Near-infrared light for phototherapy : photothermal induced drug release and tumor ablation

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Near-infrared light for phototherapy:

Photothermal induced drug release and tumor ablation

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School of Materials Science and Engineering

2015

**Near-infrared light for phototherapy:
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School of Materials Science and Engineering

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Technological University in partial fulfilment
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Abstract:

Since the introduction of nanotechnology into cancer treatment, numerous therapeutic nanoagents have been developed for the detection, imaging and therapy of various types of cancers. Amongst all these efforts, near-infrared-light-involved anticancer therapy has drawn increasing attention in recent years, due to the minimal invasiveness and high tissue penetration of near-infrared light (NIR). This thesis will focus on the utilization of NIR light in anticancer therapy in combination with inorganic and organic NIR-responsive photothermal agents, of which could absorb the incoming NIR irradiation and transform it into thermal energy. The temperature escalation is used to initiate the release of anticancer drugs from the nanocarrier or directly destroy the cancer cells. The studies listed in this thesis made a robust investigation into the potential application of NIR-driven therapeutic nanoplateforms and showed inspiring results both *in vitro* and *in vivo*.

Gold nanorods are one of the many types of anisotropic metallic nanoparticles used in biomedical research. With their highly achievable monodispersity and manipulable aspect ratio, the optical absorption wavelength of gold nanorods could be precisely controlled within the biological window of living tissues, which is ideal for controllable and efficient photothermal heat generation. Chapter 2 introduces a stimuli-responsive drug delivery system using a single gold nanorod as the core

compartment. The hydrophilic anticancer drug doxorubicin was encapsulated using ultraviolet irradiation and could be released in response to NIR illumination. Its controllable drug release behaviour has been further investigated in vivo in live zebrafish embryo models.

Polymer chemistry is one of the most important sub-branches of modern chemistry that is closely related to our daily life. It could provide us with products at various sizes with unique physical and chemical properties. Chapter 3 describes one of my attempts to combine the specifically functionalized polymer with gold nanorods for a controllable drug delivery system. A pH/temperature dual responsive polymer shell was conjugated onto the gold nanorods for the drug encapsulation and delivery. Though the eventual outcome is not satisfactory, it still provided some inspirations for my follow-up research.

The lessons learnt from the dual responsive core/shell drug nanocarrier proved substantial, which offered useful insights for the shaping of a new NIR responsive drug delivery system. To overcome those problems that are persistent to the organic/inorganic hybrid nanoparticles such as low cellular uptake and morphological uniformity, a new organic photothermal agent IR825 was used with thermoresponsive liposomal formulations. The structural characterizations and therapeutic efficacy were introduced in Chapter 4.

My studies of these NIR responsive therapeutic nanoplateforms presented a variety of applications based on nanomaterials that were capable of photothermal heat generation. Collectively these studies

provided robust insights on the application of NIR laser in cancer treatment, as well as expanded our knowledge of nanotechnology in drug delivery research.

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Chapter 1: Introduction

Cancer, a name given to a collection of related diseases, is one of the greatest threats to people's health and well-being throughout human history^[1]. For all types of cancer that have been identified, they share two major characteristics: the malignant proliferation of abnormal cells and the ability of these cells to migrate to other parts of body from its site of original occurrence, through either the lymphatic system or blood circulation^[2]. In most circumstances, the cancer cells would join together and form solid tumors consisting of massed tissues^[3].

For the treatment of cancer, the aim is to remove the tumor tissue as much as possible and at the same time prevent their recurrence at the site of origin as well as their invasive spreading^[4]. The treatment could take up many forms that are distinctively different from theory to practice. Conventional types of cancer treatment mainly are: surgery^[5], radiotherapy^[6] and chemotherapy^[7]. While they have been honed for decades by the specialists together with researchers and imbued with continuous optimization, each one has its own intrinsic disadvantages. For instance, surgery, which is the most common technique used for cancer treatment, is only capable of removing visible solid tumors, and as the tumor grows in size or enters the metastasis stage, its effectiveness significantly declines. As for chemotherapy and radiotherapy, they are always troubled by the severe collateral damage to the health organs and

tissues beyond the desired site of action^[8]. To circumvent these disadvantages, much of the research work in the field of cancer treatment is focused on developing new cancer therapies, and light-controlled therapy is one of those that are now under clinical testing. For cancer therapies where light is involved, the incoming light is mostly used to initiate the release of anticancer drugs^[9] or stimulate some other photosensitive compounds^[10, 11] to achieve the expected results. With the late development in electro-optics technology, light illumination could be precisely controlled within the diseased area with manipulable duration of treatment, thus guaranteed the great safety and versatility of the light-controlled cancer therapy.

Among all types of light that are currently used in clinical trials and research, near-infrared light is spectacularly favoured by the researchers and scientists. This chapter introduced the characteristics of NIR light and its usage in biomedical applications. A brief introduction was given on the NIR-tissue interaction and the advantage of NIR light, and finally an overview accounting on the safety issues for the implementation of NIR light in cancer treatment.

1.1 Definition of near-infrared light.

Infrared light, which is often called IR for short, is a kind of radiant energy that is invisible to human eyes. Compared with the visible light, its wavelengths were significantly longer, which ranges from 700 nm to 1 mm. The name “IR” is a general term given to any light that falls within this wavelength region, and could still be subdivided into a series of smaller

sections. A commonly accepted subdivision of the IR spectral band specified by ISO 20473 is shown below^[12]:

Designation	Wavelength range
Near infrared (NIR)	0.78-3 μ m
Mid infrared (MIR)	3-50 μ m
Far infrared (FIR)	50-1000 μ m

Table 1-1. Subdivision of the infrared region, given by ISO20473.

As is demonstrated in this table, the near infrared (NIR) light is closest to the wavelength range visible to human eyes. NIR light was traditionally used in fibre optic telecommunication^[13] and night vision devices^[14]. However in recent years, the utilization of NIR light in clinical and biological applications has also increased drastically. With its superior tissue penetration and non-invasiveness, NIR-involved biomedical applications has attracted much interest and enjoyed a booming development.

1.1.1 NIR-Tissue interaction: the biological windows

Though complete light-impermeable in human eyes, human tissues can become spectacularly transparent to incoming electromagnetic radiations at specific spectral bands where their light absorption and scattering rates were both at a minimum^[15]. The figure below shows the extinction

spectrum of a typical human tissue where the absorption and scattering effects were both taken into account.

Figure 1-1. The extinction coefficient of typical living tissue to light irradiation of various wavelength. The causes of light extinction as well as the two biological windows were also indicated. Reproduced with permission from reference ^[10]

As is seen in the figure, there are primarily two spectral bands that show extraordinarily low extinction coefficient in comparison with the other regions. These two bands are commonly referred to as the two major “Biological Windows”^[15], of which the details are shown below:

The first biological window starts from 700 nm and ends at 980 nm and is defined by the light absorption bands of hemoglobin (visible light range) and water (980 nm) combined^[16]. The absorption of the incoming light in this wavelength region is reduced to a minimum, which greatly increases

the tissue penetration of these light irritations. It is important to note that due to the residual scattering of water and other small molecules, the optical extinction would still exist, although its relevance significantly decreases as the wavelength of the incoming light increases.

The second biological window is from 1000 nm to 1400 nm^[17]. Nevertheless, it also corresponds to the light absorption of water. However, unlike the first biological window, for electromagnetic radiations in this wavelength range, it is the optical scattering that has come to a minimum as the light wavelength increases. The light absorption, while being small, cannot be completely neglected (the average absorption coefficient of water in the said range is around 0.5 cm⁻¹).

With these characteristics introduced above, using laser devices with specific wavelengths in the biological windows is quite appealing to researchers for biomedical applications. Unlike visible or ultraviolet light, light illumination in the biological windows would allow for deeper tissue penetration while attenuating the possible collateral damage to the healthy cells and tissues along the light path, thanks to the simultaneously minimized light absorption and scattering. It has been reported that in realistic situations, the maximum human tissue penetration achievable has effectively reached a few centimetres under proper optimization.^[18] With emerging optical devices such as optical fibre delivery ^[19] and endoscopes^[20], it is expected that the light irritations could reach deeper into the human body with improved precision and efficacy.

1.2 NIR-responsive materials for photothermal heat generation.

This part will introduce several typical materials that could effectively convert light into thermal energy. The interaction modes between the material and the light beam have been reviewed as well as the heating mechanism. The knowledge could help us design new nanomaterials with high-energy conversion efficiency and at the same time substantially improve the current NIR-associated therapeutics. Specifically, the interactive mode between the light beam and the material itself determines the light absorption spectrum and effectiveness of that material which in turn, affect the ways that this material is used in therapeutics.

For most of the NIR-involved nano-therapeutics which are currently used or under test, one thing they have in common is that they all operate in the first biological window. Only a few nano-therapeutics, such as specifically-tuned gold nanorods and carbon nanotube, would take the second biological window as the channel for operation^[10]. It is widely accepted that using the first biological window for photothermal generation is more advantageous than the other one, as the light irritations coming in the spectral range of the first biological window would solely heat up the NIR-absorptive nanomaterial itself other than the surrounding tissues or body fluids, due to the minimal light absorption of water in this region. While for the second biological window, as is previous mentioned, due to the enhanced light absorption from water in this spectral range, light irritations coming in this region would not only heat up the nano-

Figure 1-2. a) Schematically illustration showing typical photothermal materials and their optimal spectrum ranges of operation. Abbreviations: GNSph: spherical gold nanoparticles, GNRs: Gold nanorods, Nd:NPs: nanoparticles doped with neodymium, CNTs: Carbon nanotubes, O:NPs: Organic NIR absorptive nanoparticles, GNSs: star-like gold nanoparticles. b) Conceptual representation of the interaction mechanism between the incident light and the nano-sized photothermal agent. As is shown in the

figure, photoluminescence and photothermal heating would only occur as the result of light absorption. Reproduced with permission from reference^[10].

therapeutics but all the cells and tissues in the area of operation as well. The non-discriminative photothermal heating would impose a potential threat to the healthy tissues and reduce the efficacy and selectivity of the nano-therapeutics themselves^[21].

Upon the illumination from a proper light source, some photons from the incident beam will be inevitably scattered by the nanosized therapeutics while some others might be absorbed instead. Those photons which are absorbed by the nano-therapeutics are usually converted directly into thermal energy or luminescence at the different wavelength. As the total energy of the incident light is usually restricted in biomedical applications, it is easily understood that materials with high absorption efficiency and low luminance yield should be used if we want to maximize the photothermal generation. In these sections below, several typical NIR-responsive photothermal nanomaterials are introduced, with details on their chemical compositions, structures and light/matter interaction modes.

1.2.1 Gold nanostructures.

Gold is one of the typical noble elements and has already been used as jewels, currency and utensils for thousands of years. The first report of nanosized gold particles came out in 1857 by Michael Faraday, in which he described the formation of some “fine particles” by reducing aqueous

gold chloride with phosphorous as well as carbon disulfide as the additional stabilizer. The synthetic route developed by Michael Faraday is much the same as the methods we are using for colloidal gold nanoparticles today, where gold ions are reduced under the protection of some surfactants to prevent the aggregation of newly formed nanoparticles. Due to the chemical/physical inertness, versatile surface modification and tuneable morphology, many gold nanostructures have been developed and used in biomedical and industrial applications, such as nanospheres, nanorods, nanoshells, nanocages, *etc.*, each has its own unique structural and physical/chemical properties, and could fulfil specific goals under different circumstances.

Figure 1-3. SEM and TEM visualization of various gold nanostructures have been used in photothermal applications: (a) nanoparticles, (b)

nanorods, (c) nanobipyramids, (d) hollow nanosemishells, (e) nanocages, (f) hematite core/Au shell nanograins, (g) nanostars, (h) silica /Au core/shell nanohybrids, and (i) nanocubes. Reproduced with permission from reference^[22]

Amongst the various types of gold nanostructures, gold nanorods have been extensively studied due to the various synthetic methods available and easy achievable aspect ratio control. Gold nanorods are essentially a non-spherical gold nanostructure stabilized by ligands, usually CTAB.^[23] Due to its anisotropic nature, the optical absorption spectrum of gold nanorods could be divided into two distinctively different sections, namely the longitudinal absorption band, which is located at longer wavelength region and significantly stronger, and the cross-section absorption band, which is usually located in the visible spectrum and much weaker.^[24] It is understood that the longitudinal absorption is caused by the electron oscillation along the long axis of the gold nanorods, while the latter one is associated with the transverse electron oscillation. It has been confirmed in numerous reports that the longitudinal absorption of gold nanorods is determined by its aspect ratio, which could be finely tuned from around 600 nm to beyond even 1200 nm to meet specific wavelength demands. Specifically, the longitudinal absorption band will significantly shift towards the longer wavelength end when the aspect ratio of the nanorods increase. The wide spectrum coverage and the tuneable light absorption makes the gold nanorods a potent photothermal heat source.

Figure 1-4. A schematic presentation of the surface plasmon resonance (SPR) from a single gold nanorod upon light irritation. The two oscillation modes are demonstrated in (a) and the light absorption spectrum is shown in (b). It should be noted that the SPR along the long axis and the short axis correspond to the longitudinal and transverse absorption respectively. Reproduced with permission from reference^[24]

This figure above shows how a single gold nanorod interacts with the incident light in the right wavelength region. When the incident light first reaches the gold substrate, its electromagnetic field will cause the conduction electrons on the nanorods' surface to oscillate coherently, in resonance with the very frequency of the incoming light itself. The interaction of the free electrons and the electromagnetic field will result in a charge separation between these electrons and the charged gold atom core and Coulomb repulsion among the electrons themselves. The coulomb repulsion will act as a restorative force pushing the free electrons in the opposite direction, and in the end cause all the free electrons to oscillate resonantly. The photon energy dissipated during this inelastic

process would be then release as heat, thus complete the whole process of photothermal heat generation.

There are many issues associated with the light absorption peak and photothermal conversion efficiency of gold nanorods. Through DDA simulations it was calculated that gold nanorods with length of 45 nm and width of 12 nm were optimal for nanorod-based photothermal treatment. Those nanorods of this type gave a longitudinal absorption peak near 800 nm. And for this particular geometry and size of gold nanorods, it was predicted in the simulation that the absorption coefficient of photons at the wavelength of 800 nm could be high as 98%, which meant that nearly all the incident light energy would be absorbed. This simulative prediction was confirmed by the work of L. M. Maestro *et al.*^[25] where they found that this particular gold nanorod has a light-to-heat conversion efficiency of almost 100% through monitoring with quantum dot-based fluorescence thermometry. Additionally, gold nanorods with width of 15 nm and length over 100 nm tend to have their longitudinal absorption peak over 1000 nm, which is preferable for photothermal therapy that is conducted through the second biological window.

There are also other gold nanostructures that operate in the NIR range, such as nanoshells^[26], nanocages^[27], nanostars^[28]. In addition to the traditional photothermal capabilities that the gold nanorods possess, these new gold nanoparticles bring many new features like broadened peak of light absorption and drug loading, some of them can even be used for NIR-guided *in vivo* imaging.

1.2.2 Carbon nanostructures.

Aside from gold nanostructures, some types of carbon-based nanomaterials are also capable of generating heat when illuminated with light. Given by its chemical composition and biological inertia, carbon-based nanomaterials are considered excellent candidate for biomedical applications. Based on their structure, the various types of carbon-based nanomaterials can be divided into two sub-categories: the nanodiamonds and graphite-based nanostructures. Nanodiamonds have already been utilized extensively in biomedical applications as luminescent biomarkers.^[29] They are capable of providing high resolution tracking of bioactivities intracellularly. Moreover, it has been reported in recent researches that nanodiamonds have been proposed as nanothermometers which could be used for the thermal mapping of cells.^[30]

Nanodiamonds excel in applications such as biosensors and *in vivo* imaging, however, when it comes to photothermal heat generation, it is the graphite-based nanostructures that show better performance.

The most developed carbon nanostructure that is originated from graphite is carbon nanotube, which was first discovered by Iijima *et al* in 1991.^[31] Carbon nanotubes are essentially sheets of graphene rolling into a cylinder shape. The widths of carbon nanotubes are usually only a few nanometres, however, their lengths may range from only a dozen nanometres to even beyond tens of micrometres. The varying aspect ratios bestow carbon nanotubes with versatile physical and chemical properties, as is was observed that even a slight change in the geometry of the carbon nanotubes would confer significant change to its electronic structure. With

fine tuning of their size and geometry, it was possible to prepare carbon nanotubes with particularly high absorption coefficient in the NIR range, which is most suitable for photothermal heating.

Figure 1-5. a) Common carbon nanostructures used for photothermal applications. b) Comparison of the light extinctive characteristics of single-wall carbon nanotubes, multi-wall carbon nanotubes and graphene oxide nanoparticles. Reproduced with permission from reference^[10].

According to the simulation results of the de-excitation process of the carbon nanotubes, when the carbon nanotubes are illuminated with light at the corresponding wavelength, it could generate heat as well as luminescence, as the de-excitation of carbon nanotubes involves both luminescence and non-radiative energy dissipation^[32-34]. The relative proportion that the absorbed energy is to be released is presented as the fluorescent quantum yield of the nanotubes. In the case of multi-wall carbon nanotubes, the fluorescence would be effectively quenched by the inter-layer interactions and other mechanisms, and consequently, a great proportion of the light energy would be converted into heat. It could be seen that the photothermal mechanism of carbon nanotubes is very similar to that of gold nanostructures, that heat is mostly generated through the relaxation of surface plasmon.

In comparison with other metallic nanoparticles that are capable of photothermal heating, especially gold nanostructures, carbon nanostructures usually have lower conversion efficiency at around 50%. However, the deficiency in absorption is compensated by the wide range of irradiation spectrum. As is reported by L. M. Maestro *et al*, unlike the gold nanostructures which are highly wavelength-dependent, carbon

nanotubes could operate at several different wavelengths across over 300 nm, and were still working reliably^[25].

