Near infrared light-mediated upconversion nanocrystals for precisely theranostic applications

Ai, Xiangzhao

2018

Ai, X. (2018). Near infrared light-mediated upconversion nanocrystals for precisely
theranostic applications. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/74379>

<https://doi.org/10.32657/10356/74379>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**NEAR INFRARED LIGHT-MEDIATED
UPCONVERSION NANOCRYSTALS FOR PRECISELY
THERANOSTIC APPLICATIONS**

**AI XIANGZHAO
SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES**

2017

**NEAR INFRARED LIGHT-MEDIATED
UPCONVERSION NANOCRYSTALS FOR PRECISELY
THERANOSTIC APPLICATIONS**

AI XIANGZHAO

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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

2017

ACKNOWLEDGEMENT

Firstly, I am especially grateful to my supervisor, Assoc. Prof. Xing Bengang, for his talented guidance, insightful instruction, endless support, and great encouragement in the past four years. His conscientious attitude and passionate enthusiasm for science have inspired me a lot, which will be of great significance for my future research careers.

In addition, I also would like to appreciate Prof. Chen Xiaoyuan, Prof. Liu Gang, Prof. Olivo Malini, Prof. Gao Mingyuan, Prof. Zuo Zhenghong, Prof. Yeow Edwin K.L., Prof. Liu Xiaogang, Prof. Chen Huabing, Assoc. Prof. Yang Yanmei, Assoc. Prof. Wang Yong, Dr. Wang Xiaoyong, Dr. Wang Yu, Dr. Attia Amalina Binte Ebrahim, Dr. Tang Yanxia, Dr. Ho Chris Jun Hui, Mr. Zhang Yang, Mr. Zhou Yixi, for their generous collaboration and invaluable help on my research.

Moreover, I want to thank all the members in our group, including Dr. Li Jinming, Dr. Min Yuanzeng, Dr. Liu Fang, Dr. Shao Qing, Dr. Wu Xiangyang, Dr. Ariyasu Shinya, Dr. Zhang Zhijun, Dr. Mu Jing, Mr. Aw Junxin, Ms. Lyu Linna, Mr. Hu Ming, Mr. Cheong Haolun, Mr. Wang Zhimin, Ms. Chan Hui Ling, for their helpful discussion and kindly advice in my research. Furthermore, I would also like to express my sincere appreciation to my parents and Ms. Feng Tao for their continued understanding, support, and encouragement during my PhD study.

Finally, I would like to appreciate Nanyang Technological University for the sufficient financial support and academic opportunities during my study and research in Singapore.

TABLE OF CONTENTS

Acknowledgements	I
Table of Contents	II
Abstract	V

Chapter 1 Introduction

1.1 Light-Mediated Theranostics.....	1
1.2 Upconversion Nanocrystals (UCNs).....	3
1.2.1 Properties of UCNs.....	3
1.2.2 Preparation of UCNs.....	6
1.2.3 Luminescence Enhancement of UCNs	7
1.2.4 Surface Modification of UCNs.....	9
1.2.5 NIR Light-Controlled Theranostic Applications of UCNs.....	10
1.2.5.1 Drug and siRNA Delivery.....	10
1.2.5.2 Photodynamic Therapy (PDT).....	15
1.2.5.3 Photothermal Therapy (PTT).....	20
1.3 Photoacoustic (PA) Techniques with Deep-Tissue Penetration.....	22
1.4 Research Objectives.....	25
1.5 References.....	26

Chapter 2 Enzyme-Responsive Upconversion Nanoplatfrom for Enhanced Platinum(IV) Prodrug Activation and Real-Time Evaluation of Therapeutic Efficiency

2.1 Introduction.....	32
2.2 Materials and Methods.....	34
2.3 Results and Discussion.....	42

2.3.1 Preparation and Characterization of UCN-1 and UCN-2.....	42
2.3.2 <i>In Vitro</i> Enzyme Triggered Cross-linking between UCN-1 and UCN-2...	43
2.3.3 Enhanced Cytotoxicity and Real-Time Evaluation of Tumor Therapeutic Efficiency Based on UCNs Nanoplatfrom.....	47
2.4 Conclusion.....	51
2.5 References.....	51

Chapter 3 Intracellular Covalent Cross-linking of Upconversion Nanostructures for Tumor Localization and Theranostics

3.1 Introduction.....	54
3.2 Materials and Methods.....	56
3.3 Results and Discussion.....	68
3.3.1 Enzyme Triggered Covalent Cross-Linking of UCNs <i>In Vitro</i>	68
3.3.2 Enzyme Triggered Cross-Linking of UCN in Living Cells.....	75
3.4 Conclusion.....	80
3.5 References.....	80

Chapter 4 *In Vivo* Covalent Cross-linking of Photon-converted Rare-earth Nanostructures for Tumor Localization and Theranostics

4.1 Introduction.....	83
4.2 Materials and Methods.....	85
4.3 Results and Discussion.....	91
4.3.1 <i>In Vivo</i> Cross-Linking of CRUN in Tumor-Bearing Mice.....	91
4.3.2 <i>In Vivo</i> PDT Treatment with Affinity Ligand Modified CRUN.....	93
4.4 Conclusion.....	99
4.5 References.....	99

Chapter 5 Remote Regulation of Membrane Channel Activity by Site-Specific Localization of Lanthanide-Doped Upconversion Nanocrystals

5.1 Introduction.....	102
-----------------------	-----

5.2 Materials and Methods.....	104
5.3 Results and Discussion.....	113
5.3.1 Covalent Localization of DBCO-UCNs on Cell Membrane.....	113
5.3.2 Precise Regulation of Membrane Channel Activities <i>In Vitro</i>	119
5.3.3 Remote Regulation of Membrane Channel Activities <i>In Vivo</i>	123
5.4 Conclusion.....	127
5.5 References.....	127
 Chapter 6 Conclusion.....	 130
 List of Awards and Publications.....	 132

ABSTRACT

In recent decades, precision theranostics are greatly demanded to efficiently treat various human diseases in clinics. However, it's still challenging to accumulate the specific therapeutic agents in the targeted pathological regions and simultaneously avoid the harmful side effects to healthy tissues in human body. As a promising and powerful tool in living system, the light-mediated therapeutic strategy can precisely activate the imaging probes and reagents remotely at targeted areas based on its non-invasive properties and greatly spatiotemporal resolution *in vivo*. Despite the initial success, the established approaches for light-mediated treatment mainly focus on short-wavelength light, which is limited by several unavoidable disadvantages including serious phototoxicity, undesired absorption/scattering and low penetration depth in tissues. Thus, a long-wavelength near-infrared (NIR) light-triggered strategy at "optical window" (700-1000 nm) is highly demanded in the last decades which displays negligible photo-damage, excellent penetration depth and minimized auto-fluorescence for precisely phototherapeutic applications in clinics. As an amazing candidate in nanomedicine, NIR light-mediated lanthanide-doped upconversion nanocrystals (UCNs) have attracted considerable attention in the basis of its impressive optical properties in the NIR windows to convert NIR light excitation into UV or visible light emission through non-linear multiphoton processes. Therefore, UCNs have been considered as effective theranostic agents in living studies benefiting from their promising characteristics including excellent tissue penetration depth, tunable multicolor emission, long lifetime, high photostability, and excellent biocompatibility. In this dissertation, we fabricated some smart UCNs-based nanoplateforms to further advance their theranostic applications *in vitro* and *in vivo*, as shown in following parts.

Firstly, tumor-specific cathepsin protease-triggered method was developed to

cross-link dual peptide-functionalized-UCNs in tumor cells. Upon the formation of covalent bonds between 2-cyanobenzothiazole (CBT) and exposed cysteine residual between different UCNs, the photoactivation of Pt(IV) prodrug was enhanced and the increased reactive oxygen species (ROS) generation in the process of cell death was detected by a sensitive fluorescent dye in real time. Therefore, this UCNs nanoplatform could lead to increased antitumor efficacy and was capable of evaluating therapeutic response in an early stage.

Secondly, we presented another novel microenvironment-sensitive strategy for localization of peptide-premodified UCNs within tumor areas both *in vitro* and *in vivo*. Upon tumor-specific cathepsin protease reactions, the cleavage of peptides induces covalent cross-linking between the exposed cysteine and 2-cyanobenzothiazole on neighboring particles, thus triggering the accumulation of UCNs into tumor site. Such enzyme-triggered cross-linking of UCNs leads to enhanced upconversion emission upon 808 nm laser irradiation, and in turn amplifies the singlet oxygen generation from the photosensitizers attached on UCNs. More importantly, this design enables remarkable tumor inhibition through either intratumoral UCNs injection or intravenous injection of nanoparticles modified with the targeting ligands. The presented strategy may provide a multimodality solution for effective molecular sensing and site-specific tumor treatment.

Thirdly, we introduced a novel strategy to achieve site-specific covalent linkage of UCNs on the cell membrane through metabolic glycan biosynthesis and copper-free click cyclization. Upon NIR light irradiation (808 nm), the strong blue emission (~ 480 nm) could effectively activate a photosensitive ion channel, channelrhodopsins-2, and remotely manipulate the cation influx in living cells and zebrafish. This unique strategy could provide valuable insights about the remote and precise regulation of membrane-associated activities in living conditions.

In summary, these proposed NIR light-mediated approaches in this dissertation provided a prospective future for the precision theranostics in nanomedicine based on

the promising physical properties of UCNs. We believe the present works will lead to inspiring innovations in this research field and will be developed continuously to benefit human health in the future.

Chapter 1

Introduction

1.1 Light-mediated theranostics

In recent decades, precision medicine has received considerable attention in clinics due to its significant roles in the effective treatment of various human diseases.^{1,2} While some advances in the precision medicine have been made, issues in the low accumulation of therapeutic molecules and contrast agents in the targeted disease areas, and meanwhile, harmful effects to healthy tissues still need to be addressed.³ Thereby, the rational design of “smart” platforms with optimal performance in theranostics while minimizing systemic side effects will be of significance for clinical applications. So far, numerous stimuli-responsive platforms have been considered as ideal therapeutic approaches for effective therapy and relevant biomedical imaging.⁴ Briefly, these strategies include endogenous stimuli (such as pH, enzyme, redox reactions, *etc*) and exogenous stimuli (such as light, ionizing irradiation, magnetic field, *etc*).⁵ Among various methods, the light-mediated therapy has shown great superiority in achieving on-demand therapeutics and diagnostics in targeted areas *in vitro* and *in vivo*, owing to their non-invasiveness and spatiotemporal precision upon a specific wavelength light irradiation.⁶ Especially, with the latest innovations in chemistry and nanotechnology, numerous promising nanomaterials, such as gold nanoparticles (AuNPs), upconversion nanocrystals (UCNs), quantum dots, and carbon nanostructures, have been integrated with therapeutic agents for fabrication of photo-responsive systems based on their specific physical and chemical properties (Table 1).⁷⁻¹⁰ The great benefits from their tunable size and shape, optimal photo-physical property, and easy surface modification provide more success in the rational design of precisely light-controlled platforms

Table 1. Advantages and disadvantages of various NIR light-mediated nanomaterials.

Nanomaterials type	Advantages	Disadvantages
Metallic nanoparticles (e.g., AuNPs, AgNPs, etc)	Uniform size and shape Simple and fast preparation and bioconjugation High-contrast property	Less fluorescence signals Limited photostability Mutable emissions
Quantum dots (e.g., Ag ₂ S, PbS, etc)	Broad excitation spectra Size-tunable and narrow emission peaks High luminescence efficiency	Potential heavy metals toxicity “Blinking” behavior in bioimaging
Carbon nanomaterials (e.g., carbon nanotubes, graphene, etc)	High surface area to volume ratio Good thermal conductivity Great photostability	Limited fluorescence signals Undesired decomposition
Upconversion nanocrystals (e.g., NaYF ₄ :Yb/Er/Tm, etc)	Multiple emission spectra from UV, visible to NIR range Narrow-bandwidth and tunable luminescence Low autofluorescence High photostability	Low upconversion efficiency

toward the specific regulation of theranostic activities at targeted diseases areas both *in vitro* and *in vivo*. So far, various photolabile or photoactivatable therapeutic systems have been extensively established in the light-responsive drug delivery, photodynamic therapy (PDT), and photothermal therapy (PTT) in living subjects.¹¹ By now, the commonly used excitation wavelength in theranostics heavily relies on short-wavelength light (e.g., ultraviolet (UV) and visible light), which has limited tissue penetration depth owing to the inherent absorption/scattering of some endogenous light absorbers including water, lipids, and protein molecules including hemoglobin and oxyhemoglobin in living subjects.¹² In order to further improve the tissue penetration in the deep-seated pathological areas for light-controlled therapy and imaging, new light sources and photo-responsive reaction systems are highly demanded. Nowadays, one inspiring approach to deal with these challenges is to utilize the long-wavelength near-infrared (NIR) light in the range of 700~1000 nm, termed as “NIR window” or “optical window”.⁵ Typically, NIR light irradiation displays minimum absorption in living subjects, achieving better tissue penetration depth (e.g., < 10 cm) than UV or visible light (e.g., < 1 cm), which is also expected to exhibit minimal photodamage to biomolecules, cells, and tissues.¹³ Currently, numerous drug active agents or probe molecules with large absorbance in the NIR

window have been pursued and applied in clinics for deep-tissue therapeutics and imaging, including indocyanine green (ICG) and methylene blue (MB).¹⁴ However, several inherent drawbacks, such as tedious synthetic procedures, low water solubility, and limited accumulation properties in targeted areas, heavily restrict their further clinical applications. Fortunately, some novel nanomaterials (*e.g.*, AuNPs, UCNs, carbon nanomaterials, *etc*) with a large extinction coefficient in the NIR region, have recently become promising candidates in the field of NIR light-mediated theranostics, which could harvest NIR photons and generate cytotoxic moieties (*e.g.*, reactive oxygen species (ROS), heat, *etc*) for effective diseases treatment seated in deep tissues.¹⁵ Moreover, these nanomaterials provide significant platform for the integration of multiple functionalities in a single construct, which could thus facilitate the synergistic disease diagnosis, therapy, and real-time monitoring *in vitro* and *in vivo*.¹⁶

Among these different nanomaterials for NIR light-mediated theranostics, lanthanide-doped UCNs have attracted much attention in recent years based on their outstanding chemical and optical properties, including great biocompatibility, high resistance to photobleaching and narrow-bandwidth emission.¹⁷ More importantly, they can serve as a promising nano-transducer with excellent tissue penetration depth *in vivo* to convert NIR light excitation into a broad range of emissions from UV, visible to NIR region through a multi-photon upconversion process.¹⁸ These unique properties make lanthanide-doped UCNs particularly serve as a promising vector for biological sensing, biomedical imaging, and diseases theranostics. Herein, we will briefly introduce the properties, synthesis, surface modifications, and NIR light-controlled theranostics of UCNs in biomedical applications.

1.2 Upconversion nanocrystals (UCNs)

1.2.1 Properties of UCNs

The general components of UCNs are mainly based on the doped lanthanide ions

Figure 1. (A) Scheme of the structure and components of UCNs.¹⁹ Copyright 2013 Wiley-VCH. (B) Illustration of tuning upconverted colors of UCNs by different dopant ratios.²⁰ Copyright 2008 American Chemical Society. (C) Upconversion mechanism of UCNs with varied dopant ions: Yb³⁺ and Er³⁺, Yb³⁺ and Tm³⁺, or Yb³⁺ and Ho³⁺.²¹ Copyright 2015 American Chemical Society.

in the insulating host matrix containing a sensitizer (e.g., Yb³⁺, Nd³⁺) and an activator (e.g., Tm³⁺, Er³⁺ and Ho³⁺) within the crystal homogeneously (Figure 1A).¹⁹ Until now, various host materials have been developed, including NaYF₄, NaGdF₄, NaYbF₄, NaLaF₄, GdF₃, LaF₃, GdOF, Y₂O₂S, Y₂O₃, Lu₂O₃, La₂O₃, and others, which generally possess low lattice phonon energies to maximize radiative emission and minimize non-radiative loss. In particular, fluorides are the most attractive host matrices because of their high chemical stability and low phonon energies of about 350 cm⁻¹. Generally, Er³⁺, Tm³⁺, and Ho³⁺ ions are often used as specific activators, which present excellent upconversion luminescence (UCL) emission from the nanocrystals under NIR light excitation. The different optical emission from the nanocrystals is attributed to the localized electronic transition within the 4f orbitals of the lanthanide

dopants due to their ladder-like arranged energy level. Therefore, it is critical to precise control the size and morphology of synthesized UCNs with multicomponent lanthanide dopants. More importantly, to minimize the energy loss of cross-relaxation, the dopant ratio of activators is usually lower than 2 mol%. The Yb^{3+} ion is often used as a sensitizer and co-doped with activators to enhance the UCL efficiency owing to its large absorption cross-section at approximate 980 nm.

Table 2. Typical dopant-host combinations and multicolored upconversion emission.

Activators	Sensitizers	Hosts	Major transitions and emissions ^a (nm)				Output Colors
Tm^{3+}			$^1\text{D}_2 \rightarrow ^3\text{F}_4$	$^1\text{G}_4 \rightarrow ^3\text{H}_6$	$^1\text{G}_4 \rightarrow ^3\text{F}_4$	$^3\text{H}_4 \rightarrow ^3\text{H}_6$	
	Yb^{3+}	$\alpha\text{-NaYF}_4$	451 (w)	478 (m)	650 (w)	800 (s) (NIR)	blue
	Yb^{3+}	$\beta\text{-NaYF}_4$	450 (w)	477 (s)	646 (w)	800 (s)	blue
	Yb^{3+}	NaLuF_4	450 (w)	480 (m)	650 (w)	800 (s)	blue
	Yb^{3+}	NaYbF_4	451 (w)	476 (s)	–	800 (s)	blue
Er^{3+}		LaF_3	–	475 (m)	650 (w)	800 (s)	blue
			$^2\text{H}_{11/2} \rightarrow ^4\text{I}_{15/2}$	$^4\text{S}_{3/2} \rightarrow ^4\text{I}_{15/2}$	$^4\text{F}_{9/2} \rightarrow ^4\text{I}_{15/2}$		
	Yb^{3+}	$\beta\text{-NaYF}_4$	523 (m)	541 (s)	655 (m)		green
	Yb^{3+}	$\alpha\text{-NaYF}_4$	521 (w)	539 (s)	651 (m)		yellow
	Yb^{3+}	BaYF_5	521 (m)	544 (m)	655 (s)		red
Ho^{3+}	Yb^{3+}	NaGdF_4	520 (w)	540 (s→m)	656 (m→s)		green→red
	Yb^{3+}	YF_3	521 (s→m)	543 (m→w)	660 (w→s)		green→yellow→red
			$^5\text{S}_2, ^5\text{F}_4 \rightarrow ^5\text{I}_8$	$^5\text{F}_5 \rightarrow ^5\text{I}_8$			
	Yb^{3+}	$\beta\text{-NaYF}_4$	549 (s)	649 (w→m)			green
	Yb^{3+}	BaYF_5	540 (s)	650 (m)			green
Yb^{3+}		Y_2O_3	550 (s)	650 (w)			green
		NaYbF_4	542 (s)	645 (w)			green
		$\alpha\text{-NaYF}_4$	545 (s)	644 (m→s)			green→yellow→orange

^as, m, and w refer to strong, moderate, and weak emission intensities, respectively.

Typically, by altering the composition and concentration of doped lanthanide ions, the UCL color of UCNs can be tuned finely (Figure 1B).²⁰ Usually, the rationales for dopant ions selection in bioapplications are based on their specific UCL emissions (*e.g.*, UV and blue emissions from Tm^{3+} -doped UCNs; green and red emissions from $\text{Er}^{3+}/\text{Ho}^{3+}$ -doped UCNs, *etc*), as shown in Table 2.²¹ For instance, Yb^{3+} and Er^{3+} doped UCNs show green emissions in the range of 510-570 nm and the peaks centered at 520 and 542 nm, which originate from transitions from the $^2\text{H}_{11/2}$ and $^4\text{S}_{3/2}$ excited states to the $^4\text{I}_{15/2}$ ground state (Figure 1C).²² A red emission is centered at 660

nm, ranging from 630 and 680 nm and corresponding to transition from the $^4F_{9/2}$ excited state to the $^4I_{15/2}$ ground state. Weak blue emissions are centered at 450, 470, and 495 nm, which are from the $^4F_{5/2} \rightarrow ^4I_{15/2}$, $^2P_{3/2} \rightarrow ^4I_{11/2}$, and $^4F_{7/2} \rightarrow ^4I_{15/2}$ transitions, respectively. In addition, emissions at 390 and 410 nm can also be detected for UCNs with a high concentration of Yb^{3+} under high power excitation. For UCNs doped with Yb^{3+} and Tm^{3+} , the upconverted emissions at 345, 365, 451, 481, 646, and 800 nm are assigned to the $^3P_6 \rightarrow ^3F_4$, $^1D_2 \rightarrow ^3H_6$, $^1D_2 \rightarrow ^3F_4$, $^1G_4 \rightarrow ^3H_6$, $^1G_4 \rightarrow ^3F_4$, and $^3H_4 \rightarrow ^3H_6$ transitions, respectively. Moreover, the UCL intensity at 800 nm is usually the highest signals. For UCNs doped with Yb^{3+} and Ho^{3+} ions, the upconverted emissions at 486, 541, 647, and 751 nm result from the $^4F_3 \rightarrow ^5I_8$, $^5F_4/^5S_2 \rightarrow ^5I_8$, $^5F_5 \rightarrow ^5I_8$, $^5F_4/^5S_2 \rightarrow ^5I_7$ transitions, respectively.

1.2.2 Preparation of UCNs

Basically, UCNs can be synthesized by several classical strategies, including thermolysis, hydro(solvo)-thermal, coprecipitation, microwave-assisted approach, sol-gel process, and other strategies, among which thermolysis and hydro(solvo)-thermal methods are the most widely utilized.²¹ For example, in the

Figure 2. (A) Scheme of the growth of UCNs by thermolysis method.²² Copyright 2014 American Chemical Society. (B) Illustration of the hydro(solvo)-thermal method based on liquid-solid-solution phase transfer.²³ Copyright 2005 Nature Publishing Group.

thermolysis method, organometallic compounds are usually applied as precursors, which can decompose in an organic solvent with a high boiling point in the presence of surfactants at high temperature (Figure 2A).²² The common precursors are metallic trifluoroacetate salts, which undergo rapid decomposition to create fast nucleation for monodispersed nanophosphors production. Usually, the solvent is 1-octadecene, and the surfactants are oleylamine, oleic acid, or trioctylphosphine oxide, which can coordinate metallic elements by functional capping group and avoid nanoparticle aggregation by long hydrocarbon chain. Furthermore, high-quality UCNs can be prepared with great crystallinity, narrow size distribution, and excellent optical properties by optimizing experimental parameters, including solvents amount, precursor concentration, reaction time, and temperature. For instance, Capobianco and co-workers synthesized cubic $\text{Yb}^{3+}/\text{Er}^{3+}$ or $\text{Yb}^{3+}/\text{Tm}^{3+}$ co-doped NaYF_4 nanoparticles through thermolysis of metal trifluoroacetate precursors in 1-octadecene with the assistance of oleic acid.²⁴

Typically, in a hydro(solvo)-thermal method, reaction precursors, solvents, and surfactants are mixed and heated in a sealed autoclave under high pressure and temperature, which is usually higher than the critical point of the solvent to enhance the solubility and reactivity of inorganic substances.²² By using this method, highly crystalline phases can be obtained at much lower temperatures and various reactions occur simultaneously. Generally, surfactants, such as ethylenediaminetetraacetic acid, polyethylenimine, oleic acid, and cetyltrimethylammonium bromide, are used to regulate reacting concentration by chelation and control the size, morphology, crystalline phase, and surface functional groups of UCNs. Through the most popular liquid-solid-solution approach, various UCNs nanostructures, including NaYF_4 , NaLaF_4 , NaLuF_4 , BaGdF_5 , YF_3 , and CaF_2 , were synthesized with the reaction, phase transfer, and separation occurring at the interfaces (Figure 2B).^{22,23}

1.2.3 Luminescence enhancement of UCNs

Considering the low extinction coefficients of lanthanide dopants, UCNs often

Figure 3. (a) Scheme of different core-shell UCNs for enhanced upconversion luminescence.²⁵ Copyright 2014 Wiley-VCH. (b) Illustration of dye-sensitized NaYF₄:Yb/Er UCNs with tunable excitation wavelengths (ET: energy transfer).³¹ Copyright 2015 Royal Society of Chemistry.

possess the drawbacks of low quantum yields and strong luminescence quenching in water. In recent years, various strategies have been proposed to improve the upconverted emission of UCNs, including surface passivation, surface-plasmon coupling, and dye sensitization.²⁵ Among these methods, surface passivation approach, which is realized by coating a protecting shell (*e.g.*, amorphous shell, inert crystalline shell, and activators or sensitizers doped active-shell, *etc*) on the core UCNs nanostructures, can greatly enhance the photoluminescence of UCNs by suppressing the surface dopant-induced quenching of excitation energy (Figure 3A). For example, after coating CaF₂ shell on the surface of α -NaYF₄:Yb/Tm core nanoparticles, the UCL at 800 nm presented significant enhancement (~ 35 folds).²⁶ Moreover, surface-plasmon coupling approach, which couples UCNs surface with metallic nanoparticles (*e.g.*, AuNPs, AgNPs, *etc*), has been utilized as effective light-trapping methods to boost the energy conversion efficiency. For instance, by modifying Au sphere (~ 10 nm) on the surface of core NaYF₄:Yb/Tm nanostructures, the UCLs at 450 nm and 474 nm enhanced by ~ 49.8 folds and ~ 29.9 folds, respectively.²⁷

Furthermore, in recent decades, a new strategy (dye sensitization), which anchors organic dyes with broad and intense NIR light absorption on the surface of UCNs, has

been proposed to harvest the excitation energy and transfer to the lanthanides in UCNs through Förster or Dexter mechanisms. Interestingly, thousands-folds luminescence enhancement was observed than non-dye-sensitized UCNs, demonstrating the highly improved upconversion efficiency upon dyes sensitization.²⁸⁻³⁰ More importantly, by anchoring diverse organic dyes with different absorption wavelengths, the upconversion process can be endowed with highly tunable excitation bands more than traditional utilized 980 or 808 nm light irradiation (Figure 3B).³¹ These promising approaches may further facilitate the fundamental research of underlying physical mechanism during upconversion process and explore the biomedical application of UCN-based theranostics in the future.

1.2.4 Surface modification of UCNs

Figure 4. Scheme of surface modification to prepare hydrophilic UCNs.³² Copyright 2012 Royal Society of Chemistry.

Normally, most of UCNs nanostructures synthesized by the above methods are mainly capped with hydrophobic ligands (*e.g.*, oleic acid, oleylamine, *etc*) which

generally hinder their further bioapplications due to the limited solubility in aqueous solution. Therefore, suitable surface modification approaches are of great significance to prepare hydrophilic UCNs for further biomedical applications. So far, different methods for surface functionalization of UCNs have been developed in recent years, such as ligand exchange, silanization, ligand oxidation, hydrophobic-hydrophobic interaction, layer-by-layer self-assembly, and host-guest interaction (Figure 4).³² For examples, in the process of ligand exchange approach, various hydrophilic ligands, including poly(ethylene glycol) (PEG), polyacrylic acid (PAA), citrate, 3-mercaptopropionic acid, thioglycolic acid, and other ligands, are exchanged with hydrophobic molecules on the surface of UCNs, which can afford UCNs with great water solubility and unaltered crystallization and morphology. In ligands oxidation method, the unsaturated carbon-carbon bonds in the capping reagents are usually oxidized to the carboxylic group upon oxygen and high temperature. Another strategy is based on hydrophobic-hydrophobic interaction, in which the hydrophobic UCNs can be modified with amphiphilic polymers (*e.g.*, chitosan, maleic acid anhydride/hexadecane co-polymer, *etc*). UCNs also can be functionalized based on layer-by-layer self-assembly approach through electrostatic interaction with several oppositely charged polymers (such as poly(allylamine hydrochloride) and poly(sodium 4-styrenesulfonate)). Moreover, another interesting surface modification method is based on the host-guest interaction with some specific host molecules (*e.g.*, β -cyclodextrin, adamantaneacetic acid, *etc*).

1.2.5 NIR light-controlled theranostic applications of UCNs

1.2.5.1 Drug and siRNA delivery

For NIR light-triggered drug delivery, the upconverted emissions at UV or visible window can effectively cleavage the photo-responsive linker and activate prodrugs modified on the surface of UCNs for therapy and imaging. In 2010, Branda and co-workers firstly reported the photocaged compounds functionalized NaYF₄:Yb/Tm UCNs that could undergo a 980 nm NIR light-controlled molecules release by the

Figure 5. (A) Scheme of photocaged UCNs for remote control of D-luciferin photoactivity.³⁴ Copyright 2012 Wiley-VCH. (B) Illustration of UCNs for NIR light-controlled drug release.³⁵ Copyright 2013 Wiley-VCH.

upconverted emission at UV range.³³ Although this strategy was only carried out in organic solution, the pioneering investigation still impressed the researchers for further biomedical studies. In 2012, Xing and co-workers developed a novel NIR light-triggered UCNs nanoplatfrom to control D-luciferin photoactivity remotely (Figure 5A).³⁴ In their proposed design, the silica-coated UCNs were covalently linked with o-nitrobenzyl (ONB) photocaged D-luciferin. Upon 980 nm NIR light illumination, the upconverted UV emission at 365 nm cleaved the photoactive ONB group and induced the photorelease of D-luciferin, which resulted in significant bioluminescence enhancement upon the reaction with the firefly luciferase reporter enzyme in *in vitro* and *in vivo* studies. Similarly, they also designed a NIR light-responsive mesoporous silica modified UCNs drug delivery system for

light-controlled drug (Doxorubicin, Dox) release in cancer cells (Figure 5B).³⁵ Upon 980 nm light irradiation, Dox could be selectively activated through the photocleavage of the capped ONB cross-linker by the upconverted UV emission from UCNs, which provided a good example for effective light-triggered drug delivery in living system.

