J = 9 Hz, 1H), 7.53 (d, J = 6Hz, 2H), 7.40 (s, 1H), 7.38 (m, 2H), 6.97 (m, 1H), 6.90 (d, J = 3Hz, 1H), 6.67 (m, 1H), 6.15 (d, J = 3Hz, 1H), 5.12 (s, 2H), 4.05 (m, 1H), 1.49 (d, J = 6Hz, 3H) (**Figure 6-13**). MS (ESI): m/z = 389.79 [M + H]⁺ (**Figure 6-14**).

Synthesis of CyOH. Synthesis was carried out according to literature report.¹¹⁶ ¹H NMR (300 MHz, CDCl₃) δ : 8.18 (d, J = 15 Hz, 1H), 7.70 (m, 1H), 7.35 (s, 1H), 7.31 (s, 1H), 7.22 (s, 1H), 7.10 (t, 1H), 6.89 (t, 2H), 5.67 (d, J = 12 Hz, 1H), 2.65 (m, 3H), 1.91 (t, 2H), 1.69 (s, 6H), 1.25 (s, 4H), 0.86 (m, 3H). MS (ESI): m/z = 386.45 [M + H]⁺.

Synthesis of Compound 4. β -D-galactosamine pentaacetate (780 mg, 2 mmol) was dissolved and stirred in 6 mL anhydrous DCM in ice bath under N_2 atmosphere, and HBr (33 wt% in acetic acid, 4.7 mL, 2 mmol) was added via a syringe. The mixture was continuously stirred in ice bath for 6 h. The residue mixture was then washed with ice-cold saturated sodium bicarbonate solution for three times to remove residue acid. The organic layer was dried with anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was stored at $-20\text{ }^{\circ}\text{C}$ and directly taken for next step synthesis without further purification. MS (ESI): $m/z = 409.71 [M + H]^+$.

Synthesis of compound 5. Pure CyOH (20 mg, 0.054 mmol) and grinded potassium carbonate (28 mg, 0.2 mmol) was mixed and stirred in anhydrous DCM under N_2 atmosphere for 30 minutes before compound 4 (86 mg, 0.2 mmol) dissolved in anhydrous DCM was added via a syringe. The residue mixture was stirred overnight and concentrated under reduced pressure. The crude product was directly used for next step synthesis without further purification. MS (ESI): $m/z = 713.56 [M + H]^+$.

Synthesis of MUR3. Crude compound 5 (38 mg, 0.054 mmol) and MeONa (25 wt% in methanol [MeOH], 550 μL , 2 mmol) were dissolved and stirred in MeOH in ice bath. The reaction was continuously monitored by thin-layer chromatography, and 10-times diluted acetic acid was added dropwise until pH was adjusted to 7. The residue mixture was concentrated under reduced pressure and purified with column chromatography to yield pure MUR3 (20 mg, 64%). ^1H NMR (300 MHz, MeOD) δ : 8.78 (d, $J = 15\text{ Hz}$, 1H), 7.67 (d, $J = 8\text{ Hz}$, 1H), 7.47 (m, 3H), 7.33 (s, 1H), 7.13 (s, 1H), 7.00 (d, $J = 9\text{ Hz}$, 1H), 6.55 (d, $J = 15\text{ Hz}$, 1H), 5.21 (m, 1H), 3.96 (m, 4H), 3.73 (m, 1H), 3.59 (m, 2H), 3.42 (m, 2H),

2.75 (d, $J = 15$ Hz, 4H), 1.96 (m, 6H), 1.81 (s, 3H), 1.28 (s, 2H) (**Figure 6-15**). MS (ESI): $m/z = 587.30$ $[M + H]^+$ (**Figure 6-16**).

