Functionalised silica coated nanoparticle hybrids for drug delivery and imaging

Yang, Yanmei

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**FUNCTIONALIZED SILICA COATED
NANOPARTICLE HYBRIDS FOR DRUG
DELIVERY AND IMAGING**

YANG YANMEI

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FUNCTIONALIZED SILICA COATED NANOPARTICLE HYBRIDS FOR DRUG DELIVERY AND IMAGING

YANG YANMEI

School of Physical and Mathematical Sciences

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ABSTRACT

In recent years, nanoparticles have been extensively explored for biomedical applications due to their unique properties. Among various nanomaterials, silica-based nanoparticles exhibit superior biocompatibility which attracted much research attention. These particles also provide several advantages such as large functional surface, tunable structures and high spectroscopic transparency. In this research dissertation, we focused on developing novel and convenient drug delivery systems for theranostic applications based on silica-coated nanoparticles.

Taking the advantage of functional surface structure property of silica nanoparticles, we designed a silica-coated magnetic nanoparticle platform that doxorubicin-peptide conjugated to the surface through “*click*” chemistry. The chosen peptide could be cleaved by enzyme which only found in the intracellular compartment. This conjugate nanoparticle system demonstrated highly efficient drug release upon enzyme interactions *in vitro* and in living cells. This system also showed multiple functions that drug release in the selected cells, fluorescence imaging and magnetic resonance imaging.

Since silica nanoparticles exhibit good biocompatibility and high spectroscopic transparency, we employed silica-coated upconversion nanoparticles for light-induced therapeutic agents release in the following chapters. By linking the caged D-luciferin to the particles surface, the obtained conjugates can serve as promising platforms for the near-infrared light-responsive bioluminescent molecules release. This approach takes advantage of upconverted UV emission of upconversion nanoparticles from near-infrared light excitation to trigger the uncaging of D-luciferin from the

nanoparticle conjugates. Furthermore, the release process can be monitored either by tracking the enhanced fluorescence intensity of D-luciferin or by measuring the bioluminescence signals *in vitro* and *in vivo*.

The use of silica-coated upconversion nanoparticles can be extended to gene delivery. The particles functionalized with cationic photocaged linker were used as nanocarriers for siRNA delivery to effectively silencing enhanced green fluorescent protein gene expression. siRNA was adsorbed on the platform through electrostatic attractions which was easily internalization by cells. The release process was initiated by the upconverted UV light from upconversion nanoparticles under near-infrared light exposure.

As we known, silica-based nanoparticles have two major types, solid silica nanoparticles which we have been used to coat inorganic core nanoparticles in the previous work, the other one is mesoporous silica nanoparticles. In this final project, mesoporeous silica coated upconversion nanoparticles were demonstrated for near-infrared light-responsive drug delivery. The mesoporous silica shell was employed for drug loading and further was capped with cross-linked photocaged linkers. The cover on the pores would be opened by the UV light emitted from upconversion nanoparticles upon near-infrared light irradiation.

ABBREVIATIONS

AgNPs	silver nanoparticles
anti-EGFR	<i>anti</i> -epidermal growth factor receptor
APTES	(3-aminopropyl)trimethoxysilane
ATP	adenosine triphosphate
AuNPs	gold nanoparticles
AuNRs	gold nanorods
AuNSs	gold nanoshells
BAM	bioanchored membrane
BRET	bioluminescence resonance energy transfer
C dots	Cornell dots
CTAB	cetyltrimethylammonium bromide
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMAP	dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
Dox	doxorubicin
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
FA	folic acid
FDA	United States Food Food and Drug Administration
Fe-NPs	iron oxide nanoparticles

FITC	isothiocyanate
fLuc	firefly luciferase
FR	folate receptor
FTIR	Fourier transform infrared spectra
GFP	green fluorescent protein
GRAS	Generally Recognized As Safe
HBSS	Hank's balanced salt solution
HOSu	<i>N</i> -Hydroxylsuccinimide
HPLC	high performance liquid chromatography
ICP	Inductively Coupled Plasma
IND	Investigation New Drug
Lipo2K	lipofectamine 2000
LRET	lanthanide resonance energy transfer
MMPs	matrix metalloproteinases
MRI	magnetic resonance imaging
MS	Mass spectra
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NB	<i>o</i> -nitrobenzyl
NBS	<i>N</i> -Bromosuccinimide
NHS	<i>N</i> -Hydroxyl-succinimide
NIR	near-infrared
NP	nanoparticle
PABC	<i>p</i> -aminobenzyloxycarbonyl

PBS	phosphate buffered saline
PDT	photodynamic therapy
PEG	polyethylene glycol
PEG-b-PAMA	PEG- <i>b</i> -poly(dimethylaminoethylmethacrylate)
PMT	photomultiplier tube
PtNPs	platinum nanoparticles
PTT	photothermal therapy
QD	quantum dot
RBCs	red blood cells
siRNA	small interfering RNA
Si-UCNPs	silica-coated UCNPs
SMNPs	silica coated magnetic nanoparticles
SPR	surface plasmon resonance
TEA	triethylamine
TEM	transmission electron microscopy
TEOS	tetraethyl orthosilicate
TLC	Thin-layer chromatography
TRITC	tetramethylrhodamine isothiocyanate
UCNPs	upconversion nanoparticles
W/O	water-in-oil

Chapter 1 Introduction

1.1 Nanoparticles in biomedical applications

The application of nanoparticles (NPs) in the field of biomedical sciences has attracted much attention in recent years and continues to be a rapidly growing research area in the future. NPs often possess excellent properties, such as large surface area, amazing optical or physical activities, good photostability or magnetic properties, which have been considered as the most promising functional materials for various applications. For example, some polymeric NPs often serve as candidates in catalysis or biomolecule carriers due to their large surface area and high loading capacity.^[1] NPs with good optical properties are commonly used in fluorescence optical imaging and biosensing.^[2] Magnetic NPs have been widely used in biomedical applications such as MRI contrast agents or beads for biomolecules separation, *etc.*^[3] In biomedical field, one of the most essential requirements is that sufficient therapeutic agents should be selectively introduced to the target site. In general, many of the bioactive molecules cannot automatically target the disease areas. How to effectively enhance the localized concentrations of therapeutic agents and to provide favorable clinical outcomes is a highly important issue in clinical practice.^[4] For this purpose, various organic and inorganic NPs have been extensively developed and used for these potential applications in both clinical diagnosis and therapy (Figure 1-1). Some of these functional nanomaterials will be shown below.

1.1.1 Polymer based-NPs

Among many types of functional biomaterials, polymeric NPs including general

Figure 1-1. Various organic and inorganic nanoparticles have been used in biomedical applications. a) silica-coated upconversion nanoparticles, b) metallic gold/silver nanoparticles, c) polymeric micelles, d) iron oxide nanoparticles, e) mesoporous silica nanoparticles, f) quantum dot nanoparticles.

polymers, dendrimers, liposomes, polymeric micelles and nanogels have been commonly used in the delivery of therapeutic agents, such as drug, gene or other biomolecules. One of the examples for polymer-based NPs in nanomedicine is polyethylene glycol (PEG),^[5] a kind of flexible and water-soluble polymer, which can slow down the rate of clearance by kidneys and prolong the blood circulation time and thus greatly reduce the immunogenic response easily caused by other commonly used materials.^[6] Moreover, PEG is also a well-established functional polymer which has been extensively used to modify NPs and hence, improve their biocompatibility and colloidal stability under physiological conditions.^[7]

Liposomes, another most frequently used polymer NPs, were firstly found by Bangham and co-workers more than 40 years ago.^[8] These materials have colloidal structure formed by self-assembly of various phospholipid molecules in an aqueous medium. Due to their promising properties including biocompatibility,

biodegradability and nontoxicity, liposomes have been used as nanocarriers for effective delivery of bioactive molecules, especially for those with poor water-solubility.^[9] Despite their broad applications, some liposomes are known to be lack of stability under intracellular and extracellular environments. Some liposomes can be quickly cleared from the blood stream if their outer shells are unmodified or unprotected.^[6a, 10] Therefore, in order to increase the stability of liposomes and enhance their lifespan in blood circulation, introduction of functional polymers such as PEG chains onto liposomes surface have been widely performed. So far, such an elegant approach has already been successfully utilized in cancer therapy, for example, Doxil is one of the commercial “stealth liposome” with antitumor drug doxorubicin (Dox) encapsulated in a closed liposome.^[11] Another widely used approach to promote stability and long blood circulation is on the basis of cholesterol-terminated poly(acrylic acid), one hydrophobic polymer which can be used to insert into the liposomes’ lipid bilayer and thus induce crosslinking to enhance the stability of the liposomes while the nanocarrier function is still retained.^[12] Similar with liposomes, polymeric micelles are another type of extensively used polymer structures to enhance the adsorption and bioavailability of lipophilic drug molecules in aqueous environment. On the basis of self-assembly of some amphiphilic polymers, polymeric micelles can be easily formed and can be applied to prolong circulation times by inserting the polymers like PEG to form the outer protecting corona.^[6a]

Apart from the commonly applied liposomes and polymeric micelles, polymeric gel-based hybrid NPs are other kinds of soft materials, which are also often utilized for the target delivery of bioactive molecules. These nanogels are usually formed as a result of cross-linked or self-assembled small or polymeric molecules, which exhibit high stability and excellent loading capacity.^[13] As a result, such nanogel-based soft

material hybrids can work as suitable carriers for effective control release of payload molecules on the basis of external stimuli such as pH, ionic strength, temperature, light and enzymatic hydrolysis.^[14]

1.1.2 Inorganic NPs

Recently, the programmed self-assembly and disassembly of inorganic NPs with controlled surface chemistry have already attracted considerable attention and have been extensively studied due to their unique physical, optical and magnetic properties. For instance, inorganic iron oxide NPs have been used in many biomedical systems, either as contrast agents for magnetic resonance imaging (MRI), or as magnetic bioseparation and magnetic field guided drug delivery due to their superparamagnetic property.^[15] Apart from magnetic NPs, the noble metal nanomaterials such as gold NPs (AuNPs), gold nanorods (AuNRs) or silver NPs (AgNPs) have also been considered as promising biomaterials for drug/gene delivery or medical diagnosis based on their unique surface plasmon resonance (SPR) properties, large surface area and other unique physical properties.^[16] In addition, by changing the aspect ratio of NPs to NRs, the absorption caused by SPR can shift from visible wavelength to the long wavelength near-infrared (NIR) region. This interesting property is extremely useful for *in vivo* imaging and photothermal therapy because of the deeper penetration of such longer wavelength light and the localized thermal functions produced from NRs upon the light irradiation.^[17] Besides these magnetic and noble metal nanomaterials, the use of semiconducting nanocrystals (*e.g.*, QDs, *etc.*) with different components (*e.g.*, CdTe, ZnS, CdSe and CdS) for optical imaging is another very popular option, due to their high emission quantum yield, good photostability, narrow emission spectra and high fluorescence efficiency.^[18] Moreover, the absorption and

emission properties of these semiconducting nanomaterials can shift to NIR windows (700-900 nm) by tuning the particles size that allows for real-time *in vivo* imaging.^[19] A novel class of lanthanide-doped upconversion nanoparticles (UCNPs) has also been developed very recently. These newly created UCNPs exhibit very interesting anti-Stokes emission that can concurrently generate higher-energy emissions upon lower-energy exciting light irradiation (usually NIR (750-1400nm) or infrared (IR) radiation).^[20] Furthermore, UCNPs also show many advantages such as narrow emission bandwidth, tunable multicolor emission, long lifetime, high photostability and low cytotoxicity, which make them particularly useful for biolabeling and bioimaging, especially for those applications in deep light and tissue penetration.^[20a, 21]

1.1.3 Functionalization of NPs

Figure 1-2. Schematic illustration of the general steps involved in the application of functional nanoparticles.

As showed above, NPs have found many applications in different fields such as biocatalysis, biosensing, bioimaging, photothermal therapy and drug delivery, owing to their special and unique properties. However, due to the surfactants and other stabilizing agents used in the process of NPs fabrication, most of the prepared NPs are not stable and tend to aggregate easily in the aqueous solutions, which greatly impede

their direct applications in the physiological environment. Therefore, NPs surface modification with suitable functional groups needs to be involved to enhance their stability and biocompatibility (Figure 1-2). So far, many strategies have been developed for effective surface functionalization of NPs such as tethering bioactive ligands to the particles surface, conjugating biomolecules with NPs or coating the NPs with silica or polymers shell, *etc.*

In order to facilitate the applications of NPs in biological systems, one commonly used approach is to coat the surface of NPs with different types of ligands. For example, in the process of metallic NPs-based colorimetric biosensing and imaging, AuNPs are usually surface-modified with thiol-linker to enhance their biocompatibility and to minimize non-specific aggregation.^[22] Another approach to functionalize NPs is physically transferring nanocrystals from organic solution to aqueous solution by phase-transfer reagents. By using this method, the as-synthesized hydrophobic NPs can be changed to hydrophilic surfactants-stabilized NPs which can be easily modified and further applied in living cells.^[23] Furthermore, tethering NPs with small molecule ligands may also affect their interactions with cell membrane. Once doped with positively charged ligands, most of the modified NPs will show higher cellular internalization when compared with those neutral or negatively charged particles.^[24] Therefore, the cationic surface modified NPs have been reported as nanocarriers for the delivery of therapeutic agents.^[25] For example, positively charged iron oxide NPs which surface was functionalized with guanidine head groups exhibited remarkably strong cell-penetrating capabilities for therapeutic usage.^[26] Another example of cationic NPs for biomedical application was on the basis of positively charged polymer-coated AuNPs which were used to adhere siRNA on the surface of AuNPs *via* electrostatic interactions. These modified particles exhibited

great siRNA loading capability and high cellular uptake, and subsequently resulted in significant gene silencing efficiency.^[27]

1.1.3.1 Functionalization of NPs with biomolecules

Among the variety of molecules that have been successfully tethered onto NPs surface, the bioactive moieties including proteins, DNA and siRNA molecules coated NPs also play important roles in nanomedicine, mostly attributed to their great potential as intrinsically diagnostic agents or affinity ligands to facilitate the effective drug delivery in biological system.^[16b, 28] For instance, the modified AuNPs have been used to deliver protein such as green fluorescent protein (GFP) into human or murine cells. The fluorescence of GFP was quenched while adsorbed to the particles surface through electrostatic interactions. Upon releasing from the carriers in living cells, the fluorescence intensity of GFP was recovered and was easily monitored by fluorescence microscopy.^[22] Additionally, protein conjugated magnetic NPs can also be used as contrast agents for MRI application. During the course of MRI, the role of the coated protein moieties is to localize the particles at the specific tumor site for the purpose of targeted delivery.^[29] When capped with antibodies, the NPs can be used to target the cells with specific surface receptors. For example, in one study, AuNPs and AgNPs were engineered by loading of an antibody, Herceptin. The as-synthesized antibody-NPs conjugates could selectively bind to the targeting tumor cells with Her-2 receptor overexpression.^[30] Furthermore, the applications of antibody-NP conjugates can also be extended to bioimaging and photothermal therapy studies. One typical example is that the anti-epidermal growth factor receptor (anti-EGFR) conjugated NPs was found to exhibit higher binding to the targeted cancerous cells with six-fold greater affinity when compared to their binding to normal cells.^[31] When

these antibody conjugated NPs incubated with cells and followed by NIR laser irradiation, localized heating was generated and cell damage was caused subsequently.^[32]

DNA or RNA can also be used to functionalize the surface of NPs and to further explore their functions in biomedical science. For example, in 1996, Mirkin and Alivisatos independently reported a pioneering study for colorimetric biosensing of DNA by immobilizing short oligonucleotides to AuNPs surface through covalent gold-thiol bond.^[33] Since then, many other extensive studies with NPs surface modified with oligonucleotides have been continually conducted in diagnostic and therapeutic applications.^[34]

1.1.3.2 Core-shell NPs

Figure 1-3. Different core-shell nanoparticles: a) spherical core-shell nanoparticles; b) core-mutishell nanoparticles; c) movable core within hollow shell nanoparticles;^[35] d) core-mesoporous shell nanoparticles; e) polymer-coated multicore nanomaterials; f) hexagonal core-shell nanoparticles.^[36]

With the rapid advancement of nanomaterials science and technology in recent decades, the focus on nanoparticles preparation has shifted from the basic synthesis

methods like the preparation of homogeneous NPs to more complex systems with desired functions.^[37] In biomedical applications, the methodology to prepare biocompatible and multifunctional nanomaterials has been frequently studied. Among various types of NPs, core-shell NPs have attracted much attention because of their structural diversity and possibility to supply multiple functions (Figure 1-3). The reported methods for the synthesis of core-shell NPs are often on the basis of surface modifications of the prepared NPs core structure with metal, polymer or silica complexes.

For the coating with metals, a typical example is gold-coated NPs. Due to the inert chemical activity, gold is ideally suitable for the purpose of surface coating nanomaterials.^[38] The surface coating with gold is often prepared in reverse micromulsion by using surfactants such as cetyltrimethylammonium bromide (CTAB) and octane. This method has been successfully implemented for different types of NPs, such as Co/Pt NPs and iron NPs.^[39] One big advantage of this metal-coating method is that the gold-coated core-shell NPs can be easily functionalized through the covalent gold-thiol bond, which may enhance the stability and provides the great possibility for subsequent bioconjugation.^[38]

Other than metal materials, organic molecules have also been used to prepare core-shell NPs structures. One typical example is the surface functionalization with polymers. Among different approaches for polymers coated core-shell NPs, a simple strategy is to utilize the micelle structures, usually formed by the self-assembled polymer chains. Generally, a stable metal complex of soft acid and softer base will be produced first in the micelles core.^[40] The following chemical reduction to interact with metal NPs would lead to the formation of polymer stabilized metal NPs.^[41] In

Figure 1-4. Several examples about silica coated NPs. a) silica-coated hydrophilic Fe_3O_4 nanoparticles; b) mesoporous silica-coated hydrophobic Fe_3O_4 nanoparticles; c) silica-coated hydrophobic upconversion nanoparticles; d) mesoporous silica-coated hydrophobic upconversion nanoparticles.

most cases, polymer chains such as PEG with suitable functional groups can directly graft on the surface of the pre-synthesized NPs to supply the biocompatible polymer coated NPs.^[42] So far, several types of polymer-stabilized NPs with metal core have been currently presented. One successful example is that the copolymer, PEG-*b*-poly(dimethylaminoethylmethacrylate) (PEG-*b*-PAMA), has been used to coat AuNPs and QDs. The copolymer stabilized core-shell NPs could be easily functionalized for further biosensing applications.^[43] Similarly, some polymers with pH-sensitive or thermal-sensitive special functionalization, have also been used to coat NPs for stimuli-induced biomolecules release or other related applications. For example, Li and co-workers have prepared such an interesting multifunctional core-shell particles by using the pH-sensitive poly(4-vinylpyridine) polymer for potential biosensor application.^[44]

1.2 Silica-coated NPs

Silica coating, in contrast to other methods mentioned above, is the most widely used approach for the preparation of core-shell NPs.^[45] In 1996, Liz-Marzán and co-workers reported a systematic study of the synthesis of silica-coated metal NPs.^[46] Inspired by this pioneering work, more than one thousand scientific papers have been published on the synthesis, modification and properties study of the silica-coated core-shell NPs (Figure 1-4).^[45-46] So far, silica-coated NPs have become increasingly important and have been greatly applied for the promising biomedical applications.

In general, silica nanomaterials exhibit excellent properties, including 1) high stability in aqueous solutions, 2) optically transparent, 3) easy synthesis from commercial silane coupling agents, 4) good biocompatibility and 5) broad options for surface functionalization under both hydrophilic and hydrophobic environments.^[47] Importantly, because of the stability in aqueous media, silica coating is often used to increase the suspension stability of the core NPs, especially for those NPs synthesized in organic phase but used in aqueous solutions.^[48] In addition, silica shell is optically transparent, the intensity and position of the surface-plasmon absorbance band of certain NPs can be modulated by silica coating without affecting their intrinsic optical properties.^[49] Furthermore, the existence of silica shell around core NPs makes it easier to introduce chemical modifications on the particles surface *via* covalent bond.^[16a] In addition, the silica coating approach can be used not only for hydrophilic NPs but also for other hydrophobic NPs like Fe₃O₄, QDs and UCNPs.^[45b, 50] Therefore, silica coating on different inorganic core particles such as metals, metal oxides and metal salts have received more attention than any other kinds of NPs coating complexes.^[51]

Mesoporous silica nanoparticles were first synthesized by two different research groups in the early 1990s.^[52] From then on, amounts of research works have been performed to provide potent silica NPs for their potential applications including biosensing, imaging and drug delivery.^[53] Mesoporous silica NPs were proved to be good carriers normally for those molecules with poor water-solubility. The porous structure and large surface area ensure the facile adsorption as well as high loading of various chemical agents or therapeutic materials.^[14c, 54] In addition to these properties, mesoporous silica-based NPs also demonstrated promising properties including easy surface functionalization and good biocompatibility *in vivo*.^[55]

1.2.1 Biocompatibility of silica-coated NPs

Silica-based NPs usually exhibit higher biocompatibility than many other nanomaterials.^[56] Currently, silica have been approved as “Generally Recognized As Safe” (GRAS) by the United States Food and Drug Administration (FDA).^[6b] For example, the C dots (Cornell dots), which are dye-doped silica core-shell NPs, have recently received approval from the FDA for the first Investigative New Drug (IND) application for targeted molecular imaging of cancer in hospital.^[57]

Apart from their good biocompatibility, the cytotoxicity of silica-based NPs has been thoroughly studied in different types of cells by several research groups and the results indicated that the toxicity of silica-based NPs is highly related to size, concentration and the surface functionalized groups.^[58] Recently, Lin and co-workers have demonstrated that the biocompatibility of silica-based NPs could be affected by the structure (mesoporous silica or solid silica).^[59] The hemolytic properties of mesoporous silica NPs toward mammalian red blood cells (RBCs) were studied. The proposed mechanism indicated that the cytotoxicity was caused by the interaction

between the surface silanol groups and the *tetra*-alkyl ammonium groups on the RBCs membrane. They also found that, in mesoporous silica NPs, most of the silanol groups existed inside of the mesochannels, which resulted in nontoxic toward RBCs. On the contrary, the amorphous silica which exhibited hemolytic activity is mostly due to the silanol groups on the external surface. Haynes and co-workers further confirmed this mechanism by using mesoporous silica-coated iron oxide NPs. They have systematically evaluated the hemolytic properties of solid silica and pores-silica-NPs from several factors and found that both two kinds of silica-based NPs showed dose- and size- dependent hemolytic activity. By surface modification with a PEG-silane, the hemolytic activity of the mesoporous silica NPs would be ameliorated which ensures the safety in the subsequent biomedical applications.^[60]

The first step towards understanding the nanomaterials applications in biomedical systems is to investigate their cellular uptake and intracellular trafficking.^[61] Through functionalization with fluorescent dye molecules, the silica coated NPs can be tracked by fluorescence and confocal microscopy. A variety of fluorescent agents such as isothiocyanate (FITC),^[62] tetramethylrhodamine isothiocyanate (TRITC),^[63] and NIR dye^[64] have been doped to the surface of particles through covalent or non-covalent interactions. Several other techniques like transmission electron microscopy (TEM), MRI, flow cytometry, *etc.*, can also be used to monitor the cellular uptake progress. In brief, the cell uptake of NPs also depends on the particles size, shape,^[30, 65] surface charge, chemical groups,^[58a, 66] the dosage used and incubation time.^[55]

1.2.2 Synthesis of silica-coated NPs

Scheme 1-1. Hydrolysis mechanisms of TEOS under a) acidic condition and b) basic media.^[48]

Although many approaches have been developed to synthesize various types of silica-coated NPs in recent years, many of them were only based on the classical hydrolysis and polycondensation reactions. The hydrolysis reaction step can be initialized in both acidic and alkaline media (Scheme 1-1), the following polycondensation normally based on condensation of silicon alkoxides which will lead to the formation of silica-coated NPs. However, for the synthesis of silica NPs from TEOS, higher pH or basic condition is preferred.^[67] According to the types of core particles and structure of the coated silica shell, we will systematically classify several most recently used strategies into three types as shown below.

1) Silica coating of hydrophilic NPs

Scheme 1-2. The sol-gel process for synthesis of silica nanoparticles from organosilane.

