**NANYANG
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UNIVERSITY**

**CONSTRUCTION OF NOVEL SMALL-MOLECULE PROBES
FOR REAL-TIME VISUALIZATION OF PROTEOLYTIC
ENZYME FUNCTIONS**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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**CONSTRUCTION OF NOVEL
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FUNCTIONS**

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Abbreviations

δ	chemical shift (ppm)
ABP	activity-based probe
Boc	<i>t</i> -butoxycarbonyl
BSA	bovine serum albumin
CLSM	confocal laser scanning microscopy
d	doublet
Dabcyl	4-((4-(dimethylamino) phenyl)azo) benzoic acid
dd	doublet of doublets
DIPEA	N,N-Diisopropylethylamine
DLS	dynamic light scattering
DMAP	4-dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
EDC	N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride
DMF	N,N-dimethylformide

FBS	fetal bovine serum
Fmoc	9-fluorenylmethyl chloroformate
FRET	Förster Resonance Energy Transfer
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
ICP	Inductively Coupled Plasma
M ⁺	parent ion peak (mass spectrum)
MHz	megahertz
MMPs	matrix metalloproteinases
MS	mass spectrum
MSNs	mesoporous silica nanoparticles
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
RP-HPLC	reversed phase high performance liquid chromatography
rt	room temperature
s	singlet

SPPS	solid phase peptide synthesis
t	triplet
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
UV-Vis	ultraviolet-visible spectroscopy
Φ	quantum yield

Abstract

Proteolytic activities have received considerable attention given their vital roles in protein activation and degradation, which have been considered as extremely important pharmaceutical targets in the development of drugs and inhibitors for the treatment of cancer, bacterial or virus infection, and neurological disorders. Therefore, developing sensitive and accurate platforms to evaluate protease activities is necessary and critical in both scientific and biomedical areas.

Fluorescent imaging has been extensively used in direct and non-invasive protein visualization, facilitating the study of protein localization and protein-protein interactions in living cells and organisms. Commonly used strategies are based on genetically encoded fluorescent proteins or tags into the protein of interest (POI). However, such genetic manipulations and *in vitro* assays fail to reflect the dynamic protease activities in living systems. Moreover, genetic fusion of fluorescent protein and tag probing labeling methods possibly perturb protein functions and thus affect cell physiology. Therefore, novel and sensitive platforms capable of real-time monitoring proteolytic activities and functions, especially in the pathological states are highly desired. In this dissertation, by taking the advantages of Förster Resonance Energy Transfer (FRET)-based small-molecule probes in the simplicity, sensitivity and amplified signal generation, the author attempted to develop a series of novel probes to visualize proteases activities at specific sites or organelles.

In detail, the author firstly presents a simple and convenient method to visualize cell-surface proteolytic activities in living cells and tissues. The developed probe could

be specifically cleaved by the membrane-associated furin-like enzymes, thereby recovering the fluorescence signals on the membrane of furin-expressed cells (Chapter 2). Subsequently, the proteolytic activities were visualized in single cell and the more detailed physical processes were investigated in chapter 3. In order to improve the diagnostic precision and specificity at the tumor microenvironment, a series of stimuli-responsive probes for selectively imaging furin-like enzyme activities are constructed. By using the optimal property of constructed photocaged probe, membrane-localized and endosome-localized proteolytic activities in living cells are achieved respectively, which presented great potentials for dynamically monitoring the furin-mediated processing of pathogens, virus or toxins at different sorting compartment.

Chapter 1

General Introduction

1.1 Proteases

Enzyme activities have received considerable attention given their essential roles in catalyzing a series of biochemical reactions for cell regulation. For instance, kinases and phosphatases are implicated in the signal transduction and regulation of intracellular processes. The other important enzyme family-proteases provide a wide variety of functions in the protein digestion, intracellular protein turnover and damaged protein removal *etc.*¹ Dysregulation of enzyme activities will give rise to a series of biological disorders and subsequent unexpected diseases. Therefore, detailed investigations of enzyme functions and development of tools are highly crucial for the better understanding of biological basis of diseases, thereby greatly benefiting the rational design towards early diagnosis, drug discovery, and monitoring of treatment efficacy.²

Protease, also known as proteolytic enzyme, can catalyze the hydrolysis of peptide bonds and serve as an important role in a variety of biological processes, including cell cycle progression, cell proliferation, DNA replication, tissue remodeling, death and immune response.³ Due to its critical functions in cellular or extracellular processes, the regulatory network requires to be strictly controlled to ensure proper signaling pathways. However, dysregulation of proteolysis would result in numerous pathological processes like cancer, arthritis, atherosclerosis, neurological disorders as

well as parasitic diseases.⁴

The complete sets of proteases encoded by the human genome consist of more than 500 active members, which are generally classified as serine, cysteine, threonine, aspartic, and metalloproteases.⁵ Generally, proteases cleave peptide bonds with two fundamentally catalytic mechanisms. In the N-terminal threonine, serine and cysteine proteases, they process the covalent catalysis by nucleophilic attack of the catalytic side chain residue. By contrast, metalloproteases and aspartic proteases go through the non-covalent catalysis by generating an activated water molecule (Figure 1.1).⁶

Fig. 1.1 Catalytic mechanism of five classes of proteases.⁴

1.2 Biological functions of proteases in cancer

Since Fisher in 1946 proposed that tumor-associated proteolytic activity could be essential for the degradation of the cell matrix,⁷ the biological functions of proteases in cancer was brought into sharp focus in next few decades. Recent studies have shown that proteases are responsible for tumor progression both at primary and metastatic sites.⁸ Tumor formation from the normal cells requires several generations of mutation and natural selection. Tumor circulation, invasion and metastases have to rely on the

development of new blood vessels for the transportation of the nutrients and oxygen. Such process is facilitated by various proteases in the tumor or surrounding areas (Figure 1.2).⁹ Cells involving in these metastasis steps include fibroblasts,¹⁰ endothelial cell (EC), and inflammatory cells (IC), like the extracellular matrix and macrophages.^{5,11}

Fig. 1.2 Illustrations of tumor cells in the process of dissemination and colonization .⁵

Intracellular and extracellular proteases play essential roles in regulating various cellular processes, including cell adhesion, proliferation, differentiation, migration, angiogenesis, senescence, apoptosis and evasion of the immune system.¹² The intracellular proteases generally participate in protein digestion, intracellular protein turnover and damaged products removal, *etc.*¹³ Lysosomal caspases mediate the programmed cell-death process-apoptosis and play important roles in the processing of cytokines.¹⁴ lysosomal aspartyl protease cathepsin D is highly relevant to tumor

invasion, membrane degradation and angiogenesis.¹⁵ On the other hand, extracellular are largely responsible for the tumorigenesis and invasion. Matrix metalloproteinases (MMPs) are thought to cause the degradation of extracellular matrix (ECM), the release of growth factors and the production of bioactive molecules.¹⁶⁻¹⁸

1.3 Applications of proteolysis

Due to the hallmark of altered protease activities in diseases, proteases have aroused interest not only as pharmaceutical targets for inhibitors or drug development, but also as valuable tools for the protease-sensitive fluorescent imaging and drug delivery systems.

1.3.1 Protease-sensitive imaging probes

Owing to the benefits in low costs, high signal contrast, easy implementation and non-invasive readout, optical imaging has been considered as one of the most widely used techniques for real-time visualizing the enzyme activities and cellular events *in vitro* and *in vivo*.¹⁹⁻²¹ In order to analyze detailed and authentic enzyme processing, it is highly necessary to generate more specific and quantitative signals in the course of optical imaging. Strategies based on the principle of Förster Resonance Energy Transfer (FRET) stand out owing to its amplified signal generation, the accuracy in fluorescent intensity measurement, better contrast and high detection sensitivity.²²⁻²⁴

One simple and conventional approach to visualize protease is the use of bio-activatable imaging probes. As mentioned earlier, most proteases will interact with targeting peptide sequences before cleavage. Using the nature of substrates, a bio-activatable imaging probe can be designed by conjugating the optimal peptide

QSY 21 and Alexa Fluor 647 fluorophore at the opposite terminus of the caspase recognition sequence, to achieve the imaging of caspase activity during apoptosis.²⁹

An alternative approach to study the enzyme activity is to covalently bind to the enzymes at the catalytic site, usually known as activity-based probes (ABP).³⁰ A typical ABP consists of three basic components: a reactive group (called a warhead), a specific recognition linker and a fluorophore. The covalent binding-based FRET probes mostly rely on the quenched ABP (qABP), which the use of fluorophore/quencher pairs is allowed. The recognition probe provides selective affinity to the enzyme of interest, followed by covalent modification. Then the probe-labeled protease will become fluorescent once the quencher moiety is released from the molecule structure. So far, several types of enzyme families like protease, kinase, phosphatases *etc.* have been analyzed by using such covalent binding-based probes.³¹⁻³³

Fig. 1.4 General module of a quenched activity based probe (qABP). (A) Inhibition illustrations of cysteine proteases by an acyloxymethyl ketone. (B) Activity-based

labeling of a cysteine protease target by a qABP.³⁴

Blum *et al.* developed a variety of quenched activity-based probes (qABP), based on the epoxysuccinyl- and acyloxymethyl ketone reactive groups.³⁴ These pre-quenched probes can recover their fluorescence once activity-based covalent modification occurs. These reagents can be applied for dynamically monitoring protease activities in living cells. Subsequent near infrared versions of fluorescent probes were able to provide whole-body non-invasive imaging.³⁵

1.3.2 Protease-activatable Prodrugs

The traditional chemotherapy of human diseases, particularly cancer treatment always comes along with the non-specific cytotoxicity to normal tissues and does-limited effect at the target site. In order to improve the bioactivity of therapeutic agents while minimizing cytotoxic effects in proliferating normal cells, targeting the up-regulation of proteases or other biomarkers in the diseases areas have been attempted. Alternative drug platforms are designed to control the activation and distribution of therapeutic agents, which the concept of prodrug was develop. Prodrug could go through the structure transformation by endogenous or exogenous stimuli to liberate the bioactive drugs at the disease areas.³⁶ In this way, prodrugs can largely enhance the efficacy of the chemotherapy and reduce the adverse effects. Among the various bio-responsive stimuli, proteases have received a lot of attention due to the excessive proteolytic activities occurred in many diseases. In particular, a variety of protease-activatable prodrugs have been reported and most of them have been developed for the treatment of cancer.^{37,38}

The rational design of prodrug is mainly based on the profound understanding of disease molecular pathologies and chemical molecular properties for altering the pharmacokinetics and pharmacodynamics. To design a protease-sensitive prodrug, the most straightforward approach is to conjugate a free drug to a protease-responsive peptide sequences via the formation of amide bonds. The newly formed molecule will be less potent and pharmacologically active, which later will be hydrolyzed by selective proteases to liberate the effective drug molecules. Such peptide-caged drug can achieve the preferential release of chemotherapeutic drugs at the target site upon the hydrolytic cleavage, thereby maximizing the tumor treatment efficacy and minimizing the cytotoxic effect.³⁹ So far, a large number of peptide-caged prodrugs have been developed for targeting several proteases, most of which are early diagnostic and prognostic markers for cancer, like MMP, caspases, cathepsin B, plasmin and uPA *etc.*^{38,40}

Table 1.1 Examples of peptide-based prodrugs in response to cancer-associated proteases.³⁸

protease	Constructed sequences	Drug	Diseases
Caspase-3	Asp-Glu-Val-Asp-Pro-PABC-X ⁴¹	DOX,	Cancer
	CAR-Lys-Gly-Ser-Gly-Asp-Val-Glu-Gly-X ⁴²	PH-A	
Cathepsin B	N-L-Leu-X ^{43,44}	DNR	RA
CP	Arg-X, Ala-X, Asp-X ⁴⁵	MTX	Cancer
FAP	BHQ3-Lys-Gln-Glu-Gln-Asn-Pro-Gly-Ser-Thr-X ⁴⁶	PH-A	Cancer
Kallikrein 2	Gly-Lys-Ala-Phe-Arg-Arg-X ^{47,48}	TPG	Cancer
MMP-2/-9/-14	Glu-Pro-Cit-Gly-Hof-Tyr-Leu-X ^{49,50}	DOX	Cancer
MMP-7	BHQ3-Lys-Arg-Ala-Leu-Gly-Leu-Pro-Gly-X ^{51,52}	PH-A	Cancer
Plasmin	D-Ala-Phe-Lys-X ⁵³	ara-C,	Cancer
	D-Val-Leu-Lys-X ^{54,55}	AT-125	RA
PSA	Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-X ⁵⁶	DOX,	Cancer
	4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Ser-Pro)-X ^{57,58}	5-FudR	

TOP	β -Ala-L-Leu-L-Ala-L-Leu-X ^{59,60}	DOX	Cancer
uPA	D-Ala-Phe-Lys-PABC-X ⁵³	DOX	Cancer

Since the proteolytic imbalance or upregulation of proteases have been correlated with various types of diseases, numerous applications based on the proteolysis have been explored to assist the therapy and diagnosis. Protease-sensitive molecular reporters are designed for disease imaging and detection. Selective prodrugs or controlled delivery systems are constructed to achieve the desired therapeutic efficacy. Moreover, protease-based drug discovery and inhibition are also pursued for more specific and effective treatment.

1.4 Biological properties of Furin-like enzyme

In general, Prooprotein convertases are the subfamily of subtilases, which belongs to the family of subtilisin-like and Ca^{2+} -dependent serine proteases. These convertases associate with the activation of cellular and pathogenic precursor proteins like polypeptide hormones, growth factors and bacterial pathogens etc. They consist of nine family members: convertase 1 (PC1), PC2, furin, PC4, PC5, paired basic amino acid cleaving enzyme 4 (PACE4), PC7, subtilisin kexin isozyme 1 (SKI-1) and proprotein convertase subtilisin kexin 9 (PCSK9)⁶¹.

Furin, which belongs to the members of the proprotein convertase family, proteolytically activates a large number of proprotein substrates in the secretory pathways. It is ubiquitously expressed and cyclized through the TGN/endosomal compartments and the cell surface. To date, furin substrates include extracellular metalloproteases, hormones, cell surface receptors, secretases, bacterial toxins and

viral glycopeptides, *etc.* ⁶²

1.4.1 The specificity of furin cleavage site

From the statistical data of furin cleavage sites, sequences of 20 amino acids and their physical properties were unveiled.⁶³ The motif contains a core region with 8 amino acids at P6-P2' and two polar regions at P7-P14 and P3'-P6'. The specific properties are required at each position for proper recognition by furin (Figure 1.5). More importantly, characterization of furin cleavage sites will be crucial for rational design of various reporters for accelerating or on the contrary, temporally blocking the proteolytic activity.

Based on the data collected from the published reports, all biologically active substrates contain the arginine residue with positive charge at P1 position. In fact, various *in vitro* and *in vivo* studies have revealed that mutation of arginine at P1 results in undetectable cleavage, suggesting that arginine is essential at position P1. At position P2, positively charged residues Arg and Lys have been found in majority of substrates and small flexible residues such as glycine, alanine and serine are favored. Similar to the property at position P1, most of residues at position P4 are arginine and replacement of arginine will abolish the furin activity in cases like pro-anthrax protective antigen and insulin proreceptor. Different from position P1, aliphatic residues like valine and leucine are also accepted at position P4. The 3D structure and many known examples suggested that position P5 and P6 may have the compensatory effect in the absence of a positive charge at substrate position P4. Research results indicated that the introduction of Arg or Lys at position P6 will have several-fold

increase in cleavage in relative to the identical substrates with alanine at position P6. A majority of substrate screening reveals that furin preferentially cleaves the sequence pattern R-X-[K/R]-R↓, which is now regarded as the furin consensus cleave site. As for X at the position P3, there is no clear evidence to prove the existence of strong interaction with the furin binding site.⁶⁴

Fig. 1.5 Illustrations of the distinct physical properties of 20 residues at furin cleavage regions.⁶³

The commercially available furin substrate sequence is RVRR-MCA, which lacks the P1' contacts. Some studies found that substrates containing the P1' residues will show higher kinetic value compared with that lack the P1' contacts. At position P1', small hydrophilic residues are preferred and most formed by serine, asparagine, tyrosine. Additionally, bulky and hydrophobic residues like valine, leucine is not present at position P1'. At position P2', hydrophobic residues, like valine, seems to be

avored.

1.4.2 Biological roles of furin in diseases

Due to the wide spectrum of substrate candidates, it is not surprising that furin plays important roles in both physiological and pathological conditions, like atherosclerosis, infectious diseases, neoplastic diseases, and neuro-degenerative diseases.⁶⁵ At the cellular levels, furin at different locations process different substrates, which turns out that diverse furin-related diseases are developed. At the cell surface, furin is responsible for the cleavage of substrates like Anthrax toxin PA, Clostridium α -toxin and Proaerolysin. In the early endosome, substrates which can be recognized by furin include Shiga toxin, Diphtheria toxin, and Pseudomonas exotoxin A.⁶⁶ Moreover, many substrates like Pro- β -NGF, Insulin pro-receptor, Pro-BMP-4 and Ebola Zaire pro-GP are found in the trans-Golgi network where most mature furin enzyme exists.⁶²

Fig. 1.6 Furin-related processing of various disease-relevant substrates at different compartments, including cell-surface, trans-Golgi network (TGN) and endosome systems.²⁹

1.4.2.1 Cancer

Furin also presents high levels of expression in various cancer cell lines. Its substrates involved in cancers include growth factors, receptors and matrix metalloproteinases (MMPs). Membrane type-1 matrix metalloproteinase (MT1-MMP) is pivotal in cancer metastasis and invasion. The cellular MT1-MMP requires the proteolytic processing of the zymogen by furin-like proprotein convertases, and mature MT1-MMP further activates extracellular pro-MMP2 to induce the tumor growth or metastasis (Figure 1.7). The activation of MT1-MMP can be inhibited by furin inhibitors like α_1 -antitrypsin Portland (PDX), resulting in repressing the motility of cancer cells. Another furin substrate which is upregulated in the colon, prostate, breast, and lung cancers is insulin-like growth factor 1 (IGF-1). Interventions of this IGF-1 processing could reduce the incidence of metastases, indicating that inhibiting PCs may represent novel approaches to combating various aggressive cancers.^{62,67}

Fig. 1.7 Furin activate type-1 matrix metalloproteinase (MT1-MMP) first. Then active MT1-MMP further processes pro-MMP2 into MMP2, which is implicated in in the degradation of the extracellular matrix.²⁴

1.4.2.2 Infectious Diseases

Studies on the pathogenesis of certain infectious diseases reveal that the key roles of furin-like proprotein convertases to ensure their survival in the host cells. Bacterial toxins and viral surface glycoproteins can be activated by furin at the cell surface or endosome and toxic moieties exert harmful impact on the host cells.