1.2.3 Quantum dots

Figure 1-6. a) Comparison of the light extinction spectra of typical CuS QDs with Au doped CuS nanostructures. b) Comparison of light extinction spectra of typical CuS QDs with advanced CuS nanostructures. (Inserted image shows a CuS nanoflower under SEM). Reprinted with permission from A. M. Derfus, W. C. W. Chan, S. N. Bhatia, *Nano Lett.* 2004, 4, 11. Copyright (2004) American Chemical Society^[35]

Quantum dots (QDs) are semiconductive nanocrystals and have been established as a successful fluorescent probe in the past decade. Quantum dots have already been used in many applications for bioimaging and biosensing. CdSe, CdS, CdTe and PbSe are typical semiconductor combinations that are currently used to prepare the quantum dots^[36]. QDs are toxic in nature, but some recent reports indicate that, with proper surface engineering it is possible to mitigate their toxicity risks, and these hypotheses have already been confirmed on live small animals. These modifications greatly improved the overall reliability of QDs in biological environment and might further promote its usage in biomedical applications.^[37, 38]

In theory, the optical properties of QDs are determined by two crucial factors: the types of the semiconductor combination and size. Among the numerous types of QDs that have been developed, copper-based quantum dots have drawn particular interest for biological applications at early stages^[35]. These copper-based QDs could be used as biocompatible fluorescent probes as it doesn't contain Cd or Pb. More importantly, for the interest of NIR-induced photothermal heat generation, one of its light absorption peaks is located beyond the ultraviolet-visible light range and reaches as high as 980 nm, unlike other QDs. The origin of the abnormal NIR absorption band is yet unknown^[39]. There are also alternative method to induce photothermal hyperthermia using NIR light with QDs, which is to use semiconductors with indirect band gap. An example of this type of QDs is the hydrophilic germanium-based QDs, of which the light absorption band extends from UV-vis range to over 900 nm. The

photothermal efficiency of Ge-QDs is not yet fully studied and still remains to be explored systematically before using it as a photothermal agent. [40]

1.2.4 Organic chromophores.

The nanomaterials described above are potent candidates for the photothermal heat generation *in vivo*. However, it also suffers from the common drawback shared by almost all inorganic nanoparticulate systems, that it cannot be degraded through metabolism or excretion. This has been one of the major obstacles for the real clinical tests of gold nanorod as well as many other inorganic nanoparticulate therapeutic systems. [41] On the other hand, organic dyes have been traditionally used in biomedical

Figure 1-7. Typical organic chromophores currently used in photothermal applications. Reproduced with permission from reference^[42]

applications as the fluorescent agent for imaging and detection, due to their excellent clinical performance and low long-term toxicity concern^[43]. For instance, two NIR fluorescent contrast agents, namely the indocyanine green (ICG) and the methylene blue (MB)^[43], have already been approved by FDA for clinical fluorescence optical imaging. To fulfil their role as fluorescent contrast agent, these dyes have a high quantum yield (QY) which is preferable for clinical imaging. Enlightened by these successful trials, research has begun trying to use the chemical derivatives of these dyes for in vivo photothermal heat generation, hoping to decrease their QY to a moderate level while retain their NIR absorbing capability.

Typically, when the organic photothermal agent were administered into living tissues and illuminated with NIR laser at corresponding wavelength, it will absorb the incoming photons and reach an excited state. Instead of emitting photons like those fluorescent agents, when returning to its ground state most of the energy it absorbs is dissipated through non-radiative processes such as dynamic collisional quenching, and thus converted into thermal energy, which is then absorbed by the surrounding tissues thereby enhancing the environmental temperature in its vicinity.^[44]

In regard with biomedical applications, organic NIR chromophores boast many advantageous features including high light-absorption coefficient per unit mass, excellent biosafety and biocompatibility. However, they also have several persistent drawbacks which may severely

impair its therapeutic efficacy. Most of the current organic chromophores are highly hydrophobic and need additional organic solvent to facilitate solubilization, even though, they may still be cleared rapidly from human body with a bi-exponential plasma clearance. The photothermal ablation with organic NIR chromophores is non-discriminative for both cancer cells and healthy cells, and its actual outcome may vary due to temperature and light interference. Though they could not be metabolized within the body, their circulation time is still very limited. Usually they are removed from the circulation system by the liver within several hours of dosage and then excreted into bile.^[45]

To address these inherent problems of organic NIR chromophores, there are several major strategies that have been recently reported. Take the indocyanine green (ICG) for instance, which is an organic dye that has been approved by FDA for fluorescence imaging while also capable of photothermal heat generating. In a paper published by Jie Yu et al^[43], the ICG molecules have been loaded into nanoparticles which have an average diameter of 120 nm after antibody coating. Remarkably the preparation was completed in an all-water process. This nanopreparation could be used for tumor imaging and additionally when excited with an NIR laser source of 808 nm, it could also confer photothermal damage to cancer cells. Other approaches include using polymeric shell with vectors for the targeted co-delivery of ICG and doxorubicin, hoping to achieve the combinational chemo-photothermal cancer therapy.^[46]

1.3 NIR optical devices current available for biological applications:

After decades of development, the NIR optical technology is already mature for practical implementation in biomedical applications. Though they may be different in power, appearance or optical pathway, all these devices still share the same fundamental mechanism of photon emission. Basically, when the metal alloy crystals in the filament are energized with electric current, they could emit infrared radiations at specific wavelength in regard to the types of metals that are used. The power of IR emission is positively correlated to the current intensity within a certain limit. Below are some of the most common NIR light sources that are commercially available.

1.3.1 Conventional NIR lamps.

These lamps usually employ tungsten-halogen bubbles for the generation of photons, which is a widely-used general-purpose visible-NIR light source for many spectroscopic applications. This type of IR lamps comprise a tungsten wire as the filament with fused silica enveloping around it, a small amount of active halogen gas is also infused into the bubble, such as bromine and iodine. The tungsten-halogen bubbles are nearly blackbody radiation sources and can provide very stable spectral output. In practice they could cover a fairly large area with NIR light with adjustable power density. However, these lamps are very large in size and thus can only be used for the NIR treatment of superficial tissues, such as skin cancer.

Figure 1-8. A typical NIR lamp used in clinical trials.

1.3.2 Fiber optics for NIR illumination in vivo.

These optical devices also use the tungsten filament as the NIR light source. But instead of shining the object directly with the bubble, the NIR radiations are collected and then converge into light fibres. The light-guiding optical fibres that are currently used in clinical practice are mainly flat-cleaved fibres. With these devices it is possible to deliver NIR light to the deeper tissues inside human body. The problem with those optical fibre devices is that the laser power that can be delivered through the fibre is quite limited, as high laser irradiance at the tip of the fibre would almost certainly lead to excessive temperature escalations, which could potentially carbonize the surrounding tissues and damage the fibre itself.^[47]

To remedy this problem new fibre materials and manufacturing process have been developed which allow for a more homogeneous light distribution within the fibre and at the same time reduce the energy loss during the light transportation. With these improvements we now could deliver greater amount of therapeutically useful light energy to even larger and greater tissues.^[48]

Figure 1-9. A typical computer-operated endoscope which could be used for intrabody NIR illumination.

1.4 NIR treatment for photothermal heat generation *in vitro* and *in vivo*

Once the cancer site is located and the nanomaterials used for the NIR-induced photothermal heat generation is selected, the subsequent step will be finding out the best conditions and procedures to carry out the NIR illumination. This step is essential for the success of photothermal therapy and photothermal induced drug release both in *in vitro* evaluations and future clinical trials. In this section, some of the current experimental techniques and protocols that have been used for the identification of

photothermal efficacy are introduced, which should cover most of the relevant pre-clinical trials both *in vitro* and *in vivo*.

1.4.1 NIR treatment for photothermal heating *in vitro*.

Cellular uptake and cell-killing efficiency are the first things to consider when designing an anticancer therapy with nanomaterials. Under most circumstances, the nanotherapeutics are simply dispersed in a solution medium which should be completely biosafe and then added into the extracellular environment under proper atmosphere and temperature conditioning. There are several methods available to regulate the interaction between the cells and the nanomaterials. For instance, it is possible to set different time limit for the co-incubation so that we may control the amount of nanomaterials that have been internalized by the cells or adhered to the cell membrane that the longer the incubation lasts the greater is the amount of the nanomaterials that have been assimilated by the cells. Though simple this may seem it may be very difficult to find the optimal incubation condition in cell experiments as it is determined by many factors. Some factors that have been considered relevant are: the size, shape, charge state, concentration and surface engineering of the nanomaterial itself.

The cellular uptake is only one of the many issues associated with the photothermal efficacy of certain nanomaterial at cell level. The level of photothermal heating within cells is also dependent on other factors like the particular type of cells that is incubated, the intensity, wavelength, pulse interval and polarization of the NIR laser light source. Considering the complexity of this issue, only some of the most relevant factors will be

introduced in the section below, which are namely the intracellular nanoparticle allocation, particle size and morphology, particle concentration, duration of incubation and the illumination setup of the laser.

1.4.1.1 Effects of the intracellular particle allocation on the photothermal efficacy.

It was first discovered by W. Zhou *et al* that the allocation of the photothermal nanoparticle within the cells is closely related to the eventual outcome of the photothermal treatment.^[49] In their work, they investigated and compared the photothermal damages that could be achieved by using NIR laser to irritate gold nanorods that were outside the cells or already internalized by the cells. The gold nanorods they chose for the study were 30 nm in length and 8.4 nm in width, and the excitation wavelength of their laser source was around 800 nm. They found out that under the continuous-wave mode and with moderate laser power output, the photothermal damage caused by the internalized gold nanorods was more severe than that caused by the gold nanorods in the culture medium. A more detailed quantitative analysis was given in the work presented by M. Aeschbacher *et al*, in which they tested the photothermal damage induced by continuous-wave NIR illumination in human liver cancer cell lines (HepG2 line) using gold nanorods coated with phosphorylcholine as the photothermal agent.^[50] For the assessment of the cell damage they used the FDA dye as the fluorescent cell marker, so that the number of damaged cells could be quantified using confocal fluorescence microscope. It was observed that for cells with gold nanorods uptake, after 130 seconds of

NIR laser illumination all cells in the irritated area were virtually eradicated. While for those cells that were simply “bathed” in gold nanorod solutions, even though after a prolonged NIR treatment as long as 190 seconds, most of the cells in the illuminated area were still very much alive (without the FDA fluorescent staining). They also observed that most of the gold nanorods that were internalized were located in the endosomes and lysosomes, and they speculated that one possible mechanism of the photothermal therapy is that the hydrolytic enzymes would be released from the endosome/lysosomes upon the photothermal heating and decompose the functional proteins in the cytoplasm, thus result in the disruption of normal cell activity and disintegration of the cell structure.^[10]

Figure 1-10. Photothermal damaged conferred by gold nanorods intracellularly or extracellularly with different NIR illumination duration. Reproduced with permission from reference^[49].

It is presented in the examples shown above that to maximize the efficacy of photothermal treatment, the photothermal agent are required either being internalized or adhering to the cell membrane. It is also suggested in some literatures that for purely photothermal ablations, selectively attaching the photothermal agent to the cell membrane shows superior performance relative to the endosome/lysosome internalization design. Several factors may contribute to the enhanced photothermal damage: (i) the photothermal hyperthermia may directly disrupt the cell membrane, causing the leakage of the cytoplasm, (ii) the selective accumulation of the photothermal agent may concentrate the hyperthermia in a smaller area, rather than spreading in the whole cytoplasm and (iii) the thermal conductivity of the cell membrane is significantly lower than that of the cytoplasm, which may facilitate a sharper temperature gradient in the vicinity of the photothermal agent.^[51] Aside from these speculations, some researchers also suggest that the attaching the photothermal agent on the cell membrane may promote the cell perforation through the light-induced cavitation pathway.^[52, 53]

To control the selective subcellular accumulation of photothermal agents, many surface functionalization techniques have been developed which may fulfil different demands. For instance, polyethylene glycol coating has been commonly used various metallic photothermal nanoparticles to prevent the *in vivo* aggregation as well as prolong their circulation life. In another paper published by El-Sayed *et al.*, they conjugated gold nanorods with monoclonal antibodies which could specifically recognize a certain type of biomolecule that is overexpressed

in the cancer cell lines they used. By comparing the experimental results on the cancer cell line and healthy epithelial cell line, they found that after the conjugation the nanorods would preferably bind to the cancer cells, as even a weak NIR laser power of 80 mW would result in the massive cell death, while the laser power that was required to destroy the healthy cells was high as 320 mW. The higher gold nanorod uptake by cancer cells was also confirmed by surface plasmon resonance detection, where the cancer cells showed a much stronger peak after the incubation with antibody-conjugated gold nanorods.

Figure 1-11. The selective uptake of anti-EGFR conjugated gold nanorods by malignant cells. Three cell lines (HaCat (normal), HSC and HOC (malignant)) were chosen for this study, the circle indicated the laser-

irritated area, photothermally damaged cells were stained using a blue fluorescent marker. Reprinted with permission from *J. Am. Chem. Soc.*, 2006, 128 (6), pp 2115–2120. Copyright 2006 American Chemical Society.^[54]

Surface engineering of the photothermal agents is not only useful to enhancing the particle accumulation or selective subcellular accumulation, but it can also serve other purposes. For example, specifically modified photothermal nanoparticles could be used for the controlled release of anticancer drugs. Gold/Mesoporous silica core/shell hybrid structure is a well-established nanocarrier for the photothermal induced drug release. This system employs a single gold nanorod as the heat source and has it coated with a layer of mesoporous silica of which the thickness could be adjusted at will. The addition of the mesoporous silica shell not only eliminates the cytotoxicity issues with gold nanorods prepared with cetyltrimethylammonium bromide (CTAB) as the protective ligand, but also facilitate the subsequent surface modification of other functional groups. Moreover, the mesoporous feature of the silica layer could significantly improve the drug-encapsulation capability of the core/shell structure. A good example of using the functional gold/silica core/shell structure in drug delivery applications is reported by Xiaogang Qu *et al.*,^[55] in which they conjugated anticancer aptamer DNAs onto the surface of the mesoporous silica scaffold. They demonstrated the possibility of using the aptamer DNA as both a targeting agent for selective cellular uptake and capping agent for the controlled cargo release. The aptamer AS1411 used

in their design was a guanine-rich oligonucleotide of 26 nucleotide units and already under phase II clinical trials for the treatment of acute myeloid leukemia as well as renal cell carcinoma. In its original G-quadruplex state the AS1411 could specifically bind to nucleolin receptors with great affinity, which is overexpressed in various types of cancer cells. When the functional gold nanorods were illuminated with NIR laser of matching wavelength, the light-induced localized hyperthermia would dehybridize the duplex DNA structure and eventually remove the G-quadruplex, thus unblock the mesopores in the silica shell and effectively release the cargo molecules within. This work demonstrates that how the photothermal agent and the surface modifications could cooperate to achieve the effective photothermal-controlled drug delivery *in vitro*.

1.4.1.2 Effect of size and morphology of the photothermal nanomaterials

It is already introduced in the previous sections that the size and morphology is the dominant factor for the photothermal property of many metallic nanoparticles, as they have a crucial role in their energy conversion process and determine their photothermal efficiency. However aside from the nanomaterials themselves these factors may also affect their cellular uptake capabilities. The effects were investigated and demonstrated in the work of B. D. Chithrani *et al*^[56], where they compared the cellular uptake efficiency of spherical gold nanoparticles and the anisotropic gold nanorods with the same surface modification in the same cancer cell lines. They found that those gold nanospheres showed higher cell internalization efficiency compared to the gold nanorods. Moreover,

the greater the aspect ratio was, the more difficult for the nanoparticles to be taken in by the cells. Regarding their observations they explained that it requires more time for the cell to wrap up and fully take in nanoparticles with greater length. They also incubated the cancer cells with a series of gold nanospheres with different diameter from 10 nm to 100 nm and analysed their cellular uptake capability individually. Through the comparative study they concluded that optimal particle diameter of gold nanospheres for maximized cell uptake is at around 50 nm^[57].

1.4.1.3 Effects of particle concentration and time of incubation.

These two factors function in a very similar way so they will be discussed here together. Both of them are widely accepted as the critical parameters for the determination of cell uptake of almost all nanoparticulate systems in *in vitro* experiment. It is noted that nanoparticles are generally internalized by cells in a two-step process: the nanoparticle must first bind to the cell membrane and then be internalized through endocytosis, of which the later usually takes much longer time (roughly over 10 hours for typical nanoparticles). However it has been pointed out in many literatures recently that the overall particle uptake usually shows saturated trends against both parameters, that the number of nanoparticles that can be internalized by a single cell is in the order of several thousand at most.^[58, 59] As they have found out, prolonging the incubation time of cell lines with media containing the nanoparticle beyond 4 hours or unlimitedly increasing the particle concentration in the culture media didn't necessarily increase the number of nanoparticles that were successfully internalized.^[60] Moreover, more internalized particles

don't equal more efficient photothermal heating, so prolonged incubation duration or high particle concentration is not usually required.^[56]

When it comes to the determination of the optimal incubation time and nanoparticle concentration, cell internalization efficiency is not the only parameter that should be considered. It is important that the cytotoxicity of the particles and their possible aggregation or disintegration should be taken in account as well.^[61, 62] It is shown in many literatures that most nanoparticles show low or almost no cytotoxicity under low concentrations, while their cytotoxicity significantly increase when the incubation duration or concentration is increased. Thus, the cytotoxicity of the photothermal agent must be evaluated together with its photothermal efficacy when designing photothermal treatment. As for the particle aggregation, it is already mentioned that the optical properties of many photothermal nanomaterials are very sensitive to the state of aggregation. For instance, the NIR absorption peak of gold nanorods would shift towards longer wavelengths when several nanorods aggregated with the extinction coefficient and the energy conversion efficiency greatly deteriorated. So increasing the number of particles internalized by cells may result in a decreased photothermal efficiency in some situations.^[62]

1.4.1.4 Effect of the laser illumination setup on the efficacy of photothermal treatment

To achieve the best cost-effectiveness and optimal target localization, the light source currently utilized in NIR photothermal treatment are lasers. By the luminescence time profile of these laser sources, they could be

generally divided into two types: the pulse laser and the continuous wave laser (abbreviated to PW and CW lasers, respectively.) Typically for the PW laser, they usually have much higher peak power intensity than the CW ones, therefore their cell-damaging mechanism is also a little different from the CW lasers. Despite the difference in their luminescence mode, two factors are of uttermost importance when designing photothermal therapies using either type of laser as the light source, which are the power density and the energy fluence of the laser. The laser power density describes the photon energy the focal area receives in unit time, while the energy fluence is defined by the total photon energy delivered to the target per unit area. It is easily understood that the laser power density could be adjusted by tweaking the laser power or resizing the focal area, and the energy fluence could be manipulated by changing the illumination time or the laser power density. When it comes to the photothermal heat generation *in vitro*, due to the various parameters involved the optimal laser intensities may vary in a wide range. As is reported from various sources of literatures where CW lasers are used, the common laser power setups range from 1 to 100 W/cm².

The association between the laser fluence and the photothermal effect *in vitro* has also been thoroughly investigated, which has been summed up in a set of medical guidelines for laser safety.^[63] The photothermal damage on cells at varied energy fluences has been systematically studied in a work published by T. B. Huff *et al.*^[64], where they incubated the KB cell lines (a cell line that is originally extracted from oral epithelium) gold nanorods and subsequently illuminated the cell samples at different laser

fluences. For those cell samples with gold nanorods uptake, severe cell damage has been observed where the photothermal treatment induced intense blebbing even at the lowest energy fluence (30 J/cm^2). However in the control groups where no gold nanorods were added, no blebbing was observed even when the energy fluence has been increased to 120 J/cm^2 .