In the late 1960s, Stöber *et al.* developed the sol-gel process of silicon alkoxides for synthesis of silica NPs in the mixed aqueous and alcohol solutions.^[47] In Stöber's

Figure 1-5. Silica coating chemistry for AuNPs and Fe₃O₄ NPs

method, the base ammonia should be used to catalyze the hydrolysis and condensation reactions (scheme 1-2).^[47, 68] The silica NPs synthesized by this method are typically monodisperse and spherical in shape. In addition, the size of the obtained nanoparticles can be tuned by controlling the reaction conditions. So far, numerous investigations have been done through the well-established Stöber method for preparation of both silica NPs and silica coated core-shell NPs when a pre-synthesized core is suspended in the solution.^[69]

Liz-Marzán and co-workers first synthesized silica-coated AuNPs by using the (3-aminopropyl)trimethoxysilane (APTES) in aqueous solution. The citrate-reduced AuNPs were bound with the -NH₂ groups and followed by hydrolysis and condensation, the thin silica layer was formed on the surface (Figure 1-5a).^[46b] Silane coupling agents such as TEOS can be directly polymerized on the surface of magnetic NPs under basic conditions because of the hydroxide groups on the NPs surface which can initiate silane hydrolysis and polymerization from the NPs surface (Figure 1-5b).^[70]

2) Silica coating of hydrophobic NPs

Figure 1-6. Schematic illustration of the W/O microemulsion silica coating of NPs. Reprint with the permission.^[71] Copyright 2010, Wiley-VCH Verlag GmbH & Co. KGaA.

Water-in-oil (W/O) microemulsion, also termed as reverse microemulsion, is another promising strategy for the synthesis of monodisperse silica coated NPs (Figure 1-6).^[72] Typically, small water droplets are dispersed by a nonionic surfactant (*e.g.*, Triton x-100) in oil phase (*e.g.*, cyclohexane) in a W/O microemulsion system. This reverse microemulsion method has been found to be used as nanoreactors for the synthesis of silica coating of inorganic NPs which are unstable in the Stöber reaction medium (water-ammonia-alcohol mixture).^[71] The pre-synthesized hydrophobic NPs can be coated with silica shell using this one-pot method and no prior ligand exchange is required. Furthermore, this reverse microemulsion approach has been found to be extremely useful to control over silica nucleation because of the nanometer-sized droplets.^[73]

Many hydrophobic NPs such as QDs (*e.g.*, CdSe), magnetic Fe₃O₄ NPs and UCNPs, have also been encapsulated within silica shell by using this reverse microemulsion method.^[74] Ying and co-workers have developed a facile strategy of incorporating hydrophobic QDs into silica through using this strategy.^[75] By employing Igepal as the nonionic surfactant, the trioctylphosphine oxide-capped QDs could be coated with

silica in a direct one-pot synthesis method. The magnetic NPs such as Fe_3O_4 also can be easily encapsulated into silica using reverse microemulsion method and further functionalized for use in biomedical practice. For instance, Lai *et al* have reported the applications of the functionalized silica coated Fe_3O_4 iridium (Ir) complexes for MRI, biolabeling and photodynamic therapy.^[76] Very recently, our group has also reported a simple strategy to encapsulate the UCNPs in silica shell. Typically, the reverse micelles were formed by the surfactant Triton x-100, organic solvent, ammonia and water.^[50b] This silica coating strategy enabled us to prepare water-soluble functionalized UCNPs and further study their biomedical applications.^[77]

3) Mesoporous silica coated NPs

Figure 1-7. Synthetic procedure for encapsulation of nanoparticles within mesoporous silica shell.

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Mesoporous silica is a special form of silica with large surface area and porous structure. In 1997, Grun *et al.* first synthesized submicrometer-scaled MCM-41 particles by adding a cationic surfactant to the reaction mixture.^[79] Later on, mesoporous silica NPs of 100 nm and even below 50 nm were obtained by using different surfactant systems.^[79-80] In the process of synthesis of mesoporous silica coated NPs, many strategies have been proposed, including using base (*e.g.*, ammonia)

to breaking up the siloxane bonds,^[51h, 81] or using different polymer protection.^[55, 82]

One of the commonly used approaches is employing micelles formed by cationic surfactant as the template for synthesis of mesoporous silica coated NPs. The organosilane precursors (such as TEOS) can be directly condensed around the template into ordered silica structure.^[13]

In order to satisfy the subsequent biomedical applications, some inorganic nanocrystals (*e.g.*, iron oxide NPs) were induced to be embedded in the mesoporous silica nanoparticles shell during the synthesis process in recent years. In 2006, Hyeon and co-workers reported a simple method for encapsulation of magnetic NPs into mesoporous silica shell (Figure 1-7).^[83] In this approach, the surfactant CTAB was used not only as template but also as phase-transfer ligand. The pre-synthesized oleic acid stabilized iron oxide NPs were transferred to aqueous media by CTAB. Subsequently, the silica sol-gel reaction was carried out under base catalysis, which resulted in the production of mesoporous silica coated iron oxide nanoparticles.^[83] Employing this useful synthetic method, other hydrophobic NPs like MnO NPs, QDs and UCNPs could also be incorporated into mesoporous silica shell.

1.3 Silica-coated NPs for drug delivery

Among various successful applications of nanomaterials in the fields of biology and medicine, drug delivery have been extensively investigated because of their high capability to be internalized by cells and their meaningful physicochemical properties to biological systems.^[9] Additionally, the use of nanometer sized “carriers” for drug delivery is a very promising option for improving drug efficacy through enhanced delivery concentration to the target site.^[84] In order to achieve a controllable drug

release, several types of stimuli-responsive drug delivery systems based on pH-, temperature-, enzyme-, light- or other biomolecule-, have been studied recently.^[85] Enzyme-responsive and light-induced drug delivery strategies are directly related to the work in this dissertation, hence will be mainly introduced. Since drug molecules can be associated with nanoparticles *via* ionic interactions, hydrogen bonding or covalent attachment, prior to description of these two drug delivery approaches, the forms of covalent and non-covalent transport will be first introduced.

1.3.1 Drug delivery transport forms: covalent and non-covalent

Figure 1-8. Silica-based NPs for drug delivery: a) covalent drug delivery and b) non-covalent drug delivery.

Covalent drug delivery is the form that a direct chemical bond should be used for association between drug molecule and the transport vector (Figure 1-8a).^[86] A liable chemical linker which can be cleaved by physiological trigger is required in this covalent drug delivery system. Since the drug release efficiency is highly dependent on the linker being sufficiently cleaved in the presence of the triggers, the choice of appropriate covalent linker is extremely important.^[87] Among various potential chemical linkers, disulfide or ester linkers are commonly used for nonspecifically

drug release.^[88] Utilizing the linkers, which can be selectively cleaved by specific triggers, will provide an advantage for a better control of drug release. For example, some peptides can be cleaved by the special enzymes expressed in tumors that can serve as linkages between drug molecules and NP carriers.^[89] Moreover, linkers may also be designed to be cleaved by the environmental changes such as pH, heating or light irradiation.^[90] Silica-based NPs are suitable transports especially for these covalent drug delivery systems, mostly attributed to the silica shell that can be easily functionalized with the specific linkers for association with drug molecules.

For non-covalent drug delivery system, drug molecules could not directly bind to the NPs surface, but they could be encapsulated within NPs through physical interactions like electrostatic interactions or hydrogen bonding (Figure 1-8b).^[86] Mesoporous silica NPs are especially appropriate carriers for non-covalent based drug delivery strategy. Since their high surface area and pore volume, mesoporous silica-based NPs can incorporate numerous therapeutic agents into the pores.^[91] For the biomolecules with intrinsic charges such as DNA or RNA, another commonly used approach is the carrier NPs (both solid silica and mesoporous silica-based NPs), which are modified with positively charged linkers (Figure 1-8b).

1.3.2 Enzyme-induced drug delivery

Enzymes play important roles in drug development, including cell regulation and in many other biomedical processes.^[92] Certain enzymes are found to be overexpressed at particular stage of the disease sites which allow to develop the enzyme-induced drug release systems.^[93] The ideas of such drug delivery approaches are also based on the fact that one enzyme usually only reacts with a special substrate. In the case of silica-based NPs for drug delivery, the NPs themselves are not responsive to enzyme

Figure 1-9. Illustration of the protease-mediated payload release from mesoporous silica nanoparticles. Reprint with the permission.^[94] Copyright 2010, American Chemical Society.

reaction but their surface can be functionalized with linkers which can be recognized by special enzymes.

Many kinds of enzymes can be utilized to trigger drug release from NPs-based carriers. However, due to the facile design of the attachment of bioactive moieties to the nanocarriers, the mostly commonly used enzymes are hydrolases enzymes such as proteases, lipases and glycosidases *etc.*^[9, 95] Proteases can be used to trigger drug release when the nanocarriers are capped by specific peptides. Heise *et al.* utilized this approach to demonstrate the protease-mediated guest molecule release strategy from silica particles (Figure 1-9).^[94] In addition, a kind of protease enzyme, cathepsin B, can serve as an ideal effector biomolecule in drug delivery when utilized to release cargo in cell compartments. This cathepsin B enzyme is expressed in lysosome which can selectively cleave special peptides.^[96] Drug molecules can be linked to the nanoparticles surface through such peptide sequences. Since certain enzymes are only found in the intracellular compartments, this enzyme-regulated drug release can increase the drug activity because the drug molecules are only released and exert their

therapeutic effect only after cellular uptake.^[89, 97] Many of the reported enzyme-responsive drug delivery systems rely on peptides that mimic the nature enzyme substrates. The used peptides should not only include special cleavage sites which can be recognized by the target enzymes but also have functionalized groups used to associate with particles. Additionally, the chosen peptides are typically very short (2-20 residues) that can be easily synthesized.^[9] For example, peptide with sequences GPLGVRG can be recognized by the enzyme matrix metalloproteinase-2 (MMP-2), thus can be exploited for enzyme-induced drug delivery systems.^[98] Recently, the group of Bhatia developed a MMPs-responsive drug delivery system by using mesoporous silica-based NPs. The nanocarriers were modified with protease cleavable peptides which could be used to target tumor sites with MMPs enzyme overexpression.^[99] Another example for enzyme-triggered drug delivery by employing silica-based NPs was demonstrated by Kim *et al.* The guest molecules were loaded on particles and further blocked by cyclodextrin-based covers. The linkers between cyclodextrin and NPs could be hydrolyzed by the target enzyme lipase, thus the gatekeepers were removed and the entrapped molecules were released subsequently.^[100] Our group has also developed a facile strategy to deliver the commonly used anti-cancer drug, Dox to cancer cells by using silica-coated magnetic NPs. In our study, drug molecules with short enzyme-cleavable peptide modification were covalently tethered on the surface of uniform silica-based magnetic NPs through click chemistry. Drug molecules could be selectively released in the tumor cells with the special enzyme overexpression.^[101]

1.3.3 Light-induced drug delivery

Light-induced controlled release system has been considered to be one of the most

promising approaches for drug delivery due to its several advantages, such as time-specificity and non-invasive property.^[102] A large number of works for light-responsive drug delivery were based on nanomaterials. In this section, NPs-based light-activated drug delivery strategies including covalently binding and encapsulation forms will be briefly reviewed. Generally, according to the wavelength of light used, there are three distinct areas: 1) Low wavelength light-induced drug delivery; 2) light-to-thermal-induced drug delivery; 3) NIR light-induced drug delivery by using UCNPs.

The applications of UV light as an external stimulus to control drug release have been reported by many research groups.^[102b, 103] In order to initiate the light-responsive reactions to release biomolecules, a photolabile protecting group should be used. Among many photocaged protecting moieties, the *o*-nitrobenzyl (NB) derivatives are the most extensively used in the light-induced drug delivery systems.^[104] Wu *et al.* have reported UV excitation triggered release of fluorescent dye from silica NPs.^[102a] The light-responsive silica NPs were prepared by covalent conjugation of NB bromide groups which were used for caging of dye molecules. Recently, Cabane *et al.* demonstrated the release of both low molecular weight molecules (FITC and ATTO655 dye) and proteins (GFP) by UV irradiation.^[105] In this work, a self-assembled photocleavable polymer, which also contained NB group, was employed as photo-triggerable nanocarrier. Since the absorption wavelength of these compounds are often around 300 nm, the photocaging usually requires UV light irradiation. However, the short wavelength UV light is harmful to cells and has poor light penetration ability, which limits the applications of UV-induced drug delivery *in vivo*. Nevertheless, the use of visible light induced release of cargo molecules has been demonstrated as a promising option for drug release. Lin's team have

Figure 1-10. Illustration for NIR light-triggered release of guest molecules from the pores of aptamer-covered mesoporous silica-coated AuNRs. Reprint with the permission.^[106] Copyright 2012, Wiley-VCH Verlag GmbH & Co. KGaA.

demonstrated that Ru compound caps bound to thiol terminated mesoporous silica NPs could be opened with visible light (at 455 nm).^[90c] In addition, the use of visible light to release trapped molecules has been further studied using liposomal carriers,^[107] dendrimer molecules,^[108] and [Mn-NO] molecules.^[102c]

Another kind of approach for visible light or longer wavelength light-responsive therapy is based on the photothermal properties of metallic NPs.^[86, 109] Upon absorption of light, the metal NPs can dissipate the absorbed energy, releasing heat into the region of surrounding space. This will lead to the overheating of the local area which can be used to control drug release or directly cause cell or tissue damage subsequently.^[104a] Gold nanomaterials including AuNPs, AuNRs and gold nanoshells (AuNSs) are ideal photothermal therapy (PTT) agents because they have light absorption in the visible and NIR regions. Furthermore, these nanomaterials also exhibit other excellent properties such as high photostability and good biocompatibility. A fundamental work reported by Pitsillides *et al.* has provided the

use of AuNPs as photothermal sensitizer.^[110] Since then, several other groups have further examined the use of gold nanomaterials in PTT by visible light.^[111] In

Figure 1-11. Multicolor luminescent photos of lanthanide-doped UCNPs with varied dopant ratios: a) NaYF₄:Yb/Tm (20/0.2 mol%), b)~f) NaYF₄:Yb/Tm/Er (20/0.2/0.2 – 1.5 mol%), and g)~j) NaYF₄:Yb/Er (18 60/2 mol%). Reprint with the permission.^[112] Copyright 2008, American Chemical Society.

comparison to spherical AuNPs, AuNRs whose absorption fall in the more “biologically friendly” NIR window have been paid more attention for NIR-induced drug release and therapy. For example, Qu and co-worker have developed a NIR-triggered drug delivery system based on AuNRs (Figure 1-10). In order to enhance the stability of AuNRs in biological environment and encapsulate drug molecules, the AuNRs were coated with mesoporous silica shell. The obtained core-shell NPs were surface-modified with aptamer DNA in order to capping dye FITC and drug Dox. Upon NIR light irradiation, the local temperature would be raised by the photothermal effect of AuNRs, resulting in the opening of aptamer cover and releasing of entrapped guest molecules.^[106] A similar work, which also focused on NIR light-to-thermal-responsive drug release, was demonstrated by the Yeh’s group.^[17b]

In principle, biomedical applications prefer longer-wavelength NIR light which is less detrimental to tissue and has deeper light penetration ability. As a result, NIR-induced

drug delivery has been developed in recent years. One of the most useful approaches to NIR light-responsive drug delivery is through lanthanide-doped UCNPs. The UCNPs can absorb long wavelength NIR light (980 nm) and emit short wavelength UV and visible lights. The emission wavelength of UCNPs is usually tunable through

Figure 1-12: Silica-coated upconversion nanoparticles in aqueous solutions. a) Normal light photo, b) Luminescent photo under 980 nm light excitation.

controlling of host/dopant combination and dopant concentrations as shown in Figure 1-11.^[20a] Additionally, one of the distinguished advantages of UCNPs is their excellent photobleaching resistance since they can display no reduction in emission intensity under continually several hours 980 nm laser exposure.^[113] Because of these optical properties, UCNPs have been employed as NIR-triggered drug delivery vehicles for providing the low wavelength light needed for the photocleavable systems. A few works reported in the literatures have demonstrated that the use of NIR-to-UV light base on UCNPs for drug or other small molecules release. A fundamental work by Zheng and co-workers has shown that $\text{YF}_3\text{:Yb}^{3+}$, $\text{Tm}^{3+}/\text{TiO}_2$ core-shell NPs are good candidates for efficient NIR-to-UV photocatalyst.^[114] Moreover, Branda *et al.* have reported the use of Tm^{3+} and Yb^{3+} codoped UCNPs as NIR-activated photorelease of caged compounds.^[115] However, both of the two examples were conducted in organic environments that are not suitable for biological

purpose. In order to further study the NIR-to-UV strategies in biomedical environment, Zhao's team has used UCNPs-based micelles to release the entrapped hydrophobic molecules in aqueous solution.^[21c] An alternative for drug delivery is silica-coated UCNPs because of the biocompatibility and photostability of the silica shell (Figure 1-12). For example, the unique dimensional mesoporous silica coated UCNPs which were used for remote control of siRNA release were demonstrated by Zhang *et al.*^[116] The photocaged siRNA or photocaged plasmid was incorporated in the pores shell of mesoporous silica-coated UCNPs. These caged molecules would be photoreleased by the upconverted UV light emitted from UCNPs by 980 nm light irradiation, which is shown to be successful in living cells assay.

1.4 Silica-coated NPs in bioimaging applications

Functionalized silica-based NPs are commonly used for optical imaging, MRI and combination of both techniques. Optical imaging is a primarily used tool for research in cells and animal models. With easy functionalization of the surface, silica-based NPs can be labeled with fluorescent dyes or drug molecules with intrinsic fluorescence properties like Dox. On the other hand, some kinds of nanomaterials with excellent optical properties, such as QDs and UCNPs, can be incorporated into silica shell for optical imaging studies.

MRI is currently one of the widely considered *in vivo* imaging technologies because of its advantages of non-invasive nature and high-resolution of three-dimensional images. Recently, the use of silica-coated magnetic NPs or Cd-doped shell of silica-coated NPs for MRI application provides several other advantages like enhancement of the stability and the effective relaxivity of contrast agents.^[3a, 91]

1.4.1 Optical imaging

Fluorescent dye molecules doped silica nanoprobe are commonly used for optical imaging. The first work was presented by Van Blaaderen and co-workers that using FITC modified silane to form the target fluorescent nanomaterials.^[117] Kang *et al.* have synthesized FITC-doped silica-coated magnetic NPs *via* modified Stöber method which were further functionalized with polymer brushes.^[118] One of the famous fluorescent silica-based nanoprobe is the so-called C-dots which were developed by Wiesner and co-workers in 2005. The formed silica core-shell NPs have excellent optical properties like high quantum yield and photoresistance towards bleaching.^[63, 119] Besides normal fluorescent dyes, the novel fluorescent metal complexes such as Ir complex and $[\text{Ru}(\text{bpy})_3]^{2+}$ can also be doped into silica NPs for bioimaging applications.^[76, 120] NIR dye-doped silica NPs have received much attention since they can be “friendly” used *in vivo* imaging systems. For example, the ICG-doped mesoporous nanoparticles have been used for cancer imaging and monitoring of their *in vivo* distribution.^[64]

Another commonly used approach for fluorescence imaging is by using silica-coated the NPs which have optical properties. The highly fluorescent QDs have emerged as promising probes for cell or animal imaging.^[121] Ying *et al.* has used functionalized silica-coated QDs for living cell imaging. The surface of silica shell was modified with bioanchored membrane (BAM) which was used for targeting cell membrane, thereby imaging the cells.^[122] In addition, the investigation of silica coated CdSe/ZnS QDs NPs interacting with neural cells has also been studied by Bardi and co-workers.^[123]

Despite the promising prospects for optical imaging, the application of some of these nanomaterials is somewhat limited due to their poor photostability, short excitation

wavelength or cytotoxicity. The use of silica-coated UCNPs is such a better choice for bioimaging due to the combined advantages that are biocompatibility from silica shell and high photostability from UCNPs.^[20b, 124] Many studies have reported that silica-coated UCNPs with functionalization of specific molecules, such as antibody and folic acid, could be used for bioimaging in living systems. For example, thin silica-coated NaYF₄: Yb/Er UCNPs have been functionalized with the rabbit anti-CEA8 antibody to form antibody-UCNP conjugates which were further used as fluorescent biolabels for HeLa cells imaging.^[125] Similarly, Li and co-workers have synthesized silica-coated UCNPs with organic dye-impregnated shells. Folic acid (FA) was attached on the surface for targeting the cells with folate receptor overexpression. These FA-conjugated NPs have been used for UC luminescence imaging and down conversion imaging.^[126] Jiang *et al.* synthesized silica-coated UCNPs, which were also modified with antibody named anti-Her2 for cell imaging and siRNA target delivery.^[127] In order to further study the siRNA delivery using silica-coated UCNPs, the same group has labeled siRNA with BOBO-3 intercalating dye which would generate lanthanide resonance energy transfer (LRET) with UCNPs. By measuring LRET, the intracellular siRNA release process was tracked.^[128] Zhang and co-workers have used mesoporous silica coated UCNPs for imaging and PDT to cancer cells.^[116]

1.4.2 MR imaging

In the process of MR imaging, there are two main types of contrast agents: 1) the T₁ (longitudinal relaxation time) MRI contrast agents, paramagnetic complexes such as manganese oxide (MnO), Mn³⁺ and Gd³⁺ iron complex; 2) the T₂ (transverse relaxation time) MRI contrast agents, like superparamagnetic iron oxide NPs. Many studies incorporating magnetic NPs or other contrast agents into silica NPs for MRI

have been reported recently.^[6b, 91] The silica-coated iron oxide NPs are commonly studied core-shell nanomaterials for *in vitro* and *in vivo* MRI applications. Hyeon

Figure 1-13. a) TEM images of mesoporous silica-coated magnetic nanoparticles with different size. b) *In vivo* T_2 -weighted MRI and c) *In vivo* fluorescence images of injected cells labeled with substrates. d) *In vivo* T_2 -weighted MRI images of the tumor. Reprint with the permission.^[78]

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group has reported the use of uniform mesoporous silica coated Fe_3O_4 NPs for imaging and therapy (Figure 1-13). The average size of the core-shell NPs can be controlled to be smaller than 100 nm in order to be easily internalized by cells. In addition, the NPs consist of superparamagnetic iron oxide NPs core and mesoporous silica shell which allowed them to be used as both MRI contrast agents and drug delivery vehicles.^[78]

Although the coating with silica shell will facilitate magnetic NPs stability, it has been proved experimentally that the coating thickness will affect the saturation

magnetization of the core-shell NPs.^[3a, 129] This weakness can be overcome by clustered magnetic NPs which can enhance the T_2 contrast

Figure 1-14. TEM images of a) magnetic NPs clusters; b) iron oxide NPs-mesoporous silica NPs.

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effect (Figure 1-14a). For instance, single-iron oxide NP-containing micelles have an r_2 relaxivity of $84 \text{ Fe mM}^{-1}\text{s}^{-1}$, while multiple-iron oxide NPs-containing micelles have an r_2 of relaxivity up to $345 \text{ Fe mM}^{-1}\text{s}^{-1}$.^[130] When the NPs were assembled on silica NPs template, the higher iron oxide NP density complex displayed about 2.5 times higher T_2 relaxivity than single iron oxide NPs.^[131] Another approach to enhance MRI contrast is decoration of numerous iron oxide NPs on the surface of mesoporous silica NPs based on covalent bond (Figure 1-14b). The as-synthesized iron oxide NPs-mesoporous silica NPs showed remarkably enhanced r_2 relaxivity value by 2.8 times compared to iron oxide NPs which have been successfully used for *in vivo* MR imaging at the tumor site.^[132]

Conjugated with MRI contrast agents, silica-coated UCNPs can also be used for *in vivo* multimodal imaging. Idris *et al.* have shown that Gd-based MRI contrast agents could be encapsulated into silica shell coated UCNPs to serve as dual-functional contrast agents for both optical and MR imaging.^[133] Recently, Li and co-workers

synthesized upconversion nanophosphors on the basis of silica-coated UCNPs. The functionalized core-shell NPs were used for NIR upconversion luminescence imaging in living cells and small animals. Furthermore, the particles were modified with Gd³⁺ complex and the *in vivo* optical and MR imaging capability of this platform was demonstrated.^[77]

1.5 Research goals and thesis organizations

As discussed above, nanomaterials have been increasingly applied in the biomedical field in recent years. Development of new kinds of biospecific or multifunctional nanomaterials has received a great deal of attention. In our study, we have focused on the silica-coated nanoparticles including magnetic Fe_3O_4 NPs and UCNPs and further studied their biomedical applications in drug/siRNA delivery, MRI and optical imaging. The organizations of the thesis are as following:

Chapter 1 is the introduction of this dissertation, and provides a summary of the research background knowledge.