Anthrax toxin is a type of tripartite toxin secreted by bacterium *Bacillus anthracis*. The anthrax toxin consists of protective antigen (PA), lethal factor (LF) and edema factor (EF). The full-length PA (PA₈₃) secreted from bacterium can bind to the receptors and then processed by cell-surface furin and become a 63 kDa protein, which further heptamerizes and binds to LF or EF. Subsequent endocytosis and translocation into cytosol can disrupt the mitogen-activated protein kinase (MAPK) pathway, further causing septic shock, oedema and even death.⁶⁸⁻⁷⁰

Fig. 1.8 A schematic model of *Bacillus anthracis* toxins cell entry.⁶⁸ Copyright © 2014 Taylor & Francis.

Unlike the cell-surface furin-mediated processing, the Pseudomonas exotoxin A, Shiga and diphtheria toxins require the cleavage by furin within the early endosomes. These toxins are known as A-B toxins with the active moiety (A) and binding moiety (B). Following the receptor-mediated endocytosis, the toxins are processed by furin in the early endosome where the acidic pH is provided. The low endosomal pH can trigger the translocation of toxin molecules into cytosol, where toxic factors can prevent protein synthesis.⁷¹

Based on the understanding of host proteinase-derived activity in the infection processes. Furin-like proprotein convertases have been proposed as attractive targets for the treatment and prevention of infectious diseases. Development of furin inhibitors to block the damage caused by bacterial toxins has been taken as a promising approach and more effective therapeutic option.

Fig. 1.9 Molecular action of *E. coli* SLTs. The B5 subunit interacts with the cell-surface receptor.⁷¹ Reprinted with permission from ref [71] Copyright © 2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

1.5 Stimuli-responsive strategies

During the past decades, scientists have made extensive efforts in improving the anticancer diagnosis and therapy. Various nanoscale systems have been developed as the carriers for improving the pharmacokinetics and biodistribution of reagents through the enhanced permeability and retention (EPR) effect in tumor tissues. Meanwhile, stimuli-responsive systems, which can undergo molecular conformational changes or hydrolytic cleavage in response to the corresponding stimuli, have provided additional opportunities to improve on-demand delivery, accumulation, efficient internalization of drugs or imaging reagents.⁷² As is well-known, tumor microenvironment has intrinsic physiological characters, like lowered interstitial pH, hypoxia, a higher glutathione concentration and up-regulated levels of certain enzymes *etc.* By taking the advantages of specific micro-environmental changes associated with specific diseases, imaging and therapeutic agents can be selectively activated at the targeted areas, thereby greatly improving the diagnostic precision and antitumor effectiveness (Figure 1.10).⁷³ Besides the endogenous stimuli, Extracorporeal physical stimuli like heat, light, magnetic field and ultrasound *etc.* can also be applied to guide the behavior of the imaging and therapeutic agents.⁷⁴

Fig. 1.10 Stimulus-responsive strategies in the tumor microenvironment.⁷³ Reprinted with permission from ref [73]. Copyright © Royal Society of Chemistry 2013.

1.5.1 pH-sensitive systems.

The lowered extracellular/interstitial pH in solid tumors has been employed as an intrinsic stimulus to control the localized activation of bioactive ligands in specific organs or intracellular compartments (such as endosomes or lysosomes). The irregular angiogenesis requires more oxygen and nutrients, which turns out to produce excessive acidic metabolites (lactic acid) in the tumor interstitium.⁷⁵ Therefore, the extracellular environment of solid tumors is more acidic (pH 6.5 to 6.8) than that of healthy tissues. Besides the lowered pH in the extracellular tumor areas, the acidification of early endosomes also occurs during endocytosis owing to the vacuolar proton ATPase-mediated proton influx.⁷⁶ By taking the advantages of pH difference, researchers have exploited a variety of pH-responsive systems to improve the local

drug accumulation while minimizing the systemic toxicity to healthy tissues.

In order to achieve pH-dependent drug release, several approaches have been can be designed. One way is based on the hydrophobic-hydrophilic transition.⁷⁷ Some nanomaterials containing chemical groups like amines, carboxylic acids and phosphoric acid are subject to pH-dependent changes in their chemical and physical properties, like swelling and solubility. Such conformation changes results in either enhanced internalization or release of bioactive molecules at the target cells. Lee's group developed a pH-responsive polymeric micelles (pH-PMs) that contains the poly(ethylene glycol) methyl ether-poly(β -amino ester) (MPEG-PAE).⁷⁸ The tertiary amines that is ionized at the lower pH values induce pH-dependent micellization/demicellization transition for drug release at the tumor acidic environment.

Fig. 1.11. Illustration on the design of peptide-based pH-responsive micelles.⁷⁸

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The second method is based on the acid-labile chemical bonds. Such acid-labile groups are stable under neutral conditions while hydrolyzed under acidic conditions, thereby causing the release of drugs or imaging agents. The mostly used acid-labile

linkers include orthoester, acetal, hydrazone, cis-aconyl bonds and imine *etc* and chemical structures and respective degradation products are summarized in the table 1.2.⁷⁹ Recently, Zhang's group reported acid-responsive therapeutic nanoparticles (NPs) that cisplatin was covalently attached to the chains of two biocompatible diblock copolymer by pH-sensitive hydrazone bond.⁸⁰ The resulting NPs presented well drug release kinetics in response to the acidic environment, and enhanced *in vitro* cytotoxicity against ovarian cancer cells than free cisplatin. More importantly, these NPs could potentially avoid the drug release during the NP circulation in the blood where the neutral environment exists, but rapid drug release after the internalization by target tumor cells in responsive to the acidic intracellular environment.

Table 1.2 Lists of acid-sensitive chemical bonds and degradation products⁷⁹.

Name	Chemical bonds	Degradation products
Orthoester		HO---
Ester		HO---
Hydrazone		H ₂ N---
Imine		H ₂ N---
Cis-aconityl		H ₂ N---
Acetal/ketal		HO---

1.5.2 Redox-sensitive systems.

The reducing agent glutathione (GSH) is ubiquitous in living cells with a higher concentration (2-10 mM) in cytoplasm than that in extracellular matrix and blood pool (about 2-20 μ M).⁸¹ In particular, the concentration level of glutathione in tumor areas is higher than that in healthy ones. By taking the advantages of redox potential difference, rational design based on the widely used redox reaction of disulfide bond has been applied for the purpose of intracellular drug release and tumor targeting. For example, Li and his coworkers developed redox-sensitive hyaluronic acid-deoxycholic acid (HA-ss-DOCA) conjugates to deliver the anticancer drug paclitaxel (PTX).⁸² Such self-assembled nano-size micelles kept stable at normal physiologic conditions whereas quickly disassembled when treated with 20 mM reducing agent, e.g. glutathione. Both *in vitro* and *in vivo* investigations revealed that such HA-ss-DOCA micelles presented higher tumor targeting and improved antitumor efficacy of PTX.

1.5.3 Enzyme-sensitive systems

Enzyme involves a series of biochemical reactions for cell regulation and signal transduction. Under pathological conditions, some enzymes like such as proteases, phospholipases or glycosidases presented altered expression levels, which has been considered as biomarkers for diagnostics and therapy for cancer, inflammation, and neuron degeneration.⁸³ Meanwhile, the hallmark of the irregular enzyme expression also provides great possibility for specifically activating imaging agents and drug molecules at pathological sites through enzyme-responsive reactions. Matrix metalloproteinases (MMPs) belongs to the family of human zinc endopeptidases,

which are responsible for the degradation of extracellular matrix (ECM) and the process of tissue remodeling.^{17,18} Additionally, they are also found to be upregulated in tumor microenvironment and tumor invasion, proliferation and metastasis. MMP-responsive substrates including peptides and proteins have been widely employed for tumor-targeted biomolecule delivery, especially for MMP-2 and MMP-9.⁸⁴

Recently, the design of the multifunctional liposomal nanocarrier in response to MMP-2 in the tumor microenvironment were developed.⁸⁵ The nanocarrier was functionalized with polyethylene glycol (PEG)-lipid and tumor cell-specific antinucleosome monoclonal antibody (mAb 2C5). The MMP-2-sensitive bond between PEG and lipid that can be cleaved in tumor areas, which exposed the previously hidden cell penetrating peptide (TATp) to improve the internalization. The results indicated that such design was able to significantly enhance the tumor target ability and internalization of nanocarriers into tumor cells.

Fig. 1.12 The design of multifunctional liposomal nanocarrier in response to

MMP-2.⁸⁵ Reprinted with permission from Ref [85]. Copyright © 2012 American Chemical Society.

In a recent publication, Wang *et al.* reported a new MMP-2 targeting nanoprobe used for tumor detection and imaging.⁸⁶ Their nanoprobe design comprises of two main units, (1) low molecular weighted herapin modified quantum dots (QDs) and (2) QSY21 quencher dye with MMP-2 cleavable peptide sequence (ALMWP). These two units provided the FRET effect, thus making the QDs inactive. Once inside, the QDs are localized in the MMP-2 expressed tumor, and QSY21 will be cleaved from the QD. These will activate the QDs, thus allowing it to fluoresce. In addition, with the use of the low molecular weighted herapin, non-specific uptake of QDs by reticuloendothelial system was significantly reduced. This allows the nanoprobes to be accumulated within tumor areas due to the EPR effect. For further application, Wang's group used the nanoprobe for brain tumor imaging. It was noted that MMP-2 is overexpressed in gliomas, which is accounted for 80% of all malignant brain tumor. To overcome the poor penetration of the blood-brain barrier (BBB) of the probe, they modified their initial probe design. By incorporating the brain-targeting T7 sequence into their cleavable peptide sequence, they were able to improve the accumulation of the QDs in the brain tumor with significant fluorescence effect.

Fig. 1.13. (a) Illustration of the design and mechanism of the QD probe (b) QD/FRET ratio imaging of different cell lines.⁸⁶ Copyright © 2015, American Chemical Society.

Similar enzyme-sensitive strategies are also applied in other enzymes. For example, gate-like functional hybrid systems based on silica mesoporous nanoparticles, which saccharide derivatives were anchored on the pore outlets.⁸⁷ Such capped mesoporous silica was able to specifically deliver drugs into cells after the enzymatic-assisted cleavage of the glycosidic bond and reduction in length of saccharide chains at the surface of nanoparticles.

Despite great success has been obtained in the enzyme-responsive systems, more efforts are still needed to fully understand the precise information and substrate preferences of the target enzyme levels at the specific tumor site. Therefore, creative chemistry and approaches in resolving the specificity will be needed to ensure the correct signal measurement during the catalytic studies.

1.5.4 Light-sensitive systems

Among various external stimuli-responsive approaches, light activation stands out due to its superior advantages in the highly spatial and temporal precision and minimally invasive capability. So far, a huge number of photo active systems have been developed in response to light irradiation at different wavelength ranging from the ultraviolet to near-infrared (NIR) regions.⁸⁸ As for the design of light-sensitive platforms, several chemical groups such as o-nitrobenzyl, azobenzene, spiropyrans and diarylethenes *etc* have been established as biostable photoswitches to create biologically active compounds, which can be reversibly activated and deactivated. Additionally, some of them have been successfully applied for spatiotemporally regulating biomolecule activities and controlled cancer therapy and diagnostics in *in vitro* and *in vivo*.⁸⁹

In biological systems, the activity of therapeutic molecules can maintain dormant by introducing some photo-cleavable groups on their chemical structure. Once the photolabile group is irreversibly activated by irradiation with light, such liberated molecules will present their biological activity and therapeutic effect. By far, the most widely used photolabile caging groups rely on the 2-nitrobenzyl (NB) derivatives in research studies.⁹⁰

Fig. 1.14 (A) Chemical structures of photo-sensitive caging groups; (B) the reversible photoisomerization of molecules.⁸⁹ Reprinted with permission from Ref. [89]. Copyright © Royal Society of Chemistry 2010.

For example, Yeh and his co-workers recently developed a photocaged nanosystem by using the *o*-nitrobenzyl (ONB) group to cage the folate acid (FA) molecule on the surface of Au nanoparticle (Au NPs).⁹¹ The light-triggered uncaging and activated FA enable better targeting specificity to cancer cells. Besides, intracellular delivery was achieved by encapsulating the drug paclitaxel (Taxol) into the PLGA@lipid hybrid nanoparticles (PLGA=poly(d,l-lactide-co-glycolide)). The active FA-mediated targeting can greatly improve the cellular internalization of PLGA@lipid nanoparticles to liberate Taxol molecules under the light irradiation. Such activated platform presented enhanced cytotoxicity to tumor cells when compared to the inactivated nanoparticles which FA molecule is under the caged state.

Fig. 1.15 General scheme of the process for the photocaged folate nanosystem, which was activated when exposed to the ultraviolet light for targeting cancer cells. NBA=2-nitrobenzylamine.⁹¹ Reprinted with permission from Ref [91] Copyright © 2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Another mostly applied design is the photoisomerization of the chemical groups-reversible cis-trans switches by shining light in ultraviolet or visible region. Different strategies based on photoisomerization molecules like spiropyran, diazobenzene and dithienylethene *et al.* have been achieved to control the on-off biomolecule event by illumination with light of different wavelengths. In 2012, Tan's group developed a photon-controlled mesoporous release system on the basis of azobenzene-incorporated DNA double strands.⁹² The azobenzene-modified DNA was immobilized on the surface of MSN, capping the guest molecules within the nanopores. Changes in the light from visible to the UV light enable the release of guest molecules from the DNA/mesoporous silica hybrid nanostructures due to the conformation changes of the azobenzene group.

Fig. 1.16 Illustration of azobenzene-modified DNA-manipulated reversible release system.⁹² Adapted with permission from Ref [92] Copyright © 2012, American Chemical Society.

The reversible photoisomerization of spiropyran (SP) in response to ultraviolet light was also well-established to controlled drug release. Kohane's group designed photoswitching SP/lipid-polyethylene glycol (PEG) hybrid nanoparticle system that contains spiropyran analogue and DSPE-PEG complex, (1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-carboxy (polyethylene glycol)-5000).⁹³ Such assembled nanoparticulate system enabled not only absorption changes (E_m from 350 nm to 551 nm), but also volume changes from 150 to 40 nm when illuminated with UV light. Such light-induced size switching allowed the spatiotemporal controlled release of drugs, thereby enhancing the tissue penetration and antitumor efficacy.

1.6 Research goals

Optical imaging has been considered as a powerful tool for wide applications ranging from molecular biology to clinical diagnosis. This sensitive, fast and

non-invasive technology has a considerable influence on the prevention and treatment of a large number of human diseases. As one of the most abundant enzyme families, protease, many physiological and pathological processes such as protein catabolism, inflammation, infection, and cancer progression *etc.* Among this huge family, furin has been found to play many essential roles in the cancer progress and bacterial or virus invasion at different organelles. In this thesis, the author aims to develop various novel small-molecule based reporters for furin-like enzyme, which was chosen as the biological target owing to its importance in human health concerns.

Furin was ubiquitously expressed and cyclized between the trans-Golgi network and the cell surface. cell-membrane bound furin or furin-like proprotein is thought to involve in the invasion of bacteria and virus. Thus the development of a simple and convenient method to visualize cell-surface proteolytic activities in living cells are highly of interest and significance. Rational design of a novel small-molecule fluorescent probe to selectively visualize furin-like activities on the cell surface was described in chapter 2. What's more, the developed design was employed to visualize the single cell imaging and relevant physical modules were established in chapter 3.

Based on the design of membrane-localized probe, investigations on how to specifically switch on the proteolytic activation in response to tumor microenvironment was carried out in chapter 4. Firstly, the physical properties of furin binding pocket were systematically analyzed for the screening the enzyme-recognized substrate sequences. Then various stimuli-responsive strategies like pH-sensitive, GSH-sensitive and photoresponsive approaches were utilized in the construction of

stimuli-responsive probes for selectively visualizing furin-like enzyme activities. In the chapter 5, the well-designed photocaged probes were successfully applied for membrane-localized and endosome-localized enzyme imaging in living cells. Such promising results provide more potential for exploring the furin-mediated processing of some pathogens, virus or toxins at different sorting compartment.

1.7 References

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Chapter 2

Small-Molecule FRET Reporter for Real-Time Visualization of Cell-Surface Proteolytic Enzyme Functions

ABSTRACT. The real-time visualization of cell-surface associated proteolytic enzymes is crucial for better understanding of their performances in both physiological and pathological processes. Whereas current strategies are limited by their complexity and poor membrane anchoring properties. Here, we successfully developed and synthesized a simple and unique small molecule fluorescent probe, which integrates the principles of passive exogenous membrane insertion and Förster Resonance Energy Transfer (FRET) to image cell-surface associated proteolytic furin-like convertase activities. The membrane associated furin-like enzymatic cleavage of the developed peptide reporter molecule would lead to a highly-increased fluorescence signal which was found to be mainly localized onto the plasma membrane of the furin expressed cells upon the assistance of the carefully optimized hydrocarbon lipid moiety in the probe structure. Such rationally designed small molecule fluorescent

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probe may act as a unique and reliable probe for real-time imaging of endogenous cell-surface localized furin-like enzyme functions in living cells and tissues based on both one-photon and two-photon microscopy setups.