Enzyme kinetic studies. Various concentrations of MUR1 (10, 20, 40, 80, 160, 320, 640, 800 μ M) were incubated with GGT (5 μ g) at 37 °C for 5 min in a 100 μ L system of PBS buffer (1 $\times$, pH = 7.4) with 0.02% Tween@20. Similarly, various concentrations of MUR2 (5, 10, 20, 40, 80, 160, 240, 320 μ M) were incubated with AAP (0.1 μ g) at 37 °C for 5 min in a 100 μ L system of Tris buffer (50 mM, pH = 7.4). Lastly, various concentrations of MUR3 (10, 25, 50, 100, 150, 300, 600 μ M) were incubated with NAG (10 mU) at 37 °C for 5 min in a 100 μ L system of PBS buffer (1 $\times$, pH = 7.4). After incubation, the mixture was injected into HPLC (eluent: methanol/water) for quantification analyses. The initial reaction velocity (μ mol/s) was calculated, plotted against MURs1-3 concentration, and fitted to a Michaelis-Menten curve. The kinetic parameters were calculated using Michaelis-Menten equation: $V = V_{\max} * [S] / (K_M + [S])$, where V is initial velocity, and $[S]$ is substrate concentration.

Cell culture. HK-2 and NDF cells were cultured in Dulbecco's Modified Eagle Media (DMEM) medium supplemented with heat-inactivated fetal bovine serum (10%) and antibiotics penicillin/streptavidin (1%) in a humidified environment containing 5% CO₂ and 95% air at 37 °C. Cell media was replaced every two to three days.

Cell fluorescence imaging. For cell fluorescence imaging, HK-2 and NDF cells (10⁵ cells per well in 1 mL) were seeded in the dishes (dia. 35mm) and incubated overnight to reach 80% confluency, respectively. The cells were incubated with 30 μ M MURs cocktail (containing 10 μ M of each MUR) for 30 minutes. The medium was removed,

and the cells were washed with PBS for three times. Then the cells were fixed with 4% polyformaldehyde solution. Fluorescence images of fixed cells were acquired on a Laser Scanning Microscope LSM800 (Zeiss). The excitation and emission wavelength for cell imaging were 405/410-470nm for MUR1, 561/580-630nm for MUR2 and 640/655-710nm for MUR3. ImageJ software was utilized to remove signal background and quantify cellular fluorescence intensity.

Cytotoxicity assay. HK-2 and NDF cells were seeded in 96-well plates (10^4 cells per well) for 24 h, and then different concentrations of MUR cocktail (5, 10, 20, 30 and 40 μ M, containing equimolar amount of each MUR) were added to the cell culture medium. Cells were incubated with or without (control) MUR cocktail, followed by addition of MTS assay (Promega Cat. no. G3581, 100 mL, 0.1 mg/mL) for 4 h. The absorbance of MTS at 490 nm was measured by using a microplate reader. The cytotoxic effects (VR) of MUR cocktail were assessed using the following equation: $VR = A/A_0 \times 100\%$, where A and A_0 are the absorbance of the experimental group and control group, respectively. The assays were performed in five sets for each concentration.

Mice cisplatin-induced acute kidney injury model development. All animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Nanyang Technological University-Institutional Animal Care and Use Committee (NTU-IACUC) and approved by the Institutional Animal Care and Use Committee (IACUC) for Animal Experiment, Singapore. The choice of drug dosage and injection methods could be found in literature reports. Cisplatin (1 mg/mL in 0.9% NaCl solution) was injected once intraperitoneally into Ncr mice (18-20 g, drug dosage

20 mg/kg bw). Age-matched control Ncr mice were injected with an equal volume of isotonic saline. All mice were housed in a temperature controlled (22 °C) room with 12 h dark light cycles (0700 h on and 1900 h off). All mice were weighed twice daily. Mice were sacrificed at different drug post-treatment time points (t = 12, 24, 48 and 72 h). An overnight urine collection of spontaneously voided urine was made from each animal in metabolic cages on the night before sacrifice. Blood samples for serum creatinine (sCr) and Blood Urea Nitrogen (BUN) were obtained from central tail artery in non-anesthetized mice. Obtained blood samples were stored in ice and centrifuged at 3000 rpm for 10 minutes to separate serum.