The above discussion focused primarily on the setup of CW lasers. For PW lasers, due to their different luminescence time mode, some other techniques have been developed which function rather different from those with CW lasers. One of them is the laser-activated nano-thermolysis method (LANTCET) which is developed by Lapotko *et al.*^[65] Through the illumination of PW lasers bubbles were generated intracellularly around the gold nanoclusters, which could be used to detect and selectively destroy the cancer cells. The LANTCET method relies on the non-stationary character of the pulse heating. Theoretically speaking, when illuminated with the pulse laser, the instantaneous high energy input will be converted to localized hyperthermia by the gold nanocluster, which will then evaporate the cell sap around the cluster surface, thus result in the emergence of the short-life nanobubbles (also termed as plasmonic nanobubbles or PNB for short). These PNBs are in a constant vibrating state between expanding and collapsing, and their lifetime ranges from 20 ns to 300 ns. The mechanical stretching or shrinking caused by the bubbles when they emerge or vanish within the cancer cells will disrupt the cell membrane and other important cellular organelles, resulting in the immediate cell lysis. As was demonstrated in their work, with a relatively weak single pulse laser source (40 mJ/cm^2), they have successfully killed

97% of the cancer cells on the focal area while the collateral damage on the adjacent healthy cells was much less severe (cell viability remained above 80% after the NIR treatment). The energy fluence of the laser source used in the experiment was 4~6 orders of magnitude lower than previous reports which also used gold nanoclusters for the purely photothermal ablation of cancer cells.

1.4.2 NIR treatment for photothermal heating *in vivo*

The administration of NIR-induced photothermal heating *in vivo* is even more complicated than the *in vitro* experiments, as it involves even more factors such as the temperature gradient at the target site, the intrabody circulation of the photothermal agent and so on. A commonly used protocol for simple *in vivo* experiment consists of the following steps: First, tumors are induced on healthy animal specimens, either through chemical induction or xenograft. Subsequently, the photothermal agent was administered on these specimens by either direct injection or intravenous injection. As soon as enough photothermal agent has accumulated at the tumor site, the tumors would be illuminated with NIR laser. To evaluate the efficacy of the NIR treatment, the progression of the tumors must be monitored and plotted and the parameters used to indicate the progression are the tumor size and weight.

To achieve the optimal treatment efficacy, even though they are free from the usual wavelength issues with visible or ultraviolet light, the NIR laser source must also be carefully adjusted and positioned so that it would only heat up the selected area of tissues that has been incorporated with the photothermal agent. As a matter of fact, the temperature elevation

caused by the photothermal heating is determined by the laser power density and illumination time, so consequently the outcome of the NIR treatment should also be dependent on these two factors. The hypothesis was supported by many studies, and a relevant one is published by N. Huang *et al.*,^[66] in which they investigated how the laser intensity and treatment duration could affect the photothermal ablation with carbon nanotubes on nude mice. When the tumor volume on the mice reaches around 70 mm^3 , the mice in the experimental group were injected with $100 \text{ }\mu\text{L}$ of PBS which contained 120 mg of PEGylated SWCNT directly in the tumor. The tumor site was then irritated with continuous-wave NIR laser at 808 nm for 3 min (Power density of the NIR laser was kept at 70 W/cm^2). It could be seen from the results that for those mice in the experimental group, their tumors have been completely destroyed 20 days after the NIR treatment, and all mice were still alive even 60 days after the treatment. Moreover, they reported that those photothermally treated mice survived more than 6 months after the NIR treatment, and no sign of cancer recurrence nor any toxic effect has been observed. However, for those mice that have been illuminated with NIR laser (both those mice that have not been administered with PEGylated SWCNT and those incubated with PEGylated SWCNT but haven't been illuminated with NIR), the tumors evolved rapidly with time and 25 days after the tumor inoculation their average size reached 3000 mm^3 , also all mice in these two sample groups have died within 60 days of the inoculation.

1.4.2.1 Tracking of the photothermal agent in biosystems.

As is already discussed above, to improve the photothermal efficiency and minimize the collateral damage to healthy tissues, it is preferable that the photothermal agent could be selectively accumulated in the tumor site. Though it is possible to administer the photothermal agent through direct intratumor injection to ensure a high intratumoral concentration of the photothermal agent, it doesn't guarantee the effective cell uptake of these subcutaneously injected nanoagents. The alternative administration method is to inject the photothermal agents intravenously and let them accumulate in the tumor site actively or passively. In this regard it would be ideal if the transportation and allocation of the photothermal agents could be monitored during the *in vivo* experiment. There are several specialized analytical techniques available for the quantification of the photothermal agent *in vivo*, such as instrumental neutron activation analysis^[67], high performance liquid chromatography^[68], inductively coupled plasma-mass spectrometry^[69] and immunohistology^[70]). But the simplest way is still to use photothermal agent that has innate luminescence. A good example of this type of theranostic systems is reported by H. Dai *et al.*^[71] where they used PEGylated Ag₂S quantum dots for the photothermal ablation of grafted 4T1 murine subcutaneous tumor. This type of quantum dot has a moderate quantum yield of around 15%, which should allow the intravital monitoring of these quantum dots while doesn't sacrifice too much of their photothermal capability. The wavelength of the luminescence emission of those Ag₂S quantum dots is located at near 1200 nm, which happens to fall into the second biological

window. Moreover, the average diameter of these quantum dots is about 54 nm, which is very close to the optimal particle size for cellular uptake introduced in previous sections. It is shown in the imaging result that the fluorescence intensity at the tumor site reached the maximum 10 hours after the intravenous injection of the Ag₂S quantum dots, which indicated that the Ag₂S quantum dots could be selectively accumulated at the tumor site as the result of the so-called EPR effect.

1.4.2.2 Using the photothermal effect to facilitate other cancer therapies

As has already been mentioned before, the photothermal treatment in vivo could be used in combination with other cancer therapies to achieve better therapeutic outcome, such as chemotherapy and radiotherapy. A relevant example could be found in the work published by R. Atkinson *et al.*,^[72] where they injected breast-tumor bearing mice with gold nanorods and treated the mice with both NIR laser and ionizing radiation of 6 Gray. After the combinational treatment the authors observed that the tumor has regressed and the number of radio-resistant stem cells have decreased as well. Moreover the authors reported that after the treatment the tumor has become more dormant.

1.4.2.3 Safety precautions on the temperature control during the NIR photothermal treatment.

During the photothermal treatment, the final temperature of the tumor target site is determined by a lot of factors, of which the major ones include laser illumination setup such as laser power density, energy fluence and

laser wavelength, amount of nanoagents that were successfully internalized and the incubation environment. It is widely accepted that

Figure 1-12. Thermographic imaging showing the area spreading of NIR induced hyperthermia on mice intravenously injected with PEG protected gold nanocages. Reproduced with permission from reference^[73].

when the temperature rises above 48 °C, the hyperthermia will confer irreversible damage to the affected cells. Thus to ensure the maximized efficacy of photothermal treatment while minimizing the unwanted damage to the adjacent healthy tissues, thermal imaging is usually employed to monitor the thermal field in a real-time manner. In the work published by J. Chen *et al.*,^[73] it was revealed by the infrared thermal camera that even when the photothermal agent was completely confined within the tumor, as the laser illumination went on the hyperthermia continued to spread and eventually affected the surrounding healthy tissues. Thus to minimize the non- discriminative damage caused by the photothermal heating, new techniques and equipment must be developed which are capable of monitoring the thermal field real-time and even controlling the laser illumination dynamically to eliminate the collateral damage.

1.5 Conclusion

This chapter revolves around the photothermal effect using near-infrared light and its application in anticancer therapy. Some of the typical NIR-absorptive photothermal nanoagents and their mechanism of action have been introduced, together with the common equipment and guidelines used for the NIR treatment both at the cell level as well as on live animal specimen. In the chapters that follow, several new types of NIR-responsive nanoparticulate therapeutic systems have been developed based on these background information, where the NIR-induced

photothermal heating has been successfully used for controlled release of anticancer drugs and the ablation of the tumor tissue.

Chapter 2 Controlled Drug Release from Rotaxane-Capped Mesoporous Silica Coated Au Nanorods with NIR Light as the Trigger

2.1 Introduction

Nowadays light illumination has been widely used in various applications as an inducing signal, due to the fact that the delivery and dosage of light could be remotely controlled with high precision and operability in a localized area.^[9] In regard to drug delivery applications, by adopting to a light responsive design it could further enhance the stimulus sensitivity and drug delivery efficiency of drug nanocarriers as well as minimize the adverse side effects. In the past several years, there have been numerous reports on the designing and fabrication of light-responsive drug delivery systems, and the most notable examples include photo-isomerization of azobenzene derivatives,^[74] self-assembled pseudorotaxanes and their light-induced destabilization^[75, 76] and other types of photo-cleavable linkages.^[77] For instance, the photoisomerization of azobenzene between the *trans* and *cis* geometries has been utilized to achieve the controlled release of cargo molecules.^[74, 78] However, most of these drug delivery systems rely on ultraviolet or visible light as driving

force for the triggered drug release, which may incur undesired collateral damage on healthy cells and tissues. This issue was solved by introducing near-infrared light as the triggering signal which operates in the aforementioned “first biological window”.^[79] The adoption to NIR illumination has significantly augmented the biosafety of the light treatment, moreover the enhanced tissue penetration of NIR light allowed for the controllable drug release even at sites deep within the body.^[80]

As mentioned in the discussion above, azobenzene is a type of photo-responsive organic materials which was originally used as synthetic coloring agents in the dyestuffs and coloration industry.^[81] It was in 1937 that Hartley reported concrete evidences on the formation of *cis*-azobenzene after exposure to light, as he observed that the light-absorption of the azobenzene species has significantly changed after the light treatment.^[82] Eventually he successfully isolated the *cis*-azobenzene through solvent extraction. A tremendous amount of research efforts have been dedicated to the azobenzene derivatives and up until now, it is still one of the most-thoroughly studied organic photoswitches. *Trans*- and *cis*-azobenzenes have overlapping spectra absorption but are distinctive at some wavelengths. Specifically, *trans*-azobenzenes usually show weak n–p* absorption bands near 440 nm and strong p–p* transition band at around 320 nm. On the contrary for *cis*-azobenzenes, they usually have stronger n–p* absorption bands at the same wavelength but shorter p–p* transition bands at 280 nm and 250 nm respectively. The thermodynamic analysis of these two isomers shows that the *tran* isomers are more stable than the *cis* ones, which requires an activation energy of 2 kcal/mol.^[83]

Thus it is easily understood that at normal conditions, the *trans* isomers will be the dominant conformation (over 99.99%). When provided with UV irradiation or heated above 35 °C, the *trans*-azobenzene species will undergo the conformational change and transform into the *cis*-isomers.^[84]

The possibility of utilizing the photo-isomerization of azobenzene between its *trans* and *cis* state for the light-controlled blocking and unblocking of nanochannels has been confirmed by several reports, but as is previously mentioned all of them employ invasive triggering signal of ultraviolet and visible light, which may potentially undermine their therapeutic efficacy in some more realistic situations. Herein, we report the design and preparation of a new NIR-responsive drug delivery system that is to conjugate the azobenzene-based rotaxanes on the surface of mesoporous silica coated gold nanorods (denoted as Au@MSN-Rotaxane). Specifically in this design, the core compartment is a gold nanorod with fine-tuned longitudinal absorption wavelength, which would function as the light-to-heat converter and initiate the *cis* to *trans* isomerization of the azobenzene units.^[85] The intermediate mesoporous silica layer could be used as the substrate for the subsequent surface modification of the rotaxanes^[86, 87] while the nanochannels inside could serve as the reservoirs for the drug loading and releasing.^[88, 89] The azobenzene-based rotaxanes are anchored on the silica surface with a non-cleavable covalent linkage through click reaction.^[90] For the rotaxane^[90] it comprises of an α -cyclodextrin ring (α -CD) encapsulated on the *trans*-azobenzene site. The loading of the anticancer drug is achieved through diffusion in solution at 40°C, as the drug molecules would be enriched within the unblocked

nanochannels due to the capillary effect. After the drug loading the nanocarrier would be illuminated with UV light so that the drug molecules would be sealed within the silica nanoreservoir. The drug release after administration is achieved by illumination with NIR laser. The drug release behaviour of the hybrid nanoparticle has been investigated both in solution and on live zebrafish embryos as well. The release and distribution of the fluorescent anticancer drug *in vivo* was monitored using a confocal laser scanning microscope (CLSM).

Scheme 2-1. Graphical demonstration of the preparation process and the cargo release mechanism of the NIR-responsive functionalized Au nanorod/mesoporous silica core/shell hybrid nanostructure (Au@MSN-Rotaxane), excorporeal NIR illumination was used to control the switching of the surface-conjugated rotaxane remotely so as to regulate the cargo release.

2.2 Experimental section.

2.2.1 Materials and equipment

All the chemicals used in this project were purchased from Sigma-Aldrich and used directly without any further purification. FT-IR spectra

were monitored on a Perkin-Elmer 1760X FT-IR spectrometer with the sample powder dispersed in KBr pellets (1%). BET surface areas were measured on a Micromeritics ASAP 2020M automated sorption analyzer. The transmission electron microscopy (TEM) images were taken on a JEOL 2010 high resolution transmission electron microscope. UV-vis spectra absorption analyses were performed on a Shimadzu UV-3600 UV-vis-NIR spectrophotometer (1-mm quartz cuvette). Fluorescent spectra were measured on a Shimadzu RF-5301 spectrofluorophotometer.

2.2.2 Methods.

2.2.2.1 Preparation the Au/Mesoporous silica hybrid structure (Au@MSN)

To start with, the Au nanorods were firstly synthesized according to some previous studies with several minor modifications.^[91] Initially, the ultra-small Au seeds were prepared by reducing HAuCl_4 in aqueous environment with NaBH_4 solution. Concentrated cetyltrimethyl ammonium bromide (CTAB) solution in deionized water (0.2M, 10 mL) was first added with HAuCl_4 solution (m/V 1%, 100 μL) and subsequently, the ice-cold aqueous solution of NaBH_4 (0.01 M, 0.6 mL) was quickly injected to the above mixture. The ultra-small Au seeds were immediately formed upon the addition of reductive agent. The Au seeds were always used freshly within 3 h after the preparation.

The growth solution for the fine-tuned Au nanorods was a mixture of CTAB (0.1 M, 100 mL), HAuCl_4 (0.01 M, 5 mL), AgNO_3 (10 mM, 1 mL), H_2SO_4 solution (0.5 M, 2 mL), these chemical solutions were added

sequentially and in the end ascorbic acid solution (0.1 M, 800 μ L) was added into the above mixture to reduce the Au^{4+} ions to Au^{1+} . The growth was initiated by adding the freshly prepared seed solution to the growth medium (240 μ L). The nanorod was allowed to grow for 6 h and throughout the growth process the temperature was kept at 30 $^{\circ}\text{C}$. The synthesized Au nanorods were first washed with deionized water for several times to remove the excessive CTAB and remaining reactants and then extracted through centrifugation. The collected Au nanorods were eventually concentrated to 10 mg Au/mL.

The coating of mesoporous silica was achieved via a well-established template method. Firstly, 1 mL of the concentrated Au nanorod solution was re-dispersed in aqueous solution of CTAB (0.01 M, 100 mL), and the mixture was gently stirred for about 15 min before the next step. Ammonia water (0.01M, 1 mL) was subsequently added into the Au nanorod solution in order to adjust the solution pH to slightly basic, three injections of tetraethyl orthosilicate (TEOS, 30 μ L each) was then added into the solution with 30 min interval. The temperature of the mixture solution was also kept at 30 $^{\circ}\text{C}$. The reaction lasted about 24 h. The silica coated Au nanorods were then extracted from the solution via centrifugation and washed with ethanol for 3 times to remove the unreacted reagents.

The CTAB template in the silica layer was removed by treating with ethanol solution of hydrochloride. To be specific, the as-synthesized silica coated Au nanorods (60 mg) were dispersed in ethanol (40 mL) containing concentrated hydrochloric acid (m/V 35%, 5 mL), and the mixture was heated to 40 $^{\circ}\text{C}$. After 24 h of reaction, the activated mesoporous-silica-

coated Au nanorods were collected again through centrifugation. The extracted sample was dried under vacuum at room temperature and the product was denoted as Au@MSN.

2.2.2.2 Synthesis of N-(3-(triethoxysilyl) propyl) propiolamide

The ligand N-(3-(triethoxysilyl) propyl) propiolamide was synthesized through the protocol reported in a previous literature.^[92] N,N-Dicyclohexylcarbodiimide (2.46 g, 12.0 mmol) was added into the mixture dichloromethane solution (15 mL) of 3-aminopropyltriethoxysilane (2.40 g, 10.9 mmol) and propiolic acid (0.87 g, 11.9 mmol). The mixture was stirred at room temperature for 1 h, and then separated firstly using a filter paper. Excessive solvent in the remaining mixture was removed by co-evaporation with toluene (40 mL). The co-evaporation was successively repeated 3 times, and in the end the purified solution was diluted with toluene to 20 mL.

2.2.2.3 Grafting N-(3-(Trimethoxysilyl) propyl) propiolamide onto Au@MSN surface (Au@MSN-Alkyne)

The anhydrous Au@MSN was first homogeneously dispersed in toluene (10 mg of the dried Au nanorod powder in 20 mL anhydrous toluene) and then added with 1 mL of the N-(3-(triethoxysilyl) propyl) propiolamide in toluene solution. The mixture was refluxed at 120°C for 24 h to allow for sufficient ligand functionalization. After that, the product was extracted via centrifugation. The precipitate was washed first by acetone and then with deionized water for several times, after that the extract would undergo the anhydration under vacuum at room temperature

for another 24 hours. The product of this step was denoted as Au@MSN-Alkyne.

2.2.2.4 Synthesis of the 7-((4-(2-(2-azidoethoxy) ethoxy) phenyl) diazenyl) naphthalene-1,3-disulfonic acid and conjugation of photo-responsive rotaxane onto Au@MSN-Alkyne

The synthesis of the water-soluble azobenzene moiety, 7-((4-(2-(2-azidoethoxy) ethoxy) phenyl) diazenyl) naphthalene-1, 3-disulfonic acid, was performed following a route reported in a previous paper^[90]. The azobenzene intermediate (150 mg, 0.27 mmol) and α -cyclodextrin (525 mg, 0.54 mmol) were then dissolved together in H₂O (10 ml) and stirred at room temperature under N₂ protection for 2 h to give the complex rotaxane unit, denoted as Azidized-Switch 1.

The 5 mL water solution of Au@MSN-Alkyne (20 mg), CuSO₄•5H₂O (3.4 mg), and sodium ascorbate (3.4 mg) were added to the above mentioned complex solution for the immobilization of the rotaxane unit on the silica surface. The mixture solution was then allowed to react at room temperature for 3 days. The product was again extracted via centrifugation, and then washed by deionized water for several times to give the final product, denoted as Au@MSN-Rotaxane (Scheme 2-2).