Inspired by these pioneering studies, great possibilities for NIR light-triggered therapy and imaging have emerged in recent years. For instance, around the same

Figure 6. (A) Scheme of NIR light-mediated UCNs nanoplatform for Pt(IV) prodrug-based therapy and multimodal imaging.³⁶ Copyright 2013 American Chemical Society. (B) Schematic illustration of 980 nm light-induced Pt(IV) prodrug photoactivation and intracellular apoptosis monitoring.³⁷ Copyright 2014 Wiley-VCH. (C) NIR light-triggered drug release from the mesopores of UCNs@mSiO₂ due to trans-cis azobenzene photoisomerization.³⁸ Copyright 2013 Wiley-VCH. (D) Scheme of power density controlled photoswitching for non-invasive cell adhesion/detachment.³² Copyright 2015 American Chemical Society.

time, Lin's and Xing's groups have independently developed the novel NIR light-mediated Pt(IV) anticancer prodrug delivery nanoplateforms based on UCNs for remotely controlled prodrugs photoactivation under 980 nm light irradiation. In Lin's design (Figure 6A), a novel photoactive Pt(IV) prodrug, *trans,trans,trans*-[Pt(N₃)₂(NH₃)(py)(O₂CCH₂CH₂COOH)₂], was conjugated with a core-shell UCNs complex (NaYF₄:Yb/Tm@NaGdF₄/Yb).³⁶ Such photoactive drug delivery system not only exhibited significant *in vivo* antitumor efficacy upon 980 nm laser irradiation, but also provided multifunctional tri-modality imaging contrast agents for cancer treatment in living animals. While, in Xing's system, the silica-coated UCNs were constructed as nanocarriers to covalently combine with Pt(IV) prodrug (*trans,trans,trans*-[Pt(N₃)₂(py)₂(OH)(O₂CCH₂CH₂COOH)]) and another apoptosis imaging peptide probe consisting of a fluorescence donor (Cy5) and a quencher (Qsy21) (Figure 6B).³⁷ Upon 980 nm light illumination, the Pt(IV) prodrug was photo-activated by upconverted UV emission and induced effective cytotoxic activity in cancer cells. Moreover, the caspases enzymes triggered by cytotoxicity could effectively cleave the peptide probe to achieve optical imaging of apoptosis in living cells, making this nanocarrier an excellent biomarker for real-time cellular imaging and earlier evaluation of anticancer therapeutic efficiency.

Another effective approach to control drug delivery with deep tissue penetration is depending on the design of NIR triggered photoisomerisation, which undergoes reversible conformation change and subsequently induces controllable drug release upon NIR light excitation. For instance, Shi *et al.* demonstrated a novel NIR light-mediated drug carrier based on mesoporous silica-coated NaYF₄:Yb/Tm@NaYF₄ core-shell nanostructures (UCNs@mSiO₂) (Figure 6C).³⁸ The anticancer drug (Dox) was co-loaded with photoactive azobenzene groups into the nanoplateform. Upon 980 nm laser illumination, the upconverted UV (350 nm) and visible (450 nm) light from UCNs resulted in reversible azobenzene structural alteration from *trans* to *cis* status and induced continuous rotation-inversion migration, which could thus drive light-controlled drug release in cancer cells. Additionally,

another feasible NIR light-mediated drug delivery approach will rely on the light switchable spiropyran molecule, which can respond to converted visible emission from UCNs. In this direction, Qu's group recently reported an interesting design for NIR light-controlled cell adhesion/detachment reversibly by utilizing spiropyran conjugated multi-shell UCNs (Figure 6D).³⁹ In this system, the conformational behavior of spiropyrans was only relying on the power density of 980 nm NIR laser excitation. High power density permitted the ring-opening while reversed to ring-closing status when upon lower power density excitation. Such smart NIR light-induced design switched the reversible interaction between spiropyran and cell surface protein (fibronectin), which therefore resulted in NIR light-regulated adhesion/detachment of cancer cells.

Moreover, in recent decades, a smart strategy, gene therapy, has been an attractive therapeutic method for cancer treatment, which is based on double-stranded RNA with 21-23 nucleotides and can cause efficient gene silencing in the process of small-interfering RNA (siRNA) interference.⁴⁰ However, the practical application of this approach is limited by several shortcomings, such as poor cellular uptake, immune response, and fast degradation in living system. To overcome these

Figure 7. (A) Scheme of the synthesis of photocaged Si-UCNs for NIR light-responsive siRNA release.⁴² Copyright 2012 Royal Society of Chemistry. (B) Illustration of UCN-based siRNA delivery system for gene tracking and reversing drug resistance.⁴³ Copyright 2017 American Chemical Society.

disadvantages, siRNA can be loaded and protected by some specific nanocarriers for further targeted gene therapy. For instance, Zhang and co-workers loaded siRNA on NaYF₄:Yb,Er@SiO₂-anti-Her2 nanoparticles and studied the delivery behavior and therapeutic efficiency.⁴¹ When Her2 receptors overexpressed human breast adenocarcinoma SK-BR-3 cells were incubated with the nanoparticles, UCL was observed obviously when compared to that in human breast adenocarcinoma MCF-7 cells with low Her2 receptors expression. Moreover, the luciferase gene expression was not silenced in MCF-7 cells, while it was down-regulated by 45.5% in SK-BR-3 cells. These results confirmed the targeted siRNA delivery and effective gene silencing toward cells with high level of Her2 receptors using NaYF₄:Yb,Er@SiO₂-anti-Her2 as nanocarriers. Interesting, Xing's group developed a smart approach for NIR-light- triggered siRNA delivery. The siRNA was anchored on the surface of NaYF₄:Yb,Tm@mSiO₂ *via* electrostatic interaction based on photosensitive ONB-functionalized groups (Figure 7A).⁴² Under NIR light irradiation, siRNA could be released in living cells upon the cleavage of ONB linker by the upconverted UV light. Moreover, in Duan's work, multidrug resistance (MDR)-1 siRNA (MDR1-siRNA) was loaded on layer-by-layer engineered UCNs through electrostatic attraction (Figure 7B).⁴³ Besides tracking the loading and release of MDR1-siRNA by the alternation of fluorescence resonance energy transfer efficiency between UCNs donor and fluorophore-functionalized MDR1-siRNA acceptor, this nanocarrier could enhance cellular uptake, promote endosome escape, and avoid nuclease degradation of MDR1-siRNA, leading to efficient MDR gene silencing to reverse drug resistance in ovarian cancer.

1.2.5.2 Photodynamic therapy (PDT)

Besides NIR light-mediated drug/siRNA delivery, another well-established unique type of therapeutic modality is PDT, which relies on photosensitizers (PS) to generate ROS (*e.g.*, peroxides, superoxide, hydroxyl radical, singlet oxygen (¹O₂), *etc*) upon light irradiation. Basically, the mechanism of PDT consists of three stages: excitation of PS, generation of ROS and cell damage (Figure 8).⁴⁴ Upon light

Figure 8. Schematic illustration of the typical mechanism of photodynamic reaction.⁴⁴
Copyright 2012 Frontiers Media SA.

excitation, a PS molecule becomes activated from ground state to a short-lived excited singlet state ($^1\text{PS}^*$). The excited PS may either decay back to the ground state with fluorescence emission for imaging and detection or undergo intersystem crossing to form a relatively long-lived triplet state ($^3\text{PS}^*$). The triplet excited PS can directly interact with a surrounding substrate (*e.g.*, cell membrane, biomolecules, *etc*), and transfer the proton/electron to form radical ions, which then reacts with oxygen to produce oxygenated products such as superoxide anion radicals ($\text{O}_2^{\bullet-}$), hydroxyl radicals ($\text{OH}^\bullet$), and hydrogen peroxides (H_2O_2) (type I reaction). Alternatively, the energy of the excited PS can be directly transferred to molecular oxygen at ground state ($^3\text{O}_2$) to form $^1\text{O}_2$ (type II reaction). Due to the required energy for the transition of oxygen from $^3\text{O}_2$ to $^1\text{O}_2$ is only 22 kcal/mol, type II reaction is generally accepted as the dominant reaction during the therapeutic process in PDT. These active oxygen species from type I and type II reactions are usually associated with oxidative stress and subsequently cause significant cellular damage by degrading the components in cells, like DNA, certain enzymes, or proteins.⁴⁵

Compared to traditional therapeutic modalities such as chemotherapy and radiotherapy, PDT exhibits great advantages especially in cancer therapy due to its intrinsically non-invasive safety and highly spatiotemporal selectivity after light

irradiation. Therefore, PDT has been well developed as a viable therapeutic option for effective cancer treatment and been approved by the U.S. Food and Drug Administration (FDA) in recent decades.⁴⁶ In general, the application of PDT in the clinic can be restricted by two main aspects. First, the most PS agents employed in conventional PDT are hydrophobic molecules that contain porphyrins or their analog structures, which may usually suffer from limited accumulation efficiency at the targeted regions due to their poor water solubility. In order to improve the therapeutic efficacy of PDT, nanomaterial-based carriers have been proposed to facilitate the PS agent delivery in targeted disease areas in living system. Another potential shortcoming of traditional PDT is the limited tissue penetration of excitation light source because most of the PS agents are only responsive to short-wavelength light irradiation. In an attempt to solve this problem, NIR light-responsive PS molecules can be developed with deep tissue penetration, which significantly improves the safety of PDT treatment by effectively reducing the possible light-induced cytotoxicity in living systems.⁴⁷ However, several unavoidable drawbacks, such as troublesome synthetic procedures, low water solubility, and limited accumulation abilities in targeted areas, heavily restrict their clinical applications in deep-tissue therapeutic and imaging studies. Therefore, various kinds of nanomaterials with large extinction coefficient in the NIR window, have been extensively explored for PDT applications both *in vitro* and *in vivo*.

Typically, as a promising candidate, UCNs, which can convert NIR light into broad UV/visible emission to activate several kinds of PS agents, provide a powerful tool to overcome the major drawbacks of current PDT strategies with NIR light-triggered applications. For instance, in 2007, Zhang *et al.* firstly reported that silica-coated UCNs could act as an energy transducer to induce PDT therapeutic effect in living cells under NIR light irradiation.⁴⁸ In 2012, Zhang's group presented a smart UCNs-based PDT treatment strategy by incorporating two types of PS molecules, zinc phthalocyanine (ZnPc) and merocyanine 540 (MC540), into one single core-shell mesoporous silica coated UCNs nanoplatfrom (Figure 9A).⁴⁹ Upon

Figure 9. (A) Scheme of FA/PEG-modified UCNs@mSiO₂ loaded with ZnPc and MC540 for PDT.⁴⁹ Copyright 2012 Nature Publishing Group. (B) Schematic illustration of UCNs coated with PS-doped silica shell for PDT.⁵¹ Copyright 2015 Wiley-VCH. (C) Design of UCNs-RB for 808 nm laser-induced PDT.⁵⁴ Copyright 2015 Royal Society of Chemistry. (D) Illustration of 808 nm light-triggered dual-agent photosensitizing-UCNs for hyaluronic acid-targeted PDT and enhanced PDT.⁵⁵ Copyright 2016 Elsevier.

980 nm laser irradiation, the two main upconverted emissions, green (~540 nm) and red (~660 nm) light, simultaneously excite the loaded ZnPc and MC540 molecules respectively, thus resulted in enhanced PDT efficiency. After coating with tumor affinity ligands (*e.g.*, folic acid (FA), RGD peptide, *etc*), the UCNs nanoplatform exhibited effective tumor accumulation for promising NIR light-mediated PDT effects *in vivo*. Moreover, some interesting studies have also been proposed to improve the

PDT effect in hypoxia tumor environment. It is well known that the presence of hypoxia regions in human tumors with low levels of oxygen would be the bottleneck of PDT therapy because these deep-seated tumor cells in the hypoxic regions are resistant to both chemotherapy and light-triggered therapy, which significantly affect cancer treatment efficacy.⁵⁰ In an attempt to solve this problem, recently, Shi and co-workers constructed a double silica-shelled Gd^{3+} -doped UCNs nanoplatfrom that are able to co-delivery of PS molecules (e.g. silicon phthalocyanine dihydroxide, SPCD) and a bioreductive prodrug (tirapazamine, TPZ) (Figure 9B).⁵¹ In fact, TPZ-induced cytotoxicity was caused by various intracellular reductase enzymes to produce highly cytotoxic free-radical intermediate species when oxygen was exhausted by PDT under hypoxia tumor environment. Upon 980 nm laser illumination, a remarkable tumor growth inhibition was achieved based on the relative tumor volume after 16 days PDT treatment in the groups of TPZ-PS-UCNs (~1.8 fold increase) as compared to PS-UCNs (~3.8 fold increase) and PBS (~9.0 fold increase), respectively. The great tumor therapeutic effect in animal experiments demonstrated the highly effective synergetic cancer therapy of this bioreductive TPZ-PDT system. Chen's group also presented a unique rattle structures of organosilica shell modified $\beta\text{-NaLuF}_4\text{:Gd/Yb/Er}$ nanoprobles loaded with PS molecules for effective PDT and dual-modal imaging.⁵² Upon NIR light irradiation, the energy transfer from UCNs to PS facilitated the $^1\text{O}_2$ generation, thus inducing an enhanced PDT effect for cancer treatment.

Although the NIR light-mediated PDT approaches have attracted significant interest in recent years due to the promising optical properties of UCNs, most current UCN-based strategies mainly rely on the 980 nm NIR light, which would likely cause the concern of unavoidable overheating effect owing to the strong water absorption at this wavelength.¹² Therefore, great efforts have been made to convert the excitation light of UCNs to a shorter wavelength range in the medical spectral window (e.g., 700-900 nm). Recently, Nd^{3+} -doped UCNs with ~800 nm excitation have been investigated to minimize the undesirable overheating effect.⁵³ By taking this

promising advantage, Zhang and co-workers conjugated a photosensitizer, Rose Bengal, on NaYF₄:Yb/Ho@NaYF₄:Nd@NaYF₄ nanoparticles for PDT under an excitation wavelength of 808 nm (Figure 9C).⁵⁴ This nanoplatfrom revealed minimal overheating effect and a high PDT efficacy *in vitro*. Lin and co-workers developed a dual-PS-functionalized hyaluronic acid-targeted nanoplatfrom through integrating UV-light-excited TiO₂ PS and blue-light-excited hypocrellin A PS with NaYF₄:Yb/Tm@NaYF₄:Yb@NaNdF₄:Yb@NaYF₄ nanoparticles, which could emit upconverted UV and blue light simultaneously under 808 nm illumination (Figure 9D).⁵⁵ By targeted internalization in cancer cells and effective utilization of upconverted emission by dual-PS, this nanoplatfrom generated increased ROS and revealed enhanced PDT toward cancer cells or tumor-bearing mice, which could promote the NIR-mediated PDT to next generation.

1.2.5.3 Photothermal therapy (PTT)

Apart from NIR light-mediated drug/siRNA delivery and PDT, another outstanding strategy, PTT, has also attracted much attention in recent years, especially in the process of tumor treatment *in vitro* and *in vivo*. Generally, PTT employs photothermal conversion agents (PTCAs) to convert excited NIR light into heat energy in the localized region. Such process will lead to the overheating of disease areas, promote the damage of surrounding biological species and cause subsequent cell death.⁵⁶ The expected PTCAs should at least include several features: (1) strong NIR light absorption, (2) great photothermal conversion efficiency, (3) excellent biocompatibility and biodegradability, and (4) real-time visualization for therapy.⁵⁷ Until now, several novel PTCAs, including inorganic nanomaterials and organic polymers, have been widely employed for effective NIR light-triggered PTT *in vitro* and *in vivo*.⁵⁸

Among these promising PTCAs, noble metal nanostructures have attracted much attention for PTT applications based on their enhanced absorption cross-sections at NIR regions than conventional photo-absorbing dyes on account of their specific

Figure 10. (A) Scheme of the preparation of NaYF₄:Yb,Er@Ag nanoparticles for PTT.⁵⁹ Copyright 2011 Royal Society of Chemistry. (B) Illustration of NaYF₄:Yb,Er@Fe₃O₄@Au-PEG nanoparticles for magnetically targeted PTT under the guidance of dual-modal imaging.⁶⁰ Copyright 2012 Elsevier.

surface plasmon resonance (SPR) oscillations, which ensures effective laser therapeutic outcome at relatively lower energies to render minimal invasiveness.⁵⁸ Additionally, metallic nanostructures present higher photostability, less photobleaching and better biocompatibility than organic PTT agents. Therefore, various metallic nanostructures, including gold/silver nanospheres, gold nanorods and nanoshells, are widely used in PTT because of their ease of preparation, multiple functionalizations, and tunable optical properties. For example, Song and co-workers prepared multifunctional NaYF₄:Yb,Er@Ag nanoparticles successfully, which revealed effective UCL imaging-guided PTT toward human hepatic cancer HepG2 cells and human breast cancer BCap-37 cells upon irradiation with 980 nm laser (Figure 10A).⁵⁹ In Liu's work, multifunctional NaYF₄:Yb,Er@Fe₃O₄@Au-PEG nanoparticles were developed by layer-by-layer method through adsorbing superparamagnetic iron oxide (Fe₃O₄) on the surface of UCNs *via* electrostatic interaction and subsequent formation of a thin gold shell *via* seed-induced reduction growth (Figure 10B).⁶⁰ By further functionalization with PEG molecules, this nanoplatform could be used for dual UCL/magnetic resonance (MR) imaging-guided and magnetically targeted PTT. Upon intravenous injection of this nanoparticle to tumor-bearing mice and subsequent attachment of a magnet on the tumor, T₂-weighted MR signals lowered by 62.1% and UCL intensity enhanced by several

folds. Moreover, without the injection of nanoparticle, the temperature of 808 nm laser-irradiated tumor had no obvious change (about 38 °C), while the temperature could increase to about 50 °C by the intravenous injection of nanoparticle with magnetic targeting and NIR light irradiation, leading to the absolute ablation of tumors. These results promote the application of multifunctional UCNs for effective NIR light-mediated cancer theranostics.

In summary, these promising NIR light-mediated strategies including drug/siRNA delivery, PDT, and PTT, have been proposed for effective therapy and imaging on the basis of versatile UCN-nanoplatfroms. Considering the multiple optical properties of lanthanide-doped UCNs, different kinds of dopant ions play specific roles in the NIR light-mediated theranostics. For example, Tm^{3+} -doped UCNs are mainly utilized for photosensitive drug/siRNA delivery systems, which should be attributed to the responsiveness of most photo-cleavable or photo-reversible linkers (*e.g.*, azobenzene, spiropyran, *etc*) and photoactive prodrugs (*e.g.*, photoactive Pt(IV) prodrug, *etc*) to short-wavelength excitations at UV or blue light emissions. Moreover, Er^{3+} or Ho^{3+} doped UCNs with strong green and red emissions are mainly applied for PDT studies because of the generation of cytotoxic free radicals by most photosensitizers are responsive to visible light excitations (*e.g.*, 540 nm for MC540, 650 nm for ZnPc, *etc*). For PTT approach, the therapeutic effect is mainly attributed to the surface modified metallic nanostructures, while UCNs are usually used as contrast agent for UCL imaging to guide the PTT. These NIR light-controlled strategies present excellent therapeutic effects with deep tissue penetration in living systems.

1.3 Photoacoustic (PA) techniques with deep-tissue penetration

Recently, noninvasive ultrasound imaging technique has been widely used in clinics as effective diagnostic tool to provide valuable information of internal body structures with deep penetration, such as muscles, tendons, joints, vessels and organs. Based on these promising properties, a brand-new technique, PA imaging, which integrates ultrasound imaging and NIR light excitation into one system, has been

Figure 11. (A) Brief scheme of the mechanism of PA signal production. (a) Structure of 128-element hemisphere array-based PA tomography. (b) PA imaging of blood vessels in a living mouse ear.⁶³ Copyright 2014 Royal Society of Chemistry. (B) Design of photo-activated probe for ROS monitoring with PA imaging in mice.⁶⁶ Copyright 2014 Nature Publishing Group. (C) Illustration of MNP for multimodal imaging.⁶⁷ Copyright 2014 American Chemical Society.

developed as a new kind of imaging modality to provide deep-tissue penetration and high spatial resolution in living systems.^{61,62} As shown in Figure 11A, upon laser irradiation, a part of light is absorbed by the contrast agents and converted to localized heat, followed by the generation of a pressure rise through thermo-elastic expansion. Such pressure rise is propagated as an acoustic wave with the ultrasound frequency in the biological tissues, which can be captured by surrounding ultrasonic transducers for PA imaging in deep-seated regions.⁶³ Therefore, due to these promising properties,

PA imaging may overcome the limitations of conventional optical imaging (e.g., limited tissue penetration depth) and ultrasonic imaging (e.g., low contrast for diagnosis), making it an outstanding clinical imaging method in biomedical fields.⁶⁴ Moreover, benefited from the great absorption of NIR light, various photothermal reagents can also be utilized as ideal contrast agents for PA imaging.⁶⁵ For example, a promising semiconducting conjugated polymer nanoparticles (SPNs) that have strong NIR light absorbance have been reported recently, which could act as a new class of contrast agents for PA imaging (Figure 11B).⁶⁶ These SPNs exhibited considerable PA signal than gold nanorods and single-walled carbon nanotubes on a per mass basis, and could make them an ideal contrast agent for whole-body lymph node PA imaging in living mice. Furthermore, Chen's group also constructed a novel multimodality imaging nanoplatfrom based on ultrasmall (< 10 nm) water-soluble melanin nanoparticle (MNP), which indicated unique properties for PA imaging and innate binding abilities with various metal ions (e.g., $^{64}\text{Cu}^{2+}$, Fe^{3+} , *etc*) for multifunctional imaging (Figure 11C).⁶⁷ Moreover, they further modified cyclic-RGD peptide on MNPs surface to achieve specific conjugation with tumor overexpressed $\alpha_v\beta_3$ integrins. The animal experiments also demonstrated that these multifunctional MNPs had great potential for molecular theranostics and clinical translation. Recently, Lovell's group reported a promising probe, phosphorus phthalocyanine (P-Pc), which could reduce the light scattering based on its long wavelength absorption band beyond 1000 nm for deep-tissue and high quality PA imaging *in vitro* and *in vivo*.⁶⁸ It was demonstrated that P-Pc could accumulated passively in tumor areas by the enhanced permeability and retention (EPR) effect after intravenous injection in mice. It could also been used for non-invasive intestine PA imaging of mice by oral administration. Moreover, further studies were also conducted by external placement of the P-Pc under the arm of healthy adult human. The results demonstrated that the PA signal could be clearly visualized through the entire 5 cm limb to show strong capability for deep-tissue PA imaging in humans. Based on these inspiring results, the promising PA techniques have the potential to be used in therapeutic studies in the near future.

1.4 Research objectives

According to the above introduction, UCNs have been utilized in various NIR light-mediated theranostics with great tissue penetration depth based on their impressive optical properties, which can convert low-energy NIR light (usually at 980 nm) into high-energy emission in the range of UV, visible and NIR region. In order to further promote their biomedical applications in living condition, various UCNs-based nanoplatforms were proposed in this dissertation.

In chapter 2, to construct an enzyme-triggered UCN-nanoplatform for site-specific tumor treatment, a UV light-responsive Pt(IV) prodrug and a ROS-responsive dye were conjugated on dual peptide-functionalized-UCNs. Upon tumor-specific cathepsin protease-responsive cross-linking reaction between two different UCNs nanostructures, increased photo-release of Pt(IV) prodrug occurred and the generated ROS in the process of cell death was monitored in real time. Therefore, this strategy could enhance the anticancer efficacy and real-time evaluate the early-stage therapeutic response.

In chapters 3 and 4, in order to provide a multimodality solution for effective molecular sensing and site-specific tumor treatment, another unique micro-environment-sensitive strategy for localization of peptide-modified UCNs within tumor areas was presented. Upon tumor-specific cathepsin protease reactions, the cleavage of peptides induced covalent cross-linking between the exposed cysteine and 2-cyanobenzothiazole on neighboring particles, thus triggering the accumulation of UCNs into tumor site. Such enzyme-triggered cross-linking of UCNs led to enhanced upconversion emission upon 808 nm laser irradiation, and in turn, amplified the singlet oxygen generation from the PS molecules attached on UCNs. Importantly, this design enabled remarkable tumor inhibition through either intratumoral UCNs injection or intravenous injection of nanoparticles modified with the targeting ligand.

In chapter 5, to develop a simple and innovative strategies for the specific

attachment of photoswitches on the cell surface without modifying or genetically encoding channel structures, and to enable the remote activation of ion-channel functions within NIR spectral window in living systems, metabolic glycan biosynthesis was used to achieve site-specific covalent attachment of NIR light-mediated UCNs to the cell surface through copper-free click cyclization. Upon irradiation with 808 nm light, the converted emission at 480 nm could activate a light-gated ion channel, channelrhodopsins-2 (ChR2), and thus remotely control the cation influx. This unique strategy could provide valuable insights on the specific regulation of membrane-associated activities in living zebrafish.

1.5 References

- (1) Reichard, C. A.; Stephenson, A. J.; Klein, E. A. *Cancer* **2015**, *121*, 3403.
- (2) Friedman, A. A.; Letai, A.; Fisher, D. E.; Flaherty, K. T. *Nat. Rev. Cancer* **2015**, *15*, 747.
- (3) Mahoney, K. M.; Rennert, P. D.; Freeman, G. J. *Nat. Rev. Drug Discov.* **2015**, *14*, 561.
- (4) Thambi, T.; Park, J. H.; Lee, D. S. *Biomater. Sci.* **2016**, *4*, 55.
- (5) Bansal, A.; Zhang, Y. *Acc. Chem. Res.* **2014**, *47*, 3052.
- (6) Ai, X.; Mu, J.; Xing, B. *Theranostics* **2016**, *6*, 1899.
- (7) Vijayaraghavan, P.; Liu, C. H.; Vankayala, R.; Chiang, C. S.; Hwang, K. C. *Adv. Mater.* **2014**, *26*, 6689.
- (8) Tian, G.; Zhang, X.; Gu, Z.; Zhao, Y. *Adv. Mater.* **2015**, *27*, 7692.
- (9) Hong, G.; Robinson, J. T.; Zhang, Y.; Diao, S.; Antaris, A. L.; Wang, Q.; Dai, H. *Angew. Chem., Int. Ed.* **2012**, *51*, 9818.
- (10) Fadel, T. R.; Sharp, F. A.; Vudattu, N.; Ragheb, R.; Garyu, J.; Kim, D.; Hong, E.;

- Li, N.; Haller, G. L.; Pfefferle, L. D.; Justesen, S.; Herold, K. C.; Fahmy, T. M. *Nature Nanotech.* **2014**, *9*, 639.
- (11) Tian, J.; Ding, L.; Ju, H.; Yang, Y.; Li, X.; Shen, Z.; Zhu, Z.; Yu, J. S.; Yang, C. *J. Angew. Chem., Int. Ed.* **2014**, *53*, 9544.
- (12) Shen, J.; Chen, G.; Vu, A. M.; Fan, W.; Bilsel, O. S.; Chang, C. C.; Han, G. *Adv. Opt. Mater.* **2013**, *1*, 644.
- (13) Hudson, D. E.; Hudson, D. O.; Wininger, J. M.; Richardson, B. D. *Photomed. Laser Surg.* **2013**, *31*, 163.
- (14) Deng, K.; Hou, Z.; Deng, X.; Yang, P.; Li, C.; Lin, J. *Adv. Funct. Mater.* **2015**, *25*, 7280.
- (15) Shanmugam, V.; Selvakumar, S.; Yeh, C. S. *Chem. Soc. Rev.* **2014**, *43*, 6254.
- (16) Melancon, M. P.; Zhou, M.; Li, C. *Acc. Chem. Res.* **2011**, *44*, 947.
- (17) Tsang, M. K.; Bai, G.; Hao, J. *Chem. Soc. Rev.* **2015**, *44*, 1585.
- (18) Li, X.; Zhang, F.; Zhao, D. *Chem. Soc. Rev.* **2015**, *44*, 1346.
- (19) Gu, Z.; Yan, L.; Tian, G.; Li, S.; Chai, Z.; Zhao, Y. *Adv. Mater.* **2013**, *25*, 3758.
- (20) Wang, F.; Liu, X. *J. Am. Chem. Soc.* **2008**, *130*, 5642.
- (21) Gai, S.; Li, C.; Yang, P.; Lin, J. *Chem. Rev.* **2014**, *114*, 2343.
- (22) Chen, G.; Qiu, H.; Prasad, P. N.; Chen, X. *Chem. Rev.* **2014**, *114*, 5161.
- (23) Wang, X.; Zhuang, J.; Peng, Q.; Li, Y. *Nature* **2005**, *437*, 121.
- (24) Boyer, J. C.; Vetrone, F.; Cuccia, L. A.; Capobianco, J. A. *J. Am. Chem. Soc.* **2006**, *128*, 7444.
- (25) Han, S.; Deng, R.; Xie, X.; Liu, X. *Angew. Chem., Int. Ed.* **2014**, *53*, 11702.

