Tailoring Fluorescent Probes for Organelle-Specific Imaging and Sensing

Xin Zhang

Mitochondrion

Lipid Droplet

Lysosome

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**Tailoring Fluorescent Probes for Organelle-
Specific Imaging and Sensing**

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During the course of the research underlying this thesis, Xin Zhang was enrolled in *Forum Scientium*, a multidisciplinary doctoral program at Linköping University, Sweden.

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Cover, fluorescent probes for subcellular organelle imaging and sensing, designed by Xin Zhang

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Abstract

Exploring and visualizing biological and pathological processes in living cells are useful for both fundamental research and applications. An understanding on the molecular level for these processes with focus on tracking biomarkers, collecting information about their surroundings, and uncovering essential molecular pathways and functions in live cells are of vital interest for cell biological study and clinical diagnosis application. Fluorescent imaging technologies have become essential tools in cell biology studies, providing reliable information about the localization and quantification of the analytes in living systems that we cannot see by our naked eyes. Since the discovery of organic fluorescent dyes, unremitting efforts have been made to visualize the behaviors of specific targets by using these fluorophores as labels. Numerous of probes and nanoprobe have been developed for specific targeting and sensing. However, the current probes and nanoprobe still show some inherent deficiencies, such as poor specificity, strong photobleaching, high toxicity, low signal-to-noise ratio, poor cell membrane penetration, etc. To conquer these limitations, this thesis will focus on developing fluorescent platforms for bioimaging and biosensing with improved sensitivity, selectivity, specificity, and stability.

A red-emitting fluorescent probe is first proposed that not only tracks the dynamic changes in real-time, during migration and fusion of lipid droplets but also monitors starvation-induced lipophagy (**Paper I**). In addition to organic fluorophores, hybrid fluorescent silica nanoparticles (SiNPs) are very promising for bioimaging/sensing owing to the advantages of low toxicity, high biocompatibility, multifunctionality, hydrophilicity and accessible surface functionality. Nonetheless, to apply SiNPs for such purposes, it is mandatory to address common problems of poor cell penetration and lack of specificity. Therefore, in this thesis, an efficient membrane-penetration SiNP is tailored with the intention to enable

subcellular imaging/sensing. The proposed SiNPs are characterized by rapid cellular uptake (< 30 min) and specific subcellular targeting capabilities with surface modification. Finally, real-time tracking of dynamic changes in mitochondria and lysosomes during autophagy process is successfully performed (**Paper II**). Through the rational design of new functionalities on the established SiNPs, mitochondria- and lysosome-specific pH nanoprobe are further tailored for real-time monitoring of pH variations under various stimuli (**Paper III** and **IV**). Another ratiometric nanoprobe is developed for quantitative indication of lysosomal adenosine 5'-triphosphate (ATP) levels in living cells. The nanoprobe enables a deeper understanding of the interplay between energy metabolism and autophagy (**Paper V**).

In conclusion, throughout the studies in this thesis, the fabrication and utilization of fluorescent molecular probes and SiNP-based nanoprobe for cellular probing have been investigated and analyzed. The strategies and the fabricated SiNPs can facilitate our deeper understanding of cellular pathological processes and provide basic knowledge for the development of other functional materials for life science applications.

Keywords: Fluorescent imaging, Ratiometric Nanoprobe, Autophagy, Apoptosis, FRET

Populärvetenskaplig sammanfattning

Att utforska och visualisera biologiska och patologiska processer i levande celler är värdefullt både inom grundforskning och tillämpningar. En förståelse på molekylär nivå för dessa processer, med fokus på att spåra biomarkörer, samla information om deras omgivning och avslöja grundläggande molekylära vägar och funktioner i levande celler är av yttersta vikt för cellbiologiska tillämpningar och klinisk diagnostik. Dessutom har fluorescerande bildtekniker blivit oundgängliga verktyg inom cellbiologi eftersom de ger dynamisk information om lokalisering och kvantitet av analyterna av intresse i levande celler. Ända sedan upptäckten av organiska fluorescerande föreningar i slutet av artonhundratalet har ansträngningar gjorts för att "se" beteendet hos specifika biomolekyler i levande system genom att använda dessa färgämnen som etiketter. Idag har en mängd olika prober och nanoprober utvecklats för att spåra metalljoner, pH, enzymaktiviteter och signalmolekyler. Dagens tillgängliga prober har fortfarande begränsningar, såsom brist på specificitet, stark fotoblekning och toxicitet, höga signal-brusförhållanden, dålig cellmembranpenetration, etc. För att finna vägar att komma förbi dessa begränsningar är fokus i detta avhandlingsarbete. Detta görs genom att utveckla verktyg bestående av fluorescerande prober för bioimaging och biosensing med förbättrad känslighet, selektivitet, specificitet och stabilitet.

I denna avhandling rapporterar vi i det första arbetet om en rödemitterande fluorogen prob som selektivt kan ackumuleras i lipiddroppar (LD) (**Paper I**). Den uppvisar inte bara ett extremt högt signal/brusförhållande och stort Stokes-skift (upp till 214 nm) utan har också utmärkt motståndskraft mot fotoblekning. Det har därför tillämpats för spårning i realtid av intracellulära LD-processer, inklusive fusions-, migrations- och lipofagiprocesser. Den utvecklade proben erbjuder här nya möjligheter för att följa LD-associerade

processer, så som deras roll och mekanismer i cellulär metabolism och också studier av övergången mellan adaptiv lipidlagring och lipotoxicitet. Till skillnad från organiska fluorofores har fluorescerande nanopartiklar (FNPs) flera fördelarna såsom låg toxicitet, hög biokompatibilitet, mångsidighet, hydrofilicitet och tillgängliga ytfunktioner som gör dem till lovande verktyg för bioavbildning. Vårt nästa steg var att utveckla nya FNPs avsedda för avbildning på subcellulär nivå. Först utvecklade vi en skräddarsydd membranpenetrerande nanopartikel som lämpar sig för subcellulär inriktning och avbildning (**Papper II**). Ytfunctionalisering av denna nanopartikel möjliggör egenskaper så som snabbt cellupptag (mindre än 30 min), med fokus på -lysosom och mitokondriell imaging och visualisering. De skräddarsydda FNP används med goda resultat för att spåra autofagiprocessen. Baserat på denna mångsidiga nanoplattform uppnådde vi ratiometrisk fluorogena nanoprober genom att manipulera en fluorescensresonans energiöverföring genom rationella konstruktioner och optimeringar. De lysosom- och mitokondrie-specifikt riktade nanoproberna med anpassade pH-avkänningssenheter kan möjliggöra kvantitativ övervakning av lysosomalt och mitokondriellt pH (**Papper III** och **IV**) under flera olika biologiska processer i levande celler. På samma nanoplattform utvecklades en ratiometrisk prob för att övervaka nivån av lysosomalt adenosine 5'-triphosphate i levande celler (**Papper V**).

Sammanfattningsvis har fluorescerande molekylära nanoprober designats för cellulära avbildningstekniker. Under hela detta avhandlingsarbete har dessa nanoprober utvecklats, undersökts och analyserats i detalj. Dessa prober och metoden för att utveckla dem kan underlätta vår förståelse av normala och patologiska cellulära processer och det kan ge en teoretisk grund för utvecklingen av andra funktionella material för life science -applikationer.

Nyckelord: Fluorescerande avbildning, Ratiometric Nanoprobe, Autophagy, Apoptosis, FRET

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Researching is a big task, so it can be overwhelming to know where to start. Being with so many excellent people from Molecular Surface Physics and Nanoscience (MOLYT) and IFM would enable me to make a quick transition into being an independent researcher with the ability to deal with whatever challenges I encounter in my career. It also gives me a strong external motivation, a broad scientific perspective and creativity, as well as a scientific mind. Numerous people I have met have affected me. I would like to express my greatest gratitude and appreciation to some particular people.

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A handwritten signature in black ink, reading 'Xin Zhang'. The signature is stylized, with the first name 'Xin' and the last name 'Zhang' clearly legible.