2.1 Introduction

Plasma membrane that separates the interior of cells from outside environment consists of phospholipid and many integral membrane proteins and membrane-associated proteins.^{1,2} Membrane proteins are critical for a variety of vital functions including cellular communications, material transport and energy conversion.³ Combining the fluorescence imaging and protein specific labeling technique, the dynamic behaviors of membrane-associated proteases have been visualized and investigated.^{4,5} For instance, the endocytic internalization of membrane proteins labeled with fluorophores can be observed through stimulation by agonist. Also, the membrane redistribution to other lipid microdomains, interprotein interactions and conformational changes could be revealed by using FRET.⁶

Membrane-associated proteolytic enzymes play important roles in physiological and pathological processes ranging from growth factors and receptors' activation, extracellular matrix processing to bacteria invasion, *etc.*⁷ These proteolytic events not only have been involved in the site specific cleavage of bioactive proteins or peptide substrates within transmembrane domains, thus performing various biological functions, they also participate in the progression of various degenerative diseases including atherosclerosis, neurological disorder and cancer.^{8,9} Therefore, diagnostic targeting and regulating of intramembrane proteolysis becomes one promising option

for deeper insight into the basic pathological pathways and even for the treatment of cancer and other diseases.^{10,11}

2.1.1 Membrane-associated furin functions

Furin, a 96 kDa type-I transmembrane protein that belongs to the proprotein convertase (PC) family, is ubiquitously expressed and cyclized between the trans-Golgi network and the cell surface.¹² Furin-like convertases can activate a variety of protein precursors and proproteins in intracellular membrane systems and cell surface and process into biologically functional peptide and proteins.¹³ For example, furin has been well known to participate in the intramembrane processing of several types of matrix metalloproteinases (MMPs), especially the MMP-2 enzyme which was found to be elevated in several types of human cancers.^{14,15} The activation of α -secretase and β -secretase, which are two key enzymes in processing the generation of toxic amyloid peptides during the development of Alzheimer's disease (AD), was also mediated by the proteolytic activities of furin.¹⁶ Moreover, Although furin is widely localized in membranes systems, its distribution in different locations may indicate different actions. Due to their highly relevance to chronic pathologies, which would induce a series of cellular events and even cell death, cell surface associated furin has received a lot of attention. Besides, cell-surface furin-like pro-protein processing was also extensively found to mediate the maturation of bacterial toxins and the propagation of many non-enveloped or lipid-enveloped viral pathogens, which are prerequisite processes to mediate the bacterial or viral invasion into host cells.¹⁷⁻¹⁹ In terms of such multiple functions of furin in human

pathophysiology, effective strategies to visualize cell-surface furin activities will be of high significance, which will pave the way for elucidating cell-membrane functions and understanding furin-dependent dynamic processes, and thus making them as potential target of interests for the development of therapeutic agents. .

2.1.2 Approaches to imaging of specific cell-membrane associated proteolytic enzyme activity

Recently, researchers introduce fluorescent protein such as green fluorescence protein (GFP) labelling or genetically encoded tag-based fluorescent protein probes to image the extracellular furin activities.²⁰⁻²² Similar non-invasive investigation of cell-surface proteolytic furin activity was also achieved in single living cells on the basis of the reengineered anthrax toxin- β -lactamase fusion protein mutants with the altered protease cleavage specificity.²² Although all of these approaches have made substantial progress, these genetic manipulations before analyzing the activation process may potentially perturb the cell's physiology and cause biological responses.²³ Moreover, they are not ideal for evaluating the enzyme activities and pathological processes. In order to compensate the shortcomings of genetic manipulation, another developed approach to studying protease activity is based on the commercially available chromogenic or fluorogenic substrates (e.g. easy preparation and manipulation).²⁴⁻²⁹ Unluckily, because of the rapid diffusion and poor immobilizing ability on the cell surface, most established small-molecule fluorescent reporters for furin-like assays (such as the fluorogenic furin convertase substrate Boc-RVRR-AMC) are mainly applied in fixed cells or cell-lysate systems.³⁰

2.1.3 Rational design and principle

Rational design of simple and reliable small-molecule-based probe molecules for real-time imaging of specific cell-membrane associated proteolytic enzyme activity remains a big challenge in the fields. Herein systematic investigations on developing small-molecule probes with tractability and immobilizing property for visualizing cell-surface proteolytic activities in living cells are highly of interest and significance. Rao and his coworkers³¹ have developed a PA probe via the formation of aggregates and nanostructure to detect furin-like activities in trans-Golgi apparatus. However, to the best of our knowledge, there is no small-molecule based probes that have been reported for the detection and real-time visualization of low levels of cell-surface furin-like activity.

Herein, we provide a simple and convenient strategy to selectively visualize furin-like enzyme activities on the cell surface. This well-designed membrane-anchored and furin-responsive probe (MFP) molecule could efficiently insert into the lipid raft compartments in cell membrane, and will be barely internalized by cellular structure when upon the specific enzyme interactions, which greatly facilitate the reliable fluorescent imaging of furin-like enzyme functions on the cell membranes. Moreover, such unique enzyme responsive cell-surface insertion also provides great opportunities for real-time visualization of specific extracellular proteolytic enzyme activities in living cells and tissues through both one-photon and two-photon imaging facility. The pre-quenched reporter was also characterized with minimum auto-fluorescence and cytotoxic side effect.

Figure 2.1 illustrates the small molecularly defined peptide probe toward the imaging of the processes of furin activity on cell surface. Generally, the rational design of furin preferentially recognized probe was based on the consensus formula of (K/R)-(X)_n-(K/R) ↓ , as the basic moiety for enzyme reaction (where n = 0, 2, 4 or 6 and X is any amino acid, respectively),³¹⁻³³ and further integrated with the principle of passive exogenous insertion and Förster Resonance Energy Transfer (FRET) in order to visualize the membrane associated furin activity.³⁴⁻³⁶ As proof of concept, peptide sequences of RXRR was chosen as main binding motif, which were subsequently linked with small hydrophilic residues (e.g. serine) at position P1' and hydrophobic residues (e.g. valine) at position P2' respectively at C-terminus to improve the binding strength between furin like enzymes and the substrate. Therefore, the RVRRSV sequence was chosen in our design due to selective cleavage by furin over other enzymes.

For the purpose of real-time visualization of furin activity, the enzyme responsive RVRRSV sequence was conjugated with an activatable FRET pair consisting of fluorescein (FITC) and an effective FITC quencher Dabcyl. The introduction of FITC as the fluorophore was mainly for its optimal fluorescence quantum yield at the physiological environment and promising two-photon properties.³⁷ Dabcyl moiety is widely used to quench fluorescent signals of FITC due to their desirable spectral overlap, potentially lower fluorescence background. The 6-aminohexanoic acid unit provides the spacing between FITC and peptide sequence for MFP to be cleaved by furin.

Fig. 2.1 Imaging process of furin cleavage on cell surface and structure of membrane-anchored and furin-responsive probe (MFP). The furin cleavage site is localized between Arginine (R) and Serine(S) (dashed line).

In addition, in order to specifically immobilize the enzyme responsive fluorescent probe onto the cell surface,^{28,38-40} the hydrophobic fatty acid moieties with different length of carbon chains were carefully screened. The probes containing different hydrophobic chains and furin-responsive peptide sequences were synthesized by using the similar synthetic routes. The selected lipid moiety could serve as efficient targeting vector to associate the fluorescent probe to surface, mostly to lipid raft compartments in the cell membranes to enhance the specific enzyme interaction.

2.2 Result and discussion

In Figure 2.2, the synthesis of fatty acid tagged FITC-peptide probe was performed by Fmoc based solid phase peptide synthesis. After fluorescent labeling, the peptide was cleaved from the resin and exposed the free ϵ -amine on the Lysine, which was further reacted with Dabcyl SE. After removing all the protecting groups, the final product (MFP) was purified by reverse-phase high-performance liquid chromatography (HPLC) and further characterized by mass and spectroscopic analysis (Figure 2.3).

Fig. 2.2 Synthesis of MFP probe. a) DIPEA, 1 hr in DCM; b) HBTU/DIPEA in DMF; c) 2 % hydrazine in DMF, 20 min, then FITC/DIPEA, 4 hr; d) DIPEA, Dabcyl SE, 4 hr; e) 95 % TFA, 2.5 % H₂O, 2.5 % TIPS 2 hr.

Fig. 2.3 a) UV absorbance spectra of FITC (in black) and MFP (in red); b) Fluorescence Intensity of FITC (in black) and probe (in red).

The fluorescent properties of the MFP probe demonstrated that FITC can be initially quenched by Dabcyl moiety (Figure 2.3), making it a desirable platform with high sensitivity and low-level background noise. Subsequently, the enzymatic hydrolysis of the developed MFP probe (6.5 μ M) was studied by measuring the changes in fluorescence emission upon the addition of furin in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (0.1 M, pH 7.4). As shown in Fig 2.4A, the MFP probe was almost non-fluorescent due to efficient FRET quenching effect. After incubation with furin enzyme for 2 hours, there was 12-fold increase in the fluorescence signals observed at a maximum wavelength of 525 nm, which was attributed to the connected FITC. Similar enzyme hydrolysis was carried out in the presence of standard inhibitor (Dec-RVKR-cmk) and results demonstrated that there was no obvious fluorescence enhancement after 2 hours incubation time,⁴¹ suggesting that the specific furin interaction would broke FRET pair by releasing the fluorescence quencher Dabcyl.

Fig. 2.4 A) Fluorescence increase of MFP (6.5 μM , in HEPES buffer, pH 7.4) when incubated with furin in the presence or absence of inhibitor Dec-RVKR-cmk at different times (0 ~ 120 min, $\lambda_{\text{ex}} = 450 \text{ nm}$) at 37 $^{\circ}\text{C}$. Dec-RVKR-cmk (100 μM) was preincubated with furin enzyme for 30 min before the addition of probe. Inset: the enzymatic kinetics of furin (2U) with MFP. B) HPLC characterization of MFP alone, MFP with furin or MFP incubated with inhibitor before addition of furin. C) The LC-Mass spectroscopy of the cleavage product S-V-K (DabcyI).

Enzyme kinetics of MFP probe to furin hydrolysis were further determined ($K_{\text{m}} = 25.5 \pm 2.1 \mu\text{M}$, $K_{\text{cat}} = 2.78 \pm 0.51 \text{ s}^{-1}$) and the enzyme catalytic efficiency ($K_{\text{cat}} / K_{\text{m}}$) for furin was $1.09 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. Moreover, characterization of the furin processing was also confirmed by HPLC analysis (Figure 2.4C). There was a new peak observed to increase gradually after incubation with furin for 60 min. The retention time of furin enzyme cleavage product (S-V-K (DabcyI)) is 10 min, which was further confirmed by LC-MS analysis. The retention time of enzymatic product incubated with furin inhibitor was the same as that of MFP probe, clearly suggesting that the designed MFP

probe could be specifically cleaved by furin hydrolysis. The result is in accordance with that measured in the fluorescent microscopy.

Fig 2.5 Analysis of furin expression by western blot in U251 and Lovo cells. β -tubulin was included as an internal control.

After verifying the MFP probe in response to the furin enzyme, next we tested its feasibility on furin dependent imaging on cell membrane. In this experiment, human glioma U251 cells were chosen as our target owing to the expression of high levels of furin.^{42,43} Meanwhile, the furin-deficient Lovo cell line was selected as a negative control.⁴⁴ In order to validate the specific expression level of furin enzyme in different cell lines, both cell lysated samples were initially analyzed by immunoblotting with anti-furin antibodies. For quantitative analysis, the expression levels of furin were normalized to internal control of β -tubulin. The chemiluminescence signals in Figure 2.5 confirmed the highly expressed furin in U251 cell lines, whereas the same enzyme expression was greatly reduced in Lovo cell lines. Subsequently, we performed the real-time imaging assays in both cell lines. Basically, U251 and Lovo cells were cultured and incubated with MPF probe (130 nM) in Dulbecco's Modified Eagle

Medium at 37 °C for 15 min. The fluorescent imaging was recorded by a laser scanning confocal microscopy (LSCM) with excitation filter 360/40 nm and emission filter 535/40 nm. By contrast, cell membrane was also tracked with commercially available CellMask Deep Red which was widely applied to monitor the status of membrane staining. As shown in Figure 2.6, after 15 min incubation with MFP probe, the intense green fluorescence signals on the surface of U251 cells were clearly detected.

Fig. 2.6 Fluorescence imaging of U251 cells when treated with MFP. Living cells were incubated with MFP alone (130 nM), MFP and inhibitor (40 μ M) for 15 min at 37 °C. Cells stained with CellMask Deep Red (2.5 μ g/mL) were used as standard to stain the membrane. The relative fluorescence intensity was plotted as a function of distance along with arrows. Lovo cells incubated with MFP as control. (Scale bar: 20 μ m)

Moreover, fluorescent signals produced from MFP on the U251 cell membrane

could still be detected even with prolonged incubation time (up to 90 min) (Figure 2.7 and 2.8). On the other hand, there was no obvious fluorescence detected in the same cells with the pre-treatment of furin inhibitor (Dec-RVKR-cmk) or in the control group of furin-deficient Lovo cells even under high concentration probe incubation (Figure 2.9). Such promising imaging results demonstrated that MFP probe could serve as an effective tracer to report the cell-surface associated furin activity in living cells.

Fig. 2.7 Real-time confocal fluorescence images of U251 cells treated with MFP (130 nM) ($\lambda_{\text{ex}}=488$ nm).

Fig. 2.8 Time-lapse imaging of U251 cells after 2 min incubation with MFP (260 nM) and subsequent washing procedure. Bottom was the normalized fluorescence intensity

values plotted over time.

Fig. 2.9 Fluorescence imaging of Lovo cells and U251 cells after incubating with high concentrations of MFP (1 μ M) for 30 min at 37 $^{\circ}$ C.

In order to further verify the varying levels of furin expression between U251 and Lovo cells resulted in the difference of the observed fluorescence on the cell surface, the cell lysate based furin activity assays was carried out. Two different cells were lysed into furin reaction buffer and then incubated with MFP probe (10 μ M). In this experiment, the level of the total protein in cell lysates maintained same by using the standard Bradford method.⁴⁵ Figure 2.10 demonstrated that the detected fluorescence signals in furin deficient Lovo cells was quite low, whereas the fluorescence enhancement in furin expressed U251 cell lysates was considerably significant over all the incubation time. Moreover, the furin cleavage effect in U251 cell lysates was found to be significantly suppressed once the furin inhibitor Dec-RVKR-cmk was pre-treated, indicating that the as-prepared MFP could serve as a reliable tracer to quantitatively determine specific furin activities in complex biological environment (Figure 2.11).

Fig. 2.10 Plot indicating changes in fluorescence intensity of MFP (10 μ M) over time for the purpose of quantification of the furin activities in U251 and Lovo cell lysates (10 μ g total protein was incubated with MFP at 37 $^{\circ}$ C). The inhibitor Dec-RVKR-cmk (100 μ M) was added to U251 cell lysates for 30 minutes prior to addition of the probe.

The enzyme cleavage experiment was also performed by using the standard fluorescent furin substrate Boc-RVRR-AMC and characterized by HPLC (Figure 2.12 and 2.13). The results were consistent with that observed when treated with MFP probe.

Fig. 2.11 Standard curve for quantitative analysis for enzyme activity in U251 and Lovo cell lysates samples.

Fig. 2.12 HPLC analysis of MFP or MFP and inhibitor incubated with cell lysates. HPLC condition for the analysis of enzyme hydrolysis is a linear 20 min gradient from 30% to 95% acetonitrile in water with 0.1% TFA.

Fig. 2.13 Fluorescence signals of RVRR-CMK (10 μ M) for quantifying total furin activity in U251 and Lovo cell lysates.

To further reveal the utility of our designed probe in practice, we also evaluated the potential cytotoxicity of MFP probe by using standard MTT assay (Figure 2.14). Both U251 and Lovo cell lines were chosen to incubate with different concentrations of MFP probe. As demonstrated in Figure 2.13, even after long time incubation, MFP probe did not present obvious cytotoxicity to both U251 and Lovo cells. The result

indicated that this platform provides the possibility for visualization of furin activities without affecting the cells' physiological processes

Fig. 2.14 Cytotoxicity for different concentrations of MFP at U251 and Lovo cell lines.

One great benefit of our developed MFP probe was its capability to specifically localize on the cell surface when upon the specific reaction with surface associated furin enzyme. In order to examine whether the hydrophobic lipid anchor could assist the membrane insertion, the similar fluorescent probe (FP) but without fatty acid moiety was also synthesized and applied for live cell imaging (Figure 2.15). Although enzymatic hydrolysis result clearly demonstrated that FP could be cleaved by furin in HEPES buffer (Figure 2.16), the cell imaging study based on FP alone revealed a poor membrane staining when compared to those observed by using MFP (Figure 2.17,). This result clearly suggested that the lipid moiety would be indispensable for the localization of MFP probe on the cell surface.

Fig. 2.15 The chemical structure of FP

Fig. 2.16 Fluorescence enhancement of FP (6.5 μM), when treated with furin in the presence or absence of inhibitor Dec-RVKR-cmk at different times ($\lambda_{\text{ex}}=450$ nm, at 37 $^{\circ}\text{C}$). Inset shows HPLC analysis of FP alone, FP incubated with furin for 60 min.

Fig. 2.17 Real-time confocal fluorescent images of U251 cells treated with FP (130 nM) ($\lambda_{\text{ex}}=488$ nm). (Scale bar: 20 μM).

To further screen the possibility of chain length in lipid moiety to affect cell

membrane staining, the similar MFP peptide sequence but no Dabcyl quencher connection was used to conjugate with different fatty acids (e.g. Caproic acid, Lauric acid and Stearic acid, respectively). The as-prepared FITC-peptide fatty acid conjugates, abbreviated as C6-F, C12-F (same carbon chain with MFP) and C18-F respectively, were incubated with HEK 293 cells for different time intervals. As shown in Figure 2.17, there was almost no fluorescence observed in C6-F incubated HEK 293 cells compared to cells treated with C12-F and C18-F. The results confirmed that the increased length of carbon chain could indeed enhance the immobilization of the probe onto cell surface. Unlike the staining of C12-F, although the C18-F conjugate indeed indicated enhanced tracking ability on cell membrane, the obvious internalization into lysosome would be hard to avoid after prolonged incubation (e.g. 2 hours) (Figure 2.18 and 2.19). Therefore, the MFP with dodecanoyl moiety would be the ideal tracer to specifically localize the probe onto cell membrane, and thus significantly facilitate long-time trackable visualization of surface associated furin in living cells. Such long-time membrane anchoring would provide great potentials to monitor dynamic processes of cell-surface enzyme activities, which is normally difficult for most of existing cell permeable small-molecule probes.