In chapter 2, uniform silica-coated magnetic NPs which were doped with Dox-peptide moieties were presented. The magnetic iron oxide NPs were incorporated into silica shell using Stöber method and the as-synthesized core-shell NPs were further functionalized with azide groups for further use. The enzyme-cleavable Dox-peptide substrate was covalently tethered onto the NPs surface through click chemistry. The cathepsin B enzyme-responsive nanoparticle conjugates exhibited highly efficient drug release in the tumor cells with high enzyme expression. In addition, the drug-loaded nanoparticle complexes demonstrated multifunctional applications for real-time monitoring of intracellular drug release, tumor cell fluorescence imaging and MR imaging.

Light-induced drug and gene delivery systems have been introduced as a promising option for specific therapy due to their non-invasive property. One of the commonly used light-based strategies for drug release is photocage or photolysis. In the next chapters, we studied the silica-coated UCNPs for the use of NIR light induced therapeutic molecules release base on photocage.

In chapter 3, a system for uncaging photocaged molecules by using silica-coated UCNPs was developed. One commonly used bioluminescent probe, D-luciferin was bound to the particle platform through the photocaged linker. Upon irradiation with NIR light, D-luciferin would be uncaging by the upconverted UV light emitted from UCNPs. This method takes advantage of NIR light to trigger the caged molecules release which provides the advantages of deep light penetration and low cellular damage.

In chapter 4, the NIR light-responsive drug delivery system is extending to the use of gene delivery. Naked siRNA cannot across cellular membrane freely because of highly negative charges which limits its utility for gene therapy. Modified silica-coated UCNPs were used as siRNA delivery nanocarriers. The particles were functionalized with cationic photocaged linker and siRNA subsequently was adsorbed on the particles surface through electrostatic attractions. The upconverted UV emission from UCNPs could be used to initiate the release of siRNA by NIR light exposure, which resulted in effective gene silencing for EGFP expression.

Chapter 5 will focus on mesoporous silica coated UCNPs for NIR light-responsive drug delivery. The drug molecules were encapsulated in the pores of silica shell and covered with cross-linked photocaged linkers. The caps could be opened with the UV emission from UCNPs upon NIR light irradiation, showing great potentials for light controllable drug delivery in living cells.

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Chapter 2

Enzyme Responsive Multifunctional Magnetic Nanoparticles for Tumor Intracellular Drug Delivery and Imaging

2.1 Introduction

Multifunctional nanoparticles and their potential biomedical applications in therapy and diagnosis have received considerable attention.^[1] Among various colloidal nanostructured materials, superparamagnetic iron oxide nanoparticles (Fe-NPs) have been of great interest due to their easy preparation, biocompatibility and magnetic properties.^[1-2] Currently, Fe-NPs have been extensively employed in cell targeting, bioseparation and magnetic resonance imaging (MRI) *in vitro* and *in vivo*.^[1c, 1d, 3] Tremendous efforts have also been conducted to broaden the applications of these nanoparticles for the controlled delivery of antitumor drugs.^[4] For example, immobilization of Fe-NPs on mesoporous silica nanoparticles has been developed and the surface adsorption of anticancer drugs in the prepared silicon-Fe-NPs complex supplied a unique platform to achieve MRI detection and deliver drug to the tumors.^[4b-d, 4i] Similarly, incorporation of anticancer drug and magnetic nanoparticles within polymer or micelle structure has also been investigated to efficiently perform the magnetically-guided drug release and effective tumor imaging *in vitro* and *in vivo*.^[4f-h] However, despite their significant success in molecular imaging and drug delivery, the release of drug moieties from these complexed silica magnetic or magneto-polymeric nanohybrids was normally achieved through a passive diffusion mechanism which could potentially undermine the targeting efficiency and specificity of the nano-bioconjugates in the living system. Development of a simple, stable and

Scheme 2-1. Schematic illustration of synthesis of Dox-SMNPs and their cellular functions for drug release and tumor imaging.

covalently drug-conjugated nanoparticle hybrids system which would allow the more selective drug release to targeted locations upon specific biological stimuli and meanwhile also enable the real-time monitoring of drug delivery at single cell level is still highly desirable and remains a challenge in the field.

Herein, we present the rational design and development of a novel enzyme-responsive Doxorubicin (Dox)-peptide coated silica-magnetic nanoparticles (SMNPs) conjugate for selectively triggered intracellular delivery of Dox, a commonly used lipophilic antitumor drug into the tumor cells with specific protease enzyme expression. This nanoparticles conjugate also provides the opportunity to real-time monitor the cellular trafficking of Dox molecules in the living conditions in combination of MRI and fluorescence imaging techniques.

2.2 Results and discussion

As proof-of-concept, we designed the uniform silica coated magnetic nanoparticles (SMNPs) complex, which have been tethered with Dox-peptide substrate moiety. Dox-peptide prodrug was synthesized through the coupling the anticancer drug Dox with a peptide sequence H-Phe-Lys-OH under solution phase conditions (Scheme 2-1). A self-immolative *p*-aminobenzyloxycarbonyl (PABC) linker was required to combine the Dox with the dipeptide sequence. Typically, the dipeptide sequence of H-Phe-Lys-OH can be selectively recognized and cleaved by cathepsin B, a ubiquitous intracellular cysteine protease over-expressed in a variety of malignant tumors,^[5] which has been usually applied as an important target for tumor cell imaging and prodrug development.^[6] Upon the cathepsin B enzyme cleavage, an *p*-aminobenzyloxycarbonyl-Dox (PABC-Dox) intermediate will be exposed from the Dox-peptide substrate, followed by 1, 6-elimination and decarboxylation, the immolative PABC linker was hydrolytically decomposed and therefore released free Dox antitumor from the Dox-peptide conjugate. This enzyme responsive drug release was further confirmed by HPLC analysis (Figure 2-1a). The incubation of Dox-peptide substrate with cathepsin B resulted in an efficient enzyme cleavage over a period of 5 hr. At 485 nm, the peak at 18.2 min that corresponded to Dox-peptide substrate significantly decreased in the presence of enzyme for 5 hr, meanwhile, a new enzymatic product peak with retention time of 13.9 min increased accordingly which corresponded to the free Dox drug molecule. The chromatographic results clearly indicated that it was the enzyme mediated reaction that led to the effective release of free drug from the Dox-peptide substrate.

The silica-magnetic nanoparticle complex was synthesized by Stöber method.^[7]

Figure 2-1. a) HPLC analysis of enzymatic reaction of Dox-peptide substrate with cathepsin B. Absorbance wavelength: 485 nm. b) UV absorbance of Dox-peptide Substrate, before and after “click chemistry” reaction.

Hydrophilic iron oxide particles were first prepared in an aqueous phase. Silane-containing amine functional groups were then used to coat the iron oxide particles to form core-shell silica-magnetic structure. This core-shell silicon coated structure exhibited the advantages to stabilize the nanoparticles suspension in biological system and to enable the easy surface modification for further practical applications.^[4i, 8] In order to achieve the effective peptide prodrug loading onto the surface of the as-prepared nanoparticles and also to improve the dispersion stability of drug coated nanoparticles in aqueous solution, the Dox-peptide was modified with an alkynyl group and an azido-oligo (ethylene) glycol (dPEG₄) linker was coupled to the silica-coating magnetic nanoparticles.^[4a, 4i, 9] The alkynyl-peptide prodrug can efficiently conjugate with azido-dPEG₄ modified nanoparticles through [2+3] cycloaddition to produce the uniform drug loaded functional SMNPs (Scheme 1). The loading amount of Dox-peptide substrate on the surface of SMNPs was determined by the difference of UV absorbance spectrum (Figure 2-1b) and the optimal drug loading content of Dox to SMNPs was about 5 %, which is comparable with the results

Figure 2-2. Characterization of synthesized SMNPs. a) The size distribution of SMNPs determined by dynamic light scattering and TEM image (inset, scale bar = 20 nm). b) The specific relativity value (r_2) of SMNPs and commercially available Feridex nanoparticles.

reported previously.^[4b, 4f, 4i] The hydrodynamic diameter and size distribution of the prodrug conjugated SMNPs were then characterized by laser dynamic light scattering (DLS) and transmission electron microscopy (TEM). The results indicated the narrow size distribution of the prodrug conjugated SMNPs with an overall diameter of 50 nm (Figure 2-2a), which would be the most suitable size to achieve the effective cell penetration.^[1e, 10] The magnetic property of the Dox-conjugated SMNPs was verified by measuring transverse (T_2) relaxation times of dispersed nanoparticles on a 7.0 -T MRI equipment. T2-weighted MRI results indicated the significant signal changes at various concentrations of particles. The specific relaxivity value (r_2) was calculated to be 136 $mM^{-1} s^{-1}$, which is close to the value as determined by using the commercially available Fe_3O_4 nanocrystal feridex (142 $mM^{-1} s^{-1}$) (Figure 2-2b). Moreover, this Dox-coated SMNPs (Dox-SMNPs) conjugate exhibited good biocompatibility and was stable in buffer solution for more than one month without any changes, which enabled the Dox-SMNPs conjugate to be a stable and effective contrast agent for

Figure 2-3. a) Dox release upon cathepsin B (33 nM) enzymatic reaction with Dox-SMNPs (100 μ M) at different times (0~6.0 hours) ($\lambda_{\text{ex}} = 485$ nm, $\lambda_{\text{em}} = 590$ nm); b) Fluorescence enhancement of Dox-SMNPs when treated with cathepsin B in the absence and presence of enzyme inhibitor, antipain hydrochloride.

subsequent multifunctional applications in targeted drug delivery and intracellular tumor imaging.

The activity of Dox-SMNPs towards enzyme reaction was examined through detecting the fluorescence of Dox. Briefly, free Dox exhibited a fluorescence emission at 590 nm with an excitation at 485 nm. The fluorescence of Dox would be quenched when the drug modified peptide sequence was covalently tethered on the surface of SMNPs as the results of electronic energy transfer.^[4f] As expected, upon the incubation of Dox-SMNPs with protease cathepsin B at 37 °C in acetic buffer solution (pH = 5.0), sequential fluorescent enhancement in the intensity of native spectrum of Dox was observed corresponding to the release of drug molecules from Dox-SMNPS and approximately 80 % of free drug could be released within 6 hours. The drug release was also monitored in the buffer solution without enzyme treatment and there was no significant fluorescent enhancement observed, indicating the good stability

Figure 2-4. Confocal laser scanning microscopic images of a) NIH/3T3 cells incubated with 5.0 μ M Dox-SMNPs at 37 °C for 2 hours, b) HT-29 cells incubated with 5.0 μ M Dox-SMNPs at 37 °C for 2 hours, c) Inhibitor antipain hydrochloride pretreated HT-29 cells, incubated with 5.0 μ M Dox-SMNPs at 37 °C for 2 hours. (λ_{ex} = 535nm/50nm, λ_{em} = 610nm/75nm). And graph d): Fluorescence intensity of Dox in cell lysate shown in panels a)~c). Substrate Dox-SMNPs was chosen as control.

and minimal nonspecific drug release from Dox-SMNPs conjugate (Figure 2-3a). Similar drug release measurements were also conducted in the presence of a potent cathepsin B inhibitor, antipain hydrochloride. The enzyme activity was decreased dramatically and only limited fluorescence enhancement could be detected as shown in Figure 2-3b, clearly demonstrating the effective Dox release from Dox-SMNPs upon the specific cathepsin B enzyme interactions.

The intracellular uptake and distribution of the drug-conjugated SMNPs complex was monitored by a fluorescent imaging technique. In this study, the cancer cell line HT-29 which highly expresses protease cathepsin B was chosen as a positive target cell line.^[6b] As a negative control, NIH/3T3 cell was used since there is no cathepsin B expression in this cell line. Typically, both HT-29 cells and NIH/3T3 cells were cultured and incubated with 5.0 μ M of drug-conjugated SMNPs in Dulbecco's Modified Eagle Medium (DMEM) at 37 °C for 2 h to obtain an effective live cell

Figure 2-5. Confocal laser scanning microscopic images of HT-29 cells incubated with Dox-SMNPs (5.0 μ M) at 37 $^{\circ}$ C for different time points.

fluorescent imaging. After 2 hours incubation, a bright red fluorescence signal was observed mainly in the cytoplasm and not in the cell nuclei of HT-29 cells (Figure 2-4b), probably owing to the involvement of endocytosis during the uptake of Dox-SMNPs.^[4b-e, 4i, 11] As contrast, there was no obvious fluorescence detected in NIH/3T3 cells (Figure 2-4a) and HT-29 cells pretreated with the cathepsin B enzyme inhibitor antipain hydrochloride (Figure 2-4c and 2-4d). The results clearly indicated

Figure 2-6. Confocal laser scanning microscopic images of HT-29 cells incubated with Dox-SMNPs (5.0 μ M) and free Dox at 37 °C for 2h.

that Dox-SMNPs could easily enter into the intracellular cytoplasm and Dox could be selectively released upon the reaction of drug-conjugated SMNPs with the specific enzyme in the target cell lines. Longer time incubation (e.g., ≥ 72 h) of the drug-SMNPs complex in the targeted cell would lead to the red fluorescence accumulated more in the cell nuclei than in cytoplasm (Figure 2-5), demonstrating the dynamic process of the drug release from the Dox- conjugated SMNPs complex into the target HT-29 cells, in which enzyme cleaved Dox was firstly released to the cytoplasm and then eventually diffused into the cell nuclei where Dox is known to react with topoisomerases to induce DNA damage and cytotoxicity.^[11b-f] As a control, the cellular imaging measurements were also conducted by incubating the cell lines with free Dox (Figure 2-6). Upon two hours incubation, a strong fluorescence was observed in the cell nuclei and relatively weak fluorescence in the cytoplasm, indicating the different intracellular uptake between free Dox and drug-conjugated SMNPs. Compared to the Dox-SMNPS, the passive diffusion of free Dox towards cell nuclei would lead to the faster accumulation of the drug by intercalating into the DNA structure.^[4b-e, 4i, 11]

The similar cellular uptake of Dox-SMNPs was also evaluated by MRI technique. The Dox-SMNPs at various concentrations were incubated with HT-29 cells for 2 hours.

Figure 2-7. a) T2-weighted images of Dox-SMNPs in HT-29 cells. b) *In vitro* cellular uptake study of HT-29 cells after 2h incubation.

After the cells were washed with phosphate buffered saline (PBS) and residual nanoparticles in solution were removed, the cells were evaluated by T2-weighted MRI. The signal intensity of the MRI decreased owing to the presence of the accumulation of SMNPs and the signal was also getting darker with the increasing concentration of Dox-SMNPs as showed in Figure 2-7a. The intracellular uptake of SMNPs and Dox-SMNPs were further studied by Inductively Coupled Plasma (ICP) (Figure 2-7b). ICP analysis for the concentrations of iron confirmed the efficient cellular penetration of various concentrations of iron oxide nanoparticles and about 60% multifunctional nanoparticles were taken up by the HT-29 cells. The similar percentage of the magnetic nanoparticles uptake was also found in the control NIH/3T3 cells. These results clearly showed that Dox-SMNPs could easily enter into the cellular structures to work as an effective platform to monitor the drug release in the different cell lines.

Figure 2-8. Cell viability assay of a) HT-29 and b) NIH/3T3 cells with free Dox, SMNPs and Dox-SMNPs.

In order to evaluate the cytotoxicity and antitumor activity of Dox-SMNPs in living cells, cell viability was examined by standard MTT assay.^[11b-f] In this typical study, both HT-29 and NIH/3T3 cancer cell lines were chosen to incubate with 5.0 μ M of Dox- SMNPs, free Dox and SMNPs, respectively at different time intervals. As shown in Figure 2-8, incubation of free particle SMNPs with HT-29 and NIH/3T3 cells could not lead to obvious cytotoxicity to both of cells, indicating the little toxicity caused by the as-prepared nanoparticles itself. However, upon the incubation of Dox-SMNPs with HT-29 cells, the significant reduced cell viability could be observed. Compared to HT-29 cells, limited change in the cell viability was detected in NIH/3T3 cells. It demonstrated that Dox-SMNPs could selectively interact with HT-29 cancerous cells which have high expression of cathepsin B enzyme and thus specifically release the

anticancer drug into the target cells. As a commonly used anticancer drug, free Dox usually showed potent anticancer activity towards both HT-29 and NIH/3T3 cancer cell lines (Figure 2-8). But compared to free Dox, drug conjugated SMNPs complex displayed different cytotoxic effects towards HT-29 and NIH/3T3 cancer cells. In HT-29 cell, free Dox indicated a bit higher cytotoxicity than that of Dox-SMNPs during short time (e.g., 2h) incubation with the cells, most likely due to the faster drug accumulation of free Dox inside the cell nuclei as observed in the cell imaging measurements. The comparable cytotoxicity between free Dox and Dox-conjugated SMNPs could be detected after a prolonged incubation time with the HT-29 cells, the slightly higher cell viability of Dox-SMNPs could correspond to the efficient drug delivery property of nanoparticles and their potent functions to circumvent the multidrug resistance effect, one situation in which free antitumor drugs could be pumped out from target cells and thus inevitably decreased the therapeutic effect.^[12] In the control NIH/3T3 cell experiments, there was limited cytotoxicity of Dox-SMNPs observed in all the time points cell incubation, further confirming that Dox-SMNPs would selectively release the drug molecules and improve the drug activity significantly in the tumor cells with high cathepsin B expression, whereas the drug conjugated nanoparticle complex exhibited the lower cytotoxicity towards other cells without enzyme expression.

2.3 Conclusions

In conclusion, an enzyme-responsive multifunctional Dox-SMNPs complex has been synthesized based on “click chemistry”. This stable and non-toxic drug SMNPs conjugate exhibited the selective controlling for the release of antitumor drug

molecules in living cells in which the specific enzyme has been highly expressed. This drug loaded nanoparticle complex demonstrates the promising properties in real-time monitoring of the intracellular drug release and tumor cell imaging in combination of fluorescent imaging and MRI techniques. The multifunctional drug-coated hybrid nanoparticles provide the possibility for their potential applications in tumor-targeted drug delivery and simultaneous diagnosis or detection of therapy intervention.

2.4 Experiment section

Materials: All the Fmoc amino acids were from Bachem or GLchem. Other chemical reagents were purchased from Aldrich or Fluka. Cathepsin B and its inhibitor, Antipain hydrochloride, were purchased from Sigma-Aldrich. Azido-dPEG₄ NHS ester linker was purchased from Quanta Biodesign. Commercially available reagents were used without further purification. Anhydrous solvents for organic synthesis were purchased from Aldrich and stored over activated molecular sieves (4 Å). Thin-layer chromatography (TLC) was performed on precoated silica gel 60F₂₅₄ glass plates.

Instruments: ¹H NMR and ¹³C NMR spectra were recorded using Bruker 300. Mass spectra (MS) were measured a Thermo Polaris Q for EI and Thermo LCQ Deca XP MAX for ESI. HPLC analysis was performed on a reverse-phase column with a Shimadzu HPLC system. Analytical reverse-phase high performance liquid chromatography (HPLC) was performed on Alltima C-18 column (250 × 3.0mm) at a flow rate of 1.0 mL/min and semi-preparative HPLC was performed on the similar C-18 column (250 × 10mm) at a flow rate of 3 mL/min. UV absorption spectra were recorded in a 5-mm path quartz cell on a Beckman coulter DU800 spectrometer.

Scheme 2-2. Scheme of synthesis Dox-peptide substrate. a) HOSu, EDC, THF, 0 °C to rt; b) Piperidine, DMF, rt; c) Et₃N, THF/DMF, rt; d) PABOH, EEDQ, DCM, rt; e) Diethylamine, DCM, rt; f) 4-Pentynoic acid, HOSu, EDC; Et₃N, THF, 0 °C; g) Bis-PNP carbonate, DIPEA; h) Dox.HCl, Et₃N, DMF, rt; i) 0.5% TFA, DCM, 0 °C

Fluorescence emission spectra were performed on a Varian Cary eclipse Fluorescence spectrophotometer. TEM images were collected from an FEI EM208S Transmission Electron Microscopy (Philips) operated at 120 kV. Fourier transform infrared spectra (FTIR) were recorded on Shimadzu Iprestige-21 FTIR spectrophotometer. The size distribution of SMNPs was performed using photon correlation spectroscopy (Brookhaven Instruments Corporation).

Synthesis and characterization of Dox-peptide substrate**Preparation of H-Phe-Lys(Mtt)-OH (3)**

Fmoc-Phe-OH (315 mg, 0.80 mmol) was dissolved in THF (5 ml) at 0 °C to form a colourless solution. The solution was then treated with N-Hydroxysuccinimide (HOSu) (96.5 mg, 0.83 mmol) and subsequently, 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (159.5 mg, 0.83 mmol), was added into the mixture and allowed to stir for 20 hours. The generated product 1 was used in the next step synthesis without further purification.

A stirred solution of Fmoc-lysine (Mtt)-OH (500 mg, 0.80 mmol) in DMF (N, N-methyl formamide) (2 ml) at room temperature was treated with piperidine/DMF (2 ml, 40%) for 2 hours. The mixture was added to cold diethyl ether to obtain precipitate, then centrifuged and washed by diethyl ether (3 × 10 ml). A white precipitate 2 was collected and dried *in vacuo*. A mixture of 2 was dissolved in DMF (5 ml) and triethylamine (TEA) (115 µl) was slowly added into the mixture of solution. 1 was slowly added into the mixture from its crude product in THF to form a colorless solution. The reaction mixture was allowed to continue stirring at room temperature for 22 hours. The mixture was then dry under rotary evaporation and extracted with ethyl acetate (2 × 20 ml), washed by water (2 × 20 ml) and brine (20 ml). The crude product was purified with flash chromatography with eluent DCM and methanol (20:1) to afford white precipitate 3 (290 mg, 70.1%). ¹H NMR (300 MHz; [D₆]DMSO, 25 °C, TMS): δ=1.30 (2H, s), 1.45 (2H, s), 1.60 (2H, s), 1.95 (2H, m), 2.21 (3H, s); 2.80 (1H, t, *J* 12.90), 3.03 (1H, d, *J* 9.00), 4.05~4.33 (5H, m), 7.01~7.38 (23H, m), 7.74 (2H, d), 7.85 (2H, d), 8.08 (1H, br-s). MS (70eV): *m/z*: calcd for C₅₀H₄₉N₃O₅: 771.37; found: 772.06 [M+H].

Preparation of H-Phe-Lys(Mtt)-PABOH (5)

A solution of 3 (290 mg, 0.376 mmol) and *p*-aminobenzyl alcohol (PABOH) (55.5 mg, 0.451 mmol) in anhydrous DCM (8 mL) was treated at room temperature with 2-ethoxy-1-ethoxycarbonyl-1, 2 dihydroquinoline (EEDQ) (139.5 mg, 0.564 mmol). The mixture was then stirred for 20 hours. Finally, the mixture was concentrated at reduced pressure and washed with cold diethyl ether (3 × 5 ml). A white solid residue 4 (329 mg, 99.0%) was formed and was used for the next step reaction without any further purification. *m/z*: calcd for C₅₇H₅₆N₄O₅: 876.43; found 876.85 [M+H]⁺.