2021 October, at Linköping

Papers Included in the Thesis

Paper I

Light-Up Lipid Droplets Dynamic Behaviors Using a Red-Emitting Fluorogenic Probe

X. Zhang, L. Yuan, J. Jiang, J. Hu, A. Du Rietz, H. Cao, R. Zhang, X. Tian, F. Zhang, Y. Ma, Z. Zhang, K. Uvdal, Z. Hu.

Anal. Chem. **2020**, 92 (5), 3613-3619.

Paper II

Tailorable Membrane-Penetrating Nanoplatfrom for Highly Efficient Organelle-Specific Localization

X. Zhang, C. Wang, G. Feng, J. Jiang, J. Hu, A. Du Rietz, C. Brommesson, X. Zhang, Y. Ma, K. Roberg, F. Zhang, H. Shen, K. Uvdal, Z. Hu.

Small, **2021**, 17, 110114.

Paper III

A Ratiometric Fluorogenic Nanoprobe for Real-Time Quantitative Monitoring of Lysosomal pH

X. Zhang, A. Du Rietz, J. Hu, C. Brommesson, X. Wu, K. Uvdal, Z. Hu.

Sens. Actuators, B, **2021**, 130350.

Paper IV

Ratiometric Tracking of Mitophagy by a Broad pH-Responsive Mitochondria-Specific Fluorogenic Nanoprobe

X. Zhang, J. Chen, J. Hu, A. du Rietz, X. Wu, Z. Zhang, R. Zhang, K. Uvdal, Z. Hu.

In Manuscript.

Paper V

Real-time Monitoring of Lysosomal Adenosine Triphosphate in Autophagy Process

X. Zhang, A. Du Rietz, C. Wang, J. Hu, C. Brommesson, X. Zhang, X. Wu, K. Uvdal, Z. Hu.

In Manuscript.

Author contribution

Paper I: **X. Zhang** participated in the planning of the project; performed the experimental works, and data analysis, except for the molecule synthesis and PLIN5-GFP protein imaging study; was responsible for the manuscript draft, writing, review & editing.

Paper II: **X. Zhang** participated in the planning of the project; performed the fabrication and characterization of the nanoprobe, the photophysical properties studies, co-localization experiments, and data analysis, except for western blot, autophagy and mitophagy imaging experiments; was responsible for the manuscript draft, writing, review & editing.

Paper III: **X. Zhang** participated in the planning of the project; performed the design and fabrication of the nanoprobe, properties studies, cellular imaging experiments, and all data analysis; was responsible for the manuscript draft, writing, review & editing.

Paper IV: **X. Zhang** planned the project; performed the fabrication of the nanoprobe, properties studies, cellular imaging studies, and all data analysis, except for western blot experiments; wrote the manuscript draft.

Paper V: **X. Zhang** planned the project; performed the design and fabrication of the nanoprobe, the studies of properties, cellular imaging studies, and data analysis, except for western blot experiments; wrote the manuscript draft.

Papers Not Included in the Thesis

Paper I

Ratiometric Fluorogenic Determination of Endogenous Hypochlorous Acid in Living Cells.

J. Hu, **X. Zhang**, T. Liu, H. Gao, S. Lu, K. Uvdal, Z. Hu.

Spectrochim. Acta, Part A, **2019**, 219, 232-239.

Paper II

Rapid Detection of Mercury (II) Ions and Water Content by a New Rhodamine B-based Fluorescent Chemosensor

J. Hu, X. Yu, **X. Zhang**, C. Jing, T. Liu, X. Hu, S. Lu, K. Uvdal, H. Gao, Z. Hu.

Spectrochim. Acta, Part A, **2020**, 241, 118657.

Paper III

Real-time Tracking of Mitochondrial Dynamics by a Dual-sensitive Probe

H. Wang, J. Hu, G. Yang, **X. Zhang**, R. Zhang, K. Uvdal, Z. Zhang, X. Wu, Z. Hu.

Sens. Actuators, B, **2020**, 320, 128418.

Paper IV

Selective Colorimetric Detection of Copper (II) by a Protein-based Nanoprobe

J. Hu, L. Wang, **X. Zhang**, W. Yu, H. Gao, N. Solin, Z. Hu, K. Uvdal.

Spectrochim. Acta, Part A, **2021**, 252, 119462.

Paper V

Impact of Amine Additives on Perovskite Precursor Aging: A Case Study of Light-Emitting Diodes

Y. Xu, W. Xu, Z. Hu, J. A. Steele, Y. Wang, R. Zhang, G. Zheng, H. Wang, **X. Zhang**, E. Solano, M. B. J. Roeffaers, K. Uvdal, J. Qing, W. Zhang, and F. Gao.

J. Phys. Chem. Lett. **2021**, 12, 5836-5843.

Author contribution. For these papers not included in the thesis, **X. Zhang** contributed to partial experimental characterization and data analysis.

List of Abbreviations

Abbreviation	Full form
TPA	Triphenylamine
LDs	Lipid droplets
D	Donor
A	Acceptor
S/N	Signal to noise
SiNPs	Silica-based nanoparticles
SiNP	Silica-based nanoparticle
TEOS	Tetraethyl orthosilicate
PEO	Ethylene oxide
PPO	Propylene oxide
AIE	Aggregation-induced emission
ACQ	Aggregation-caused quenching
APTES	(3-aminopropyl) triethoxysilane
EDC	1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide
NHS	N-hydroxysuccinimide
TPP	Triphenylphosphonium
MP	Morpholine
CPCDs	Cell-penetration cyclic disulfides
CDs	Cyclic disulfides
ICT	Intramolecular charge transfer
TICT	Twist intramolecular charge transfer
PET	Photo-induced electron transfer
FRET	Förster resonance energy transfer
TPE-OMe	1,2-Bis(4-methoxyphenyl)-1,2-diphenylethene
HPS	1,1,2,3,4,5-hexaphenyl-1H-silole
ATP	Adenosine triphosphate

TEM	Transmission electron microscopy
DLS	Dynamic light scattering
Uv-Vis	Ultraviolet and visible spectrophotometry
FRC	Fluorescein
RDM	Rhodamine B
LMP	Lysosomal membrane permeability
Rr	The Pearson's colocalization coefficients
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
3-MA	3-methyladenine
CQ	Chloroquine
BafA1	Bafilomycin A1
NEM	N-Ethylmaleimide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
MDR	Mitotracker Deep Red
LDR	Lysotracker Deep Red
GSH	Glutathione

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Chapter 1. Introduction

It has been said that humans rely on vision for around approximately 80 % of all the information received from the external world.^[1] Therefore, as the saying “seeing is believing” suggests, even in the scientific world, visual information is the most effective and informative way. However, there is not directly detectable visual information in living systems to observe specific biological events, *e.g.*, cell interactions and signaling. In general, cells contain multitudinous biomolecules whose concentrations, modifications, and interactions are precisely regulated, both spatially and temporally. One of the ultimate goals of biology is to decipher the complex system of interacting molecules inside and among the cells. Tools that allow us to observe the dynamics of cells are becoming therefore essential. For this reason, scientists must rely on special techniques to visualize the behavior of the molecules of interest, as most biomolecules lack distinguishable characteristics allowing the differentiation from the others by simple optical observations.

Such a technique must meet the following general merits: 1) be sensitive enough to observe biological substrates and events at physiological concentrations, which in most cases range from nanomolar to micromolar; 2) have sufficient spatial and temporal resolution to analyze the dynamic processes; 3) be reliable and not invasive; 4) being best, not rely on too special or expensive instruments.