Fig. 2.18 Fluorescence imaging of live HEK 293 cells after incubation with FITC-peptide conjugates (C6-F, C12-F, and C18-F) (1 μ M) for 2 hrs at 37 °C. (FITC: green; LysoTracker, red; H33258, blue.) The arrow shows the co-localization of C18-F and LysoTracker (yellow). Scale bar =20 μ m.

Fig. 2.19 Fluorescence imaging of live HEK 293 cells after incubation with higher concentration of MFP (2.5 μ M) for 2 hrs at 37 °C. (FITC: green; LysoTracker, red; H33258, blue).

In terms of two-photon characteristic of FITC,^{46,47} we also investigated the possibility of two-photon imaging based on the specific interactions between the developed MFP probe and cell-surface associated furin enzyme in living cells or tissues. As proof of concept, U251 cells were incubated with MFP probe (1.0 μ M) at 37 °C for 30 min, and two-photon imaging was carried out upon the excitation at

wavelength of 800 nm. As shown in Figure 2.20, intense fluorescent signals on the surface of U251 was observed. Similar two-photon imaging was also conducted by incubation of MFP with mouse ear tissues and the images were monitored at depths of $\sim 40\ \mu\text{m}$. The bright fluorescence observed in the tissues clearly indicated that the developed probe molecule could work properly to monitor furin enzyme activities in *in vivo* systems.

Fig. 2.20 Fluorescence imaging of live Two-photon imaging in U251 cells and mouse ear tissue after incubation with the MFP probe. Channel: FITC (A), membrane tracker CellMask Deep Red (B), merged images (C). D) MFP signals (green) in tissues and second harmonic generation from collagen (blue). Scale bar=20 μm .

2.3 Conclusions

In conclusion, the specific localization of our developed MFP probe on the plasma membrane first represents a simple and novel small molecule-based method for direct visualization of furin-like enzyme activity on the cell surface. In this study, the carefully optimized lipid moiety in the molecule structure can efficiently facilitate the

immobilization of the probe on the extracellular plasma membrane for a relative long time. Upon the specific enzyme hydrolysis, a strong fluorescence turn-on effect can be observed which shows the great promising real-time imaging of surface-localized furin activities in living cells and fresh tissues using one-photon and two-photon microscopy. Such rational design could be also expanded as a reliable and general method to visualize other membrane-associated proteolytic enzymes in their functions and biological roles without the need of invasive procedures. Furthermore, optimization of this type of probe to trace the enzyme dynamics and location in specific sub-cellular organelles will provide deeper insight into mechanisms of viral or pathogen invasion, which is currently under the process. More importantly, such a unique MFP probe can also be used to screen potential proteolytic enzyme inhibitors to selectively block furin-dependent cell-surface processing without interfering with the normal functions, which will definitely facilitate the new discovery of specific probes or drug molecules towards theranostics of furin related diseases.

2.4 Experimental details

Materials: 2-Chlorotriethyl Chloride Resin and all Fmoc amino Acids were purchased from GL Biochem Ltd (Shanghai). All chemical reagents were purchased from Sigma-Aldrich. Commercially available reagents were used without further purification, unless noted otherwise. Anhydrous solvents for organic synthesis were stored over activated molecular sieves (4 Å). 4-((4-(dimethylamino) phenyl)azo) benzoic acid, succinimidyl ester (Dabcyl SE) and Fluorescein isothiocyanate (FITC) were purchased from Life Technologies. Furin was ordered from Biolabs (2000 U

mL⁻¹), The definition is one unit will release 1 pmol of AMC from the fluorogenic peptide Boc-RVRR-MCA (ITS Science & Medical Pte Ltd) per minute at 30°C. The furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-cmk) was purchased from Bachem.

Instruments: Mass spectra (ESI-MS) were measured with a Thermo LCQ Deca XP Max for ESI. Analytical reverse-phase HPLC analysis was performed on a Shimadzu HPLC system with an Alltima C-18 (250 x 10 mm) column at a flow rate of 3.0 mL / min. UV-Vis absorption spectra were recorded in a 10-mm path quartz cell on Cary 60 UV-Vis Spectrophotometer. Fluorescence emission spectra were performed on a Varian Cary eclipse fluorescence spectrometer. Two-photon images were acquired on A TriM Scope II single-beam two-photon microscope (LaVision BioTec) with a laser (Coherent Chameleon Ultra II One Box Ti:sapphire) and water-dipping objectives (20×, Olympus). The fluorescent signals were detected with a filter at 525/50 BP (Chroma, cat. no. ET525/50m).⁴⁸

Synthesis and characterization of MFP Probe

Synthesis of compound 1: The peptide sequence was synthesized by Fmoc based solid phase peptide synthesis (SPPS) on a 2-Chlorotrityl Chloride. The coupling reaction of two amino acids lasted 2-4 hours at room temperature in the presence of HBTU as a coupling reagent and N, N-Diisopropylethylamine (DIPEA) as a basic catalyst respectively. The reaction was allowed to last for 4 hours for coupling with arginine and 2 hours for other amino acids. The deprotection of Fmoc group was performed within 20% piperidine in Dimethylformamide (DMF) for 20 min.

Synthesis of compound 2: The Dodecanoic acid was coupled to the N-terminus of the peptide using the same SPPS protocol. Then the Dde protecting group was removed in 2% hydrazine in DMF for 20 min. Then FITC was reacted with the amino group of the lysine for 4h. Then the product was cleaved from resin using 2 % trifluoroacetic acid TFA in Dichloromethane (DCM) for 10 minutes. The Mtt protecting group was also removed during this process. After removing DCM, the diethyl ether was added to obtain the precipitate. The crude product was purified by HPLC and characterized by MS (ESI): m/z: $[M+H]^+$ calcd: 2524.27; found: 1263.50 ($[M+2H]^{2+}$) , 842.97 ($[M+3H]^{3+}$).

Compound Purification: After the mixture was cleaved from the resin, it was purified by RP-HPLC. The eluting system consists of A (water with 0.1% TFA) and B (acetonitrile with 0.1% TFA) under a liner gradient monitored by UV-Vis absorbance at 450 nm. The liner gradient stretched over 25 min from t=0 min at 30% solution B to t=15 min at 100% solution B with a flow rate of 3.0 mL/min. The retention time of the desired product was 17 min.

Synthesis of MFP probe: Compound 2 (6 mg, 2.3 μ mol) was dissolved in 100 μ L of dry DMF. Meanwhile, 20 μ L DIPEA was added and reacted with Dabcyl SE (3 mg, 8.1 μ mol) for 4 hr. The crude product was obtained by precipitation with diethyl ether to remove the redundant Dabcyl SE to get compound 3. Then Pbf and OtBu protection groups were cleaved by 95% TFA for 2 hr. The final product was purified by HPLC monitored by UV absorbance at 500 nm. The liner gradient stretched over 25 min from t=0 min at 30% solution B to t=20 min at 95% solution B with a flow rate of 3.0 mL /

min. The retention time of the desired product was 15.4 min. ESI-MS: m/z $[M+H]^+$ calcd:1963.07, found 1964.88, 982.95 ($[M+2H]^{2+}$), 655.96 ($[M+3H]^{3+}$).

Stability testing: The prepared MFP (100 μ M) was incubated in 0.15 mL of buffer or serum solution (Fetal Bovine Serum, Standard Quality solution, EU approved from GE Healthcare) for different time intervals at room temperature. At different time point, the mixture was re-suspended in methanol (0.3 mL) and then centrifuged at 16,000 g for 2 min. The supernatant was analyzed by HPLC to evaluate the stability. There was no obvious MFP probe degradation observed in PBS (for more than one week) and serum (for 24 hours) indicated the sufficient stability of MFP in in vitro and living cell experiments.

Synthesis of FP probe: FP was synthesized following the procedure of synthesis of MFP. ESI-MS: m/z $[M+H]^+$ calcd:1652.80, found 1654.44, 827.88 ($[M+2H]^{2+}$), 552.52 ($[M+3H]^{3+}$).

Synthesis of FITC-peptide conjugates:

X-Lys(FITC)-e-Acp-Arg-Val-Arg-Arg-Ser-Val-Lys-COOH (X= hexanoyl, dodecanoyl, octadecanoyl) abbreviated as C6-F, C12-F, C18-F. They were synthesized following the procedure of synthesis of compound 2. Then all the protection groups were cleaved by 95% TFA for 2 h. The final products were purified by HPLC monitored by UV absorbance at 450 nm. C6-F, ESI-MS: m/z $[M+H]^+$ calcd:1267.87, found 1969.38, 815.55 ($[M+2H]^{2+}$), 544.28($[M+3H]^{3+}$), 408.50 ($[M+4H]^{4+}$). C12-F, ESI-MS: m/z $[M+H]^+$ calcd:1711.96, found 1713.60, 857.25 ($[M+2H]^{2+}$), 572.08($[M+3H]^{3+}$), 429.36 ($[M+4H]^{4+}$). C18-F, ESI-MS: m/z $[M+H]^+$ calcd:1796.05, found 1797.48,

899.64 ($[M+2H]^{2+}$), 600.43($[M+3H]^{3+}$), 450.61 ($[M+4H]^{4+}$).

UV-Vis absorbance and fluorescence spectra characterization.

The fluorescence spectra of FITC and MFP (10 μ M) probe were recorded in PBS (pH=7.4) buffer. The fluorescence quenching efficiency is about 99.7 %, indicating the fluorescence of FITC is almost quenched by Dabcyl moiety.

General methods for cell culture, cell lysates assays and live cell imaging.

Human Neuronal Glioblastoma U251 Cells and human embryonic kidney cells HEK293 were grown in Dulbecco's modified eagle medium (Gibico) supplemented with 10% fetal bovine serum (FBS, HyClone) at 5% CO₂ and 37°C. Human colorectal adenocarcinoma Lovo cells (from ATCC) were grown in F-12K medium with 10% FBS.

Imaging and quantification of furin-like activity in live cells.

U251 and Lovo cells were seeded at 35-mm diameter μ -dish plastic-bottom (ibidi GmbH, Germany) in complete medium for 24 h incubation at 5% CO₂ and 37 °C. Then cells were incubated with the probe (130 nM) or probe together with the inhibitor Dec-RVKR-cmk (40 μ M) for 15 min incubation. Subsequently, cells incubated with CellMask Deep Red (2.5 μ g/mL) for 5 min and washed three times with PBS. Live cell imaging was performed under confocal laser scanning microscope (CLSM) (Nikon, Eclipse TE2000-E). (MFP/FP: λ_{ex} = 488 nm, λ_{em} = 525/50 nm; CellMask Deep Red: λ_{ex} = 633 nm, λ_{em} = 650/50 nm). To quantify the fluorescence intensity generated by the MFP or FP probe on the cell surface, images were analyzed by Image J software following the standard guide and references. Briefly, a ROI

(region of interest) was defined by manual selection using line tools on CellMask Deep Red channel. After defining the ROI from the red channel, the threshold of images was transferred to the FITC channel to measure the related intensity.

Incubation of MFP probe in cell lysates

U251 and Lovo Cells were carefully trypsinized and centrifuged ($3000 \times g$ for 5 min) to remove medium, and washed with PBS buffer. Then RIPA buffer (Sigma, containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, and pH 8.0) was added and kept on ice for 1h. The lysates were collected and centrifuged at $14,000 \times g$ for 5 min. The supernatant of the cell lysates was quantified by Bio-Rad Protein Assay (Bio-Rad Laboratories, USA) according to its standard procedure. Briefly, the dye reagent was diluted for 5 times with distilled water before use. 20 μ L of the tested sample was mixed with 200 μ L of the diluted reagent in 96-well plates. After incubation at room temperature for 5 minutes, the absorbance at 595 nm was recorded by a Tecan's Infinite M200 microplate reader. The 10 μ g total protein was incubated with probe (10 μ M) for 2.5 h. For the control experiment, the U251 supernatant was preincubated with the inhibitor Dec-RVKR-cmk (100 μ M) for 30 min before the addition of the probe. Then the fluorescence was measured at 523 nm using the microplate reader.

Quantitative analysis of enzyme activity in cell lysates.

By following the established procedure, the standard curves on the basis of the interactions between furin enzyme and MFP probe were constructed by plotting pure enzyme concentration (Unit) against fluorescence at an end point of 1 hour reaction.

Such end-point method was used to determine the furin activities in U251 and Lovo cell lysates samples. Our results indicated that the amount of furin activities (per 10^5 cells) in U251 cell lysates samples is 0.21 ± 0.05 Unit, whereas the negligible furin activities were found in Lovo cell lysates samples.

Immunoblotting

The immunoblotting experiments were based on the protocols provided by Abcam Company. In brief, 1.2×10^6 Lovo and U251 cells were lysed with 50 μ L 2 $\times$ SDS buffer and boiled at 90 °C for 5 min. 20 μ L of each samples were analyzed by 10 % SDS-PAGE and transferred to 0.22 μ m PVDF membrane (100 V, 110 min, 4°C). Then the membrane was blocked with milk (5% PBST: PBS + 5% Tween-20)) for 1 h, and incubated with primary antibody (anti-furin ab3467, Abcam, Cambridge, UK, 1:1000 dilutions) at 4 °C overnight. After that, the membrane was washed 3 times with PBST, then incubated with secondary antibody (Goat Anti-Mouse IgG (H + L)-HRP Conjugate #170-6516) (1: 10000 dilutions) for 1h at RT and washed 3 times with PBST. The samples were detected with LAS-4000 machine using ECL exposure buffer. After that, another 20 μ L (the same amount as the above) of each samples were analysed in 10% SDS-PAGE and transferred to 0.22 μ m PVDF membrane (100V, 110 min, 4°C). The membrane was incubated with mouse β -tubulin antibody (1:1000 dilutions) 1h at RT, washed 3 times with PBST, then incubated with goat anti-mouse secondary antibody (1: 10000 dilutions).

Cell viability assay

Glioblastoma U251 Cells and Lovo cells were seeded in 96-wells plates with a density

of 10^5 cells per well. After 24-hour incubation at 5% CO_2 and 37°C , the medium was replaced with fresh medium containing different concentrations of MFP. After 24 hour incubation, the cytotoxicity was evaluated by the standard MTT assay. The absorbance at 570 nm was measured by a Tecan's Infinite M200 microplate reader.

***In vitro* furin enzyme assays.**

The MFP or FP probe ($6.5\ \mu\text{M}$) was incubated with pure furin enzyme for 120 min at 37°C in 0.1 mL assay buffer. The assay buffer contains 100 mM HEPES, pH 7.4, 0.5 % Triton X-100, 1 mM CaCl_2 , and 1mM 2-mercaptoethanol. The emission spectrum ($\lambda_{\text{ex}} = 450\ \text{nm}$) was recorded over time. HPLC condition for the analysis of products and enzyme hydrolysis is a linear 20 min gradient from 30% to 95% acetonitrile in water with 0.1% TFA.

Live Cell imaging of FITC-peptide conjugates.

HEK293 cells were seeded at 15 μ -slide 4 well (ibidi GmbH, Germany) in complete medium for 24 h incubation at 5% CO_2 and 37°C . Then the medium was changed to fresh serum free medium in the presence of $1\ \mu\text{M}$ C6-F, C12-F, and C18-F. Cell nuclei were stained with bisBenzimide H 33258 (Sigma) and lysosomes were stained with LysoTracker® Red DND-99 (Life technologies) according to the manufacturer's protocols. The images acquired after 2 hr incubation by confocal laser scanning microscope (LSM710, Carl Zeiss Inc., Jena, Germany) The laser setting were as follows: H33258 ($\lambda_{\text{ex}} = 405\ \text{nm}$); FITC ($\lambda_{\text{ex}} = 488\ \text{nm}$) and LysoTraker ($\lambda_{\text{ex}} = 563\ \text{nm}$).

Quantum yield measurement.

The fluorescence quantum yield of MFP was measured by using fluorescein in NaOH

aqueous solution (pH=13) as the standard reference.⁴⁹ Quantum yield was calculated from equation:

$$\frac{\phi_2}{\phi_1} = \frac{I_2}{I_1} \times \frac{OD_1}{OD_2} \times \frac{n_2^2}{n_1^2}$$

Where ϕ_1 and ϕ_2 are the quantum yields, I_1 and I_2 are the integrated 1PE fluorescence intensities, OD_1 and OD_2 are the optical densities, n_1 and n_2 are the refractive indexes of solvents. The quantum yield of MFP in PBS 1X is about 44%.

Two-photon Absorption (TPA) cross sections measurement.

Two-photon excitation properties of the samples were measured by using a Avesta TiF-100M femtosecond (fs) Ti:sapphire oscillator as the excitation source. The output laser pulses have a central wavelength from 760 nm to 850 nm. Fluorescein in water (pH=13), which have been well characterized in the literature⁵⁰, was used as reference. TPA cross section was calculated from the below equation:⁵¹

$$\frac{\delta_2}{\delta_1} = \frac{I_2}{I_1} \times \frac{\phi_1}{\phi_2} \times \frac{c_1}{c_2}$$

where δ_1 and δ_2 are the TPA cross sections, I_1 and I_2 are the integrated 2PE fluorescence intensities measured at the same power of the excitation beam, ϕ_1 and ϕ_2 are the fluorescence quantum yields, c_1 and c_2 are the concentrations. The maximum two-photon action cross-section value upon excitation from 760 to 850 nm is 13 GM (1 GM = $10^{-50} \text{ cm}^4 \text{ s photon}^{-1}$).

Two photon Live cell and tissue imaging.