- (26) Chen, G.; Shen, J.; Ohulchanskyy, T. Y.; Patel, N. J.; Kutikov, A.; Li, Z.; Song, J.; Pandey, R. K.; Agren, H.; Prasad, P. N.; Han, G. *ACS Nano* **2012**, *6*, 8280.
- (27) Liu, N.; Qin, W.; Qin, G.; Jiang, T.; Zhao, D. *Chem. Commun.* **2011**, *47*, 7671.
- (28) Wang, X.; Valiev, R. R.; Ohulchanskyy, T. Y.; Ågren, H.; Yang, C.; Chen, G. *Chem. Soc. Rev.* **2017**, *46*, 4150.
- (29) Wu, X.; Zhang, Y.; Takle, K.; Bilsel, O.; Li, Z.; Lee, H.; Zhang, Z.; Li, D.; Fan, W.; Duan, C.; Chan, E. M.; Lois, C.; Xiang, Y.; Han, G. *ACS Nano* **2016**, *10*, 1060.
- (30) Huang, L.; Zhao, Y.; Zhang, H.; Huang, K.; Yang, J.; Han, G. *Angew. Chem., Int. Ed.* **2017**, *56*, 14400.
- (31) Wu, X.; Lee, H.; Bilsel, O.; Zhang, Y.; Li, Z.; Chen, T.; Liu, Y.; Duan, C.; Shen, J.; Punjabi, A.; Han, G. *Nanoscale* **2015**, *7*, 18424.
- (32) Zhou, J.; Liu, Z.; Li, F. *Chem. Soc. Rev.* **2012**, *41*, 1323.
- (33) Carling, C. J.; Nourmohammadian, F.; Boyer, J. C.; Branda, N. R. *Angew. Chem., Int. Ed.* **2010**, *49*, 3782.
- (34) Yang, Y.; Shao, Q.; Deng, R.; Wang, C.; Teng, X.; Cheng, K.; Cheng, Z.; Huang, L.; Liu, Z.; Liu, X.; Xing, B. *Angew. Chem., Int. Ed.* **2012**, *51*, 3125.
- (35) Yang, Y.; Velmurugan, B.; Liu, X.; Xing, B. *Small* **2013**, *9*, 2937.
- (36) Dai, Y.; Xiao, H.; Liu, J.; Yuan, Q.; Ma, P.; Yang, D.; Li, C.; Cheng, Z.; Hou, Z.; Yang, P.; Lin, J. *J. Am. Chem. Soc.* **2013**, *135*, 18920.
- (37) Min, Y.; Li, J.; Liu, F.; Yeow, E. K.; Xing, B. *Angew. Chem., Int. Ed.* **2014**, *53*, 1012.
- (38) Liu, J.; Bu, W.; Pan, L.; Shi, J. *Angew. Chem., Int. Ed.* **2013**, *52*, 4375.
- (39) Li, W.; Chen, Z.; Zhou, L.; Li, Z.; Ren, J.; Qu X. *J. Am. Chem. Soc.* **2015**, *137*,

8199.

(40) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494.

(41) Jiang, S.; Zhang, Y.; Lim, K. M.; Sim, E. K. W.; Ye, L. *Nanotechnology* **2009**, *20*, 155101.

(42) Yang, Y.; Liu, F.; Liu, X.; Xing, B. *Nanoscale* **2013**, *5*, 231.

(43) Lin, M.; Gao, Y.; Diefenbach, T. J.; Shen, J. K.; Hornicek, F. J.; Park, Y. I.; Xu, F.; Lu, T. J.; Amiji, M.; Duan, Z. *ACS Appl. Mater. Interfaces* **2017**, *9*, 7941.

(44) Dai, T.; Fuchs, B. B.; Coleman, J. J.; Prates, R. A.; Astrakas, C.; St Denis, T. G.; Ribeiro, M. S.; Mylonakis, E.; Hamblin, M. R.; Tegos, G. P. *Front Microbiol.* **2012**, *3*, 120.

(45) Zou, W.; Visser, C.; Maduro, J. A.; Pshenichnikov, M. S.; Hummelen, J. C. *Nature Photon.* **2012**, *6*, 560.

(46) Lucky, S. S.; Soo, K. C.; Zhang, Y. *Chem. Rev.* **2015**, *115*, 1990.

(47) Mehraban, N.; Freeman, H. S. *Materials* **2015**, *8*, 4421.

(48) Zhang, P.; Steelant, W.; Kumar, M.; Scholfield, M. J. *Am. Chem. Soc.* **2007**, *129*, 4526.

(49) Idris, N. M.; Gnanasammandhan, M. K.; Zhang, J.; Ho, P. C.; Mahendran, R.; Zhang, Y. *Nat. Med.* **2012**, *18*, 1580.

(50) Brown, J. M.; Wilson, W. R. *Nat. Rev. Cancer* **2004**, *4*, 437.

(51) Liu, Y.; Liu, Y.; Bu, W.; Cheng, C.; Zuo, C.; Xiao, Q.; Sun, Y.; Ni, D.; Zhang, C.; Liu, J.; Shi, J. *Angew. Chem., Int. Ed.* **2015**, *54*, 8105.

(52) Lu, S.; Tu, D.; Hu, P.; Xu, J.; Li, R.; Wang, M.; Chen, Z.; Huang, M.; Chen, X.

Angew. Chem., Int. Ed. **2015**, *54*, 7915.

(53) Lai, J.; Zhang, Y.; Pasquale, N.; Lee, K. B. *Angew. Chem., Int. Ed.* **2014**, *53*, 14419.

(54) Wang, D.; Xue, B.; Kong, X.; Tu, L.; Liu, X.; Zhang, Y.; Chang, Y.; Luo, Y.; Zhao, H.; Zhang, H. *Nanoscale* **2015**, *7*, 190.

(55) Hou, Z.; Deng, K.; Li, C.; Deng, X.; Lian, H.; Cheng, Z.; Jin, D.; Lin, J. *Biomaterials* **2016**, *101*, 32.

(56) Wang, X.; Wang, C.; Cheng, L.; Lee, S. T.; Liu, Z. *J. Am. Chem. Soc.* **2012**, *134*, 7414.

(57) Melancon, M. P.; Zhou, M.; Li, C. *Acc. Chem. Res.* **2011**, *44*, 947.

(58) Jaque, D.; Martínez Maestro, L.; del Rosal, B.; Haro-Gonzalez, P.; Benayas, A.; Plaza, J. L.; Martín Rodríguez, E.; García Solé, J. *Nanoscale* **2014**, *6*, 9494.

(59) Dong, B.; Xu, S.; Sun, J.; Bi, S.; Li, D.; Bai, X.; Wang, Y.; Wang, L.; Song, H. *J. Mater. Chem.* **2011**, *21*, 6193.

(60) Cheng, L.; Yang, K.; Li, Y.; Zeng, X.; Shao, M.; Lee, S. T.; Liu, Z. *Biomaterials* **2012**, *33*, 2215.

(61) Kim, C.; Favazza, C.; Wang, L. V. *Chem. Rev.* **2010**, *110*, 2756.

(62) Fried, N. M.; Burnett, A. L. *Nat. Rev. Urol.* **2015**, *12*, 451.

(63) Nie, L.; Chen, X. *Chem. Soc. Rev.* **2014**, *43*, 7132.

(64) Dragulescu-Andrasi, A.; Kothapalli, S. R.; Tikhomirov, G. A.; Rao, J.; Gambhir, S. S. *J. Am. Chem. Soc.* **2013**, *135*, 11015.

(65) Li, K.; Liu, B. *Chem. Soc. Rev.* **2014**, *43*, 6570.

- (66) Pu, K.; Shuhendler, A. J.; Jokerst, J. V.; Mei, J.; Gambhir, S. S.; Bao, Z.; Rao, J. *Nature Nanotech.* **2014**, *9*, 233.
- (67) Fan, Q.; Cheng, K.; Hu, X.; Ma, X.; Zhang, R.; Yang, M.; Lu, X.; Xing, L.; Huang, W.; Gambhir, S. S.; Cheng, Z. *J. Am. Chem. Soc.* **2014**, *136*, 15185.
- (68) Zhou, Y.; Wang, D.; Zhang, Y.; Chitgupi, U.; Geng, J.; Wang, Y.; Zhang, Y.; Cook, T. R.; Xia, J.; Lovell, J. F. *Theranostics* **2016**, *6*, 688.

Chapter 2

Enzyme-Responsive Upconversion Nanoplatfom for Enhanced Platinum(IV) Prodrug Activation and Real-Time Evaluation of Therapeutic Efficiency

2.1 Introduction

In recent decades, cancer has been a significant challenge to health all over the world.¹ Until now, the accurate diagnostic techniques and effective therapeutic strategies have already been focused for the treatment of various cancers in clinics.^{2,3} Traditional diagnosis for cancer therapeutic effect is primarily evaluated based on relative volume and morphology of tumor, which is limited by early and reliable prediction of therapeutic efficiency. Hence, new strategies that achieve non-invasive and precise evaluation of tumor status in early stages and indicate delicate response in cancer therapeutic process, are highly desirable in clinics for effective cancer treatment. In recent years, imaging technologies have been demonstrated to be such a potential way to meet the requirement in this field.⁴ Several effective imaging modalities, including fluorescent imaging, magnetic resonance imaging, photoacoustic imaging, positron emission tomography, and X-ray computed tomography, have been powerful techniques for biomedical research and clinical diagnostics. Compared with other expensive and complex techniques, fluorescent imaging represents a simple, rapid, sensitive, and economic modality for the study of cancer diagnosis and therapy.⁵ Usually, the progress of fluorescent imaging of tumor is depending on the availability of novel imaging contrast agents, which should be non-toxic, selective, and sensitive for accurate evaluation of cancer treatment. However, conventional imaging probes, such as organic fluorophores and semiconducting quantum dots, suffer from their inherent limitations including photobleaching, auto-fluorescence, and cytotoxicity.^{6,7} More importantly, their

down-conversion properties result in limited tissue penetration depth and weak signal intensity in living system. Therefore, the development of innovative materials with promising bioimaging properties is highly demanded.

Recently, UCNs have emerged as an outstanding nanomaterial in biomedical applications due to their unique optical properties.⁸⁻¹⁰ In particular, UCNs exhibit efficient and broad emissions from UV, visible, to NIR light upon NIR light irradiation (usually at 980 nm). Such promising properties enable UCNs to be ideal NIR light-responsive imaging contrast agent which can greatly suppress auto-fluorescence, and importantly, allow deep tissue penetration for photo-stable tracking therapeutics *in vivo*. Currently, most of the conventional studies on UCNs for cancer targeted imaging or therapy are based on nonspecific enhanced EPR effect, pH-sensitive polymer encapsulation, and direct conjugation of tumor affinity ligands (such as FA and the tripeptide Arg-Gly-Asp (RGD) sequence) onto UCNs surfaces. However, the complex and dynamic physiological environment present significant barriers to such routine tumor recognition, and great challenges still remain to design innovative UCNs nanoplatform for more specific localization and effective tumor treatment.

Herein, we proposed a smart NIR light-controlled strategy for enhanced Pt(IV) prodrug activation based on selective localization of UCNs into targeted tumor cells *via* covalent cross-linking reaction.¹¹⁻¹⁴ Two different kinds of UCNs nanoplatforms were constructed through involving tumor over-expressed Cathepsin B enzyme (CtsB)¹⁵ responsive peptide (peptide-1) on the surface of UCNs (UCN-1), and cross-linking 2-cyanobenzothiazole (CBT) group containing peptide (peptide-2) on the exterior of UCNs (UCN-2), respectively (Figure 1). Such unique enzyme triggered condensation of UCNs could greatly improve the specificity by selectively aggregating drugs or imaging probes (UCN-1 and UCN-2) into tumor areas, and thus largely improve antitumor therapeutic and imaging effects through enhanced energy transfer efficiency than those individual UCNs. More significantly, the increased release of localized Pt(II) drug could largely induce the death of tumor cells, and the

generated ROS could be monitored in real-time by the ROS-responsive probe on UCNs.¹⁶ Therefore, this novel UCNs nanoplatform could greatly enhance the efficiency of tumor therapeutic effect based on enhanced photoactivation of Pt(IV) prodrug, and evaluate the antitumor activities owing to the generated ROS by real-time imaging during the treatment period.

Figure 1. Schematic illustration of enzyme-responsive cross-linking of UCN-1 and UCN-2 for enhanced photoactivation of Pt(IV) prodrug upon NIR light irradiation and real-time evaluation of therapeutic efficiency during the treatment period.

2.2 Materials and methods

Materials. Mal-dPEG₂-NHS was obtained from Quanta Biodesign. Other chemical reagents were purchased from Sigma-Aldrich. The solvents for synthesizing peptides were dehydrated and kept with activated 4 Å molecular sieves. The commercial reagents were used as received. The human lung adenocarcinoma epithelial cell line (A549) and mouse embryonic fibroblast cell line (NIH/3T3) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂. All the commercially reagents were used as received unless otherwise noted.

Instruments. Transmission electron microscope (TEM) images were measured

on an FEI EM208S TEM (Philips) at 100 kV. ^1H -NMR and mass spectra were respectively recorded on a 300 MHz Bruker spectrometer and a Thermo LCQ Deca XP MAX instrument (ESI measurements). Semi-preparative high-performance liquid chromatography (HPLC, Shimadzu) system was performed on an Alltima C-18 column (250×10 mm) at a flow rate of 3 mL min⁻¹. UV-vis absorption, fluorescence emission, and Fourier-transform infrared (FTIR) spectra were respectively measured on a Beckman Coulter DU800 spectrometer, a Varian Cary Eclipse fluorescence spectrophotometer and a Shimadzu prestige-21 FTIR spectrophotometer. The upconversion fluorescence emission spectra of UCNs excited at 980 nm were performed on a DM150i monochromator fitted with an R928 photon-counting photomultiplier tube (PMT) and a diode laser of 980 nm. Platinum content was recorded using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7700). Confocal laser scanning microscopy (CLSM) imaging was carried out on an LSM 510 instrument (Zeiss). Flow cytometry (FCM) was performed using LSR II instrument. Photo-irradiation experiments were done with an MDL-H-980 nm laser (0-2.6 W cm⁻²). The cell viability was evaluated by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on a Tecan's Infinite M200 microplate reader.

Synthesis of NaYF₄:Yb/Tm (20/0.2%) upconversion nanoparticles (UCNs).

The UCNs were obtained following a reference published before.¹⁷ In a 50 mL flask, methanol (2 mL) containing RECl₃ (RE = Y, Yb, and Tm, 0.2 M), oleic acid (3 mL) and 1-octadecene (7 mL) were added. The mixture was heated to 160 °C for 30 min before cooling down to room temperature. Methanol (5 mL) containing NH₄F (1.6 mmol) and NaOH (1 mmol) was added before stirring for 30 min. Then methanol was evaporated and the solution was kept at 300 °C for 1.5 h under nitrogen atmosphere. After cooling down to room temperature, the nanoparticles were precipitated using ethanol and collected by centrifugation several times.

Synthesis of silica-coated UCNs (UCNs@SiO₂). UCNs@SiO₂ was synthesized by using the classical Stöber method.¹⁸ Briefly, in cyclohexane (16 mL) dissolving

UCNs (4 mg), Triton X-100 (2 mL), 1-hexanol (2 mL), and DI water (680 μ L) were added. After stirring for 0.5 h, tetraethyl orthosilicate (TEOS, 10 μ L) and (3-aminopropyl)triethoxysilane (APTES, 2 μ L) were added. Then NH_4OH (200 μ L) was added after 6 h. After reaction for 12 h, the UCNs@SiO_2 was acquired using centrifugation and washed with ethanol thrice and dried under vacuum.

Synthesis of UV light-photoactive Pt(IV) prodrug (*trans,trans,trans*-[Pt(N₃)₂(OH)(O₂CCH₂CH₂CO₂H)(py)₂]). The complex *trans,trans,trans*-[Pt(N₃)₂(OH)₂(py)₂] was synthesized as previously described (Figure 2).¹⁹ Succinic anhydride (0.15 mmol) and *trans,trans,trans*-[Pt(N₃)₂(OH)₂(py)₂] (0.10 mmol) were added to dimethylformamide (DMF, 1 mL) for stirring at 75 °C for 4 h. After drying under vacuum, the remaining oil was dissolved in acetone (1 mL). Yellow solid was precipitated by adding diethyl ether and dried under vacuum (yield: 61%). ¹H-NMR (CD₃OD): δ = 8.94 (m, 4H), 8.31 (t, 2H), 7.80 (t, 4H), 2.64 (m, 2H), 2.58 (m, 2H). ESI-MS (MeOH/H₂O): m/z: 594.01 ([M+Na]⁺); 1164.55 ([M₂+Na]⁺).

Figure 2. Synthesis of UV light-photosensitive Pt(IV) prodrug.

Synthesis of peptide-1. The peptide Phe-Lys-Cys(StBu)-Gly-Lys-Lys-Lys-Lys-Lys-Cys (FKC(StBu)GKKKKKC, peptide-1) was obtained using Fmoc-chemistry based solid phase peptide synthesis (SPPS) with 2-chlorotrityl chloride resin (Figure 3). 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/diisopropylethylamine

(DIPEA) was used for coupling reaction at room temperature and 20% piperidine/DMF solution was used to deprotect Fmoc group in the terminal of the peptide. After washing with DMF and dichloromethane (DCM) several times, 95% trifluoroacetic acid (TFA) was used to cleave the peptide-1 from the resin and remove Boc and Trt protecting group under stirring for 2 h. The peptide-1 was purified using reverse-phase HPLC and then frozen-dried. ESI-MS ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$): m/z : 1286.41 ($[\text{M}+\text{H}]^+$, calcd. 1285.72); 643.29 ($[\text{M}+2\text{H}]^{2+}/2$, calcd. 642.86).

Figure 3. Chemical structure of peptide-1.

Synthesis of enzyme responsive peptide-2. The synthetic route of Glu(Lys-Lys-Lys-Lys-Lys-Cys)-CBT (E(KKKKKK)-CBT, peptide-2) was shown in Figure 4. Isobutyl chloroformate (IBCF, 21.6 μL , 0.171 mmol) was added to anhydrous tetrahydrofuran (5.0 mL) containing Fmoc-Glu(OtBu)-OH (72.9 mg, 0.171 mmol) and 4-methylmorpholine (MMP, 37.6 μL , 0.342 mmol) at 0 $^{\circ}\text{C}$ under N_2 and the mixture was stirred for 20 min, then added with 2-cyano-6-aminobenzothiazole (CBT, 20.0 mg, 0.114 mmol) for further stirring at 0 $^{\circ}\text{C}$ for 2 h before overnight at room temperature. Water (30 mL) was added to the reaction mixture by extracting with ethyl acetate (3 $\times$ 30 mL). The organic phase was combined and dried using Na_2SO_4 . After filtration, the solvent was evaporated. The pure product Fmoc-Glu(OtBu)-CBT was obtained by normal flash chromatography with ethyl acetate/hexane (1:1). After drying by a rotary evaporator, TFA/ H_2O (95%, 2 mL) solution was added with stirring at room temperature for 2 h. The white product Fmoc-Glu-CBT was precipitated using diethyl ether and purified by HPLC. The Resin-CKKKKK-NH₂ was synthesized by SPPS and coupled with excess

Fmoc-Glu-CBT in the presence of HBTU/DIPEA. After cleaving the Fmoc group by 20% piperidine, all the side chain protect groups (Boc and Trt) were deprotected using 95% TFA (4 mL). The final product peptide-2 was precipitated by diethyl ether and purified by HPLC. ESI-MS ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$): m/z : 1049.86 ($[\text{M}+\text{H}]^+$, calcd. 1048.33); 526.93 ($[\text{M}+4\text{H}]^{2+}/2$, calcd. 526.18).

Figure 4. Synthesis of enzyme responsive peptide-2.

Synthesis of peptide functionalized UCNs (UCNs@SiO₂-peptide). UCNs@SiO₂ (10 mg) was dissolved in DMF (3 mL) before adding with Mal-dPEG₂-NHS ester linker (10 mg) and DIPEA (2 μL). After stirring for 12 h, the UCNs@SiO₂ functionalized with Mal-dPEG₂ was centrifuged and washed with DMF thrice. After dispersing the Mal-dPEG₂-functionalized UCNs in DMF, peptide-1 (or peptide-2) (10 mM in 1 mL DMF) and DIPEA (2 μL) were added. The mixture was reacted for 24 h at room temperature under dark. The UCNs@SiO₂-peptide was centrifuged and washed with DMF several times.

Synthesis of Pt(IV) prodrug functionalized UCNs (UCNs@SiO₂-peptide-Pt(IV)). The Pt(IV) prodrug was first activated before conjugating to the amino group of the peptide on the surface of UCNs@SiO₂. Photoactive Pt(IV) prodrug (5 mg, 0.0088 mmol), 1-ethyl-3-[3-dimethylaminopropyl]- carbodiimide hydrochloride (EDC) (4.2 mg, 0.022 mmol) and *N*-hydroxysuccinimide (NHS) (1.5 mg, 0.013 mmol) were mixed in anhydrous DMF (0.5 mL). After reacting overnight at room temperature under dark,

the product Pt(IV)-NHS was precipitated with diethyl ether and collected. Then the yellow precipitation dissolved in DMF (0.5 mL) was added to DMF (0.5 mL) containing UCNs@SiO₂-peptide (5 mg) and DIPEA (3 μ L). After stirring for 24 h at room temperature under dark, the resulting UCNs@SiO₂-peptide-Pt(IV) were centrifuged and washed by DMF several times in the dark condition. The optimal loading amount of Pt(IV) prodrug on UCNs surface was determined after reaction with a different concentration of Pt(IV) prodrug (0.1-6 mg) at similar experimental conditions.

Synthesis of DCFDA functionalized UCNs (UCNs@SiO₂-peptide-Pt(IV)-DCFDA). ROS-responsive 5'-(6')-carboxy-2,7-dichlorofluorescein diacetate (COOH-DCFDA, 2.3 mg, 0.0022 mmol) was dissolved in DCM (3 mL) at 0 °C, added with IBCF (10 μ L).²⁰ After stirring for 2 h at 0 °C, the mixture was concentrated to a colorless semisolid under dark. The as-prepared UCNs@SiO₂-peptide-Pt(IV) (10 mg) dissolving in anhydrous DMF (4 mL) under nitrogen at 0 °C was added dropwise with anhydrous DMF (1 mL) containing IBCF-activated DCFDA over 0.5 h under stirring. After stirring for 2 h under dark, the resulting nanoparticles (UCNs@SiO₂-peptide-Pt(IV)-DCFDA) were collected after precipitation by diethyl ether and dried with nitrogen in dark condition. The optimal loading amount of DCFDA on UCNs surface was determined after reaction with a different concentration of DCFDA dye (0.1-4 mg) at similar experimental conditions.

Photoactivation of Pt(IV) prodrug on the UCNs@SiO₂-peptide-Pt(IV)-DCFDA. In order to release active platinum component under NIR light irradiation, the UCNs@SiO₂-peptide-Pt(IV)-DCFDA (1 mg in 50 μ L dimethyl sulfoxide (DMSO)) was irradiated with 980 nm laser under different time in the dark condition at room temperature. After centrifugation to remove nanoparticles, the supernatant containing released activated platinum component was measured by ICP-MS. The samples without NIR light irradiation were used as a control. In addition, in order to detect the loading amount of Pt(IV)

prodrug on the surface of UCNs nanoplatfrom, UCNs@SiO₂-peptide-Pt(IV)-DCFDA was incubated in 70% nitric acid and digestion was allowed at 70 °C for 12 h until the solution was clear. After diluted with water to 2% acid solution, the solution was filtered with 0.22 µm filter and analyzed by ICP-MS for platinum ions (Pt²⁺) determination.

***In vitro* fluorescence assay with ROS (H₂O₂).** Stock solutions of UCNs@SiO₂-peptide-Pt(IV)-DCFDA (1 mg mL⁻¹ in phosphate buffered saline (PBS), pH 7.4) was purged with nitrogen and stored at -20 °C. First, the DCFDA was hydrolyzed to DCFH by NaOH (0.01 M) at room temperature for 20 min, followed by readjusting the pH to 7.4 using HCl. Then the fluorescence enhancement from the oxidation of DCFH was determined by addition of H₂O₂ (0-150 µM). The excitation and emission wavelengths were 490 and 530 nm, respectively. The loading amount of DCFDA on the surface of UCNs nanoplatfrom was determined based on the strong fluorescence of DCF molecules after treatment by overdose H₂O₂ (300 µM) oxidation.

***In vitro* enzyme triggered condensation of UCNs.** The stock solution of CtsB was dissolved in acetate buffer (25 mM)/ethylenediaminetetraacetic acid (EDTA, 1 mM, pH 6.0). CtsB (10 µL, 100 nM) was first activated in DL-dithiothreitol (DTT, 60 mM)/EDTA (15 mM, 5 µL) for 30 min at 37 °C and then incubated with UCNs@SiO₂-peptide-1-Pt(IV)-DCFDA (UCN-1, 1 mg) dissolving in acetate buffer (25 mM)/EDTA (1 mM, 1% DMSO) for different time. Then UCNs@SiO₂-peptide-2-Pt(IV)-DCFDA (UCN-2, 1 mg) was added for 4 h under stirring. The aggregated UCNs were separated by centrifugation and re-dispersed in ethanol for TEM test. For the inhibitor experiment, the antipain hydrochloride inhibitor (100 µM) was pretreated with CtsB for 2 h at 37 °C.

MTT assay. A549 and NIH/3T3 cells were seeded with a cell density of 1×10⁴ cells per well on a 96-well plate in DMEM (100 µL) and cultured for 24 h at 37 °C. For NIR light irradiation experiments, the cells were treated with UCN-1 or UCN-2 at a platinum concentration of 20 µM for 8 h and then irradiated with NIR light for

different time (2 W cm⁻², 10 min break after 10 min irradiation) followed by incubation for 24 h. For cytotoxicity study, the cells were treated with UCN-1 or UCN-2 at different platinum concentrations for 8 h and then irradiated with NIR light for 1 h (2 W cm⁻², 10 min break after 10 min irradiation) followed by incubation for 24 h. The medium was removed carefully and cells were washed with PBS (pH 7.4). Then 20% MTT (100 µL) in DMEM was added to 96-well plate and incubated for 4 h at 37 °C. After removing the medium, DMSO (100 µL) was used to lyse the cells and the absorbance of formazan crystals was measured at 570 nm by a Tecan's Infinite M200 microplate reader. The cell viability was calculated according to the following equation:

$$\text{Cell viability (\%)} = \frac{\text{OD}_{570}(\text{sample}) - \text{OD}_{570}(0)}{\text{OD}_{570}(\text{control}) - \text{OD}_{570}(0)} \times 100\%$$

in which OD₅₇₀ (sample) is the absorbance from the well treated with Pt(IV) prodrug, OD₅₇₀ (control) is the absorbance from the well treated without Pt(IV) prodrug, and OD₅₇₀ (0) represents the blank absorbance. Each experiment was repeated thrice and the average values were used for analysis.

Fluorescence imaging of cells. Cells (including A549 and NIH/3T3 cells) were seeded in an ibidi confocal µ-dish (35 mm, plastic bottom) with a cell density of 1×10⁵ cells per well in DMEM (1 mL) and incubated at 37 °C for 24 h. For NIR light irradiation experiments, the cells were incubated with UCN-1 or UCN-2 at a platinum concentration of 20 µM for 8 h and then irradiated with NIR light for 1 h (2 W cm⁻², during the light irradiation process, 10 min break after 10 min irradiation). Then the treated cells were maintained for 24 h. After removing the medium carefully, cells were rinsed with DMEM and PBS (pH 7.4). The propidium iodide (PI) staining was performed on standard detection kit (Life Technologies) according to manufacturer's protocol. After cell nuclei stained by DAPI (5 µg mL⁻¹), the cells were washed with DMEM and PBS (pH 7.4). Then the samples were applied for imaging measurements on LSM510 confocal laser scanning microscope.

Similarly, the cellular imaging was also performed using CtsB inhibitor (antipain

hydrochloride, 100 μ M) for 2 h before the addition of UCN-1 or UCN-2 for 8 h incubation. Then the cells were irradiated with NIR light for 1 h (2 W cm^{-2} , during the light irradiation process, 10 min break after 10 min irradiation) and treated as the same procedures above.

FCM analysis. A549 or NIH/3T3 cells were seeded with a cell density of 1×10^5 cells per well in 24-well plates within DMEM (1 mL) and maintained at 37 °C for 24 h. Then the cells were treated with UCN-1 or UCN-2 at a platinum concentration of 20 μ M for 8 h incubation and irradiated with NIR light for 1 h (2 W cm^{-2} , during the light irradiation process, 10 min break after 10 min irradiation). After 24 h incubation, the cells were washed three times with DMEM and then trypsinized. The cells were diluted by PBS (2 mL) for flow cytometry measurement.

2.3 Results and discussion

2.3.1 Preparation and characterization of UCN-1 and UCN-2

To construct the enzyme-responsive UCNs nanoplatform for NIR light-mediated Pt(IV) prodrug activation and real-time evaluation of therapeutic efficiency (Figure 5), the oleic acid coated UCNs were prepared with an average size of around 20 nm as confirmed by TEM and UCL spectra in Figure 6. After coating with silica shell, the average size of UCNs@SiO₂ was around 40 nm with silica shell thickness of 10 nm and a great size distribution. A short PEG-maleimide linker (Mal-dPEG₂-NHS) was used to modify the surface of UCNs@SiO₂ with maleimide group which could react with the thiol group of peptide-1 or peptide-2. For the photo-controlled drug release, a photoactive Pt(IV) prodrug was prepared and activated to NHS ester first *via* an EDC-based coupling reaction, and then anchored on the surface of UCNs@SiO₂-peptide. Simultaneously, a ROS sensitive dye (DCFDA) was also linked with the amino groups on the peptide-1 or peptide-2 to afford UCN-1 or UCN-2, respectively. In order to optimize the loading concentration of labelling of Pt(IV) prodrug and DCFDA on the surface of UCNs, ICP-MS analysis and

Figure 5. Schematic illustration of (A) synthesis of UCN-1 and UCN-2, (B) enzyme-triggered cross-linking of UCN-1 and UCN-2, and (C) NIR light-triggered Pt(IV) prodrug activation and ROS-induced DCFDA fluorescence increase in tumor cells during the treatment period.

fluorescence spectra were utilized, which demonstrated that the loading amounts of DCFDA on the surface of UCN-1 and UCN-2 were determined as 5.1 wt% and 4.9 wt%, and there was $3.5 \mu\text{M mg}^{-1}$ and $3.2 \mu\text{M mg}^{-1}$ on UCN-1 and UCN-2, respectively.