There is no doubt that the fluorescence imaging technique stands out from biomedical imaging technologies based on radioactivity, bioluminescence, electromagnetism, and electrochemistry.^[2] It benefits from high sensitivity, real-time mode, facile accessibility, and excellent spatiotemporal resolution. We should also emphasize here that another advantage of this technique is that the fluorescence signal of the probe can be flexibly modulated by using

proper mechanism so that probes can be utilized relying on the target-‘activation’, not just accumulation.^[3] The fluorescent probes including molecular fluorophores, fluorescent proteins, nanoparticles, and other fluorescent nanocomposites have recently been developed and widely used. They generally display favorable properties, such as strong brightness, high photostability, good biocompatibility, etc., and can track and quantify specific molecules, as well as biochemical reactions within cells or light up intracellular milieu by means of fluorescence imaging technique.^[4]

This thesis, therefore, focuses on the development of organic small molecular probes and hybrid fluorescent silica-based nanoparticles (SiNPs) for intended cellular imaging and sensing. It has 6 chapters. the fundamental knowledge of molecules and nanoparticles-based fluorescent imaging and sensing, especially the general principles, and guidelines for the construction of SiNPs are presented in **Chapter 2**. A systematic summary of the synthesis, encapsulation, and surface modification strategies of SiNPs, as well as the sensing mechanisms, fabrication methods of ratiometric nanoprobe are displayed in detail. A brief introduction of the basics of autophagy and apoptosis, probes and design strategies used to track these processes are given in **Chapter 3**. The related methodologies are discussed in **Chapter 4**. The summaries of the included papers are listed in **Chapter 5**. The conclusions and outlook of fluorescent probes are discussed in **Chapter 6**.

Chapter 2. Fluorescent Imaging and Sensing

Fluorescent imaging and sensing are essential for tracking biological events and visualizing dynamic details in living cells. This is because they offer considerable advantages over other methods, including high sensitivity and selectivity, great spatial and temporal sampling capability, easy operation, and low cell invasivity, etc.^[5] They are widely applied to unravel the molecular mechanisms, underlying the cells to better understand biological functions and dysfunctions. Over the past decades, the development of molecule and nanoparticle-based fluorescent probes has facilitated significant advances in cell biology studies.^[6, 7]

2.1 Molecule-based fluorescent imaging and sensing

Figure 2.1 Representative common fluorophores that are applied for fabricating fluorescent probes, sorted by structure and emission color.

Fluorescent imaging methods based on synthetic small molecules are frequently used for real-time observation of biological events in live cells, due to their outstanding features, including smaller size, greater emissivity,

and a broad accessible spectrum range.^[8, 9] Many fluorophore and chromophore probes have been well designed and used exactly for this purpose (**Figure 2.1**). Rhodamine-derived dyes, for example, have aroused great attention due to their outstanding photophysical properties including high absorption coefficient, fluorescence in the visible spectrum region, high fluorescence quantum yield, and good photostability.^[10] However, some inherent limitations in the photophysical properties of these common organic fluorophores hinder the full potential for fluorescent imaging and sensing, such as weak fluorescence intensity, very small Stokes shift, poor water solubility, lack of subcellular specificity, high toxicity, etc.^[11] In particular, for biomedical imaging and sensing, probes emitting in the long-wavelength region, i.e. red- and near-infrared (NIR) are preferred over the short emission wavelength probes since fluorescence in the long-wavelength region would result in the least phototoxicity, the deepest tissue penetration, and the smallest background interference, rendering them suitable for imaging *in vivo*.^[12] Therefore, an ideal fluorescent probe should demand the requirements mentioned above.

In **Paper I**, a red-emitting probe, TPA-LD, is proposed for the dynamic lipid droplets (LDs) behaviors study.^[13] The probe can be selectively trapped into the waterless core of LDs through hydrophobic interactions (**Figure 2.2 a**). As a result of the donor (D)–acceptor (A) architecture and molecular single bond rotation, TPA-LD gives low fluorescence in a high-polarity medium but switches to strong fluorescent once it is incorporated into the core of LDs, certainly providing a high signal to the background ratio. Combined with other properties, including a large Stokes shift, as well as low toxicity and improved photostability compared to the commercial probe BODIPYTM 493/503 (Thermo Fisher) (**Figure 2.2 b-c**), making TPA-LD a promising candidate for real-time and long-term dynamic monitoring of LDs-associated processes *in vitro* and *in vivo* (**Figure 2.2 d-f**).

Figure 2.2 (a) Structure of TPA-LD. (b) Comparison of cell viabilities of TPA-LD and commercial tracker BODIPY 493/503. (c) Comparison of photostability of TPA-LD and BODIPY 493/503 in HeLa Cells. (d) Fluorescence image of HeLa cells stained only with TPA-LD. (e) Fluorescence image of HeLa cells stained only with BODIPY 493/503. (f) *In vivo* imaging of LDs in a zebrafish model. (a from the section of Scheme 1; b, c from Figure S6; d and e from Figure 2; f from Figure 6, in **Paper I**).

2.2 SiNPs-based fluorescent imaging and sensing

Recently, an increasing number of molecular mechanisms strongly correlated to underlying disease have been successfully revealed.^[14] As a result, clinical diagnosis is coming to rely on the monitoring of individual chemical interactions of smaller targets and specific events in living cells.^[15] To this end, fluorescent nanoparticles stand out for their enhanced fluorescence signal, higher selectivity and sensitivity, as well as ease of surface modification, thus showing great potential for cell biology analysis.^[16, 17] In particular, fluorescent silica-based nanoparticles (SiNPs) are being extensively applied mostly owing to the following features:

- (1) SiNPs with hydrophilic nature, easy to produce, synthesize and purify.^[18]
- (2) SiNPs with light transparency allow for the pass-through of excitation and emission lights.
- (3) SiNPs with biocompatibility and non-toxic degradability cause minimal damage to living cells.^[19]
- (4) SiNPs with specific surface area allow for the surface decoration with various functional groups, such as carboxyl (-COOH), amine (-NH₂), azide (-N₃), and phosphonate (-CH₃HPO₂), and the decoration does not cause obvious cytotoxicity (**Figure 2.5 d**).^[20]
- (5) SiNPs with cross-linked silica matrix shells allow for a wide range of dyes to be encapsulated without altering the optical properties but adding extra properties.^[21]
- (6) SiNPs with controlled nano size are feasible for bioimaging and sensing.

2.2.1 Fabrication of fluorescent SiNPs

1. Synthesis

The synthesis of SiNPs in solution is usually carried out with the sol-gel process, which involves acid/base-catalyzed hydrolysis and condensation of the alkoxide precursors. Currently, the most widely used synthetic

approaches can be divided into three categories, the Stöber method^[22], the reverse microemulsion method^[23-25], and the direct micelle-assisted method.^[26] In all situations, tetraethyl orthosilicate (TEOS) is commonly applied as a silica precursor, offering simplicity, low costs, versatility, and controllable size (**Figure 2.3**). Recently, the direct micelle-assisted method has been demonstrated as a very efficient way to achieve hybrid fluorescent SiNPs for imaging and sensing purposes, where two main common methods are often used to achieve fluorescence properties: 1) encapsulation of fluorophores/dyes into the nanoparticles through molecular stacking; 2) modification of fluorophores/dyes with trialkoxysilanes, which can be further covalently bonded to the silica matrix through hydrolysis of trialkoxysilane (**Figure 2.4**).^[27]

Hydrolysis:

Condensation:

Figure 2.3 Schematic diagram of TEOS hydrolysis and condensation reactions.

Commercially available Pluronic 127, a visible light transparent and amphiphilic tri-block copolymer consisting of poly (ethylene oxide) (PEO) - poly (propylene oxide) (PPO) - (ethylene oxide) (PEO), is commonly utilized as soft micelle template to achieve SiNPs. The hydrophobic core of the formed micelle then allows the lipophilic TEOS used as a silica precursor.^[26, 28] As a result, rigid SiNPs are created based on self-assembly of the F127, and hydrolysis/condensation of TEOS at hydrophobic/hydrophilic interface

in the micelles in acidic solutions (**Figure 2.4**). The hydrophilic PEGylation moieties are oriented outwards from the SiNPs surface, which can effectively prevent aggregation of the resulting SiNPs, ultimately forming a monodisperse silica-PEG core-shell architecture. This silica cross-linking strategy exhibits many advantages, such as mild reaction condition; relative quick cross-linking reaction; a cross-linked silica network can efficiently lock the encapsulated dyes/drugs minimizing their leakage; not affected by the harsh external environments; the presence of a high density of reactive sites on the surface allows for additional modifications.^[29] Most importantly, SiNPs from the direct micelle-assisted method exhibits very good monodispersity and biocompatibility, as well as not subject to microbial attack, making them very suitable for biological imaging and sensing.^[30, 31]

Figure 2.4 Representation of direct micelle-assisted method to obtain hybrid fluorescent SiNPs. (a) Encapsulation of hydrophobic dyes. (b) Covalently incorporation of trialkoxysilane-modified dyes onto the silica matrix. (c) Combination of the methods described in (a) and (b).