A solution of 4 (329 mg, 0.376 mmol) in DCM (8 ml) at room temperature was treated with diethylamine (16 ml). The mixture was allowed to stir at room temperature for 2 hours. The solvent was evaporated and was purified with column chromatography MeOH:DCM (20:1) to afford a white solid 5 (198.9 mg, 81.1%). ¹H NMR (300 MHz; [D₆]DMSO, 25°C, TMS): δ=1.21 (2H, s), 1.36 (2H, s), 1.90 (2H, t), 1.90 (2H, m), 2.13 (2H, m), 2.22 (3H, s), 2.74 (1H, dd, *J* 9.0, 13.5), 3.21 (1H, dd, *J* 3.5, 13.5), 3.64 (1H, d, *J* 3.6, 9.0), 4.60 (3H, m), 7.03 (2H, m) 7.12~ 7.45 (21H, m), 7.95 (1H, d, *J* 8), 9.16 (1H, s). ¹³C NMR (75 MHz; [D₆]DMSO, 25°C, TMS): δ=175.3, 170.0, 146.4, 143.2, 137.3, 137.2, 136.8, 135.7, 129.2, 128.8, 128.6, 128.5, 127.7, 127.6, 127.0, 126.1, 120.0, 70.6, 64.8, 60.4, 56.2, 53.8, 43.4, 40.8, 31.8, 30.7, 29.7, 23.5, 21.1, 20.9, 14.2. *m/z* : calcd for C₄₂H₄₆N₄O₃: 654.36; found 654.87 [M+H]⁺.

Preparation of Alkynyl-Phe-Lys(Mtt)-PABOH (6)

4-Pentynoic acid (500 mg, 5.09 mmol) was dissolved in THF (5 ml) at 0 °C to form a colorless solution. The solution was then treated with HOSu (591.8 mg, 5.09 mmol) and subsequently EDC (974.7 mg, 5.09 mmol) was added into the mixture and stirred for 22 hours. The product was used directly for the next step synthesis without further purification. 5 (198.9 mg, 0.304 mmol) was dissolved in THF (5 ml) and 4-pentynoic acidsuccinimide ester (118.6 mg, 0.608 mmol) was added dropwise into the mixture

under ice bath. The mixture was then treated with TEA (50 μ l) and stirred overnight. The mixture was then purified with flash chromatography with MeOH and DCM as the eluent with gradient (20:1) to afford a yellow precipitate **6** (156.3 mg, 70.0%). ^1H NMR (300 MHz; $[\text{D}_6]\text{DMSO}$, 25°C, TMS): δ =1.24~1.69 (6H, m), 1.96 (2H, m), 2.24 (3H, s), 2.41 (2H, t, J 3.00), 2.69~2.80 (3H, m), 3.04 (1H, d, J 9.84), 4.38~4.57 (4H, m), 7.05~7.53 (21H, m), 8.11 (2H, m), 9.94 (1H, s). ^{13}C NMR (75 MHz; $[\text{D}_6]\text{DMSO}$, 25°C, TMS): δ =171.6, 170.8, 170.7, 146.9, 143.7, 138.2, 138.0, 137.9, 135.4, 129.7, 128.8, 128.7, 128.4, 128.1, 127.4, 126.6, 126.4, 119.5, 84.1, 71.8, 71.7, 70.6, 63.0, 54.3, 54.0, 43.8, 38.3, 34.6, 32.6, 30.2, 24.3, 20.5, 14.8. m/z : calcd for $\text{C}_{46}\text{H}_{48}\text{N}_4\text{O}_4$: 734.38; found 734.97 $[\text{M}+\text{H}]^+$.

Preparation of Alkynyl-Phe-Lys(Mtt)-PAB-PNP (**7**)

Compound **6** (156.3 mg, 0.213 mmol) was dissolved into DCM (7 ml) and Bis-PNP carbonate (325.1 mg, 1.07 mmol) was added into the mixture. Next, *N,N*-Diisopropylethylamine (DIPEA) (50 μ l) was added into the reaction mixture and allowed to stir vigorously at room temperature for 2 days. After the spot of starting material of **6** was disappeared from the TLC plate, the mixture was concentrated under reduced pressure and purified with flash chromatography with eluent ethyl acetate and hexane (1:1). A white precipitate product was collected as the final product **7** (174.2 mg, 91.0%). ^1H NMR (300 MHz; $[\text{D}_6]\text{DMSO}$, 25°C, TMS): δ =1.31~1.45 (4H, m), 1.63 (2H, m), 1.87 (1H, s), 2.06 (2H, m), 2.23 (3H, s), 2.47 (4H, m), 3.09 (2H, d, J 6.00), 4.68 (1H, q, J 6.00), 5.02 (1H, dd, J 6.0, 9.0), 5.29 (2H, s), 7.01~7.45 (23H, m), 7.63 (2H, d, J 8.00), 8.25 (2H, d, J 9.00), 9.18 (1H, s). ^{13}C NMR (75 MHz; $[\text{D}_6]\text{DMSO}$, 25°C, TMS): δ =171.5, 171.2, 170.1, 155.5, 152.5, 146.3, 145.4, 143.2, 138.6, 136.0, 135.7, 130.2, 129.7, 129.3, 128.6, 128.5, 128.4, 127.7, 126.1, 125.3, 121.8, 120.6, 82.8, 70.7, 70.6, 69.8, 54.4, 43.4, 39.0, 35.2, 30.8, 29.7,

23.7, 20.9, 15.0. m/z: calcd for $C_{54}H_{53}N_5O_8$: 899.39; found 899.99 $[M+H]^+$.

Preparation of Dox-peptide substrate (9)

TEA (100 μ l) was added to a solution of **7** (174.2 mg, 0.193 mmol) and Dox.HCl (112.1 mg, 0.193 mmol) in 8 ml of dry DMF. The solution was stirred at room temperature for 18 h and was extracted with ethyl acetate (3×10 ml), water (2×3 ml) and brine (1×3 ml). After concentrating the mixture under reduced pressure, the mixture **8** was used for the next step without further purification. m/z: calcd for $C_{75}H_{77}N_5O_{16}$: 1303.54; found 1304.07 $[M+H]^+$.

The mixture of **8** was dissolved in of DCM (5 ml) and 1% of TFA in DCM (5 ml) was added in dropwise into the suspension to form a clear red solution.^[6b] The solution was stirred at room temperature for 4 hours and finally purified with HPLC. A red solid product **9** was obtained after lyophilization (81.1 mg, 40.7%). m/z: calcd for $C_{55}H_{62}N_5O_{16}$: 1047.41; found 1048.26 $[M+H]^+$.

Preparation of colloidal iron oxide nanoparticles

Scheme 2-3. Scheme of synthesis Dox-SMNPs of substrate.

The magnetic iron oxide nanoparticles were prepared via as described previously.^[8b] Typically, magnetic nanoparticles were prepared based on the chemical co-precipitation of Fe^{2+} and Fe^{3+} by adding a concentrated base, sodium hydroxide

Figure 2-9. FTIR spectra analysis of a) Azido-dPEG₄-NHS ester linker, b) Azido-dPEG₄ Functionalized SMNPs and c) free SMNPs.

(10 M) into the mixture of iron salts with a molar ratio ($\text{FeCl}_2/\text{FeCl}_3$) of 1:2. The mixture was stirred vigorously for 1 hour at room temperature and was further heated to 90 °C for another 1 hour. Trisodium citrate solution (0.3 M, 200 ml) was added in to the mixture and continued heating for another 30 minutes. The mixture was then cooled to room temperature and the magnetic particles were precipitated with acetone, the supernatant was decanted with a magnet. Water was then added to re-disperse the magnetic particles and sent for dialysis for one week.

Preparation of SMNPs

SMNPs were synthesized *via* Stöber method. The coating of the iron oxide with silica was carried out in ethanol and Milli-Q water in a ratio of (4:1). Iron oxide in Milli-Q water (1.5 wt %, 2 ml) was suspended in ethanol (160 ml) and Milli-Q water (40 ml) and follow by adding aqueous ammonia (25%, 5 ml). This suspension was then homogenized by ultrasonic vibration in a water bath for 15 minutes. Next, tetraethyl orthosilicate/3-(aminopropyl) triethoxysilane (10:1) (500 μl) was added dropwise into the mixture under continuous mechanical stirring. After 12 hours reaction, the mixture

was washed thoroughly with ethanol and dried *in vacuo* to obtain the desired iron oxide coated with silica (46.0 mg). The surface amino groups were quantified at 1.5 mmol/g by ninhydrin test.^[13]

Preparation of azido-dPEG₄ functionalized SMNPs

The azido-dPEG₄ functionalized silica coated iron oxide nanoparticles (Azido-dPEG₄ Functionalized SMNPs) was synthesized by dissolving the SMNPs (46.0 mg) into 2 ml of DMF. Next, Azido-dPEG₄ NHS ester (0.14 mmol) in DMF was added dropwise into the suspension. While under continuous stirring, TEA (20 μ l) was added into the mixture and stirred overnight at room temperature. The suspension was washed with ethanol 5 times and centrifuged at 1×10^4 rpm in order to obtain the supernatant. Azido-dPEG₄ functionalized SMNPs were analyzed by FTIR (Figure 2-9).

Preparation of Dox-SMNPs via click chemistry

The azido-dPEG₄ functionalized SMNPs were clicked with acetylene-functionalized Dox-peptide substrate by dissolving the azido-dPEG₄ SMNPs into water/tert-butanol (4 ml, 1:1). Then Dox-peptide substrate (3.1 mg, 0.003 mmol) was added into the mixture. Subsequently, ascorbic acid (200 mM, 100 μ l) and copper (II) sulfate (200 mM, 50 μ l) were added. The mixture was allowed to stir for 1 day at room temperature and was washed for 5 times with deionized water and centrifuged at 1×10^4 rpm in order to obtain the final product Dox-SMNPs, the supernatant and washed solution were collected. The loading amount of Dox-peptide substrate was calculated by the difference of UV absorbance spectrum ($\epsilon_{495}=8030 \text{ cm}^{-1} \text{ M}^{-1}$).^[4b, 4f, 4i, 14]

***In vitro* enzymatic assays**

Enzymatic activity for Dox-peptide Substrate The stock solution of cathepsin B was dissolved in 25 mM of acetate buffer/1 mM EDTA (pH=5.0). Dox-peptide substrate

Figure 2-10. Fluorescence enhancement for Dox-SMNPs, cathepsin B (33 nM) incubated with Dox-SMNPs in different concentration (0~100 μM).

was also dissolved in 25 mM of acetate buffer /1mM EDTA (pH=5.00) (0.5 % DMSO). Cathepsin B (10 μl) was first activated in 5 μl 60 mM DTT/15mM EDTA for 30 minutes under room temperature then incubated with 85 μl of Dox-peptide substrate for different times, the final concentration of Dox-peptide substrate and cathepsin B were 100 μM and 33 nM respectively. The enzyme reaction was analyzed by HPLC at wavelength 485 nm .

Enzymatic activity for Dox-SMNPs

The stock solution of cathepsin B was dissolved in 25 mM of acetate buffer/1mM EDTA at pH=5.0. Dox-SMNPs was similarly dissolved in 25 mM of acetate buffer /1mM EDTA at pH=5.0. Cathepsin B (10 μl) was first activated in 60 mM DTT/15 mM EDTA (5 μl) for 30 minutes under room temperature then incubated with 85 μl of Dox-SMNPs (concentration: 0 ~ 100 μM) at different time intervals (0 ~ 6 hours). The fluorescent enhancement was monitored by Fluorescence spectrophotometer with excitation wavelength at 485 nm (Figure 2-10).

In vitro enzyme inhibition assays

The enzyme cathepsin B was pretreated with inhibitor (100 μ M), antipain hydrochloride, for 2 hours at 37 °C, Then incubated with Dox-SMNPs (final concentration 100 μ M) for another 2 hours for fluorescent measurement.

Living cells assays

HT-29 cell line was bought from American Type Culture Collection (ATCC Cat No: HTB-38) and maintained in McCoy's 5a medium (ATCC, Manassas VA) containing 10% FBS (Invitrogen, Burlington, Canada. HT-29 cell line were seeded at a density of 2×10^5 in a 35 mm diameter μ -dish plastic bottom (ibidi GmbH, Germany) and cultured for 2 days in McCoy' 5a medium with L-Glutamine (ATCC). Control NIH/3T3 cell line was also bought from American Type Culture Collection (ATCC Cat No.: CRL-2795) and cultured with the same protocol as HT-29 except the medium used was Dulbecco's Modified Eagle Medium cell lines (DMEM).

Cell viability measurement^[12]

HT-29 was seeded in a cell density of 1×10^4 per well in a 96 wells plates in McCoy's 5a medium. The cells were cultured at 37°C for 2 days. Dox -SMNPs (5 μ M), Dox (5 μ M) and free SMNPs were incubated in McCoy's 5a medium in a range of (0 ~ 120 hours) respectively. At the end of the experiment, the cells were replaced with fresh McCoy's 5a medium and 1.0 mg/ml of MTT solution in medium was then added. The plates were incubated for 4 hours at 37C, then MTT -containing medium was removed and 100 μ l of DMSO was added to dissolve the formazan crystal formed by the living cells. Absorbance was measured at 560nm using a Bio-Tek EL-311 micro plate. The cell viability (%) was calculated according to the following equation:

$$\text{Cell Viability (\%)} = (\text{OD}_{560} (\text{sample}) - \text{OD}_{560} (0)) / (\text{OD}_{560} (\text{control}) - \text{OD}_{560}(0)) \times 100\%$$

Where the OD_{560} (sample) represents the measurement from the wells treated with

Figure 2-11. Fluorescence intensity of cell lysates after incubation of Dox-SMNPs (5.0 μ M) with HT-29 cells in the absence and presence of 5.0 μ M of enzyme inhibitor antipain hydrochloride at different time points. Excitation: 485 nm and Emission: 590 nm.

Dox-SMNPs, Dox and SMNPs and the OD560 (control) represents the measurement from the wells treated with 25 mM of acetate buffer /1mM EDTA at pH=5.0 in medium. OD₅₆₀ (0) represents the blank absorbance. Control NIH/3T3 cell lines were performed using the same protocol except with a different culture medium, DMEM.

Cell imaging

The living HT-29 and NIH/3T3 cells were cultured in 35 mm diameter μ -dish plastic bottom for 2 days and then cells washed twice with McCoy's 5a and DMEM respectively. The cells were treated with Dox-SMNPs (5 μ M) in McCoy's 5a medium (HT-29) and DMEM (NIH/3T3) and incubated for in a range of time (0 ~ 96 hours) at 37 °C. After washing twice with Hank's Balanced Salt solution (HBSS), the cell imaging was conducted under confocal fluorescence microscope (Nikon, Eclipse TE2000-E) with Excitation filter: 535/50 nm; emission filter: 610/75 nm.

Cell inhibition assay

The live HT-29 cell lines were washed twice with McCoy' 5a medium and pretreated

separately with 5 μ M inhibitor in McCoy' 5a: Antipain hydrochloride for the required time points (0.5 or 2 hours) at 37 $^{\circ}$ C. Dox-SMNPs was then added with the final concentration of 5 μ M and incubated for another 0.5 or 2 hours. The cells were washed twice with HBSS solution for the living cell imaging and cell lysates fluorescent measurement (Figure 2-11).

For the cell lysates fluorescent measurement, typically, the Dox-SMNPs incubated HT-29 cell lines (in the absence and presence of enzyme inhibitor) were treated with trypsin and washed three times by HBSS solution. Then the mixture underwent a cycle of freeze and thaw (water bath, 37 $^{\circ}$ C) for 5 times. The supernatant was then sonicated at 4 $^{\circ}$ C for 10 minutes and subsequently centrifuge at 10000 rpm for 10 minutes for the fluorescent detection at 590 nm.

MRI *in vitro* cellular uptake study

Phantom study: SMNPs were suspended in 0.5 % agarose gel in 300 μ l PCR tubes. The tubes were embedded in a home-made tank, which was designed to fit the MRI coil and was filled with 0.5% agarose gel. T2-weighted MRI images were acquired on a GE 7.0 T small animal MRI system with the following parameters: TR 3000 ms; TE 20, 40, 60, 80, 100, 120 ms; flip angle 30 $^{\circ}$; FOV 6 $\times$ 6, 256 $\times$ 256 matrix; slice thickness 1 mm.

The different cells were cultured in 10 cm culture dish in incubator. When cells reach 90% confluency, two kinds of particles, SMNPs and Dox-SMNPs with different iron concentration ^[5a] in culture medium without FBS were added and incubated for 2 h at 37 $^{\circ}$ C. After twice washing with PBS, the cells were collected and suspended in 150 μ l PBS and were sonicated for 2 hours. The sonicated cell lysates containing particles were suspended in 0.5% agarose gel in 300 μ l PCR tubes and phantom study were conducted as above procedure. After cell phantom study, the cell lysates were

dissolved in nitric acid and heated at 90°C for two hours. After heating, cell lysates were diluted in distilled water and added into sample tubes. The iron concentration was measured by Inductively Coupled Plasma (ICP) Spectrometer. Cell uptake of particles were calculated based on input Fe amount and remained amount in cells.

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Chapter 3

***In Vitro* and *In Vivo* Uncaging and Bioluminescence Imaging through Photocaged Upconversion Nanoparticles**

3.1 Introduction

High temporal and spatial regulation of cellular activities, biological pathways, and gene expression is critical for understanding complex biological processes.^[1] One remarkable technique that enables such control is the use of light to manipulate photoactivatable (or photocaged) compounds in various biological systems.^[2] Previously, this strategy has been used to map cellular functions, monitor transgene expression, and image dynamic processes of cell-cell interactions *in vitro* and *in vivo*.^[3] Although all of these attempts were successful in principle, there were significant limitations associated with the photoactivation with high-intensity UV or visible light. Excessive exposure to UV light can cause photoreactions in nucleic acids and result in cellular damage. In addition, the short-wavelength UV–Vis light has low power of tissue penetration, limiting their utility for deep-tissue imaging and photoactivation of the caged compounds. Alternatively, multiphoton photolysis with a long-wavelength excitation has been used to enable deep-tissue imaging and target gene expression.^[4] Despite its usefulness, the multiphoton photolytic process typically requires a complexed experimental set-up and has a low conversion efficiency due to narrow absorption cross-sections. Therefore, the development of a simple and effective approach that allows high depth of penetration and precise control of photocaged systems with limited cellular damage will be highly desirable.

Scheme 3-1. Experimental design for realizing D-luciferin uncaging and bioluminescence through use of photocaged core-shell upconversion nanoparticles.

Recently, lanthanide-doped upconversion nanoparticles (UCNPs) have received considerable attention for applications ranging from biolabeling to optical data storage.^[5] These nanoparticles offer high photostability and enable high tissue-penetration depths (up to 10 mm) by irradiation with near-infrared light (NIR), which makes them particularly attractive for bioimaging applications.^[6] Herein, we demonstrated a novel method by combining versatile photocaged compounds with the UCNPs for *in vitro* and *in vivo* uncaging and bioluminescence imaging studies.

3.2 Results and discussion

Scheme 3-1 illustrates a proof-of-concept design for photolysis of caged D-luciferin by using bioconjugated UCNPs. As a commonly used bioluminescent probe, D-luciferin can recognize firefly luciferase (fLuc) reporter genes and produce bioluminescence in the presence of oxygen, magnesium ions, and adenosine triphosphate (ATP), thereby providing opportunities for extensive applications in molecular imaging *in vitro* and *in vivo*.^[7] In our design, Tm/Yb co-doped

Figure 3-1. a) and b) TEM images of the as-prepared core-shell nanoparticles and silica-coated nanoparticles, respectively. c) and d) corresponding emission spectra of the core-shell nanoparticles and silica-coated nanoparticles (Insets: corresponding luminescent photos of the colloidal solutions). The particle concentration for both samples is 1 mg/mL in DMSO.

$\text{NaYF}_4@\text{NaYF}_4$ core-shell nanoparticles^[8] with a reduced surface-quenching effect will be chosen as the platform for D-luciferin conjugation. The core-shell nanoparticles will be subject to coating by thiolated silane molecules and subsequently coupled to 1-(2-nitrophenyl)ethyl caged D-luciferin. As the absorption band of photocaged D-luciferin overlaps with the upconverted emission band of the nanoparticle in the UV region ($^1\text{I}_6 \rightarrow ^4\text{F}_3$, $^1\text{D}_2 \rightarrow ^3\text{H}_6$ transitions of Tm^{3+}), NIR excitation of the photocaged UCNPs can trigger disassociation of D-luciferin molecules from the surface of nanoparticle. Importantly, the uncaging process can be monitored either by tracking luminescence intensity of D-luciferin or using a fLuc enzyme reporter, thus providing the possibility of bioluminescence imaging studies without the need of UV or visible light resources.

Figure 3-2. a) TEM image of photocaged silica-UCNPs. b) UV-Vis absorption spectra of silica coated-UCNPs (black line) and photocaged (50 μ M in DMSO) UCNPs (blue line).

In a typical experiment, silica-coated upconversion nanoparticles with reactive thiol terminal groups were first synthesized by using a water-in-oil microemulsion method (Experiment section).^[6e-g] Transmission electron microscope (TEM) imaging results demonstrated that the silica-modified nanoparticles exhibited a narrow size distribution of about 50 nm (Figure 3-1a and 3-1b). Both solutions of unmodified and silica-coated UCNPs revealed similar emissions in the UV, visible and NIR spectral regions under NIR irradiation (Figure 3-1c and 3-1d), demonstrating that decoration of UCNPs with silica shell would not significantly affect the upconversion properties. To ensure a high-efficiency coupling of photocaged molecules to the particle surface, 1-(2-nitrophenyl)ethyl photocaged D-luciferin was pre-modified with a thiol-reactive maleimide-terminated oligo(ethylene glycol) linker.^[9] The loading density of photocaged D-luciferin on the surface of a nanoparticle after the cross-coupling reaction was measured to be approximately 490 molecules by UV-Vis absorption spectroscopy (Figure 3-2b).

We next examined the feasibility of D-luciferin uncaging through the use of UCNPs.

Figure 3-3. a) Time-dependent fluorescence increment from D-luciferin released from photocaged (0.5 μ M) silica-coated nanoparticles under 980 nm irradiation (0-3 h). b) Time-dependent bioluminescence generated by adding the fLuc enzyme to uncaged D-luciferin under 980 nm irradiation (0-3 h)

The oligo(ethylene glycol)-modified D-luciferin derivative was chosen as a control. Without NIR irradiation, both the caged D-luciferin derivative and the D-luciferin/nanoparticle conjugate did not exhibit D-luciferin fluorescence, indicating that D-luciferin molecules were effectively blocked in the photocaged systems. However, upon 980 nm laser excitation, the solution of photocaged UCNPs showed significantly enhanced D-luciferin fluorescence, which can be attributed to photodeprotection of 1-(2-nitrophenyl)ethyl group by upconverted UV light (Figure 3-3a). 980 nm laser irradiation of caged D-luciferin derivative would not lead to significant fluorescence enhancement from D-luciferin (Figure 3-3a), indicating that this photocaged molecule itself was not sensitive to NIR light exposure. Similar fluorescence enhancement profiles were also observed for the caged D-luciferin derivative and photocaged UCNPs when directly exposed to a UV lamp (Figure 3-4). The photolysis of caged D-luciferin silica-UCNPs conjugate was further confirmed by

Figure 3-4. Fluorescence spectra of photoreleased D-luciferin upon UV irradiation at 365 nm: a) photocaged D-luciferin and b) photocaged UCNPs (Sample concentration: 0.5 μ M in 10 mM PBS). c) Photoreleased D-luciferin from photocaged UCNPs and photocaged D-luciferin.

analysis of fLuc enzyme-mediated bioluminescence in a PBS buffer solution (10 mM, pH=7.2). Prior to NIR photoactivation, there were no bioluminescence signals detectable in either caged luciferin derivative or luciferin-tethered nanoparticles in the presence of fLuc enzyme, Mg^{2+} and ATP. Similarly, we did not observe bioluminescence signals from the caged luciferin derivative under NIR excitation for 2 h. In stark contrast, upon NIR excitation we observed significantly enhanced bioluminescence signals at 560 nm (Figure 3-3b), attributing to the enzymatic oxidation of photoreleased D-luciferin substrate accompanied by light emission in the process.