2.3.2 *In vitro* enzyme triggered cross-linking between UCN-1 and UCN-2

To study the enzyme-triggered cross-linking between UCN-1 and UCN-2, they

Figure 6. TEM images of hydrophobic UCNs (A), UCNs@SiO₂ (B), UCNs@SiO₂-peptide-1 (C), UCN-1 (D), and corresponding UCL spectra (E-H) of these nanostructures (1 mg mL⁻¹ in DMF), respectively.

were co-incubated with tumor over-expressed CtsB and glutathione (GSH) simultaneously. The H-Phe-Lys-OH peptide on UCN-1 could be selectively recognized and cleaved by CtsB enzyme to expose the cysteine residual on peptide-1. After the breakage of disulfide to remove StBu protecting group by GSH reductant, the cysteine residual could react with CBT group on UCN-2, leading to the cross-linking between UCN-1 and UCN-2 (Figure 5). The nanoparticles condensation was demonstrated by TEM imaging and dynamic light scattering (DLS) analysis. As shown in Figure 7A, upon enzyme treatment for 4 h, the significant aggregation between UCN-1 and UCN-2 was observed in PBS (pH = 5.0), while negligible condensation was determined in the presence of CtsB enzyme inhibitor (antipain

Figure 7. TEM images (A-B) and DLS analysis (C) of 100 nM CtsB-triggered aggregation between UCN-1 and UCN-2 at the same concentration (1 mg mL^{-1}) in the absence (A) and presence (B) of inhibitor (antipain hydrochloride, $100 \mu\text{M}$).

hydrochloride) (Figure 7B). Moreover, these results were also demonstrated by DLS results at different time after reaction of UCN-1 and UCN-2 upon enzyme treatment. As shown in Figure 7C, the size of DLS results presented significant increment after enzyme-triggered aggregation of UCN-1 and UCN-2 until the incubation time was more than 4 h, indicating the obvious time-dependent cross-linking occurred between

Figure 8. Time-dependent photoactivation of Pt(IV) prodrug upon NIR light irradiation at different conditions: (1) UCN-1 (1 mg mL^{-1}), (2) UCN-2 (1 mg mL^{-1}), (3) UCN-1 and UCN-2 (1 mg mL^{-1}), (4) UCN-1 and UCN-2 with CtsB (100 nM), and (5) UCN-1 and UCN-2 with CtsB and inhibitor ($100 \mu\text{M}$). UCN-1 and UCN-2 without NIR light treatment (NIR(-)) were used as controls.

different UCNs nanoplatfrom. While negligible aggregation was observed when the enzyme was pretreated with CtsB inhibitor, indicating that the UCNs condensation was specifically responsible for CtsB enzyme.

Inspired by these results, we investigated the enhanced photo-release of Pt(IV) prodrug by ICP-MS analysis from UCNs nanostructures upon NIR light irradiation. As shown in Figure 8, negligible Pt(II) component was released from UCN-1 or UCN-2 without NIR light illumination, indicating the great stability of this Pt(IV) prodrug in the dark condition. Upon 980 nm laser illumination, effective Pt(II) drug release was observed on UCN-1 and UCN-2 and the released amount increased with extended irradiation time. Importantly, when UCN-1 and UCN-2 were treated with CtsB enzyme, the enhanced release of Pt(II) drug was determined compared with UCN-1 or UCN-2 alone at the same conditions (e.g., $\sim 22.5\%$ increase after 6h NIR light irradiation), which might be attributed to the enhanced energy transfer efficiency by utilizing ambient upconverted UV light from the cross-linked UCNs nanoplatfroms. Moreover, in the presence of specific CtsB enzyme inhibitor, negligible change of Pt(II) drug release was detected on the surface of UCNs. These results indicated that the effective cross-linking between UCN-1 and UCN-2 could improve the photoactivation of Pt(IV) prodrug upon NIR light illumination.

Figure 9. The DCFDA fluorescence response of (A) UCN-1 (1 mg mL^{-1}) and (B) UCN-2 (1 mg mL^{-1}) in the presence of different concentration of H_2O_2 (0-150 μM).

In order to demonstrate the ROS response properties of UCNs nanoplatfrom, we

Figure 10. The UCL spectra of UCN-1 and UCN-2 (1 mg mL^{-1}) in the presence of H_2O_2 ($100 \text{ }\mu\text{M}$) and CtsB (100 nM) at different incubation time.

further determined the enhanced fluorescence of DCFDA in the presence of H_2O_2 at different concentrations. As shown in Figure 9, the fluorescence of DCFDA showed a significant increase at the emission wavelength of 530 nm under H_2O_2 treatment, indicating that the present UCNs nanoplateforms were responsive to the ROS levels in buffers. Moreover, the UCL spectra of cross-linked UCNs nanoplateform in the presence of H_2O_2 was also determined upon incubating with UCN-1 and UCN-2 with CtsB at a different time. As shown in Figure 10, the decreased UCL intensities of UCNs at 340 , 360 , 460 and 480 nm at different enzyme incubation time were observed along with the remarkable increase of ROS-responsive DCFDA emission at 540 nm (2.1-fold), which could monitor the ROS levels during the tumor cell death process. These results strongly demonstrated the enhanced energy transfer occurred between cross-linked UCNs nanoplateform and modified moieties including Pt(IV) prodrug and DCFDA chromophores upon NIR light irradiation.

2.3.3 Enhanced cytotoxicity and real-time evaluation of tumor therapeutic efficiency based on UCNs nanoplateform

In order to further demonstrate their potential cytotoxicity upon NIR light irradiation, a standard MTT assay was evaluated after cross-linking of UCN-1 and UCN-2 in CtsB over-expressed A549 cells and enzyme non-expressed NIH/3T3 cells. As shown in Figure 11, little cytotoxicity was observed for these two cell lines

Figure 11. The cytotoxicity of UCN-1 and UCN-2 toward (A, C) A549 cells and (B, D) NIH/3T3 cells with (A, B) different NIR laser irradiation time and (C, D) UCNs nanoplatform with different platinum concentrations under 980 nm laser irradiation for 60 min (2 W cm^{-2}).

without UCNs nanoparticles or incubation with UCNs@SiO₂, demonstrating that the nanoparticle itself and NIR light have no cytotoxic effects to the tumor cells. However, in the A549 cells with CtsB enzyme overexpression, the significant enhancement of cell death ($\sim 52\%$ cell viability) was detected compared with single UCN-1 ($\sim 72\%$ cell viability) or UCN-2 ($\sim 73\%$ cell viability) alone upon NIR light irradiation for 60 min. Moreover, in the enzyme non-expressed NIH/3T3 cells, no obvious difference was monitored in the cell viability after treatment with UCN-1, UCN-2, or UCN-1 and UCN-2, respectively, indicating that the proposed UCNs nanoplatform could effectively improve the therapeutic effects in the CtsB-overexpressed tumor cells under NIR light irradiation.

To evaluate the therapeutic response during the process of tumor cells death,

Figure 12. Confocal imaging of (A) A549 and (B) NIH/3T3 cells in the presence of (1) UCN-1, (2) UCN-2, (3) UCN-1 and UCN-2, and (4) UCN-1 and UCN-2 with inhibitor after NIR light irradiation for 60 min (2 W cm^{-2}). The platinum amounts on UCNs nanoplatform was $20 \mu\text{M}$ for all samples. Blue: DAPI (E_x : 405 nm, E_m : 460/50 nm), green: ROS-sensitive DCFDA (E_x : 488 nm, E_m : 530/50 nm), red: propidium iodide (PI) (E_x : 543 nm, E_m : 580/50 nm).

Figure 13. UCL imaging of (A) A549 and (B) NIH/3T3 cells in the presence of (1) UCN-1, (2) UCN-2, (3) UCN-1 and UCN-2, and (4) UCN-1 and UCN-2 with inhibitor upon NIR light irradiation for 60 min (2 W/cm^{-2}). The concentration of platinum was $20 \mu\text{M}$ for all samples. DAPI (E_x : 405 nm, E_m : 460/50 nm), UCL (E_x : 980 nm, E_m : short-pass 750 nm), UCL-G (E_x : 980 nm, E_m : 530/50 nm).

confocal imaging was also utilized to investigate the enhanced fluorescence of ROS-sensitive DCFDA molecules. As shown in Figure 12, few green fluorescence of DCFDA was observed, indicating the negligible ROS was produced when A549 cells were incubated with UCN-1 or UCN-2 alone upon 980 nm laser irradiation. However, significant enhancement of DCFDA fluorescence was determined after incubation of UCN-1 and UCN-2 together in A549 cells, which could be inhibited by the pre-treatment with specific CtsB inhibitor (antipain hydrochloride, 10 μ M). Moreover, there was no obvious change of green fluorescence in the enzyme non-expressed NIH/3T3 cells, and the UCL imaging with more aggregated UCNs in A549 cell than NIH/3T3 also were indicated the significant cross-linking of UCNs nanoplatform in tumor cells (Figure 13). These results demonstrated that the enhanced cell cytotoxicity of UCNs nanoplatforms after enzyme-triggered cross-linking could amplify the generation of ROS in the process of tumor cell death. Furthermore, quantitative FCM analysis also indicated that more significant increase of DCFDA fluorescence was observed in enzyme-overexpressed A549 cells than that in the CtsB non-expressed NIH/3T3 cells (Figure 14). All of these results strongly demonstrated that the established UCNs nanoplatform could not only improve the therapeutic effects by enhanced Pt(IV) prodrug photoactivation in the CtsB-overexpressed tumor cells under

Figure 14. Quantitative FCM analysis of (A) A549 and (B) NIH/3T3 cells upon NIR light irradiation for 60 min (2 W cm^{-2}) in the presence of UCN-1 (red), UCN-2 (green), UCN-1 and UCN-2 (blue), UCN-1 and UCN-2 with inhibitor (cyan), respectively. The concentration of platinum was 20 μ M for all samples.

NIR light irradiation, but also evaluate the antitumor activities owing to the generated ROS by real-time imaging during the treatment period.

2.4 Conclusion

In summary, we have successfully presented a CtsB enzyme-responsive UCNs nanoplatform for enhanced Pt(IV) prodrug activation upon NIR light illumination. This unique tumor over-expressed CtsB enzyme could induce the effective cross-linking of UCNs in tumor cells, which could further improve the tumor specificity by aggregating drugs and imaging probes in targeted tumor areas, and thus improve therapeutic and imaging effects based on the enhanced energy transfer efficiency than that in single UCNs nanostructures. Moreover, under NIR light irradiation, the localized photoactive Pt(IV) prodrugs were released to induce the tumor cell death, which could be monitored by the loaded ROS-sensitive dye (DCFDA) to evaluate the antitumor activities during the therapeutic period. Such rational design provided an innovative strategy to further improve the treatment effects in enzyme-overexpressed tumor regions upon NIR light irradiation, and evaluate the tumor response to therapeutic drugs in an early stage. The corresponding experiments in living animals are in progress to further confirm the biomedical applications of the present design *in vivo*.

Despite the initial results are successfully achieved in cellular studies, it should be noted that the present design is based on the covalent crosslinking of two different kinds of UCNs nanoplatforms, which are limited by their complicated preparation for further bioapplications. More importantly, the utilization of 980 nm NIR light would induce unavoidable overheating effect due to the strong water absorption at this wavelength. Thus, the further design of simple UCNs-based nanosystems which could achieve covalent crosslinking of UCNs *in vivo* and overcome these undesired shortcomings in biomedical applications, still remain in high-demand.

2.5 References

- (1) Bray, F.; Jemal, A.; Grey, N.; Ferlay, J.; Forman, D. *Lancet Oncol.* **2012**, *13*, 790.
- (2) Ng, K. K.; Lovell, J. F.; Zheng, G. *Acc. Chem. Res.* **2011**, *44*, 1105.
- (3) Melancon, M. P.; Zhou, M.; Li, C. *Acc. Chem. Res.* **2011**, *44*, 947.
- (4) Ding, H.; Wu, F. *Theranostics* **2012**, *2*, 1040.
- (5) Kobayashi, H.; Ogawa, M.; Alford, R.; Choyke, P. L.; Urano, Y. *Chem. Rev.* **2009**, *110*, 2620.
- (6) Park, J. H.; von Maltzahn, G.; Ruoslahti, E.; Bhatia, S. N.; Sailor, M. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 7284.
- (7) Yezhelyev, M. V.; Qi, L.; O'Regan, R. M.; Nie, S.; Gao, X. *J. Am. Chem. Soc.* **2008**, *130*, 9006.
- (8) Wang, C.; Cheng, L.; Liu, Z. *Theranostics* **2013**, *3*, 317.
- (9) Li, X.; Zhao, D.; Zhang, F. *Theranostics* **2013**, *3*, 292.
- (10) Wang, F.; Liu, X. *Acc. Chem. Res.* **2014**, *47*, 1378.
- (11) Farrer, N. J.; Woods, J. A.; Salassa, L.; Zhao, Y.; Robinson, K. S.; Clarkson, G.; Mackay, F. S.; Sadler, P. J. *Angew. Chem. Int. Ed.* **2010**, *49*, 8905.
- (12) Dai, Y.; Xiao, H.; Liu, J.; Yuan, Q. et al. *J. Am. Chem. Soc.* **2013**, *135*, 18920.
- (13) Cao, C.; Chen, Y.; Wu, F.; Deng, Y.; Liang, G. *Chem. Commun.* **2011**, *47*, 10320.
- (14) Ye, D.; Shuhendler, A. J.; Cui, L.; Tong, L.; Tee, S. S.; Tikhomirov, G. ; Felsher, D. W.; Rao, J. *Nature Chem.* **2014**, *6*, 519.
- (15) Yang, Y.; Shao, Q.; Deng, R.; Wang, C.; Teng, X.; Cheng, K.; Cheng, Z.; Huang, L.; Liu, Z.; Liu, X.; Xing, B. *Angew. Chem. Int. Ed.* **2012**, *51*, 3125.
- (16) Simon, H. U.; Haj-Yehia, A.; Levi-Schaffer, F. *Apoptosis* **2000**, *5*, 415.

- (17) Wang, F.; Han, Y.; Lim, C.; Lu, Y.; Wang, J.; Xu, J.; Chen, H.; Zhang, C.; Hong, M.; Liu, X. *Nature* **2010**, *463*, 1061.
- (18) Qian, H.; Guo, H.; Ho, P.; Mahendran, R.; Zhang, Y. *Small* **2009**, *5*, 2285.
- (19) Min, Y.; Li, J.; Liu, F.; Yeow, E. K. L.; Xing, B. *Angew. Chem. Int. Ed.* **2014**, *53*, 1012.
- (20) Lloyd, Q.; Kuhn, M. A.; Gay, C. V. *J. Biol. Chem.* **1995**, *270*, 22445.

Chapter 3

Intracellular Covalent Cross-linking of Upconversion Nanostructures for Tumor Localization and Theranostics

3.1 Introduction

Currently, therapeutic and diagnostic techniques based on supramolecular assemblies and functional nanomaterials have been extensively recognized as promising nanomedicine platforms for the battle against many urgent health concerns including cancer, cardiovascular, and neurodegenerative diseases as well as other life-threatening illnesses.^{1,2} The remarkable biomedical application of nanomaterials could be mainly attributed to their unique photo-physical properties, high surface area, and multivalent binding ability.³ Despite the leap forward in the continuous breakthroughs in biomedical research, critical challenge still remains in designing targeted nanoplatforms that are capable of selectively localizing at the specific diseases - in particular - tumor sites for early-stage diagnosis and effective treatment.⁴ One emerging strategy to achieve high targeting selectivity is to conjugate the nanomaterials with affinity ligands including small organic moieties or bioactive molecules that can bind to receptors in the tumor cells.⁵ However, varying expression levels of the receptors, complex and dynamic physiological cell environments may potentially pose the issue of nonspecific recognition for this ligand-mediated tumor affinity. Therefore, more specific targeting approaches are demanded that do not solely rely on receptors to differentiate tumor and normal cells.⁶ Indeed, some bioorthogonal reactions provide feasibility to locate functional nanostructures into tumor cells mostly through their electrostatic or covalent binding to biomolecules in living system.⁷ Nevertheless, the effective bioorthogonal functionalities that can selectively respond to the dynamic processes of native environment are still ongoing

challenges for *in vivo* applications.⁸ Hence, different approaches that enable sensitive recognition of dynamic tumor micro-environment, and more importantly, can further trigger the tumor-specific localization of theranostic nanomaterials *in vivo* are highly desirable, and extensive studies still need to be further investigated.

Recently, rare-earth doped UCNs have been widely demonstrated for use in biomedical applications. In general, UCNs particles offer deep tissue penetration capability for enhanced bioimaging and better tumor treatment arising from their unique non-linear photon upconverting process upon light irradiation at NIR window.⁹⁻¹² As with the majority of nanomaterials for theranostic tumor studies, the effective targeting of upconversion materials mainly relies on receptor-mediated interactions, and the specific cellular localization of UCNs nanostructures at the tumor site upon the sensitive response to micro-environment stimulation have not been fully solved.^{13,14} Moreover, despite the great potential of UCNs in meeting biomedical demands *in vitro* and *in vivo*, most of the conventionally used UCNs employ long wavelength laser irradiation at 980 nm. This often leads to an inevitable overheating effect because of significant water absorption.¹⁵ To this end, the development of new strategies to selectively direct UCNs into targeted tumor areas, while notably minimizing undesired side effects is highly essential. Unfortunately, such relevant studies are still limited so far.

In this chapter, a specific tumor micro-environment-sensitive UCNs platform has been fabricated and prepared to perform covalent localization of UCNs in tumor regions, which could further improve the proposed design in chapter 2 in living systems. Different from the process involving nonspecific tumor cells targeting, such unique platform can respond to tumor-specific enzyme and undergo cross-linking reaction, which thus enables the selective tumor accumulation. More significantly, compared to the particles that cannot undergo cross-linking reaction, the enzyme-triggered covalent cross-linking of UCNs possesses an enhanced light upconverting emission when illuminated at 808 nm. Such enhancement can effectively amplify the production of reactive singlet oxygen (e.g. $^1\text{O}_2$) from the

photosensitizers loaded on the particle surface, which therefore represents promising nanomedicine for improved photodynamic tumor treatment as well as non-invasive fluorescence and PA imaging *in vitro* and *in vivo*.

3.2 Materials and methods

Chemicals and reagents. $\text{Y}(\text{CH}_3\text{CO}_2)_3$, $\text{Yb}(\text{CH}_3\text{CO}_2)_3$, $\text{Er}(\text{CH}_3\text{CO}_2)_3$, $\text{Nd}(\text{CH}_3\text{CO}_2)_3$, oleic acid, 1-octadecene, NH_4F , NaOH , polyacrylic acid (PAA, Mw 1800), diethylene glycol (DEG), polyethylenimine (PEI, Mw 25000, branched), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride ($\text{EDC}\cdot\text{HCl}$), N-hydroxysuccinimide (NHS), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), isobutyl chloroformate (IBCF), 2-cyano-6-aminobenzothiazole (CBT), 4-methylmorpholine, folic acid (FA), tris(2-carboxyethyl) phosphine (TCEP), ethylenediaminetetraacetic acid (EDTA), *In vitro* toxicology assay kit (TOX8, resazurin based), cathepsin B (CtsB) from human liver, antipain hydrochloride inhibitor and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. Chlorin e6 (Ce6) was purchased from Precision Technologies Pte Ltd (Singapore). Mal-dPEGTM₂-NHS and NH_2 -PEG₅₀₀₀-SH (Mw 5000) were obtained from Quanta Biodesign and Laysan Bio, Inc., USA, respectively. Singlet oxygen sensor green (SOSG), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA, USA). The solvents for synthesizing peptide were dehydrated and kept with activated 4 Å molecular sieves. All the commercially reagents were used as received unless otherwise noted.

Instruments. ^1H -NMR and ^{13}C -NMR spectra were measured on a 300 MHz Bruker spectrometer. High-resolution mass spectra were measured on a Waters Q-ToF Premier mass spectrometer (ESI measurements). Semi-preparative high performance liquid chromatography (HPLC, Shimadzu) system was performed on an Alltima C-18 column of 250×10 mm at a flow rate of 3 mL min⁻¹. UV-vis absorption spectra were

measured using a Beckman coulter DU800 spectrometer. Fluorescence emission spectra were captured with a Varian Cary eclipse fluorescence spectrophotometer. The fluorescence emission spectra of UCN was recorded at an angle of 90° to the excitation laser (808 nm) and an optical SEC-2000 spectrometer coupled 2048 pixels CCD assay (ALS Co., Ltd, Japan). A filter with short pass 750 nm was placed before the spectrometer to minimize scattering from the excitation laser beam. The luminescence lifetime decay curves and quantum yield were measured with a phosphorescence lifetime spectrometer (FSP920, Edinburgh) equipped with a microsecond flash lamp as the excitation source and an integrating sphere for efficiency data measurement. The resonance light scattering (RLS) spectra were recorded on a RF-5301PC spectrofluorophotometer (Shimadzu, Japan) at room temperature. Transmission electron microscope (TEM) images were recorded using a FEI EM208S TEM (Philips) operated at 100 kV. Dynamic light scattering (DLS) measurements were performed by Brookhaven 90 Plus Nanoparticle Size Analyzer. The cell viabilities were measured by a Bio-Tek EL-311 microplate reader. Cell imaging was carried out on a confocal fluorescence microscope (Nikon, Eclipses TE2000-E) and upconversion imaging was also recorded on the same machine equipped with 980 nm laser wide-field fluorescence add-on (EINST Technology Pte Ltd, Singapore) in our lab. Photo-irradiation experiments were performed with a near-infrared (808 nm) and visible (660 nm) diode laser (Changchun New Industries Optoelectronics Technology Co., Ltd., China). *In vitro* PA imaging experiments were performed using a real-time multispectral optoacoustic tomographic (MSOT) imaging system (iThera Medical GmbH, Neuherberg, Germany).

Synthesis and characterization of enzyme responsive cross-linking of rare-earth upconversion nanostructures (CRUN) and controlled non-cross-linking particle conjugate (NCRUN). The illustration of design and synthesis of enzyme triggered cross-linking CRUN and control NCRUN were shown in Figure 1 with the following synthesis steps.

Synthesis of NaYF₄:Yb/Er/Nd (18/0.5/1%) core UCNs. The UCNs were

Figure 1. Design and synthesis of enzyme triggered cross-linking of CRUN and control NCRUN.

obtained following a reference published before.⁹ Typically, 2 mL methanol containing $\text{RE}(\text{CH}_3\text{CO}_2)_3$ ($\text{RE} = \text{Y}, \text{Yb}, \text{Er}$ and Nd), 3 mL oleic acid and 7 mL 1-octadecene were added in a 50 mL three-neck flask. The ratio of $\text{Yb}/\text{Er}/\text{Nd}$ is 18/0.5/1% and the total lanthanide amount is 0.4 mmol. The mixture was heated to 150 °C for 60 min before cooling down to room temperature. Subsequently, a methanol solution (6 mL) containing NH_4F (59.3 mg) and NaOH (40.0 mg) was added and stirred for 30 min at 50 °C. Then methanol was evaporated and the solution was kept at 290 °C for 1.5 h under nitrogen atmosphere. After cooling down to room temperature, the core particles were precipitated using ethanol and collected through centrifugation several times after ethanol washing. Finally, the core particles were re-dispersed in 4 mL hexane.

Synthesis of $\text{NaYF}_4\text{:Yb/Er/Nd}$ (18/0.5/1%)@ $\text{NaYF}_4\text{:Nd}$ (20%) core-shell UCNs. Typically, 3 mL oleic acid and 7 mL 1-octadecene were added in a 50 mL three-neck flask, then 2 mL methanol containing 0.4 mmol $\text{RE}(\text{CH}_3\text{CO}_2)_3$ ($\text{RE} = \text{Y}/\text{Nd}$ (80/20%)) was also added in the solution. The mixture was heated to 150 °C for 60 min before cooling down to room temperature. The as-synthesized $\text{NaYF}_4\text{:Yb/Er/Nd}$ core particles in 4 mL hexane were added along with a methanol

solution (6 mL) containing NH_4F (59.3 mg) and NaOH (40.0 mg). The resulting mixture was stirred for 30 min at 50 °C. Then methanol was evaporated and the solution was kept at 290 °C for 1.5 h under nitrogen atmosphere. After cooling down to room temperature, the core-shell particles were precipitated using ethanol and collected through centrifugation several times after ethanol washing. The absolute quantum yield of core-shell UCN was 0.22 % under excitation at 808 nm (80 W cm^{-2}) according to the standard process.¹⁰

Synthesis of PAA@UCNs. The PAA@UCNs synthesis was carried out following a literature protocol reported previously.¹¹ Briefly, 300 mg of PAA (1800 Da) and 30 mL DEG were added to a three-neck flask. The mixture was heated to 110 °C to form a clear solution. Then a hexane solution containing 100 mg core-shell UCNs was added slowly and the mixture was maintained at 110 °C for 1 h under nitrogen protection. After heating at 240 °C for 1.5 h, the resultant solution was cooled down to room temperature and precipitated with ethanol. The obtained PAA@UCNs was recovered by centrifugation (18000 rpm for 10 min) and washed three times with ethanol/water (1:1 v/v).

Synthesis of PEI/PAA@UCNs. The PAA-capped UCNs were covalently encapsulated with branched PEI as reported previously.¹² Briefly, 30 mg of PAA@UCNs in 5 mL water were activated by $\text{EDC}\cdot\text{HCl}$ (50 mg) and NHS (5 mg) to form the active succinimidyl ester for 30 min. Then the mixture was added slowly to 10 mL PEI (2 mg mL^{-1}) in PBS (pH 7.4) with 30 min sonication. After 24 h magnetic stirring, the PEI/PAA@UCNs was purified by centrifugation (18000 rpm for 10 min) and washed three times with ethanol/water (1:1 v/v) to remove excess reactant.

Synthesis of enzyme responsive cross-linking peptide sequence and negative control peptide. All peptide sequences were synthesized by using standard Fmoc-chemistry based solid phase peptide synthesis (SPPS) with 2-Chlorotrityl Chloride Resin. HBTU/DIPEA was used for coupling reaction at room temperature and 20% piperidine/DMF solution was used to deprotect Fmoc group in the terminal

of peptide. The synthetic route of enzyme responsive cross-linking peptide sequence Ac-FKC(StBu)AC(SH)-CBT was shown Figure 2 as the following process.¹⁶

Figure 2. Synthesis of enzyme responsive cross-linking peptide and control non-cross-linking peptide. (a) CBT, NMP, IBCF, 0°C-r.t. (b) 20% piperidine in DMF. (c) Ac-FK(Boc)C(StBu)A-OH, HBTU, DIPEA. (d) TFA/TIPS/DCM (95/2.5/2.5%).

Synthesis of substrate 1. The isobutyl chloroformate (IBCF, 23.4 mg, 0.17 mmol) was added the mixture of Fmoc-Cys(Trt)-OH (72.9 mg, 0.12 mmol) and 4-methylmorpholine (MMP, 34.6 mg, 0.34 mmol) in 3 mL anhydrous THF at 0 °C under N₂ atmosphere and the mixture was stirred for 20 min. Then CBT (20.0 mg,

0.11 mmol) was added to the mixture for further stirring at 0 °C for 2 h before overnight at room temperature. After evaporating the THF solvent under reduced pressure, 10 mL water was added to the reaction mixture with extracting with 10 mL ethyl acetate for three times. The organic phase was combined and dried using Na₂SO₄. The mixture was concentrated under reduced pressure and subsequently a flash column chromatography with eluent ethyl acetate and hexane is performed to obtain a white product (53.1 mg, 65%). ¹H NMR (300 MHz, (CD₃)₂SO): δ 8.74 (s, 1H), 8.21 (d, 1H, *J* = 6Hz), 7.97-7.85 (m, 6H), 7.38-7.25 (m, 19H), 4.44 (d, 1H, *J* = 3Hz), 4.34-4.25 (m, 3H), 3.38 (s, 2H), 3.20 (d, *J* = 3Hz, 1H). ¹³C NMR (75 MHz, (CD₃)₂SO): 170.0, 156.2, 148.3, 144.6, 144.2, 144.1, 141.2, 139.5, 137.1, 135.7, 129.6, 128.6, 128.3, 128.1, 127.5, 127.3, 125.8, 125.3, 121.4, 120.5, 114.0, 112.2, 66.6, 66.4, 55.1, 49.1, 47.1, 34.3; HRMS (*m/z*): calcd. for C₄₅H₃₄N₄O₃S₂Na⁺ [M+Na]⁺: 765.1970, found 765.1966.

Synthesis substrate 2. Substrate **1** (40.0 mg, 0.05 mmol) of was added into 5 % piperidine dissolved in 3 ml of DMF and allowed to stir under ambient temperature for 20 min. Next, 5 mL of water was added to the reaction mixture and extracted with 10 mL ethyl acetate for three times. The organic phase was combined and dried using Na₂SO₄. After concentrating the mixture under reduced pressure, the sample was purified using flash chromatography with choice of eluent as ethyl acetate and petroleum ether to obtain substrate **2** (19 mg, 73%). ¹H NMR (300 MHz, (CD₃)₂SO): δ 8.76 (s, 1H), 8.18 (d, 1H, *J* = 6Hz), 7.79 (dd, 1H, *J* = 6Hz, 3Hz), 7.35 (m, 16H), 3.42 (t, 1H, *J* = 6Hz), 3.31 (s, 2H). ¹³C NMR (75 MHz, (CD₃)₂SO): 173.2, 148.2, 144.9, 139.7, 137.2, 135.5, 129.6, 128.5, 127.2, 125.2, 121.4, 114.0, 111.8, 66.4, 55.5, 37.3; HRMS (*m/z*): calcd. for C₃₀H₂₄N₄OS₂Na⁺ [M+Na]⁺: 543.1289, found 543.1293.

Synthesis substrate 3 and enzyme responsive cross-linking peptide of Ac-FKC(StBu)AC(SH)-CBT. Substrate **2** (17.3 mg, 0.033 mmol) was dissolved in 1 mL of DMF solution containing Ac-Fk(Boc)C(StBu)A-OH peptide (28.9 mg, 0.041 mmol). EDC (10.3 mg, 0.066 mmol) of and NHS (10.1 mg, 0.066 mmol) were then added into the reaction mixture. Finally, DIPEA (14.5 μl, 0.083 mmol) was added

into the reaction flask and allowed to stir over night under ambient condition. After monitoring the starting materials has been consumed in the reaction through TLC, the reaction mixture was extracted using 10 ml of ethyl acetate, water and brine and finally dried over anhydrous sodium sulphate. After the reaction mixture has been concentrated under reduced pressure. The crude mixture of **3** obtained was dissolved in 1ml of DCM and subsequently TFA and TIPS were added into the reaction flask. The mixture was then purified using reverse phase HPLC to obtain enzyme responsive cross-linking peptide (28.3 mg, 42% in two steps). ^1H NMR (300 MHz, $(\text{CD}_3)_2\text{SO}$): δ 8.74 (s, 1H), 8.21 (d, $J=3\text{Hz}$, 4H), 8.18 (d, $J=3\text{Hz}$, 2H), 7.80-7.73 (m, 4H), 7.25-7.16 (m, 6H), 4.56-4.54 (m, 3H), 4.34-4.29 (m, 2H), 3.16-3.12 (m, 2H), 2.98-2.87 (m, 8H), 1.75 (s, 3H), 1.55 -1.47 (m, 4H), 1.26 (s, 9H), 1.22 (s, 3H); ^{13}C NMR (75 MHz, $(\text{CD}_3)_2\text{SO}$): 172.6, 172.1, 169.9, 169.6, 148.3, 139.6, 138.5, 137.1, 135.4, 129.6, 128.5, 126.7, 125.3, 121.4, 114.0, 112.0, 56.5, 54.5, 53.0, 52.8, 49.0, 48.2, 42.9, 37.7, 31.7, 30.0, 29.9, 27.0, 26.3, 22.9, 22.6, 18.3; HRMS (m/z): calcd. for $\text{C}_{38}\text{H}_{52}\text{N}_9\text{O}_6\text{S}_4$ $[\text{M}+\text{H}]^+$: 858.2923, found: 858.2914.