Figure 2.5 (a) A scheme showing the structure of fluorophore DTPA-based SiNPs (DTPA-NPs) generated by encapsulation strategy described in Figure 2.4 a. (b) AIE characteristics of DTPA studied by UV-Vis absorption spectra (dashed line) and fluorescence spectra (solid line) in THF/water mixtures. (c) Normalized absorption intensity (dashed line) and emission intensity (solid line) of DTPA-NPs. (d) Cytotoxicity of various surface-modified DTPA-NPs in HeLa cells. (a-c from the section of Figure 1; d from Figure 2; in **Paper II**).

As mentioned, one of the main convenient approaches to obtain fluorescent SiNPs is to introduce fluorescent components into SiNPs by "physical doping" (**Figure 2.4**).^[32] Since the amphiphilic SiNPs are able to dope dyes with lipophilic properties in the central hydrophobic core domain. Ideally, the brightness of dye-doped SiNPs would be proportional to the amount of dye molecules encapsulated. However, π -conjugated fluorophores are tend to aggregate, which usually results in quenching fluorescence intensity by

well-known photophysical phenomena, aggregation-caused quenching (ACQ).^[33] Obviously, the ACQ effect is an obstacle in the development of such fluorescent SiNPs. Therefore, today, the most compelling example is to incorporate aggregation-induced emission (AIE) luminogens (AIEgens) to enhance fluorescence contrast for imaging and sensing. The AIE phenomenon, where the fluorescence of organic dyes increased as they aggregate, can effectively avoid ACQ effects.^[34] The encapsulation of AIEgens inside SiNPs opens up the radiative channel and results in strong fluorescence.^[35] Furthermore, the resulting fluorescent SiNPs exhibit high resistance to photobleaching, which can facilitate the long-term tracking of dynamic biological processes.^[36] By using this strategy, we developed highly red-emitting SiNPs through encapsulating an AIEgen of DTPA (**Figure 2.5**).^[28] The resulting SiNPs exhibit bright red fluorescence, small size (≈ 50 nm), high chemical stability, good photostability, and biocompatibility as well as high cell-retainability. After essential surface modifications, SiNPs could provide a versatile and promising nanosystem for improved cell imaging and sensing.

Another approach is to covalently link the fluorescent dyes/probes to the silica matrix through hydrolysis and condensation of the trialkoxysilane group (**Figure 2.4 b**).^[37] Technically, this method demonstrates an improved ability to reduce the leaching risk of the fluorescent components from SiNPs in the complicated biological system, compared to the "physical doping" method. The first task of this method is to derive the fluorophores with the trialkoxysilanes group. Afterward, the fluorophores can be immobilized into the silica matrix of SiNPs. Of course, multiple fluorophores could be installed into a single nanoparticle simultaneously depending on the application. For instance, in **Paper IV**, two acidic and basic pH-responsive spectra-distinguishable fluorophores (rhodamine B and fluorescein) are individually fabricated and then derived with trialkoxysilane. Their immobilizations into SiNPs enable the achievement of a broad range of pH-

responsive nanoprobe with fluorescence in dual-color that are very useful for tracking the pH variations during mitophagy (**Figure 2.6**).

Figure 2.6 Schematic incorporation of two trialkoxysilanes-modified dyes into a single SiNP, and its pH-sensing mechanism. (Figure 2.6 from the section of Figure 2, in **Paper IV**).

In addition, depending on the application requirements, the above two main approaches could be rationally combined to deliver fluorescent SiNPs (**Figure 2.4 c**). For instance, in **Paper III** and **V**, we have fabricated ratiometric fluorogenic nanoprobe by this combination. Briefly, hydrophobic AIEgens are physically doped in the core of SiNPs, and sensitive fluorophores are covalently linked onto the silica matrix of SiNPs. The details are presented in section 2.2.3 *SiNPs-based fluorescent sensing*.

2. Surface modification

The practicability of SiNPs in biological applications has been even further extended by surface modification due to their high surface-to-volume ratio. Various functional groups ($-\text{COOH}$, $-\text{NH}_2$, $-\text{N}_3$ and $-\text{SH}$)-based trialkoxysilanes could be used to incorporate these reactive groups onto silica matrix layers, which can provide reactive sites for the surface modification of SiNPs.^[38, 39] For instance, to provide surface amino groups, (3-aminopropyl) triethoxysilane (APTES) could be used, and to stimulate the amine-carboxy coupling and allow the production of extended ligands, the

classical covalent coupling agents, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), could be applied for this purpose.^[40] In **Paper II**, this strategy has been applied to provide the SiNPs with extra functionalities. Firstly, through hydrolysis and condensation of triethoxysilane, APTES can provide the reactive amine sites on the surface of the tailored SiNPs. It can be followed by EDC/NHS coupling with the specific chemical moieties including subcellular targeting ligands triphenylphosphonium (TPP) and morpholinyl (MP), cell-penetration cyclic disulfides (CPCDs), endowing the SiNPs with additional functionalities (**Figure 2.7**).^[41, 42]

Figure 2.7 Surface modification of amino groups on SiNPs through condensation of APTES, and further functionalization through EDC/NHS coupling reaction of specific chemical moieties.

2.2.2 SiNPs-based fluorescent imaging

So far, many fluorescent SiNPs have been strategized for bioimaging and bioanalysis purposes.^[43] With ingenious and innovative design, fluorescent SiNPs with emission bands at various wavelengths, enhanced emission intensity, and smaller size can be constructed with non-demanding synthetic efforts. It has been demonstrated that SiNPs are promising in the field of effective real-time intracellular imaging and biomedical diagnostic applications. As a matter of fact, novel SiNPs have driven unprecedented bioimaging and biosensing prospects that were unimaginable a decade ago. There is no doubt that the advent of fluorescent SiNPs has contributed to our

understanding of the complex physiological processes in biological systems.^[26]

Figure 2.8 Fluorescence images of starvation-induced autophagy in HeLa cells stained with two tailored spectra-distinguishable SiNPs (DTPA-MP-CDs and TPE-TPP-CDs). Scale bar = 10 μm . (Figure 2.8 from the section of Figure 5, in **Paper II**).

In this thesis, a tailorable membrane-penetrating SiNP is established to enable highly efficient organelle-specific targeting, especially for mitochondria and lysosomes. With rational modulation of functionalities in a single nanoparticle, the resulting SiNPs can be delivered to subcellular organelles for biological activity studies, *e.g.*, the dynamics of related subcellular organelles during autophagy (**Figure 2.8, Paper II**), pH variations during cell activities at subcellular levels (**Paper III** and **IV**), the correlation between lysosomal autophagy and energy metabolism (**Paper V**), etc.

2.2.3 SiNPs-based fluorescent sensing

Exploring and understanding the biomolecular changes in biology and pathology are crucial prerequisites for the early diagnosis and treatment of diseases, as well as for fundamental biology and medical study.^[44, 45] Fluorescent SiNPs-based nanoprobes have also been widely utilized in this field.^[46] It is demonstrated that SiNPs are not only possible to track distributions of biologically relevant molecules in cells, but also to give various fluorescent signal readouts depending on the fluctuations of targets, allowing to accurately visualize these changes.^[47] SiNPs-based nanosensors have numerous advantages as an analytical tool with great potentials, such as enhanced sensitivity and selectivity, improved water solubility, higher targeting ability, etc.^[48] Additionally, the surface modification can further offer extra functions to meet individual requirements as mentioned above. However, modulation of sensing fluorescent signals in a single particle is still a challenging work. For instance, intensity-dependent (“turn-on” or “turn-off”) probes/nanoprobes may result in inaccurate analysis results owing to various analyte-independent factors such as instrumental parameters, microenvironments, probe concentrations, and photobleaching (**Figure 2.9 a-b**).^[49]

It has been known that the ratiometric measurement is a perfect solution to address the drawbacks associated with the intensity-based one. It relies on the variations in the intensity ratio of two or more emission bands caused by analytes.^[50] The ratio is directly related to the concentration of the target analyte, and therefore provides built-in self-calibration for signal correction, giving more accurate and trustable outcomes (**Figure 2.9 c-d**).^[51] SiNPs are recognized as powerful scaffolds to obtain the ratiometric fluorogenic nanoprobes, as this nanostructure can effectively isolate donor and acceptor in the core and shell, thereby eliminating serious self-quenching caused by the overpopulation of dye molecules. On the contrary, donor and acceptor can be easily incorporated into a single particle and the ratio can be fairly tuned by adjusting the doping amount of the two components.^[52] Rational

designs of SiNPs are of course needed to obtain such ratiometric nanoprobe that are more sensitive and reliable, resulting in the precise detection of analytes.