Encouraged by the results of *in vitro* uncaging and bioluminescence studies, we further investigated intracellular uptake and uncaging of D-luciferin by using

Figure 3-5. Fluorescence imaging of C6 glioma cells incubated with photocaged D-luciferin (0.5 μM) and photocaged silica-UCNPs conjugate (0.5 μM) in the absence (a and c) and presence (b and d) of 980 nm illumination. Excitation filter: 460/40 nm and emission filter: 535/ 50nm (Exposure time by 980 nm: 2 h). e) Fluorescent intensity of uncaged D-luciferin in cell lysate. The cell lines without sample incubation were used as control. f) Bioluminescence visualization of different concentration of photocaged silica-UCNPs in living cell (MCF-7/fLuc). g) Bioluminescence visualization of photocaged UCNPs in living cell (MCF-7/fLuc) with or without 980 nm excitation.

photocaged UCNPs in living cells. In a typical cell imaging study, photocaged nanoparticles ([D-luciferin]: 0.5 μM) were incubated with C6 glioma cells in DMEM (Dulbecco's Modified Eagle Medium) at 37°C for 2 h. As a control, the caged D-luciferin derivative was used for incubation with the living cells under the same conditions. These two samples were then treated with NIR irradiation for intracellular uncaging studies (Figure 3-5b and 3-5d).

Figure 3-6. a) Bioluminescence plotted as a function of cell number (MCF-7/fLuc). Irradiation time is 4 h. b) Cell viability of silica-UCNPs and photocaged silica-UCNPs conjugate. As a control, cells without sample incubation were also treated with UV and 980 nm irradiation.

Before exposure to NIR light, both the caged D-luciferin derivative and the photocaged nanoparticles displayed weak fluorescence signals within the cells (Figure 3-5a and 3-5c), indicating the possible fluorescence quenching of D-luciferin by the 1-(2-nitrophenyl)ethyl group or by the silica shell of UCNPs.^[6g-i] The NIR-activated uncaging event was further confirmed by bioluminescence studies on the basis of MCF-7 cells with fLuc expression. Upon 980 nm irradiation, strong bioluminescent emission at 560 nm could be detected (Figure 3-5f and 3-5g). The emission signals from the fLuc-transfected cells were found to be proportionally related to the number of cells used (Figure 3-6a). As a negative control, there was no bioluminescence observed in the nanoparticle-incubated MCF-7 cells without NIR irradiation, clearly suggesting that the use of cell-permeable photocaged nanoparticles enables effective identification of fLuc enzymatic activity in living cells. More importantly, MTT assays showed that no noticeable cytotoxicity was observed for the nanoparticle-treated C6 glioma cells after two hours of NIR irradiation. This was in a stark contrast to a parallel experiment with UV illumination that showed significant

Figure 3-7. Bioluminescent imaging of fLuc activity in D-luciferin-treated living mice. a) Left: injection with D-luciferin (20 μ M, 20 μ L) and right: injection with photocaged nanoparticles without light irradiation. b) Left: injection with photocaged nanoparticles and irradiation by UV for 10 min and right: injection with photocaged nanoparticles and irradiation by 980 nm for 1 h.

Figure 3-8. Bioluminescent imaging of fLuc activity in living mice. a) The animal photo taken under normal light. b) Bottom left: injection of photocaged (20 μ M, 20 μ L) UCNP, but no UV excitation; bottom right: injection of photocaged (20 μ M, 20 μ L) UCNP with 10 min of UV illumination; and top center: photocaged UCNP (20 μ M, 20 μ L) dropcasted on the surface of the mouse, followed by 10 min of UV excitation.

cellular damage after a short time (5 min) light exposure (Figure 3-6b).

Another unique advantage of using photocaged silica-UCNPs is the ability to carry out bioimaging studies at substantial tissue depths. As a proof-of-concept experiment,

we subcutaneously injected caged nanoparticles and fLuc enzyme into living mice at a tissue depth of 2 mm and then measured bioluminescence by using a modified EvolveTM in vivo imaging system. Contrasting to the observable bioluminescence from the left thigh of a mouse directly injected with D-luciferin, there was no bioluminescence signal detectable from the point of injection at the right thigh of the mouse without 980 nm light irradiation (Figure 3-7a). By comparison, we observed strong bioluminescence signals from the mouse injected with photocaged nanoparticles and irradiated by NIR light (Figure 3-7b). It should be noted that under UV irradiation we could not detect any notable bioluminescence (Figure 3-7b and Figure 3-8b). Taken together, these data strongly indicate the power and versatility of using photocaged UCNP for bioluminescence studies at substantial tissue depths.

3.3 Conclusions

In conclusion, we have reported a rational design, based on the photocaged upconversion nanoparticles, for controlled uncaging and bioluminescence imaging studies. This method takes advantage of photon upconversion from the caged D-luciferin- conjugated UCNP when excited with NIR light. The upconverted UV emission effectively triggered the uncaging of D-luciferin and conferred enhanced fluorescence and bioluminescence signals in vitro and in vivo with deep light penetration and low cellular damage. With compositional or structural tuning of UCNP and the availability of diverse photocaged functional groups, this UCNP-based photolysis could be readily extended to other biomedical studies. These results may also offer new possibilities for non-invasively monitoring the dynamic functions of cells and selectively delivering the drug molecules at targeted areas in

vitro and in vivo on the basis of photocaged techniques.

3.4 Experiment section

Materials and general methods

Materials: All reagents were purchased from Aldrich or Fluka. Anhydrous solvents for organic synthesis were purchased from Aldrich and stored over activated molecular sieves (4 Å). Firefly luciferase and ATP were purchased from Sigma-Aldrich. Mal-dPEGTM₂-NHS ester linker was purchased from Quanta Biodesign. Unless noted otherwise, commercially available reagents were used without further purification. Thin-layer chromatography (TLC) was performed on glass plates pre-coated with silica gel 60F-254. The NaYF₄:Yb/Tm (20/0.2 mol%)/NaYF₄ core-shell nanoparticles (UCNPs) were synthesized according to the previously reported method.^[10]

Instruments: ¹H NMR spectra were recorded on a JEOL 400 MHz spectrometer. Chemical shifts (δ, ppm) were reported using TMS as an internal reference. EI and ESI mass spectra were performed on a Thermo Polaris Q and a Thermo LCQ Deca XP MAX, respectively. UV-Vis absorption spectroscopy was performed using a 5-mm path quartz cell on a Beckman coulter DU800 spectrometer. TEM images were recorded on an FEI EM208S Transmission Electron Microscope (Philips) operated at 100 kV. Fluorescence studies were carried out on a Varian Cary Eclipse fluorescence spectrophotometer. The size distribution of UCNPs was measured using photon correlation spectroscopy (Brookhaven Instruments Corporation). Photo-irradiation experiments were carried out using a MDL-H-980 nm laser (500 W cm⁻²) and a UV lamp (Blak-Ray, B-100AP/R, 100 W/365 nm, intensity: 8.9 mW cm⁻²).

Bioluminescence studies were carried out on a Turner Biosystems 20/20ⁿ Luminometer. Living animal studies were conducted using EvolveTM *in vivo* imaging system from Photometrics with the software of IMAGE-PRO PLUS Version 6.3.

Scheme 3-2. Synthesis of photocaged D-luciferin.

Synthesis and characterization of the photocaged D-luciferin derivative.

4-Ethyl-3-nitrobenzoic acid (1). 4-Ethylbenzoic acid (0.75 g, 5 mmol) was slowly added to fuming nitric acid and sulfuric acid at 0°C. The reaction was stirred at this temperature for 1 h. The mixture was then poured into ice. The resulting precipitate was collected by filtration, washed with water, and dried under vacuum to yield a white solid (0.9 g; 90%). ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 8.59 (d, J = 1.8 Hz, 1H), 8.24 (dd, J = 8.2, 1.8 Hz, 1H), 7.52 (d, J = 8.2 Hz, 1H), 3.00 (q, J = 7.6 Hz, 2H), 1.33 (t, J = 7.5 Hz, 3H); MS (EI): m/z : 195.05, calculated for $[\text{M}]^+$: 195.05.

2-(Trimethylsilyl)ethyl-4-ethyl-3-nitrobenzoate (2). 4-Ethyl-3-nitrobenzoic acid (500 mg, 2.56 mmol) was dissolved in anhydrous THF (3 mL) and refluxed with thionyl chloride (0.75 mL) for 2 h. The mixture was concentrated under vacuum and used without further purification. The crude product was dissolved in anhydrous THF (3 mL) and cooled in an ice bath, followed by addition of 2-(trimethylsilyl) ethanol (455 mg, 3.84 mmol) and DIPEA (663 mg, 5.13 mmol). The mixture was stirred for 3h and concentrated. The residue was purified by column chromatography on silica gel with eluent EA/hexane (1:10) to give a yellow oil (400 mg; 80%). ¹H NMR (400 MHz, CDCl₃, 25°C, TMS): δ=8.43 (d, *J* = 1.8 Hz, 1H), 8.11 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.40 (d, *J* = 8.2 Hz, 1H), 4.39 (m, 2H), 2.90 (q, *J* = 7.4 Hz, 2H), 1.25 (t, *J* = 7.4 Hz, 3H), 1.10 (m, 2H), 0.03 (s, 9H); MS (EI): *m/z*: 295.09, calculated for [M]⁺: 295.12.

2-(Trimethylsilyl)ethyl-4-(1-bromoethyl)-3-nitrobenzoate (3). *N*-Bromosuccinimide (NBS, 271 mg, 1.52 mmol) and benzoyl peroxide (30 mg) were added to a benzene solution of **2** (300 mg, 1.02 mmol). The reaction mixture was refluxed overnight and then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with eluent EA/hexane (1:50) to give rise to a yellow solid (255 mg; 85%). ¹H NMR (400 MHz, CDCl₃, 25°C, TMS): δ = 8.45 (d, *J* = 1.8 Hz, 1H), 8.25 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.96 (d, *J* = 8.2 Hz, 1H), 5.78 (q, *J* = 6.8 Hz, 1H), 4.46 (m, 2H), 2.09 (d, *J* = 6.8 Hz, 3H), 1.15 (m, 2H), 0.09 (s, 9H); MS (EI): *m/z*: 373.26, calculated for [M]⁺: 373.03.

2-(Trimethylsilyl)ethyl-4-(1-(2-cyanobenzo[d]thiazol-6-yloxy)ethyl)-3-nitrobenzoate (4). 2-Cyano-6-hydroxy-benzothiazole (60 mg, 0.34 mmol) and potassium carbonate (235 mg, 1.70 mmol) were added to a solution of **3** (191 mg, 0.51 mmol) in 20 ml of acetone. The mixture was refluxed for 2h. After cooling, the mixture was concentrated and purified by column chromatography on silica gel with eluent

EtOAc/hexane (1:10) to give a white solid (140 mg; 88%). ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 8.65 (d, J = 1.8 Hz, 1H), 8.21 (dd, J = 8.7, 1.8 Hz, 1H), 8.02 (d, J = 8.7 Hz, 1H), 7.83 (d, J = 8.2 Hz, 1H), 7.23 (m, 2H), 6.17 (q, J = 6.4 Hz, 1H), 4.42 (m, 2H), 1.76 (d, J = 6.4 Hz, 3H), 1.11 (m, 2H), 0.06 (s, 9H); MS (ESI): m/z : 470.13, calculated for $[\text{M}+\text{H}]^+$: 470.11.

4-(1-(2-Cyanobenzo[d]thiazol-6-yloxy)ethyl)-3-nitrobenzoic acid (5). TBAF (565 mg, 2.16 mmol) was added to a solution of **4** (100 mg, 0.72 mmol) in THF (3 mL). The reaction mixture was stirred at room temperature for 2h and concentrated. The residue was dissolved in ethyl acetate, washed with 1M HCl and dried over Na_2SO_4 . The filtrate was concentrated and purified by column chromatography on silica gel with eluent MeOH/DCM (1:20) to give a white solid (240 mg; 90%). ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ =9.78 (br, 1H), 8.75 (d, J = 1.4 Hz, 1H), 8.30 (dd, J = 8.2, 1.4 Hz, 1H), 8.07 (d, J = 9.2 Hz, 1H), 7.92 (d, J = 8.2 Hz, 1H), 7.24 (m, 2H), 6.22 (q, J = 6.4 Hz, 1H), 1.79 (d, J = 6.4 Hz, 3H); MS (ESI): m/z : 370.33, calculated for $[\text{M}+\text{H}]^+$: 370.04.

***tert*-Butyl-2-(2-(2-(4-(1-(2-cyanobenzo[d]thiazol-6-yloxy)ethyl)-3-nitrobenzamido)ethoxy)ethoxy)ethylcarbamate (6).** EDC-HCl (30 mg, 0.16 mmol) and HOBt (11 mg, 0.08 mmol) were added to a solution of **5** (29 mg, 0.08 mmol) in DMF and the mixture was stirred for 1h. Then *N*-Boc-1,8-diamino-3,6-dioxaoctane (38 mg, 0.15 mmol) and DIPEA were added and the mixture was stirred for another 5h. The mixture was concentrated and the residue was purified by column chromatography on silica gel with eluent MeOH/DCM (1:20) to give 37 mg (77%) of product. ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 8.52 (m, 1H), 8.06 (m, 2H), 7.82 (m, 1H), 7.24 (m, 2H), 6.17 (m, 1H), 5.97 (br, 1H), 4.97 (br, 1H), 3.56-3.70 (m, 10H), 3.28 (m, 2H), 1.76 (d, J = 6.4 Hz, 3H), 1.40 (s, 9H); MS (ESI): m/z : 622.05, calculated for $[\text{M}+\text{Na}]^+$:

622.19.

2-(6-(1-(4-((2-(2-(2-(Tert-butoxycarbonyl)ethoxy)ethoxy)ethyl)carbamoyl)-2-nitrophenyl)ethoxy)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (7).

D-Cysteine hydrochloride monohydrate (11 mg, 0.09 mmol) was added to a solution of **6** (37 mg, 0.06 mmol) in DCM (2 mL) and MeOH (1 mL). The mixture was stirred for 15 minutes and 10% NaHCO₃ (1 mL) was added. The mixture was stirred for another 6h and then acidified with HCl (1M) to pH 2-3 and extracted with DCM. The combined organic layer was washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The product was obtained as a pale yellow solid (39 mg; 92%). ¹H NMR (400MHz, CDCl₃, 25 °C, TMS): δ = 8.49 (s, 1H), 8.03 (s, 1H), 7.95 (m, 1H), 7.83 (m, 1H), 7.07-7.19 (m, 2H), 6.13 (m, 1H), 5.38 (m, 1H), 5.01 (br, 1H), 3.51-3.80 (m, 12H), 3.28 (m, 2H), 1.74 (t, *J* = 6.0 Hz, 3H), 1.40 (s, 9H); MS (ESI): *m/z*: 703.87, calculated for [M+H]⁺: 704.20.

Photocaged D-luciferin. Compound **7** (20 mg, 0.03 mmol) was dissolved in DCM (2 mL) and cooled in an ice bath. Then TFA (2 mL) was added and the mixture was stirred for 2h. The solvent was removed at reduced pressure and the product was washed with hexane to give a yellow solid (18 mg; 85%). ¹H NMR (400MHz, [D₄] MeOH, 25 °C, TMS): δ=8.37 (d, *J* = 1.4 Hz, 1H), 7.96 (dd, *J* = 8.2, 1.4 Hz, 1H), 7.75 (m, 2H), 7.23 (m, 1H), 7.03 (m, 1H), 6.51 (s, 2H), 6.02 (q, *J* = 6.0 Hz, 1H), 5.25 (m, 1H), 3.45-3.64 (m, 12H), 3.20 (m, 2H), 2.98 (t, *J* = 4.6 Hz, 2H), 1.63 (d, *J* = 6.0 Hz, 3H); MS (ESI) : *m/z*: 604.37, calculated for [M+H]⁺: 604.15.

Synthesis and characterization of UCNPs and silica coated-UCNPs

Synthesis of NaYF₄:Yb/Tm (20/0.2 mol%) *core* nanoparticles

Figure 3-9. Size distributions determined by DLS: a) UCNPs and b) silica coated-UCNPs.

$\text{RE}(\text{CH}_3\text{CO}_2)_3$ solution (0.2 M, RE = Y, Yb, and Tm) in DI water (2 mL) was added to a 50 mL flask containing oleic acid (3 mL) and 1-octadecene (7 mL) and the solution was heated to 150 °C for 1 h with magnetic stirring and then cooled down to room temperature. Thereafter, methanol solution (5 mL) containing NH_4F (1.6 mmol) and NaOH (1 mmol) was added and the solution was stirred for 30 min at 50 °C. The reaction temperature was then increased to 100 °C and the methanol evaporated from the reaction mixture. After that, the solution was heated to 290 °C and maintained at this temperature under argon flow for 1.5 h and then cooled down to room temperature. The resulting nanoparticles were precipitated by the addition of ethanol, collected by centrifugation, washed with ethanol several times, and finally re-dispersed in cyclohexane for the subsequent shell growth procedure.

Synthesis of $\text{NaYF}_4\text{:Yb/Tm}$ (20/0.2 mol%)@ NaYF_4 core-shell nanoparticles

$\text{Y}(\text{CH}_3\text{CO}_2)_3$ solution (0.2 M, 2 mL) in DI water were added to a 50 mL flask containing oleic acid (3 mL) and 1-octadecene (7 mL) and the solution was heated to 150 °C for 1 h with magnetic stirring and then cooled down to 50 °C. $\text{NaYF}_4\text{:Yb/Tm}$ core nanoparticles in cyclohexane (4 mL) were added along with methanol solution (5 mL) of NH_4F (1.6 mmol) and NaOH (1 mmol). The resulting mixture was stirred at

50 °C for 30 min, at which time the reaction temperature was increased and the methanol evaporated. After methanol evaporated, the solution was heated to 290 °C under argon flow for 1.5 h and then cooled to room temperature. The resulting nanoparticles were precipitated by the addition of ethanol, collected by centrifugation, washed with ethanol several times, and dry in oven overnight to obtain pure powders.

Synthesis of silica-coated UCNPs

Silica coated-UCNPs were prepared from reverse micelles also using a previous reported procedure.^[11] Briefly, UCNPs (4 mg) was dissolved in cyclohexane (16 mL), and followed by adding Triton X-100 (4 mL), 1-hexanol (4 mL) and DI water (680 μ L). Then TEOS (tetraethyl orthosilicate) (10 μ L) and (3-mercaptopropyl)-methyl dimethoxy silane (2 μ L) was added into the mixture. After 6 hours of stirring, NH_4OH (200 μ L) was added. After 12 hours reaction, the product was obtained by centrifuge and washed thoroughly with ethanol. The as-prepared silica-UCNPs were characterized by DLS and TEM (Figure 3-1 and 3-9). The fluorescence emission spectrum ($\lambda_{\text{ex}} = 980 \text{ nm}$) of the upconversion particles (1 mg/mL, assume the yield of the coating step was 100%) were recorded on a DM150i monochromator equipped with a R928 photon counting photomultiplier tube (PMT), in conjunction with a 980 nm diode laser.

Preparation of photocaged silica-UCNPs

Silica coated-UCNPs (20 mg) were dissolved in DMF (300 μ L) and then added to Mal-dPEGTM₂-NHS ester (100 μ L, 20 mM, from Quanta Biodesign) and N, N-Diisopropylethylamine (DIPEA: 2 μ L). After overnight, the NHS-functionalized nanoparticle suspension was collected and washed by DMF three times. Then the NHS-modified nanoparticles were dissolved in photocaged D-luciferin derivative (2

Figure 3-10. Fluorescence spectra of photoreleased D-luciferin upon 980 nm irradiation: a) photocaged D-luciferin and b) photocaged UCNPs (Sample concentration: 0.5 μ M, in 10mM PBS).

mM in DMF, 400 μ L) and DIPEA (2 μ L). The reaction was allowed to continue for 24 h under dark conditions at room temperature. The photocaged UCNPs were obtained by centrifugation and washed by DMF/EtOH (v:v = 1:5). The as-prepared photocaged UCNPs were further characterized by TEM and UV-vis spectroscopy. Finally, the product was suspended in DMSO and stored in a fridge prior to uncaging and bioluminescence studies. The loading efficiency for photocaged UCNPs was estimated by measuring the absorbance of at 325 nm. A calibration curve was determined with known concentrations of photocaged D-luciferin derivative.

***In vitro* photo irradiation of photocaged D-luciferin derivative and photocaged UCNPs**

Photorelease of D-luciferin by UV irradiation: A solution (100 μ L, 0.5 μ M, in 10 mM PBS) of each sample was transferred to a 96-well plate. The plate containing the sample solutions was illuminated at 365 nm for a specified time period. After each

Figure 3-11. Measured bioluminescence from photocaged ($0.5 \mu\text{M}$) UCNPs treated with fLuc under 365 nm irradiation.

irradiation, the fluorescence spectrum was recorded in the range of 425 nm to 675 nm by using 350-nm excitation. The integrated fluorescence intensity was plotted against irradiation time.

Luciferin release by 980 nm laser irradiation: A solution ($10 \mu\text{L}$, $5 \mu\text{M}$ in 10 mM PBS) of each sample was transferred to a 1.5-mL Eppendorf tube. The solution was irradiated at 980 nm for a specified time period. After each irradiation, each sample was diluted to $0.5 \mu\text{M}$. The fluorescence spectrum was recorded in the range of 425 nm to 675 nm by using 350-nm excitation. (Figure 3-10)

***In vitro* firefly luciferase enzyme assay:** Each sample was irradiated with respective light source (365 nm UV (Figure 3-11) and 980 nm NIR laser (Figure 3-3)) to induce D-luciferin release. Upon irradiation of samples including photocaged UCNPs or photocaged D-luciferin derivative, fLuc, ATP, MgCl_2 were added into each solution. The final concentration for each sample was maintained at $0.5 \mu\text{M}$. For firefly luciferase, ATP and MgCl_2 , their final concentrations were $75 \mu\text{g/mL}$, 1 mM and 2.5 mM , respectively. Luminescence intensity was recorded by a luminometer (Turner

Figure 3-12. Fluorescence imaging of C6 glioma cells incubated with photocaged D-luciferin (0.5 μ M) and photocaged (0.5 μ M) UCNPs without/with UV irradiation. a) and b) Cells incubated with photocaged UCNPs. c) and d) Cells incubated with photocaged D-luciferin.

BioSystems, 20/20n Luminometer; Turner Biosystems, Sunnyvale, CA, USA).

Living cell assay for photo irradiation of photocaged D-luciferin derivative and photocaged UCNPs

C6 glioma cell line was brought from ATCC (ATCC Cat No.: CCL-107, Manassas, VA) and maintained in DMEM medium containing 10% FBS (Invitrogen, Burlington, Canada).

Cell imaging: C6 glioma cells were plated in a 35-mm diameter μ -dish plastic-bottom (ibidi GmbH, Germany) and cultured 1 day in DMEM medium without FBS. The medium was removed and the cells were washed twice with DMEM medium. Then DMEM medium (1mL) solution containing 0.5 μ M photocaged D-luciferin or photocaged silica-UCNPs was added to the cells and incubated for 2 h at 37 °C with 5 % CO₂. Then the cells were washed twice with PBS (1 mL) and illuminated with a 980-nm laser (with a 5-min break for each 30 min exposure for 2 hours). After exposure, the fluorescence imaging was acquired with a confocal fluorescence

Figure 3-13. Fluorescence intensity of uncaged D-luciferin in cell lysate. The cells without sample incubation were used as control.

microscope (Nikon, Eclipse TE2000-E) equipped with an excitation filter (460/40 nm) and emission filter (535/50 nm). After cell imaging experiments, the cells were harvested and washed twice with PBS. Finally, the cells were suspended in 100 μ L of PBS, followed by cell lysis. After centrifugation, the supernatant was collected and the fluorescence spectra were recorded (Figure 3-13).

Bioluminescence images of MCF-7/fLuc Cell line

Generally, MCF-7 cells with stably transfected firefly luciferase (fLuc) were obtained from Agilent Technologies (Stratagene Products Division, La Jolla, CA, USA). MCF-7/fLuc cell line were plated in a 25-cm² flask with a density of 1×10^6 per flask and cultured overnight in DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS. The medium was removed and washed twice with fresh medium. Then the cells were incubated with photocaged silica-UCNPs for 2 hours with different concentrations at 37 °C. After incubation, the cell were washed twice with medium and collected, followed by centrifugation and washing by PBS for two times. Then the

cells were suspended in 50 μL PBS and irradiated by 980 nm NIR for 2 hours in cold room ($0\sim 4\text{ }^{\circ}\text{C}$). Finally, each sample was added with another 50 μL PBS buffer and the bioluminescence was recorded using the same luminometer. For Bioluminescence visualization, the cells were transferred to a 96-well plate.