U251 cells were seeded in complete medium for 24 h incubation at 5% CO₂ and 37°C. Then the medium was replaced with fresh serum free medium in the presence of 1 μM

MFP for 30 min incubation. Next cells were stained with CellMask Deep Red (2.5 µg/mL) for 5 min and fixed. Images were acquired under one photon and two photon microscopy. As Furin exhibits a widespread tissue distribution, we chose the mouse ear tissues as the model. The ear tissues were prepared from NCr nude mice and unfolded to get monolayer tissues. Then the tissues were incubated with 5 µM MFP at 37°C for 2 h and washed with PBS for three times. Images were observed under TriM Scope II single-beam two-photon microscope at depths of ~ 40 µm.

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Chapter 3

Measuring Membrane-Associated Enzyme Activities in Single Cells via a Small-Molecule-Based Probe

ABSTRACT. Approaches to measure enzyme activities in single cells recently received great attention. More physical processes and kinetics could be disclosed when compared to the bulk measurement. In this chapter, the single cell imaging and relevant physical processes of membrane-anchored probes were explored by using the wide field microscopy. Preliminary results indicated that the membrane insertion process of the developed probe may occur faster than the furin-mediated hydrolysis.

3.1 Introduction

Single molecule biophysics is quite an important and fundamental area in precise understanding the intracellular events like the biomolecule transportations, dynamics of molecular diffusion and enzyme reaction kinetics *etc.* Till now, single molecule spectroscopy and imaging have been treated as a vital part in the physical chemistry and biophysics due to its ability to measure the distributions of behavior instead of the ensemble averaging, which can provide more information about the underlying mechanisms. The internal state and transitions of one molecule and its kinetic pathway can be measured. Also single molecule imaging also allows to observe the intermediate states or rare events due to the sufficient time resolution.¹⁻³ In recent years, physicists and biologists have made great progress in the deciphering the cell

complexity, and visualizing the individual molecules in the biological context. Especially, more comprehensive models have been established to gain deeper insight into the underlying behaviors of molecule motions and transport in live cells.⁴⁻⁶ Unlike the previous used confocal laser microscopy based technology, which mainly can provide ensemble signal changes of fluorescence in bulk measurements, single-molecule measurements can provide more dynamic and quantitative information in single cells and observe the diffusion and motion of individual probe molecules.⁷⁻⁹

As we know, various biological processes take place on the cell surface and many of them are involved in the enzymatic catalysis. Understanding of the enzyme acting on the substrates on the cell membrane and tracking the diffusion process would be of significance to get closer to understand overall mechanisms of interfacial enzymatic process.¹⁰⁻¹² Hence here we employed our current unique design to investigate the enzymatic dynamics and the underlying interpretations in the single molecule levels. The more detailed information would show some light on the issues how probes could get close to the enzyme, how fast the probes interact with the enzyme and have the capability to anchor on the cell membrane. In order to answer these questions, we apply our current system into the single molecule based real-time imaging though more precise microscopy-wide field microscopy set-up. In the meantime, we incorporate Atto647N into this probe mainly attributed to its great advantages including strong absorption, high photostability and excellent fluorescence quantum yield. For the corresponding quencher, BHQ3 was chosen due to its broad absorption

curves and well overlap with the emission spectrum of Atto647N fluorophore.

Fig. 3.1 Schematic illustration of imaging experimental setups by using wide field microscopy.

3.2 Result and discussion

Fig 3.1 illustrated the general imaging setups by using the wide field microscopy. The laser light passes through the filter and objective lens to get the sample on the dish. Then fluorescence signals from the sample go through the dichroic mirror and can be detected by CCD camera with a magnified camera lens. The fluorescence signals were recorded under the 633 nm laser irradiation by using standard protocols.^{13,14} HEK293 cells were seeded on the glass bottom petri dish. Then the focal plane was located on the top of cells, which can be considered as two-dimensional plane and all experiments were under the same condition.

The probe Atto-MP1 in the single cell imaging was synthesized using the similar method of that in the probe MFP (chapter 2). Except the Cy5.5 / BHQ3 as the FRET pair, all the peptide sequences and chemical linkers are the same as those in the MFP molecule. In order to get more information about the probe diffusion into the

membrane area, we synthesized the control molecule Atto-MP2, which was the cleavage fragment of Atto-MP1 by furin.

Fig. 3.2 (A) Fluorescence intensity of Atto-MP1 and Atto-MP2 (4 μ M); (B) Fluorescence increase of Atto-MP1 (4 μ M, in HEPES buffer, pH 7.4) when treated with furin

Initially, the fluorescence spectrum of Atto-MP1 and Atto-MP2 in PBS buffer were obtained and Atto-MP2 has the maximum fluorescence at 662 nm (as shown in Fig. 3.2). However, there was almost no fluorescence observed in the probe Atto-MP1 and quenching efficiency was calculated as 98.8%, clearly indicating the excellent quenching effect of fluorescence by BHQ3. Next, we tested whether the designed probe can be recognized by furin in *in vitro* conditions. As shown in Figure 3.2B, the intense fluorescence enhancement when incubated with furin within 20 min demonstrated that furin was responsible for the recovered fluorescence, similar to the previous MFP design. It is noted that the fluorescence increase reached the plateau within a shorter incubation time (30 min) when compared to the enzyme hydrolysis of the probe MFP. We assume the different molecular charges between these two FRET pairs would be the possible reason to cause the different enzymatic hydrolysis.

Fig. 3.3 Single cell imaging when treated with the Atto-MP2 by using the wild field microscopy. The probe Atto-MP2 was diluted to the extreme low concentrations ($\sim$ nM) to get the single molecule imaging.

Fig. 3.4 Quantitative analysis of the fluorescent signals of Atto-MP2 on the upper plasma membrane.

Subsequently, real-time imaging of HEK293 cells when treated with Atto-MP2 (300 nM) was evaluated under the confocal microscopy. The fluorescence of the probe Atto-MP1 was quenched before incubation with cells. Within a short time, the

fluorescence was recovered gradually. Not surprisingly, the turn-on fluorescence was mostly distributed on the cell surface, which was consistent with our previous imaging results.

In order to monitor the cell-surface associated furin activities in the single molecule level, the fluorescence imaging of the cells was performed with wide-field microscopy using laser illumination at 633 nm and each images were taken at 20 s intervals. As shown in figure 3.3, The number of the “fluorescent dots” increased very fast when cells were treated with the control molecule Atto-MP2. Further quantitative measurement of the fluorescent signals on the upper plasma membrane was obtained and average values were acquired by analysis of ten cells under same conditions. As shown in figure 3.4, after 3 min incubation with HEK293 cells, there was no obvious increase in the number of fluorescent dots, indicating a very fast physical process. The results demonstrated that the molecule Atto-MP2 presented faster motion into the cell membrane and can reach a steady and constant state within 2 min. Results from single cell imaging were consistent with those obtained bulk measurements.

Fig. 3.5 Single cell imaging when treated with the Atto-MP1 ($\sim$ nM) by using the wild field microscopy.

Fig. 3.6 Quantitative analysis of the fluorescent signals of Atto-MP1 on the upper plasma membrane.

Subsequently, we evaluated the molecular behavior of the pre-quenched molecule Atto-MP1 in single cells. As shown in figure 3.5, there was no obvious fluorescence signals within 2 min incubation. Then fluorescence increased gradually with prolonged incubation time and reached the plateau at around 8 min. Quantitative analysis of the increased fluorescent signals were determined and shown in figure 3.6. Based on the

excellent quenching efficiency of the molecule itself, we believe the observed fluorescence signals came from the unquenched molecule state. Since Atto-MP1 contains the furin-sensitive peptide sequences and the dissociation of the FRET donor Cy5.5 and acceptor BHQ3 were most likely attributed to the furin proteolytic cleavage. One of the cleavage fragment of molecule Atto-MP1 is the same structure as the molecule Atto-MP2. Different from the molecule Atto-MP2, which mainly involves in the membrane insertion process, diffusing from the solutions into the cell membrane environment, the molecule Atto-MP1 participates in both membrane insertion and enzyme cleavage processes. In order to simplify the physical module, we assume both molecules when in the homogenous solutions can be described as unconstrained Brownian motion. From our result, the lipid-moiety assisted membrane insertion process was very fast and can reach a steady state within 3 min. However, most signals arising from enzyme cleavage can only be observed after 3 min. The results indicated that membrane insertion is a faster process when compared to the cell-surface furin proteolytic process and such hydrophobic anchor would help the probe to have more chances to interact with the furin enzyme.

3.3 Conclusions and future work

In this work, we first reported the small molecule interactions with cell-surface furin enzyme in single living cells. The designed molecules involved two processes, passive membrane insertion and enzyme cleavage. The dynamics of both process were evaluated by using the wide field microscopy setups. The single living cell imaging was studied and clear individual signals were obtained. Although more experiments

are required to verify the physical phenomena, our results indicated that the membrane insertion process may occur faster than the furin hydrolysis.

Based on these preliminary results, we are undergoing to map the single-molecule positional trajectories on the cell membrane. The individual single-molecule trajectory can be further used to estimate the diffusion coefficient and test for heterogeneity on the cell membrane. By comparing the diffusion difference between the off-on probe and all-on probe (Atto-MP2), we could get more evidence about the probe diffusion process and enzyme involved catalytic process.

3.4 Experimental details

Scheme 3.1 Synthesis of Atto-MP1 probe. a) DIPEA, 1 hr in DCM; b) HBTU/DIPEA in DMF; c) 2% hydrazine in DMF, 20 min, then Atto647N-NHS/DIPEA, 4 hr; d) DIPEA, BHQ₃-NHS, 4 hr; e) 95% TFA, 2.5% H₂O, 2.5% TIPS 2 hr.

Synthesis of Atto-MP1 probe. Atto-MP1 was synthesized by following the similar

synthetic procedure of MFP probe (Scheme 3.1). Briefly, compound 2 was synthesized by SPPS, and purified by HPLC. Subsequently, Compound 2 (1 mg, 0.3 μmol) was dissolved in 100 μL of dry DMF. Meanwhile, 2 μL DIPEA was added and reacted with BHQ-3 Succinimidyl Ester (BHQ₃-NHS) (0.7 mg, 0.9 μmol) for 4 h. The crude product was obtained by precipitation with diethyl ether to remove the redundant BHQ₃-NHS. Then all protection groups were cleaved by 95% TFA for 2 h. The final product (Fig 3.8) was purified by HPLC monitored by UV absorbance at 520 nm. ESI-MS: m/z $[\text{M}+\text{H}]^+$ calcd:2480.58, found 1241.38 ($[\text{M}+2\text{H}]^{2+}$).

Figure 3.7 The chemical structure of Atto-MP1 probe

Synthesis of Atto-MP2

Scheme 3.2 Synthesis of Atto-MP2 probe. a) DIPEA, 1 hr in DCM; b) HBTU/DIPEA in DMF; c) 2% hydrazine in DMF, 20 min, then Atto647N-NHS/DIPEA, 4 hr; d) 95% TFA, 2.5% H₂O, 2.5% TIPS 2 hr.

Synthesis of Atto-MP2 probe. Atto-MP2 was synthesized by following the similar synthetic procedure of MFP probe (Scheme 3.2). Briefly, Atto 647N-NHS ester was conjugated on the solid phase, followed by the addition of 95% TFA to cleave the product from the resin and remove all protection group. The final product (Fig 3.8) was purified by HPLC monitored by UV absorbance at 520 nm. ESI-MS: m/z $[\text{M}+\text{H}]^+$ calcd: 1637.12, found 819.65 ($[\text{M}+2\text{H}]^{2+}$).

Figure 3.8 The chemical structure of Atto-MP2 probe

Single cell imaging by using wide field microscopy

HEK293 cells were seeded at 35-mm diameter μ -dish plastic-bottom in complete medium for 24 h incubation at 5% CO₂ and 37 °C. Then fresh medium with the Atto-MP1 or Atto-MP2 probe (1 nM) was added and images were taken immediately by the wide field microscopy. The wide-field fluorescence microscopy was set as follows: 633 nm HeNe laser source (35 mW, Melles Griot), an oil immersion objective lens (60 $\times$, N.A. 1.4, Olympus) and a highly sensitive CCD camera (CascadeII 512B, Photometrics) after magnification with a 3.3 $\times$ camera lens. Images was measured to be 40 $\times$ 40 μ m. All images were analysed by using the Image J software.

3.5 References

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Chapter 4

Construction of stimuli-responsive probes for selectively visualizing the proteolytic enzyme functions

ABSTRACT. Recently, a variety of small-molecule based probes have been developed for monitoring numerous protease activities, However, cellular background interference and unsatisfactory selectivity in the disease area may hinder their further *in vivo* and clinic application. In attempts to improve the specificity and selectivity of probe selectivity to the tumor areas for more precise diagnosis on disease progression, stimuli-responsive strategies provide promising solutions to diagnosis and therapy. Herein, a series of stimuli-responsive probes were properly designed and different enzyme-recognized sequences were screened. Enzymatic assays revealed that nitrobenzyl-caged probe could successfully block its hydrolysis by furin enzyme. Upon the specific light irradiation, such designed probe could be effectively uncaged and provide strong fluorescence signals in response to furin.

4.1 Introduction

Proprotein convertases play diverse and essential roles in regulating protein functions under both physiological and pathological conditions, including immune responses and malignancies. The protein expression levels of proteases and protease inhibitors usually can be determined by the real-time polymerase chain reaction (RT-PCR)¹⁻³ and Western blot.⁴ However, these methods cannot be applied for

real-time imaging of proteolytic enzymes in living cells and animals. Small-molecule based probes provide the promising solutions to dynamic visualization of cell-surface and intracellular protease activities.

Current approaches to detecting protease activities are mainly based on the conjugation of optimal substrate sequences with reporters that reflect the signal changes once the substrates are cleaved by enzymes.⁵ Despite the expression levels of these enzymes are relatively higher in the disease area, the activated fluorescence signals in the healthy tissues will inevitably give rise to background interference, leading to the inaccurate detection. In order to improve the probe selectivity to the tumor areas for more precise diagnosis on disease progression, stimuli-responsive strategies may provide the possibility for *in situ* protease activities.⁶⁻⁸ If the designed probes can temporarily become “inert” without any stimuli, while “active” in response to the different microenvironment, the precise control of enzyme activities at the sites of interest would become feasible.⁹

In our previous work, we have successfully designed the small-molecule FRET reporter for imaging of cell-surface proteolytic activities. Based on this optimal peptide sequence, in this chapter, we explored different stimuli-responsive strategies to spatiotemporally control the protease activities by involving a variety of cleavable chemical bonds. Based on the comprehensive analysis of the physical properties of furin cleavage site, we have designed and synthesized ten different probes by changing the amino residues at the P3 and P1' positions. We aimed to conjugate various stimuli-responsive chemical groups adjacent to the enzyme-targeted scissile bond,

resulting in blocking protease activity. Meanwhile, we evaluated the enzyme hydrolysis of the prepared probes in HEPES buffer. Through the screening of the peptide sequences, we successfully obtained the photo-responsive furin probes by conjugating the photolabile molecules into the P3 positions of the binding pocket, thereby preventing the furin cleavage by possible steric hindrance. Such well-designed probes would have great potentials to be applied on dynamically monitoring the furin sorting and trafficking, furin-mediated pathogen invasion, and effectively screening membrane furin inhibitors in living cells.

4.2 Result and discussion

4.2.1 Construction of endogenous stimuli-responsive probe

Compared to the pH 7.4 in normal blood or most normal tissues, the extracellular environment of solid tumors presents a lowered pH value, usually at 6.5-7.2. The reason is fast-growing tumors will result in the excessive metabolite and an increased production of lactic acid. The pH value continues to drop in the cellular cells, such as in endosomes (5.5) and lysosomes (below 5.5).^{10,11} Therefore, the pH changes in the tumor microenvironment has been widely exploited as intrinsic stimulus in the design of stimuli-responsive systems for localized exposure of targeting ligands.

Among a variety of acid-labile bonds, hydrazone bond has been widely applied in preparing the pH-responsive imaging probes and drug delivery nanoconjugates.¹²⁻¹⁴ The hydrazone bond is formed by reactions between hydrazine and ketone or aldehyde. In order to introduce the bulky group near the enzyme cleavage site through the hydrazone bond while maintaining the whole structure of probe as possibly, we slightly

change the serine at the P1' position with the 2-amino-4-oxopentanoic acid during the SPPS, which the ketone group will be introduced to the probe. In order to quickly test whether enzyme can recognize newly designed substrates, compound S1 and S2 are synthesized as the model probe and Fmoc group was regarded as the bulky group for the steric hindrance effect.

Fig. 4.1 Molecular structure of substrates S1-S4.

Subsequently, the probe cleavage by furin enzyme can be monitored by the turn-on fluorescence intensity through the microplate reader. As shown in Figure 4.2, after incubation with furin enzyme for 2 hours, the fluorescence of compound S1 was recovered gradually while its kinetic activity was decreased compared to our previous probe MFP. By contrast, the compound S2 with Fmoc masking group presented lower fluorescence enhancement, indicating the steric hindrance caused by Fmoc group indeed can have the obvious impact on the proteolytic activity. By flowing this

promising and preliminary result, we further synthesized the desired probe with lipid moiety as the membrane targeting ligand. Unfortunately, the *in vitro* enzyme assays revealed that both compound S3 and S4 were unable to be cleaved effectively by furin enzyme. By comparing the structure difference from compound S1 and S2, we believe that the lipid moiety which occupying the solvent accessible regions may also potentially influence the affinity to the furin binding pocket. Considering the indispensable role of such lipid moiety in the probe design, the subsequent substrate screening will consider all factors as a whole part to evaluate whether the probe can be recognized by the enzyme.

Fig. 4.2 Fluorescence enhancement of MFP (5 μ M, in HEPES buffer, pH 7.4) when treated with furin enzyme at different times (0 ~ 120 min, $\lambda_{\text{ex}} = 450$ nm, $\lambda_{\text{em}} = 523$ nm)

Besides the proposed modification in the P1' position, another possible solution is to modify the key binding pocket at P3 position, due to the flexible requirement. Meanwhile, according to the previously reported physical properties of substrate

positions, the side chain of the residue at position P3 orients outward and there seems no obvious specific interaction with the furin binding pocket. Based on this hypothesis, we put the 2-amino-4-oxopentanoic acid at the P3 position and obtained the compound S5. The enzyme hydrolysis results indicated that there was no improvement for the recognition. From the results obtained, we believed that the unnatural amino acid may not be the ideal option for the design of the stimuli-responsive enzyme probes.