Scheme 2-2. Procedure for the preparation of the rotaxane conjugated Au/Silica hybrid structure.

2.2.2.5 Characterizing the conformation transition of Au@MSN-Rotaxane

100 μg of the Au@MSN-Rotaxane was first dispersed in deionized water, which was stored at 35°C for 10 min to allow for complete *trans-cis* conformation transition. The UV-vis absorbance was subsequently recorded and the obtained absorption spectrum was denoted as the “on” state. The sample solution was then cooled down to room temperature and subsequently irradiated with UV light of 365 nm for 15 min. When the illumination is complete the UV-vis spectrum was measured again, which was denoted as the “off” state. The cuvette that bearing the same sample solution was subsequently illuminated with NIR laser (808 nm, 200mW/cm²) for 30 min before the UV-vis spectrum was again measured (Scheme 2-3). The UV-NIR illumination processes were repeated for 5 cycles using the same cuvette and sample solution so as to eliminate the interference of other potential factors.

2.2.2.6 Photothermal effect of Au@MSN-Rotaxane

For the characterization of the photothermal capability of the Au@MSN-Rotaxane, 500 μg of Au@MSN-Rotaxane was dispersed in a cuvette containing 0.5 mL of deionized water. To achieve the maximum light absorption efficiency, the cuvette was wrapped in tin foil. Temperature of the solution was measured using a digital thermometer. The lens module of the NIR laser was placed right above the cuvette and from the spot size and the total laser power output the power density of the NIR illumination was calculated to be $1.5\text{W}/\text{cm}^2$. Deionized water of same volume was used as the experiment control, which has also been continuously illuminated with NIR laser using the same setup. Initial temperature of all the sample solutions was all at around $25\text{ }^\circ\text{C}$.

2.2.2.7 Drug loading and measurement of the release profile

FITC, a widely used fluorophore, was used as the model drug and loaded into Au@MSN-Rotaxane in aqueous solution via diffusion at $40\text{ }^\circ\text{C}$. The loading solution was placed under dark environment and the whole process lasted for 1 day. FITC-loaded Au@MSN-Rotaxane was then irritated with UV light at 365 nm for 15 min, where the *trans*-to-*cis* isomerization of the rotaxane in Au@MSN-Rotaxane should block the mesopores. The FITC-loaded Au@MSN-Rotaxane was then extracted through centrifugation, and has been washed with deionized water for several times to remove those FITC molecules attached on the particle surface. For the release profile measurement, each cuvette was poured into 20 μg of FITC-loaded Au@MSN-Rotaxane, and deionized water was added to make the total volumes of all these solutions at 1mL. Noting that

all the sample solutions were kept undisturbed for 30 minutes until all the nanoparticles have settled at the bottom. Three sample groups of the same setup were prepared for each condition. The release behaviors of FITC-loaded Au@MSN-Rotaxane under heat and NIR irradiation were monitored by the spectrofluorophotometer. The excitation and emission wavelengths chosen for the experiment were 495 nm and 519 nm, respectively. A blank sample where no external trigger (25 °C, placed in dark environment) was used as the control group. For the temperature induced release, the temperature of the sample solution was kept at 25 °C for the first 5 min and subsequently heated up to 45 °C using a digital heat source. For the NIR triggered release, the sample solution was illuminated with NIR laser continuously (power density of the laser: 200mW/cm²). The cuvette was also wrapped with tin foil to ensure the NIR absorption. For all the three samples, the fluorescent intensity readings were taken after 5 min, 11 min, 19 min, 45 min, 100 min and 180 min, respectively. The release tests under each condition have all been repeated 3 times to eliminate accidental error.

2.2.2.8 Evaluation of the controlled release on zebrafish embryo models

Doxorubicin (DOX), which is one of the typical clinical anticancer drug, was used as the cargo and at the same time the fluorescent indicator. Au@MSN-Rotaxane (40 mg) was dissolved in aqueous DOX solution (1mg/mL, 10 mL), and the mixture was stirred at 40 °C in dark environment for 1 day to ensure the maximized amount of drug loading. DOX-loaded Au@MSN-Rotaxane was also extracted through

centrifugation. It was first illuminated with UV light for 15 min and subsequently stored in a sealed container out of light at 25 °C. For every 5-day old zebrafish embryo, a dose of concentrated DOX-loaded Au@MSN-Rotaxane solution (40 mg/mL, 5 μ L) was injected into its brain ventricle. Each mounted zebrafish embryo was imaged at bright field with HeNe 543 nm laser attached to a 560/615 nm emission band-pass filter, which were used to simultaneously detect optically opaque gold nanorods and the intrinsic red fluorescent of the DOX molecules. For the release test on the zebrafish embryo models, the particle-injected embryos were illuminated with NIR light (power density: 1.5 W/cm²) for 1 h. The experimental samples were then incubated together with the controls in a dark environment at 25 °C for another 2 days before final imaging. This process was to evaluate the long-term drug release effect in vivo. For the study of the drug-release kinetics, the two experimental groups were illuminated with NIR light for 10 min and 5 min respectively, one day after the injection under the same power density (1.5W/cm²). The embryos were then immobilized and mounted, and simultaneously imaged for 2 h after the NIR illumination. In another experimental group, the injected embryos were incubated under the physiological temperature of 37 °C for 3h and subsequently imaged. The image size and corresponding positions in the imaged area were all kept the same, and a fixed line where the terminal position was demarcated by an arrowhead was first drawn across the bright field image. Bright field intensity profile, detecting the distance occupied by the opaque Au@MSN-Rotaxane, with the minimum gray value along the demarcated line was measured and depicted. The DOX intensity

profile was subsequently examined across the same position and length using Image J. A red serrated box was drawn to delimit the region that corresponded to the DOX trapped in the mesoporous silica layer of the Au nanorods. Equal distance was selected and compared across all samples and control group.

2.3 Results and discussion

2.3.1 Morphological and optical characterization of the functional Au/Silica hybrid structure.

The Au@MSN-Rotaxane nanocarrier was successfully prepared and the relevant physical/chemical characterization results were shown below. As shown in Figure 2-1a, it is confirmed in the TEM observations that the as-prepared Au nanorods have controlled size and homogeneous morphology, and it has been maintained in the Au/silica hybrid structure(Au@MSN, Figure 2-1b) and Au@MSN-Rotaxane (Figure 2-1c). The length and width of the original Au nanorods were 75 ± 15 nm and 18 ± 5 nm, respectively. The thickness of the mesoporous silica coating added through the template method was between 10-20 nm. In comparison to the bare Au@MSN species, the appearance of those Au@MSN-Rotaxane has become more blurry, which might be explained by the addition of the rotaxane.

Figure 2-1. TEM characterization of the morphological evolution during the synthetic process. a) Au nanorod, b) Au@MSN, and c) Au@MSN-Rotaxane. Scale bar: 100nm

The optical absorption of the nanomaterial was characterized using Uv-vis spectra. For the original gold nanorods, they had a longitudinal absorption peak at around 808 nm and a cross-sectional absorption band at around 520 nm. After the coating of the mesoporous silica shell, the cross-sectional band remained almost the same while the longitudinal peak has slightly shifted towards longer wavelength. The discrepancy could be explained by the dependence of surface plasmon patterns on the material interface, where the spectral shifts depends roughly linearly on the refractive index of the substrate.^[93] As the refractive index of the particle surface has increased significantly after the silica coating (the refractive index of monocrystalline gold surface is 0.28,^[94] while that of the mesoporous silica is high as 1.54.^[95]) which would result in the enhanced localized refraction of the incident light. When the alkyne groups were grafted onto the silica surface, the longitudinal absorption band of the hybrid structure shifted to a shorter wavelength at 780 nm, and eventually shifted to 830 nm after the surface conjugation of the photo-switchable rotaxane. Additionally, a new absorption peak at 380 nm was also

observed after the rotaxane conjugation, which was attributed to the characteristic UV absorption characteristics of the *trans* azobenzene unit.^[96]

Figure 2-2. UV-vis spectrum characterization showing the changes in the optical absorption of the hybrid nanostructure at different stages. 1: Naked Au nanorods, 2: Au@MSN, 3: Au@MSN-Alkyne, and 4: Au@MSN-Rotaxane.

2.3.2 BET characterizations of the porous feature of this hybrid nanostructure.

The N₂ adsorption/desorption isotherm characterizations and the derivative pore size distribution curves shown in Figure 2-3, which was used to demonstrate the porous properties of Au@MSN. It was shown that the total surface area of the Au@MSN hybrid nanostructure was 535.110 m² g⁻¹, the total pore volume was 0.8215 cm³/g. The average pore diameter was found to be 1.45 nm, which was typical for mesoporous

silica nanochannels prepared through the CTAB-template method. It was also noteworthy that the surface area of the as-prepared nanostructure was slightly less than other pure mesoporous silica nanoparticles, which could be explained by the implementation of the high-density gold nanorod as the core compartment.

Figure 2-3. BET characterizations showing the a) isotherms and b) the pore size distribution of Au@MSN.

2.3.3 Characterization of the surface modification process for the functional Au/Silica hybrid structure.

The surface modification process for the preparation of the functional Au/Silica hybrid structure was monitored with FTIR spectrum. As is depicted in Figure 2-4, when the mesoporous silica layer was coated onto the bare gold nanorods, a strong peak emerged in the vicinity of 1100 cm^{-1} , which could be explained by the Si-O bond in the mesoporous silica structure. After the surface conjugation of the ligand with alkyne end groups, several new IR peaks were detected at 1536, 2100, 2860 and 2930 cm^{-1} , of which the first one was assigned to the amide bond, the two in the middle to the alkyne groups and the last one to the alkyl stretching. As is proposed in Scheme 2-2, in the subsequent conjugation of the azobenzene-based rotaxanes through click reactions, these alkyne groups would be exhausted. This process was also reflected in the comparative spectrum analysis from Au@MSN-Alkyne to Au@MSN-Rotaxane, as the characteristic alkyne IR peak at 2100 cm^{-1} has disappeared, while a series of new peaks emerged at 760 and 989 cm^{-1} . These two peaks were assigned to the benzene rings and the sulfonic groups^[97] modified on the azobenzene moiety, respectively. The disappearance of the alkyne peak and the emerging of azobenzene peaks indicated that the azobenzene-based rotaxane has been successfully conjugated onto the silica surface through click reaction.

Figure 2-4. FTIR spectra of the hybrid nanostructure after major modification steps. 1: Au@MSN, 2: Au@MSN-Alkyne, 3: Au@MSN-Rotaxane.

2.3.4 Photothermal capability of the Au@MSN-Rotaxane.

As was shown in Figure 2-5a, the NIR laser illumination at 808 nm resulted in the rapid temperature increasing of the Au@MSN-Rotaxane solution that after 1 hour of illumination the temperature of the sample solution maintained at 42°C, while the temperature variation found in the control sample (deionized water) under the same illumination condition was very limited (no more than 2°C even after 1 hour of direct illumination). The difference observed in the temperatures of the two samples indicated that the Au@MSN-Rotaxane species has excellent photothermal efficiency. Furthermore, the photothermal-stability of the Au@MSN-Rotaxane has also been investigated and the results were summarized in Figure 2-5b, as it was observed that no loss of photothermal

efficiency for the Au@MSN-Rotaxane was detected even after 5 rounds of continuous cyclic heating-cooling.

Figure 2-5. Photothermal efficiency of the Au@MSN-Rotaxane monitored using a thermometer. a) Comparison of the temperature elevation of the Au@MSN-Rotaxane sample to deionized water upon NIR

laser illumination. b) Cyclic photothermal-heating/cooling of the Au@MSN-Rotaxane solution. The sample solution was illuminated with NIR laser for 1 hour and then allowed to cool down to room temperature. The temperature reading of the sample solution after each step was immediately recorded and plotted against the number of cycles.

2.3.5 Characterization of the light-induced conformation transition of the azobenzene unit.

The light-induced conformation transition of the azobenzene unit that were conjugated on the surface of the hybrid structure was monitored using Uv-vis spectroscopy. It has been reported in numerous literatures that UV irradiation at specific wavelength could effectively induce the *trans*-to-*cis* isomerization at the nitrogen-nitrogen double bond of the azobenzene unit, while the *cis*-isomers of azobenzene could undergo the thermal relaxation process and return to the thermodynamically stable *trans* state.^[84] Limited by the size of its central cavity, α -CD could effectively encapsulate the *trans*-state azobenzene units but not its *cis*-isomers, thus resulted in the forced α -CD movement along the azobenzene molecule. It could also be observed that under UV irradiation at 365 nm, the optical absorption of the Au@MSN-Rotaxane has evidently decreased due to the *trans*-to-*cis* isomerization of the azobenzene moiety. However, after the exposure to NIR illuminations, a significant recovery of light absorption in the same region has been observed, indicating that the *cis*-azobenzene isomers has been changed back to the *trans* state. Additionally, the changes in the absorption intensity have been further plotted against

the numbers of the “on/off” illumination cycles. As shown in Figure 2-6, it could be observed that the interconversion capability of the azobenzene unit under different light irritations has been maintained even after 5 cycles. These evidences above collectively proved that the NIR irritation could effectively initiate the *cis*-to-*trans* isomerization of the azobenzene unit.

Scheme 2-3. The light-induced isomerization of the azobenzene unit and the resultant “on/off” switching of Au@MSN-Rotaxane.

Figure 2-6. a) Comparison of the UV-vis spectra of Au@MSN-Rotaxane between different irritation states. The Au@MSN-Rotaxane was treated

either with UV light at 365nm (labelled as the “on” state) or NIR light at 808nm (labelled as the “off” state). b) The effect of periodically altered UV and NIR irradiations on the optical absorption at 380nm of the Au@MSN-Rotaxane species.

2.3.6 Release profile of the cargo molecules.

Based on the findings above, the cargo release profile was then monitored using fluorescent microscopy. Fluorescein isothiocyanate (FITC), a widely used fluorophore in biological applications, has been loaded into the Au@MSN-Rotaxane in advance and functioned as a model drug. At a nonstimulated condition of 25 °C, it was found in the release profile (Figure 2-7a) that there were barely any FITC molecules that escaped the nanochannels, as the relative increase of FITC fluorescence intensity was less than 5% even after 180 minutes of incubation. At the physiological temperature of 37 °C (Figure 2-7b), the amount of FITC leakage has slightly increased to less than 20%, which was still within the acceptable range matched well with the kinetic features reported in previous studies.^[98] The results above indicated that the release of the cargo molecules could be blocked by the “off-state” rotaxane. In another sample group the solution was heated with an external digital heater (Figure 2-7c), and it has been observed that for the first 5 minutes when the heating was still not initiated and the temperature was kept at 25 °C, the increase in the fluorescence intensity was almost negligible (less than 5%), which was consistent with the results from the controlled group. However, as soon as the incubation temperature was elevated to 45 °C, the percentage of accumulative cargo release increased rapidly and it has eventually reached

around 50% after 180 minutes. This was explained by the fact that the *cis*-to-*trans* transformation of the azobenzene unit would be accelerated under higher temperature. For the sample group where the solutions have been treated with NIR laser (Figure 2-7d), the FITC fluorescence intensity began to drastically increase after around 5 minutes of NIR illumination. The delay between the starting of the laser treatment and the bursting release of FITC was attributed to the core/shell hybrid structure of the nanocarrier, where the gold nanorod “heaters” would have to heat up the whole surrounding environment thus to effectively evoke the thermal relaxation of the azobenzene unit that resided on the exterior silica surface, and eventually resulted in the delayed cargo release in comparison with that of the externally heating condition, where the temperature was highest in the solution not in the gold nanorods. The release profiles of these sample groups collectively confirmed that the Au@MSN-Rotaxane nanocarriers could readily respond to the external NIR illuminations and effectively release the cargo molecules.

Figure 2-7. Release kinetics of FITC molecules from the Au@MSN-Rotaxane carrier in aqueous solution under different stimuli. a) Control 1

at 25 °C, b) Control 2 at 37 °C, c) Externally heated to 45 °C, and d) upon illumination with NIR laser at 808nm.

2.3.7 Characterization of the controlled drug release in vivo

Based on the FITC release profiles of the Au@MSN-Rotaxane in solution, the controlled drug release capability of the nanocarrier was further investigated *in vivo* on zebrafish embryo models through confocal laser microscope. Doxorubicin (DOX), which is one of the most common anticancer drugs, was loaded inside the nanocarrier as the cargo.^[99] The CLSM images gave a visualized demonstration of the biodistribution of the DOX molecules that have been released, as the DOX species has intrinsic red fluorescence which could greatly facilitate the CLSM visualizations. As was shown in Figure 2-8, it could be found that in comparison with the embryos in the control group which haven't been illuminated with NIR light, the NIR-illuminated embryos showed higher DOX fluorescence intensity and wider fluorescence spread along the vertebral canal, indicating higher extent of DOX release. In a quantitative analysis the integral DOX fluorescence intensity of the NIR illuminated sample group is 3 times that of the unilluminated control. To eliminate the possible interference from the residue DOX molecules that were still encapsulated within the Au@MSN-Rotaxane, it is worth noting that when doing the integration the area of the injected Au@MSN-Rotaxane has been subtracted from the total area. The huge difference between the integral fluorescence intensity of the two sample groups indicated that the Au@MSN-Rotaxane was not only capable of retaining the encapsulated drug molecules when no external stimulus was applied, but more

importantly they could release them steadily and rapidly in response to the NIR laser irritation. In addition to the comparative study of fluorescence intensity between the NIR illuminated and unilluminated samples, the drug-release kinetics was further studied by changing the duration of the laser illumination. As was shown in Figure 2-9, for the zebrafish embryo specimens that have been illuminated with NIR light for 10 min, the distance of the spreading of DOX fluorescence was significantly higher than that of the unilluminated samples. The increasing distance of fluorescence spreading indicated that the DOX inside Au@MSN-Rotaxane has been efficiently released into the tissues surrounding the vertebrate canal under the NIR irradiation for 10 min. However, when the NIR illumination duration was reduced to 5 min, the band width of the DOX fluorescent area still overlapped completely with those optically opaque Au@MSN-Rotaxanes, as was shown in the merged image. The spatial overlap indicated that most of the encapsulated DOX was still entrapped within the nanochannels even after the 5 minutes of NIR irritation, which was similar to the situation observed in the unilluminated embryo controls. These results were also consistent with the kinetic feature described in the previous release profiles, where the nanocarrier showed a delayed release of about 5 minutes after the starting of the NIR irradiation. Additional *in vivo* experiment with an incubation temperature of 37°C (Figure 2-10) showed a similar trend, where the maximum duration of the NIR illumination has been prolonged to 30 min. The observations collectively supported the hypothesis that the DOX release from the

nanocarriers could be effectively controlled by the external NIR illumination.