Synthesis of control non-cross-linking peptide. The control peptide sequence Ac-FKC(StBu)AC without cross-linking property was obtained using the conventional SPPS with 2-Chlorotrityl Chloride Resin. HBTU/DIPEA was used for coupling reaction at room temperature and 20% piperidine/DMF solution was used to deprotect Fmoc group in the terminal of peptide. After washing with DMF and DCM several times, 95% TFA was used to cleave the peptide from the resin and remove Boc and Trt protecting group under stirring for 2 h. The negative control peptide was purified using reverse-phase HPLC and then frozen-dried with lyophilizer. ^1H NMR (300 MHz, $(\text{CD}_3)_2\text{SO}$): δ 8.20 (s, 2H), 8.10 (t, $J=6\text{Hz}$, 3H), 7.80 (s, 3H), 7.26 (s, 4H), 7.20-7.18 (m, 1H), 4.52-4.40 (m, 2H), 4.35-4.29 (m, 3H), 3.43-3.37 (m, 3H), 3.17-3.98 (m, 5H), 2.39 (t, $J=9\text{Hz}$, 1H), 1.77 (s, 3H), 1.70 (s, 1H), 1.57-1.55 (m, 3H), 1.29 (s, 9H), 1.24 (s, 3H), 1.00-0.99 (m, 1H). ^{13}C NMR (75 MHz, $(\text{CD}_3)_2\text{SO}$): δ 172.3, 172.0, 171.9, 171.8, 169.9, 169.6, 138.5, 129.6, 128.5, 126.6, 54.7, 54.5, 53.0, 52.7, 48.6, 48.1, 42.9, 42.6, 37.8, 31.7, 31.0, 30.0, 27.1, 26.0, 22.9, 22.6, 18.5, 18.3, 12.5;

HRMS (m/z): calcd. for $C_{30}H_{48}N_6O_7S_3[M+H]^+$: 701.2785, found 701.2780.

Synthesis of CRUN. Firstly, Ce6 (14.4 mg) was activated with EDC (23.0 mg) and NHS (13.8 mg) for 30 min. Then PEI/PAA@UCNs (20.0 mg) was dissolved in PBS (10 mM, pH=7.4) with sonication containing Mal-dPEGTM₂-NHS (10.0 mg) ester linker and active Ce6-NHS ester. After stirring for 24 h, the mixture was precipitated by centrifugation and washed with water thrice, and then it was dissolved in 10 mL PBS (10 mM, pH=7.4) containing enzyme responsive cross-linking peptide sequence Ac-FKC(StBu)AC(SH)-CBT (20.6 mg) in 200 μ L DMSO. Accordingly, the controlled NCRUN was prepared following the same protocol with Ac-FKC(StBu)AC. The final products CRUN and NCRUN were obtained by centrifugation and washed with water three times. The successful formation of enzyme responsive cross-linking peptide and Ce6 modified UCNs conjugate (CRUN) was confirmed by UV-vis spectroscopic characterization. The loading percentage of Ce6 on UCNs was determined by UV-vis absorbance peak at 665 nm. The amount of enzyme responsive cross-linking peptide on UCN was determined by monitoring the fluorescence emission of CBT group (λ_{ex} = 320 nm, λ_{em} = 450 nm).

Enzyme triggered cross-linking reaction of CRUN in buffer. The reaction scheme of enzyme triggered cross-linking was shown in Figure 3. Briefly, the stock solution of CtsB protease enzyme was dissolved in 25 mM of acetate buffer solution (pH 5.0) with 1 mM EDTA. The upconverted luminescence (UCL) spectrum in quartz

Figure 3. The reaction scheme of enzyme triggered cross-linking of CRUN.

cuvette was recorded after incubating CtsB (55 nM) and CRUN (1.5 mg mL⁻¹) in working buffer (0.1 M sodium phosphate, 0.05 M NaCl, 1mM EDTA, pH 6.0) at 37 °C containing TCEP (5 mM, a commonly used reductive agent) for different time.¹⁷ In the negative control experiment, the typical CtsB inhibitor, antipain hydrochloride (100 μM), was pretreated with CtsB for 2 h at 37 °C and then incubated with CRUN for different time as above. After enzyme triggered cross-linking reaction, the CRUN were separated by centrifugation and re-dispersed in ethanol for TEM test.

The UV-vis absorption and luminescence spectra of CRUN in the presence of CtsB and antipain inhibitor were measured after 4 hours incubation. In order to confirm the possibility of the cross-linking reaction, the formation of the covalent cross-linking product, firefly luciferin, was monitored by the fluorescence change at 489 nm (320 nm light as excitation wavelength).¹⁶

In the meantime, the lanthanide UCL of CRUN with CtsB treatment was also measured with 808 nm excitation at different time intervals. The luminescence photographs of different CRUN samples in the absence of CtsB, in the presence of CtsB, or with both CtsB enzyme and inhibitor treatment were recorded in the dark, respectively. The relevant fluorescence intensity was measured by Image J software. The luminescence decay curves of lanthanide dopants (Er³⁺, Nd³⁺ and Yb³⁺) in CRUN were measured in the absence and presence of CtsB enzyme after 4 h incubation. After enzyme triggered cross-linking reaction (4 h), the CRUN were separated by centrifugation and the precipitates were re-dispersed in 0.1 mL DMSO solution. RLS spectra were obtained by scanning synchronously with the same excitation and emission wavelength ($\lambda_{\text{ex}} = \lambda_{\text{em}}$) from 808 to 900 nm. Both the excitation and emission slit widths were kept at 10.0 nm.

Quantification of CtsB and folate receptor expression in HT-29 and NIH/3T3 cells. The human colorectal adenocarcinoma cell line (HT-29, cat. no. HTB-38) and mouse embryonic fibroblast cell line (NIH/3T3, cat. no. CRL-1658) were provided from American-type culture collection (ATCC) and checked for mycoplasma

contamination. These cells are not listed by ICLAC as misidentified cell lines (October 3rd, 2014). Both these two cells were cultured in high-glucose DMEM containing 10% FBS, penicillin (100 I.U. mL^{-1}) and streptomycin ($100 \mu\text{g mL}^{-1}$) at 37°C in a humidified atmosphere with 5% CO_2 . The quantification of CtsB in HT-29 cells ($40.1 \pm 4.4 \text{ ng mg}^{-1} \text{ protein}$) and NIH/3T3 cells ($2.2 \pm 1.4 \text{ ng mg}^{-1} \text{ protein}$) were performed by Human Cathepsin B ELISA (Enzyme-linked immunosorbent Assay) kit according to the procedure described by the manufacturer (CUSABIO, USA). The protein content was determined by using bicinchoninic acid (BCA) method after protein extraction from 1×10^5 cells based on the standard protocol (Thermo Scientific, USA). The quantification of folate receptor levels in HT-29 cells ($3.8 \pm 0.3 \times 10^5$ folate receptors per cell) was also performed by Human FOLR1/Folate Receptor Alpha ELISA kit according to the procedure described by the manufacturer (CUSABIO, USA).

Western blot assay was also performed to compare the difference expression levels of CtsB in these two cell lines. Briefly, 1.2×10^6 HT-29 or NIH/3T3 cells were lysed with $50 \mu\text{L}$ 2 $\times$ SDS sample buffer and boiled at 90°C for 5 min. Then $20 \mu\text{L}$ of each samples were analyzed by 10% SDS-PAGE and transferred to $0.22 \mu\text{m}$ PVDF membrane (100 V, 110 min, 4°C). The membrane was blocked with 5% milk (PBST) for 1h, and incubated with rabbit anti-Cathepsin (ab125067, Abcam) antibody (1:750 dilutions) at 4°C overnight. The membrane was washed 3 times with PBST, then incubated with goat anti-rabbit 2nd antibody (1: 10000 dilutions) for 1h at RT and washed 3 times with PBST. The samples were detected with LAS-4000 machine using enhanced chemiluminescence (ECL) exposure buffer. After that, another $20 \mu\text{L}$ (the same amount as the above) of each samples were analyzed in 10% SDS-PAGE and transferred to $0.22 \mu\text{m}$ PVDF membrane (100 V, 110 min, 4°C). The membrane was incubated with rabbit anti-GAPDH antibody (sc-25778, Santa Cruz, 1:1000 dilutions) for 1 h at RT, washed 3 times with PBST, then incubated with goat anti-mouse 2nd antibody (1: 10000 dilutions) and revealed by ECL.

Fluorescence imaging of enzyme triggered particle cross-linking in living cells.

CtsB-over-expressing HT-29 cells and CtsB-deficient NIH/3T3 cells were seeded with a cell density of 1×10^5 in an ibidi confocal μ -dish (35 mm, plastic bottom) in 1 mL DMEM media which was incubated for 24 h at 37 °C. The seeded cells were subsequently treated with CRUN ($50 \mu\text{g mL}^{-1}$) and incubated for 4 h. The cell nuclei were stained by DAPI ($1 \mu\text{g mL}^{-1}$) for 20 min and then the cells were washed with PBS (pH 7.4) for three times. As controls, the HT-29 cells and NIH/3T3 cells were treated with CRUN in the present of CtsB inhibitor. These two cells were also treated with non-cross linking NCRUN conjugates, which were used as another control. For the cell inhibition assay, the cells were first pretreated with CtsB inhibitor (antipain hydrochloride, $100 \mu\text{M}$ in DMEM) for 2 h at 37 °C follow by the addition of CRUN and incubated for a further 4 h. Fluorescent imaging of the samples were then carried out under confocal microscopy system with a continuous-wave 980nm laser as excitation wavelength (EINST Technology Pte Ltd). The fluorescence analysis of enzyme triggered particle cross-linking in these two cells were measured by Image J.

$^1\text{O}_2$ analysis in buffer solution. $^1\text{O}_2$ was detected based on a highly sensitive probe SOSG according to the protocol reported previously.¹⁸ Briefly, SOSG ($100 \mu\text{g}$ per tube) was dissolved in $33 \mu\text{L}$ methanol as stock solution (5 mM). Different samples containing Ce6 ($1 \mu\text{M}$) were mixed with SOSG ($2.5 \mu\text{M}$) in PBS (10 mM , pH 7.4) and then irradiated by an 808 nm or 660 nm laser (0.4 W cm^{-2}) for different periods of time in the dark.

In addition, a 2-mm thick pork tissue (adipose tissue) was used to mimic living condition for further investigation of the different penetration between 808 nm (NIR) and 660 nm light irradiation. The generation of $^1\text{O}_2$ was based on the enhanced fluorescence of SOSG at 530 nm compared with background signals under the excitation wavelength at 494 nm.

***In vitro* photodynamic cytotoxicity assay of CRUN in HT-29 and NIH/3T3 cells.** The HT-29 and NIH/3T3 cell lines were seeded in a 96-well plate (1×10^4 cells per well in $100 \mu\text{L}$ DMEM) and cultured for 24 hours at 37 °C. The cells were

incubated with CRUN at various concentrations for 4 h and then washed with fresh medium to remove free nanoparticles. For *in vitro* PDT treatment, cells were exposed to 808 nm or 660 nm irradiation at the same power density of 0.4 W cm^{-2} for 60 min in the dark (10 min break after 10 min irradiation). Similar control experiments were employed as following: cells treated with CRUN in the absence of laser illumination; with CRUN in the presence of CtsB inhibitor (antipain hydrochloride, $100 \text{ }\mu\text{M}$); with controlled NCRUN conjugates in the presence of laser irradiation; and cells treatment by NIR light irradiation only but without particle incubation.

Moreover, to demonstrate the deep-tissue penetration advantage of 808 nm (NIR) light for PDT treatment, CRUN was incubated with enzyme over-expressed HT-29 cells and treated with 660 nm or 808 nm light irradiation in the absence or presence of 2 mm thick pork tissue for 1 h under the same optical power density. After PDT treatment, the cells were incubated for another 48 h in the dark. The cell viabilities were evaluated by *in vitro* toxicology assay kit (TOX8, resazurin based) according to standard manufacturer's protocol. Each experiment was repeated three times and the average values were used for analysis.

PA imaging experimental protocol and parameters. Phantom *in vitro* PA imaging experiments were performed using MSOT imaging system. Typically, the optical excitation was provided by an optical parametric oscillator (OPO) with a tunable NIR wavelength which ranges from 680 nm to 980 nm that is in turn pumped by a Q-switched Nd:YAG laser with an average pulse duration of about 10 ns and repetition rate of 10 Hz. The PA signals were acquired using a 128-element concave transducer array spanning a circular arc of 270° . This transducer array has a central frequency of 5 MHz, which is used to provide a transverse spatial resolution in the range of 150-200 μm . During the process of image acquisition, the sample is being translated *via* the transducer array along its axis across the volume region of interest (ROI). Considering the multiple absorption peaks of lanthanide doped upconversion nanostructures due to their specific f-f transitions, in our study, the multi-wavelength PA signals were recorded from 680 nm to 980 nm illumination, and the maximum

contrasted PA signal can be obtained when the excitation wavelength is 680 nm during PA imaging experiments.

***In vitro* PA imaging of enzyme triggered particle cross-linking in buffer solution.** The stock solution of CRUN (1.5 mg mL⁻¹) and CtsB (55 nM) were first incubated in 200 μ L working buffer (0.1 M sodium phosphate, 0.05 M NaCl, 1 mM EDTA, pH 6.0) at 37 °C containing TCEP (5 mM) as reduction agent for different time. Then the solution was loaded into a PA phantom containing two-channel polyurethane cylindrical for PA testing, one for holding the control medium (working buffer) and the other for holding CRUN after incubation with enzyme for different time. The PA signal was recorded using a 128-element concave transducer array spanning a circular arc of 270° with the optimal excitation wavelength at 680 nm. In the negative control experiment, a typical inhibitor, antipain hydrochloride (100 μ M) was first pretreated with CtsB for 2 h at 37 °C and then incubated with CRUN for different time as same protocol.

***In vitro* PA imaging of enzyme triggered particle cross-linking in living cells.** HT-29 cells and NIH/3T3 cells were cultured in a 65 mm dish with a density of 4 million cells per well in 2 mL DMEM media and incubated for 24 h at 37 °C. The cells were incubated with CRUN (1.5 mg mL⁻¹) for 4 h at 37 °C incubator. In the control experiment, the cells were pretreated with CtsB inhibitor (antipain hydrochloride, 100 μ M in DMEM) for 2 h at 37 °C before the addition of CRUN for 4 h incubation. Then cells were harvested using trypsin and washed three times with PBS. The cells were re-dispersed in 0.2 mL PBS for PA imaging test in two-channel cylindrical.

3.3 Results and discussion

3.3.1 Enzyme triggered covalent cross-linking of UCNs *in vitro*

Figure 5 illustrates the rational design of our enzyme responsive CRUN for tumor localization and targeted therapeutics. Generally, the core-shell UCNs were

Figure 5. Illustration of the micro-environment sensitive strategy for covalent cross-linking of peptide-premodified UCNs in tumor areas (a). Upon tumor-specific CtsB reactions, the peptide cleavage on the particle surface induces covalent cross-linking between the exposed free cysteine and CBT on neighboring particles (b), which triggers the accumulation of UCNs into the tumor site. Such enzyme-triggered covalent cross-linking of UCNs leads to an enhanced upconversion emission after 808 nm laser irradiation (c), and in turn amplifies the $^1\text{O}_2$ generation from the photosensitizers (e.g. Ce6) attached on UCNs for enhanced PDT treatment *in vitro* and *in vivo*.

synthesized according to an established thermal decomposition method.⁹ In order to minimize undesired overheating effect typically associated with 980 nm laser irradiation, we constructed lanthanide platform doped with Nd^{3+} ions for improved pumping efficiency at 808 nm. These Nd^{3+} -doped nanocrystals were further coated with PAA and PEI to improve biocompatibility and to facilitate subsequent surface modification (Figure 1). TEM and spectroscopic analysis revealed a cubic morphology of the particles with a narrow size distribution of 40 ± 2 nm. Both core-shell UCNs and polymer-coated UCNs exhibited similar UCL with visible emission at 545 nm and 655 nm upon excitation at 808 nm (Figure 6).

Figure 6. (a-d) TEM images and (e-h) corresponding UCL spectra of the as-prepared materials of (a, e) NaYF₄:Yb/Er/Nd (18/0.5/1%) core UCNs, (b, f) NaYF₄:Yb/Er/Nd (18/0.5/1%)@NaYF₄:Nd (20%) core-shell UCNs, (c, g) PAA@UCNs and (d, h) PEI/PAA@UCNs, respectively. (h) The UV-vis absorption of Ce6. Scale bar: 100 nm. The particle concentration of samples of (e)-(h) was 2 mg mL⁻¹.

To ensure the theranostic efficacy, an effective photosensitizer, Ce6, with favorable optical properties was chosen and coupled to the PEI/PAA@UCNs. In order to achieve selective tumor localization, an enzyme responsive peptide, Ac-FKC(StBu)AC(SH)-CBT (Figure 2) containing a side protected cysteine and CBT was designed for specific reaction with CtsB, one important lysosomal cysteine protease over-expressed in various malignant tumors to process intracellular protein degradation and regulate cancer pathology.²⁰ The selected sequence was further connected to Ce6-PEI/PAA@UCNs through the reaction of free thiol in terminal cysteine and short maleimide oligo (ethyl glycol) (dPEG₂) linker on the particle surface. The successful formation of peptide and Ce6-modified UCNs (CRUN) conjugate was confirmed by spectroscopic characterization (Figure 7a). The optimal amount of peptide on UCNs was finally determined to be 11.4 nmol mg⁻¹, and there was 2.1% Ce6 found on the UCN surface (Figures 7b and c). In principle, cellular uptake of the peptide and Ce6-conjugated UCNs may first reduce the disulfide bond of protected cysteine due to the reductive cellular environment. The subsequent lysosomal enzyme processing will cleave the peptide and expose a free 1,2-aminothiol

Figure 7. (a) UV-vis absorption spectra of Ce6, enzyme responsive cross-linking peptide, PEI/PAA@UCNs and Ce6-peptide-PEI/PAA@UCNs (CRUN, 1 mg mL⁻¹) in aqueous solution. (b, c) Quantification of Ce6 and enzyme responsive cross-linking peptide on UCNs surface. (b) UV-vis absorbance spectra of Ce6 at different concentration (0.84-167 μM). Inset: the standard curve for Ce6 detection at absorbance wavelength of 663 nm. (c) Fluorescence spectra of enzyme responsive cross-linking peptide (Ac-FKC(StBu)AC(SH)-CBT) at different concentrations (1-12 μM). Inset: the standard curve for monitoring of the peptide at the emission wavelength of 450 nm ($\lambda_{\text{ex}} = 320 \text{ nm}$ excitation). Data bars showed mean $\pm$ s.d. ($n = 3$ technical replicates).

group in cysteine, which easily undergoes condensation reaction with -CN moiety in CBT structure,¹⁶ and thus triggers the localization of cross-linked UCNs at the tumor region (Figure 3). During the reaction, great care needs to be taken to avoid non-specific cross-linking by providing optimal spacing in the enzyme responsive peptide and the short PEG linker to control the distance between the peptide and

particle surface.

Figure 8. (a-c) TEM images and DLS characterization of CtsB enzyme triggered cross-linking of CRUN: (a) in the absence of CtsB, (b) in the presence of CtsB (55 nM), and (c) in the presence of CtsB (55 nM) and antipain inhibitor (100 μ M) respectively. (d) UV-vis absorption and fluorescence spectra of enzyme responsive cross-linking peptide (100 μ M). (e) Fluorescence emission of CRUN (1.5 mg mL⁻¹) in the absence of CtsB, in the presence of CtsB (55 nM), and with CtsB and inhibitor treatment (100 μ M).

First, we examined the covalent cross-linking reactions of CRUN upon enzyme treatment in buffer through particle morphology and spectroscopic analysis. After incubation of CRUN (1.5 mg mL⁻¹) with CtsB (55 nM), TEM and DLS analysis indicated significant particle aggregation in solutions (Figures 8a-c), while CRUN alone or the enzyme treated CRUN together with CtsB inhibitor (antipain), remained unchanged over time. Furthermore, the absorption spectrum showed a new peak at 489 nm during the enzyme reaction, indicating the formation of firefly luciferin structure in solutions.¹⁶ These results clearly suggest that the particles aggregation is mainly due to the specific CtsB enzyme triggered cross-linking reaction (Figures 8d

Figure 9. (a) UCL of CRUN at different time intervals with CtsB treatment. The inset showed the luminescence photograph of CRUN incubated with CtsB for 4h. (b) PA imaging and UCL signal after 4 h incubation of CRUN with CtsB. (c) Luminescence photographs of CRUN (1.5 mg mL⁻¹) in the absence of CtsB (left), in the presence of CtsB (middle), and with CtsB and inhibitor treatment (right) and (d) corresponding fluorescence intensity measured by Image J. The concentration of CtsB enzyme and antipain inhibitor were 55 nM and 100 μM.

and e). Compared to CRUN without CtsB treatment, the covalent cross-linking of CRUN could lead to an enhanced upconversion luminescence. There was up to 2.2-fold luminescence emission intensity enhancement observed at 655 nm after 4 h reaction (Figures 9a and b). Similar enzyme triggered CRUN cross-linking was also displayed by the luminescence color change in the absence and presence of CtsB inhibitor (Figures 9c and d), further confirming that it is the specific enzyme reaction that induces the enhanced luminescence from CRUN particles.

Considering deep tissue penetration capacity and great advantages of the PA imaging technique that integrates both functions of ultrasound and optical imaging,

Figure 10. (a) Luminescence decay curves of the emission at 655 nm of UCNs in the absence and presence of Ce6 excited by 808 nm laser. (b) Lifetime of CRUN in the absence and presence of CtsB treatment. (c, d) Luminescence decay curves of lanthanide dopants of (c) Nd^{3+} ($\lambda_{\text{em}} = 864 \text{ nm}$) and (d) Yb^{3+} ($\lambda_{\text{em}} = 977 \text{ nm}$) in CRUN before and after enzyme triggered cross-linking reaction under 808 nm light irradiation. (e) RLS spectra of CRUN upon tumor specific enzyme treatment.

we assessed the difference in PA signal during the specific enzyme cross-linking reactions. Contrary to the increased luminescence observed, there was an obvious PA phantom signal decrease (~40 % in intensity) detected in solution after 4 h reaction (Figure 9b). Generally, laser illuminations on enzyme-responsive UCN conjugates would excite the internal energy levels of lanthanide dopants. The excited states can relax to the ground state through both radiative and nonradiative processes. The enhanced radiative emission relaxation may correspondingly lead to a deactivation of the nonradiative relaxation in PA, therefore resulting in the decreased PA signals as observed in the experiments.²¹ These results demonstrate that such inverse trend observed in PA may also be attributed to the specific enzyme triggered cross-linking reaction.

Furthermore, we also investigated the underlying reason of the enzyme-responsive luminescence enhancement of CRUN by monitoring the decays of the luminescence lifetime. As shown in Figure 10a, the presence of Ce6 on CRUN particles would lead to a sharp decay (from 500 μ s to 205 μ s) in lifetime at 655 nm, indicating the strong absorption of Ce6 at around 660 nm.¹⁵ Moreover, upon enzyme treatment, there was no significant lifetime change of lanthanide dopants in CRUN (Figures 10b-d). In addition, we also examined the RLS properties of CRUN before and after CstB reactions (Figure 10e). There was obvious scattering increment observed upon 808 nm laser excitation, mostly due to the covalent cross-linking of UCN conjugates triggered by enzyme reaction. The increase in scattering efficiency enhanced the amount of light absorption among the cross-linked particles, which would be the possible factors contributing to the increased upconversion luminescence.²²

3.3.2 Enzyme triggered cross-linking of UCN in living cells

The intracellular uptake and enzyme-triggered cross-linking of UCNs in living cells were further investigated by fluorescence and PA imaging techniques. Typically, CtsB over-expressing HT-29 tumor cell and CtsB deficient NIH/3T3 cell were chosen

Figure 11. (a) Analysis of CtsB enzyme expression by Western blot in HT-29 and NIH/3T3 cells. Anti-GAPDH was included as an internal control. (b, c) Confocal imaging and (d, e) fluorescence analysis of (b, d) HT-29 cells and (c, e) NIH/3T3 cells incubated with CRUN, CRUN and inhibitor, and control NCRUN, respectively. The concentration of CRUN or NCRUN and inhibitor were $50 \mu\text{g mL}^{-1}$ and $100 \mu\text{M}$. Blue: DAPI ($\lambda_{\text{ex}} = 350\text{nm}/50\text{nm}$, $\lambda_{\text{em}} = 460 \text{ nm}/50 \text{ nm}$), Red: Ce6 ($\lambda_{\text{ex}} = 545 \text{ nm}/25 \text{ nm}$, $\lambda_{\text{em}} = 610 \text{ nm}/75 \text{ nm}$), Yellow: UCN ($\lambda_{\text{ex}} = 980 \text{ nm}$, $\lambda_{\text{em}} = 350 \text{ nm}-690 \text{ nm}$). Scale bar: $20 \mu\text{m}$. Fluorescence intensity was measured by Image J. Data bars showed mean $\pm$ s.d. ($n = 3$ technical replicates).

as models to incubate with enzyme responsive CRUN ($50 \mu\text{g mL}^{-1}$) at 37°C for 4 h. The different expression levels of CtsB were verified by Western blot and quantitative

human CtsB ELISA kit in HT-29 ($40.1 \pm 4.4 \text{ ng mg}^{-1} \text{ protein}$) and NIH/3T3 ($2.2 \pm 1.4 \text{ ng mg}^{-1} \text{ protein}$) cells, respectively (Figure 11a).²³ As shown in Figure 11b, the yellow fluorescence contributed from light-upconversion emissions at 545 nm and 655 nm was clearly observed in the HT-29 cells, indicating the effective cellular internalization of CRUN. Moreover, compared to cell incubated with CtsB inhibitor or control NCRUN conjugates (the control particles with surface modified by non-cross-linking Ac-FKC(StBu)AC sequence), HT-29 cell treated with enzyme responsive CRUN exhibited an increased luminescence. Additionally, there was no obvious fluorescence enhancement detected in CtsB-deficient NIH/3T3 cells after incubation (Figures 11c-e). These results clearly suggested that the over-expressed CtsB enzyme in HT-29 cell could induce covalent cross-linking of CRUN and such cross-linking leads to the desired particle localization in the tumor micro-environment.

Figure 12. (a) PA signals of HT-29 and NIH/3T3 living cells without (left) and with (right) CtsB inhibitor. The PA signals were shown with a laser excitation at 680 nm. (b) Quantification of $^1\text{O}_2$ generation before and after enzyme triggered CRUN or covered by 2 mm thick pork tissue when exposed to 808 nm or 660 nm laser irradiation for 1h (0.4 W cm^{-2}). Data bars showed mean $\pm$ s.d. ($n = 3$ technical replicates).

Similar cellular studies based on PA imaging were also carried out to monitor CRUN localization in living cells. As shown in Figure 12a, the enzyme responsive CRUN, when incubated with HT-29 cells, exhibited less PA signal ($\sim 56\%$) than that incubated with NIH/3T3 cells (CtsB-deficient). Moreover, processing of CRUN in

HT-29 cells upon blocking the CtsB activity with inhibitors prevented the decrease in PA signal, corresponding to the specific enzyme reactivity. Moreover, incubation of CRUN with NIH/3T3 cells would not induce obvious PA signal change, further confirming that the enzyme processing can cause the localization of CRUN particles within HT-29 cell and the enhanced upconverting emissions resulted in the PA signal decrease. This was in agreement with the observation tested in buffer.

We also evaluated the capability of CRUN to generate cytotoxic $^1\text{O}_2$ through the standard SOSG.¹⁸ Basically, 808 nm laser irradiation of CRUN conjugates resulted in the converted emission 655 nm, corresponding to the absorption of photosensitizer Ce6 labeled on UCNs surface. Compared to CRUN alone or enzyme treated CRUN with inhibitor, the CtsB enzyme induced cross-linking can cause enhanced light converting emission, which thus amplifies the production of $^1\text{O}_2$ from the activated photosensitizers after 1 h illumination (Figure 12b). Importantly, a 2-mm thick pork tissue (adipose tissue) was also used to mimic clinical skin for further investigation of the different penetration of 808 nm (NIR) and 660 nm light irradiation. As shown in Figure 12b, the presence of pork tissue significantly decreased the efficiency of $^1\text{O}_2$ generation (2.9-fold decrease) with 660 nm irradiation, while there was only 0.6-fold decrease in $^1\text{O}_2$ generation when illuminated at 808 nm. The higher $^1\text{O}_2$ production with 808 nm excitation in the presence of pork tissue clearly indicated a better penetration depth as compared to that with irradiation at 660 nm, which makes UCN-based PDT suitable for effective tumor treatment in living system.