Mice doxorubicin-induced chronic kidney injury model development. The choice of drug dosage and injection methods could be found in literature reports. Doxorubicin (1 mg/mL in 0.9% NaCl solution) was intravenously injected into BALB/c mice (18-20 g, 10 mg/kg bw). Age-matched control BALB/c mice were injected with an equal volume of isotonic saline. All mice were housed in a temperature controlled (22 °C) room with 12 h dark light cycles (0700 h on and 1900 h off). All mice were weighed twice daily. Mice were sacrificed at different drug post-treatment time points (t = 5, 8, 10, 14, 21, 28 days). An overnight urine collection of spontaneously voided urine was made from each animal in metabolic cages on the night before sacrifice. Blood samples for serum albumin and creatinine (Cr) were obtained from central tail artery in non-anesthetized mice. Obtained blood samples were stored in ice and centrifuged at 3000 rpm for 10 minutes to separate serum.

Statistics analysis. Statistical comparisons between the two groups were determined by Student's t-test (1-tailed, unpaired). For all tests, $p < 0.05$ was considered as

statistically significant. For analysis of diagnostic performance of MURs, ROC curves were drawn and the AUCs were calculated and compared according to Hanley & McNeil.²³⁴ The combination of MURs is calculated by a linear relationship of MURs1-3 derived from logistic regression: $MURs = -2.16 + 0.32 \times [MUR1] + 0.37 \times [MUR2] + 0.17 \times [MUR3]$, [MUR] indicates normalized fluorescence enhancement for the three MURs, respectively. All statistical calculations were performed using GraphPad Prism v.6 (GraphPad Software Inc., CA, USA).

6.3. Results and Discussions

The sensing mechanism of MURs1-3 are shown in Scheme 1. MUR1 is L-Glutamic acid γ -(7-amido-4-methylcoumarin), which is a specific GGT-activatable probe and emits blue fluorescence signals after enzymatic cleavage. MUR2 was derived from an orange-fluorescent dye, resorufin, by caging its optically tunable hydroxyl group with a self-immolative linker connected to AAP-cleavable alanine amino acid (Ala). Upon AAP cleavage at the terminal alanyl group, the linker is released by 1,6-immolation, leading to free resorufin unit with enhanced electron-donating ability from the oxygen atom. Similarly, MUR3 was synthesized from a NIR fluorescent hemicyanine dye (CyOH), with its hydroxyl group caged by a NAG-cleavable responsive group (N-acetyl- β -D-glucosaminide). In the presence of NAG, the terminal glycosidic bond is cleaved, forming free CyOH unit with enhanced electron-donating ability from the oxygen atom. Therefore, MURs2-3 are able to turn on their orange and NIR fluorescence signals in the presence of AAP and NAG, respectively.

MURs2-3 were synthesized through a synergistic approach (**Scheme 6-1**). The amino acid alanine was first coupled to p-aminobenzyl alcohol with subsequent 1° bromination to yield a highly active compound 2. Compound 2 was then conjugated to the hydroxyl group on resorufin followed by Fmoc deprotection on Alanyl amino group to afford MUR2. As for MUR3, its precursor CyOH was obtained by reacting IR775-Cl with resorcinol via a retro-Knoevenagel reaction. For its biomarker responsive compartment, a highly reactive compound 4 was afforded by brominating N-acetyl- β -D-glucosamine tetraacetate at its anomeric center, it was then reacted with the hydroxyl group of CyOH to afford compound 5, and compound 5 was deprotected of acetate groups to yield MUR3. The structures of these compounds were confirmed by NMR and mass spectra (Supporting Information).

To investigate detection sensitivity and specificity of MURs, their UV-vis absorption and fluorescence spectra were measured in the absence or presence of biomarkers (**Figure 6-1** and **Table 6-1**). Note that all MURs were barely fluorescent at their intrinsic states. After incubation of MUR1 with GGT, its absorption maximum shifted from 325 to 345 nm, and fluorescence signals at 440 nm enhanced 67-times (**Figure 6-1a**). Similarly, upon treatment of MUR2 with AAP, its absorption maximum shifted from 480 to 570 nm, which is the characteristic peak for released resorufin (**Figure 6-1b**). At this time point, MUR2 showed 60-fold fluorescence enhancement. Moreover, when MUR3 was incubated with NAG, its absorption maximum shifted from 600 to 690 nm, due to enzymatic cleavage at the ester bond and generation of free CyOH unit (**Figure 6-1c**). Meanwhile, MUR3 showed 33-fold fluorescence enhancement. High-performance liquid chromatography (HPLC) analysis confirmed that in the presence of