Figure 2-8. Comparison of the fluorescence imaging results of the zebrafish embryo head after NIR illumination. The DOX-loaded Au@MSN-Rotaxanes were injected into the spinal canal in equal amount.

Figure 2-9. Bright field/fluorescence merged images demonstrating the release of DOX from Au@MSN-Rotaxane nanocarrier at room temperature. The spreading of the DOX fluorescence was quantified using the intensity plot of gray value (Smaller figures on the right). DOX-loaded Au@MSN-Rotaxane was injected into the spinal canal of the embryos in each sample group and was highlighted by a white box. Sample groups a and c are the blank controls under each condition. In group b the embryos were treated with NIR laser for 10 min, while in group d they were treated with NIR of the same power density for 5 min. In Row 1 the images were taken under bright field. Row 2 showed the DOX fluorescence, and Row

3 was the merged image of 1 and 2, and finally Row 4 Intensity plot of the gray value across the arrow.

Figure 2-10. Additional *in vivo* study of the DOX-release kinetics under 37°C using zebrafish embryos. Sample group a was excluded from NIR light, and sample group b was illuminated with NIR laser for 10 min, in group c the duration of NIR illumination was prolonged to 20 min, and eventually in group d the NIR illumination lasted 30 min. In Row 1 the images were also taken under bright field and Row 2 showed the DOX

fluorescence imaging. Row 3 are the gray value intensity plots across the blue line selected in Row 1 and 2.

2.4 Conclusion.

In the project, a novel NIR-responsive nanoplatform for anticancer drug delivery has been developed by combining the Au nanorods, mesoporous silica reservoir and azobenzene-based rotaxane together. With the photothermal heating using NIR laser illumination, the *cis*-to-*trans* isomerization of the azobenzene moiety in the rotaxane could be readily initiated, which would result in remotely controlled cargo release from the mesoporous silica nanostructures. The drug delivery capability of Au@MSN-Rotaxane has been demonstrated by a series of release tests in solution as well as on live zebrafish embryo models *in vivo* using the external NIR laser irritation. The Au/silica core/shell hybrid structure used in this study shows a good example for the realization of NIR-controlled drug release with nanoparticles *in vivo*.

Chapter 3 Functional gold nanorods with dual responsive polymer coating for the controlled release of anticancer drugs.

3.1 Introduction

Cancer is among the leading causes of death and morbidity globally. In the year of 2012 alone, 14 million people have been diagnosed with cancer and 8.2 million people have died from cancer and cancer related disease. It is estimated by the WHO that in the next two decades, the number of new cancer cases will continue to rise at about 70% worldwide. For long years chemotherapy is one of the major treatments to fight cancer, which essentially refers to the use of anticancer agents to suppress the tumor growth or even completely remove the cancer cell.^[100] However, using free drug for the medication of cancer doesn't always lead to satisfactory results, as the chemotherapeutic efficacy is usually attenuated by a variety of factors such as the non-discriminative cell and tissue distribution, deactivation due to antibody binding or metabolism and the rapid excretion from the body, which all prevent the drug accumulation at the desired site of action.^[101] Thanks to the recent progress in nanotechnology, numerous nanocarriers of different functionalities have been developed, which allow for the improved efficacy of drug delivery while don't bring

other problems itself. Specifically, the current surface-engineering techniques have bestowed the nanocarriers with the ability to partially control the drug release behavior and biodistribution, which may further alter the pharmacokinetics of the chemotherapy.^[102]

A good example of those surface-engineered drug nanocarrier is the stimuli-responsive nanoparticles,^[103] which was originally developed against the random Fickian diffusion of drug molecules that accounts for the drug leakage from the nanoreservoir.^[104] Theoretically, these stimuli-responsive nanocarrier should be able to recognize one or more specific microenvironments or signals and then react dynamically with sufficient sensitivity. The history of stimuli-responsive nanomaterials can be dated back to as early as the 1970s when the thermoresponsive liposomal formulations have been first proposed for the localized drug release through the stimulation of hyperthermia.^[105] Since then, many researches have been dedicated to the investigation of the stimuli-responsive mechanisms and the design and preparation of relevant stimuli-responsive nanomaterials, especially those which may be used as nanocarriers for the controlled drug delivery.

According to an analysis of previous studies, many exogenous and endogenous signals can be used to stimulate the controlled drug release.^[106] For instance, the acid pH in the tumor cells and tissues (pH 6.5~7.2), endosomes (pH 5.0-6.5) and lysosomes (pH 4.5-5.0) could be used to trigger the cargo release, while the enriched glutathione in the intracellular environment could be used to disassociate the nanostructures. In regard to the extracorporeal signals, localized hyperthermia^[107], light irritation^[108],

magnetic oscillation^[109] and ultrasound^[110] have all been utilized to actively control the cargo release from the nanocarriers. Despite the large amount of stimuli-responsive nanocarriers that are currently available, most of them are unfortunately mono-responsive which may potentially abate their therapeutic efficiency under complex biological conditions like the incubation medium or the living tissues. Thus it is expected that a nanosized drug delivery system that could readily respond to more than a single stimulus signal can further improve the outcome of the chemotherapy.

Among the multistimuli-responsive drug delivery system designs, functional nanoparticles coated with biocompatible polymer shells are of special interest.^[111] Due to the advantageous versatility of the polymer coating, it is possible to prepare drug nanocarriers with specific functionalities by simply changing the polymer formulation used for the surface coating. For instance, in a study published by Sahoo *et al*, the authors have reported a dual responsive core/shell nanoparticle with smart polymer coating which has high temperature sensitivity (LCST of the polymer shell was at 31 °C) and acid pH responsiveness (around 5) and investigated its usage as a nanocarrier for the anticancer drug doxorubicin (DOX).^[112] In another study reported by Wuli Yang *et al*, they successfully prepared the thermo/pH dual-sensitive mesoporous silica nanoparticles with functional poly(N-isopropylacrylamide-*co*-methacrylic acid) coating and used it for the controlled drug delivery of DOX *in vitro*. They observed that this composite nanostructure has fairly low drug leakage below the LCST while was also capable of rapidly releasing the cargo molecules

when the temperature was the LCST.^[113] These examples collectively demonstrate the feasibility of using multi-responsive nanoparticulate systems to improve the therapeutic efficacy of anticancer drugs. However, still no example of multi-responsive drug delivery systems that can respond to external NIR signals has been reported, which could further extend the application range of these composite systems.

Scheme 3-1. Typical swelling/collapsing mechanism of responsive polymers. Reproduced from reference^[114].

Therefore, in this chapter we introduce a novel NIR/pH dual-responsive core/shell nanoparticle by coating the monodispersed gold nanorods with cross-linked poly (N-vinylcaprolactam-*co*-methacrylic acid) shells. The gold nanorods would serve as the internal heat source while the polymer

shells would function as the gatekeeper for the controlled drug release. To achieve the conjugation of the polymer shell, the gold nanorods were first coated with a layer of mesoporous silica and then modified with alkene end groups, which could be used as the active site for the subsequent coating of polymer. When the polymerization was complete the thermos-resistant intermediate silica layer was then removed which could enhance the temperature sensitivity of the whole system. It is demonstrated that the hybrid nanostructure could be loaded with the anticancer drug doxorubicin (DOX) when heated at acid conditions and readily release the drug payload in response to the small changes in the pH of the surrounding environment as well as external triggering signal of NIR illumination.

3.2 Experimental section.

3.2.1 Materials and equipment.

All the chemicals used in the experiment were purchased from Sigma-Aldrich. N-Vinylcaprolactam (VCL, $\geq 99\%$), Potassium persulfate (KPS) Methacrylic acid (MAA) were purified individually before using, *N*, *N*'-Methylenebisacrylamide (MBA), 3-(Trimethoxysilyl) propyl methacrylate (MPS), tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), ammonium nitrate (NH₄NO₃) and sodium dodecyl sulfate (SDS) were all of analytical grade and used as received. For the purification of the first three reagents, VCL has been recrystallized for three times in hexane from room temperature to 4°C, and the obtained crystal was then washed carefully with cold methanol and anhydrated under vacuum, and ultimately stored at -20 °C. KPS was

recrystallized thrice in water also from room temperature to 4°C and then collected through lyophilization. The polymerization inhibitors in the MAA was removed through distillation under reduced pressure, and the purified MAA was stored at -80 °C. In regard to the equipment for the characterization, FT-IR spectra were monitored on a Perkin-Elmer 1760X FT-IR spectrometer with the sample powder dispersed in KBr pellets (1%). DLS size characterization was monitored on a Malvern Nanosizer. BET surface areas were measured on a Micromeritics ASAP 2020M automated sorption analyser. The transmission electron microscopy (TEM) images were taken on a JEOL 2010 high resolution transmission electron microscope. UV-vis spectra absorption analyses were performed on a Shimadzu UV-3600 UV-Vis-NIR spectrophotometer (1-mm quartz cuvette). Fluorescent spectra were measured on a Shimadzu RF-5301 spectrofluorophotometer.

3.2.2 Methods.

3.2.2.1 Preparation of the gold nanorods.

The preparation procedures of the gold nanorod substrates were adapted from section 2.2.2.1 within minor modifications. The Au seeds were also prepared by the reduction of HAuCl_4 in aqueous environment by the addition of NaBH_4 solution. Basically, HAuCl_4 solution (m/V 1%, 100 μL) was added into the concentrated cetyltrimethyl ammonium bromide (CTAB) solution in deionized water (0.2 M, 10 mL) and subsequently, a dose of ice-cold aqueous solution of NaBH_4 (0.01 M, 0.6 mL) was quickly injected to the above mixture. The ultra-small Au seeds

were immediately formed upon the addition of NaBH₄ solution. The Au seeds were always used freshly within 3 h after the preparation.

The growth solution for the Au nanorods with high absorption at 808 nm was prepared by mixing CTAB (0.1 M, 100 mL), HAuCl₄ (0.01 M, 5 mL), AgNO₃ (10 mM, 1 mL), H₂SO₄ solution (0.5 M, 2 mL), these chemical solutions were added in sequence and eventually ascorbic acid solution (0.1 M, 800 μ L) was added into the above mixture readily reducing the Au⁴⁺ ions to Au¹⁺. The growth was initiated by adding the freshly prepared seed solution to the growth medium (240 μ L). The nanorod was allowed to grow for 6 more hours and the temperature was kept at 30 °C throughout the whole process. The as-prepared Au nanorods were then centrifuged washed with deionized water for several times to remove the excessive surfactants in the solution as well as those remaining reactants and then extracted through centrifugation. The collected Au nanorods were eventually centrifuged and concentrated to 10 mg Au/mL.

3.2.2.2 Coating of intermediate mesoporous silica shell

The coating of mesoporous silica was achieved using the procedures introduced in section 2.2.2.1 with several modifications. Firstly, 3mL of the concentrated Au nanorod solution was re-dispersed and sonicated in the aqueous solution of CTAB (0.01 M, 300 mL), and the mixture was stirred at 500 rpm for about 15 min before moving onto the next step. Ammonia water (0.01 M, 1 mL) was subsequently added into the Au nanorod solution so as to adjust the solution pH to slightly basic, three injections of TEOS (60 μ L each) was then added into the solution with 30 min interval. The temperature of the mixture solution was kept at 30°C.

The reaction lasted about 24 h. The silica coated Au nanorods were then extracted from the solution via centrifugation and washed with ethanol for 3 times to remove the unreacted reagents.

3.2.2.3 Modification of the silica surface and the removal of the CTAB template

The mesoporous silica coated gold nanorod extracts were re-dispersed in water-free ethanol and extensively sonication to ensure the monodispersity of the alkene-modified intermediate product. 1 mL MPS was added into the suspension and the mixture was allowed to be further stirred at room temperature for another 24 hours. The resultant alkene-modified Au/mesoporous silica hybrid structure were then transferred to the ethanol solution of hydrochloride to remove the CTAB template. In this step, Au nanorods (60 mg) were dispersed in ethanol (40 mL) containing concentrated hydrochloric acid (m/V 35%, 5 mL), and the mixture was heated to 40 °C. The CTAB surfactant would be thusly removed through the ion exchange. After 24 h of reaction, the activated mesoporous-silica-coated alkene-modified Au nanorods were collected again through centrifugation. These modified gold nanorods were washed with anhydrous ethanol for 3 times and then changed to deionized water for another 3 times to ensure that no residual MPS has left or attached.

3.2.2.4 Coating of the poly (N-vinylcaprolactam-*co*-methacrylic acid) shell

The coating of poly (N-vinylcaprolactam-*co*-methacrylic acid) onto the silica surface was achieved by precipitation polymerization.^[115] In a single batch run, 60 mg of VCL, 18 uL of MAA, 5 mg of MBA, 5 mg of NaHCO₃

and 3 mg of the SDS surfactant were all dissolved in 45 mL of deionized water together with 30 mg of the alkene-modified Au/mesoporous silica core/shell nanoparticles. The solution was heated up to 70 °C and then gently stirred for 30 minutes. After that, 0.5 mL of the KPS aqueous solution (2.5 mg/mL) was rapidly injected into the solution and thusly initiate the precipitation polymerization. The polymerization lasted for another 2 hour since the injection of the KPS solution while be constantly stirred. Eventually the container was cooled down to room temperature and the polymer coated Au/Mesoporous silica hybrid nanostructure was extracted through centrifugation. The nanoparticle extracts were first washed with tetrahydrofuran (THF) for 3 times to remove the remaining chemicals and then changed to deionized water for another 3 times. The product was denoted as Au@MSN-p(VCL-co-MAA).

3.2.2.5 Removal of the intermediate mesoporous silica layer

In this step, the mesoporous silica shell was selectively etched using Na_2CO_3 . Specifically, 50 mg of the aforementioned Au@MSN-p(VCL-co-MAA) was redispersed in 30 mL of deionized water and sonicated for 15 minutes to minimize the particle aggregation. 600 mg of the Na_2CO_3 powder was subsequently added into the solution to remove the silica content. The mixture solution was further stirred at room temperature for 24 hours and then collected through low-speed centrifugation at 4000 rpm. The extracted polymer coated Au nanorods were washed with the aqueous solution of Na_2CO_3 (5% w/w) for 3 times and then deionized water for 3 more times. The resultant product was denoted as Au@p(VCL-co-MAA).

3.2.2.6 Loading of the anticancer drug into the Au@p(VCL-co-MAA)

The drug loading into the hybrid nanostructure was achieved through the spontaneous diffusion at acid condition under mild heating. Basically, 15 mg of the Au@p(VCL-co-MAA) was dispersed in 10 mL aqueous solution of DOX (DOX concentration was 5 mg/mL). The pH value of the loading solution was then adjusted to around 4.5 with diluted sodium citrate aqueous solution (0.1M). When all the pretreatment was complete the solution was incubated at 45°C with water bath for 24 hours under slow stirring (200 rpm). It is noteworthy that the container was wrapped with aluminum foil to avoid light exposure, which was to eliminate the possible light-induced decomposition of DOX when exposed to UV irradiation. When the drug loading was complete the nanoparticle was extracted again via centrifugation. The pellet was washed with PBS with a pH of around 7.2 for another 2 times to remove the DOX molecules that were loosely attached to the particle surface.

3.2.2.7 Characterizing the photothermal capability of the dual responsive nanocarrier

1 mL of the as-prepared Au@p(VCL-co-MAA) solution (weight fraction: 10%) was transferred to a transparent cuvette, which was then capped and wrapped with parafilms for an airtight sealing to eliminate the possible variation of particle concentration caused by the solvent evaporation during the photothermal heating. To maximize the effective absorbing area the cuvette was positioned at a 45° angle. The NIR laser source was placed directly above the cuvette and the power density of the incoming NIR laser was fixed at 1.5W/cm². Equal volume of deionized water was used as the experimental control. The temperature of each

sample was recorded using a non-contact infrared thermometer to avoid the possible heat loss. Sample temperature were recorded at 0 min, 2.5 min, 5 min, 10 min and 15 min, respectively.

To characterize the photothermal stability of the Au@p(VCL-*co*-MAA) species, 1 mL of the Au@p(VCL-*co*-MAA) aqueous solution (weight fraction: 10%) was poured into a quartz cuvette and air-tightly sealed as previously mentioned. The cuvette were then illuminated with NIR laser for 30 minutes using the same setup and the temperature was also measured using the non-contact infrared thermometer. The cuvette containing the Au@p(VCL-*co*-MAA) solution were then cooled down to room temperature and again subjected under illumination. The cyclic NIR laser treatment was repeated for 5 times and the temperature reading of each time was plotted against the cycle numbers.

3.2.2.8 Characterizing the thermally/NIR induced shrinking of the polymer shells

Dynamic laser scattering (DLS) was used to monitor the size reduction of the thermos/pH dual sensitive polymer shell in response to the external heating or NIR illumination at different pH conditions. Basically, for the thermo-induced size reduction, 20 μg of the Au@p(VCL-*co*-MAA) were dispersed in 1 mL of either sodium citrate (pH 4.5) or PBS (pH 7.2) and then heated for 15 minutes in the water bath of varied temperatures (30 °C, 35 °C, 40 °C, 45 °C, 50 °C). For the NIR groups, the other parameters were kept the same while the water bath was changed to continuous NIR laser illumination, and the hydrodynamic diameters of the sample solutions were measured at 0 min, 5 min, 10 min, 20 min, 30 min, 50 min.

All results were normalized with regard to the initial hydrodynamic diameter of the hybrid nanoparticle individually. Each measurement has been repeated for 3 times and the mean value with the standard error was used for the plotting of the size-reduction curve

3.2.2.9 Monitoring the DOX release from the dual-responsive nanocarriers

To investigate the NIR/pH dual responsive release behavior of DOX from the nanocarrier. Two types of buffer solutions with different pH values were prepared for the study of the pH selectivity, which were namely the sodium citrate solution (pH 4.5) and the PBS (pH 7.2). These two buffer solution were used to mimic the pH conditions at the cancerous site or the normal tissues respectively. The stimuli-responsive release behaviors of the DOX-loaded the Au@p(VCL-co-MAA) were monitored using the spectrofluorophotometer. The excitation and emission wavelengths used for the fluorescence detection were 488 nm and 560 nm, respectively. 4 conditions were chosen for the study the of pharmacokinetics of the nanocarrier, which included PBS without NIR illumination (double negative control), PBS with NIR and sodium citrate solution without NIR illumination (negative control) as well as sodium citrate solution with NIR illumination. For the NIR treatment, the sample solution was illuminated with continuous NIR laser (laser power density: 1.5 W/cm^2). The cuvette was wrapped in aluminum foil to maximize the NIR absorption by the sample solution. For all the 4 conditions, the fluorescent intensity readings were measured and recorded after 0 min, 5 min, 10 min, 20 min, 30 min, 60 min, 90 min and 120 min, respectively.

The release profile under each condition has been repeated 3 times and the mean value with the standard error was used for the plotting of the release curve.