The potential photodynamic cytotoxicity of CRUN was further evaluated through an *in vitro* toxicology assay (TOX8). As shown in Figure 13a, there was negligible cytotoxicity in the CRUN incubated HT-29 cell without laser exposure at 808 nm, suggesting minimum toxicity caused by the particle itself. However, upon 808 nm laser illumination of CRUN ($200\ \mu\text{g mL}^{-1}$) incubated HT-29 cells, there was higher cytotoxicity (~53 % cell viability) observed in HT-29 cells than that of cells treated with the control NCRUN (~64 % cell viability). Additionally, similar photodynamic inactivation was also studied in CRUN incubated HT-29 cells but with CtsB inhibitor

Figure 13. (a, b) The cell viability of (a) HT-29 and (b) NIH/3T3 cells incubated with different concentration of CRUN and the controlled non-cross-linking NCRUN with NIR light treatment (0.4 W cm^{-2}) for 1 h. The cells treated with CRUN but no light illumination and in the present of inhibitor ($100 \mu\text{M}$) were used as controls. (c, d) The phototoxicity of NIR light (at 808 nm) irradiation over increasing time on (c) HT-29 and (d) NIH/3T3 cells after incubating with CRUN ($100 \mu\text{g mL}^{-1}$) for 4 h. (e) The photodynamic cytotoxicity assay of HT-29 cells incubated with CRUN in the absence and presence of 2-mm thick pork tissue upon 808 or 660 nm laser irradiation. Data bars showed mean $\pm$ s.d. ($n = 3$ technical replicates).

or in the negative control (CtsB-deficient NIH/3T3 cells). Compared to the laser excited HT-29 cells with CRUN, there was no significant enhancement of cytotoxicity

detected in these control experiments. Meanwhile, 808 nm laser irradiation alone did not induce obvious cytotoxicity in both HT-29 and NIH/3T3 cells (Figures 13b-d), indicating that the covalent cross-linking of CRUN triggered by specific enzyme would increase the $^1\text{O}_2$ generation, and thus induce the enhanced cell death in the targeted tumor cells with over-expression of the CstB enzyme. Moreover, the cytotoxicity of HT-29 cells incubated with CRUN was also evaluated upon 660 nm or 808 nm light irradiation. Although the photodynamic cytotoxicity was found to be higher at direct 660 nm excitation (~86 % in cytotoxicity) than that at 808 nm irradiation (~39 % in cytotoxicity) (Figure 13e), the presence of pork tissue can significantly lower the cytotoxicity at 660 nm illumination (~13 %, ~6.6 fold decrease), while only less cytotoxicity decreases at 808 nm irradiation (~28 %, ~1.4 fold decrease). Such cytotoxicity studies were consistent with the trends of $^1\text{O}_2$ generation as above, clearly demonstrating the great advantage of deep-tissue penetration of 808 nm (NIR) light for living cell PDT treatment.

3.4 Conclusion

In summary, in this chapter, we fabricated a novel targeting strategy based on specific enzymatic reaction to effectively localize light converting UCN nanocarriers in living cells. Such tumor micro-environment responsive enzyme reactions would trigger the covalent cross-linking of single UCN particles, and thus display unique particle localization to facilitate promising multimodality imaging (fluorescence and PA imaging) and amplified PDT therapeutic effects in living cells upon NIR light illumination at 808 nm. This well-defined rational design could be further extended into the studies in living mice, which may represents effectively therapeutic approaches for improved photodynamic tumor treatment as well as non-invasive multifunctional cancer diagnosis for precision clinical nanomedicine in the future.

3.5 Reference

(1) Gao, Y.; Shi, J.; Yuan, D.; Xu, B. *Nat. Commun.* **2012**, 3, 1033.

- (2) Williams, R. J.; Smith, A. M.; Collins, R.; Hodson, N.; Das, A. K.; Ulijn, R. V. *Nat. Nanotechnol.* **2009**, *4*, 19.
- (3) Petros, R. A.; Desimone, M. J. *Nat. Rev. Drug. Discov.* **2010**, *9*, 615.
- (4) Giljohann, D. A.; Mirkin, C. A. *Nature* **2009**, *580*, 461.
- (5) Thaker, A. S.; Gambhir, S. S. *CA: Cancer J. Clin.* **2013**, *63*, 395.
- (6) Mura, S.; Nicolas, J.; Couvreur, P. *Nat. Mater.* **2013**, *12*, 991.
- (7) Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Nature* **2004**, *430*, 873.
- (8) Lang, K.; Chin, J. W. *Chem. Rev.* **2014**, *114*, 4764.
- (9) Xie, X.; Gao, N.; Deng, R.; Sun, Q.; Xu, Q. H.; Liu, X. *J. Am. Chem. Soc.* **2013**, *135*, 12608.
- (10) Zhong, Y.; Tian, G.; Gu, Z.; Yang, Y.; Gu, L.; Zhao, Y.; Ma, Y.; Yao, J. *Adv. Mater.* **2014**, *26*, 2831.
- (11) Wang, C.; Cheng, L.; Liu, Y.; Wang, X.; Ma, X.; Deng, Z.; Li, Y.; Liu, Z. *Adv. Funct. Mater.* **2013**, *23*, 3077.
- (12) Zhao, L.; Kutikov, A.; Shen, J.; Duan, C.; Song, J.; Han, G. *Theranostic*, **2013**, *3*, 249.
- (13) Wang, C.; Cheng, L.; Liu, Z. *Biomaterials* **2011**, *32*, 1110.
- (14) Li, L. L.; Zhang, R.; Yin, L.; Zheng, K.; Qin, W.; Selvin, P. R.; Lu, Y. *Angew. Chem., Int. Ed.* **2012**, *51*, 6121.
- (15) Ai, F.; Ju, Q.; Zhang, X.; Chen, X.; Wang, F.; Zhu, G. *Sci. Rep.* **2015**, *5*, 10785.
- (16) Liang, G.; Ren, H.; Rao, J. *Nat. Chem.* **2010**, *2*, 54.
- (17) Dragulescu-Andrasi, A.; Kothapalli, S. R.; Tikhomirov, G. A.; Rao, J.; Gambhir,

S. S. J. Am. Chem. Soc. **2013**, *135*, 11015.

(18) Huang, P.; Lin, J.; Wang, X.; Wang, Z.; Zhang, C.; He, M.; Wang, K.; Chen, F.; Li, Z.; Shen, G.; Cui, D.; Chen X. *Adv. Mater.* **2012**, *24*, 5104.

(19) Li, J.; Zheng, L.; Cai, H.; Sun, W.; Shen, M.; Zhang, G.; Shi, X. *Biomaterials* **2013**, *34*, 8382.

(20) Lecaille, F.; Kaleta, J.; Bromme, D. *Chem. Rev.* **2002**, *102*, 4459.

(21) Nie, L.; Chen, X. *Chem. Soc. Rev.* **2014**, *43*, 7132.

(22) Atwater, H. A.; Polman, A. *Nat. Mater.* **2010**, *9*, 205.

(23) Talieri, M.; Papadopoulou, S.; Scorilas, A.; Xynopoulos, D.; Arnogianaki, N.; Plataniotis, G.; Yotis, J.; Agnanti, N. *Cancer Lett.* **2004**, *205*, 97.

Chapter 4

***In Vivo* Covalent Cross-linking of Photon-converted Rare-earth Nanostructures for Tumor Localization and Theranostics**

4.1 Introduction

Currently, nanomedicine based on lanthanide doped UCNs towards cancer imaging and therapy has received considerable attention due to their intrinsic photon-converting nature upon exposure to long wavelength light irradiation.¹⁻⁴ Despite such exceptional optical capabilities in the near-infrared ranges offered by UCN nanoparticles, the ability to provide specific targeting strategy for effective localization of UCNs within pathological areas, especially at the tumor site, remains a critical challenge. So far, the ligand-mediated recognition strategies based on the conjugation of affinity moieties to the particle surface have been mostly applied to achieve tumor targeting specificity.⁵⁻⁸ Considering the complex and dynamic physiological cell conditions, the results along with the stochastic nature of ligand-receptor interactions between normal and tumor cells are not so satisfactory. Alternative to develop “smart” conjugating nanoplatfroms that are sensitive to local tumor environment conditions (*e.g.*, a particular pH, temperature, or enzyme level *etc*), and then facilitate specific localization of UCN particles at tumor sites would be thus a highly desirable option.^{9,10}

So far, photodynamic antitumor therapy has been regarded as a minimally invasive treatment modality, and has been widely applied in clinics for various types of cancer treatment.^{5,7} Compared with conventional chemotherapy, light-mediated PDT exhibits some unique advantages, in which cytotoxic photosensitizers can be selectively activated by manipulating the location of light exposure. Currently, the

most clinically used photosensitizers are excited at a shorter wavelength range from 630 to 700 nm, which unfortunately has limited penetration depth in biological tissues. One promising strategy to avoid this limitation is to shift the excitation wavelength to the NIR region (700-1000 nm), in which biological tissues have maximum light transparency, and thus ideal for *in vivo* optical imaging and phototherapy.^{11,12} Actually, latest advances in rare-earth doped UCNs have witnessed unique upconverting optical properties, which can transduce low-energy NIR light illumination into high-energy emissions from UV to Vis range. Such exceptional light converting capabilities could greatly match the demands and thus achieve deeper-tissue penetration to benefit biomedical applications including photodynamic therapy, biological imaging and remotely controlled release of therapeutic agents *in vitro* and *in vivo*.^{5,7}

By right, current progress of UCN-based PDT system mostly utilizes the NIR light excitation wavelength at 980 nm, at which, however, the absorption of water is significant.^{3,4} Such 980 nm light-mediated PDT is facing tremendous hurdles to travel into deeper tissue and bypass the inevitable heat effect.⁵⁻⁸ Therefore, efforts have been intensively made to adjust the excitation range of UCNs to a shorter wavelength to meet the demands of the medical spectral window (i.e. $\sim$ 700-900 nm).^{11,12} Very recently, Nd³⁺-doped upconversion processes based on a new excitation wavelength around 808 nm have been investigated to manipulate optical properties of lanthanide UCN nanocrystals to minimize this photothermal effect.¹² Therefore, 808 nm light-mediated UCNs exhibited deeper tissue penetration and better antitumor effect than those in the conventional PDT treatment system. Meanwhile, during the process of effective UCN-based PDT treatment, the prolonged 808 nm laser irradiation would not induce significant overheating and tissue damage, thus providing promising prospect for their safer applications in the future.

In chapter 3, by exploiting the advantages of intrinsic enzymatic stimulus to specifically target the tumor areas, we developed such a “smart” upconversion nanoconjugate that can selectively react with protease enzyme cathepsin B (CtsB), which has been considered as one important biomarker in many types and stages of

cancers.¹³ Upon tumor-specific enzymatic CtsB reaction, the CtsB-sensitive peptide on the UCN particle surface can be cleaved, which exposes free cysteine and induces its condensation reaction with 2-cyanobenzothiazole (CBT) groups on the surface of neighboring particles, therefore triggering the covalent cross-linking of UCNs particles. Therefore, in this chapter, our extensive fluorescence and PA imaging studies also have validated the successful enzyme triggered cross-linking reaction *in vivo*, which easily realized the enhanced light-converting emission and unique targeting of enzyme sensitive UCNs at tumor areas. Moreover, relevant theranostic studies *in vivo* are presented to demonstrate that the enzyme-triggered interparticle cross-linking reaction can exhibit enhanced light-converting emission and amplify the singlet oxygen generation in process of PDT treatment in living conditions.

4.2 Materials and methods

Chemicals and reagents. All the commercially reagents were used as received unless otherwise noted.

Instruments. Yttrium element content was recorded using an Agilent 7700 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) in the present of standard yttrium nitric acid solution. Photo-irradiation experiments were performed with a near-infrared (808 nm) and visible (660 nm) diode laser (Changchun New Industries Optoelectronics Technology Co., Ltd., China). *In vivo* PA imaging experiments were performed using a real-time multispectral optoacoustic tomographic (MSOT) imaging system (iThera Medical GmbH, Neuherberg, Germany). The animal fluorescence imaging was performed with an IVIS Lumina II imaging system (Caliper Life Sciences, Roissy, France).

PA imaging experimental protocol and parameters. In the process of *in vivo* PA imaging, the ultrasound gel was applied on the surface of the mouse skin and subsequent measurements were acquired in temperature-controlled water for a good acoustic coupling. For the PA experiments of i.v. injection, a similar PA imaging

experiment was conducted under the same condition of acoustic signals were acquired using a 256-element tomographic ultrasound detector array spanning a circular arc of 270° with a central frequency of 5 MHz in the same system whole body imaging were operated under conditions of 0.3 mm step distance along the long axis of animal and 10 repeat pulse per position in designed time point. The multi-wavelength PA signals were recorded from 680 nm to 980 nm illumination, with the maximum contrasted PA signal obtained under excitation wavelength of 680 nm, and PA signal intensity of tumor was measured with ROI assay.

***In vivo* animal studies.** All animal experimental procedures were performed in accordance with the protocol #120774 approved by the Institutional Animal Care and Use Committee (IACUC). Female Balb/c nude mice (~6-8 week old) were purchased from Charles River Laboratories (Shanghai, China). Xenograft mice models were established by injecting 0.1 mL of tumor cells suspension (PSB/matrigel, BD biosciences 1:1, v/v) containing 3×10^6 HT-29 into both flanks or only right side of mouse. When the tumor volume reached a palpable size, the mouse was used for further studies.

***In vivo* PDT treatment through intratumoral injection of enzyme triggered cross-linking of CRUN in tumor bearing mice.** Xenograft mice models were treated when the tumor volumes approached to 3-5 mm in diameter. For *in vivo* PDT treatment, the tumor-bearing mice were randomized into four groups ($n = 8$, each group) and treated by intratumoral injection of particle samples. In group 1, CRUN (3 mg in 100 μ L saline) was directly injected into implanted tumors, followed by an 808 nm laser irradiation after 4 h injection. In group 2, same amounts of controlled NCRUN conjugate was injected with subsequent laser exposure. Control experiments with injection of CRUN but no NIR light irradiation (group 3) and saline with laser treatment (group 4) were employed, respectively. The effective exposure time for each mouse was 45 min (5 min interval after 5 min irradiation with total treatment time of 90 min) with power density at 0.4 W cm^{-2} (fluence 1080 J cm^{-2}). A second and third dose injection followed with PDT treatment described above was repeated at 4 d and

8 d after the first dose injection, respectively. Tumor size was measured three times a week using a vernier caliper upon PDT treatment. The tumor volume was calculated using the following equation: tumor volume (V) = length $\times$ width²/2. Relative tumor volume was calculated as V/V_0 (V_0 was the initial tumor volume before PDT treatment).

***In vivo* PA imaging of enzyme triggered cross-linking in tumor region.** For *in vivo* PA imaging of intratumoral injection of CRUN and NCRUN (3 mg in 100 μ L saline), the tumor bearing mice was anaesthetized with 3% isoflurane and ultrasound gel was applied on the surface of the mouse skin. Next, the distribution of the probe in tumor region was monitored over a period of time using PA imaging. In order to obtain an image acquisition, several slices with a step size of about 0.3 mm spanning from the liver all the way to abdomen was manually selected by live MSOT images with 8 laser excitation wavelengths from 680 nm to 980 nm (50 nm interval). Multispectral imaging was then performed with 10 signal averages per wavelength per transverse slice before injection and 4, 8 and 24 h post-injection.

Histology examination of tumor tissues after treatment. Mice were sacrificed after the multiple intratumoral injections with NIR light mediated PDT treatment. The tumor tissues were collected and fixed with 4% paraformaldehyde solution, processed routinely into paraffin and sectioned into 8 microns thick slices. After stained with hematoxylin & eosin (H&E) followed by standard protocol,¹⁴ the slices were examined by a digital optical microscope (BX 51, Olympus, USA). Damaged cells in tumor region was performed using the terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) assay using an *in situ* damaged cell detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The extent of damaged cells was evaluated by counting the number of TUNEL-positive (brown-stained) cells. The damaged index was calculated as the number of TUNEL-positive cells divided by the total number of cells in 10 randomly selected high-power fields.

Synthesis of tumor affinity ligand conjugated CRUN (FA-PEG@CRUN). The illustration of synthetic scheme of FA-PEG@CRUN and PEG@CRUN were shown in Figure 1. First, the FA-PEG₅₀₀₀-SH conjugate was synthesized according to previous study.^{15,16} Briefly, FA (44.2 mg) was activated with EDC (48.0 mg) and NHS (28.8 mg) in DMSO (6 mL) under N₂ for 30 min. The resulting activated FA solution was added dropwise to the DMSO solution (2 mL) of NH₂-PEG₅₀₀₀-SH (Mw 5000, 100 mg) with triethylamine (TEA, 145.7 μ L) under vigorous magnetic stirring in the dark at room temperature for 24 h. Then the reaction mixture was dialyzed in water using a dialysis membrane with molecular weight cut-off (MWCO) of 3500 Da for 3 days and followed by lyophilization to obtain the product FA-PEG₅₀₀₀-SH. Second, the as-prepared CRUN (40.0 mg) was activated by Mal-dPEGTM₂-NHS (20.0 mg) ester linker in PBS (10 mM, pH = 7.4) for 12 h. After remove the excess reactants, the Mal-activated UCN complex was re-dissolved in PBS (10 mM, pH = 7.4) and then FA-PEG₅₀₀₀-SH (25.0 mg) was added into the solution with vigorous magnetic stirring in the dark at room temperature for 24 h. Accordingly, the PEG modified CRUN particles but without FA as affinity ligand (PEG@CRUN) were prepared as controlled conjugate following the same protocols with NH₂-PEG₅₀₀₀-SH (25.0 mg) modification. The final products FA-PEG@CRUN and PEG@CRUN were obtained by centrifugation and washed with water for three times. The luminescence analysis of FA-PEG@CRUN (1.5 mg mL⁻¹) at different time intervals after CtsB enzyme treatment (55 nM) was followed the same protocol as previous CRUN platform

Figure 1. Illustration of synthetic scheme for FA-PEG@CRUN and PEG@CRUN.

without FA modification.

***In vivo* targeted PDT treatment with intravenous injection of enzyme triggered FA-PEG@CRUN.** Xenograft HT-29 tumor model in the right side of female Balb/c mice were first developed as described above. For *in vivo* tumor targeting PDT treatment, the tumor-bearing mice have been randomly arranged into six different groups ($n = 8$, each group) to perform a series of intravenous injection of saline (group 1), PEG@CRUN (group 2) and FA-PEG@CRUN (group 3-6) respectively (3 mg in 100 μ l saline). After 4 h of i.v. injection, laser treatment was performed on groups 1-6 by irradiation the tumor region with a continuous 808 nm or 660 nm laser. The effective exposure time for each mouse was 45 min (5 min interval after 5 min irradiation with total treatment time of 90 min) with power density at 0.4 W cm⁻² (fluence 1080 J cm⁻²). The tumor in group 4 and 6 were covered by 2 mm thick pork tissue and then irradiated with 808 nm or 660 nm lights under the same condition.^{17,18} The PDT treatment with laser irradiation was repeated every two days. A second and third dose injection of the PDT treatment described above was repeated 4 d and 8 d after the first dose injection, respectively. Tumor size was measured and calculated as described above.

***In vivo* fluorescence imaging in living mice.** For *in vivo* luminescence imaging of live animals, the stock solution of PEG@CRUN and FA-PEG@CRUN were prepared by dissolving the substrates in saline (3 mg in 100 μ l saline). The tumor bearing mice was anaesthetized with isoflurane and imaged immediately after tail vein injection at the indicated time points of 0, 4, 8 and 24 h post-injection. The optical imaging of Ce6 molecules ($\lambda_{em} = 670$ nm) loading at UCNs were collected using an IVIS Lumina II imaging system (Caliper Life Sciences, Roissy, France) with the excitation wavelength of 640 nm and analyzed with the living Image 2.11 software package. For quantification of measured signals, regions of interest were drawn over the tumor region with blue circle and final values were expressed by averaging the obtained signals from the all the mice scanned in each experiment.

***In vivo* PA imaging of enzyme triggered cross-linking in tumor region.** For *in vivo* PA imaging of intravenous injection of FA-PEG@CRUN and PEG@CRUN (3 mg in 100 μ L saline), the tumor bearing mice was anaesthetized with 3% isoflurane and ultrasound gel was applied on the surface of the mouse skin. The location of the probe in tumor region was monitored over a period of time using PA imaging. In order to obtain an image acquisition, several slices with a step size of about 0.3 mm spanning from the liver all the way to abdomen was manually selected by live MSOT images with 8 laser excitation wavelengths from 680 nm to 980 nm (50 nm interval). PA imaging was performed with 10 signal averages per wavelength per transverse slice before and 1 h injection.

Histology examination of organs and tumor tissues with intravenous injection. Mice were sacrificed after the multiple intravenous injections with NIR light mediated PDT treatment. The liver, lung, kidney, heart, spleen and tumor tissues were collected and fixed with 4% paraformaldehyde solutions. For histology analysis, all tumors and organs were processed routinely into paraffin and sectioned into 8 microns thick slices. Damaged cells in tumor region was performed using TUNEL assay using an *in situ* damaged cell detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The extent of damaged was evaluated by counting the number of TUNEL-positive (brown-stained) cells. The slices from various organs were also stained with H&E and examined by a digital optical microscope (BX 51, Olympus, USA).

Bio-distribution studies. Mice were sacrificed after the multiple intravenous injections with NIR light-mediated PDT treatment. The corresponding dissected organs (liver, lung, kidney, heart, spleen and tumor) were collected and weighted. The tissues samples were incubated in 70% nitric acid and digestion was allowed at 70 $^{\circ}$ C for several hours until the solution was clear.^{19,20} After diluted with water to 2% acid solution, the solution was filtered with 0.22 μ m filter and analyzed for yttrium (Y^{3+}) content by ICP-OES.

Statistical analysis. Quantitative data are expressed as mean $\pm$ standard deviations (s.d.) unless specifically described. Significant differences can be determined using the Student's two-tailed t test where differences were considered significant ($p < 0.05$).

4.3 Results and discussion

4.3.1 *In vivo* cross-linking of CRUN in tumor bearing mice

Figure 2. PA imaging of living mice upon intratumoral injection of CRUN and control NCRUN (3 mg in 100 μ L saline) at different time. Data bars showed mean $\pm$ s.d. ($n = 3$ technical replicates).

Inspired by the promising imaging and PDT results in living cells at chapter 1, we examined the *in vivo* theranostic efficacy *via* intratumoral injection of CRUN into living female Balb/c nude mice. We implanted HT-29 cells (CtsB over-expressing) into the left and right flanks of nude mice. At 2-3 weeks post-implantation, the tumors grew to 3-5 mm in diameter and the living mice underwent PA imaging and PDT studies. Typically, one group of xenograft mice bearing two tumors were subjected to CRUN and controlled NCRUN conjugates respectively (3 mg in 100 μ L saline), followed by PA imaging. As compared to the PA intensity before injection, the mice exhibited a significant increase in PA signals at 4 h post-injections for both conjugates. Moreover, the PA signals in the tumor injected with CRUN were found lower ($\sim 30\%$ decrease in intensity) than that subjected to NCRUN (Figure 2). These results

suggested the feasibility of the enzyme processing to trigger the localization of CRUN at the tumor site and it was the covalent cross-linking that led to the decreased PA signals.

Figure 3. (a) Photographs of tumor-bearing mice after 11 d treatment with CRUN (group 1), NCRUN (group 2), CRUN without NIR light irradiation (group 3) and saline (group 4). (b) Relative tumor volumes in the treated groups after PDT therapy under 808 nm laser irradiation. The treatment after 4 h of intratumoral injection (3 mg in 100 μ L saline) was started on day 0 and repeated twice at day 4 and day 8 (arrows) followed by 808 nm laser irradiation. Data were means $\pm$ s.d. ($n = 8$ mice per group), $*p < 0.05$. (c) TUNEL histology of tumor tissues and (d) corresponding damaged index after NIR light-mediated tumor treatment, scale bar: 100 μ m. Data bars showed mean $\pm$ s.d. ($n = 3$ technical replicates), $***p < 0.001$.

Furthermore, we also investigated the therapeutic effect of CRUN as a PDT agent for tumor therapy in living mice. As shown in Figure 3a, in group 1 ($n = 8$), CRUN was injected into the implanted tumor on the left flank, and followed by an 808 nm

laser irradiation at the tumors after 4h injection. As contrast, group 2 ($n = 8$) mice were intratumorally injected with the control NCRUN on the right flank and subsequent light exposure under identical conditions. Tumor bearing mice with direct injection of CRUN but no NIR light irradiation were assigned as group 3 ($n = 8$). The last group (group 4, $n = 8$) consisted of mice injected with saline only. The tumor sizes in the different groups were measured to assess the PDT efficacy over a period of 2 weeks. After CRUN-mediated PDT treatment ($p = 0.038$), the tumor growth in group 1 had been significantly inhibited as compared to that observed in group 2 with control NCRUN ($p = 0.033$, Figure 3b), indicating that the enzyme triggered cross-linking of UCNs particles at the tumor region played an important role in enhancing therapeutic outcomes. On the other hand, in group 3 ($p = 0.021$) and 4, the tumor sizes grew exponentially over the period of time, suggesting that the treatment with 808 nm laser only or CRUN without light irradiation induced minimum inhibition of tumor growth. To further evaluate the therapeutic efficacy, we also performed TUNEL staining on the tissue sections from different groups after 11 days of PDT treatment (Figures 3c and d). As expected, the treated tumors in group 1 mice displayed significant damage than those in group 2 mice. The mice treated with NIR light alone (group 4) and probe without light (group 3) displayed no detectable damage. This is good accordance with the trends observed in tumor inhibition after NIR light-mediated PDT treatment. More importantly, there were no significant burnt scars on the tumor skins in similar light-mediated treatment by conventionally used 980 nm laser illuminations, clearly implying that the novel enzyme responsive CRUN conjugates with laser irradiation at 808 nm could serve as a reliable platform for effective *in vivo* antitumor treatment.

4.3.2 *In vivo* PDT treatment with affinity ligand modified CRUN

To further improve the tumor uptake and PDT efficiency, we conjugate an affinity ligand, FA, to the surface of CRUN for their recognition to folate receptors that over-express in most tumors. Meanwhile, considering the fact to enhance the biocompatibility of CRUN in living system, the FA was first coupled with polyethylene glycol (PEG₅₀₀₀) spacer containing amino and thiol group at each terminus. Such

Figure 4. (a) Illustration of the targeting strategy of FA-PEG@CRUN through the conjugation of FA and PEG₅₀₀₀ on CRUN. (b) Fluorescence enhancement of FA-PEG@CRUN (1.5 mg mL⁻¹) when treated with CtsB (55 nM) at different time intervals (0-4 h).

FA-PEG₅₀₀₀-SH linkers were then coupled to enzyme responsive CRUN conjugate through NHS-dPEG₂-Mal as a linkage moiety to afford final product of FA-PEG@CRUN (Figures 1 and 4a). Upon reaction with CtsB enzyme, FA-PEG@CRUN indicated similar luminescence enhancement as compared to previous CRUN platform without FA modification, suggesting that introduction of FA and PEG linker into CRUN platform would not influence its activity for enzyme recognition (Figure 4b).

To study whether FA conjugation can aid tumor uptake in living mice, we used fluorescence imaging to real-time monitor the targeting effect *in vivo*. The female Balb/c nude mice bearing HT-29 tumors were divided into three groups. The group 1 was the mice with intravenous injection of saline. The mice subjected to PEG modified CRUN particles but no FA ligands were assigned to group 2. In group 3, the tumor bearing mice were injected with FA-PEG@CRUN *via* the tail vein. The fluorescent images were recorded at different time intervals after the first injection.

Figure 5. (a) Fluorescence imaging of tumors (blue circle) in living mice and (b) corresponding quantitative fluorescence signals at different time intervals after injection (from left to right: Saline, PEG@CRUN, FA-PEG@CRUN), respectively; ($\lambda_{\text{ex}} = 640 \text{ nm}$, $\lambda_{\text{em}} = 670 \text{ nm}$). Data bars showed mean $\pm$ s.d. ($n = 3$ technical replicates).

Relative to control studies, a significant increase in fluorescence was observed in living mice at 4 h post-injection of probes (Figures 5a and b). In contrast, the tumors in group 3 with injection of FA-PEG@CRUN displayed stronger fluorescence than that in group 2 subjected to PEG@CRUN injection. Both fluorescence signals gradually decreased 24 h post-administration through the process of circulation. Additionally, similar tumor targeting was also validated by PA imaging in living mice. After administration of CRUN conjugates at 1 h, the mice showed obvious PA signal increase in tumors. Moreover, the observed PA signals in the tumors of group 3 were

Figure 6. PA imaging signals in the tumor region at different time intervals after *i.v.* injection (top: PEG@CRUN, bottom: FA-PEG@CRUN, 3 mg in 100 μL saline).

higher than that in group 2 (Figure 6). The higher fluorescence and PA signals present in tumors confirmed the possible efficacy of the FA ligand in improving the targeting of CRUN conjugates at the tumor region.

Figure 7. Tumor volumes change as a function of time in treated groups to evaluate the effectiveness of PDT treatment *in vivo*. The PDT treatment was carried out after 4 h drug-light interval of intravenous injection (3 mg in 100 μ L saline) and periodically repeated at indicated time points (arrows) following by 808 nm laser irradiation every two days. Data were means $\pm$ s.d. ($n = 8$ mice per group), * $p < 0.05$, ** $p < 0.01$.

Encouraged by the imaging data that revealed the effective tumor uptake of CRUN particles, we continued the light mediated PDT therapy *via* intravenous injection of surface modified CRUN conjugates or saline into six different groups of mice, and *in vivo* tumor inhibition effect was evaluated upon 808 nm or 660 nm light illumination. As shown in Figure 7, after 808 nm laser illumination, the tumor sizes in group 2 ($n = 8$, $p = 0.050$, with injection of PEG@CRUN) and 3 ($n = 8$, $p = 0.038$, injected with FA-PEG@CRUN) were found to be reduced significantly over the therapeutic period, whereas the tumor sizes in control group 1 ($n = 8$) with injection of saline revealed a fast growth rate. In addition, during the treatment, the tumors in group 3 displayed more effective inhibition than those in group 2. These data suggested that the facilitated tumor uptake by FA ligands and effective tumor reduction was mainly from the production of $^1\text{O}_2$ from Ce6 anchored on the CRUN

surface.

Notably, direct 808 nm (group 3) and 660 nm (group 5, $p = 0.008$) laser irradiation of living animals with i.v. injection of FA-PEG@CRUN resulted in comparable PDT efficiency, in which tumor growth has been greatly suppressed (Figure 7). However, considering the different light penetration, similar *in vivo* PDT treatments were also performed when the tumors were covered with a 2 mm thick pork tissue. Compared to mice with FA-PEG@CRUN injection and laser illumination at 808 nm (group 4, $p = 0.019$), the presence of pork tissue could significantly compromise tumor therapy for the mice treated with FA-PEG@CRUN and 660 nm laser excitation (group 6, $p = 0.029$), further demonstrating the benefits of deep tissue penetration in 808 nm laser-mediated tumor treatment.