Figure 2.9 Schematic spectra of “turn-on” (a), “turn-off” (b), and ratiometric probes (c) upon the addition of analytes. (d) The relationship between the intensity ratio from the two wavelengths (I_{W2}/I_{W1}) obtained from panel c vs the variations of the analyte.

The most commonly sensing mechanisms currently used for the ratiometric probes/nanoprobes are based on intramolecular charge transfer (ICT)^[53], photo-induced electron transfer (PET)^[54], and Förster resonance energy transfer (FRET)^[55], of which FERT is the most popular strategy for constructing ratiometric nanoprobe.^[56] Herein, only a brief description of the FRET mechanism used in this thesis will be given here. If two or more fluorescent units are very close together, fluorescent energy can be

transferred between them. This is the basic principle behind FRET, which has been extensively used to construct fluorescent sensing nanoprobe. FRET is a process that describes non-radiative energy transfer between the donor-acceptor pair through long-range dipole-dipole interactions. During the excitation, the donor fluorophore absorbs the photon and transitions from the ground energy level (S_0) to the excited energy level (S_1) (blue line). If they jump back to give fluorescence of donor, there is no FRET process (green line). When FRET occurs, the energy will be absorbed and transferred to the acceptor fluorophore, which emits fluorescence from the acceptor as it returns to the ground state (orange line) (**Figure 2.10 a**).

Figure 2.10 Schematic representation of FRET sensing mechanisms. (a) Jablonski diagram for the principle of FRET. (b) Schematic diagram of the basic FRET system composed of donor and acceptor. (c) Schematic representation of the spectral overlap required between donor and acceptor. (Inspired by ref. ^[56])

Figure 2.11 Diagrammatic representation of three common strategies of FRET-based sensing systems. (a) Analyte-induced reaction at the acceptor, switching on the FRET. (b) Analyte-induced reaction at the receptor, switching off the FRET. (c) Analyte-induced cleavage of the spacer, switching off the FRET. (Inspired by ref. ^[60])

Generally, three prerequisites are required for constructing the FRET process: 1) distance, donor-receptor pairs should be in close proximity to each other (< 10 nm) (**Figure 2.10 b**); 2) spectral overlap, the absorption spectrum of the acceptor and the emission spectrum of the donor should have a certain overlap (**Figure 2.10 c**); 3) orientation, the donor and acceptor must be in the correct orientation.^[58] Compared to PET and ICT, the FRET process is widely used in the development of ratiometric fluorescent nanoprobes.^[59]

There are three main common designs used to manufacture ratiometric fluorescent nanosystems. One method is to design a weak/non-fluorescent acceptor, whose fluorescence could be selectively restored by the analyte of interest, consequently switching on the FRET to give ratiometric readouts (**Figure 2.11 a**). On the contrary, design a fluorescent acceptor, whose fluorescence could be selectively suppressed by the analyte of interest, then switching off the FRET (**Figure 2.11 b**). Another strategy is to design a reactive spacer between FRET donor and acceptor. The third is that analyte-induced selective cleavage will switch off the FRET by destructing the proper distance between donor and acceptor (**Figure 2.11 c**).^[60]

Figure 2.12 (a) Schematic illustration of the structure of SiNPs-based nanoprobe TR-MP for pH sensing, and its ratiometric responsive mechanism to pH. (b) Ratiometric fluorescent intensity changes of TR-MP towards pH from 3.0 to 9.0. (c) Schematic illustration of the structure of SiNPs-based nanoprobe HR-MP for ATP sensing, and its ratiometric responsive mechanism to ATP. (d) Ratiometric fluorescent intensity changes of HR-MP toward ATP variations. (a, b from the section of Figure 2, in **Paper III**; d from Figure 2; in **Paper V**).

Based on these principles, many SiNPs-based ratiometric nanoprobe have been elaborated for intended sensing, imaging, and biomedical purposes.^[61-63] In this thesis work, based on our established fluorescent SiNPs scaffold, we tailored several ratiometric nanoprobe for subcellular detections. For instance, a ratiometric nanoprobe for lysosomal pH sensing is presented in **Figure 2.12 a**, where hydrophobic AIEgen TPE-OMe is encapsulated in the core of SiNPs as a donor, acidic pH-sensitive fluorophore as an acceptor is attached to the silica matrix layer of SiNPs to minimize their distance, thereby increasing the energy transfer efficiency.

There are no spectra overlap between the donor and acceptor at basic pH, FRET does not occur, giving fluorescence of the donor. Once the pH turns acidic, an efficient energy transfer happens, therefore gradually giving a fluorescence of the acceptor (**Figure 2.11 a**). The ratio of such two emission bands then can indicate the pH variations and visualize the intracellular pH fluctuations (**Figure 2.12 b**). With a similar design, we also tailored a ratiometric nanoprobe for ATP sensing. The main difference is that an ATP-sensitive fluorophore is fabricated and grafted to the surface of SiNPs instead of the pH-sensitive one (**Figure 2.12 c**). The fabricated nanoprobe allows for selective, rapid, and quantitative detection of ATP changes in living cells (**Figure 2.12 d**).

2.3 CDs-assisted intracellular internalization

Normally, hydrophilic nanoparticles have difficulty penetrating cell membrane and often require long-time incubation.^[64] In addition, the entry path of larger species such as nanoparticles is through endocytosis, and yet they are usually trapped in endosomes and later delivered to the lysosome for degradation without being released into the cytosol.^[65] This significantly limits the high-efficient and precise delivery of nanomaterials for biological application. To date, several methods including grafting cell-penetrating peptides (CPP)^[66], proteins^[67] and poly(disulfide)s (CPPDs)^[68] on the surface of the nanoparticles have been identified for their effective intracellular

delivery.^[40] Although CPP-mediated strategies require only a short membrane penetration time, they are still easily trapped in endocytic vesicles.^[69, 70] Antibody-antigen interactions for protein-mediated delivery also show some disadvantages, including cost, complex synthesis, limited shelf life, and immunogenicity.^[69, 70] Recently, dynamic covalent disulfide bond exchange at the cell surface has emerged as an innovative concept to improve the cell penetration of the nanoparticles.^[71, 72] In this thesis, the lipoamido-dPEG₈-acid with a high tension cyclic disulfide (CDs) ring is coupled to the surface amino of SiNPs through EDC/NHS coupling reaction to equip the SiNPs with CDs. When the grafted CDs approached the cell surface, the CDs will have a disulfide exchange with thiols at the cell membrane, then the towed SiNPs are readily transported into the cells, effectively avoiding the endocytosis path (**Figure 2.13 a**).^[73]

Figure 2.13 (a) A scheme showing the cell-penetrating cyclic disulfides (CDs)-promoted cellular uptake of SiNPs. (b) Fluorescence images of HeLa cells treated with DTPA-NPs and DTPA-CDs for 30 min, respectively. Scale bar = 10 μ m. (b from the section of Figures 3 and 4, in **Paper II**).

Cell imaging studies have demonstrated that the cell uptake of pristine SiNPs almost required 24 h. It is unacceptable for bioimaging and biosensing, particularly for tracking subcellular events in real-time. While CDs-grafted SiNPs exhibit much faster cell internalization through the thiol-mediate cell uptake. It is clearly seen in **Figure 2.13 b** that CDs-decorated SiNPs (DTPA-CDs) give more significant fluorescence intensity compared with pristine DTPA-NPs after 30 min of incubation. And more importantly, further studies demonstrated that this efficient cellular association does not affect their subcellular specificity endowed by targeting ligands of TPP and MP.