3.2.2.10 Confocal imaging of the NIR-induced drug release *in vitro*.

The cellular internalization of the nanocarrier and the subsequent DOX NIR-induced release were monitored on HeLa cell lines using confocal laser scanning microscope (CLSM). The red-fluorescence DOX molecules were used as the fluorescent marker to indicate the intracellular allocation of the nanoparticle and the cargo release. Specifically, the DOX-loaded Au@p(VCL-co-MAA) nanoparticles were dispersed in cell culture medium and then injected into the 6-well plated where the cells were incubated. The HeLa cells were seeded at a density of 10^5 cells per well and the particle concentration used in the experiment was 5 mg/mL. The particle incubation would last 24 hours to allow for sufficient amount of particle intake. When the co-incubation was complete the old medium was removed and the cell film was washed with PBS for 2 times to remove the residual DOX-loaded Au@p(VCL-co-MAA). The experimental groups were then treated with NIR laser at $1.5\text{W}/\text{cm}^2$ for 15 minutes. All cell samples were monitored on a Zeiss LSM 510 META confocal laser scanning microscope, and the DOX fluorescence was excited by the 488 nm laser channel.

3.3 Results and discussion

3.3.1 Morphological study of the hybrid nanoparticles.

Figure 3-1: Synthetic procedure for the hybrid nanoparticle.

As was shown in Figure 3-2, the Au@MSN-p(VCL-co-MAA) prepared through the previously described strategy has excellent morphological homogeneity. The thicknesses of the mesoporous silica mid-layer and p(VCL-co-MAA) shell were around 20 nm and 50 nm, respectively. It could also be observed that polymer shell prepared from the precipitation polymerization didn't affect the monodispersity of the silica coated Au nanorods. After the Na₂CO₃ etching, several significant changes were found about the morphology of the core/shell nanoparticle. It could also be seen that the nanochannels have completely disappeared after rinsing in the Na₂CO₃ solution, indicating that the mesoporous silica layer has been successfully removed in that process. Without the rigid silica substrate the free-standing polymer shells were prone to swelling and dispersion, and their morphology has become much more irregular.

Figure 3-2. a) Au@MSN-p(VCL-co-MAA), before the silica removal. b) Au@p(VCL-co-MAA), after silica removal. Scale bar represents 100 nm.

3.3.2 Characterization of the porous structure.

The coating of the cross-linked p(VCL-co-MAA) shell and the removal of the intermediate silica layer substantially changed the structure of the hybrid nanoparticle, and their effects on the porosity of the particle were studied by using BET. For the Au@MSN core/shell structures the mesoporous silica layer has an average pore diameter of around 2.2 nm, which was consistent with that of the MSNs that were also prepared via the CTAB template method. The pore volume of the nanochannels was around 400 cm³/g STP, which could be explained by the attenuated thickness of the silica layer. However, as was shown in table 3-1, after the surface coating of the p(VCL-co-MAA) shell, no significant peak value was detected for the pore size and the pore volume has decreased drastically as well (from 400 cm³/g STP to no more than 70 cm³/g STP, indicating that the nanochannels in the mesoporous silica layer have been successfully sealed after the polymer conjugation. After the removal of the silica layer, the pore volume was further reduced to less than 30 cm³/g STP,

which was due to the anhydration of the free-standing polymer shell. These results collectively demonstrated that the polymer shell in the swelling state could effectively prevent the pre-releasing of the cargo molecules.

	Pore volume/STP	Pore diameter
Au@MSN	400	2.2 nm
Au@MSN-p(VCL- <i>co</i> -MAA)	<70	No peak
Au@p(VCL- <i>co</i> - MAA)	<30	No peak

Table 3-1. BET results showing the effect of major synthetic steps on the pore volumes and the mean pore diameters of the hybrid nanoparticle.

3.3.3 Optical characterization of the hybrid nanomaterial

The optical characteristics of the Au@p(VCL-*co*-MAA) hybrid nanoparticles were investigated using UV-vis spectroscopy. It could be seen in Figure 3-3 that the longitudinal absorption peak of the Au nanorods was located at around 810 nm, which matched well the 808 nm NIR laser source used for the photothermal heating. However, the absorption peak for the Au@MSN-p(VCL-*co*-MAA) has slightly shifted towards the shorter wavelengths in comparison with that of the naked Au nanorods, possibly due to the full coverage of the polymer shell which has a

relatively smaller refractive index (around 1.33)^[116] than mesoporous silica (1.54) and the reshaping of the Au nanorods at 70°C during the precipitation polymerization.^[117] For the Au@p(VCL-*co*-MAA) species, their main absorption peak was further blue-shifted, which could be explained by the removal of silica layer that has higher refractive index. Overall, the longitudinal absorption of the Au@p(VCL-*co*-MAA) has been still maintained at around 780 nm, which ensured the excellent photothermal efficiency of the hybrid nanostructure when illuminated with the 808 nm NIR laser.

Figure 3-3. The optical absorption of key intermediate and the final product, monitored by UV-vis spectroscopy.

3.3.4 Investigation on the removal of the silica shell.

In this part, the removal of the mesoporous silica layer was investigated and confirmed by using X-ray photoelectron spectroscopy (XPS). As was shown in Figure 3-4, for the Au@MSN-p(VCL-co-MAA) samples two peaks have been observed at 149 eV and 102 eV, which were characteristic for the 2s and 2p electrons of silicon atoms under XPS mapping. However for those samples that have been rinsed with Na_2CO_3 , the intensities of these two peaks have been significantly attenuated while several new peaks were detected at around 110 eV, 87 eV and 83 eV, which corresponded to the 5s and 4f electrons of Au atoms respectively. More direct evidence was shown in the area integration that after the etching process the area percentage of the Si 2p electrons dropped from 89.57% to 6.48%. It is also well known that the X-ray used in the XPS mapping has an effective analysis depth of only about 20 atoms. The decreasing intensity of the Si electron signal and the emerging of the Au electrons indicated that the mesoporous silica layer has been removed successfully and completely.

Figure 3-4. X-ray photoelectron spectroscopy for the confirmation of the removal of the intermediate silica layer. Graph a1 was the survey results of the Au@MSN-p(VCL-*co*-MAA) and a2 was the enlarged area from 180 eV to 70 eV in a1. Similarly, b1 was the survey results of Au@p(VCL-*co*-MAA) and b2 was the 180 eV-70 eV region in b1 enlarged.

3.3.5 Characterization of the surface modification of the hybrid nanoparticles

Fourier transform infrared (FTIR) spectrometer was used to monitor the changes in the surface functional groups during the preparation processes. It could be seen in Figure 3-5 that compared to the Au@MSNs, a small peak appeared around 1650 cm^{-1} after the conjugation of MPS, which was typical for the alkene functional groups. Though relatively weak due to the small amount of MPS used in the experiment, this peak indicated that the Au@MSNs have been successfully modified with alkene end groups. When the precipitation polymerization was complete, several new peaks emerged at around 2850 cm^{-1} , 1730 cm^{-1} , 1650 cm^{-1} and 1250 cm^{-1} , which were assigned to the C–H stretches, C=O stretches and C–N stretches in the p(VCL-*co*-MAA) shells respectively.^[115, 118] When the silica shell was finally etched away with Na_2CO_3 , the IR absorption at around 1100 cm^{-1} has been significantly reduced in comparison with the Au@MSN-p(VCL-*co*-MAA) species, thus consistently confirmed the removal of the silica mid-layer.

Figure 3-5. FTIR characterization of the key intermediates and final product. From the bottom up: Au@MSN, Au@MSN-Alkene, Au@MSN-p(VCL-co-MAA) and Au@p(VCL-co-MAA).

3.3.6 Investigation on the photothermal capability of the Au@p(VCL-co-MAA)

As was described in the experimental sections, the temperature elevation of the Au@p(VCL-co-MAA) solution upon NIR laser illumination was monitored and compared with deionize water. It was observed that the Au@p(VCL-co-MAA) had excellent photothermal efficacy when exposed to NIR laser, as after only 7.5 minutes of NIR illumination, the temperature of the sample solution increased rapidly from 22°C to more than 36°C, while that of the deionized water still remained at around 25°C. Moreover, after 25 minutes of NIR laser illumination, the temperature of the sample

solution eventually reached 53°C, while that of the deionized water has stabilized at 25°C. The comparative analysis revealed that the

Figure 3-6. Photothermal heating curve of the Au@p(VCL-co-MAA) in aqueous solution (upper), as well as the cyclic photothermal-

heating/cooling of the Au@p(VCL-*co*-MAA) solution (bottom), where the highest temperature reading in each cycle was plotted against the cycle number.

Au@p(VCL-*co*-MAA) hybrid nanostructure could readily respond to the external NIR irritations and effectively convert the photon energy into heat. Based on the above results, the photothermal stability of the Au@p(VCL-*co*-MAA) species were also studied using almost the same NIR illumination setup, except that the laser illumination duration has been extended to 30 minutes which was to investigate the long-term effect of the photothermal heating on the Au@p(VCL-*co*-MAA) species. As was demonstrated in Figure 3-5b, it was evident that the Au@p(VCL-*co*-MAA) hybrid structure was very resistant to the photothermal heating upon NIR illumination. It was revealed that the solution temperature always rose up to around 55°C from the same starting temperature after 30 minutes of laser illumination, even after 5 photothermal heating and cooling cycles. The results indicated the NIR illumination didn't incur any exhaustion of the photothermal capability of the Au@p(VCL-*co*-MAA) species, and that the hybrid structure could be photothermally heated repeatedly with impaired energy conversion efficiency.

3.3.7 NIR/pH dual-sensitive phase transitional behavior of the p(VCL-*co*-MAA) shells.

As is reported in previous literatures, PVCL is a type of thermoresponsive polymers that have a temperature-dependent water

solubility. Formally termed as the lower critical solution temperature (LCST) behaviour, ^[119, 120] PVCL may undergo a phase transition process when the temperature increases above a certain point, resulting in the collapsing and aggregation of the polymer content. In comparison of the earlier LCST polymers like Poly(N-isopropylacrylamide) (PNIPAM), PVCL has a critical phase transition temperature close to the body temperature (Pure PVCL: around 34°C) and attenuated cytotoxicity,^[121] which makes it an excellent candidate for biological applications.

Scheme 3-2 describes the mechanism behind the controlled drug release from the LCST polymer matrix when the temperature increases. When the temperature is below the LCST, the polymer matrix exists in a swollen state, where the inner space could serve as reservoirs for the drug loading. However, when the polymer matrix is heated and the temperature increases above the LCST, the decreased water solubility of the polymer would cause the matrix to shrink, thus effectively building up a hydrostatic pressure within the inner space. The additional pressure from the hydrophobic interaction between the matrix and the solvent would eventually squeeze the drug molecules out into the surrounding environment. In the study of Wu *et al*,^[122] they discovered that the drug release from the polymeric substrate could be precisely controlled by the changing the heating and cooling conditions, moreover, they also found that the drug release rate was associated with several variables, including the solubility of the drug molecule in the hydration solvent, relative size of the drug molecule, loading amount of drug in the matrix, the interaction

between the polymer and the drug molecule, hydration of the polymer matrix as well as its swelling-shrinking kinetics.

Scheme 3-2. One of the proposed release mechanism of cargo release from LCST polymer substrates as the temperature increases. Reproduced from reference^[120] with permission.

In most situations, the drug type and the operational environment are already fixed, thus necessary modification is required on the polymer matrix itself to regulate its drug release kinetics. For instance, it is well known that hydrophilic comonomers like PEO tend to increase the LCST while the hydrophobic ones would work oppositely.^[123] Specifically in this

study, MAA units have been employed as a comonomer to introduce the pH selectivity into this system as well as to increase the overall hydrophilicity.^[124] Theoretically, in neutral or basic conditions most of the carboxyl groups in the MAA unit would be disassociated and thus become negatively charged. The electrostatic repulsion between these charged carboxyl groups could be used as restraining forces to alleviate the shrinking of the polymer shell when it is compressed by the surface tension of water, which would then suppress the drug release from the polymer matrix. However, in an acidic environment with a pH around 4~5, the carboxyl groups (pKa of MAA monomer: 4.6) would be protonated and thus become neutrally charged, so that they are no longer capable of stretching the polymeric matrix. It is also speculated that the weakened repulsion could facilitate the drug release from the nanocarrier.

In this study, the changes in the hydrodynamic diameter of the Au@p(VCL-*co*-MAA) were investigated using dynamic laser scattering (DLS). As is shown in Figure 3-7a, the size of the hybrid nanostructure has decreased significantly in response to the external heating at pH 4.5. It was observed that when the temperature increased to 40°C, the relative hydrodynamic diameter of the nanoparticle decreased to 70% of the initial size, and it was eventually reduced to 55% when the temperature reached 50 °C. However, in comparison with the rapid size reduction observed at pH 4.5, the thermos-induced polymer shell shrinkage at pH 7.4 was not very remarkable, that even at 50 °C the total size reduction was no more than 25%. The comparative results indicated that the p(VCL-*co*-MAA) coating could readily respond to the temperature rise and undergo a rapid

spatial contraction, while the polymer coating showed less temperature sensitivity at neutral or basic conditions.

Based on the pH selective temperature-induced contractive behaviour of the polymer matrix, the Au@p(VCL-*co*-MAA) hybrid structure was treated with NIR laser to investigate if the photothermal heating could also effectively initiate the polymer shrinkage. It is demonstrated in Figure 3-7b that the polymer matrix began to rapidly collapse when illuminated with NIR laser under acidic condition of pH 4.5, and eventually the effective hydrodynamic diameter of the hybrid nanostructure was almost halved after 50 minutes of laser illumination. Nonetheless the hybrid structure showed enhanced reshaping resistance to the NIR illumination under neutral or basic conditions, as the ultimate contraction was only around 23%. The results above consistently demonstrated that external NIR signal could also be used to triggered the shrinking of the p(VCL-*co*-MAA) matrix in acidic environment and that the contraction of the polymer shell would be impeded in neutral or basic pH.

Figure 3-7. Collapsing of the p(VCL-*co*-MAA) shell upon external heating (a) or NIR illumination (b) at different pH conditions.

3.3.8 Investigation of the DOX release from the nanocarrier under NIR illumination at different pH conditions.

The drug loading and encapsulation was realized through the electrostatic attraction between the amino groups in DOX and the carboxylic groups in the MAA unit, thus forming a sub-stable complex under physiological pH. The NIR-stimulated DOX release profile was measured using fluorescent spectroscopy to evaluate whether the Au@p(VCL-co-MAA) hybrid structure could effectively control the drug release from the nanocarrier. Before the release test, the DOX loading amount was determined through the typical calibration curve method, and it was found that the loading capacity of DOX in the nanocarrier was around 1.91%, which was acceptable due to the existence of the high-density Au core. As was shown in Figure 3-8, the drug release amount was lowest at pH 7.4 and 37 °C that only 3% of the encapsulated DOX has been released after 120 minutes of incubation, indicating that the DOX molecules could be reliably encapsulated within the polymer matrix when no stimuli were applied. Likewise, it was observed that no significant drug leakage occurred when the DOX-loaded Au@p(VCL-co-MAA) was either dispersed in sodium citrate solution or illuminated with NIR laser, of which the drug release amounts were 7% and 9% respectively. The inefficiency of singular stimulation demonstrated that the p(VCL-co-MAA) shell could reduce the amount of drug leakage in undesired conditions. Finally, when the pH was set at 4.5 and the NIR illumination was imposed, a rapid increase in the DOX fluorescence intensity was observed, that nearly 70% of the total encapsulated DOX has been released

after 120 minutes of NIR laser treatment. The different DOX release profiles could be explained by the fact that the electrostatic repulsion between the disassociated carboxyl groups could effectively impede the contraction of the polymer substrate from the photothermal heating and maintain it at a loosely swollen state, thus resulting in the delayed DOX release under NIR illumination in neutral or basic pH. These results collectively demonstrated that the Au@p(VCL-*co*-MAA) nanocarriers could selectively release the anticancer drug payload in response to the external triggering signal as well as the endogenous acidic environment, which is optimal for the controlled delivery of anticancer drugs to the cancer sites. Overall, both the acidic pH and the photothermal heating through NIR illumination may enhance the release of encapsulated DOX; however, significant drug release would only be observed when both stimuli were simultaneously applied. Therefore, instead of the proposed synergistic effect, the simultaneous implementation of both stimuli is an essential prerequisite to efficient drug release.

Figure 3-8. DOX release profiles from the Au@p(VCL-co-MAA) substrate under different conditions.

3.3.9 Evaluation of the NIR-induced DOX release *in vitro*

The NIR-induced DOX release from the hybrid nanocarrier after the cellular internalization was evaluated on live HeLa cell lines with confocal laser scanning microscope (CLSM). For the unilluminated samples, it could be observed that after 24 hours of co-incubation, the red DOX fluorescence was enriched in the cytoplasm, while no DOX were detected in the cell nucleus. The intracellular distribution of DOX fluorescence indicated that the DOX cargo was still encapsulated within the Au@p(VCL-co-MAA) hybrid nanostructures, as the nanoparticles could be internalized by HeLa cells through endocytosis but couldn't enter the

cell nucleus. However, for those samples that have been illuminated with NIR laser for 15 minutes, DOX fluorescence was not only detected in the

Figure 3-9. Confocal study of the intracellular release of DOX from the Au@p(VCL-*co*-MAA) hybrid structure.

cytoplasm but in the nucleus as well, indicating that active DOX species have successfully entered the nucleus from the cytoplasm. The changes observed after the NIR illumination confirmed that the combination of the acidic endosomal pH and NIR laser illumination could readily initiate the DOX release from the nanocarrier, effectively causing the accumulation of DOX in the cell nucleus.

3.4 Conclusion.

In this study, a novel NIR/pH dual responsive drug nanocarrier has been successfully developed. This system employs a single gold nanorod as the photothermal inner heater and coated it with thermos/pH dual responsive p(VCL-*co*-MAA) shells, which would undergo a reversible collapsing only at acidic pH and elevated temperature. The hydrophilic anticancer drug DOX could be encapsulated within the loosely packed polymer matrix and rapidly release into the surrounding environment when illuminated with NIR laser at acidic conditions. The controlled DOX release from the nanocarrier has been confirmed in buffer solution and further *in vitro* on HeLa cell lines. It is expected that this core/shell hybrid nanostructure could minimize the premature drug leakage during intrabody transportation as well as release the drug molecules steadily at the desired site of action in response to extracorporeal NIR illuminations.