Bioluminescence of MCF-7/fLuc cell line

According to the standard protocol,^[12] different numbers of MCF-7 cells were collected and washed twice by PBS after cell culturing for several days. Then, the lysis buffer solutions (25 mM Tris-phosphate, 2 mM DTT, 2 mM EDTA, 10 % glycerol, 1% Triton X-100) was added and incubated with different numbers of cell samples for 15 min at room temperature. After centrifugation, the supernatant was collected and added to photocaged (2 μM) UCNPs or photocaged D-luciferin solution (2 μM) in the presence of 1mM ATP and 2.5 mM MgCl_2 . The bioluminescence was recorded by the same luminometer.

***In vivo* bioluminescent imaging under UV and NIR light irradiation:**

D-luciferin caged (20 μM) UCNPs and fLuc enzyme (0.3 mg/mL) were injected subcutaneously into the left and right thighs of nude mice. In a typical experiment, the mouse was injected with D-luciferin caged UCNPs (20 μL), followed by 980 nm excitation for 1 h (power=0.8 W, power density= 255 W cm^{-2}) or by UV irradiation for 10 min. After the injection of fLuc enzyme (10 μL), ATP and Mg^{2+} (final concentration: 0.3 mg/mL, 1 mM and 2.5 mM, respectively), bioluminescence scanning experiments were conducted. By comparison, similar imaging experiments were also carried out for living mice administrated by subcutaneous injection of the caged substrates but without light illumination. As a control, the same amount of D-luciferin was also injected to a living mouse to screen the fLuc activity under the living animal imaging facility.

Cell viability Assay: C6 glioma cells (or MCF-7/fLuc cell) were seeded in a 96-wells plates with a density of 1×10^4 per well in DMEM medium. After 2-day incubation, the cells were treated with 0.5 μM of silica-modified UCNPs and photocaged UCNPs, respectively. After 2 h incubation and respective irradiation (365 nm UV for 5 m or 980 nm laser for 2 h with a 5-min break after 30 min), the cytotoxicity was evaluated by the MTT assay as previously described.^[31]

3.5 References

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Chapter 4

NIR Light Controlled Photorelease of siRNA and Its Targeted Intracellular Delivery Based on Upconversion Nanoparticles

4.1 Introduction

Small interfering RNA (siRNA) has been emerged as gene-based therapy due to their highly desirable roles in RNA interference and gene silencing effects in biomedical research. One of the key requirements for an effective siRNA therapy is that sufficient siRNA need to be introduced into cells or organs.^[1] However, because of the highly negatively charged nature of “naked” siRNA, it cannot spontaneously diffuse across cellular membranes, which becomes the most restrictive factor in siRNA-based therapy.^[2] Currently, many types of siRNA-carrier complexes have been developed for the purpose of effective siRNA delivery and targeted gene knockdown.^[3] Moreover, a number of nanomaterial conjugates such as gold nanoparticles, mesoporous silica nanoparticles and polymeric nanoparticles with their surface modification by the positively charged functional groups, have also been explored as delivery vehicles for transporting siRNA into living cells.^[4] Despite the promising progress with respect to their efficient siRNA delivery and specific gene silencing in living cells, remote control the release of siRNA or unpacking siRNA inside target cells in a highly spatial and temporal precision remains a critical challenge and systematic studies still need to be fully exploited.

So far, several intracellular controlled release strategies on the basis of light

irradiation, low pH, enzymatic hydrolysis, redox reaction, *etc.*, have been extensively established to direct the targeted delivery of bioactive moieties including therapeutic oligonucleotides into the living cells.^[5] Among various strategies, the photolysis or the use of a beam of light represents a unique approach to achieve time- and site-specific control of effective release of payload molecules from the carrier systems, which have been currently recognized as new directions for their promising biomedical practices in gene, drug or other related biomolecules delivery *in vitro* and *in vivo*.^[6] However, one potential problem existed in most of current photolysis delivery systems is that the activation of bioactive molecules must rely on the short wavelength UV irradiation. The unavoidable cellular damage and less tissue penetration would largely impede their further applications *in vivo*. Therefore, the search for appealing alternatives for effective photorelease while maintaining minimum side effects in living cells will be highly desirable. Recently, the core-shell lanthanide-doped upconversion nanoparticles (UCNPs) as emerging stars have attracted much attention in the fields of biological imaging and targeted delivery mostly attributed to their novel optical property.^[7] In general, UCNPs can absorb long wavelength NIR light and give out narrow and sharp emissions ranged from UV to visible or even NIR region. Such unique upconversion property observed in UCNPs has provide major advantages to suppress autofluorescence and to minimize photo damage to living cells, which enabled UCNPs to work as promising NIR-responsive delivery cargos for biological studies. Indeed, there are a few reports exploiting the converted UV or visible light from UCNPs to activate photocaged therapeutic agents or imaging probes *in vitro* and *in vivo*.^[8] Very recently, NIR-induced UCNPs delivery system has also been reported to successfully photo release of caged nucleic acids in

Scheme 4-1. Schematic illustration of a) the synthesis processes of cationic photocaged linker functionalized silica-coated UCNP (Si-UCNP); b) siRNA were adsorbed on the particles surface and then photo-released by upconverted UV light from UCNP.

cells and living animals.^[9] However, the extra chemical modification of target nucleic acids with photocaged groups has to be involved in advance in order to functionalize the delivered nucleic acids and enhance their physical adsorption onto nanoparticles surface. Extensive studies to develop simple and reliable NIR photoactivatable nanoparticle platforms with higher payload of more diverse and intact DNA/siRNA moieties remain highly desirable in the field of gene therapy and have been minimally investigated so far.

Herein, we presented such a simple and effective NIR light reactive nanoparticle carrier system to exert target siRNA delivery in host living cells on the basis of silica coated UCNP. In our system, lanthanide ytterbium (Yb^{3+}) and thulium (Tm^{3+}) co-doped sodium yttrium fluoride (NaYF_4) nanocrystals were chosen as templates,

Figure 4-1. Characterization of UCNPs and Si-UCNPs: a) and b), TEM image and DLS of UCNPs; c) and d), TEM image and DLS of Si-UCNPs; e) luminescence emission spectrum of UCNPs; f), luminescence emission spectrum of Si-UCNPs.

which were coated with silica layer for the purpose of increasing the biocompatibility and facilitating the subsequent chemical modification.^[10] The as-synthesized Si-UCNPs were then functionalized by cationic photocaged linkers through covalent bonding, which would greatly promote the physical adsorption of target DNA or siRNA on the particles surface and thus facilitated the cell membrane penetration for effective delivery in living cells. Scheme 4-1a illustrated the experimental design of the silica coated UCNPs carrier for the target siRNA delivery by using photoactive *o*-nitrobenzyl ester functionalized thiol-linker with the positively charged alkyl amine at the end of structure. The constructed Si-UCNPs conjugate will associate with siRNA through electrostatic interactions between the phosphate ions in siRNA and the cationic ammonium on the surface of Si-UCNPs. Upon 980 nm laser irradiation, the upconverted UV light emitted from UCNPs will cleave the photosensitive

Figure 4-2. UV-Vis spectra of silica coated UCNPs, photocaged linker and photocaged Si-UCNPs.

o-nitrobenzyl linker and split the positively charged amino group from nanoparticles surface, and thus resulted in the efficient release of siRNA to silence target gene expression in living cells (Scheme 4-1b). The *in vitro* agarose gel electrophoresis and intracellular imaging experiments indicated that this constructed NIR light-induced siRNA carrier system could efficiently eliminate the membrane barrier and provided a simpler and more effective approach for target gene delivery with less photo damage in living cells.

4.2 Results and discussion

In a typical experiment, the $\text{Yb}^{3+}/\text{Tm}^{3+}$ co-doped UCNPs were prepared by according to the protocol from the literature.^[8c, 8d, 11] The obtained nanocrystals were suspended in cyclohexane and further encapsulated in silica shell with amine groups on the surface by using water-in-oil microemulsion method.^[8d, 12] Both of the UCNPs and Si-UCNPs were characterized by TEM and DLS. As shown in Figure 4-1a~d, the average size of UCNPs and Si-UCNPs were approximately about 65 nm and 74 nm

Figure 4-3. *In vitro* characterization of Si-UCNPs/Alexa-siRNA complex. a) Agarose gel electrophoresis analysis of Alexa-siRNA (1 μ M) loading with cationic Si-UCNPs with different ratios: 1:5, 1:10 and 1:15. b) Time course of fluorescence spectra of Alexa-siRNA loading: (a~g: 0, 1 min, 2 min, 5min, 10min, 15min, 20 min). c) UV absorbance spectra of Alexa-siRNA and Si-UCNPs/Alexa-siRNA complex. (1 μ M). d) Absorbance spectra of Alexa-siRNA (black), photocaged Si-UCNPs (red), Alexa-siRNA loaded on photocaged Si-UCNPs (blue), the Si-UCNPs/siRNA complex irradiated with UV (dark cyan) 5 minutes and the Si-UCNPs/siRNA complex irradiated with NIR light (magenta) 2 hours.

respectively, which are suitable for the effective cellular uptake. The coating of a silica layer is on the purpose of increasing biocompatibility of UCNPs. Moreover, this silica shell can also facilitate easy surface functionalization for further biomedical study. As contrast, the luminescence spectra shown in Figure 4-1e and 1f indicated that all the post-modifications of the UCNPs would not affect their optical properties. In other words, the modified UCNPs exhibited same optical properties to the “naked” UCNPs, including UV, visible and NIR light emission.

In order to achieve high thiol-photolinker loading efficiency, the Si-UCNPs were firstly modified with maleimide-terminated oligo(ethylene glycol) linker. It is well-known that addition of PEG linker will improve the dispersion stability of nanoparticles in aqueous solutions. The simple and specific thiol-maleimide conjugation between the cationic photocaged linker and PEG modified Si-UCNPs led to the formation of NIR-responsive nanoparticle delivery cargos. The loading efficiency of photocaged linker on the surface of silica-UCNPs was determined by the UV-vis absorbance spectrum (Figure 4-2) and the loading amount was estimated to be about 8000 molecules per nanoparticle. These photoactive linker functionalized Si-UCNPs were suitable for loading of siRNA because of the positive charge of the cationic linker.

We next studied the loading and light-induced release of siRNA from the photocaged UCNPs platform. As a proof-of-concept, the Alexa Fluor@546 labeled siRNA (Alexa-siRNA) was loaded on the nanoparticles by mixing the particles with the siRNA (ratio: cationic linker/siRNA=5:1, 10:1, 15:1) in PBS for 20 minutes at room temperature. As shown in Figure 4-3a, the fluorescence intensity in gel electrophoresis indicated that about 60 % siRNA was adsorbed on the particles surface in the case of 5:1 mixing ratio. When the ratio increased to 15:1, nearly all of the siRNA was found to be adsorbed onto the cationic photocaged UCNPs. Moreover, we have also monitored the loading procedure by measuring fluorescence spectrum. The fluorescence intensity was decreased during the loading process (Figure 4-3b), most likely due to the intermolecular interactions between dye molecules when they were densely packed on the nanoparticles surface.^[13] The loading capacity of Alexa-siRNA was characterized by measuring UV-vis absorption spectrum (Figure 4-3c) and running agarose gel electrophoresis (Figure 4-3a). The results showed that

Figure 4-4. a) and b), cells incubated with siRNA/UCNPs complex before NIR light irradiation. c) and d), cells incubated with siRNA/UCNPs complex after NIR light irradiation. e) and f), using lipofectamine 2000 (Lipo 2K) to transfer siRNA. The concentration of Alexa-siRNA was 25nM.

about 0.7 wt % Alexa-siRNA in total was adhered on the surface of the platform.

After the adsorption of the siRNA on the particle surface, NIR light-induced siRNA release from photocaged UCNPs was investigated. The solution of photoactivated Si-UCNPs/Alexa-siRNA complex was exposed with light and the photo-cleaved process was monitored by UV-vis spectroscopy. As proof-of-concept, UV light was firstly used to confirm the uncaging properties of the developed system. As shown in Figure 4-3d, upon irradiation with UV light for 5 min, the peak (dark cyan line) of photocaged functional groups at 321 nm was significantly decreased, indicating the effective uncaging of photocaged linker caused by UV irradiation which subsequently resulted in the release of siRNA. With this information in hand, the Si-UCNPs/Alexa-siRNA complex was exposed with NIR light. Under this light irradiation, the cationic part of photocaged linker was cleaved and a similar phenomenon was observed (magenta line), in which the absorption at 321 nm was also dropped (Figure 4-3d). The decreased absorption at 321 nm indicated that the

Figure 4-5. a) cell images of HeLa incubated with Si-UCNPs/Alexa-siRNA complex for 3 hours, and stained by DAPI. b) cell images of HeLa incubated with Si-UCNPs/Alexa-siRNA complex for 1 hour and followed by 2 hours NIR light irradiation, and stained by DAPI. The concentration of Alexa-siRNA was 25nM. c) analysis of fluorescence colocalization of Alexa-siRNA fluorescence in a) and b) with ImageJ software.^[14] d) cell viability of Si-UCNPs/Alexa-siRNA without or with NIR light irradiation. As controls, cells without any treatment, cells incubated with siRNA and cells incubated with lipofectamine 2000 (Lipo2k)/siRNA were also performed.

breakage of the ester bond and the positively charged alkyl amine part was released from the platform. Due to the change of charged properties on the surface of nanoparticles, the siRNA can be easily escaped from nanoparticles. These results demonstrated the fact that the photoactivated Si-UCNPs/Alexa-siRNA complex can be

uncaged and the adsorbed siRNA can be easily released from the platform by NIR light irradiation.

Encouraged by the promising results of effective siRNA release with NIR light irradiation *in vitro*, we further examined the potential transfection of Si-UCNPs/Alexa-siRNA complex in living cells. HeLa cell lines were utilized for living cell experimental study. Typically, the Si-UCNPs/Alexa-siRNA conjugate (siRNA concentration, 25 nM) was incubated with cells for 1 hour followed by another 2 hours of NIR light irradiation. As we mentioned above, the fluorescence intensity was quenched by about 75% when Alexa-siRNA was loaded on the particles surface. After incubation with cells, weak red fluorescence signals were detected from the cells, which reflected the fact that the complex was efficiently uptaken by living cells. As shown in Figure 4-4c, the red fluorescence intensity was significantly increased after NIR light irradiation. Due to the converted UV light emitted from UCNPs after 980 nm laser exposure, the *o*-nitrobenzyl groups were cleaved which induced the Alexa-siRNA release from the nanoparticles platform, whereas the cells treated with the Lipo2K/siRNA only showed very weak fluorescence signals under the same concentration of Alexa-siRNA, which further confirmed the effective siRNA delivery from the setup system (Figure 4-4e).

In order to further study the efficiency of siRNA delivery from photocaged UCNPs through NIR light irradiation, another two comparative cell experiments were also conducted accordingly. Typically, in the first experiment, the cells were incubated with silica-UCNPs/Alexa-siRNA complex for 3 hours but no light illumination, while the sample in the second experiment was incubated for 1 hour followed by 2 hours NIR light irradiation. The fluorescence microscopy images of two samples were shown in Figure 4-5a and 5b, from which we can see that both of the cell samples

Figure 4-6. Light cytotoxicity of HeLa with UV light (365 nm) and NIR light (980 nm) irradiation.

showed red fluorescence signals. However, the cells which have been irradiated with 980 nm light exhibited much stronger fluorescence intensity than the one without light exposure. These images also further confirmed the efficiency of NIR light induced siRNA delivery in living cells.

In order to evaluate the potential toxicity from NIR light irradiation, the cytotoxicity experiments were performed by standard MTT assay. Meanwhile, the Lipo2K/siRNA was also chosen as control and viability percentage of the cells without any treatment was set as 100%. From Figure 4-5d, we can see that the UCNPs/Alexa-siRNA complex without NIR light irradiation showed no significant cytotoxicity when incubated with HeLa cells, indicating that the developed siRNA delivery platform will not significantly affect the living cells viability and could be used for further biological studies safely. Similarly, the UCNPs/Alexa-siRNA complex also showed very weak cell cytotoxicity as well upon NIR light irradiation, which should be attributed to light-induced thermal effect. However, this NIR light generated cytotoxicity was much less weak than that caused by UV light exposure (Figure 4-6).

Figure 4-7. a) fluorescence and b) overlapped bright field cell images of HeLa cells treated with EGFP plasmid; cells were treated with Lipo2K/siRNA after transferred with EGFP plasmids: c) fluorescence and d) overlapped bright field cell images; cells were treated with Si-UCNPs/siRNA no light treatment after transferred with EGFP plasmids: e) fluorescence and f) overlapped bright field cell images; cells were treated with Si-UCNPs/siRNA with NIR light irradiation after transferred with EGFP plasmids: g) fluorescence and h) overlapped bright field cell images. For i), fluorescence spectra of the cells with different treatments. And j), cell viability treated with EGFP plasmids, EGFP plasmids+siRNA/Lipo2k, EGFP plasmids+ Si-UCNPs/siRNA, EGFP plasmids+ Si-UCNPs/siRNA +NIR light.

The cellular imaging experiments have shown that siRNA could be successfully

delivered into cells by the photocaged Si-UCNPs nanocarriers. When evaluating the effectiveness of the gene delivery pipeline, one of the most important concerns is if the delivered siRNA remains sufficient activity. The most notable role of siRNA is in their interference toward RNA pathway, where it interferes with the expression of some specific genes. To this purpose, another experiment has been conducted for the gene silencing measurements. In this typical experiment, plasmid encoding with enhanced green fluorescence protein (EGFP) gene was chosen for the imaging study. The Si-UCNPs/EGFP siRNA (Si-UCNPs/siRNA) complex was prepared by using the same method which was mentioned previously in section 2.4. Four parallel HeLa cells samples were chosen to be transferred with the Lipo2K/EGFP plasmids in order to express the GFP. In the mean time of GFP expression, the sample 2 was also treated with Lipo2K/siRNA as a control by using the standard gene silencing method (Figure 4-7c and 7d). While the cell samples 3 and 4 were treated with Si-UCNPs/siRNA complex, but only the last cells (sample 4) were further exposed with NIR light after 2 hours incubation (Figure 4-7g). The gene knockdown was checked after 24 hours incubation by taking the confocal microscopy images. As expected, the strong green fluorescence observed in Figure 4-7a and no fluorescence signal observed in Figure 4-7c clearly indicated that GFP could be effectively expressed by using standard cargo on the basis of Lipo2K, and meanwhile, siRNA transferred by Lipo2K could also significantly knockdown EGFP gene expression. With this information, then we discussed the phenomenon indicated in the rest of fluorescence images. As shown in Figure 4-7e, very strong green fluorescence intensity was detected from cells which has been treated with Si-UCNPs/siRNA but there was no 980 nm laser irradiation. However, the cells treated with Si-UCNPs/siRNA and followed by 2 hours NIR light irradiation displayed very weak fluorescence signals (Figure 4-7g). These results

suggested that photocaged Si-UCNPs could effectively deliver siRNA into target cells, and NIR laser illumination could cleave photocaged functional groups and greatly activate the release of siRNA for the subsequent gene silencing in host cells. In addition, in order to confirm the details of the expected gene silencing results, all of the cell samples were harvested and related cell lysates were prepared for the further fluorescent analysis. To our delight, the trend of observed fluorescent intensity was consistent with the results shown in cell images (Figure 4-7i). Similarly, during the course of gene silence, the cytotoxicity of applied photoactive Si-UCNPs/siRNA conjugates was also investigated by MTT assay. As shown in Figure 4-7j, the cell viability observed in the samples with NIR light irradiation was quite similar with those treated with siRNA/Lipo2k. All of these results unequivocally suggested that the developed photocaged Si-UCNPs conjugate could work as a safe and reliable nanoplatform for the NIR-light controllable gene and siRNA delivery. Apart from the effective siRNA delivery to silence EGFP gene expression, such simple nanoparticle delivery platform would be easily extended to transport other therapeutic oligonucleotides without chemical modification of target nucleic acids structures.

4.3 Conclusions

In summary, we have developed the effective strategies for target siRNA delivery by using photocaged silica coated UCNPs as the nanocarriers. The uploading of negatively charged siRNA to photocaged silica coated UCNPs can be easily achieved through the complementary electrostatic interactions caused by the cationic linkers on particles surface. After cellular uptake and followed by 980 nm light exposure, the functionalized Si-UCNPs could emit UV light which effectively triggered siRNA

photorelease from UCNPs platform. Compared to the existing carrier systems for UV-controlled siRNA delivery, the use of unique NIR-to-UV property of UCNPs would result in negligible phototoxicity and guarantee high tissue penetrating by NIR irradiation. Most importantly, the siRNA delivered here is physically adsorbed on the UCNPs surface. The NIR irradiation released siRNA inside cells remains good biological-activity in the RNA interference pathway, which has been confirmed by gene silencing test. The multiple advantages of cationic functionalized UCNPs in gene delivery, cell imaging and potential therapeutic applications make it serve as outstanding template in future biomedical research and therapeutic applications.

4.4 Experiment section

Materials and general methods:

Materials: Anhydrous solvents for organic synthesis were purchased from Aldrich and stored over activated molecular sieves (4 Å). All reagents were purchased from Aldrich or Fluka. Mal-dPEGTM₂-NHS ester linker was purchased from Quanta Biodesign. Unless noted otherwise, commercially available reagents were used without further purification. Thin-layer chromatography (TLC) was performed on glass plates pre-coated with silica gel 60F-254. The PBS buffer for siRNA test was autoclaved at 121 °C for two times. Alexa Fluor@546 labeled siRNA from 1st BASE. EGFP siRNA, from Life Technologies.

Instruments: ¹H NMR spectra were recorded on a JEOL 400 MHz spectrometer. Chemical shifts (δ, ppm) were reported using TMS as an internal reference. EI mass spectra were performed on a Thermo Polaris Q. TEM images were recorded on an FEI

EM208S Transmission Electron Microscope (Philips) operated at 100 kV. The size distribution of UCNPs was measured using photon correlation spectroscopy (Brookhaven Instruments Corporation). UV-Vis absorption spectroscopy was performed using a 10-mm path quartz cell on a Beckman coulter DU800 spectrometer. Fluorescence studies were carried out on a Varian Cary Eclipse fluorescence spectrophotometer. Photo-irradiation experiments were carried out using a MDL-H-980 nm laser (5.6 W/cm^2) and a UV lamp (Blak-Ray, B-100AP/R, 100 w/365 nm, intensity: 8.9 mW/cm^2).

Scheme 4-2. Synthesis of photocaged linker.

Preparation of photocaged linker (Scheme 4-2)

Preparation of compound 1

5-Hydroxy-2-nitrobenzyl alcohol (340 mg, 2 mmol) and 2-(dimethylamino)propyl chloride hydrochloride (380 mg, 2.4 mmol) were dissolved in DMF (20 mL). Subsequently, sodium carbonate (424 mg, 4 mmol) were added. The reaction mixture was allowed to warm to 80°C and stirred for 24 hours. After reaction, the DMF solvent was removed under a reduced pressure. The residue was dissolved in ethyl acetate (EA) and washed thoroughly with sodium bicarbonate solution, water and

brine. The organic layer was dried and further purified by column (eluent: DCM/methanol = 95:5). Yield: 165.1mg (65%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 8.18 (d, $J=8.0$ Hz, 1H), 7.44 (s, 1H), 7.0 (d, $J=7.2$ Hz, 1H), 4.87 (s, 2H), 4.35 (t, $J=7.2$ Hz, 2H), 3.92 (m, 2H), 2.93 (s, 6H), 2.47 (t, $J=8.4$ Hz, 2H), MS (EI): m/z calcd for $\text{C}_{12}\text{H}_{18}\text{N}_2\text{O}_4$ 254.28, found 254 $[\text{M}]^+$.

Preparation of compound 2

3-mercaptopropanoic acid (233.5 mg, 2.2 mmol) was added to the solution of chloro triphenylmethane (556 mg, 2 mmol) in 1.0 ml of anhydrous dichloromethane (DCM). Then trifluoroacetic acid (TFA, 1.0 ml) was added. The solution was stirred for 2 hours under nitrogen condition. The reaction was quenched by 1M NaOH (3 mL). The product was collected and washed by brine (10 mL $\times$ 3) and dried over anhydrous sodium sulfate. The residue was purified by flash chromatography on silica gel with DCM/MeOH (95:5) as eluent to addord 598 mg product (yield: 86%).