Given the possibility that the unnatural amino acid like 2-amino-4-oxopentanoic acid may potentially impede the enzyme cleavage, more stimuli-responsive probes will be designed based on the functionalization of nature amino acids. The side chain of lysine contains the amino group, which can interact with ketone to form the acid-labile imine bond. Therefore, compound S6 was synthesized as the potential candidate for pH-responsive probe.

Besides the pH-sensitive linkers, disulphide bond, prone to be cleaved by glutathione (GSH), is another option for the design of endogenous release of bioactive molecules at the tumor site. In tumor tissue, the glutathione concentration is much higher than that in healthy ones.¹⁵⁻¹⁷ Based on the difference of GSH concentration, cysteine can be utilized for getting the redox-responsive probes. Therefore, the compound S7 were synthesized by involving the cysteine at the P1' position. However, the enzyme assays indicated that both compound S6 and S7 could not be able to be cleaved by furin enzyme.

Table 4.1 Structures of substrates and proteolytic cleavage by furin enzyme

Name	Sequence	P3	P1'	F. I. Increase fold	Enzyme recognition
MFP	(C ₁₂)-K(FITC)-Acp-RVRR-S-V-K(Dabcyl)	V	S	5.61	Yes
S3	(C ₁₂)-C(FITC)-Acp-RVRR-X-V-K(Dabcyl)	V	X	1.15	No
S5	(C ₁₂)-C(FITC)-Acp-RXRR-S-V-K(Dabcyl)	X	S	1.11	No
S6	(C ₁₂)-C(FITC)-Acp-RVRR-K-V-K(Dabcyl)	V	K	1.07	No
S7	(C ₁₂)-K(FITC)-Acp-RXRR-S-V-K(Dabcyl)	V	C	1.17	No

4.2.2 Construction of light-responsive probe with enzyme-recognition sequences

Among the different internal and external stimuli-responsive approaches, light-responsive systems have attracted considerable attention recently, owing to their non-invasiveness, highly spatial and temporal control, and the ability of photo-activation of drug or imaging agents. Until now, a large variety of photo-responsive systems in response to the light from ultraviolet to near infrared have been engineered to achieve the photo-activated diagnosis and therapy.¹⁸⁻²⁰ In the principle of molecule design, ortho-nitrobenzyl derivatives are by far the most common photolabile caging groups in biological studies owing to the virtue of good quantum yield and rate release.^{21,22} Meanwhile, when the nitrobenzyl moiety is attached to an amino acid, such protected building blocks support the solid phase synthesis of peptide and oligonucleotides. When exposed to the near-visible UV light (>350 nm), selective deprotection will occur followed by the recovery of caged amino acids or oligonucleotides.

According to the proposed mechanism of nitrobenzyl moiety-based photolytic release, efficient and fast leaving groups include RO-, RHN-, or RS- etc, among which the photocaged serine would be the optimal choice in our project. In our studies, based on the adjustable position at P3 and P1', we rationally designed and synthesized

compound S8 with caged serine at P1' and S10 with caged serine at P3 by introducing the 4,5-Dimethoxy-2-nitrobenzyl (DMNB) group. As the control molecule, we also synthesized compound S9 to evaluate the enzyme recognition of such designed peptide sequence.

Table 4.2 Structures of photocaged probes and proteolytic cleavage by furin enzyme

Name	Sequence	P3	P1'	F. I. Increase fold	Enzyme recognition
MFP	(C ₁₂)-K(FITC)-Acp-RVRR-S-V-K(Dabcyl)	V	S	5.61	Yes
S8	(C ₁₂)-K(FITC)-Acp-RVRR-S(pho)-V-K(Dabcyl)	V	S(pho)	2.75	Yes
S9	(C ₁₂)-K(FITC)-Acp-RSRR-S-V-K(Dabcyl)	S	S	2.59	Yes
S10	(C ₁₂)-K(FITC)-Acp-RS(pho)RR-S-V-K(Dabcyl)	S(pho)	S	1.05	No

4.2.3 Photolysis of caged-probe S10 (pho-SFuP)

After the synthesis of the caged probe S10 (also named pho-SFuP), we analyzed whether the probe can be activated by the light irradiation. The slight difference of the chemical structure between the caged probe and uncaged probe leads to the different retention time (peak 1 at 24.3 min, peak 2 at 25.7 min). Subsequently, the photolysis of caged probe was examined by the HPLC based on the same method. Surprisingly, a new peak with a retention time around 24 min was observed after 1 min light irradiation, which matched well with the uncaged peptide(SFuP). Moreover, the increase of the irradiation time can result in the increased conversion of peak 2 to peak 1. The HPLC quantitative results were obtained under different light irradiation time. HPLC analysis revealed that the conversion can reach approximately 85% after 7 min light exposure, which indicated that it has great potential as an ideal platform to

achieve fast and efficient photoactivation for dynamically monitoring the biological processes.

Fig. 4.3 Characterization of the photolysis of probe pho-SFuP. (A) HPLC characterization of caged probe (pho-SFuP, peak 2) with or without UV irradiation and uncaged peptide (SFuP, peak 1). The retention time of new peak after irradiation matched well with that of the uncaged peptide; (B) Time-dependent photolysis of pho-SFuP under increasing irradiation time of lamp at 365 nm.

4.2.4 *In vitro* furin enzyme assays

After characterization of compound S8, S9, S10, we examined the enzyme hydrolysis of these three probes by measuring changes in the fluorescence intensity upon the addition of furin in the HEPES buffer. The result demonstrated that compound S9 without any caging groups could be effectively cleaved by enzyme after 2-hour incubation (2.59 folds increase). Surprisingly, the prepared compound S8, of which the serine at the P1' position was caged by the nitrobenzyl moiety also can be recognized by enzyme (2.75 folds increase). On the other hand, when the caging group

was inserted in the P3 position, almost no fluorescence enhancement is observed even 2 hours' incubation treated with the enzyme. The results demonstrated that compound S10 (pho-SFuP) presented the inactive property in the presence of furin enzyme. Next, we test whether the photo-caged probe could have response to enzyme after the light activation. The exposure of the DMNB veiled probe to 365 nm light has been proved to make the uncaged peptide substrate available for proteolytic cleavage. After 5 min light irradiation, the elevated fluorescence intensity was clearly observed with increasing incubation time (shown in Figure 4.4). Compared to the proteolytic activity of the SFuP probe, the increase folds of the pho-SFuP when exposed to the light was less due to the slightly lowered concentration of activated peptide (incomplete photoactivation in 5 min). Therefore, the results demonstrated that such constructed caged probe have the great potential to enable spatiotemporal control of the accessibility of the substrate during visualization of proteolytic activities.

Fig. 4.4 Photolysis of probes pho-SFuP in the presence or absence of 365 nm light irradiation (5 min). The probe SFuP without the caging group was used as the control.

4.3 Conclusions

In this chapter, a series of stimuli-responsive probes have been properly designed. Based on the physical properties of furin binding pocket, we screened ten different enzyme-recognized substrate sequences by introducing the functional natural and unnatural amino acids. Hydrolytic studies revealed that the photo-caged probe pho-SFuP can effectively block the furin enzyme cleavage when the DMNB group was conjugated to the serine at the key binding site (P3 position). Once the light exposure unveiled the enzyme-recognized peptide, the increase of proteolytic activities was clearly observed by the enhancement of the fluorescence intensity. Such novel design provided great potentials to dynamically and spatially probe the proteolytic activities in *in vitro* and *in vivo*. Moreover, it would not be far for further applications of such developed probe to allow the precise control of monitoring enzyme activities at the disease sites of interest.

4.4 Future work

4.4.1 Screening protease inhibitors in biological context

In order to gain a better insight of protease functions and biological roles is to develop effective and specific inhibitors as drugs for intervention of many related diseases. However, there are many challenges for developing the small-molecule protease inhibitors. One of them is its non-selectivity as protease that belongs to the same subfamily usually cleaves the similar peptide bonds, which leads to the substrate and inhibitors overlaps for different proprotein enzymes.²³ Secondly, inhibitions assays *in vitro* using purified enzyme are always not in agreement with those *in vivo*

environment. Nonspecific association in cell levels, cell permeability issues and pH, salt concentration and proteins interference may be responsible for the reduced inhibition effectiveness. Considering the limitations of developing potent inhibitors in vitro, scientists are still struggling to peruse the techniques of screening protease inhibitors at cell levels or *in vivo*.²⁴

Currently, there are three commercially available furin inhibitors that have now been used by several scientific research groups: suicide inhibitor PDX, irreversible inhibitor decanoyl-Arg-Val-Lys-Arg-CMK, and competitive inhibitor hexa-D-arginine peptide. As far as the irreversible inhibitors are concerned, although they are effective to block furin-like enzyme activities, there is a lack of promising factor for drug design because of their large size (PDX) and high toxic properties coming from chloromethyl derivatives. Therefore, as an ideal inhibitor, it should be based on non-covalent binding combined with low toxicity, small size and high specificity. In contrast, the reversible inhibitor II and hexa-D-arginines are of smaller size and less toxicity, but a very high concentration (>100 μ M) during the inhibition process limits its effectiveness on cell surface or in trans-Golgi network.^{25,26} Therefore, the identification and selection of small molecule inhibitors of furin has drawn a widely attention among researchers.

Our substrate-based probes can offer a simple and convenient method to evaluate enzyme activity and inhibitor effectiveness on cell levels or even in living animals. And it is also possible to evaluate whether inhibitors could block the membrane-associated furin activities. This study may open a window to visualize the

dynamic process of furin-related activities and difference of normal and pathological functions of proteases.

4.4.2 Design of new reversible membrane-associated furin inhibitors

Recently one proposed approach in the design of fluorescent probes for targeting proteases are based on the “reverse design”. This approach takes the advantages of the potent binding of optimized inhibitors and specific inhibitors are transformed to the cleavable and selective substrates by linking to the peptide bond. Therefore, such cleavable substrates can be used for the design of small-molecule based probes for proteolytic imaging. Considering the undesirable effect of current furin inhibitors, the exploration of potential furin-inhibitors is also necessary and significant.

In this chapter, our results demonstrated that the involvement of lysine and cysteine in the position P1' will impede the enzyme cleavage. In the meantime, the conscious sequence RVRR will provide strong affinity to furin enzymes. Therefore, such designed peptide sequences may potentially be applied as the competitive inhibitors of furin. What's more, the existence of the lipid moiety will keep the probe on the membrane, which therefore, could provide the possibility to selectively inhibit the cell-membrane-associated furin activity and minimum interference with the normal cellular function of furin.

4.5 Experimental details

Scheme 4.1 Synthesis of compound S3. a) Boc_2O , 0°C ; b) 1. Maleic anhydride, Et_3N , CH_2Cl_2 , 0°C ; 2. Ac_2O , Na_2OAc , 70°C ; c) TFA, CH_2Cl_2 ; d) DIPEA/DMF, 4 hr; e) Fmoc-Cl, NaHCO_3 in acetone; f) DIPEA/HBTU, 2hr; g) NMM, 10% DTT in DMF; h) DIPEA/DMF, 4 hr; i) 2% TFA; Then DIPEA, Dabcyl SE, 4 hr; Then 95% TFA, 2.5% H_2O , 2.5% TIPS 2 hr.

N-Boc-Ethylenediamine (1). Di-tert-butyl dicarbonate (Boc_2O) (1.83 mL, 8 mmol) was added into a mixture of ethylenediamine (5.4 mL, 80 mmol) in 80 mL of chloroform at 0°C . The solution was stirred overnight at room temperature. Then solutions were extracted with brine and 3M Sodium carbonate. Extraction was collected by removing the solvent and used in the next step without further purification (41 %). ^1H NMR (400 MHz, CDCl_3): δ 1.44 (9H, s), 2.76 (2H, t), 3.14 (2H, t), 5.07 (1H, s).

N-(2-[(t-Boc)amino]ethyl Maleimide (2). Maleic anhydride (300 mg, 3.12 mmol) in CH_2Cl_2 (30 mL) was added dropwise into a solution of 1 (1.2 equiv) and triethylamine in 30 mL CH_2Cl_2 at 0°C and stirred for 4 hours. Solvent was removed and the mixture was dissolved in acetic anhydride (3 mL). Sodium acetate (120 mg) was added into solution and the solution was heated to 70°C for 30 min. When the temperature was cooled down to room temperature, saturated sodium bicarbonate was added and the product was extracted with CH_2Cl_2 . And washed with brine for three times. The product was purified with flash chromatography (EA/hexanes, 1:3) to give a white solid 2 (40 %). ^1H NMR (400 MHz, CDCl_3): δ 1.41 (9H, s), 3.32 (2H, d), 3.66 (2H, t), 4.72 (1H, s), 6.71 (2H, s).

Synthesis of compound (4) and (5). The synthesis of compound 4 and 5 was referred to the established method. FITC (15 mg) was dissolved in 600 μL DMF, compound 3 (18 mg) and DIPEA (20 μL) was added and stirred overnight. The product was precipitated with ether and purified with flash column chromatography to get compound 4. m/z calcd: 529.09 $[\text{M}+\text{H}]^+$; found 530.16 $[\text{M}+\text{H}]^+$. A solution of Fmoc-Cl (98.64 mg, 0.38 mmol) in acetone (5 mL) was added dropwise to a stirred solution of 2-amino-4-oxopentanoic acid hydrochloride (50 mg, 0.38 mmol) NaHCO_3 (0.95 mmol) in water (5 mL). After overnight reaction, solvent was removed and the product was extracted with CH_2Cl_2 and 1M HCl (pH = 2) and purified with flash column chromatography.

Compound S3. The synthesis of compound 3 was shown in Scheme 4.1. Generally, Compound 6 was synthesized according to standard solid peptide synthesis procedure.

After that the StBu group was cleaved on resin by using 0.1 M N-methylmorpholine (NMM) and 10 % Dithiothreitol (DTT) in DMF. The following synthetic procedure can be referred to the synthesis of the probe MFP in chapter 2. The crude product was purified by HPLC and characterized by MS (ESI): m/z : $[M+H]^+$ calcd: 2106.07; found: 1053.28 ($[M+2H]^{2+}$), 703.05 ($[M+3H]^{3+}$).

9-Fluorenylmethyl carbazate. 9-Fluorenylmethoxycarbonyl chloride solution in ether (40 mL) was added dropwise into hydrazine (3 mL) in ether (40 mL) at at 0 °C and the reaction was stirred overnight. Solvent was removed and the product was exacted with ethyl acetate and washed with brine for 3 times. The product was purified with flash column chromatography to afford 9-Fluorenylmethyl carbazate. ^1H NMR (400 MHz, CDCl_3): δ 3.76 (2H, s), 4.23 (1H, t), 4.46 (2H, d), 7.29-7.33 (4H, m), 7.41 (2H, t), 7.57(2H, d,).

Compound S4. 9-Fluorenylmethyl carbazate was dissolved in methanol (500 μL) and reacted with compound S3 for 48 hours. The product was purified by RP-HPLC (buffer A: 0.004% TFA in water, buffer B: 0.004% TFA in acetonitrile, C18 analytical column, flow rate 1 ml/min. 100% buffer A to 100% buffer B in 50 min) and compound S4 was collected at 35 min. MS (ESI): m/z : $[M+H]^+$ calcd: 2342.16; found: 1171.52 ($[M+2H]^{2+}$).

Compound S1. The crude product was purified by HPLC and characterized by MS (ESI): m/z : $[M+H]^+$ calcd: 1680.83; found: 840.70 ($[M+2H]^{2+}$), 560.99 ($[M+3H]^{3+}$).

Compound S2. The crude product was purified by HPLC. The liner gradient stretched over 50 min from $t=0$ min at 0 % solution B to $t=50$ min at 100 % solution B (buffer A:

0.004% TFA in water, buffer B: 0.004% TFA in acetonitrile, C18 analytical column) with a flow rate of 1.0 mL / min. The retention time of the desired product was 26 min. MS (ESI): m/z : $[M+H]^+$ calcd: 1916.93; found: 958.76 ($[M+2H]^{2+}$), 639.79 ($[M+3H]^{3+}$).

Compound S6. The crude product was purified by HPLC and characterized by MS (ESI): m/z : $[M+H]^+$ calcd: 2119.10; found: 1060.81 ($[M+2H]^{2+}$), 707.86 ($[M+3H]^{3+}$).

Scheme 4.2 Synthesis of compound 11. (a) triflic acid/ CH_2Cl_2 ; (b) $\text{Pd}(\text{PPh}_3)_4$ / $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CO}_2\text{H}$.

N-(9-Fluorenylmethyloxycarbonyl)-L-Serine allyl ester (8). The synthesis of Compound 8 (shown in Scheme 4.2) was followed the reported method.²⁷ Generally, N- α -FMOC-L-Serine (1.64 g, 5.00 mmol) and NaHCO_3 (0.44 g, 5.23 mmol) in water (16 mL) were combined with a solution of tricaprylmethylammonium chloride [aliquot 336] (2.00 g) and allyl bromide (3.20 g, 26.5 mmol) in 30 mL of CH_2Cl_2 , and the suspension was vigorously stirred at r.t for 24 h. Water (50 mL) was added to the reaction mixture, and the suspension was extracted with CH_2Cl_2 (3×50 mL). The combined organics were dried (Na_2SO_4) and concentrated in *vacuo*. The product is purified by flash column chromatography to afford **8** as a white solid (0.96 g, 52%): ^1H NMR (300MHz, CDCl_3) δ 3.05 (s, 1H), 3.93 (d, $J = 9.7$ Hz, 1H), 4.03 (d, $J = 9.7$

Hz, 1H), 4.18 (t, J = 7.0 Hz, 1H), 4.39 (m, 3H), 4.68 (d, J = 5.1 Hz, 2H), 5.25 (dd, J = 10.4, 0.7 Hz, 1H), 5.34 (dd, J = 17.2, 0.7 Hz, 1H), 6.03 (d, J = 7.6 Hz, 1H), 5.91 (ddt, J = 16.6, 10.9, 5.7 Hz, 1H), 7.31 (t, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.61 (s, 2H), 7.77 (d, J = 7.47 Hz, 2H). ESI-MS: m/z calcd: 368.14[M+H]⁺; found 368.40 [M+H]⁺.