Chapter 4 Near-infrared light-absorptive stealth liposomes for localized photothermal ablation of tumors with combined chemotherapy

4.1 Introduction

The emergence of nanotechnology has profoundly affected the anticancer research. The advent of nanomaterials greatly augmented the efficacy of conventional chemotherapy while enabling many alternative treatment strategies which were traditionally considered unreliable. Photothermal therapy is one of those techniques that have been reactivated recently thanks to the development of nanotherapeutics^[54]. By using photoinduced localized hyperthermia to destroy the cancer cells, the therapeutic efficacy of photothermal ablation is theoretically unaffected by the drug resistance of cancer cells evolved through somatic evolution, unlike the chemotherapy^[57, 125]. However, the translation of photothermal ablation therapy from research to clinical trials has long been hampered by the non-discriminative light absorption from both the healthy tissue and tumor. This issue was eventually solved by the introduction of nanosized photothermal agents and new optical devices. These innovations could provide precise localization and intensity control on the photothermal heating^[20, 126, 127], which substantially improved the outcomes of

photothermal ablation therapy. Amongst all the photothermal agents that have been developed, NIR-absorbing nanomaterials have drawn extensive interest ^[128-130]. These materials could interact with the deep-penetrating NIR light which allowed for the localized heat deposition in the target site and minimized collateral damage to the adjacent healthy tissues. However, most of the photothermal agents that operate in the NIR range are inorganic nanostructures that cannot be easily degraded or excreted in biological environment ^[42]. Thus, it is of great importance to develop organic NIR-absorbing nanoagents to reduce the long-term risks of photothermal treatment.

IR825 is a cationic heptamethine indocyanine dye with high absorption coefficient in the NIR range as well as abysmal quantum yield, indicating that most of the photon energy it absorbs would be released through nonradiative relaxation processes^[131]. The potent light-to-heat conversion efficiency of IR825 makes it a potential candidate for photothermal therapy. However, its utilization in anticancer applications has been limited by several inherent issues, such as poor water solubility, inefficient intrabody recirculation and negligible tissue uptake. To overcome these drawbacks, new strategies of IR825 modification and delivery have been proposed and put into practice. In the pioneering work of Liu et al ^[131, 132], the IR825 molecules were encapsulated within PEGylated micelles which significantly enhanced their water solubility and bioavailability. These works provide robust and complementary insights into the design and fabrication of photothermal nanomaterials with IR825, enabling the development of more advanced therapeutic systems.

Due to the extremely hydrophobic nature of IR825, it is very difficult to be used in some of the common nanoparticle-based drug delivery systems without substantial modification at molecular level^[133]. However, as it could be readily dissolved in short-chain alcohols and fatty acids, it is possible to incorporate IR825 into nanosized drug vehicles that comprise at least one hydrophobic oleic phase. Amongst the current drug nanocarriers that meet these criteria, liposome stands out for its versatility and biosafety. Liposomes are usually spherical nanovesicles that comprise highly biocompatible lipid bilayer shells enclosing aqueous interior compartments, which can be easily degraded in physiological conditions and cleared rapidly from the body^[134]. Currently, liposomes have been used as an effective and versatile nanodelivery system for a variety of bioactive substances in clinical practice, due to their lipid/aqueous biphasic nature^[135]. Both hydrophilic and lipophilic compounds could be accommodated in liposomes depending on their solubility in each liposome components^[136]. For instance, asymmetric hydrophobic molecules like paclitaxel and camptothecin could be reliably entrapped within the lipid bilayer of liposomes ^[136, 137], while the hydrophilic molecules like doxorubicin (DOX) could be contained in the aqueous interior^[138]. Thus it is theoretically possible that liposomes could accommodate the IR825 and DOX molecules simultaneously for combinational cancer therapy.

Ever since the first discovery of liposomes in 1961 by Alec Bangham^[139], many liposomal formulations have emerged which offered advanced and versatile features aside from the basic drug encapsulation

capability. Stealth liposomes has been developed as a countermeasure against the fast clearance of liposomes in vivo,^[140, 141] which includes biocompatible polymers or polymer-lipid conjugates in the liposomal formulation. The corona of the attached polymers may cause repulsion as the entropy of the polymeric chains would be reduced upon the approach of antibodies or immunocytes, thus significantly prolongs the circulation time of liposomes in physiological environment while imposes negligible impact on their release behaviour^[142]. Additionally, the polymer corona could reduce the susceptibility of liposomes to self-aggregation^[143]. Poly (ethylene) glycol conjugated lipids (PEGylated lipids) are by far the most widely used polymer-lipid conjugates to impart steric stabilization on liposomal nanocarriers. Another noteworthy example is the thermoresponsive liposomal formulation^[144, 145]. These liposomes comprise primarily two chemical components which are namely DPPC and DSPC, and have a phase transition temperature of around 39-42°C^[146], above which the gel-like lipid bilayer will become more disordered, causing disruptions in the domain boundaries so that the aqueous content may escape through the lipid membrane. This unique temperature sensitivity could reduce the drug leakage during the transportation of liposomes in physiological environment, which are preferable for successful chemotherapy^[147]. Overall, these technical advances could improve the delivery efficiency of liposomal nanocarrier as well as their pharmacokinetics.

In this work, an NIR-absorptive stealth liposome (NSL) has been successfully prepared by entrapping IR825 within the liposomal

phospholipid bilayer of PEG-protected DPPC/DSPC thermoresponsive liposome, and its physical/chemical properties have been thoroughly investigated. In addition to the photothermal agent IR825, a widely used anticancer drug DOX was loaded into the aqueous interior as well. We hypothesized that this liposome structure could stably complex large amount of IR825 and facilitate cellular uptake of its content primarily through robust endocytosis as well as liposome fusion with the cell membrane ^[148], which would ensure subsequent efficiency of NIR photothermal therapy. Simultaneous chemotherapy is achieved by the release of the second encapsulated cargo DOX post the intracellular degradation of the NSLs. This combinational therapeutic effect of the DOX-loaded NSLs was evaluated in HeLa cells and further validated in the zebrafish liver hyperplasia model^[149].

4.2 Experimental Section.

4.2.1 Materials.

The cationic heptamethine indocyanine dye IR825 was prepared using the procedures reported by Liu *et al* ^[11]. All chemicals were used as received without further purification. The phospholipids 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from NOF (Japan), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG2000 (DSPE-PEG2000) and the liposome mini-extruder (pore size: 100 nm) were provided by Avanti Polar Lipids. Chloroform and methanol were purchased from Merck. All the other chemicals not mentioned here were

purchased from Sigma-Aldrich. The Sephadex PD-10 columns were purchased from GE healthcare.

4.2.2. Methods.

4.2.2.1 Preparation of the NSL.

The procedures for the preparation of the NIR absorptive photothermal agent IR825 was adapted from the reported by Liu *et al.* In brief, 10.0 g of 2,3,3-trimethyl-4,5-benzo-3H-indole (48 mmol, denoted as TMBI) were co-dissolved with 12.3 g of alpha-bromo-p-toluicacid (57 mmol, denoted as BTA) in 40 mL of dichlorobenzene and reacted under stirring at 110 °C for 4 h. The raw product was later extracted through filtration and purified through recrystallization in methanol. The product was then dried under vacuum and denoted as intermediate 1.

For the preparation of the other intermediate, 80 mL of the solvent mixture of DMF and dichloromethane (v/v: 1:1) was added drop-wise into the mixture solution of 37 mL of phosphoryl chloride and 35 mL of anhydrous dichloromethane and stirred continuously in ice/water bath, 10 g of cyclohexanone was subsequently added drop-wise. When the addition of cyclohexanone was complete the ice/water bath was replaced with oil bath and further refluxed for 3 h at its boiling point. The mixture was then cooled with ice through which the solid product was precipitated and collected through filtration. The raw product was then washed extensively with diethyl ether at 4°C and gave the yellowish powder, which was denoted as intermediated 2.

1.64 g of intermediate 1 (3.9 mmol) and 0.34 g of intermediate 2 (1.9 mmol) were co-dissolved in 260 mL of the mixture solution of butanol and toluene (v/v: 7:3), the reaction was carried out at 115 °C and lasted for 4 h. The organic solvent was subsequently removed through rotary evaporation. The resulting powder like raw-product of IR825 was dissolved in DMF and re-precipitated in diethyl ether to remove the impurities. This process would be repeated for several times until the upper fluids in the precipitation beaker became almost colorless. The purified IR825 was eventually collected through filtration and dried in a vacuum drying oven.

The NSL contained DPPC: DSPC: DSPE-PEG2000:IR825 in a weight ratio of 55 mg: 20 mg: 27mg: 10mg and was prepared with the conventional film-hydration and sonication method. All the lipids were pre-dissolved in the mixture solution of chloroform and methanol (volume ratio 4:1) together with IR825. The mixture was subsequently evaporated under vacuum on a rotary evaporator to form a homogenous lipid film. The lipid film was then hydrated with 19 ml aqueous solution containing 1 g of dextrose and 95 mg of doxorubicin at pH 7.2 and 60 °C for 30 min. The as-prepared multilaminar liposomes were then sonicated using a probe tip sonicator for 30 min and subsequently extruded 21 times through the polycarbonate filter of 100 nm (the barrel of the extruder has been heated to 60 °C) and resulted in the small unilaminar liposomes with an average diameter of less than 100 nm. Noting that the raw product has been centrifuged under 10000 rpm for 10 min before extrusion to remove the titanium scraps and liposome aggregates. Non-entrapped DOX was

removed from the liposome solution by passing through a PD-10 Sephadex column just before usage. Size distribution and zeta potential (ζ) were measured by dynamic light scattering using a Malvern Zetasizer Nano ZS. Transmission electron microscopy (TEM) images of the NIR-absorptive stealth liposomes were taken on a JEOL 2010 analytical TEM.

4.2.2.2 Differential scanning calorimetry.

The critical temperature for the phase transition of the lipid bilayer was measured using differential scanning calorimetry (DSC) with a Capillary Cell MicroCalorimeter (MicroCal VP-DSC). Empty aluminum hermetic sample pans of the same model were airtightly sealed and used as reference. The liposome solution was heated from 30 °C to 70 °C with a heating rate of 1 °C/min.

4.2.2.3 Optical characterization of IR825 and the NSL.

The Uv-vis absorption spectra of the photothermal dye IR825 and liposome series were recorded on a Shimadzu UV-3600 Uv-Vis-NIR spectrophotometer in a 1-mm quartz cell. Specifically for the dye IR825 itself, it was first dissolved in methanol (IR825 concentration: 0.01 mg/mL) and then measured on the spectrophotometer as it could not be solubilized in water. For the blank and DOX-loaded liposomes, they were diluted with the aqueous solution of dextrose (dextrose concentration: 5% w/w) in a 1:4 volume ratio. It is noteworthy that the DOX-loaded liposomes were purified through the PD-10 column passage using the gravity protocol before measurement, which was to eliminate the optical interference of non-trapped DOX molecules in the solution.

4.2.2.4 Determination of the DOX encapsulation amount of the NSL.

The loading amount of DOX into the NSLs was determined by the standard-curve calibration method. Basically, 10 μL purified DOX-NSL solution that contained 60 μg of lipids was added into 990 μL of detergent solution (Deionized water that contained 2% Triton-100) and subsequently incubated at 55 $^{\circ}\text{C}$ for 30 min under dark environment. The DOX fluorescence reading of the solution was then quantified using a standard curve of DOX in water, which would give the actual DOX concentration in the liposome/detergent mixture. The NSL encapsulation efficiency was then calculated as the ratio of $[\text{DOX}]/[\text{NSL}]$.

4.2.2.5 Characterization of the photothermal capability of the NSL.

250 μL of the original liposome solution was diluted to 1 mL with dextrose aqueous solution (5% w/w) and transferred to a quartz cuvette, which was then capped and wrapped with parafilms airtightly. These preparations were used to eliminate the possible increase of liposome concentration caused by the solvent evaporation during the photothermal heating. To maximize the effective absorbing area the cuvette was positioned at a 45 $^{\circ}$ angle. The NIR laser source was placed directly above the cuvette and the power density of the incoming NIR laser was fixed at 0.5W/cm². Equal volume of dextrose solution was used as the experimental control. The temperature of each sample was recorded using a non-contact infrared thermometer to ensure the accuracy of the measurement.

4.2.2.6 Evaluation of the thermos-stability and photothermal-induced drug release.

The release profile measurement was achieved using the dialysis method. In brief, 400 μL of the DOX-loaded liposome solution was first passed through a PD-10 column and subsequently poured into a cuvette. The cuvette was then illuminated with NIR laser for varied durations using the same protocol as in the previous photothermal measurements. When the illumination was complete the solution was again transferred into a dialysis bag (molecular weight cut off: 10 kDa, SpetrumLabs). The dialysis system of the photothermal-treated liposomes was suspended in a release medium of 40 mL of dextrose solution (weight fraction 5%) at 4 °C and gently rotated at 100 rpm, which was to ensure that the DOX concentrations in the donor and acceptor compartments were at equilibrium while preventing the continuous DOX release during dialysis. After 20 min, 1 mL of the release medium was collected for the fluorescence detection. For the control group, instead of laser illumination the cuvette with the purified liposome solution was heated at 37°C in equal duration, and the sample processing was the same with the experimental groups.

4.2.2.7 Investigation of cellular uptake of the NSLs.

The cellular uptake of the NSLs was qualitatively investigated by confocal laser microscope and the fluorescent anticancer drug DOX was used as the tracer agent. Hela cells were first incubated either with DOX-NSLs at the concentration of 200 $\mu\text{g/mL}$ or free DOX of (3 $\mu\text{g/mL}$) for 4 h. Subsequently the drug-containing media were decanted and the cell

films were washed twice with PBS to remove the liposomes or DOX that were not yet internalized by the cells during the incubation. The washed cells were then fixed with 4% paraformaldehyde for 15 min and washed twice again with PBS. The nuclei of the fixed cells were stained with H33258 (w/v: 2 μ g/mL) for 20 min at room temperature and the cell sample was inspected by CLSM within 30 min.

4.2.2.8 Flow cytometry analysis of the NSL internalization and DOX-induced apoptosis.

The cellular uptake of the NSLs and their pro-apoptotic effects were quantified by flow cytometry. HeLa cells were used in the study and seeded in a six-well plate with a density of 2×10^5 cells per well. To ensure the adequate cell adhesion to the plate surface, the seeded cells were first incubated for 24 h at 37 °C. Subsequently, for the experimental groups the culture media were replaced with media containing DOX-NSL, where the liposome concentration has been adjusted to 200 μ g/mL. For the control groups the media were just replaced with fresh ones. Throughout the incubation the total volumes of the culture media were kept at 2 mL. After another 4 h of incubation, the cells were washed with PBS twice and detached with trypsin solution. The detached cells were then stained following the protocol provided in the Dead Cell Apoptosis Kit with Annexin V FITC by Invitrogen, and had their fluorescence intensity measured on a flow cytometer. All samples were measured within 30 min after the staining and 1×10^4 events were recorded for each sample.

4.2.2.9 Cytotoxicity assessment of the NSLs in vitro.

The assessment of the NSLs' cytotoxicity was also conducted on the HeLa cell line using MTT assay. HeLa cells were incubated with either DOX-free NSL or DOX-loaded NSL at different concentrations for 4 hours and then washed with fresh medium. For the experimental groups the cells were subsequently illuminated with NIR laser at 808 nm for 5 or 10 minutes, and the power density of the NIR laser was fixed at 0.5 W/cm². Cell samples without NIR treatment were used as the negative control. Both sample groups were re-incubated at 37°C for another 24 hours. The optical density of samples at 570 nm were recorded using a plate reader.

4.2.2.10 Combinational therapy on zebrafish hyperplasia model.

Transgenic zebrafish embryos expressing EGFP-krasv12 oncogene were treated with mifepristone to induce the liver hyperplasia as described in Nguyen et al, 2012^[150]. Induction with 1μM mifepristone (Sigma Aldrich) was carried out for 4 days where liver tumors can be observed in most treated larvae. Liver tumor-bearing larvae were then mounted in 1% low melting agarose, with the liver and heart facing upward for direct injection of respective cargoes into the liver. Injected cargoes include NSL stained with rhodamine to assess liver uptake, free DOX and DOX-NSL at given concentrations. Confocal microscopy images of the liver tumor were acquired using an upright Zeiss Axiovert 200M laser scanning microscope (LSM Meta 510; Carl Zeiss). Detection of the tracing agent and EGFP is enabled with two excitation laser lines (30mW Argon and 1mW HeNe) and emission band-pass filters 505/530 nm and 560/615 nm that visualize EGFP-krasv12 and internalized fluorescence tracer

respectively. All mounted zebrafish were imaged immediately after injection. The same larvae were imaged a second time 1 day after injection. All images were acquired under the same acquisition settings. Quantification of liver tumor volume was achieved with the Volumest plugin in Image J.

Scheme 4-1. A schematic illustration showing the structure and mechanism of action of the NIR-absorptive stealth liposome.

4.3 Results and discussion.

4.3.1 Size distribution, zeta potential and the morphological analysis of the prepared NSLs.

As was shown in the DLS results (Figure 4-2a), the NSLs prepared *via* the film-hydration and sonication/extrusion strategy showed excellent morphological homogeneity with an average diameter below 100 nm, which was also consistent with the TEM characterizations (Figure 4-1).

These liposomes have a slight negative surface charge of -3.68 mv (Figure 4-2b), which is common for liposomal formulations that contains DSPE-PEG due to those negatively charged phosphate groups^[151].

Figure 4-1. TEM images of the IR825-containing stealth liposomes. (a): Liposomes freshly prepared through ultra-sonication. (b) Liposomes that have been thermally extruded 21 times at 60°C. All scale bars are 200 nm.

Figure 4-2. Size distribution (a) and zeta potential (ζ) (b) of the thermally extruded liposomes.

4.3.2 Characterization of the optical absorption of the photothermal agent IR825 and NSL series.

The incorporation of IR825 in the lipid bilayer was confirmed by the Uv-vis spectrum. As the IR825 dye is not water soluble, it was dissolved in methanol instead when monitoring its optical absorption. It could be observed in Figure 4-3 that the main absorption peak of IR825 located at around 825 nm. For blank liposomes without IR825, no significant optical absorption was observed in the scan range. However, when IR825 was added into the formulation one intense peak appeared in the vicinity of 840 nm, with several other peaks at around 400 nm and 600 nm. The observation of those characteristic IR825 peaks in the Uv-vis spectrum of the liposome solution demonstrated that the proposed IR825-containing stealth liposomes have been successfully prepared. It is important to note that the main absorption peak of the IR825 species when incorporated in liposomes has slightly red-shifted in comparison with that of the free dye in methanol. This could be explained by the increasing dielectric constant from methanol (32.7) to water (80.1)^[131]. Moreover, the DOX

encapsulation was confirmed and quantified by the fluorescence detection after liposome disruption. With a DOX concentration of 5 mg/mL in the hydration solution, the DOX encapsulation capacity in the NSL was found to be around 1.5%.

Figure 4-3. Uv-vis absorption spectra of the IR825/methanol (0.01 mg/mL), non-IR825 stealth liposome (1mg/mL), NSL (1mg/mL), DOX-NSL (1mg/mL) solution.

Figure 4-4. Photograph of the conventional thermoresponsive liposomes (a), NSLs (b) and DOX-NSLs (c).