2.4 Chemical moiety-directed subcellular localization

Studying the precise key events at a specific subcellular organelle is essential for the detection and diagnosis of relevant diseases. Hence, it is indispensable to develop a variety of probes/nanoprobes with organelles specificity to enter the given regions for imaging and analysis. Benefitting from the diverse physicochemical properties of distinct organelles, decoration of the available targeting groups always allows for organelle-specific delivery of probes/nanoprobes. As a result, the analyte of interest in the targeted organelle can be indicated through fluorescent signals.^[74] In this thesis work, three subcellular organelles of mitochondria, lysosomes, and lipid droplets are mainly concerned.

2.4.1 Mitochondria localization.

As cellular powerhouses in eukaryotic cells, mitochondria are involved in many other important metabolic processes, such as cell signaling, storage of calcium ions, apoptosis, and death.^[75] They are the main compartments for aerobic respiration (oxidative phosphorylation) and as a result, this, in turn, gives them highly negative mitochondrial transmembrane potentials.^[76, 77] Inspired by that, numerous positively charged chemicals including triphenylphosphonium (TPP),^[78] positively charged quinoline and pyridine derivatives, dequalinium, rhodamine and cyanine,^[79] and cationic peptides^[80], as well as mitochondrial peptides^[81] can accumulate in the mitochondrial

matrix against their concentration gradient. Generally, TPP-assisted mitochondrial targeting is the most popular method, because it offers significant advantages over other approaches, including the high stability and

Figure 2.14 (a) Schematic illustration of TPP, MP, and hydrophobic moiety-assisted subcellular organelles targeting. (b) Colocalization of the tailored specific probes/nanoprobes of DTPA-TPP, DTPA-MP, and TPA-LD in this thesis with mitochondria, lysosomes, and lipid droplets, respectively. Scale bar = 10 μm . Red channels are from the probes; green channels are from the commercial trackers. (b from the section of Figure 2, in **Paper II** and Figure S8, in **Paper I**).

low reactivity in biological systems, as well as simple synthesis and purification (**Figure 2.14**).^[79] In this thesis work, TPP is then rationally used to localize SiNPs into mitochondrial for the intended sensing and imaging.

2.4.2 Lysosome localization.

As one of the vital organelles, lysosomes have an acidic environment of pH 3.8-5.0 and play a pivotal role in cellular homeostasis and in mediating a variety of physiological processes.^[82] Today, modification of molecules or nanoparticles with lipophilic amines (Morpholine (MP) and N, N-dimethylethylenediamine) are the most frequently adopted method for localizing them into the acidic lysosomal domain. In the case of MP, for example, MP-modified SiNPs can only be protonated ($pK_a \approx 5-6$) in the lysosome, and the protonated MPH^+ cannot cross the membrane into the cytoplasm, which contributes to their selective accumulation in the lysosomes. In this thesis work, MP is then rationally used to direct the tailored fluorescent for lysosomal detections and imaging (**Figure 2.14**).^[83, 84]

2.4.3 Lipid droplet localization.

As one of the highly dynamic organelles, lipid droplets (LDs) are monolayers and act as a reservoir for cholesterol and acyl-glycerol for membrane formation and maintenance of energy homeostasis.^[85] The most significant component in LDs is the hydrophobic core in neutral lipids, so the preferred strategy for lipid droplets targeting is to construct the fluorescent probes with lipophilic properties while minimizing the aggregation quenching effects (**Figure 2.14**).^[86]

Chapter 3. Fluorescent Monitoring of Cellular Dynamics

Fluorescent imaging techniques based on fluorescent probes have been widely explored in recent years to monitor dynamic changes in living cells, including autophagy and apoptosis processes.^[87] They are indispensable tools to unravel the molecular mechanisms underlying these processes.^[88]

3.1 Monitoring autophagy

Figure 3.1 A diagram showing macroautophagy (autophagy) response to the degradation of cytosolic content and organelles in the lysosome.

Macroautophagy (henceforth referred to as autophagy) is a natural, conserved degradation process that happened in all eukaryotic cells, by removing unnecessary or dysfunctional organelles and aggregated proteins through regulatory mechanisms that rely on lysosomes (**Figure 3.1**). It has a critical role in keeping intracellular balance and responding to stress on living cells including starvation, hypoxia, etc. Without a doubt that defects

of this metabolic pathway have been commonly associated with diverse human diseases,^[89] including the earliest stages of cancers, neurodegenerative disorders,^[90] infectious diseases,^[91] and inflammatory bowel disorders.^[92] Monitoring the occurrence and dynamics in autophagy in living cells can therefore provide important guidance for biological research and disease diagnosis.^[93] During autophagy, the microenvironments of subcellular organelles involved in this activity will change owing to the fusion process.^[94] Therefore, to monitor autophagy, fluorescent probes that can track the changes of such microenvironments have to be cleverly customized.^[95, 96]

3.1.1 Tracking lysosomal ATP

Lysosomes have a central role in the autophagic process by providing an acidic environment and lysosomal hydrolases for the degradation of the cargoes that come from the autophagosome (**Figure 3.1**). Tracking changes in the lysosomal microenvironment during autophagy is therefore of importance. So far, many fluorogenic probes have been developed for this aim, *e.g.*, tracking lysosomal pH,^[97] viscosity^[98], polarity^[99] during autophagy. In **Paper V**, a ratiometric nanoprobe is fabricated for tracking lysosomal ATP changes during autophagy. It is well known that autophagy will provide energy for cellular functions under stressful situations.^[100] This process, in turn, necessitates a great amount of energy.^[101] Tracking their exact correlations between autophagy and energy metabolism in detail will give us a deeper understanding of the autophagic process. A ratiometric lysosome-specific nanoprobe is tailored to reach the fluorescent sensing of lysosomal ATP in living cells (**Figure 2.12 c-d** and **Figure 3.2**) (in **Paper V**). The changes of lysosomal ATP during starvation-induced autophagy and drug-induced mitophagy (a selective form of autophagy that degrades mitochondria in a lysosome-dependent manner) are systematically studied. All the results demonstrated that the occurrence of autophagy is critical for the maintenance of lysosomal ATP levels, while mitophagy increases

lysosomal ATP more rapidly by directly replenishing the ATP contained in mitochondria.

Figure 3.2 (a) Lysosomal ATP variations under starvation-induced autophagy. (b) Lysosomal ATP variations under carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP)-induced mitophagy. Summarized data (ratio, F_{red}/F_{green}) for lysosomal ATP changed during autophagy (c) and mitophagy (d). (a-d from the section of Figure 4, in **Paper V**).

3.1.2 Tracking mitochondrial pH

Defective mitochondria, if not removed, will cause oxidative stress and can result in several pathological processes, including aging^[102], apoptosis^[103], and cellular injury^[104]. The removal of damaged mitochondria is through the mitophagy process. Hence, defects in the mitophagy process also underlie a variety of diseases.^[105, 106] Monitoring mitophagy is therefore crucial in order to improve the in-depth understanding of this process, highlighting its physiological and pathological roles, and potentially contributing to the study of new therapies for various age-related diseases. Similarly, for this

purpose, probes that can respond to mitochondrial pH^[107], polarity,^[108] as well as viscosity^[109] have been well developed.

Figure 3.3 (a) Fluorescence images of MCF-7 cells stained with FR-TPP under CCCP treatment. (b) Data represent the pH changes during mitophagy. (c) Rr changes between the red and LDR channels during mitophagy. Scale bar: 10 μ m. (a-c from the section of Figure 4; in **Paper IV**).

In this thesis of **Paper IV**, a broad pH ratiometric nanoprobe is fabricated that can selectively accumulate in mitochondria and be further delivered to lysosomes, giving distinguishable fluorescence signal indication of pH during mitophagy (**Figure 2.6**). The whole autophagic process can be visualized through this nanoprobe. In the beginning, only strong green fluorescence from mitochondria is observed (**Figure 3.3**). After mitophagy is initiated, nanoprobe accumulated in mitochondria will deliver into the lysosomes, and green fluorescence decreased with red fluorescence increased. By utilizing the single nanoprobe, mitochondria, lysosomes, different stages of mitophagy as well as mitochondrial pH changes, could be discriminatively visualized.