Figure 8. H&E and TUNEL histology of tumor tissues after 808 nm mediated tumor treatment by intravenous injection of FA-PEG@CRUN, PEG@CRUN, and saline. In TUNEL histology, brown-stained cell nucleus represented damaged cells and blue-stained cell nucleus represented normal cells.

Besides the promising antitumor therapy *in vivo*, the histological tumor tissues analysis also revealed the efficacy of PDT treatment. After 12 days of light-mediated PDT at 808 nm, H&E and TUNEL staining of tumor tissues displayed more significant damage in the mice with i.v. injection of FA-PEG@CRUN (group 3) than

Figure 9. Pathological H&E stained images of tissue sections from heart, liver, spleen, lung and kidney of the mice treated with FA-PEG@CRUN, PEG@CRUN, and saline. The tumor tissue sections were harvested in 12 days after intravenous injection. Scale bar: 200 μm .

Figure 10. ICP analysis of bio-distribution of FA-PEG@CRUN and control PEG@CRUN in various organs. The living mice were sacrificed after 12 days PDT treatment with tail vein injection. The yttrium (Y^{3+}) concentration was analyzed by ICP-OES. Data bars showed mean $\pm$ s.d. ($n = 3$ technical replicates).

those tumors treatment in control groups (Figure 8). Moreover, there was no obvious damage observed in other organs during the treatment (Figure 9). And our ICP spectral analysis also validated more Y^{3+} ion distribution in the tumor region (Figure 10). These results clearly indicated that CRUN conjugates modified with active

targeting agents can significantly improve the therapeutic efficacy of *in vivo* tumor treatment with a desirable PDT outcome.

4.4 Conclusion

In summary, in this chapter, we have carried out a series of UCN-based PDT experiments in living mice, and systematically compared the differences of photodynamic antitumor effect *in vivo* through 808 nm and 660 nm laser irradiation. Our data clearly demonstrated that the PDT treatment efficacy upon 808 nm light illumination did not show significant decrease when the pork tissue (e.g. 2-mm thickness) was used to increase the tissue depth, however, the dramatic drop of therapeutic effect was observed in the similar treatment under 660 nm laser excitation, suggesting that 808 nm light-mediated UCNs exhibit deeper tissue penetration and better antitumor effect than those in the conventional PDT treatment system. Meanwhile, during the process of effective UCN-based PDT treatment, the prolonged 808 nm laser irradiation would not induce significant overheating and tissue damage, thus providing promising prospect for their safer applications in the future. More significantly, this study presented a novel targeting strategy based on specific enzymatic reaction to effectively localize light converting UCN nanocarriers at tumor area in living tumor-bearing mice. Such tumor micro-environment responsive enzyme reactions would trigger the covalent cross-linking of single UCN particles, and thus display unique particle localization to facilitate promising multimodality imaging and amplified PDT therapy of malignant tumors *in vivo* upon NIR light illumination at 808 nm. This well-defined rational design could be easily extended into other unique functional materials and open new doors for precision nanomedicine in the future clinical applications.

4.5 Reference

- (1) Deng, R.; Qin, F.; Chen, R.; Huang, W.; Hong, M.; Liu, X. *Nat. Nanotech.* **2015**, *10*, 237.

- (2) Feng, W.; Zhu, X.; Li, F. *NPG Asia Mater.* **2013**, *5*, e75.
- (3) Kachynski, A. V.; Pliss, A.; Kuzmin, A. N.; Ohulchanskyy, T. Y.; Baev, A.; Qu, J.; Prasad, P. N. *Nat. Photon.* **2014**, *8*, 455.
- (4) Zou, W.; Visser, C.; Maduro, J. A.; Pshenichnikov, M. S.; Hummelen, J. C. *Nat. Photon.* **2012**, *6*, 560.
- (5) Wang, C.; Cheng, L.; Liu, Z. *Biomaterials.* **2011**, *32*, 1110.
- (6) Zhou, L.; Wang, R.; Yao, C.; Li, X.; Wang, C.; Zhang, X.; Xu, C.; Zeng, A.; Zhao, D.; Zhang, F. *Nat. Commun.* **2015**, *6*, 6938.
- (7) Lu, S.; Tu, D.; Hu, P.; Xu, J.; Li, R.; Wang, M.; Chen, Z.; Huang, M.; Chen, X. *Angew. Chem., Int. Ed.* **2015**, *54*, 7915.
- (8) Xiao, Q.; Zheng, X.; Bu, W.; Ge, W.; Zhang, S.; Chen, F.; Xing, H.; Ren, Q.; Fan, W.; Zhao, K.; Hua, Y.; Shi, J. *J. Am. Chem. Soc.* **2013**, *135*, 13041.
- (9) Yu, M. K.; Park, J.; Jon, S. Y. *Theranostics* **2012**, *2*, 3.
- (10) Thaker, A. S.; Gambhir, S. S. *CA: Cancer J. Clin.* **2013**, *63*, 395.
- (11) Ai, F.; Ju, Q.; Zhang, X.; Chen, X.; Wang, F.; Zhu, G. *Sci. Rep.* **2015**, *5*, 10785.
- (12) Shen, J.; Chen, G.; Vu, A. M.; Fan, W.; Bilsel, O. S.; Chang, C. C.; Han, G. *Adv. Optical Mater.* **2013**, *1*, 644.
- (13) Lecaille, F.; Kaleta, J.; Bromme, D. *Chem. Rev.* **2002**, *102*, 4459.
- (14) Rong, P.; Yang, K.; Srivastan, A.; Kieseewetter, D. O.; Yue, X.; Wang, F.; Nie, L.; Bhirde, A.; Wang, Z.; Liu, Z.; Niu, G.; Wang, W.; Chen, X. *Theranostics* **2014**, *4*, 229.
- (15) Li, J.; Zheng, L.; Cai, H.; Sun, W.; Shen, M.; Zhang, G.; Shi, X. *Biomaterials* **2013**, *34*, 8382.

- (16) Ye, W. L.; Du, J. B.; Zhang, B. L.; Na, R.; Song, Y. F.; Mei, Q. B.; Zhao, M. G.; Zhou, S. Y. *PLoS One* **2014**, *9*, e97358.
- (17) Cui, S.; Yin, D.; Chen, Y.; Di, Y.; Chen, H.; Ma, Y.; Achilefu, S.; Gu, Y. *ACS Nano* **2013**, *7*, 676.
- (18) Wang, C.; Tao, H.; Cheng, L.; Liu, Z. *Biomaterials* **2011**, *32*, 6145.
- (19) Crayton, S. H.; Elias, D. R.; Al Zaki, A.; Cheng, Z.; Tsourkas, A. *Biomaterials* **2012**, *33*, 1509.
- (20) Cheng, L.; Yang, K.; Li, Y.; Zeng, X.; Shao, M.; Lee, S. T.; Liu, Z. *Biomaterials* **2012**, *33*, 2215.

Chapter 5

Remote Regulation of Membrane Channel Activity by Site-Specific Localization of Lanthanide-Doped Upconversion Nanocrystals

5.1 Introduction

Light-gated ion channels are transmembrane protein complexes that can spatially and temporally regulate cellular activities through optical control of the movement of ions across the cell membrane. They have been identified as valuable tools to explore basic physiological processes on the cellular level and to facilitate the development of therapeutic agents for disease treatment.^{1,2} By right, several naturally occurring light-dependent regulatory proteins have been converted into genetically encoded light-sensitive effectors for the specific manipulation of cell signaling and gene expression in various types of cells and tissues. In addition to utilizing natural photoreceptor proteins, alternative light-gated channel systems have also been constructed by chemical modification or mutagenesis of photoswitchable ligands on channel structures in order to modulate cell functions with spatiotemporal precision.³ Currently, most of the established light-gated channels are mainly activated by UV or visible light, which may raise concerns regarding photodamage and limited light penetration, as well as inherent absorption and scattering caused by endogenous chromophores including hemoglobin, flavins, and melanin.⁴ Although two-photon optogenetic stimulation has been suggested to overcome these obstacles in part, such long-wavelength excitation usually requires complex experimental setups and there is limited conductivity for light-sensitive channels arising from narrow absorption cross-sections.⁵ Recently, some initial studies demonstrated the possibility of lanthanide-doped UCNs to enable the activation of channel functions with excitation at 980 nm,^{6,7} mainly due to their unique capability to convert tissue-penetrable NIR

light irradiation into UV or visible emissions.⁸⁻¹⁰ Despite successful attempts, in principle the manipulation of cellular activities requires genetically engineering UCNs onto membrane channel structures,⁶ or the activation can be realized by randomly mixing hybrid UCN scaffolds within targeted cells.⁷ Simple and rational design that allows specific localization of photoswitchable platforms on the cell surface without altering channel proteins, and more importantly, the precise regulation of ion-channel activities under both *in vitro* and *in vivo* conditions *via* a more secure and effective light source at the NIR window remains a technical challenge.

Figure 1. Metabolic labeling strategy for site-specific covalent localization of NIR-light-responsive UCNs on the cell membrane to achieve precise regulation of ion channel function.

Herein, we present a simple and unique approach based on native metabolic glycol-biosynthesis pathways to covalently localize NIR (808 nm)-light-responsive UCNs on the cell surface to achieve accurate manipulation of the light-gated channel activities in living cells and animals. As shown in Figure 1, through an intrinsic metabolic process, we incorporate a monosaccharide precursor, peracetylated *N*-azidoacetylmannosamine (Ac₄ManNAz) functionalized with a bioorthogonal azido tag, into glycoconjugates (e.g. sialic acids etc) on the cell membrane.^{11,12} NIR-light-sensitive UCNs doped with neodymium (Nd³⁺) ions that can respond to 808

nm laser excitation were attached to the tag through a modified dibenzyl cyclooctyne (DBCO) moiety. At this spectral window, UCNs are known to display minimized water absorption and overheating effects as compared to UCNs irradiated at 980 nm.¹³ Based on successive copper-free click reactions, the UCN conjugates could be site-specifically anchored onto the plasma membrane.¹⁴ Upon 808 nm light illumination, the upconverted emission at 480 nm from UCNs could activate the light-gated channelrhodopsins-2 proteins (ChR2),¹⁵ and therefore manipulate cation influx (e.g., Ca^{2+}) across the cell membrane to remotely regulate physiological processes under living conditions.

5.2 Materials and Methods.

Materials. $\text{Y}(\text{CH}_3\text{CO}_2)_3$, $\text{Yb}(\text{CH}_3\text{CO}_2)_3$, $\text{Tm}(\text{CH}_3\text{CO}_2)_3$, $\text{Nd}(\text{CH}_3\text{CO}_2)_3$, oleic acid, 1-octadecene, NH_4F , NaOH , polyacrylic acid (PAA, Mw 1,800), 1-hydroxybenzotriazole hydrate (HOBT), diisopropylethylamine (DIPEA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz), Hoechst 33342 (bisBenzimideH 33342 trihydrochloride), dibenzylcyclooctyne-amine (DBCO- NH_2), DBCO-Cy3, 5-carboxytetramethylrhodamine-azide (Rhod- N_3 or Azide-fluor 545), ethyl 3-aminobenzoate methanesulfonate (EABMS), 5-carboxyfluorescein-azide (FAM- N_3), 0.5% phenol red solution, and Annexin V-Cy3TM apoptosis detection kit were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, Cell MaskTM deep red plasma membrane stain kit (CMSK-Cy5), Rhod-3 AM calcium imaging kit, Lipofectamine[®] 3000 transfection reagent, and PureLink[®] HiPure plasmid miniprep kit were obtained from Invitrogen (Carlsbad, CA, USA). All the commercially reagents were used as received unless otherwise noted.

Instruments. Fluorescence emission spectra were recorded on a RF-5301PC spectrofluorophotometer (Shimadzu, Japan) at room temperature. The upconversion

luminescence spectra of UCNs and their conjugates were measured at an angle of 90° to the excitation laser (808 nm) and an optical SEC-2000 spectrometer coupled 2048 pixels CCD assay (ALS Co., Ltd, Japan). A filter with short pass 750 nm was placed before the spectrometer to minimize scattering from the excitation laser beam. Transmission electron microscope (TEM) images were obtained using a FEI EM208S TEM (Philips) operated at 100 kV. Dynamic light scattering (DLS) and zeta potential measurements were performed by Brookhaven 90 Plus Nanoparticle Size Analyzer. The cell viabilities were measured by a Bio-Tek EL-311 microplate reader. Confocal imaging of cells and zebrafish were carried out on Carl Zeiss LSM 800 confocal laser microscope (Germany) and Olympus U-TB190 confocal laser microscope (Japan). The upconversion imaging of cells were recorded on a Nikon confocal fluorescence microscope (Nikon, Eclipses TE2000-E, Japan) equipped with 980 nm NIR laser wide-field fluorescence add-on (EINST Technology Pte Ltd, Singapore) in our lab. Flow cytometry (FCM) analysis was performed using a BD LSRFortessa™ X-20 cell analyzer (USA). The whole-cell patch clamp recordings were performed using an upright microscope (Olympus BX61WI, Japan). Photo-irradiation experiments were performed with an 808 nm NIR diode laser (Changchun New Industries Optoelectronics Technology Co., Ltd., China). Yttrium (Y³⁺) content was measured on an Agilent 7500ce Inductively Coupled Plasma Mass Spectrometer (ICP-MS, USA).

Synthesis of NaYF₄:Yb/Tm/Nd (30/0.5/1%) core UCNs. The UCNs were prepared following the references published before.¹⁶ Typically, 2 mL methanol containing RE(CH₃CO₂)₃ (RE = Y, Yb, Tm, and Nd), 3 mL oleic acid and 7 mL 1-octadecene were added in a 50 mL three-neck flask. The ratio of Yb/Tm/Nd was 30/0.5/1% and the total lanthanide amount was 0.4 mmol. The mixture was heated to 150 °C for 60 min before cooling down to room temperature. Subsequently, a methanol solution (6 mL) containing NH₄F (59.3 mg) and NaOH (40.0 mg) was added dropwise and stirred for 30 min at 50 °C. Then methanol was evaporated and the solution was kept at 290 °C for 1.5 h under nitrogen atmosphere. After cooling down to room temperature, the core particles were precipitated using ethanol for

several times by centrifugation and re-dispersed in 4 mL hexane for next step.

Synthesis of NaYF₄:Yb/Tm/Nd (30/0.5/1%)@NaYF₄:Nd (20%) core-shell UCNs. Typically, 3 mL oleic acid and 7 mL 1-octadecene were added in a 50 mL three-neck flask, then 2 mL methanol containing 0.4 mmol RE(CH₃CO₂)₃ (RE = Y/Nd (80/20%)) was also added in the solution. The mixture was heated to 150 °C for 60 min before cooling down to room temperature. The as-synthesized NaYF₄:Yb/Tm/Nd core particles in 4 mL hexane were added along with a methanol solution (6 mL) containing NH₄F (59.3 mg) and NaOH (40.0 mg). The resulting mixture was stirred for 30 min at 50 °C. Then methanol was evaporated and the solution was kept at 290 °C for 1.5 h under nitrogen atmosphere. After cooling down to room temperature, the core-shell particles were precipitated using ethanol and collected through centrifugation several times after ethanol washing. Finally, the core-shell UCNs were re-dispersed in 4 mL cyclohexane and conserved in fridge.

Synthesis of PAA-UCNs. The as-prepared oleate-capped UCNs were precipitated using ethanol and re-dispersed in a 10 mL acid aqueous solution (pH = 4) adjusted by HCl (0.1 M). Then the solution was sonicated for 30 min following with vigorous stirring for 2 h. During this reaction the carboxylate groups of the oleate ligands were protonated (to yield oleic acid). Then the aqueous solution was extracted with 30 mL diethyl ether to remove the oleic acid for three times. The combined ether layers were re-extracted with 10 mL water. Finally, the dispersible ligand-free UCNs in the water were recuperated by centrifugation (20,000 rpm, 10 min) after precipitation with 20 mL acetone. The bare UCNs were re-dispersed in 20 mL water containing 200 mg of PAA (Mw = 1,800) by sonication. The pH value was adjusted to 7.4 by NaOH (1 M) solution and stirred for 24 h at room temperature. After the reaction, the products (PAA-UCNs) were collected by centrifugation (20,000 rpm for 10 min), washed with water for several time, and re-dispersed in water.

Synthesis of DBCO-UCNs. The COOH-capped PAA-UCNs was covalently modified with NH₂-DBCO by amide condensation reaction. Briefly, the as-prepared

PAA-UCNs (1 mg) were dissolved in dry DMF (200 μ L) containing HOBt (12.2 mg, 0.09 mmol), EDC (14 mg, 0.09 mmol), DBCO-NH₂ (5 mg, 0.018 mmol) and DIPEA (16 μ L, 0.09 mmol). After 24 h magnetic stirring, the DBCO-UCNs was obtained by centrifugation (20,000 rpm, 10 min) and washed by DMSO three times to remove excess reactant. The precipitate was re-dispersed in DMSO and stored in fridge before use. The amount of DBCO moiety on the surface of UCNs was determined based on the fluorescence of conjugated FAM-N₃ molecules by copper-free click chemistry reaction in DMSO. Briefly, the as-prepared DBCO-UCNs (1 mg) were dissolved in DMSO (200 μ L) containing FAM-N₃ (0.2 mg, 43.6 μ mol) and stirred in the dark for 24 h. The nanoparticle was centrifuged (20,000 rpm, 10 min) and washed by DMSO three times to remove excess reactant. The precipitate was re-dispersed in 1 mL DMSO and determined by spectrofluorophotometer.

Synthesis of DBCO/Cy5.5-UCNs and PAA/Cy5.5-UCNs. The as-prepared PAA-UCNs (1 mg) were dissolved in dry DMF (200 μ L) containing HOBt (12.2 mg, 90 μ mol), EDC (14 mg, 90 μ mol), DBCO-NH₂ (5 mg, 18 μ mol), ethanediamine (0.02 mg, 0.36 μ mol) and DIPEA (16 μ L, 90 μ mol). After 24 h magnetic stirring, the nanoparticles were obtained by centrifugation (20,000 rpm, 10 min) and washed by DMF three times to remove excess reactant. Then the precipitate were re-dissolved in dry DMF (200 μ L) containing Cy5.5-NHS ester (0.2 mg, 0.36 μ mol) and DIPEA (1 μ L, 5.6 μ mol) and stirred overnight in the dark at room temperature. The free reagents were removed by washing with DMSO and EtOH for three times. The final precipitate was dried in nitrogen gas and stored in fridge before use.

Cell culture and metabolic glycan labeling on cell membrane. The human embryonic kidney 293 (HEK293) cell lines were purchased from American Type Culture Collection (ATCC). The cells were cultured in DMEM medium supplemented with 10% FBS, 100 units mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin and maintained in a humidified incubator with 5% CO₂ at 37 °C. The cells were seeded at a density of 1×10^5 in 12-well plate and incubated with different concentration of Ac₄ManNAz for 2 days. The cells were trypsinized and re-cultured in an ibidi dish

(35 mm, plastic bottom) at 1×10^5 cells/well in 1 mL DMEM medium overnight. The azido-labeled cell membrane was stained with DBCO-Cy3 (10 μ M, 30 min) and CMSK-Cy5 (5 μ M, 10 min) separately. The azido-labeled cell membrane blocked with DBCO-NH₂ (50 μ M, 30 min) was used as control experiment. The cell nuclei were stained with Hoechst 33342 by following the standard protocols for confocal imaging (Carl Zeiss LSM 800, Germany). The labeling efficiency of DBCO-Cy3 on cell membrane was performed on flow cytometry analysis (E_x: 561nm, E_m: 585/15 nm). The mean fluorescence intensity (MFI) was determined by counting 10,000 cells.

ChR2 transfection and covalent labeling of DBCO-UCNs on HEK293 cell membrane. Typically, the pCAGGS-ChR2-Venus plasmid was purchased from Addgene (#15753). This plasmid (in *E. coli*) was expanded and purified using plasmid miniprep kit (Invitrogen) by following the standard protocol. For ChR2 transfection, the log-phase HEK293 cells were seeded in 12-well plate at a density of 1×10^5 per well for 24 h. Then a commercial plasmid transfection reagent, Lipofectamine[®] 3000 (Invitrogen), was used by following the manufacturer's protocol in serum-free MEM medium. After 4 h incubation, the medium was replaced with fresh DMEM medium containing Ac₄ManNAz (50 μ M). The cells were cultured in incubator for 48 h and re-plated in an ibidi dish (35 mm, plastic bottom) with a density of 1×10^5 cells per well in 1 mL DMEM for another 24 h. For confocal imaging experiments, cell samples were washed twice with DMEM medium and incubated with DBCO-UCNs or PAA-UCNs at a concentration of 100 μ g mL⁻¹ for 2 h at 37 °C. Then the cells were washed twice with DMEM medium and then stained with Rhod-N₃ (10 μ M, 30 min) and CMSK-Cy5 (5 μ M, 10 min) respectively for confocal imaging of DBCO-UCNs and cell membrane (UCNs: Rhod-N₃ (E_x: 543 nm, E_m: 580/50 nm), cell membrane: CMSK-Cy5 (E_x: 633 nm, E_m: 670/50 nm)). For FCM analysis of ChR2-Venus on cell membrane, the transfected HEK293 cells were harvested with 0.25% trypsin-EDTA and re-suspended in 1 mL PBS (1 million cells per 1 mL PBS). The data was recorded by BD LSR Fortessa[™] X-20 flow cytometer (E_x: 488 nm, E_m: 510/21 nm).

Intracellular calcium analysis by Rhod-3 AM. Calcium imaging kit (Rhod-3 AM) was dissolved in 100 μ L DMSO to prepare a stock solution (10 mM). After DBCO-UCNs labeling on ChR2-Venus transfected HEK293 cells, the samples in dishes (1×10^5 cells per well) were incubated with Rhod-3 AM (10 μ M) for 30 min in the dark by following the standard protocol. Then the cells were washed twice with serum-free DMEM medium and irradiated with 808 nm NIR light at the dosage of 0.8 W cm^{-2} for 20 min (5 min break after 5 min illumination). The intracellular calcium imaging was recorded on Carl Zeiss LSM 800 confocal laser microscope (E_x : 561 nm, E_m : 610/75 nm). For FCM analysis, the log-phase HEK293 cells were first seeded in 12-well plate at a density of 1×10^5 cells per well for 24 h. After genetic transfection and DBCO-UCNs labeling on HEK293 cells, the medium was replaced with fresh DMEM medium and treated with different dosage of 808 nm NIR light illumination (0 - 0.8 W cm^{-2} for 20 min, 5 min break after 5 min irradiation). The prepared cells were harvested with 0.25% trypsin-EDTA and re-suspended in 1 mL PBS for intracellular calcium analysis by BD LSR FortessaTM X-20 cell analyzer (E_x : 561 nm, E_m : 582/15 nm).

Electrophysiological measurement by patch-clamping technique. Whole-cell patch-clamping recordings were performed using an upright microscope (Olympus BX61WI) at room temperature in a recording chamber perfused with 95% O₂/5% CO₂ aerated extracellular solution. The extracellular artificial cerebrospinal fluid (ACSF) contained (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 20 D-(+)-glucose, 2.5 CaCl₂, 1.25 MgCl₂, equilibrated with mixed gas containing 95% O₂ and 5% CO₂. The internal pipette solution for the whole-cell patch-clamping recordings from HEK293 cells contained (in mM): 130 K-gluconate, 2 NaCl, 4 MgCl₂, 20 HEPES, 4Na₂ATP, 0.4 Na₃GTP, 0.25-0.5 EGTA, and 0-0.5 Na₂-phosphocreatine, pH 7.25 with 1M KOH. Light irradiation was carried out using a blue light $\sim$ 480 nm and NIR light at $\sim$ 808 nm controlled by computer software. Unless otherwise indicated, all current measurements were made at a holding potential of -70 mV. Electrical responses were acquired with a patch-clamping amplifier (Multiclamp 700B, Axoclamp 1B, or

Axoclamp 2B; all Molecular Devices), digitized at 20 kHz via a Digidata 1440A interface (Molecular Devices), acquired using pClamp software (Molecular Devices), and analyzed using Clampfit, Igor Pro and Origin 8 software.

Living cell apoptosis measurement. The Ca^{2+} -dependent apoptosis process induced by 808 nm NIR light-mediated cations influx on cell membrane was confirmed by using a standard Annexin V-Cy3TM apoptosis detection kit.⁹ Briefly, the DBCO-UCNs labeled HEK293 cells with Chr2 expression (1×10^5 cells per dish) were irradiated with 808 nm NIR light at the dosage of 0.8 W cm^{-2} for 40 min (5 min break after 5 min irradiation). After 24 h incubation, the cell samples were washed with serum-free DMEM medium and $1 \times$ annexin-binding buffer respectively. Then the cells were incubated with 0.5 mL binding buffer containing 5 μL Annexin V-Cy3 solutions ($100 \mu\text{g mL}^{-1}$) for 15 min at room temperature. After washed with buffer solution, the cells were covered with 1 mL DMEM medium for imaging at Carl Zeiss LSM 800 confocal laser microscope. In the control experiments, the Chr2-Venus expressed HEK293 cells were incubated with DBCO-free UCNs (PAA-UCNs), or the Chr2-absent cells were incubated with DBCO-UCNs at same concentration ($100 \mu\text{g mL}^{-1}$) for 2 h at 37°C . Then the cell samples were irradiated with 808 nm NIR light at the similar condition. After 24 h incubation, the cells were treated and imaged following the above methods.

For FCM analysis, HEK293 cells were seeded in 12-well plate (1×10^5 cells per well) for genetic transfection and Ac₄ManNAz incubation as mentioned before. After labeling with DBCO-UCNs ($100 \mu\text{g mL}^{-1}$) for 2 h, the cell samples were washed and irradiated with different dosage of 808 nm NIR light (0.8 W cm^{-2} for 0-60 min irradiation). After 24 h incubation, the prepared cells were harvested with 0.25% trypsin-EDTA and re-suspended in $1 \times$ annexin-binding buffer to afford the dilute cells density (1×10^6 cells per mL). Then 5 μL Annexin V-Cy3 solutions ($100 \mu\text{g mL}^{-1}$ protein in 50 mM Tris-HCl solution, pH 7.5, containing 100 mM NaCl) were added to each 100 μL of cell suspension for 15 min incubation at room temperature. The samples were mixed gently with 400 μL binding buffer and kept on ice for FCM

analysis. The apoptotic cells were enumerated by BD LSRFortessa™ X-20 flow cytometer (Ex: 561 nm, Em: 582/15 nm). The results were processed with FlowJo 7.6.1 software.

In vivo animals studies and preparation. All animal experimental procedures were performed in compliance with the approved guidelines of the Institutional Animal Care Committee of Xiamen University. Wide type zebrafish (*Danio rerio*) were maintained following the standards of zebrafish care. Briefly, the adult zebrafish were cultured in recycled water (control the conductivity to $\sim 500 \Omega \text{ cm}^{-1}$ with NaCl and NaHCO_3 , pH 7.0-7.5) at 27-30 °C with a 14/10-h dark/light photoperiod, and fed three times a day with newly hatched brine shrimp. One mature female zebrafish and one mature male zebrafish were selected before fertilization, and placed in different sides of a 1.5 L breeding cage separated using a divider at 8:00 P.M. The divider was removed from the breeding cage at 8:00 A.M on the next morning. Half an hour later, the embryos were harvested from the breeding cage by a strainer and raised with standard E3 medium containing the following (in mM): 5 NaCl, 0.17 KCl, 0.33 CaCl_2 , 0.33 MgSO_4 , pH 7.0-7.2. The embryos were kept at 28.5 °C incubator after removing the unfertilized embryos and debris in culture dishes.

The zebrafish larvae were dechorionated and anesthetized with 0.2 mg mL^{-1} EABMS in E3 medium at 48 hour post fertilization (hpf). ChR2-expressed HEK293 cells were harvested at a concentration of 1×10^7 cells/mL with 0.05% phenol red. This mixture was loaded into a borosilicate glass needle pulled by a Flaming/Brown micropipette puller (Narishige, Japan, PN-30). About 500 cells were implanted into the yolk sac of each zebrafish larvae in a single injection by using an electronically regulated air-pressure micro-injector (Narishige, Japan, IM-31). After cells implantation, the larvae were washed twice with E3 medium and examined for the presence of fluorescent cells in the yolk sac. The living larvae were randomized into three groups ($n = 20$, each group) and incubated with nanoparticle samples (DBCO/Cy5.5-UCNs or PAA/Cy5.5-UCNs, 2 mg mL^{-1} in E3 medium) in 24-well plate for 12 h. Then the zebrafish were washed with E3 medium for several times to

remove the unlabeled nanoparticles. The confocal fluorescence imaging was recorded at different hours post-incubation (hpi) to track the location of particles in the body of zebrafish larvae. Moreover, ICP analysis was utilized to record the yttrium ion (Y^{3+}) from UCNs particles and evaluate their distribution in zebrafish. The larvae ($n = 20$) were sacrificed at different time points (0, 12, 24, 48, and 72 hpi) in E3 medium by high-speed centrifuge (10,000 rpm, 10 min) and treated with 70% nitric acid for 24 h. The clear solution was diluted in water (2% nitric acid), filtered with 0.22 μ m filter and analyzed for yttrium (Y^{3+}) concentration by ICP-MS.

For calcium imaging experiments *in vivo*, the membrane-permeable Ca^{2+} indicator dyes (Rhod-3 AM) was dissolved in DMSO with 20% Pluronic F-127 to yield a 10 mM stock solution and further diluted to a final concentration of 1 mM working solution in revised Evans solution (in mM): 134 NaCl, 2.9 KCl, 21 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose). A small volume of the Ca^{2+} indicator was then pressure-injected into the yolk sac (20 times, 60 ms pulse time and 8 psi positive pressure) at 0 hpi using a fine patch-clamp micropipette. Imaging recordings were performed at room temperature after the indicator injection for 60 min. Individual zebrafish larvae were anesthetized and embedded in 2% low-melting agarose in a confocal dish (35 mm, plastic bottom), and covered with 2 mL Evans solution (in mM): 134 NaCl, 2.9 KCl, 2.1 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.8). The NIR light treatment with 808 nm laser for the zebrafish was performed at the dosage of 0.8 W cm^{-2} for 2 h (10 min break after 10 min irradiation). In addition, pork tissue (adipose tissue, 4 mm) was used to mimic clinical skin for further investigation of the different penetration depth between 808 nm NIR and 480 nm blue light illumination (20 mW cm^{-2} for 30 min). The high-resolution fluorescence imaging in living larvae were recorded before and after 808 nm NIR light and 480 nm blue light irradiation by using an Olympus U-TB190 confocal laser microscope (Japan), and data analysis was performed using Olympus FluoView software and ImageJ software (NIH).