4,5-dimethoxy-2-nitrobenzyl trichloroacetimidate (9). The synthesis of Compound 9 was followed the reported method.²⁸ 4,5-dimethoxy-2-nitrobenzyl alcohol (1.24 g, 5.79 mmol) was dissolved in 40 mL of anhydrous CH₂Cl₂ and stirred at r.t under N₂. Anhydrous K₂CO₃ (2.06 g, 14.87 mmol), trichloroacetonitrile (2.10 g, 14.54 mmol) and anhydrous triethylamine (0.72 g, 7.12 mmol) were added to the solution and the reaction mixture stirred at r.t for 24 h. CH₂Cl₂ (60 mL) was added to the reaction mixture, and the suspension sequentially washed with 1M HCl (20 mL) and saturated NaCl (20 mL). The organic layer was dried (Na₂SO₄) and concentrated in *vacuo*. The product was purified by flash column chromatography to afford **9** as a yellow solid (1.72 g, 83%): ¹H NMR (300MHz, CDCl₃) δ 3.86 (s, 3H), 3.86 (s, 3H), 5.66 (s, 2H), 7.13 (s, 1H), 7.64 (s, 1H), 8.46 (s, 1H).

N-(9-Fluorenylmethyloxycarbonyl)-O-(4,5-dimethoxy-2-nitrobenzyl)-L-Serine allyl ester (10). To a solution of **8** (0.35 g, 0.95 mmol) in anhydrous CH₂Cl₂ (8 mL) was added **9** (0.70 g, 1.96 mmol) and mixed under N₂. To the mixture, triflic acid (60 μL, in 1 mL of CH₂Cl₂) was added gradually. The resulting dark green solution was left to stir at room temperature for 30min. After the reaction, DIPEA (40 μL) in CH₂Cl₂ (1 mL) and silica gel (100-200 mg) was added to the reaction mixture. The

dark brown solution was concentrated in *vacuo* and the resulting crude residue purified by flash column chromatography to afford **10** as a pale yellow solid (0.15g, 29%). ESI-MS: *m/z* calcd: 563.20; found: 563.57 [M+H]⁺.

N-(9-Fluorenylmethyloxycarbonyl)-O-(4,5-dimethoxy-2-nitrobenzyl)-L-Serine(11).

To a solution of **10** (0.56 g, 1.00 mmol) in CH₂Cl₂ (6 mL) containing acetic acid (54 μL) and N-methylmorpholine (1.44 mL). Pd(PPh₃)₄ (0.12 g, 0.10 mmol) was then added and the reaction mixture was left to stir for 4 h at r.t. 1M HCl was added to the resulting solution until pH 4 was reached. The solution was then extracted with CH₂Cl₂ (4 × 30 mL) and the combined organics were dried (Na₂SO₄) and concentrated in *vacuo*. The product was purified by flash column chromatography to afford **11** as a yellow solid (0.47g, 90%). ESI-MS: *m/z* calcd: 523.16 [M+H]⁺; found: 523.50 [M+H]⁺.

Scheme 4.3 Synthesis of S7 probe. a) DIPEA, 1 hr in DCM; b) HBTU/DIPEA in DMF; c) 2 % hydrazine in DMF, 20 min, then FITC/DIPEA, 4 hr; d) DIPEA, Dabcyl

SE, 4 hr; e) 95% TFA, 2.5% H₂O, 2.5% TIPS 2 hr. Then NMM, 10% DTT in DMF, 10 hr.

Compound S7. Compound 7 was synthesized by following the similar synthetic procedure of MFP probe. As shown in scheme 4.3, crude compound 14 were deprotected 95% TFA for 2 h to remove the pbf group, following the deprotection of StBu group by using 0.1 M N-methylmorpholine (NMM) and 10 % Dithiothreitol (DTT) in DMF for 10 h. The final product (Fig 4.5) was purified by HPLC monitored by UV absorbance at 220 nm. ESI-MS: m/z [M+H]⁺ calcd:1981.06, found 991.78 ([M+2H]²⁺).

Fig. 4.5 Chemical structure of compound S7

Compound S8. Following the standard SPPS, the potocage serine (Compound 11) was conjugated on the resin through the coupling reaction over four hours at room temperature in the presence of HBTU as a coupling reagent and N, N-Diisopropylethylamine (DIPEA) as a basic catalyst respectively. The subsequent

reactions were referred to the similar synthetic procedure of MFP probe (chapter 2).

The crude product was purified by HPLC to get S8 (Fig. 4.6) and characterized by MS

(ESI): m/z : $[M+H]^+$ calcd: 2158.12; found: 1080.42 ($[M+2H]^{2+}$), 721.07 ($[M+3H]^{3+}$).

Fig. 4.6 Chemical structure of compound S8

Compound S9 (SFuP). Compound S9 was synthesized by using the similar synthetic procedure of MFP probe (chapter 2). The crude product was purified by HPLC get S9 (Fig. 4.7) and characterized by MS (ESI): m/z : $[M+H]^+$ calcd: 1953.04; found: 1952.78 ($[M+H]^+$), 976.77 ($[M+2H]^{2+}$).

Fig. 4.7 Chemical structure of compound S9

Compound S10 (pho-SFuP). Compound S10 was synthesized by using the similar synthetic procedure of compound S8. The crude product was purified by HPLC get S9 (Fig. 4.8) and characterized by MS (ESI): m/z : $[M+H]^+$ calcd: 2205.16; found: 1102.21 ($[M+2H]^{2+}$), 735.61 ($[M+3H]^{3+}$).

Fig. 4.8 Chemical structure of compound S10

Irradiation of the caged probe (pho-SFuP) for various times intervals and the extent of conversion to uncaged probe.

The caged probe pho-SFuP (5 μ M, 100 μ L) was placed in 96 well plate and sample was irradiated using the UV lamp (Blak-Ray, B-100AP/R, 100 w/365 nm) for 0 s, 30 s, 1 min, 2 min, 5 min, 7 min. Then solution was diluted to 1 mL (50 % water and 50% acetonitrile) solution. Then 500 μ L aliquot was analyzed by the analytical scale reverse-phase HPLC (gradient A: 0.1% TFA in H_2O ; solvent B: 0.1% TFA in acetonitrile; 1 mL/min) The liner gradient stretched over 50 min from $t=0$ min at 25 % solution B to $t=40$ min at 90 % solution B. Peptide 2 and its uncaged analogue 1 was

quantified by their absorbance at 220 nm.

Proteolytic hydrolysis studies on probe SFuP and pho-SFuP

Proteolytic activity was monitored using microplate reader to detect the fluorescence in the presence of pure furin enzyme (2U) in at 37°C in 0.1 mL assay buffer. The assay buffer contains 100 mM HEPES, pH 7.4, 0.5 % Triton X-100, 1 mM CaCl₂, and 1mM 2-mercaptoethanol. The emission spectrum ($\lambda_{\text{ex}}=450$ nm, $\lambda_{\text{ex}} = 523$ nm) was recorded over time. As comparison, the caged probe pho-SFuP was irradiated for 5 min to be activated, followed by monitoring the fluorescence changes when treated with the furin enzyme.

4.6 References

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Chapter 5

Photoactivated probes for monitoring organelle-specific proteolysis-plasma membrane and early endosome

ABSTRACT. Cell organelles like plasma membrane, mitochondria, Lysosomes/endosomes and Golgi apparatus et al are critical for supporting the normal functions of cells in signaling transduction and energy production. Effective distinction of biological activities in subcellular organelles could help better understand the basic physiological process and more importantly, could greatly facilitate the precise diagnosis and effective therapeutics. Here, we successfully designed the photoactivated probes for visualizing the organelle-specific proteolysis in living cells. Such unique probe could selectively monitor furin-like activities at the cell membrane and endosome respectively, which may provide a promising strategy for a deeper understanding of cellular process.

5.1 Introduction

Imaging of disease-related events in particular organelles is critical for precise diagnosis and for studies on the effective therapeutics. Fluorescent imaging currently provides a direct, non-invasive and sensitive imaging tool for visualizing local distributions of biomolecules and for unraveling cellular activities as well as pathological processes.¹⁻³ Among various biomolecules in the cell structure, proteases

received considerable attention due to its pivotal biological functions in the protein digestion, intracellular protein turnover and damaged protein removal *etc.* Additionally, increasing studies uncover that dysregulation of proteolytic activities is highly relevant to the progression of numerous human diseases, like inflammation, osteoarthritis, neurodegeneration, and cancer *etc.*⁴⁻⁶ Therefore, development of valid tools for visualizing and monitoring the protease activities, especially at the specific organelle would be crucial for an in-depth understanding of biological basis conferring the disease progression⁷⁻¹⁰.

5.1.1 Genetic manipulation

In many cases, proteases, like other protein biomolecules can be monitored by genetic manipulation with the fusion of fluorescent proteins or the modification of fluorescent tag molecules. However, such labeling system has many intrinsic limitations. Their relatively large size (~238 amino acids) of the fluorescent protein tag may potentially interfere with the structure or function of POI.¹¹⁻¹³ Also, the barrel structure of fluorescent proteins impede their quick movement, which reduces environmental sensitivity as probes.¹⁴ Therefore, the specific chemical probing with small organic fluorophores offers an alternative and attractive approach to compensate the shortcomings of genetically encoded fluorescent proteins.¹⁵

The first widely used labeling strategy is the receptor-protein-based method.¹⁶ Initially the receptor protein is genetically fused to the target protein which is located in cytosol or membranes; Then fluorophore-conjugated ligand can covalently or electrostatically bind to receptor proteins, which realizes the introduction of

fluorophores into POI. Compared to GFP and its genetic mutants, small molecule fluorophores can provide a wide range of emission spectra.¹⁷ However, there is no difference of fluorescence signals before and after the ligation of fluorophores, an extra washing step to remove the unbound ligand is very necessary.^{18,19} Secondly, enzyme-mediated ligation method is commonly used as well.²⁰ Through enzyme catalytic reactions, the small molecule substrate can covalently bind to the peptide tag. Specific enzyme-catalyzed reaction and relative small size of tags indeed provide many advantages for this approach, but the complexity of the labeling process allows the target proteins essentially on the cell membrane.²¹ The third strategy is fluorogenic labeling.²² The fluorescence intensity or spectrum of fluorophores will change once the labeling process of target proteins occurs. For example, Tisen and co-workers²³ developed the fluorescein arsenical hairpin binder (FIAsH) and resorufin arsenical hairpin binder (ReAsH) to successfully label recombinant proteins containing tetracysteine tags. Once probes bind specifically to tetracysteine motif, strong green fluorescence could be observed in the fusion protein.

These genetic protein approaches, despite very promising in principle, however, have shown their disadvantages. Genetic manipulation before analyzing the protein possibly perturbs the cell's physiology and causes biological response.^{24,25} Also, expected results only from limited number of cell lines and the insufficient yield of genetic tag proteins hamper this method to a variety of applications. In contrast, bio-activatable targeting has shown promising benefits by controlled emission output corresponding to changes of the probe in structural conformation and chemical

environments^{26,27} This method has been extensively used for the functional imaging of protease activities.^{28,29}

Although some developed probes can penetrate into living cells, targeting the specific sub-cellular locations and monitoring the dynamic protease processes are still not fully explored. Therefore, it is highly desirable and important to track the protease activation in living systems and determine the protease signaling at the specific organelle, which will provide more valuable information about the pathological causes and disease progression.

5.1.2 Physiological functions of endosomal proteolysis

Endosome is a membrane-bound compartment, located spatially between the plasma membrane and lysosomes. It plays important roles in nutrient uptake, intracellular signaling, turnover of membrane lipids and defense against toxins and pathogens. During the process of endocytosis, signaling receptors on the cell surface, components of the plasma membrane and nutrients are internalized into small vesicles, followed by being fused with early endosome. These cargoes can translocate into the late endosome and further to trans-Golgi network or lysosome. Some of them also can be recycled back to the plasma membrane.^{30,31}

Investigations on the endocytic pathway have benefited to better understand the roles of early and late endosome in the sorting and segregation. Moreover, recent evidence indicates that a lot of biologically important processing also occurs in this compartment.³² There are a variety of proteases located in the endosome, which have been validated to involve in the degradation of internalized proteins or peptides, the

control of the metabolic pathways and processing of antigens.³³ For example, the growth factors, hormones and antigens are cleaved by endosomal proteases. Many endopeptidases like cathepsins B and H, and the aspartic protease cathepsin D assist the turnover of divergent intracellular proteins in the endosome. More importantly, some proteases also involve in the angiogenesis, apoptosis, and tumor cell invasion. Therefore, it is high of significance to explore functions and dynamics of the endosomal proteolysis for elucidating these fundamental cellular processes, especially the pathological processes.

5.1.3 Movement of lipids in the endocytic membrane transport and sorting

It is well-known that lipids provide the physical support for the structural integrity of cell membranes. The lipids composition of the plasma membrane has been well characterized over the past years. Moreover, besides the functions in the signal transduction, they are also found to participate in the protein transport and membrane traffic. During the endocytic process, proteins, receptors as well as plasma membrane lipids will be internalized and first appear in the early endosome (Fig 5.1).³⁴ It is reported that internalized lipids and proteins (e.g. transferrin) also can be recycled back to the cell surface for reutilization. Although mechanisms of membrane traffic and protein sorting are not fully understood, a lot of progress has been made in understanding the movement of internalized lipids.³¹

Fluorescent analogs of lipids have been widely used to explore the lipid trafficking and metabolism. Fluorophores including NBD, pyrene, BODIPY³⁵, rhodamine, and polyenes are modified with various types of lipids to mimic the behavior of the natural

lipids. For instance, bodipy-labeled sphingolipid analogs have been applied for elucidating intracellular trafficking of these lipids from the plasma membrane to the Golgi network via endosomes³⁶. Recently, polyene lipid consisting of hydrocarbons with five conjugated double bonds were designed for microscopic visualization of ether lipids³⁷. However, the poor quantum yield and photobleaching, concentration-dependent spectra shift and complicated washing steps have limited their further applications in studying intracellular lipid trafficking and lipid-involved endocytic pathway.

Fig. 5.1. The lipid internalization and recycling in the endocytic pathway of animal cells. Arrows indicate routes of membrane transport. PM, plasma membrane; EE, early endosomes; RE, recycling endosomes; ECV/MVB, endosomal carrier vesicle/multivesicular body; LE, late endosomes; LYS, lysosome; GC, Golgi complex. Copyright © 1998 Academic Press.

5.1.4 Endocytic uptake pathways utilized by nanoparticles

With the continuous development and progress in nanomedicine, there is a drastic increase in the use of nanomaterials for therapeutic and diagnostic applications. Most

small molecules enter cells via passive diffusion or active transport while most nanoparticles, proteins and virus are mediated by endocytosis. In recent year, scientists have shown great interest in the endocytic process and subsequent intracellular fate of nanomedicine.^{38,39} Among various nanosystems, mesoporous silica nanoparticles (MSNs) has become an ideal platform for biomedical applications due to its great advantages in the uniform porosity, stable aqueous dispersion, well biocompatibility and *in vivo* biodegradability.^{40,41} In this project, fluorescent labelled MSNs would be applied as a model to proceed the endocytic pathway.

5.2 Results and discussion

5.2.1 The principle of photoactivated probe (pho-SFuP) to monitor endosome-specific furin proteolysis

In this chapter, the as-prepared photo-caged probe was successfully applied to specifically monitor furin-like activity at the cell membrane and endosome respectively. Initially, the caged probe will be inserted into the plasma membrane due to the strong lipid-mediated hydrophobic interactions. Caged probes will be enriched in the plasma membrane with minimal internalization and keep inactive in the biological context with the presence of furin enzyme. Once the nanoparticles activate the endocytic process, probes in the membrane will be also internalized into the vesicles and transported into the intracellular compartment. Under the light irradiation, the probe will be uncaged and enzyme-responsive peptide sequence will be exposed, thereby being cleaved by endosomal furin enzyme (Fig 5.2). The increased fluorescence demonstrated that such designed platform can be applied not only to

monitor the cell surface furin-like activity, but also to monitor both lipid-involved endocytic pathway and endosomal proteolysis during the process.

Fig. 5.2 schematic illustrations of photoactivated probe (pho-SFuP) to monitor endosome-specific furin proteolysis.

5.2.2 Photo-controlled probe for membrane-localized furin imaging

The preparation of small-molecule probes, pho-SFuP and SFuP, was included in the chapter 4. Additionally, the enzyme hydrolysis of these two probes was also examined in buffer solutions. After the verification in response to light and furin enzyme, we then test the applicability of pho-SFuP and SFuP in the live cell imaging. As shown in Fig 5.3, the images at 0 min were taken immediately after probes were added in the culture media. Results demonstrated that there was no any fluorescence detected in the cells, indicating no any autofluorescence background caused by cell and inactive probes. After 15 min incubation, strong fluorescence signals around the cell membrane area when treated with SFuP. By contrast, there was no obvious fluorescence observed when cells incubated with pho-SFuP molecule. The results indicated that the peptide

sequence in the SFuP can be cleaved to turn on the fluorescence in living cells whereas the sequence in the pho-SFuP was unable to be recognized by furin. Moreover, the cleaved part can be specifically located on the cell membrane.

Fig. 5.3 Fluorescence imaging of HEK293 cells using the pho-SFuP and SFuP probe (300 nM) for 15 min at room temperature.