their respective biomarkers, MURs1-3 were totally converted into the free fluorescent dyes AMC (quantum yield, $\Phi_f = 0.70$), resorufin ($\Phi_f = 0.39$), and CyOH ($\Phi_f = 0.21$), respectively (**Figure 6-1d-f**). All MURs showed negligible fluorescence changes in the presence of interfering enzymes (**Figure 6-1g-i**). Besides, the catalytic efficiencies of GGT, AAP and NAG towards MURs1-3 were respectively calculated to be 31.8, 0.24 and $0.0198 \mu\text{M}^{-1}\text{s}^{-1}$, and linear relationships existed between fluorescence intensity and the concentration of biomarkers (**Figure 6-2** and **Table 6-2**).

Scheme 6-1. Synthetic route for MUR2 and MUR3. Reagents and conditions: (i) *p*-aminobenzyl alcohol, *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, rt, 12 h; (ii) PBr_3 , THF, 0°C , 2h; (iii) K_2CO_3 , DMF, rt, 5h; (iv) 5% piperidine/DMF, rt, 10 min; (v) resorcinol, K_2CO_3 , ACN, 55°C ; (vi) HBr, DCM, 0°C , 6h; (vii) K_2CO_3 , DCM, 12h; (viii) MeONa/MeOH , ice, 30 min.

Figure 6-1. Evaluation of MURs sensing capabilities. (a-c) UV-vis absorption and fluorescence spectra of MURs1-3 (20 μ M) in the absence or presence of their respective biomarkers (0.8 μ g/mL GGT, 0.8 μ g/mL AAP and 60 mU/mL NAG) in PBS (10mM, pH = 7.4 with 0.02% Tween@20), Tris (50 mM, pH = 7.4) and PBS (10mM, pH = 7.4), respectively. (d-f) HPLC traces of MURs1-3 in the absence or presence of their respective biomarkers, and traces of pure AMC (7-amino-4-methylcoumarin), resorufin and CyOH. (g-i) Fluorescence signal changes of MUR1 (440 nm), MUR2 (590 nm) and MUR3 (710 nm) relative to the blank in the presence of different enzymes. Error bars are standard deviation from three separate measurements. Fibroblast activation protein-alpha (FAP α), matrix metalloproteinase-2 (MMP2), dipeptidyl peptidase-4 (DPP4).

Figure 6-2. Enzyme kinetics and calibration curve for MURs. (a) Enzyme kinetics of GGT towards MUR1, [MUR1] = 10 to 800 μM ; (b) Enzyme kinetics of AAP towards MUR2. [MUR2] = 5 to 320 μM ; (c) Enzyme kinetics of NAG towards MUR3, [MUR3] = 10 to 600 μM ; (d) Fluorescence intensity of MUR1 changes towards different GGT concentrations, [GGT] = 0 to 4 $\mu\text{g/mL}$; (e) Fluorescence intensity of MUR2 changes towards different AAP concentrations, [AAP] = 0 to 0.2 $\mu\text{g/mL}$; (f) Fluorescence

intensity of MUR3 changes towards different NAG concentrations, [NAG] = 0 to 40 mU/mL.

Table 6-1. Photophysical properties of MURs1-3 and their activated fluorescent forms.

	λ_{abs} (nm) ^[a]	λ_{em} (nm) ^[a]	Stokes shift (nm)	Φ_f ^[b]
MUR1	325	440	115	0.03
MUR1 + GGT	345	440	95	0.70
MUR2	480	590	110	0.03
MUR2 + AAP	570	590	20	0.39
MUR3	600	710	110	0.01
MUR3 + NAG	690	710	20	0.21

[a] λ_{abs} and λ_{em} : wavelength of maximum absorbance and emission, respectively. [b]

Φ_f is the relative fluorescence quantum yield estimated by using Dansyl Chloride ($\Phi_f = 0.70$ in H₂O) for MUR1 and MUR1 + GGT, rhodamine B ($\Phi_f = 0.70$ in ethanol) for MUR2 and MUR2 + AAP and indocyanine green (ICG, $\Phi_f = 0.13$ in H₂O) for MUR3 and MUR3 + NAG as a fluorescence standard.