Figure 3.4 Monitoring lipophagy by a combination of TPA-LD and Lysotracker Deep Red (LDR) in real-time. (a) Fluorescence images of HeLa cells stained with TPA-LD and LDR under starvation conditions. Scale bar: 7 μm . (b) R_r changes during starvation-induced lipophagy that were calculated from panel a. (c) Average diameter and number of LDs change during starvation-induced lipophagy that were calculated from panel a. (a-c from the section of Figure 5, in **Paper I**).

3.1.3 Tracking dynamics of lipid droplets

Lipophagy has recently been identified, which can selectively digest excessive or impaired LDs in lysosomal areas.^[110] Monitoring the dynamic

changes of lipophagy will give us a better understanding their physiological role, as massive accumulated LDs are highly related to many diseases.^[111, 112] As mentioned in section 2.1 *molecule-based fluorescent imaging and sensing*, we have successfully proposed an red-emitting probe for LDs targeting. Combined with other properties, the probe is successfully applied to track lipid droplet dynamics in real-time, including the occurrence of lipophagy under a starvation model. As shown in **Figure 3.4**, the real-time changes in morphology, size, and number of LDs can be observed by this probe.

3.2 Monitoring apoptosis

Apoptosis is another highly-regulated cell death process that is distinct from autophagy.^[113] The primarily responsible of apoptosis is involved a variety of biological activities, such as cell renewal, hormone-dependent atrophy, development, and function of the immune system, the embryo, and brain.^[114] Hence, abnormal changes in apoptosis may lead to various diseases.^[115] Real-time imaging and study of the apoptotic process, therefore, is highly important for early diagnosis of apoptosis-related diseases, assessment of disease progression and therapeutic efficiency, and also help to screen apoptosis-related drugs.^[116-118] Many fluorogenic probes that have been developed so far to elucidate the apoptosis process based on their specific fluorescence respond to caspase^[114], cysteine^[119], etc^[120].

In **Paper III**, a novel ratiometric nanoprobe (TR-MP) is present for monitoring apoptosis by tracking the lysosomal pH changes in living cells (**Figure 2.12**). The nanoprobe can rapidly target the lysosomes, and give ratiometric behavior toward pH variations, allowing it to quantify dexamethasone-induced lysosomal pH changes (**Figure 3.5**). The results revealed that the lysosomal pH tended to decrease over time during this process, which is consistent as reported, as lysosomal membrane permeability (LMP) increases during apoptosis, leading to the lysosomal pH change.^[121]

Figure 3.5 a) Real-time visualization of lysosomal pH changes under dexamethasone-induced cell apoptosis using TR-MP. (b) Summarized pH changes during apoptosis. (c) Fluorescence images of MCF-7 cells only using TR-MP. Scale bar: 10 μ m. (a-c from the section of Figure 5, in **Paper III**).

Chapter 4. Main Methodologies

4.1 Dynamic light scattering

Dynamic Light Scattering (DLS) is a prevalent, non-invasive technique for characterizing small particles in suspension or the size distribution profile of polymers in solution. DLS allows the characterization of particles in dispersion and permits monitoring of dispersion effects, for example, time and pH effects on colloid stability.^[122] In this thesis work, DLS is frequently used to characterize the size distribution of aggregated molecular probes and SiNPs (**Paper I-V**).

4.2 Zeta potential

Zeta potential measurement is an essential and simple nanoparticle characterization technique for estimating the surface charge of particles and can also be used to indicate the physical stability of a colloidal dispersion. In the thesis work, Zeta potentials are used to study potential changes following surface modification and glutathione (GSH)-assisted disulfide bond cleavage (**Paper II, IV**).

4.3 Ultraviolet and visible spectrophotometry

Ultraviolet-Visible light spectroscopy (Uv-Vis) can be absorption or reflectance-based. It probes the adjacent ultraviolet and visible regions of the electromagnetic spectrum. It is an inexpensive, simple, flexible, non-destructive, and fast analytical method that measures the absorbance or transmittance of light. The method is mostly used to determine the concentration of an absorbing species in solution, by using Lambert Beer's law (**eq. 4.1**).

$$A = \log_{10} \frac{I_0}{I} = \epsilon bc \quad 4.1$$

Where A is the absorbance, I_0 is the intensity of the incident light at a given wavelength, I is the transmitted intensity, b is the path length of light through the sample, c is the concentration of the samples, ϵ is a constant for each molecular species and wavelength, known as the molar absorptivity or extinction coefficient.

Lambert Beer's Law allows for the characterization of many compounds as each chromophore possesses a characteristic absorbance band. Furthermore, changing their environment either by modifying the molecular structure or altering the physical environment (*e.g.*, pH, polarity, viscosity), may change their energy levels and thus the wavelength and the intensity of maximum absorbance.^[123] In this thesis work, Uv-Vis is used to indicate the absorption bands of the proposed molecular probes (**Paper I-V**).

4.4 Fluorescence spectroscopy

The fluorescence spectroscopy mainly includes the excitation spectrum and emission spectrum. The excitation spectrum is the intensity of a certain spectral line or band of luminescence with the wavelength (or frequency) of the excitation light changes curve. The horizontal axis is the wavelength of the excitation light, the vertical axis is the relative intensity of luminescence. The excitation spectrum is a spectrum that measures the fluorescence intensity of a fluorophore at a fixed fluorescence wavelength as a function of the excitation wavelength. The emission spectrum is the distribution of luminescence energy by wavelength or frequency. Usually, the experimental measurement is the relative energy of luminescence. In the emission spectrum, the horizontal coordinate is the wavelength, and the vertical coordinate is the relative intensity of luminescence.

Generally, fluorescence analysis features with high sensitivity and high selectivity, usually much more sensitive than Uv-Vis. Moreover, it requires a very small number of samples and a very simple method of operation. In this thesis work, fluorescence spectroscopy is frequently used to determine the optical properties of molecular probes and SiNPs (**Paper I-V**).

4.5 MTT assay

Figure 4.1 The structure changes of MTT in living cells.

In this thesis, cell viability experiments are carried out by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT is a yellow tetrazole molecule, it will be converted into purple Formazan by living cells (**Figure 4.1**). The addition of a solution usually DMSO dissolves the insoluble Formazan into a colored solution. The absorption can be measured spectrophotometrically at a specified wavelength (normally between 500 and 600 nm). The degree of light absorption is determined by the amount of Formazan within the cells. The greater amount of Formazan, the deeper the purple color and hence the greater the absorption. The cell viability (%) is calculated based on the **eq. 4.2**:

$$\text{Cell viability (\%)} = \frac{OD_{\text{wavelength}}(\text{sample})}{OD_{\text{wavelength}}(\text{control})} \times 100 \quad 4.2$$

Where $OD_{\text{wavelength}}(\text{sample})$ indicates the optical density (OD) of the cells treated with probes/SiNPs and $OD_{\text{wavelength}}(\text{control})$ indicates the cells only treated with growth medium.

In the thesis work, MTT assay is frequently used to determine the possibility of toxicity of the proposed molecular probe and SiNPs (**Paper I-V**).

4.6 Confocal fluorescence microscopy

Figure 4.2 The principle and light paths of confocal microscopy.

Fluorescent imaging technique have evolved as an indispensable experimental tool in the study of biological systems in biological, biomedical, and material sciences. Confocal fluorescence microscopy, also known as confocal laser scanning microscopy (CLSM) is the most advanced analytical tool for molecular cell biology research, which uses computer, laser and image processing technologies to acquire three-dimensional data from biological samples. In a confocal fluorescence microscopy, light is emitted by a laser. A specific wavelength of light passes through a pinhole and is reflected by the beam splitter into the sample (**Figure 4.2**). Fluorescent light

emitted from this point is focused, *via* a pinhole, onto a detector (solid lines). The pinhole will reject fluorescence that arises from other points in the sample (dotted lines). Hence, only light from the focal plane reaches the detector. This reduces the acquisition of out-of-focus light, resulting in better image quality. Confocal fluorescence microscopy combined with a variety of fluorescent probes/nanoprobes allows the assessment of not only the presence or location of molecules of interest within cells, but also many dynamic features of cellular function. In this thesis work, confocal fluorescence microscopy is routinely used to observe the cellular dynamics and the biological changes of specific molecules, *e.g.*, pH, ATP in living cells (**Paper I-V**).