Trityl protected 3-mercaptopropanoic acid (70 mg, 0.2 mmol) was dissolved in dry THF (4 mL) with ice-bath. The dicyclohexylcarbodiimide (DCC, 53 mg, 0.2 mmol) and 4-dimethylaminopyridine (DMAP, 10 mg) were added. Finally, compound **1** was added to the reaction mixture. After the reaction finished indicated by TLC, the formed precipitate was removed by filtration and the filtrate was collect. The organic solvent was removed and the residue was purified by silica gel column (eluent: DCM:MeOH=95:5). Yield 39 mg (36.8%). ^1H NMR (400 MHz, CDCl_3) δ (ppm): 8.19 (d, $J=8.0$ Hz, 1H), 7.44~7.21 (m, 15 H), 6.99 (s, 1H), 6.90 (s, 1H), 5.50 (s, 1H), 4.07~4.11 (t, $J=8$ Hz, 2H), 2.51-2.55 (t, $J=8$ Hz, 2H), 2.34 (s, 2H), 2.31 (s, 6H), 1.98 (m, 2H), 1.24 (m, 2H), MS (EI): m/z calcd for $\text{C}_{34}\text{H}_{36}\text{N}_2\text{O}_5\text{S}$ 584.73, found 585.04 $[\text{M}]^+$.

Preparation of compound 3

Bromoethane (100 μ L, 1.3 mmol) and compound 2 (29 mg, 0.05 mmol) were dissolved in DCM (1 mL). The reaction mixture was stirred under dark condition for 2 days at room temperature. Then the excess bromoethane and solvent were removed under *vacuo*. The residue was washed thoroughly with diethyl ether and dried, the product (26.7 mg, 77.6%) was obtained. ^1H NMR (400 MHz, MeOH) δ (ppm): 8.16~8.18 (d, $J=8.8$ Hz, 1H), 7.4~7.14 (m, 15H), 7.09 (s, 1H), 7.07 (s, 1H), 5.44 (s, 2H), 4.2 (t, $J=6.0$ Hz, 2H), 3.51~3.41 (m, 4H), 3.30 (s, 6H), 2.49 (m, 2H), 2.33 (m, 2H), 2.15 (s, 2H), 1.34 (t, $J=7.2$ Hz, 3H), MS (EI): m/z calcd for $\text{C}_{36}\text{H}_{41}\text{N}_2\text{O}_5\text{S}^+$ 613.79, found 613.22 $[\text{M}]^+$.

Preparation of compound 4

The solution of 3 (15 mg, 0.021 mmol) in DCM (0.5 mL) was cooled with ice bath. To this solution, TFA (1 mL) and triisopropylsilane (50 μ L) were added. The mixture was stirred at the same temperature under dark condition for 4 hours. Then the solvent was removed under reduced pressure. The residue was washed with cold diethyl ether (1 mL $\times$ 3). After dried, product 4 was obtained (8 mg, 81%). ^1H NMR (400 MHz, $d\text{-H}_2\text{O}$) δ (ppm): 8.19 (m, 1H), 7.01~7.02 (m, 2H), 5.51 (s, 2H) 4.2~4.3 (4, 2H), 3.54~3.41 (m, 4H), 3.1 (s, 6H), 2.85 (s, 2H), 2.73 (m, 2H), 2.35 (s, 2H), 1.35 (m, 2H), MS (EI): m/z calcd for $\text{C}_{17}\text{H}_{27}\text{N}_2\text{O}_5\text{S}^+$ 371.47, found 371.24 $[\text{M}]^+$.

Preparation of Si-UCNPs

The monodispersed core-shell UCNPs composed of NaYF_4 nanocrystals doped with Yb^{3+} and Tm^{3+} were synthesized according to the reported protocols.^[8c, 8d, 11] Then the silica coated UCNPs were prepared by following the literature procedure.^[12] Briefly, UCNPs (5 mg) were firstly dissolved in cyclohexane (20 mL). Triton X-100 (5 mL), 1-hexanol (5 mL) and Milli-Q water (850 μ L) were added to the solution subsequently. Then TEOS (tetraethyl orthosilicate, 8 μ L) and (3-aminopropyl)triethoxy- silane

(APTES, 2 μ L) were added into the mixture for 6 hours and followed by further addition of NH_4OH (200 μ L). After stirring for 12 hours, the final product was collected by centrifugation and washed thoroughly with ethanol. Both of the prepared UCNPs and of silica coated UCNPs were characterized by dynamic light scattering (DLS) and transmission electron microscope (TEM), respectively. The luminescence emission spectrum ($\lambda_{\text{ex}} = 980$ nm) of the UCNPs (0.05 mg/mL, assume the yield of the coating step was 100%) were recorded on a DM150i monochromator equipped with a R928 photon counting photomultiplier tube (PMT), in conjunction with a 980 nm diode laser.

Preparation of NIR-responsive photoactivated Si-UCNPs

The as-prepared Si-UCNPs (5 mg) were dissolved in dimethyl formamide (DMF, 100 μ L) and then the solution of 100 μ L of Mal-dPEGTM₂-NHS ester in DMF (20 mM, from Quanta Biodesign) and triethylamine (TEA, 2 μ L) were added. The reaction mixture was stirred under dark condition for 20 hours. Finally, the Mal-PEG functionalized nanoparticles were collected and washed by DMF for three times.

To the suspension of Mal-PEG-modified nanoparticles (5 mg) in DMF (100 μ L), *o*-nitrobenzyl photocaged linker and TEA (2 μ L) were added. The reaction continued for 24 hours under dark condition at room temperature. After collection of photoactivated Si-UCNPs by centrifugation, the product was washed by DMF/EtOH (v:v = 1:5) for three times and followed by washing with the phosphate buffered saline (PBS, 0.1M, pH=7.2) for three times to remove the organic solvent. The obtained product was resuspended in PBS and stored for further use. The loading efficiency for photoactive linker was estimated by measuring the absorbance of photocaged group at 325 nm. A calibration curve determined with different concentrations of photocaged linker was used to calculate the value.

Loading of siRNA onto the surface of silica coated UCNPs

The loading of siRNA to the Si-UCNPs was conducted according to the method reported previously.^[4a, 5d] The Alexa-siRNA was used to mix with cationic photocaged Si-UCNPs in PBS solution with different ratios (1:5, 1:10 and 1:15). The final concentrations of siRNA were maintained at 1 μ M. After 20 minutes incubation, the mixture was centrifuged and the pellet was gently washed with PBS for two times to remove free Alexa-siRNA. The Alexa-siRNA adsorbed Si-UCNPs complex was resuspended in PBS and the uploading of siRNA onto nanoparticles surface was evaluated by agarose gel electrophoresis experiment.

Gel electrophoresis

The agarose gels were prepared with 2% agarose in Tris-acetate-EDTA buffer (TAE buffer). All of the samples were suspended in buffer (siRNA concentration: 1 μ M, 5 μ L PBS buffer + 5 μ L Loading Buffer) and added to agarose gel subsequently. The supernatant was also added to agarose gel (5 μ L + 5 μ L Loading Buffer). The gel electrophoresis was carried out at 100V for 25minutes and then for gel imaging.

***In vitro* photoactivation of Si-UCNPs/Alexa-siRNA by 980 nm light irradiation**

A solution (10 μ L, 10 μ M of siRNA in PBS) of Si-UCNPs/ Alexa-siRNA (15:1) sample was transferred to a 1.5-mL Eppendorf tube for 980 nm light exposure. After 2 hours irradiation, the sample was diluted to 1 μ M for UV-Vis absorption measurement. The solution of Si-UCNPs/Alexa-siRNA (15:1) (100 μ L, 1 μ M of siRNA in PBS) was irradiated by UV light for 5 minutes which was used as control experiment.

Cell imaging and gene silencing experiments**Cell imaging for the photolysis of Si-UCNPs/Alexa-siRNA**

HeLa cells were purchased from ATCC (ATCC No.: CCL-2) and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. All cultures were incubated at 37 °C in atmosphere environment with 5% CO₂.

HeLa cells were seeded in a 35 mm diameter μ -dish plastic-bottom (ibidi GmbH, Germany) and cultured for 24 hours in DMEM medium without FBS. After washing with DMEM medium for three times, 0.1 μ M of freshly prepared Si-UCNPs/Alexa-siRNA complex in 1 mL of DMEM medium was added to the cells and incubated at 37 °C. After 1 hour incubation, cells were washed twice with DMEM medium and illuminated with a 980 nm laser (with a 5-min break after each 30 min exposure, 2 hours irradiation). After removal of culture medium and followed by washing with Hank's Buffered Salt Solution (HBSS buffer) for two times, the cellular imaging measurements were conducted by using fluorescence microscopy (Nikon, Eclipse TE2000-E). In the process of photo controlled siRNA delivery, the standard Alexa-siRNA releasing experiment transferred by lipofectamine 2000 (Lipo2K, from Invitrogen) was used as positive control. Similarly, HeLa cells incubated with Si-UCNPs/Alexa-siRNA complex but without light irradiation were also conducted, which was applied as a negative control.

Moreover, cellular imaging experiments with prolonged incubation times were also carried out for further study of the efficiency of NIR light-induced siRNA delivery. Basically, HeLa cells were incubated with Si-UCNPs/Alexa-siRNA complex for 1 hour and followed by 2 hours 980 nm light irradiation. After washing with HBSS buffer for two times, the cell samples were used for fluorescence microscopy imaging studies. As a control, imaging measurements were also performed by using HeLa cells incubated with Si-UCNPs/Alexa-siRNA complex for 3 hours but without any light irradiation.

Gene expression and silencing experiments

In order to evaluate if both gene expression and gene silencing would be effectively regulated by the developed NIR-responsive UCNP transducers, the photo-controlled siRNA release in the target cell lines with standard gene transfection was further performed. In this typical experiment, plasmid encoding with EGFP gene was chosen for the imaging study. The transfection process was carried out according to the standard protocol provided from Invitrogen. Basically, HeLa cells were seeded in 35 mm diameter μ -dish plastic bottom for 20 hours to give about 70-80% cell confluency, culture medium was replaced with Opti-MEM medium (1 mL). 0.6 ng EGFP DNA plasmid was gently mixed with 100 μ L Opti-MEM for 5 minutes. Meanwhile, 4 μ L Lipo2K was gently diluted in 100 μ L Opti-MEM medium for another 5 minutes. After the two solutions mixed together for 20 minutes at room temperature, the mixed solution was added into cells and followed by incubation for 5 hours. The medium was changed to DMEM with 10 % FBS. Cell images were taken after 24 hours incubation.

After transferring EGFP plasmid into HeLa cells, Si-UCNPs/EGFP siRNA (Si-UCNPs/siRNA) complex (15:1, concentration of EGFP siRNA 40 pmol) in Opti-MEM was added subsequently. Then NIR irradiation was taken for 2 hours to release the siRNA. After replacing the medium with DMEM containing 10% FBS and followed by incubation for 24 hours, fluorescent imaging experiments were performed to evaluate the process of gene silencing.

As contrast, EGFP siRNA transferred by Lipo2K was used as a positive control. Briefly, 40 pmol siRNA and 2 μ L Lipo2K were diluted in 100 μ L Opti-MEM respectively. After 5 minutes incubation, the two Opti-MEM solutions were mixed for another 20 minutes. Finally, the mixed Opti-MEM solutions were added into the

EGFP plasmid transferred cell dish. Medium was changed after 5 hours incubation and cells were incubated for another 24 hours for further imaging measurements.

MTT assay

Light cytotoxicity

The light cytotoxicity was carried out using a standard methyl thiazolyltetrazolium (MTT) assay. HeLa was seeded in a 96-wells plates with a density of 1×10^4 per well in culture medium. The medium was changed with fresh DMEM medium after 24 hours incubation. The cells were treated with light irradiation (UV or 980 nm light) for different time. The cytotoxicity activity was evaluated by standard 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay.

Cell viability assay

The cell viability measurements were also performed on the basis of the standard MTT assay. Similarly, HeLa was seeded in a 96-wells plates with a density of 1×10^4 per well in DMEM medium. After 24 hours incubation, the medium was changed with fresh culture and the cells were treated with different substrates: Alexa-siRNA only, Lipo2K/Alexa-siRNA, Si-UCNPs/Alexa- siRNA without NIR light irradiation and Si-UCNPs/Alexa-siRNA with NIR light irradiation (2 hours), respectively. All the incubation time is 1 hour and the concentration of Alexa-siRNA and UCNPs was 25 nM and 0.03mg/mL. The cells without any treatment were served as control.

For gene silencing experiment, the cell viability measurements were evaluated by using the same protocol. All of the HeLa cells were transfected with EGFP plasmid except the control cells. Then the cells were incubated with different siRNA substrates: Lipo2K/siRNA (5 hours incubation time), Si-UCNPs/siRNA without light irradiation (5 hours incubation), and Si-UCNPs/siRNA with 3 hours incubation time followed by

light irradiation (2 hours). The medium was replaced with fresh culture medium and conducted the MTT assay.

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Chapter 5

NIR Light Photo-responsive Crosslinked Upconversion Nanocarriers Toward Selective Intracellular Drug Release

5.1 Introduction

The recent advances in the fabrication and self-assembly of nanostructure carrier systems have gained extensive attention for their potential biomedical applications in drug delivery, disease diagnosis and medical treatment.^[1] Controlled-release of bioactive molecules at desired location, timing and dosage will be of great importance to guarantee the determinative therapeutic and diagnostic consequence while minimizing the side effects in the process of the treatment. Currently, a series of controlled release strategies upon external stimulation have been proposed, in which the triggered release has been systematically driven by a simple physical or biochemical perturbation^[2] such as light, ultrasound, pH, temperature, enzyme or oligonucleotids treatment *etc.* Among the proposed approaches to selectively control the release of bioactive molecules, the photolysis of photoactivable or “caged” molecules has been proven to be one of the most elegant strategies, by which the release process can be readily triggered by a beam of light with non-invasive property and high spatiotemporal precision.^[3] So far, several classes of “photocaged” systems have been reported to effectively control the release of drug molecules or imaging probes *in vitro* and *in vivo*.^[4] However, most of the existing photocaged systems have to heavily rely on high energy UV or visible light to initiate the active states of caged molecules. The inevitable cellular damage and less tissue-penetration will be the potential limitations for

Scheme 5-1. Experimental design for photo-controlled Dox delivery through photocaged mesoporous silica coated UCNPs.

their further biomedical applications in living systems. Therefore, the development of simple and specific photo-responsive systems that enable spatial and temporal control of payload molecule release with limited cellular damage and deeper light penetration will be still highly desirable and the systematic investigation remains the challenge in the field.

Recently, lanthanide-doped upconversion nanoparticles (UCNPs) have received considerable attention in nanobiotechnology and nanomedicine due to their unique photophysical properties.^[5] Generally, the lower energy near-infrared (NIR) light irradiation of lanthanide-doped upconversion nanoparticles would result in the multiplexed emission with the shorter wavelengths ranged from UV to visible region through sequential absorption of multiple photons or energy transfer process. Currently, various kinds of synthetic strategies have been established to supply the unique UCNPs with precise control over particle morphology and

converted emission wavelength. The subsequent NIR light excitation of these developed UCNP s usually exhibit narrow emission and good photostability, which have been extensively applied for biological labeling, molecular imaging and effective therapeutics *in vitro* and *in vivo*.^[6] Very recently, a few research groups including ours have demonstrated the rational design of remote control of photo-release of imaging probes and payload molecules on the basis of UCNP s.^[7] Herein, we present another simple and specific light-responsive drug delivery nanocarrier by conjugation of antitumor drug Dox with the crosslinked photosensitive linker molecules on the surface of mesoporous silica-coated UCNP s. This photo-controlled nanoparticle drug carrier system provides great opportunity for the real-time imaging and selective intracellular drug release with less photo-damage and increased tissue penetration depths in the targeted living system.

5.2 Results and discussion

Scheme 5-1 illustrated the principal design of mesoporous silica coated UCNP s nanocarrier for the photo-controlled drug release. In this design, the monodispersed Yb/Tm co-doped NaYF₄ @ NaYF₄ core-shell UCNP s will be selected as the platform for the further conjugation of antitumor drug molecules. The lanthanides-doped UCNP s will be first coated with silica shell and then coupled to 1-(2-nitrophenyl) ethyl photocaged oligo(ethylene) glycol linker with the vinyl group at the end of the structure.^[8] The simple intramolecular ring-closing metathesis reaction induced by Grubbs' 2nd generation catalyst and subsequent surface adsorption of antitumor drug molecules, Dox, in the prepared

Figure 5-1. a) and b) Size distributions of UCNPs and silica-UCNPs, determined by DLS; The insets are TEM images of UCNPs and mesoporous silica-UCNPs, respectively. c) Luminescence emission spectra of the UCNPs and silica-UCNPs. d) UV-Vis absorption spectra of silica-UCNPs, photocaged linker and photocaged linker functionalized silica-UCNPs.

mesoporous silica-UCNPs complex would facilitate the formation of crosslinked nanocarriers with Dox encapsulated into the photocaged mesoporous nanostructures. Due to the spectrum overlap between the absorption band of photocaged Dox nanoconjugate and the upconverted emission band of the UCNPs in the UV region, NIR light irradiation of UCNPs can trigger the cleavage of crosslinked photocaged linker and thus precisely control the targeted drug release from the surface of nanocarriers.

In the typical experiments, the mesoporous silica coated UCNPs complex was synthesized by using sol-gel reaction as reported previously.^[9] The dynamic light

Figure 5-2. UV-Vis absorbance of Dox before and after loading to nanocarrier. (solvent: DMSO)

scattering (DLS) and transmission electron microscope (TEM) image demonstrated that the mesoporous silica coated UCNPs exhibited a narrow size distributions of about 150 nm (Figure 5-1b), while the average size of unmodified UCNPs itself was about 55 nm (Figure 5-1a). The thicker layer of silica shell on the surface of UCNPs may enhance the biocompatibility and subsequent drug loading into nanoparticles structures. Both of unmodified and photocaged silica UCNPs revealed similar emissions in the UV, visible and NIR spectral regions upon 980 nm laser irradiation (Figure 5-1c), suggesting that modification of UCNPs with mesoporous silica shell would not significantly affect the upconversion properties. The mesoporous UCNPs were further functionalized with amino groups and subsequently coupled to 1-(2-nitrophenyl) ethyl caged oligo(ethylene)glycol linker which contained vinyl group at the end of the linkage. Absorption measurement shown in Figure 5-1d demonstrated the successful conjugation of 1.6×10^4 photocaged linker molecules on the particle surface. Finally, intramolecular polymerization of photocaged linker using a ring-closing metathesis reaction under Grubbs' 2nd generation catalyst would result in the

Figure 5-3. a) Fluorescence spectra of Dox and crosslinked nanocarrier solution with the same concentration (5 μ M in PBS) under 480 nm excitation. b) Percentage of Dox released from crosslinked Dox-nanocarrier with different NIR light irradiation. Control: photocaged linker functionalized silica-UCNPs with Dox loading but no crosslinking on the surface.

formation of the crosslinked nanocarrier which could be loaded with drug molecules for the selective photo-controlled release in the living system. The ability of cross-linked nanocarrier to encapsulate drug molecules was further studied with the commonly used antitumor drug Dox. Typically, the cross-linked nanocarrier was first soaked in a DMSO solution of Dox for 12 h. After removal of unbound drug molecules by centrifugation and repeated washing in PBS (10 mM, pH 7.2), the Dox uptake in the nanocarrier was determined by the difference in UV-Vis absorbance of Dox at ~ 480 nm (Figure 5-2) and the loading efficiency was thus calculated to be about 2.33 % (w/w), which was comparable with the results reported previously.^[6d, 9-10]

After encapsulation of Dox drug molecules into nanocarriers, the photo-controlled drug release from the crosslinked mesoporous silica-UCNPs was investigated by analysis of fluorescence of Dox in a PBS buffer solution (10 mM, pH 7.2) in the

Figure 5-4. UV-Vis absorbance spectra of photocaged silica-UCNPs after different times of NIR light irradiation.

presence or absence of NIR laser (980 nm) illumination. In general, Dox exhibited a red fluorescence emission at 590 nm with an excitation at 485 nm. The red fluorescence would be easily quenched when the Dox drug molecules were encapsulated or anchored into silica coated UCNPs nanocarriers, mostly attributed to the result of electronic energy transfer^[11] (Figure 5-3a). In the absence of NIR laser irradiation, there was very weak fluorescence of Dox observed in PBS buffer solution, suggesting the stability of the drug loaded silica-UCNPs in the buffer solution and only the very limited drug release observed from the cross-linked nanocarriers. As expected, in the presence of 980 nm light excitation, the solution of photocaged silica-UCNPs conjugate showed the significantly enhanced fluorescence, which could be due to the photo cleavage of caging 1-(2-nitrophenyl) ethyl group done by upconverted UV light from UCNPs. Figure 2b illustrated the correlation of integral fluorescence intensity of the released Dox drug molecules with the duration of NIR light irradiation. More than 75% of Dox release could be detected from silica-UCNPs nanocarriers within 20 hours upon 2 h NIR laser irradiation and even more Dox

Figure 5-5. Bright field and confocal microscopy images of A-498 cell line. Cells incubated with photocaged nanocarrier for 2 hours, a) before NIR light irradiation; b) after 2 hours NIR light irradiation. c) Fluorescence intensity of cellular incubation before NIR irradiation and after NIR irradiation. d) Cell viability assay of A-498 cells upon the treatment with different nanocarriers.

molecules could be removed from the crosslinked silica-UCNPs nanocarriers when prolonged light excitation was conducted. As a contrast, similar control experiments on the basis of the mesoporous silica-UCNPs nanocarriers but without crosslinked photocaged linker on the surface were also performed to investigate the release of Dox. As shown in Figure 5-3b, with time increasing, most of drug molecules could be

directly released from silica-UCNPs through the process of uncontrolled passive diffusion. These results clearly indicated the fact that the photocaged mesoporous silica-UCNPs could provide a stable and reliable platform toward the controlled release of drug payload molecules upon 980 nm NIR photoactivation (Figure 5-3b and Figure 5-4).

Encouraged by the promising results of effective drug release *in vitro*, we further evaluated the activities of crosslinked silica-UCNPs conjugate as a photo-controlled nanocarrier for the light-responsive drug delivery into the living cells. In this typical study, A-498 tumor cells were chosen to incubate with Dox encapsulated mesoporous silica-UCNPs conjugate (5 μ M). After 2 hours incubation and followed by 2 hours of 980 nm light excitation, the fluorescence signals observed in the cell cultures were used to investigate the process of controlled drug release. As shown in Figure 5-5a, before light irradiation, there was weak fluorescence signal detected in A-498 cells, mostly owing to the limited Dox release from the crosslinked silica-UCNPs and the fluorescence of capped Dox in the tiny silica pores was quenched as mentioned previously. But after 980 nm exposure, the crosslinked photocaged linker groups on the surface of nanocarriers were cleaved by the upconverted UV light and such efficient photolysis triggered the release of Dox molecules, which exhibited more significant red fluorescence in the cells when compared to the incubation system with Dox stored in the mesoporous silica-UCNPs but no light irradiation (Figure 5-5b and 5-5c). The significant difference in fluorescence indicated the controlled drug release in living cells upon the effective NIR light excitation.

To investigate the drug activities of photocaged silica-UCNPs nanocarriers in A-498 cells, we also performed the cell viability measurements based on the

Figure 5-6. Cytotoxicity of A-498 cell line after treated with UV light (5 min) irradiation and 980 nm light (2h) irradiation.

standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Figure 5-5d, incubation of free mesoporous silica-UCNPs with cells could not lead to obvious cell viabilities. Similarly, after incubation of Dox encapsulated mesoporous silica-UCNPs nanocarrier with A-498 cells, there was no significant cytotoxicity observed in the absence of NIR light irradiation of the nanocarriers and only the limited Dox would be removed from the surface of mesoporous silica-UCNPs. However, upon 2 hours light irradiation of drug encapsulated nanocarriers, upcaging of crosslinked surface linkers would result in the controlled release of Dox payload molecules which led to the significant cytotoxicity in the cells. As controls, the similar MTT measurements were also performed by incubation of A-498 cells with free Dox and Dox adsorbed mesoporous silica-UCNPs without crosslinked photocaged linkers on the surface. Both free Dox and Dox loaded nanocarriers without photocaged linkers demonstrated the comparable cell death under the same drug concentration. The higher cell viabilities could be observed in both of their cellular incubation when

compared to the results in the Dox loaded nanocarrier systems with photocaged linker on the particle surface. There was limited cell viability detected in the photocaged nanocarriers throughout all the time points of cell incubation, and effective antitumor drug activity could be observed only after NIR light irradiation. Moreover, another negative control experiment with only 980 nm laser irradiation but no nanocarriers incubation in the cells indicated no obvious cytotoxicity in A-498 cells (Figure 5-6). These cell viability experiments suggested that this photocaged nanocarrier could work as a reliable platform for the controlled release of drug in living cells and NIR photolysis itself would not cause the notable cell viability.