Table 6-2. Enzyme kinetics parameters for MURs1-3.

	K_m (μM) ^[a]	k_{cat} (s^{-1}) ^[b]	k_{cat}/K_m ($\mu\text{M}^{-1}\text{s}^{-1}$)
MUR1	58.6	1864	31.80
MUR2	76	18.70	0.24
MUR3	126	2.49	0.0198

[a] Michealis constant obtained from the Michaelis-Menten equation $V = V_{\text{max}}[S]/(K_m + [S])$. [b]: $k_{\text{cat}} = V_{\text{max}}/[E]$.

To test the capability of MURs for multiplex imaging, MUR1-3 were mixed at an optimized equimolar ratio (MURs cocktail) and the fluorescence spectra was measured in the absence or presence of all three biomarkers by selective excitation at their respective absorption wavelengths (380, 550, and 670 nm). Upon excitation at 380 nm, only fluorescence signal of MUR1 at 440nm was detected, but signals of

MURs2-3 were negligible. This was attributed to the absence of fluorescence resonance energy transfer (FRET) among MURs, because individual MUR molecules were free and away from each other in solutions and their spectra had minimal overlap. Similar spectral behaviours were upon excitation at 550 or 670 nm, only showing the signal of MUR2 or MUR3. Thus, the MURs cocktail could realize simultaneous detection of these biomarkers with minimal signal interference (**Figure 6-3a**).

The MURs cocktail was then used for multiplex imaging of endogenous biomarkers in kidney proximal tubule epithelial cells (HK-2) after confirming their low cytotoxicity in living cells (**Figure 6-4**). Compared to normal dermal fibroblasts (NDF) as control, the fluorescence intensities of MURs1-3 in HK-2 were ~20-times higher, because of the high expression levels of these enzymes in HK-2 cells (**Figure 6-3 b-c**). These results confirmed the feasibility of MURs cocktail for multiplex fluorescence imaging of multiple biomarkers.

Figure 6-3. Multiplex fluorescence imaging in solution and renal cells. (a) Fluorescence spectra of MURs cocktail (containing 15 μ M of each MUR) in the absence or presence of all three biomarkers—GGT, AAP and NAG in PBS (1 $\times$, pH = 7.4) containing 5% DMSO. Arrows indicate respective excitation wavelength for MURs1-3. (b) Fluorescence intensity enhancement of single HK-2 relative to NDF cells in Figure 2c ($n = 3$, mean $\pm$ s.d.). (c) Multiplex fluorescence images of human primary dermal fibroblasts (NDF) and kidney proximal tubule epithelial cells (HK-2) after incubation with MURs cocktail (containing 10 μ M of each MUR).

Figure 6-4. Cytotoxicity studies of MURs cocktail (containing equimolar concentration of each MUR) by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) in NDF and HK-2 living cells. Error bars represent the standard deviation of 5 trials. The concentration of MURs cocktail is the sum of MURs1-3 concentrations.

Figure 6-5. Plasma stability of MURs 1-3 (20 μM) incubated in mice plasma up to 2 hours.

After confirming their plasma stability (**Figure 6-5**), MURs were then used to detect urinary biomarkers in murine models of DIKI with two nephrotoxicants – cisplatin and doxorubicin. Cisplatin is an antineoplastic drug with side effects of acute kidney injury (AKI), which is characterized by sequential physiological events: acute tubular necrosis, rapid GFR decline and kidney dysfunction.²³⁵ Differently, doxorubicin-induced kidney injury is a mouse model that mimics human chronic kidney disease (CKD) caused by

focal segmental glomerulosclerosis.²³⁶ Specifically, healthy mice received one-time drug treatment of cisplatin (20 mg/kg bw) or doxorubicin (10 mg/kg bw) (**Figure 6-6a**). At different post-treatment timepoints, urine and blood samples were collected, and mice were sacrificed to collect kidneys for histological analysis. The urine samples were incubated with MURs cocktail before multiplex fluorescence analysis, and serum biomarkers were tested using commercial assays. In the context of CKD, proteinuria was also tested as a clinical urinary biomarker reflecting glomerulosclerosis.²³⁷