Western blot analysis. The western blot analysis of apoptosis-associated key executioner enzyme, caspase-3, in zebrafish larvae upon 808 nm NIR light treatment

were performed by using a standard protocol. Briefly, the larvae were firstly incubated with different kinds of nanoparticles (DBCO/Cy5.5-UCNs or DBCO/Cy5.5-UCNs, 2 mg mL⁻¹) for 12 h and injected with Ca²⁺ indicator (Rhod-3 AM) in the yolk sac of zebrafish for 60 min. The zebrafish was anesthetized and embedded in 2% low-melting agarose in a confocal dish (35 mm, plastic bottom) containing 2 mL Evans solution. The NIR light treatment at 808 nm (0.8 W cm⁻²) was performed on the zebrafish for 2 h (10 min break after 10 min irradiation). After light treatment, about 50 zebrafish larvae were continually cultured for 24 h and sacrificed by high-speed centrifuge (10,000 rpm, 10 min). The zebrafish carcasses were homogenized by sonication in 100 µL of RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS and 1 mM PMSF with the addition of phosphatase inhibitors. The lysates were collected and centrifuged at 12,000×g for 10 min at 4 °C. Protein levels in supernatants were determined using the bicinchoninic acid (BCA) method (Bio-Rad, Hercules, CA, USA) and equalized to the same concentration and boiled for 10 min with SDS-PAGE sample loading buffer. A total of 50 mg proteins were separated using SDS-PAGE and transferred to the PVDF membrane, followed by blocking in the blocking buffer (Tris-buffered saline containing 10% BSA and 0.1% Tween-20) for 2 h at room temperature (RT). Incubation with the primary antibodies of cleaved caspase-3 (1:5000) (Cell Signaling Technology, Danvers, MA, USA) was performed overnight at 4 °C, followed by a series of washes and incubation with the HRP-conjugated secondary antibody (1 h, RT). The membranes were stripped using stripping buffer (62.5 mM Tris base, 0.7% β-mercaptoethanol, 2% SDS, pH 6.7) and probed with β-actin antibody (1:4000) which was used for data normalization. All signals were developed using Super Signal West Femto kit, visualized using myECL imager and quantified with ImageJ Analysis software (NIH).

5.3 Results and discussion

5.3.1 Covalent localization of DBCO-UCNs on cell membrane

Figure 2. (A) Confocal imaging of azido groups on the membrane of HEK293 cells stained with DBCO-Cy3 (10 μM) after feeding with or without Ac_4ManNAz (50 μM). As controls, the cells were blocked with DBCO- NH_2 (50 μM). Blue: Hoechst 33342 (E_x : 405 nm, E_m : 460/50 nm), red: DBCO-Cy3 (E_x : 543 nm, E_m : 580/50 nm), violet: CMSK-Cy5 (E_x : 633 nm, E_m : 670/50 nm). Scale bar: 20 μm . (B) FCM analysis of DBCO-Cy3 stained HEK293 cells after incubation with or without Ac_4ManNAz (50 μM). As controls, the cells were blocked with DBCO- NH_2 (50 μM). (C) Quantitative FCM analysis of the MFI value of azido-tagged HEK293 cells after treatment with Ac_4ManNAz at various concentrations. E_x : 561 nm, E_m : 582/15 nm.

First, we started the covalent glycan labeling approach by incorporation of azido groups into the living cell membrane *via* native metabolic biosynthesis. Basically, the

N_3 -tagged glycans could be introduced to HEK293 cells through intrinsic metabolism by simply feeding the Ac₄ManNAz precursor. A copper-free click reaction based on the DBCO-conjugated fluorophore (e.g. DBCO-Cy3) as a bioorthogonal linkage was then performed in Ac₄ManNAz-treated HEK293 cells. The covalent labeling of the cell surface was monitored by confocal microscopy and FCM analysis. As shown in Figure 2A, after feeding the cells with Ac₄ManNAz (50 mM), the bright red fluorescence from Cy3 was visualized on the HEK293 cell membrane. Moreover, similar cell-surface staining was also confirmed by a standard membrane tracker, CMSK-Cy5 (Figure 2A).¹⁷ In control experiments, no significant signal was detected in the cells that had not been incubated with Ac₄ManNAz or in the cells that had been pretreated with reagent (e.g. DBCO-NH₂) to block the N_3 groups on the cell surface

Figure 3. (A) Confocal imaging of HEK293 cells after transfection with pCAGGS-ChR2-Venus plasmid for 3 days. Green: ChR2-Venus (E_x : 488 nm, E_m : 530/50 nm). Scale bar: 20 μ m. (B-D) FCM analysis of HEK293 cells (B) before and (C) after transfection with pCAGGS-ChR2-Venus plasmid for 3 days. (D) Quantitative FCM analysis of MFI value in transfected HEK293 cells (E_x : 488 nm, E_m : 510/21 nm).

Figure 4. Schematic illustration of the synthesis process of UCNs, PAA-UCNs and DBCO-UCNs, respectively.

Figure 5. (A) Schematic illustration for the detection of DBCO moiety on the surface of DBCO-UCNs based on FAM-N₃. (B) Fluorescence spectra of FAM-N₃ at different concentration (0.1-0.5 mM) in DMSO. Inset: the standard curve for the detection of DBCO moiety concentration in DBCO-UCNs solution (1 mg mL⁻¹) (Ex: 490 nm, E_m: 545 nm).

(Figures 2B and C), suggesting the high feasibility of the covalent conjugation of functional moieties on the membrane through the native metabolic biosynthetic pathways.

Furthermore, in order to achieve precise regulation of membrane channel activities, a light-gated ion-channel protein, ChR2, was engineered on the cell surface to mediate the influx of essential signaling ions (e.g. Ca^{2+}) in the cytoplasm. The successful expression of ChR2 on the membrane was confirmed by the presence of the green fluorescent protein (GFP) marker (Venus) using confocal microscopy (Figure 3). Moreover, to conduct NIR-light-mediated regulation of ion-channel functions, UCNs doped with Nd^{3+} ions were used as novel transducers owing to their promising light-converting property at 808 nm. Basically, the Nd^{3+} -doped UCNs were

Figure 6. TEM images of (A) core UCNs: $\text{NaYF}_4:\text{Yb/Tm/Nd}$ (30/0.5/1%); (B) core-shell UCNs: $\text{NaYF}_4:\text{Yb/Tm/Nd}$ (30/0.5/1%)@ $\text{NaYF}_4:\text{Nd}$ (20%); (C) PAA-UCNs, and (D) DBCO-UCNs, respectively. Scale bar: 50 μm . (E) DLS result of DBCO-UCNs. (F) Upconversion emissions of UCNs, PAA-UCNs, and DBCO-UCNs (1 mg mL^{-1}) under 808 nm excitation. Inset: luminescence photograph of UCNs with 808 nm irradiation.

functionalized with PAA to improve their dispersion stability in buffer and to facilitate subsequent chemical modification (Figure 4). In order to covalently anchor UCNs on the azido-tagged cell surface, DBCO moieties were coupled with a carboxyl group in PAA to afford DBCO-UCNs (Figure 4). The fluorescent conjugation through FAM-N₃ confirmed the effective coating of DBCO on the UCNs surface and the optimal amount of DBCO on the UCNs surface was determined as ca. 320 nmol mg⁻¹ (Figures 5A and B). TEM revealed the spherical morphology of core-shell DBCO-UCNs particles with an average diameter of roughly 40 nm (Figures 6A-D).

Figure 7. Confocal imaging of azido-expressed HEK293 cells incubated with DBCO-UCNs and PAA-UCNs for 2 h at 100 µg mL⁻¹. As controls, Ac₄ManNAz-absent HEK293 cells and Ac₄ManNAz-treated HEK293 cells blocked with 50 µM DBCO-NH₂ were incubated with DBCO-UCNs (100 µg mL⁻¹) for 2 h. Green: ChR2-Venus (E_x: 488 nm, E_m: 530/50 nm), blue: UCNs (E_x: 543 nm, E_m: 580/50 nm), violet: CMSK-Cy5 (E_x: 633 nm, E_m: 670/50 nm). Scale bar: 10 µm.

Meanwhile, the hydrodynamic diameter in buffer solution was also determined as 96.4 ± 10.4 nm on the basis of DLS analysis (Figure 6E). The DBCO-UCNs particles displayed strong luminescence at 456 nm and 480 nm upon light excitation at 808 nm (Figure 6F), which matched well with the maximum absorption of ChR2 at 480 nm.¹⁵ Finally, the subsequent copper-free click cyclization enabled the effective linkage of DBCO-UCNs onto the N₃-tagged HEK293 cell membrane. As shown in Figure 7, the UCNs were obviously visualized on cell surface (blue) after 2 h incubation, and exhibited similar membrane staining with ChR2-Venus (green) and the standard membrane tracker (CMSK-Cy5, red). Furthermore, there were negligible signals observed in control studies when the N₃-tagged cells were incubated with DBCO-free UCNs (PAA-UCNs), or when the cells without N₃ groups on surface were treated with DBCO-UCNs (Figure 7). These results clearly indicated the metabolic glyco-biosynthesis can site-specifically localize DBCO-UCNs on living cell membranes through the copper-free bioorthogonal click reaction.

5.3.2 Precise regulation of membrane channel activities *in vitro*

Figure 8. (A) Ca²⁺ imaging in living cells loaded with the Rhod-3 AM before and after 808 nm NIR light irradiation (0.8 W cm⁻² for 20 min): (1-2) Ac₄ManNAz-treated HEK293 cells incubated with DBCO-UCNs (100 μg mL⁻¹) in the absence and presence of NIR light, (3) Ac₄ManNAz-treated HEK293 cells incubated with PAA-UCNs (100 μg mL⁻¹), and (3) Ac₄ManNAz-absent cells incubated with DBCO-UCNs (100 μg mL⁻¹). E_x: 561 nm, E_m: 610/75 nm. Scale bar: 20 μm. (B) FCM analysis of cellular Ca²⁺ with different light dosages.

To further investigate the possibility of NIR-light-responsive UCNs for remote manipulation of membrane channel activities, 808 nm light was utilized to irradiate the covalently localized UCNs transducers on the cell surface. The upconverted blue emission was used to activate the light-gated ChR2 channels to facilitate the Ca^{2+} ion influx across the membrane. The level of intracellular Ca^{2+} ions was measured through a standard fluorescent Ca^{2+} indicator, Rhod-3 AM.¹⁸ As shown in Figure 8A, 808 nm light irradiation of DBCO-UCNs-labeled HEK293 cells with ChR2 expression could lead to a significant increase of red fluorescence in the cytosol, while no apparent fluorescence increment was recorded in control experiments for either N_3 -tagged cells treated with DBCO-free UCNs, or N_3 -absent cells incubated with DBCO-UCNs. Moreover, the quantitative FCM analysis also showed that such NIR-light-responsive fluorescence enhancement was light-dose-dependent, clearly suggesting that the intracellular Ca^{2+} influx could be precisely regulated by the DBCO-UCNs located on the membrane upon NIR-light irradiation (Figure 8B). We also carried out standard whole-cell patch-clamping studies to measure the NIR-light-mediated ionic photocurrent flow across the membrane.¹⁵ As indicated in Figure 9, after 808 nm light illumination, a remarkable inward photocurrent response was immediately observed (ca. 280 pA) in the DBCO-UCNs-labeled HEK293 cells

Figure 9. Photocurrents in ChR2-expressing HEK293 cells incubated with DBCO-UCNs for 2 h under 808 nm (red) and 480 nm (blue) excitation. ChR2-expressing cells only (black) and UCNs-labeled cells without ChR2 expression (green) were used as controls.

with ChR2 expression, which was similar to the results obtained upon illumination at 480 nm. Meanwhile, there were no significant signals observed in the ChR2-expressing HEK293 cells without DBCO-UCNs labeling and the ChR2-absent cells treated with DBCO-UCNs (Figure 9).

Moreover, as a ubiquitous intracellular messenger, Ca^{2+} ions play significant roles in many physiological processes including cell proliferation, differentiation, and

Figure 10. (A, B) Live-cell apoptosis imaging after DBCO-UCNs (100 mg mL^{-1}) incubation (A) with and (B) without 808 nm light irradiation (0.8 W cm^{-2} for 40 min). (C) ChR2-present HEK293 cells incubated with PAA-UCNs and (D) ChR2-absent HEK293 cells incubated with DBCO-UCNs after 808 nm NIR light irradiation (0.8 W cm^{-2} for 40 min) were used as controls. Blue: Hoechst 33342 (E_x : 405 nm, E_m : 460/50 nm), green: ChR2-Venus (E_x : 488 nm, E_m : 530/50 nm), red: Annexin V-Cy3 (E_x : 543 nm, E_m : 580/50 nm). Scale bar: 20 μm .

apoptosis.¹⁹ To this direction, we examined the ability of cell-surface-localized DBCO-UCNs to mediate Ca^{2+} -dependent apoptosis upon their illumination at 808 nm. Typically, Cy3-tagged Annexin V was applied as an indicator to study the apoptosis in HEK293 cells. As shown in Figure 10A, the DBCO-UCNs-labeled cells presented an

Figure 11. (A) Apoptotic FCM analysis of ChR2-expressed HEK293 cells stained by Annexin V after 808 nm NIR light treatment (0.8 W cm^{-2} for 40 min) in different groups: (1) DBCO-UCNs labeled HEK293 cells without NIR light irradiation; (2) PAA-UCNs incubated cells upon NIR light irradiation; (3) ChR2-absent HEK293 cells with DBCO-UCNs labeling and NIR light irradiation; (4) ChR2-present cells with DBCO-UCNs labeling and NIR light irradiation, respectively. (B) Quantitative FCM analysis of MFI value in different groups (E_x : 561 nm, E_m : 582/15 nm). (C) Apoptotic FCM analysis of DBCO-UCNs labeled HEK293 cells with ChR2 expression after 808 nm NIR light irradiation (0.8 W cm^{-2}) for different time: (1) 0, (2) 10; (3) 20; (4) 40; (5) 60 min, respectively. (D) Quantitative FCM analysis of MFI value in different groups (E_x : 561 nm, E_m : 582/15 nm).

obvious red fluorescence on the cell membrane after 808 nm light excitation. In control experiments, there was no significant fluorescence in cells treated with DBCO-free UCNs under irradiation at 808 nm, and in the cells incubated with DBCO-UCNs but without NIR light illumination (Figures 10B-D). Similar NIR-light-responsive fluorescence enhancement was also obtained through FCM analysis (Figure 11), which confirmed the occurrence of apoptosis with 808 nm light irradiation of DBCO-UCNs localized on cell membrane. These results unequivocally indicated that cell-surface-localized UCNs could serve as a unique photoswitch to precisely regulate cation movement for the specific manipulation of cellular functions.

5.3.3 Remote regulation of membrane channel activities *in vivo*

Furthermore, we also exploited the *in vivo* metabolic glycan labeling in zebrafish, one commonly used animal model for human disease screening and sophisticated imaging studies.²⁰ Typically, we implanted N₃-tagged HEK293 cells with ChR2 expression into the yolk sac of zebrafish larvae at 48 hpf. The bright green fluorescence in the larvae abdomen indicated the successful implantation of cells in zebrafish (Figures 12A and B). To study the feasibility of the metabolic glycan labeling for localized UCNs attachment *in vivo*, the larvae were further incubated with DBCO/Cy5.5-UCNs for 12 h, which enabled the effective click linkage of UCNs onto the N₃-tagged cells in the abdominal cavity of the larvae (n = 20). Moreover, an NIR dye, Cy5.5, was coated on the UCNs surface to monitor the localization of particles *in vivo*. The systematic imaging studies indicated the bright fluorescence at 0 hpi, suggesting the effective uptake of UCNs in larvae (Figures 12C and D). Upon 72 h clearance, the obvious fluorescence in zebrafish incubated with DBCO/Cy5.5-UCNs could be still observed, while only a very weak signal was detected in fish treated with the control PAA/Cy5.5-UCNs without DBCO on the surface. Similarly, the effective cellular uptake was also confirmed by inductively coupled plasma (ICP) analysis; a higher Y³⁺ ion distribution in zebrafish with the incubation of DBCO/Cy5.5-UCNs was found at 72 hpi (Figure 12E), clearly indicating that the metabolic glycan labeling could greatly enhance the efficiency of UCNs accumulation

Figure 12. (A, B) *In vivo* fluorescence imaging of zebrafish larvae in the (A) presence and (B) absence of ChR2-expressed HEK293 cells injection in the yolk sac of wide type zebrafish. Green: ChR2-Venus (E_x : 488 nm, E_m : 530/50 nm). Scale bars: 400 μ m. (C) *In vivo* fluorescence imaging of zebrafish larvae at different hpi with DBCO/Cy5.5-UCNs and PAA/Cy5.5-UCNs (n = 20). Red: Cy5.5 (E_x : 635 nm, E_m : 680/30 nm). Scale bars: 400 μ m. (D) The mean fluorescence intensity in zebrafish at 72 hpi (n = 20). (E) ICP analysis of the Y^{3+} content in zebrafish larvae at different hpi with DBCO/Cy5.5-UCNs and PAA/Cy5.5-UCNs (n = 20).

under *in vivo* conditions.

More significantly, in order to remotely regulate the membrane channel activities *in vivo*, we utilized 808 nm NIR light to irradiate the larvae and monitored the cation

Figure 13. *In vivo* fluorescence imaging with and without NIR light treatment (0.8 W cm^{-2} for 2 h) in zebrafish incubated with DBCO/Cy5.5-UCNs and PAA/Cy5.5-UCNs ($n = 20$) in different groups. The N_3 -absent larva incubated with DBCO/Cy5.5-UCNs were used as control groups. The Ca^{2+} response in fish was monitored by Rhod-3 AM and analyzed with pseudo-colored imaging. Green: ChR2-Venus (E_x : 488 nm, E_m : 530/50 nm), red: Cy5.5 (E_x : 635 nm, E_m : 680/30 nm), yellow and pseudo-color: Rhod-3 AM (E_x : 561 nm, E_m : 610/75 nm). Scale bar: 400 μm .

influx in zebrafish ($n = 20$) by using a Ca^{2+} indicator (Rhod-3 AM). As shown in Figure 13, after 808 nm light irradiation for 2 h, the increased fluorescence in the zebrafish that had been treated with DBCO/Cy5.5-UCNs could be observed, while little fluorescence change could be found in zebrafish that had been incubated with PAA/Cy5.5-UCNs but had no DBCO on the cell surface or N_3 -absent larva incubated with DBCO/Cy5.5-UCNs. This clearly suggests that the localized DBCO/Cy5.5-UCNs labeling could effectively manipulate ion-channel activities in living animals after NIR light illumination. Furthermore, western blot analysis also

Figure 14. (A) Western blot analysis of caspase-3 expression in the zebrafish larvae incubated with (1) DBCO/Cy5.5-UCNs with NIR light treatment; (2) DBCO/Cy5.5-UCNs without NIR light treatment; (3) PAA/Cy5.5-UCNs with NIR light treatment, respectively. NIR light irradiation at 808 nm (0.8 W cm^{-2}) was performed on zebrafish for 2 h. β -Actin was used as an internal control. (B) The relative mean intensity of gray values in different groups of western blot ($n = 3$). (C) Schematic illustration for the investigation of different penetration depth upon NIR light (808 nm) and blue light (480 nm) irradiation. (D) Fluorescence pseudo-colored imaging of Rhod-3 AM with NIR light and blue light in the presence of different thickness of pork tissue (E_x : 561 nm, E_m : 610/75 nm).

demonstrated that such NIR-light-gated Ca^{2+} influx in DBCO/Cy5.5-UCNs-incubated zebrafish could lead to the enhanced expression of caspase-3, one key executioner enzyme in cell apoptosis (Figures 14A and B), indicating the feasibility of NIR-light-mediated activation of ion channels to manipulate apoptosis in living animals. Additionally, we also used different thickness of pork adipose tissue to further investigate the difference in penetration of 808 nm and 480 nm light irradiation. As expected, the presence of tissue significantly blocked the fluorescence

of the Ca^{2+} indicator upon illumination at 480 nm, while the obvious fluorescence could still be found when the tissue was excited at 808 nm (Figures 14C and D), clearly showing that NIR light irradiation could penetrate more deeply to activate the ChR2 channel than the excitation in the conventional visible region. This makes our UCNs-based metabolic strategy suitable for the effective regulation of physiological activities in living systems.

5.4 Conclusion

In summary, we introduced a novel and simple strategy based on the native metabolic glyco-biosynthesis to achieve site-specific covalent localization of NIR-light-responsive UCNs on the cell surface through a copper-free click reaction under living conditions. Upon 808 nm light irradiation, the blue emission (at 480 nm) from UCNs could remotely activate the photosensitive ion channel (ChR2) and effectively manipulate the cation influx in living cells and zebrafish. The present strategy not only provides a site-specific and effective approach for the covalently labeling of nanoparticles on the cell membrane, it also holds great potential to precisely regulate the membrane-associated activities under *in vivo* conditions. Thus, these nanoparticles could act as personalized nanomedicines for various pathological disorders in the future.

5.5 References

- (1) Jentsch, T. J.; Hübner, C. A.; Fuhrmann, J. C. *Nat. Cell Biol.* **2004**, 6, 1039.
- (2) Fehrentz, T.; Schönberger, M.; Trauner, D. *Angew. Chem., Int. Ed.* **2011**, 50, 12156.
- (3) Zhang, K.; Cui, B. *Trends Biotechnol.* **2015**, 33, 92.
- (4) Lin, J.; Knutsen, P. M.; Muller, A.; Kleinfeld, D.; Tsien, R. Y. *Nat. Neurosci.* **2013**, 16, 1499.

- (5) Peng, H.; Chiu, D. T. *Chem. Soc. Rev.* **2015**, *44*, 4699.
- (6) He, L.; Zhang, Y.; Ma, G.; Tan, P.; Li, Z.; Zang, S.; Wu, X.; Jing, J.; Fang, S.; Zhou, L.; Wang, Y.; Huang, Y.; Hogan, P. G.; Han, G.; Zhou, Y. *eLife* **2015**, *4*, e10024.
- (7) Wu, X.; Zhang, Y.; Takle, K.; Bilsel, O.; Li, Z.; Lee, H.; Zhang, Z.; Li, D.; Fan, W.; Duan, C.; Chan, E. M.; Lois, C.; Xiang, Y.; Han, G. *ACS Nano* **2016**, *10*, 1060.
- (8) Wang, F.; Deng, R.; Wang, J.; Wang, Q.; Han, Y.; Zhu, H.; Chen, X.; Liu, X. *Nat. Mater.* **2011**, *10*, 968.
- (9) Yang, Y.; Shao, Q.; Deng, R.; Wang, C.; Teng, X.; Cheng, K.; Cheng, Z.; Huang, L.; Liu, Z.; Liu, X.; Xing, B. *Angew. Chem., Int. Ed.* **2012**, *51*, 3125.
- (10) Min, Y.; Li, J.; Liu, F.; Yeow, E. K.; Xing, B. *Angew. Chem., Int. Ed.* **2014**, *53*, 1012.
- (11) Jiang, H.; English, B. P.; Hazan, R. B.; Wu, P.; Ovryn, B. *Angew. Chem., Int. Ed.* **2015**, *54*, 1765.
- (12) Xie, R.; Dong, L.; Du, Y.; Zhu, Y.; Hua, R.; Zhang, C.; Chen, X. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 5173.
- (13) Ai, X.; Ho, C. J. H.; Aw, J.; Attia, A. B. E.; Mu, J.; Wang, Y.; Wang, X.; Wang, Y.; Liu, X.; Chen, H.; Gao, M.; Chen, X.; Yeow, E. K. L.; Liu, G.; Olivo, M.; Xing, B. *Nat. Commun.* **2016**, *7*, 10432.
- (14) Li, J.; Chen, P. R. *Nat. Chem. Biol.* **2016**, *12*, 129.
- (15) Nagel, G.; Szellas, T.; Huhn, W.; Kateriya, S.; Adeishvili, N.; Berthold, P.; Ollig, D.; Hegemann, P.; Bamberg, E. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 13940.
- (16) Xie, X.; Gao, N.; Deng, R.; Sun, Q.; Xu, Q.; Liu, X. *J. Am. Chem. Soc.* **2013**, *135*, 12608.

- (17) Gao, Y.; Shi, J.; Yuan, D.; Xu, B. *Nat. Commun.* **2012**, *3*, 1033.
- (18) Park, S. A.; Lee, S.; Tung, L.; Yue, D. T. *Sci. Rep.* **2014**, *4*, 6125.
- (19) Orrenius, S.; Zhivotovsky, B.; Nicotera, P. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 552.
- (20) White, R.; Rose, K.; Zon, L. *Nat. Rev. Cancer* **2013**, *13*, 624.

Chapter 6

Conclusion

Currently, UCNs have attracted considerable attention in recent decades due to their outstanding chemical and physical properties, which make UCNs serve as a promising nanoplatform for precisely theranostic applications. In this thesis, some smart UCNs-based nanoplatforms were developed successfully to promote the further NIR light-mediated precise theranostics *in vitro* and *in vivo*.

Firstly, a UV light-activatable Pt(IV) prodrug and a ROS-sensitive dye were anchored on the surface of dual peptide-functionalized UCNs to construct the enzyme-responsive UCNs nanoplatform, which was capable of NIR light-mediated enhanced Pt(IV) prodrug activation and real-time evaluation of therapeutic efficiency. In the presence of tumor-specific cathepsin protease, the upconverted UV emission could be utilized more efficiently to photo-activate Pt(IV) prodrug after enzyme triggered cross-linking of different UCNs, resulting in enhanced release of Pt(II) antitumor drug for better tumor therapeutic effect. Moreover, the ROS-sensitive dye could be used for real-time imaging of over-produced ROS in the process of cell death and evaluation of the therapeutic response in an early stage.

Moreover, in another design, to provide a multimodal strategy for site-specifically and effective cancer diagnosis and therapy, novel and sample UCNs nanostructures were constructed successfully, which could undergo tumor-specific enzyme-responsive covalent cross-linking to selectively accumulate UCNs at the targeted tumor region. Upon NIR light illumination *in situ* at 808 nm, the light-converting emission was enhanced and the singlet oxygen generation was amplified according. Moreover, in comparison to the non-cross-linking ones, the cross-linking UCNs could significantly inhibit the tumor growth in tumor-bearing mice in the process of PDT treatment. This present design

could be utilized as a unique nanoplatform for targeted cancer therapy, which opened new doors for precision theranostics in clinical nanomedicine in the future.

Finally, we developed a novel and innovative strategy to remotely activate membrane ion-channel under NIR light irradiation in living zebrafish models. The UCNs were specifically attached to the cell surface through metabolic glycan biosynthesis and copper-free click cyclization without modifying or genetically encoding channel structures. Upon illumination with 808 nm light, the light-gated ion channel, ChR2, could be activated under the converted blue emission at 480 nm, leading to the spatiotemporal activation of cation influx *in vitro* and *in vivo*, which offered insightful understanding about the regulation of membrane-associated activities in a valuable NIR spectral window and could act as personalized nanomedicines for various pathological disorders in the future.

List of Awards and Publications

Honors and awards

- 2016.12 Chinese Government Award for Outstanding Self-financed Students Abroad (2016)
- 2016.09 Reaxys PhD Prize Finalist in 2016, which was selected out of nearly 450 excellent candidates worldwide.
- 2014.10 Best Poster Prize at Singapore International Chemistry Conference 8 (SICC-8), Singapore

Peer-review papers

1. **Xiangzhao Ai**, Chris Jun Hui Ho, Junxin Aw, Amalina Binte Ebrahim Attia, Jing Mu, Yu Wang, Xiaoyong Wang, Yong Wang, Xiaogang Liu, Huabing Chen, Mingyuan Gao, Xiaoyuan Chen, Edwin K.L. Yeow, Gang Liu,* Malini Olivo,* Bengang Xing.* In Vivo Covalent Cross-Linking of Photon-Converted Rare-Earth Nanostructures for Tumor Localization and Theranostics. *Nat. Commun.* **2016**, 7, 10432.
2. **Xiangzhao Ai**, Linna Lyu, Yang Zhang, Yanxia Tang, Jing Mu, Fang Liu, Yixi Zhou, Zhenghong Zuo, Gang Liu,* Bengang Xing.* Remote Regulation of Membrane Channel Activity by Site-Specific Localization of Lanthanide-Doped Upconversion Nanocrystals. *Angew. Chem., Int. Ed.* **2017**, 56, 3031-3035.
3. **Xiangzhao Ai**, Jing Mu, Bengang Xing.* Recent Advances of Light-Mediated Theranostics. *Theranostics* **2016**, 6, 1899-1917.
4. **Xiangzhao Ai**, Linna Lyu, Jing Mu, Ming Hu, Zhimin Wang, Bengang Xing.* Synthesis of Core-Shell Lanthanide-Doped Upconversion Nanocrystals for Cellular

Applications. *J. Vis. Exp.* **2017**, 129, e56416.

5. **Xiangzhao Ai**, Junxin Aw, Bengang Xing.* Upconversion Nanoparticles for Bioimaging. In *Phosphors, Upconversion Nanoparticles, Quantum Dots and Their Applications*. Book chapter 28, Singapore: Springer, 2016.

6. Ming Hu, Jixian Zhao, **Xiangzhao Ai**, Maja Budanovic, Jing Mu, Richard D. Webster, Qian Cao,* Zongwan Mao,* Bengang Xing.* Near Infrared Light-Mediated Photoactivation of Cytotoxic Re(I) Complexes by Using Lanthanide-doped Upconversion Nanoparticles. *Dalton Trans.* **2016**, 45, 14101-14108.