Furthermore, in order to enhance the selective photo-controlled drug release in the targeted tumor cell lines, we also attach folic acid unit onto the surface of crosslinked mesoporous silica-UCNPs nanocarriers. One major reason to select FA is mostly attributed to its higher binding affinity toward the folate receptor, one tumor-associated protein commonly found on the surfaces of various tumor cell lines with higher level expression while with less expression in normal cells.^[12] In this study, FA was conjugated to the surface of crosslinked photocaged silica-UCNPs through condensation reaction. The subsequent quantification based on UV-Visible measurements indicated that there were ~ 500 FA molecules loaded on the nanoparticles. The FA functionalized photoactive nanocarriers were finally loaded with Dox molecules under the same condition as compared to the previous nanocarrier system and drug loading percentage determined by UV-Vis absorption measurement was about 2.1 % w/w, which is similar to the photocaged nanocarriers without FA ligand conjugation on the surface.

In the process of cellular studies, HeLa cell lines were chosen as our main target due

Figure 5-7. a)~f): Confocal microscopy and bright field images of different cell lines. HeLa cells incubated with photoactive nanocarriers without FA a) before and b) after NIR light irradiation, HeLa cell line incubated with FA-Dox-nanocarriers c) before and d) after NIR light irradiation, NIH/3T3 cells incubated with FA-Dox-nanocarriers e) before and f) after NIR light irradiation. Time course of the cell viability of g) HeLa cells and h) NIH/3T3 cells treated with Dox-nanocarriers and FA-Dox-nanocarriers, respectively; All the samples were carried out with 980 nm laser irradiation and incubated for another 20 h incubation before MTT assay. The concentration of Dox was 5 μ M in all samples.

to the high expression of folic acid receptor in the cells. As a negative control, NIH/3T3 cells were used as there is no significant expression of folate receptor reported in this cell structure.^[13] Both HeLa and NIH/3T3 cells were cultured and then incubated with FA-Dox-nanocarriers in Dulbecco's modified eagle medium (DMEM) at 37 °C to investigate the effective live cell fluorescent imaging and antitumor drug activities. Similar cell incubation with the Dox-nanocarriers but no FA affinity ligand

on particle surface was also conducted, which was used as another negative control to further evaluate the cellular selectivity towards imaging and drug release. As shown in Figure 5-7d, significant red fluorescence signals could be observed into the HeLa cells upon the short incubation with photocaged FA-Dox-nanocarrier and subsequent NIR light irradiation. The similar intracellular incubation and following 980 nm laser irradiation of HeLa cells with photocaged Dox-nanocarrier but no FA ligand on the surface showed some fluorescence signals, however, the fluorescence was weaker than the HeLa cells incubated with FA-Dox- nanocarrier. These results suggested that FA target units on the surface of photocaged silica UCNPs nanocarriers can work as affinity ligand to selectively direct the photo-controlled drug release in the folate receptor-rich tumor cell lines. The weak fluorescence signals were also found in the control experiment with the incubation of FA-Dox-nanocarrier in NIH/3T3 cells. There was no significant cellular uptake enhanced by the specific recognition between FA and folate receptor in NIH/3T3 cells and such limited fluorescence observed could be mostly contributed by the nonspecific binding of released Dox from nanocarriers upon NIR laser photolysis.

Apart from the imaging results to investigate the photo-controlled drug release in living cells, the targeted intracellular antitumor activities were also studied in HeLa and NIH/3T3 cells by using two photocaged mesoporous nanocarriers with and without FA ligand, respectively. As shown in Figure 5-7g and 5-7h, cellular incubation and subsequent photolysis of photocaged nanocarrier without FA ligand in both HeLa and NIH/3T3 cells would lead to the cell viabilities in the cells, indicating the similar nonspecific cellular uptake of nanocarriers and photo-controlled Dox release in these two cells. Compare to the photocaged nanocarrier without FA units, the intracellular incubation and following light irradiation by using FA-Dox nanocarrier exhibited

more significant cytotoxicity in the folic acid receptor-rich HeLa cells (Figure 5-7g), which was consistent with the results obtained in the imaging measurement. In the cell studies by using NIH/3T3 cell lines, introduction of both FA-Dox-nanocarrier and Dox-nanocarrier with no FA conjugation would not indicate the different cell viability even after NIR laser irradiation. Unlike the higher drug activities obtained in the HeLa cells with FA-Dox-nanocarriers, there were less antitumor activities observed in NIH/3T3 cells when the cells were treated with FA-Dox-nanocarriers and followed by 980 nm laser irradiation. The low cell viability was mostly owing to the less cellular uptake of FA-Dox-UCNPs nanocarriers caused by the limited folic acid receptor expression in the NIH/3T3 cells. The targeted intracellular antitumor activities and imaging results unequivocally showed that FA-Dox-silica UCNPs may serve as an effective platform to selectively photo-control the drug release into the folate receptor over-expressed tumor cell lines without significant cellular damage caused by the NIR light irradiation. Moreover, the longer wavelength of NIR light applied here may also allow the deeper tissue penetration which could significantly benefit the further biomedical studies in the living systems with minimum unwanted side effects.

5.3 Conclusions

In this work, a NIR light-responsive crosslinked mesoporous silica-UCNPs drug delivery conjugate has been designed and synthesized. By capping the mesoporous silica-UCNPs with the crosslinked *o*-nitrobenyl photoactivatable linker, the modified silica-UCNPs complex can serve as photocaged nanocarriers to encapsulate the payload molecules within the mesopores. After loading with antitumor drug molecules of Dox and followed by NIR light irradiation, the crosslinked *o*-nitrobenyl

photoactive linker on the particle surface could be efficiently cleaved by the converted UV light from UCNPs, which could effectively trigger the photo-controlled drug release into the living cells. Moreover, upon the functionalization of the photocaged nanocarriers with FA units, the selective drug delivery can be easily achieved in the targeted tumor cell lines in which folate receptor has been highly expressed. This novel and effective drug loaded photocaged nanocarrier may demonstrate new possibility for the selective cell imaging and controlled drug release in the living system with less photo damage and deeper light penetration. Such promising applications greatly encourage us to conduct the targeted tumor imaging and drug delivery *in vivo*, which is currently under the process in the animal models.

5.4 Experiment Section

Materials: All reagents were purchased from Aldrich or Fluka. Anhydrous solvents for organic synthesis were purchased from Sigma-Aldrich and stored over activated molecular sieves (4 Å). Unless noted otherwise, commercially available reagents were used without further purification. The NaYF₄:Yb/Tm (20/0.2 mol%)@NaYF₄ core-shell nanoparticles (UCNPs) were synthesized according to the previously reported method.^[5n, 14]

Instruments: ¹H NMR spectra were recorded on a JEOL 400 MHz spectrometer. Chemical shifts (δ, ppm) were reported using TMS as an internal reference. EI mass spectra were performed on a Thermo Polaris Q. UV-Vis absorption spectroscopy was performed using a 10-mm path quartz cell on a Beckman coulter DU800 spectrometer. TEM images were recorded on an FEI EM208S Transmission Electron Microscope (Philips) operated at 100 kV. Fluorescence

Scheme 5-2. Synthesis of the photocaged linker and photocaged nanocarrier.

studies were carried out on a Varian Cary Eclipse fluorescence spectrophotometer. The size distribution of UCNPs was measured using photon correlation spectroscopy (Brookhaven Instruments Corporation). Photo-irradiation experiments were carried out using a MDL-H-980 nm laser (5.6 W/cm²) and a UV lamp (Blak-Ray, B-100AP/R, 100 W/365 nm, intensity: 8.9 mW/cm²).

Preparation of photocaged linker (Scheme 5-2)

2-(2-(allyloxy)ethoxy)ethyl 4-methylbenzenesulfonate (**1**)

2-(2-(allyloxy)ethoxy) ethanol (600 mg, 4 mmol) was dissolved in dichloromethane (DCM) (10 mL) with an ice bath. To this solution were added triethylamine (TEA, 0.75 mL). Then *p*-toluenesulfonyl chloride (900 mg, 4.8 mmol) was slowly added. The reaction mixture was allowed to warm to room temperature and stirred until the reaction was completed as indicated by TLC.

Then the reaction mixture was diluted with DCM (10 mL) and washed thoroughly with water, HCl (1 M), NaHCO₃ (1 M), and brine. The organic layer was dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The product was purified by column chromatography with EA/hexane (1:1) to afford 1 g of **1** (83 %). ¹H NMR (400 MHz, CDCl₃, δ) : 7.71 (d, J=6.8 Hz, 2H), 7.26 (d, J=8.0 Hz, 2H), 5.8 (m, 1H), 5.06 (m, 2H), 4.08 (t, J=4.8 Hz, 2H), 3.88 (m, 2H), 3.42~3.6 (m, 6H), 2.35 (s, 3H); EIMS (*m/z*): calcd for C₁₄H₂₀O₅S, 300.37, found 300.88 [*M*⁺], 323.05 [*M*+Na]⁺.

2-(2-(allyloxy) ethoxy) ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy) butanoate (**3**) To 3 mL of DMF with 4-(4-(1-hydroxyethyl)-2-methoxy-6-nitrophenoxy) butanoic acid (**2**) (200 mg, 0.66 mmol) and KF (77 mg, 1.3 mmol) were added. The suspension was stirred for 10 min, after which a solution of 380 mg (0.796 mmol) of **1** in DMF (1 mL) was added. The reaction was then continued for 48 h at 50 °C. When the reaction completed as indicated by TLC, 10 mL of EtOAc was added. The organic layer was washed with water, brine, dried with Na₂SO₄, filtered and concentrated under reduced pressure. The resulting residue was purified by silica gel column with DCM/MeOH (v:v=1:50). The product was dried under vacuum overnight to afford 220 mg (77%) of **3**. ¹H NMR (400 MHz, CDCl₃, δ): 7.57 (s, 1H), 7.31 (s, 1H), 5.9 (m, 1H), 5.57 (m, 1H), 5.17~5.3 (m, 2H), 4.26 (t, J₁=4.4 Hz, J₂=5.2 Hz, 2H), 4.11 (t, J=6.0 Hz, 2H), 3.98 (s, 3H), 3.59~3.73 (m, 6H), 2.57~2.61 (t, J=7.2 Hz, 2H), 2.2 (m, 2H), 1.55 (m, 3H); EIMS (*m/z*): calcd for C₂₀H₂₉NO₉, 427.45, found, 450.18 [*M*+Na]⁺.

4-(1-(4-(4-(2-(2-(allyloxy) ethoxy) ethoxy)-4-oxobutoxy)-5-methoxy-2-nitrophenyl) ethoxy)-4-oxobutanoic acid (**4**)

Compound **3** (100 mg, 0.234 mmol) was dissolved in 2 mL of DCM. To this

Figure 5-8. TEM image of CTAB-UCNPs in DI water solution.

solution were added 25 mg (0.25 mmol) of succinic acid anhydride, 43 μ L (0.4 mmol) of triethylamine (TEA), and 1.2 mg (0.1 mmol) of dimethylaminopyridine (DMAP). The reaction was heated to 50 $^{\circ}$ C and stirred for 12 h. Then the reaction mixture was washed with water, dried over sodium sulfate, and filtered. The filtrate was concentrated under reduced pressure and dried under vacuum overnight to afford 89 mg (72 %) of **4** as pale yellow oil. ^1H NMR (400 MHz, CDCl_3 , δ): 7.57 (s, 1H), 7.02 (s, 1H), 6.48~6.46 (m, 1H), 5.9 (m, 1H), 5.3~5.2 (m, 2H), 4.26 (d, 2H), 4.11 (t, 2H), 4.03 (m, 2H), 3.96 (s, 3H), 3.70 (t, $J=4.8$ Hz, 2H), 3.59~3.67 (m, 4H), 2.66 (m, 4H), 2.56~2.6 (t, $J=7.2$ Hz, 2H), 2.2 (m, 2H), 1.61 (d, $J=6.4$ Hz, 3H); EIMS (m/z): calcd for $\text{C}_{24}\text{H}_{33}\text{NO}_{12}$ 527.52, found, 550.15 $[\text{M}+\text{Na}]^+$.

Synthesis of mesoporous silica-UCNPs

$\text{NaYF}_4\text{:Yb,Tm}$ (20, 0.2 mol%) @ NaYF_4 core@shell nanoparticles were prepared according to the method which have been reported. The mesoporous silica-UCNPs were prepared also using a previous reported procedure. Generally, UCNPs in

chloroform (1 mL, 10 mg/mL) was mixed with cetyltrimethylammonium bromide (CTAB) aqua solution (0.4 g in 20 mL DI water). The mixture was stirred for 3 h at 60 °C. After that, the mixture was filtered by using 0.44 µm syringe filter. The filtered solution (2.5 ml) was diluted with 20 mL DI water and followed by adding NaOH (2 M, 175 µL). After stirring for 10 min at 60 °C, tetraethyl orthosilicate (TEOS) (70 µL) and ethyl acetate (1.5 mL) were added successively. After stirring for another 5 min, (3-aminopropyl)- triethoxysilane (APTES) (15 µL) was added. The reaction was maintained for 2 h. Finally the mesoporous silica-UCNPs-NH₂ product was collected by centrifugation and washed thoroughly by ethanol. Then the nanoparticles were re-suspended in ethanol (20 mL) and mixed with NH₄NO₃ (80 mg) at 60 °C for 2 h in order to remove the excess CTAB. This remove CTAB step was repeated for one more time for further use. The number of amine groups per mesoporous silica coated UCNPs was measured by ninhydrin test.^[15] There about 3.0×10^5 NH₂ on the particles surface.

The size distribution of the UCNPs and silica-UCNPs was determined by using dynamic light scattering (DLS) on a Brookhaven particle size analyzer. The morphology of the particle platform was examined with TEM (Figure 5-8). The luminescence emission spectrum ($\lambda_{\text{ex}} = 980$ nm) of the upconversion particles (0.1 mg/mL, assume the yield of the coating step was 100%) were recorded on a DM150i monochromator equipped with a R928 photon counting photomultiplier tube (PMT), in conjunction with a 980 nm diode laser.

Preparation of photocaged crosslinked nanocarrier

To a solution of photocaged linker (10 mg, 0.019 mmol) in DMF (800 µL) was added *N*-Hydroxyl-succinimide (NHS) (2.61 mg, 0.023 mmol) with an ice/water bath. Then 1-Ethyl-3-(3-dimethylaminopropyl)- carbodiimide (EDC) (4.35 mg, 0.023 mmol) was

added into the mixture and allowed to stir for 20 hours. The generated photocaged linker-NHS ester was used in the next step synthesis without further purification.

Mesoporous silica-UCNP-NH₂ (20 mg) was suspended in DMF (400 μ L), then the photocaged linker-NHS ester (1.2 mL, 20 mM) and *N,N*-Diisopropylethylamine (DIPEA) (8 μ L) was added. After overnight reaction, the photocaged linker functionalized mesoporous silica-UCNPs were collected by centrifugation and washed with DMF for three times. After re-suspension of surface functionalized silica-UCNPs together with Grubbs' 2nd generation catalyst (2 mg) in DCM (400 μ L), the mixture was allowed to stir for 24 hours in dark condition under room temperature. Finally, the crosslinked mesoporous silica-UCNPs were obtained and washed with DCM and ethanol, respectively. The loading efficiency of crosslinked photocaged linker on the surface of mesoporous silica-UCNPs was estimated based on the absorbance of 350 nm. A calibration curve determined by the known concentration of photocaged linkage group was used as a standard.

Preparation of FA target crosslinked nanocarrier

Folic acid (1 mg, 2.2 μ mol) was mixed with NHS (0.35 mg, 3 μ mol) and EDC (0.45 mg, 2.35 μ mol) in DMSO at room temperature. After 12 hours reaction, the surface crosslinked photocaged silica-UCNPs (10 mg) in DMSO were added and the reaction mixture was stirred for 12 hours at room temperature in the dark. Finally, the folic acid target crosslinked nanocarrier was collected by centrifugation and washed three times in DMSO. The as-prepared FA target crosslinked nanocarrier was used for further investigation.

Dox loading on crosslinked nanocarrier/FA-crosslinked nanocarrier

200 μ L of Dox in DMSO (10 mg/mL) solution was mixed with 6.7 mg photocaged crosslinked nanocarrier or FA-crosslinked nanocarrier. The mixture was allowed to

stir for 24 hours in the dark. After the excessive Dox was removed by centrifugation at 10,000 rpm and washed with PBS buffer solutions for three times, the final products (Dox loaded nanocarrier with or without folic acid on the surface) was dispersed in PBS for the subsequent *in vitro* and cellular experiments. During the process of drug loading, the supernatant and washed solutions were collected and the loading efficiency was determined by measuring the UV absorbance of Dox at 480 nm.

Dox release from photocaged nanocarriers by 980 nm laser irradiation

The stock solutions (20 μ L, 25 μ M, in 10 mM PBS) of as-prepared drug loaded silica-UCNPs nanocarriers were transferred to 1.5 ml Eppendorf tube and then the solutions were illuminated with 980 nm laser at different time intervals (1 ~ 2 h). After light irradiation, the samples were diluted to 5 μ M and the amount of released Dox in the supernatant solution was monitored by using a fluorescence spectrophotometer with an excitation wavelength of 485 nm.

NIR light irradiation of nanocarriers without drug loading

Samples (crosslinked nanocarrier, 75 μ M, 100 μ L) were transferred to 96-well plate followed by NIR light irradiation. Then the UV-Vis absorbance was measured in 10-mm quartz cuvettes.

Cell culture

A-498 cell line was purchased from the American-type culture collection (ATCC Cat No.: HTB-44) and maintained in DMEM medium containing 10% FBS (Invitrogen, Burlington, Canada). HeLa cells (ATCC No.: CCL-2) were cultured in RPMI-1640 medium with 10% FBS. NIH/3T3 cells (ATCC No.: CRL-1658) were cultured in DMEM medium with 10% FBS. All of the cell lines were cultured under humidified atmosphere of 5% CO₂ at 37 °C.

Cellular imaging

A-498 cells (20×10^4) were placed in a 35-mm diameter μ -dish plastic-bottom (ibidi GmbH, Germany) and cultured in medium for one day before the imaging measurement. After washing the cells twice with DMEM medium and followed by the treatment of DMEM medium (1 mL) containing 5 μ M of photocaged drug nanocarriers, the cells were further incubated for 2 h at 37 °C with 5% CO₂. Then the cells were illuminated with 980 nm laser for 2 hours (with 5 min break for each 30 min exposure). Upon light irradiation, cells were washed by Hank's balanced salt solution (HBSS) for three times. The fluorescence imaging measurements were conducted under a confocal fluorescence microscope (Nikon, Eclipse TE2000-E) with an excitation filter (535/50nm) and an emission filter (610/75nm).

For the FA target drug release experiments, HeLa and NIH/3T3 cell lines were seeded in 35-mm diameter μ -dish plastic-bottom and cultured for 24 hours in medium with density of 20×10^4 per dish. After the cells were washed twice with culture medium, the cells were incubated with FA-Dox silica-UCNPs nanocarrier (5 μ M) for half an hour. Then the cells were irradiated with 980 nm light for 2 hours (with 5 min break after each 30 min light excitation). After washing with medium and HBSS buffer solutions, the cells were ready for fluorescent imaging measurement.

Cytotoxicity assay

The *in vitro* intracellular cytotoxicity was measured using a standard methyl thiazolyltetrazolium (MTT) assay. A-498 cells were seeded in a 96-wells plates with a density of 1×10^4 per well in DMEM medium. After 24 hours, the cells were treated with 5 μ M of photocaged drug nanocarriers in DMEM medium and then were further incubated for 2 hours. Upon the appropriate light irradiation of cell samples and followed by addition of MTT solution (1.0 mg mL^{-1}), the cell viability

was evaluated by MTT assay as previously described.^[16]

For FA targeted drug release experiments, HeLa and NIH/3T3 cells were cultured in 96-wells plates with a density of 1×10^4 per well in medium for 24 hours. The cells was washed with fresh medium and incubated with FA-crosslinked Dox mesoporous silica-UCNPs for half an hour. After incubation, the cells were washed with fresh medium and irradiated with 980 nm laser for 2 hours. Then the cells were cultured for 20 hours at 37 °C with 5% CO₂ for the further MTT assays.

Light cytotoxicity MTT assays

The light cytotoxicity was measured using a standard methyl thiazolyltetrazolium (MTT) assay. A-498 was seeded in a 96-wells plates with a density of 1×10^4 per well in DMEM medium. After 24 hours cultured, the cells were treated with light irradiation (UV or 980nm light). The cytotoxicity activity was evaluated by MTT assay (Figure 5-6).

5.5 References

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Chapter 6 Summary and Outlook

Functionalized silica based nanoparticles with unique properties can be used for various biomedical applications. In this dissertation, several systems related to silica coated core-shell nanoparticles have been developed for intracellular bioactive molecules release and imaging.

As described in chapter 2, take the advantages of good biocompatibility and easy functionalization of silica, the magnetic nanoparticles were coated with silica shell and further linked with the commonly used anti-cancer drug, doxorubicin through “*click chemistry*”. The peptide linker which was used to associate doxorubicin with nanoparticle could be recognized and cleaved by enzyme cathepsin B. The Dox-peptide nanoparticle conjugates showed high selectivity and good efficiency of drug delivery in living cells.

Photocage has emerged as a remarkable technique that usage of light to manipulate photoactivatable compounds in biological systems. However, many of these attempts were using high-intensity UV or other short wavelength light which may cause photodamage to cells. Recently, lanthanide-doped upconversion nanoparticles have obtained considerable attention for biological applications. In the following chapters, the silica coated UCNPs were employed to study the NIR-induced photocaged therapeutic agents release.

A novel probe, photocaged upconversion nanoparticles for controlled uncaging and bioluminescence imaging studies was designed in chapter 3. One commonly used bioluminescent probe, D-luciferin, was bound to the particle surface through a photocaged linker. The uncaging of D-luciferin from the conjugates could be

initialized by upconverted UV emission from upconversion nanoparticles upon NIR light excitation. This method exhibited several advantages, such as deep light penetration and low cellular damage.

The use of NIR light-responsive guest molecules delivery based on UCNPs could be extended to gene/siRNA release. In chapter 4, cationic photocaged linker modified silica coated UCNPs were served as siRNA nanocarriers. The upconverted UV light from UCNPs was used to release siRNA through NIR light exposure. Additionally, siRNA was adsorbed on the particles surface through electrostatic interaction which can assist siRNA remain its bioactive in the RNA interference pathway.

In chapter 5, mesoporous silica coated UCNPs were synthesized and used for NIR light-responsive drug delivery. Drug molecules were stored in pores of mesoporous silica shell and further capped by the cross-linked photocaged linkers. All of the results indicated that these nanocarriers could effectively transport and release drug into the targeted cells under NIR light irradiation.

Despite the great progress in biomedical applications, however, the use of silica-based nanomaterials in nanomedicine field still need to be further investigated. The cytotoxicity and efficiency should be first concerned when design such delivery systems. Thorough understanding of the interactions between the cells and nanoparticles (including the functionalized nanoparticles) are still lacking. Although there have been tremendous efforts in the cell cytotoxicity studies of silica-based nanoparticles, more efforts are still needed in the living systems. An encouraging advance in this field is the Cornell-dots have been approved by FDA for the clinical trial recently. On the other hand, to overcome the efficiency, development of theranostic platforms based on nanomaterials with higher payload is still urgently needed. Furthermore, detailed studies of cellular uptake mechanism of the

functionalized nanoparticles are important to improve their theranostic abilities.

List of Publications

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