In the cisplatin-induced AKI model, the first statistically significant fluorescence enhancement of MURs1-3 was observed at 24 (1.52-fold), 48 (4.05-fold) and 72 h (3.02-fold), respectively (**Figure 6-6b** and **Figure 6-7**). In contrast, the statistically significant increase of serum sCr and BUN was observed at a later timepoint, 72 h (2.81 & 2.15-fold) after cisplatin treatment, when histological injuries like loss of brush border and renal cell debris could be readily observed (**Figure 6-8**). In the doxorubicin-induced CKD model, the first statistically significant fluorescence enhancement of MURs1-3 was observed at 5 (1.74-fold), 5 (2.06-fold) and 8 days (1.37-fold). Proteinuria and serum creatinine had statistically significant increase at later timepoints, 10 (2.17-fold) and 21 days (2.45-fold). Histological injuries were observed from 14 days onwards (**Figure 6-9**). Meanwhile, significant mice weight loss for cisplatin and doxorubicin-induced kidney injury models were observed at 48 h and 8 days, respectively (**Figure 6-10**). Thereafter, MURs1-3 detected cisplatin and doxorubicin-induced kidney injury at 24 h and 5 days post-treatment, which are respectively 48 h and 5 days earlier than traditional diagnostic methods including sCr, BUN and proteinuria. The differences in statistically significant timepoints among

MURs1-3 could be probably attributed to the biomarkers' individual regulation pathways in renal cells after drug challenge.

In order to quantitatively study the diagnostic performance (sensitivity and specificity), receiver operating characteristic (ROC) exclusion analysis was conducted by comparing the diagnosis results of MURs between two groups – healthy mice without histology injuries and drug-treated mice with obvious histology injuries. Besides, the area under curve (AUC) has statistical meaning of diagnostic accuracy and enables comparison between MURs and traditional diagnostic methods. MURs1-3 showed significantly higher AUC (≥ 0.85) compared with the tested traditional diagnostic approaches (AUC ~ 0.75). However, the performance differences among MURs1-3 were not statistically significant. Additionally, combination of MURs1-3 had the highest AUC (0.94), proving the better diagnostic performance than the individual MURs and the tested traditional clinical diagnostic measures. Thus, MURs were competent for early and accurate diagnosis of DIKI in both acute and chronic injuries.

Figure 6-6. Multiplex urinalysis for detection of DIKI. (a) Timeline for development of cisplatin and doxorubicin-induced kidney injury. Mice were treated with cisplatin (intraperitoneal injection, 20 mg/kg bw) or doxorubicin (intravenous injection, 10 mg/kg bw), and urine samples that were collected from drug-treated mice at indicated post-treatment timepoints were then incubated with MURs cocktail (containing 10 μ M of each MUR) for 1 h at 37 $^{\circ}$ C in PBS (1 $\times$, pH = 7.4) containing 5% DMSO. Commercial assays were used to measure Cr, BUN in serum and proteinuria in urine. (b-c) Fluorescence enhancement of MURs1-3 after incubation with urine samples

collected from drug-treated living mice, and fold changes in urinary and serum biomarkers at different post-treatment times. *Statistically significant difference in fluorescence enhancement and biomarkers fold change between pre-treatment and indicated statistically significant timepoints post-treatment of drugs. (n=5, mean $\pm$ s.d.)

(d) ROC curves for the exclusion analysis (nephrotoxicant-treated animals with observed renal injuries versus animals not-treated with nephrotoxicant and without renal injuries) with the two nephrotoxicant studies. MURs is linear combination of MURs1-3 derived from logistic regression. (kidney injury [n = 28] vs control [n = 13])

n.s.: not significant; *p<0.05; **p<0.01; ***p<0.001.

Figure 6-7. Fluorescence spectra of MURs (10 μ M) after incubation with urine samples from both healthy and kidney-injured mice for 2 hours.

Figure 6-8. Representative haematoxylin&eosin (H&E) staining and fluorescence images of kidney slices from healthy mice as control group and $t = 12, 24, 48$ and 72 h after cisplatin treatment (20 mg/kg bw), respectively. The blue and green signals are from DAPI and caspase-3 antibody staining. Histological changes were observed at post-treatment time $t = 72$ h, where loss of brush border (green arrowhead) and renal cell debris (blue arrow) could be observed.