Chapter 5. Summary of The Papers Included

Paper I. Light-Up Lipid Droplets Dynamic Behaviors Using a Red-Emitting Fluorogenic Probe

A probe with red emission, large Stokes shift, strong specificity to lipid droplets and anti-ACQ properties, would be very beneficial for the dynamic tracking of lipid droplets in living cells. In this paper, an LDs-specific red-emitting molecular probe TPA-LD has been successfully proposed and studied (**Figure 2.2**). The studies of the photophysical properties demonstrate that TPA-LD has effective AIE properties, high signal-to-noise ratio (S/N), large Stokes shifts (ca. 214 nm), and high resistance to photobleaching. Importantly, it can be physically trapped into the waterless core of lipid droplets through hydrophobic interaction and giving a strong fluorescence intensity. It has been successfully utilized to tracking of intracellular LDs dynamics in real-time, including fusion and migration, and starvation-induced lipophagy in living cells, as well as *in vivo* imaging of lipid-rich tissue in live zebrafish (**Figure 3.4**).

Paper II. Tailorable Membrane-Penetrating Nanoplatfom for Highly Efficient Organelle-Specific Localization

As discussed in *Sections 2.2* and *2.3*, SiNPs show many advantages when they are used as tools for bioimaging studies. However, two main issues of inefficient cellular uptake and poor specificity inevitably hinder their potential applications. In this paper, copolymer Pluronic F127 serves as a nanocarrier to load high emissive AIEgen and TEOS as fixates to generate a rigid cross-linked core, giving high emission fluorescent hydrophilic nanoprobe (SiNPs) (**Figure 2.5**). Various functional ligands (TPP or MP, and CDs) are grafted onto the surfaces to promote cell internalization and specific subcellular delivery, respectively (**Figure 2.7**). As the result, the fabricated nanoprobe can effectively be internalized into cells and further localized into different subcellular organelles within 30 min. By using a dual-color imaging strategy, the SiNPs are successfully used to track dynamic changes of mitochondria and the correlated lysosomes under autophagy process (**Figure 2.8**). Importantly, the tailorable SiNPs allow for the various possible functional modifications towards subcellular targets.

Paper III. A Ratiometric Fluorogenic Nanoprobe for Real-Time Quantitative Monitoring of Lysosomal pH

Lysosomes has a central role in cell functions, where acidic pH is key to this role. Tracking lysosomal pH fluctuations would be necessary for the study of lysosomal function and dysfunction. In this work, based on the established tailorable SiNPs, a lysosomal ratiometric fluorescent nanoprobe is fabricated for reliable quantitative measurement of lysosomal pH. Highly luminescent AIEgen TPE-OMe is encapsulated to serve as a donor, pH-sensitive rhodamine derivative (RDM-TEOS) is covalently linked into the silica shell to serve as acceptor (**Figure 2.12 a**). CPCDs and MP are further decorated on the SiNPs to ensure effective cell-internalization and lysosomal targeting, respectively. The results exhibit that the nanoprobe can real-time quantitatively monitor lysosomal pH fluctuations in live cells under various stimuli (**Figure 3.5**).

Paper IV. Ratiometric Tracking of Mitophagy by a Broad pH-Responsive Mitochondria-Specific Fluorogenic Nanoprobe

As described in section 3.12 *Tracking mitochondrial pH*, a good approach to track the dynamics of mitophagy is to monitor the changes of mitochondrial pH under this process. There is a large pH difference between the organelles of mitochondria (~ 8.0) and lysosomes (~ 5.0), so an ideal probe that can effectively track the mitophagy should be sensitive to a broad pH range (at least 5.0-8.0). Therefore, in this work, mitochondrial-specific ligand TPP and two pH-sensitive fluorophores with discriminable emissions are integrated into the established tailorable SiNPs, of which one is sensitive to acidic pH, and one is sensitive to basic pH (**Figure 2.6**). The resulting broad pH nanoprobe FR-TPP allows quantitative responses with a wide pH range from 5.0 to 8.0, thus enabling the monitoring of pH variations during mitophagy (**Figure 3.3**). As a result, mitochondria, lysosomes, and different mitophagy stages could be discriminatively visualized and analyzed by a dual-color imaging strategy.

Paper V. Quantitative Monitoring of Lysosomal Adenosine Triphosphate in the Autophagy Processes

Regulation and maintenance of normal Adenosine Triphosphate (ATP) homeostasis are crucial for cell survival. To achieve a fluorescent probe that can selectively, rapidly, and quantitatively respond to varying ATP levels, we then fabricate a lysosome-specific ratiometric fluorescent nanoprobe by rationally manipulating FRET in the established tailorable SiNPs (**Figure 2.12**). The resulting nanoprobe HR-MP can therefore monitor lysosomal ATP fluctuations in the live cells (**Figure 3.2**). The roles of autophagy in the control of lysosomal ATP levels under starvation and drug inductions are studied. It is convinced that the rational usage of HR-MP could lead to an in-depth understanding autophagy process.

Chapter 6. Conclusions and Outlook

This thesis demonstrates that red and near-infrared (NIR) probes with a promising strong anti-ACQ ability, S/N ratio, large Stokes's shift, high quantum yield and photostability, and good biocompatibility as well as strong organelles specificity will be favored for the study of dynamic processes and functions in living cells. In future work, fluorescent imaging and sensing in the second near-infrared (NIR-II, 1000-1700 nm) range deserve considerable efforts, providing higher spatial resolution. In addition, the proposed probe in Paper I is originally designed for photovoltage application but its specific chemical and photophysical properties obsessively deserve interesting bioimaging studies. It suggests that innovative potential bio probes can be screened from the existing available materials, even not initially designed for such purpose.

The rational design of the nanomaterials and proper incorporation of functionalities that could be either encapsulated into or surface engineered onto the nano can always deliver the intended nanoprobe for bioimaging and biosensing. Based on the established nano scaffold, in the future we can explore new sensing components with high selectivity and sensitivity to achieve the new probes, extending the imaging and sensing “toolbox”. In addition, the nanomaterials have great flexibility in the integration of multi-functionalities into a single probe (nanoprobe), which is very useful for tailoring the multifunctional nanoprobe that can satisfy various biomedical applications. For instance, a single multiple-channel fluorescent nanoparticle can be tailored used to real-time image/analyze intracellular correlated analytes or processes at cellular and subcellular levels simultaneously.

Considering the close relationship between autophagy (self-eating) and apoptosis (self-killing), future efforts could be made to construct functional SiNPs that can indicate the interplay of autophagy and apoptosis, with the scope of gaining a deeper understanding of the cell functions and providing

new insights for screening potential cancer therapeutics. A single nanoparticle can also be tailored to incorporate the functionalities of targeting (specific to biomarker), diagnosis (imaging), and therapy (drug delivery), which are required by the precision treatments. This methodology may also be extended to facilitate the cell internalization of other substances (*e.g.*, protein, drugs, large particles) that have trouble penetrating the hydrophobic biomembrane barriers.

Nowadays, much research interest has been devoted to the development of different targeting strategies for fluorescent nanoprobe with different organelle targeting capabilities. This thesis mainly focused on mitochondria and lysosome-based targeting, imaging, and analyzing. In later studies, more specific nanoprobe could be derived for other important subcellular organelles for imaging and sensing, including endoplasmic reticulum (ER), nucleus, Golgi apparatus, etc. For instance, phagophore formation is an important process in the autophagic process. As reported, the biogenesis of phagophore membrane comes from ER, but what chemical mechanisms drive the formation of phagophore is still unclear. Fabricating fluorescent nanoprobe for ER labeling and sensing will offer a new powerful fluorescence tool for unraveling more fundamental mechanisms underlying the phagophore biogenesis.

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