5.2.3 Light activation of the pho-SFuP probe in live cells

Next, we tested whether the pho-SFuP molecule can be activated in living cells. Firstly, we confirmed that the light will cause minimum cell deformation or toxicity under the experimental conditions. Then after incubation with pho-SFuP (300 nM) for 30 min at 37 °C, HEK 293 cells were irradiated with 365 nm light for 5 min. As shown in Fig 5.4A, the CLSM images indicated that the fluorescence signal is weak without light irradiation, but the green fluorescence is increased along with the light irradiation. Meanwhile, the localization of the probe was further confirmed by co-staining cells with nuclear, lysosome and membrane tracker. As shown in the Fig 5.4A, the green fluorescence signals could not be found in the lysosome or some other intracellular organelles. However, overlay images revealed that the activated probes were located as the same place of signals from CellMask Deep Red. Quantitative co-localization

analysis of probe with membrane tracker clearly demonstrated that such designed probes have the specific staining capability on cell membranes (Fig 5.4 B).

Fig. 5.4 (A) CLSM images of live HEK 293 cells after incubation with pho-SFuP with and without 365 nm light irradiation (5 min). FITC=green fluorescence signal; (E_x : 488 nm; E_m : 500-550 nm); Lysotracker=red signal (E_x : 561 nm, E_m : 578-621 nm); Hoechst dye H33258 (nuclear stain) =blue signal (E_x : 405 nm, E_m : 400–500 nm). CellMask Deep Red (membrane stain) =purple (E_x : 640 nm, E_m : > 670 nm); (B) Pearson co-localization analysis of Pho-SFuP with CellMask Deep Red with or without UV light irradiation. Pearson values were calculated with Zeiss software and error bars were obtained by analysis of three different cells.

5.2.4 Cellular uptake of Cy 5.5-labeled Mesoporous Silica Nanoparticles (MSNs)

Mesoporous silica nanoparticles have been characterized by TEM and SEM and the average size is around 100 nm. Early studies mentioned that the uptake and trafficking

of nanoparticles will be affected by a lot of factors including the size, shape and surface modifications. In our experiment, we firstly examined the early event of uptake when silica nanoparticles were exposed into cells in the first 1 hour. Confocal microscope images showed that Cy5.5-MSNs were able to be internalized into the HEK293 cells after one-hour incubation. The evidence of well overlay with commercially available early endosome marker EEA1 indicated that Cy5.5-MSNs mainly located in the endosome compartment (Fig. 5.5).

Fig. 5.5 CLSM images of HEK293 cells treated for 1 h with Cy5.5-MSNs (40 ug / mL. a) Alex 594: EEA1 staining of the early endosomes; b) Cyan: Cy5.5-labeled MSNs; c) overlay of a) and b), Blue: Hoechst stained nuclei; d) Enlarged images of the square area in c). Scale bars = 20 μm

5.2.5 Energy-dependent endocytosis of Cy5.5-MSNs and endocytosis-mediated probe translocation

In most cases, the extracellular substances enter cells via diffusion, phagocytosis

and receptor mediated or fluid phase endocytosis. To determine whether the uptake mechanism of the MSNs is similar to those of most nano-size particles by energy-dependent endocytosis, we carried out the uptake experiments at 37 °C and 4 °C respectively. Generally, Hek293 cells were incubated with dye-labeled nanoparticles at 37 °C and 4 °C and the uptake efficiency was evaluated by confocal microscopy. The result of the very weak fluorescence signals at 4 °C and very bright fluorescence observed at 37 °C incubation clearly demonstrated that the cellular uptake of Cy5.5-MSNs were mainly due to the energy-dependent endocytosis, which is well consistent with the cellular uptake mechanism of silica-based nanomaterials. Next, we tested how the membrane-localized probe will be proceeded when the endocytosis occurs. Cells were incubated with inactive probe at 37 °C for 30 min. The free probe molecules were removed before the addition of Cy5.5-MSNs. Then the endocytic pathway was initialized in the following 1-hour incubation. The subsequence light irradiation at 365 nm will activate the photo-caged probe within cells, thereby being recognized by furin enzyme to liberate the fluorescence signals. As shown in the Fig 5.6, strong green fluorescence could be observed in HEK293 cells at 37 °C, indicating furin-mediated proteolytic hydrolysis. Meanwhile, we surprisingly observed that such activated probes were internalized into cellular organelles where most nanoparticles located. By contrast, when the NPs-induced endocytosis was prohibited under 4 °C, most of activated probes were found to be trapped on the cell membrane, which is mainly involved in the lipid-mediated passive insertion. All the results provided the evidence that such photo-caged probe can only be internalized

into cells when cells engulfed the external nanoparticles through the endocytic pathways.

Fig. 5.6 (A) Confocal laser scanning microscope (CLSM) images of Cy5.5-MSNs uptake along with the probe translocation. Cells were incubated at 37 °C and 4 °C respectively. The pho-SFuP was added into cells for 30 min, followed by the addition of Cy5.5-MSNs for 1-hour incubation. Scale bars = 20 μ m. (B) Pearson co-localization analysis of pho-SFuP with Cy5.5-MSNs at 37 °C and 4 °C.

5.2.6 Intracellular distribution of MSNs and photocaged probe molecules

In the cellular uptake process, the analysis of nanoparticle and probe distribution is high of significance for better understanding the trafficking pathway, the pharmacological activity and rational design of specific organelle targeting. As shown

in the Fig 5.6, the MSNs alone will be transported into early endosome in the first one hour. Moreover, the internalization of pho-SFuP following the endocytosis of the MSNs at 37 °C was also observed. This phenomenon is likely attributed to the involvement of cell membrane-involved transport during the endocytosis. In the endocytic process, some vesicles coming from the cell surface will be formed and wrap the nanoparticles into the cells. Given the probes have been stably located on the cell surface, there is a high chance that probe will go along with the vesicles and fuse into early endosome. Also, the observed fluorescence light-up indicated the existence of furin processing. In order to confirm the intracellular distribution, the early endosome was stained with EEA1 marker and the cell nucleus was stained with Hoechst dye. As shown in the Fig 5.7, light-activated probes were overlapped with EEA1 marker judging from the yellow color in the merged-1 images, indicating that both probes and MSNs were located in the early endosome. We also stained the lysosome compartment with LysoTracker, nearly minimum signal overlap with either activated probes or MSNs was detected (Fig 5.8 and Fig 5.9).

Fig. 5.7 Confocal laser scanning microscope (CLSM) images of intracellular distribution after cell treated with Cy5.5-MSNs at 37 °C. pho-SFuP=green

fluorescence signal; EEA1=red signal; Hoechst dye H33258 (nuclear stain) =blue signal. Cy5.5-MSNs =purple; The merged 1 images indicated the overlay signals of green and red fluorescence. The merged 2 images indicated the overlay of four channels. Scale bars = 20 μ m

Fig. 5.8 Confocal laser scanning microscope (CLSM) images of intracellular distribution after cell treated with Cy5.5-MSNs at 37 °C. pho-SFuP=green fluorescence signal; LysoTracker=red signal; Hoechst dye H33258 (nuclear stain) =blue signal. Cy5.5-MSNs =purple; The merged 1 images indicated the overlay signals of green and red fluorescence. The merged 2 images indicated the overlay of four channels. Scale bars = 20 μ m

Fig. 5.9 Co-localization analysis of pho-SFuP at different channels. The figure presented the Pearson values of Pho-SFuP with EEA1 staining of the early endosome (second antibody conjugated with Alexa-594) or LysoTracker in the presence or absence of light irradiation.

The results clearly demonstrated intracellular distribution of activated probes during endocytosis was in the early endosome, almost same site with the MSNs and such developed reporter could be applied as the tracker of MSNs in living cells. In addition, no obvious fluorescence was observed when HEK293 cells were not treated with light irradiation. This data suggested the fluorescent signals can be locally controlled by light irradiation. Therefore, the turn-on fluorescence is most likely attributed to the endosomal furin activity. Based on all the experimental results, we first use the small-molecule tools to provide the evidence that the proteolytic activities of furin-like enzymes in the early endosome.

5.3 Conclusions

In this chapter, we have presented the applications of the photoactivated probes for both cell-membrane and endosomal proteolytic activities in living cells. For cell-membrane associated furin activity, such photo-responsive probe enabled control of the accessibility of the substrate during measurements of protease activity. Not only such design can greatly reduce the nonspecific background, but also have great potential for spatiotemporal control of the activity at the target disease area by strict control of light irradiation. More importantly, such unique design enabled specific visualization of early endosomal furin-related proteolysis upon the

nanoparticle-induced endocytosis. By taking the advantage of lipid-mediated membrane insertion of photocaged probe, movement and internalization of cell membrane in the endocytic pathway was clearly elucidated. Given a lot of bacterial or viral invasion process is highly relevant to the furin process and undergo the endocytic pathway, such developed probe provides great chances to dynamically monitor the cellular entry process, which is high of significance for uncovering the trafficking pathways of bacterial pathogens in cells and more useful information for the design of innovative tools against these life-threatening diseases.

5.4 Future work

In this chapter, we used the amine-modified MSNs as the model to monitor the endocytic pathway. Next, we want to examine whether our designed probes can evaluate the varying levels of the cellular internalization. It is known that cytomembrane possess negative charge.³⁸ Therefore, cationic nanoparticle, neutral nanoparticle and anionic nanoparticle may exhibit different interactions with cells, leading to different internalization and cellular routes. So it would be meaningful to investigate more detailed mechanism of the vesicle formation during the internalization of the different nanoparticles. Common cellular entry for nanomedicine includes clathrin-dependent endocytosis and caveolae-dependent endocytosis. Investigating the endocytic pathways is not only important for the design of novel multifunctional and effective nanomedicine, but also essential for better understanding of trafficking routes of many disease-related proteins like toxins or pathogens. By using different models which are involved in the different endocytic mechanisms, the

performance of our developed probes can be characterized, hence more detailed membrane trafficking clues may be obtained.

More importantly, pathogenic organisms such as bacteria, viruses and parasites enter cell via endocytic pathway. Cell-surface lipids and proteins can be internalized into endosome during the pathogen invasion. Also, furin is known to process a variety of pathogens for survival, replication and immune-system invasion at different compartment. Therefore, it is highly promising that our designed platform can be used to investigate the dynamic invasion process of various pathogens and their processing mechanism at different compartments, like endosome, cell membrane or the trans-Golgi network, which would open new ways to precision diagnosis and the following disease treatment.

5.5 Experimental details

Synthesis of Cy 5.5-labeled Silica Nanoparticles

The monodispersed Silica nanoparticles (SiO_2 NPs) were synthesized by Stöber method.⁴² Then prepared nanoparticles (0.1306 g) were dispersed in 120 mL of ethanol. 2 mL 3-aminopropyltrimethoxysilane (APTMS) was added to the stirring mixture at room temperature. The reaction was stirred overnight and then heated to reflux for 1 hour. To remove the excess APTMS, the NPs were washed by centrifugation in ethanol and then dispersed in 10 mL ethanol for further use. 1 mL of functionalized Silica NPs were centrifuged and dispersed in 1 mL DMF, Cy 5.5-NHS and DIPEA were added. The reaction was stirred at room temperature for overnight. Then Cy5.5-labeled NPs were washed with DMF water for 5 times and dispersed in water for further use.

Transmission electron microscopy (TEM) was carried out on a JEOL JEM-1400 electron microscope at an accelerating voltage of 100 kV. MSNs-Cy5.5 was characterized using scanning electron microscopy (SEM) (JEOL-JSM-7600F) at an accelerating voltage of 5 kV.

Imaging of membrane-localized furin-like activity in live cells

Human embryonic kidney cells HEK293 were grown in Dulbecco's modified eagle medium (Gibico) supplemented with 10% fetal bovine serum (FBS, HyClone) at 5% CO₂ and 37°C. HEK293 cells were seeded (1.5×10^5 cells / per well) at 35-mm diameter μ -dish plastic-bottom (ibidi GmbH, Germany) in complete medium for 24 hours at 5% CO₂ and 37 °C. Then cells were treated with the probes, pho-SFuP and SFuP, (300 nM) for 15 min at room temperature. Live cell imaging was taken under confocal laser scanning microscope (CLSM) (Nikon, Eclipse TE2000-E). ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 525/50$ nm). For the experiment about light activation of the pho-SFuP probe on cell surface, HEK293 cells were firstly incubated with pho-SFuP (300 nM), bisBenzimide H 33258 (Sigma) and LysoTracker® Red DND-99 (Life technologies) in the fresh medium for 30 min. After that, cells were washed with medium for three times and irradiated with 365 nm laser for 7 min. Subsequently cells were stained with CellMask Deep Red (2.5 $\mu\text{g/mL}$) for 5 min. The images were acquired by confocal laser scanning microscope (LSM 800, Carl Zeiss Inc., Jena, Germany) The laser setting were as follows: H33258 ($\lambda_{\text{ex}} = 405$ nm); FITC ($\lambda_{\text{ex}} = 488$ nm) and LysoTraker ($\lambda_{\text{ex}} = 561$ nm), CellMask Deep Red ($\lambda_{\text{ex}} = 640$ nm).

Cellular uptake of Cy 5.5-labeled Mesoporous Silica Nanoparticles (MSNs)

HEK293 cells were seeded in a 35-mm Petri dish and were cultured in DMEM containing 10% FBS at 37 °C in 5% CO₂ for 24 hr. Cells were treated with Cy5.5-MSNs (40 ug /mL) for 1 hour and washed with PBS for 3 times. For the endosomal staining, cells were fixed by incubating in 4% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized by incubating in 0.2% Triton X-100 for 5 min. Then cells were rinsed gently in PBS with four changes over 5 minutes. Samples were incubated for 1 hour at room temperature with a primary antibody 1: 500 monoclonal mouse anti-EEA1 antibody (610456, BD Bioscience). washed with 3×1 ml PBS, and then incubated at room temperature for 1 h with 1: 500 dilutions of AlexaFluor 594 goat anti-mouse IgG (H+L) as a secondary antibody. Samples were washed with 3×1 ml PBS. The images were acquired by confocal laser scanning microscope LSM 800. To study the uptake mechanism, the energy dependence of cell-particles interaction was assessed at 37 °C and 4 °C respectively. Then cells were washed with PBS for three times before the process for further use.

Endocytosis-mediated photocaged probe Pho-SFuP translocation

HEK293 cells were seeded at 35-mm Petri dish and were cultured in complete DMEM medium for 24 hours at 5% CO₂ and 37 °C. Then cells were incubated with the probe pho-SFuP (300 nM) in fresh medium for 30 min under dark conditions at 37 °C. Then free probe molecules in the medium were removed and 1 mL complete medium containing MSNs (40 µg / mL) was added for further 1 hour incubation. Then cells were washed for three times before the light irradiation with 365 nm laser. Then cells were fixed as above mentioned before the endosomal and nuclei staining. All

images were acquired by confocal laser scanning microscope LSM 800.

5.6 References

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Summary and Perspectives

Proteolysis has increasingly received more attention and many proteases have become the interest of targets in numerous diseases. The research works in this dissertation mainly focused on development various types of reporters for monitoring the biological functions of furin, one of the most important proteases that is implicated in many pathological processes such as cancer and infectious diseases. Since furin-like enzymes are ubiquitously expressed in various kinds of cells and cyclized from trans Golgi to endosome and cell surface, it is essential and important to monitor the furin functions at specific organelle for unraveling its roles in different stages of diseases.

Initially, by taking the advantages of the passive membrane insertion strategies, we designed a small-molecule FRET reporter for specifically visualizing the furin-like enzyme activities on cell surface. Such developed probes with the assistance of certain length of hydrophobic lipid moieties have strong capability for anchoring on the membrane up to two hours. Moreover, such fluorescent reporter was successfully applied in the living tissues by using the two-photon setups. Subsequently, the single cell imaging and relevant physical process was evaluated by wide field microscopy. The preliminary results indicated that the membrane insertion process of the developed probe may occur faster than the furin-mediated hydrolysis.

Based on the previous design, subsequently we want to explore more unique probes which can selectively respond to the tumor microenvironment. A variety of stimuli-responsive strategies have been employed to construct the different probes in response to endogenous or external stimuli. Though screening of ten different

substrates, we finally obtained the light-sensitive probes which contains the caging moiety in the enzyme binding pocket. Such developed probe can effectively block the proteolytic hydrolysis due to the possible steric hindrance. Once the probe is uncaged when exposed to the light irradiation, the active probe will present enhanced fluorescent signals which are induced by the enzyme cleavage.

In order to continuously explore the vital rules of furin in the living cells, we applied the photo-caged probe to monitor the plasma and endosome-associated proteolysis respectively. Especially, we found that our probe can effectively follow the endocytic process of the module target-mesoporous silica nanoparticles. More importantly, the recovered fluorescence provides strong evidence of furin processing in the endosome organelle.

Although we have developed several unique reporters in monitoring the furin-related proteolytic processes, more efforts are required to explore the potential applications in the living animals and clinic phase. Regarding the applications of membrane-associated probe in living animals, the improved photophysical properties of fluorophores are required to get higher deep tissue penetration. Alternative two-photon fluorophores may offer the possible solution. In future, we continue to develop more fluorophores with good biocompatibility, greater cross-sections and resistance to photobleaching for the reporter purpose.

In the efforts to construct various types of stimuli-responsive probes, we would provide more theoretical studies on the orientations and binding energies for these developed probes. Currently, the docking and molecular dynamic simulation (MSD) is

under processing in the laboratory of our collaborators. We believe such theoretical information would shed light on the binding difference and orientations between caged probes and uncaged probes, which would help disclose the detailed interactions between the probe and furin protein and underlying proteolytic mechanism. Meanwhile, more clues would be provided for the other substrates which were unable to be cleaved by furin enzyme. Such clues would greatly help us to design more sensitive probes as well as potential reversible inhibitors.

Since we already successfully tracked the endocytic pathways of the nanoparticles, it is also possible to explore the internalization pathway of many other proteins and even virus by using such developed probes. The pioneering studies present that the endopeptidase furin involve a lot of sorting and trafficking process. It would be high of significance by using the simpler and more direct method to monitor such pivotal biological phenomenon. More importantly, understanding of the regulation of furin activity would greatly help to provide insight on the disease progresses and facilitate the development of more safe and precise therapy

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