Figure 6-9. Representative haematoxylin&eosin (H&E) staining and fluorescence images of kidney slices from healthy mice as control group and $t = 5, 8, 10, 14, 21$ and 28 days after doxorubicin treatment.

28 days after Doxorubicin treatment (10 mg/kg bw), respectively. The blue and green signals are from DAPI and caspase-3 antibody staining. Histological changes were observed from post-treatment time $t = 14d$, where mesangium expansion (orange arrow head), hyaline casts (black arrow), glomerular hypertrophy (blue arrowhead) and renal cell debris (green arrowhead) could be observed.

Figure 6-10. Mice body weight changes with drug post-treatment time for two nephrotoxicant models.

Figure 6-11. ¹H NMR spectra of compound 1 in CDCl₃.

Figure 6-12. ¹H NMR spectra of compound 3 in DMSO-d₆.

Figure 6-13. ¹H NMR spectra of compound MUR2 in CD₃CN.

Figure 6-14. ESI-MS spectra of compound MUR2.

Figure 6-15. ¹H NMR spectra of compound MUR3 in CD₃OD.

Figure 6-16. ESI-MS spectra of compound MUR3.

6.4. Conclusions

In conclusion, molecular urinary reporters (MURs) were developed and their potential as the cocktail sensor in early detection of DIKI in mouse models was demonstrated. MURs1-3 sensitively and specifically turned on their fluorescence signals in the presence of their respective urinary biomarkers. Because MURs has minimum spectral overlap, they served as a cocktail for multiplex detection of three urinary biomarkers in both living renal cells and the mouse urine samples. In particularly, MURs detected cisplatin-induced AKI and doxorubicin-induced CKD at 24 h and 5 days after drug challenge, which were respectively 48 h and 5 days earlier than tested clinical diagnostic methods. Furthermore, an ROC exclusion analysis highlighted that MURs had better diagnostic accuracy than traditional diagnostic methods. However, in this study, MURs are only tested in drug-induced kidney injury models. To further validate the specificity of MURs, future studies should be conducted to include urine samples from patients with some common diseases like diabetes, cardiovascular disease, and other urinary disease like bladder cancer. Thus, this study exemplified a pioneering multiplex molecular detection method with a great potential for real-time monitoring of kidney function in preclinical drug screening and clinical diagnosis.

In Chapter 7, a comprehensive summary of Chapters 3 to 6 was provided. In addition, some unmet goals and future research directions were discussed.

Chapter 7. Conclusions and Future Works

7.1. Conclusions

In this thesis, the application of organic fluorophores in disease diagnosis was explored via *in vivo* real-time imaging or *in vitro* urinalysis. By fine-tuning their optical features and pharmacokinetic properties, organic fluorophores were successfully applied for diagnosis of malignant skin lesions, drug-induced liver injury, acute and chronic kidney diseases, and psoriasis.

Firstly, a biomarker-activatable near-infrared fluorophore (CyTF) that specifically emit signals at 720 nm in the presence of reactive oxygen and nitrogen species was developed. The detection sensitivity and specificity of CyTF was validated both *in vitro* and in living cells. As compared to normal dermal fibroblasts, CyTF showed 2.2-fold higher fluorescence intensity in keloid fibroblasts. Besides, CyTF was applied to detect keloid in mice model, and sensitive detection with 3-fold fluorescence enhancement was achieved at 3.5 h after systematic administration.

Secondly, a chemo-fluoro-luminescent probe (CFR) was developed to detection $O_2^{\bullet-}$ and caspase-3 with activation of chemiluminescent and fluorescent signals, respectively. The molecular design of CFR serves as a proof-of-concept platform for multiplex imaging of multiple biomarkers for disease diagnosis and disease progression monitoring.

Thirdly, a fluoro-photoacoustic renal reporter (FPRR) was developed to detect GGT in drug-induced acute kidney injury. FPRR could simultaneously activate fluorescent and

photoacoustic signals in injured kidney. As compared to fluorescent probes, FPRR offers higher signal-to-noise ratio deeper tissue penetration, demonstrating high potential for clinical translation.

Finally, the application of multiplex urinary reporters (MURs) for disease diagnosis via in vitro urinalysis in addition to real-time imaging was demonstrated. In diagnosis of drug-induced kidney injuries, MURs demonstrate high diagnosis accuracy with AUC = 0.94 in ROC analysis, which is higher than clinical diagnostic approaches (serum creatinine, proteinuria).

7.2. Future Works

The successful application of biomarker-activatable optical agents was demonstrated for disease diagnosis via real-time imaging and noninvasive urinalysis. Although diagnosis plays a vital role, disease treatment and monitoring of recurrence or progression is also important. Furthermore, disease diagnosis usually requires professional personnel and instrumentations at the clinics and laboratories. Point-of-care testing should be developed to provide easily accessible healthcare to all patients, for self-monitoring and prevention of disease recurrence. Therefore, future works can be conducted in the following direction:

(1) Development of sensitive methods for disease detection at an incipient stage will not only facilitates drug screening in preclinical studies, but also allows for early clinical intervention and better therapeutic efficacy. In our work, we have demonstrated the application of biomarker-activatable optical probes for detection of skin diseases, organ failures in mice models. However, such probes fail to identify

injury to primary sites, for example, glomerulus or proximal tubule in the kidney. Identification of specific site of injury could not only facilitate us to understand drug toxicity mechanisms, but also help clinicians with more effective therapeutic interventions. Therefore, biomarker-activatable optical probes with active-targeting groups could be developed as different regions of the nephron are characterized by different transporters.

(2) Taking advantages of their high detection sensitivity and specificity, biomarker-activatable optical agents could be applied to intraoperative fluorescence-guided surgery. In the clinic, fluorescence-guided surgery has proven an effective tool for dissecting primary and metastatic tumors, resulting in improved tumor identification, more complete tumor resection and reduced cancer recurrence, compared to visual inspection.⁴⁵ Currently, FDA-approved optical agents are restricted to “always-on” and disease-targeting optical agents. However, such optical agents usually lack the sensitivity to detect carcinoma-in-situ (Stage 0 carcinoma). For example, pancreatic intraepithelial neoplasia (PanIN) is a histological well-defined precursor to pancreatic intraepithelial adenocarcinoma. The disease diagnosis is challenging by imaging and relies heavily on biopsy, which is invasive and provides only regional information. Therefore, developing biomarker-activatable probe for sensitive PanIN detection is in urgent need.

Besides, cancer recurrence refers to the return of cancer after completion of treatment. Based on the site of recurrence, cancer recurrence could be classified into local, regional, distant recurrence; the uncertainty of both time and site of recurrence have complicated the diagnosis process. Moreover, due to the lack of sensitive

biomarkers, cancer diagnosis relies mostly on anatomical imaging, microscopic studies of tissue biopsy, and so on. Therefore, it is important to develop activatable optical probe with high sensitivity to detect tumor nodules of ultrasmall size.

(3) Point-of-care testing (POCT), defined as “any investigation carried out in a clinical setting or the patient’s home for which the result is available without reference to a laboratory and perhaps rapidly enough to affect patient management”, provides a convenient approach for patients to do self-check at home or community. For example, portable blood analysers that proficiently measures level of glucose, cardiac troponin T (cTnT) and cardiac troponin I (cTnI) allow patients to self-monitor diabetes and cardiovascular diseases. However, such POCT devices has been limited and has not been applied for kidney disease.

Singapore ranks 1st in the world for diabetes-induced kidney failure and 4th in the world for prevalence of kidney failure. Every day, 5 new kidney failure patients are diagnosed; every 5 hours, 1 new dialysis patient is diagnosed. Therefore, developing POCT for sensitive detection of kidney failure remains an unmet need. Taking advantages of the rapid body excretion, high biocompatibility and sensitive disease detection, renal-clearable optical agents provide a platform for developing POCT to detect kidney failure. The POCT is conducted in three simple steps: (i) administration of renal-clearable optical agents; (ii) urine collection; (iii) signal read-out with handphone-based portable fluorometer.

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