4.3.3 Characterization of the photothermal capability of the NSLs.

Following the optical characterizations, the temperature rising of the liposome solution under NIR laser illumination was monitored to investigate its photothermal capability (Figure 4-6a). It has been found that after only 1 min of direct laser illumination the temperature of the liposome solution increased drastically by almost 20°C. Moreover, after 4 min of continuous illumination the temperature of the sample solution rose up to 58°C and remained at this level, while that of the dextrose solution was still only 2°C above the room temperature. By comparing the temperature increments of the sample solution and control, it was revealed the NSLs retained the high light-to-heat conversion efficacy of IR825, as well as its photothermal capability in the first biological window.

4.3.4 Characterization of the temperature-dependent phase transition behavior of the lipid bilayers of the NSLs.

To evaluate the thermo-stability of the NSL, the critical phase transition temperature of its lipid bilayer was investigated by using the differential scanning calorimeter (DSC). The heat flow plot revealed that for the IR825 containing NSLs, the melting of their lipid bilayer initiated at 45°C (Figure 4-5), which was slightly higher than the phase transition temperature reported for conventional thermoresponsive DPPC/DSPC liposomes (39~42°C)^[145, 146, 152-154]. The elevated melting temperature (T_m) indicated that the introduction of IR825 molecules had augmented the stability of the lipid membrane against the structural disruption caused by temperature escalations. More importantly, with a T_m above the physiological temperature of 37°C, it was promising that the liposomes could reduce the leakage of encapsulated anticancer drugs under unstimulated conditions.

Figure 4-5. DSC analysis of the NSL in dextrose solution (w/w: 5%).

4.3.5 Monitoring the drug release profile from the NSLs.

Based on the DSC results, the time-dependent release profile of the NSL was measured to investigate its structural stability at physiological temperature conditions (Figure 4-6b). The release profile was monitored using the conventional dialysis method^[155]. The DOX-loaded NSL solution was contained inside dialysis membranes with an MWCO of 3000, which could effectively isolate the released DOX from the NSLs. It was observed that the NSL was capable of retaining its DOX cargo in the isotonic buffer solution (5% aqueous dextrose solution) at physiological temperature, that no more than 10% of the total encapsulated DOX was leaked after 30 min of incubation at 37°C, similar to the previously reported thermoresponsive liposomal formulations^[103, 105, 147, 156]. However, when the NIR laser illumination was applied a drastic increase of DOX fluorescence was detected in the release medium. With an NIR illumination density of 0.5 W/cm², the NSL released more than 20% percent of the total encapsulated DOX in the first 10 min, and eventually the release percentage reached more than 80% in 30 min. These results demonstrated the stability of the liposomes at normal body temperature as well as the capability of enhanced cargo release when the liposome was stimulated with photothermal heating.

Figure 4-6. a) Heating curves of the hydration solution and the NSL solution upon continuous NIR laser illumination at a laser power density of $0.5\text{W}/\text{cm}^2$. b) The release profiles of liposomal DOX at physiological temperature and NIR irradiation (Power density: $0.5\text{W}/\text{cm}^2$) respectively.

4.3.6 CLSM imaging of the cellular uptake of the NSLs by HeLa cell lines.

The photothermal heating curve and the release profiles of the NSL in isotonic buffer solution implied the potential use of the NSL in both the photothermal therapy and chemotherapy. To confirm our hypothesis additional *in vitro* experiments with NSLs were carried out in HeLa cell line. Liposomes have excellent cellular bioavailability due to the chemical and structural similarities between liposomes lipid bilayer and the cell membranes. This facilitates their cellular uptake by multiple pathways such as direct liposome-cell fusion, lipid exchange, phagocytosis and endocytosis^[148]. The uptake of NSLs in live Hela cells was assessed using confocal laser fluorescence spectroscopy. The anticancer drug, DOX, with its intrinsic red fluorescence was used as an indicator for successful cellular internalization. The uptake of free Dox was compared with DOX-loaded NSLs in Hela cells incubated with respective Dox preparation in

the culture medium. As was shown in Figure 4-7a, after 4 hours of incubation with free DOX of 3 $\mu\text{g/mL}$, red fluorescence was enriched in the nucleus and its vicinity, which was consistent with the findings in the previous studies [87, 157]. In contrast, for HeLa cells that were exposed to DOX-NSLs of equivalent DOX concentration, DOX fluorescence has been detected throughout the cell cytoplasm and the nuclei. Although the nucleic DOX intensity was much less in those HeLa cells that were exposed to DOX-NSLs. The distinctive Dox fluorescence pattern for DOX-NSLs suggested some DOX cargo remains encapsulated within the liposomes after 4 hours of incubation. The data also suggested that the DOX-NSLs have been successfully internalized by the HeLa cells through the predominant endocytic pathway as well as direct membrane fusion.

4.3.7 Flow cytometric study of the cellular uptake and pro-apoptotic effect of the DOX-loaded NSLs.

In order to correlate the internalization of DOX-NSLs with their pro-apoptotic effect on HeLa cells, the DOX-NSL treated cell samples were stained with Annexin V-FITC probe to assess the extent of apoptosis using flow cytometry. According to Figure 4-7b, the flow cytometric analysis of the cells that underwent 4h of incubation with the NSL-DOX solution at 37°C revealed a drastic increase in the median DOX fluorescence intensity that was almost 13-fold stronger than that in the blank control, which was suggestive that the internalization of the NSL-DOX into the HeLa cells has occurred. Correspondingly, the chemotherapeutic capability of released Dox was revealed in Figure 5c that the median intensity of FITC fluorescence of the whole cell population in the NSL-DOX group has

increased significantly by around 14 fold after incubation for 4h. The CLSM and flow cytometry results collectively demonstrated that the NSLs could be readily internalized by the HeLa cell lines and induce the cell-apoptosis attributed to the release of liposomal DOX.

Figure 4-7. CLSM imaging of live HeLa cells incubated with free DOX (3 $\mu\text{g/mL}$) or DOX-NSL solution (200 $\mu\text{g/mL}$) were shown in (a). The cell nuclei were stained with H33258. Scale bar is 10 μm . The two profiles on the bottom showed the results of flow cytometric analysis on the changes of DOX (b) and FITC (c) fluorescence intensity of HeLa cell lines after incubation with DOX-NSL solution (200 $\mu\text{g/mL}$), the median DOX/FITC

fluorescence intensities of each sample group were also shown in the figures.

4.3.8 Investigation on the efficacy of photothermal ablation and combinational treatment of the NSLs on HeLa cell lines using MTT assay.

MTT assay was subsequently carried out to quantitatively assess the tumoricidal effects of the NSLs and firstly their photothermal ablation capability was studied. As depicted in Figure 4-8a, no cytotoxicity (cell viability >90%) was detected in HeLa cells before NIR illumination even at an NSL concentration of 120 $\mu\text{g/ml}$, which was a good indicator for the biosafety of the NSLs. Contrastingly, a sharp increase in the cell death rate was observed when the cell samples were illuminated with NIR laser for 5 minutes, with down to only 14% of the cells surviving the treatment at the NSL concentration of 120 $\mu\text{g/ml}$. When the duration of laser illumination was prolonged to 10 min, the cell viability at the NSL concentration of 120 $\mu\text{g/ml}$ further dropped to 9%. It should also be noted that at concentration zero the exposure to NIR laser illumination resulted in an abnormal cell proliferation, of which the extent was roughly positively correlated to the duration of the NIR treatment. This was consistent to the experiment results in some previous reports and would not compromise the validity of the MTT assay^[158]. The comparative analysis of the MTT results supported the *in vitro* efficacy of NIR triggered photothermal cancer cell ablation with internalized NSLs.

By using the same experimental setups, the combinational therapeutic efficacy of the DOX-NSL has been further inspected on HeLa cell lines (Figure 4-8b). Despite the absence of NIR illumination, a concentration-dependent toxicity was detected on HeLa cells treated with DOX-NSLs, where the cell viability eventually fell below 60% upon incubation with 120 $\mu\text{g/mL}$ of DOX-NSLs. The observed toxicity was attributed to release of liposomal DOX. Additionally, when the NIR laser illumination was applied a further drop in the cell viability was detected, as the survival rate came down to 9% after 10 minutes of laser illumination at the DOX-NSL concentration of 120 $\mu\text{g/mL}$. Even though the anticancer property of the liposomal DOX was made less obvious by the concurrent NIR-induced photothermal ablation, nevertheless the cell viability in the DOX-NSL treated groups were on average lower than that of DOX-free NSL groups of a similar concentration and illumination condition. The *in vitro* result suggested that enhanced tumoricidal outcome can be achieved with combined photothermal and chemotherapeutic properties of DOX-NSLs.

Figure 4-8. Relative viabilities of HeLa cell lines after each treatment, where HeLa cell lines were incubated with DOX-free NSLs (a) and DOX-loaded NSLs (b) at various concentrations and treated with NIR laser for 0 min, 5 min and 10 min, respectively. All optical density (OD) readings

at 570 nm were normalized to the mean OD of the non-NIR-illuminated control group at concentration zero.

4.3.9 Characterization of the combinational therapeutic efficacy of the NSLs *in vivo* on live zebrafish models.

Additional proof of combinational therapeutic efficacy on solid tumors *in vivo* was assessed using the transgenic zebrafish based mifepristone inducible liver tumor model expressing the EGFP-kras^{v12} oncogene [149, 150]. External development and optical translucence of the zebrafish larvae facilitates real time monitoring of liver tumor progression post the anticancer treatment. Liver tumor-bearing larvae were first divided into 6 subgroups. Group 1 was the blank control/untreated liver tumor bearing larvae, Group 2, 3 and 4 were injected with NSL, free DOX or DOX-NSL solution of equivalent concentration respectively, Group 5 was injected with DOX-NSL solution and illuminated with 5 minutes of NIR laser while Group 6 was injected with DOX-NSL solution and treated with NIR laser for 5 minutes as well. Each tumor volume was then assessed by confocal imaging where images detailing tumor depth in the z direction were compiled and thickness of each optical slice is fixed at 5.5 μm . Three dimensional fluorescent images of liver tumor in all treatment groups are summarized in Figure 4-9a. Liver tumor volume was followed at day 0 and day 1 for all larvae in each treated group to detect the variations in tumor volume. Tumor progression associated with increased liver tumor volume was observed in Groups 1 and 2. Decreased tumor volume was detected in Groups 3 and 4 which supported modest tumor regression from the DOX medication. The most severe tumor damage was observed on

embryos from Group 5 and 6, where much of the tumor tissue has disappeared a day after the NIR mediated ablation. Moreover the tumor tissue in these two sample groups have acquired a dissociated morphology post the NIR treatment. The observations in these two groups demonstrated that the tumor cells have suffered severe cellular damage (necrosis and pyknosis) compared to the unilluminated sample groups. Specifically, the hyperthermia incurred by the NIR treatment would disrupt the cell membrane and degrade the proteins, as well as causing irreversible damage to the cytoskeletal filaments, all of which would contribute to the prompt cell death^[57].

Based on the qualitative results of the three dimensional fluorescent tumor mapping on live zebrafish embryos, the relative tumor size change in each sample group was further quantified to reveal the therapeutic efficacy of the NSL *in vivo* and the result was shown in Figure 4-9b. The quantitation of liver tumor volume was achieved by summing the volume of each optical slices that made up the liver tumor, using the volume estimation plugin in Image J (Volumest). It was found that there was no significant difference between the tumor volume changes of Group 1 and 2 (significance level: 5%), which evidently indicated that the NSL itself has no apparent toxicity to the living cells. It should also be noted that the average tumor size in Group 4 has decreased significantly, which was even more effective than that the free DOX solution at equivalent concentration. This was attributed to the fact that due to their high hydrophilicity and small size, free DOX molecules would easily diffuse from the injection site and result in the abatement of the bioactive drug concentration in

tumor, while the liposomal DOX could be retained in the tumor for an extended period of time due to the ineffective lymphatic drainage and thus create an area with high local drug concentration. In group 6 it was found that the tumor regression was the greatest among all the sample groups while little collateral damage has been observed on the living larvae, which revealed the enhanced combinational therapeutic effect with the DOX-NSL under NIR illumination. All these results collectively demonstrated that the combination of DOX-NSL and NIR laser illumination has improved the eventual therapeutic outcome on solid tumors *in vivo*.

Figure 4-9. a) Three-dimensional fluorescent imaging of the liver tumor immediately before and 1 day after treatment. The same liver tumor was followed at day 0 and 1 day post respective treatments. Images represent typical tumor size detected in each sample group. Only Groups 5 and 6 are treated illuminated with NIR laser (Power density: 1 W/cm^2) for 5 minutes. Group 1: blank control, Group 2: NSL, Group 3: Free DOX, Group 4: DOX-NSL, Group 5: NSL/5 minutes of NIR illumination, Group 6: DOX-NSL/5 minutes of NIR illumination. b) Changes in the relative tumor volume in each sample group, measured 1 day after the treatment.

Group 1 Day 0

Group 1 Day 1

Group 2 Day 0

Group 2 Day 1

Group 3 Day 0

Group 3 Day 1

Group 4 Day 0

Group 4 Day 1

Group 5 Day 0

Group 5 Day 1

Group 6 Day 0

Group 6 Day 1

Figure 4-10. Tumor volume measurement for typical embryos in each sample group using Image J.

4.4 Conclusion

In summary, the NIR-absorptive stealth liposome has been successfully prepared as a combinational therapeutic agent for cancer treatment. The hydrophobic organic dye IR825 has been successfully incorporated into these liposomes through which its bioavailability has been significantly improved. The liposomes could serve as the nanocarrier for the intracellular delivery of the anticancer drug DOX and at the same time as an effective photothermal agent upon NIR laser illumination. It has been confirmed in both in vitro and in vivo assays that the NIR treatment of the DOX-NSLs resulted in better tumoricidal efficacy than either the liposomal DOX medication or sole photothermal ablation. The characterization results suggest that the NSL system developed in this study is promising for safe and effective combinational anticancer therapy, and could be of clinical interest in phototherapeutic applications.

Chapter 5 Summary and Perspective

5.1 Summary.

This thesis introduced the research projects that I carried out on the NIR-responsive nanotherapeutics during my four years of study. Both the inorganic gold nanorods and the organic heptamethine indocyanine dye IR825 have been used as the bioactive photothermal species. These nanoagents could specifically absorb light in the NIR range through different mechanisms and convert the photons into thermal energy, thus resulting in a temperature rising in the near-field. We also purchased a continuous-wave NIR laser diode to stimulate these photothermal agents *in vitro* and *in vivo*. The as-prepared nanotherapeutics have exhibited excellent performance at cellular level and even on live animal specimens.

The NIR light source is favored for the phototherapeutic applications for several reasons. In comparison with ultra-violet ray or visible light, the extinction coefficient of NIR light in human tissues is much lower (provided that they don't contain the photothermal agents), which indicates that the NIR treatment would incur less collateral damage on the healthy tissues along the light path while capable of reaching places deeper beneath the irritation surface. This would significantly facilitate the treatment for some internal malignancies as well as their *in situ* visualization. Overall, by using NIR light as the irritation signal, the

anticancer therapies could be adjusted precisely to the desired site of action, which may further improve their tumoricidal outcome.

To begin with, I first studied the controlled drug release from functional mesoporous-silica coated gold nanorods. These Au/Silica hybrid nanostructures have been conjugated with azobenzene-based rotaxanes on the surface, of which the conformation could change rapidly upon different irritation signal. These rotaxanes served as the capping agent in this system so that the mesoporous silica nanochannels could be switched between the “on” state and the “off” state using remote signals. It was found in the subsequent characterizations in aqueous solution that drug molecules could be encapsulated within the mesoporous silica shells under UV irradiation and released steadily under NIR illumination. The controllable drug release was further validated *in vivo* on live zebrafish models.

Based on the successful example of NIR-induced drug release through the combination of gold nanorods and azobenzene-based nanovalves, new surface modification design was proposed through which I would like to add new functionalities aside from the NIR-responsiveness. In this study the gold nanorods were coated with poly (N-vinylcaprolactam-*co*-methacrylic acid) shell while the intermediate mesoporous silica layer has been etched away. The removal of the thermal-resistant silica layer significantly improved the overall NIR-sensitivity of the nanopreparation, while the p(VCL-*co*-MAA) coating allowed the encapsulated drug molecule to be released only under acidic environment (tumor microenvironment).

The two projects above relied on the implementation of the inorganic gold nanorods as the NIR-absorptive heat source, which could not be excreted or degraded in live cells or tissues, thus their usage may bring about long-term risk of health problems. To avoid this issue later I changed to organic photothermal agents and biodegradable nanocarriers. In this project, a recently developed photothermal dye IR825 was successfully incorporated with the biocompatible liposomes. The liposomes greatly improved the bioavailability of the non-water soluble IR825 species as well as retained their original capability of delivering anticancer drugs. A combinational therapeutic effect was achieved with this modified liposome formulation *in vitro* on HeLa cell lines and further *in vivo* on zebrafish liver hyperplasia models.

5.2 Perspective

On account of the successful examples of NIR-dependent nanotherapeutics, several structural and chemical modifications have been proposed which may further improve the anticancer efficacy.

5.2.1 Enhancing the tumor specificity with immunoliposome formulations.

One drawback of the nanotherapeutics systems introduced above is the lack of targeting functional group which allows for the specific binding with certain type of cancer cells. The insufficient tumor specificity could be solved by the introduction of immunoliposomes. Currently there are two major methods available for the preparation of PEG-stabilized immunoliposomes: One is to conjugate the targeting group on the end of

the phospholipids and then mix with the PEGylated lipids to form the liposome, and the other is to conjugate the targeting group at the end of the PEG chain. With the additional targeting groups it is possible to improve the specific cellular uptake of the liposomes by tumor tissue while minimizing their adverse side effects to healthy tissues.

5.2.2 Designing fabrication of NIR-responsive theranostic systems.

Up until now the discussion is focused on the direct medication and photothermal ablation of the tumor tissue, while the detection of the tumor and the monitoring of its progression, which has almost equal importance to anticancer treatment has been much neglected. To address the need of NIR-responsive theranostic systems, I would like to modify some of the lipid components with fluorescent markers through covalent bonding, which could reveal the cells that have liposome uptake through *in vivo* fluorescent imaging. In a more advanced design Annexin conjugated with FITC units would be encapsulated within the liposomes, which could be used to fluorescently label those apoptotic cells after the anticancer treatment. By using this theranostic NIR responsive nanopreparation, it is expected that the disease diagnose, cargo delivery and monitoring of the therapeutic response could be combined to realize the goal of personalized nanomedicine.

5.3 Conclusion.

In summary, NIR light is a powerful tool which could be used for the control of medication and thermal removal of tumor tissues. Its high

transmittance in living tissue could guarantee the sufficient depth of penetration while causing minimal photothermal damage to the adjacent healthy tissues. During my four years of postgraduate study I have successfully fabricated a series of NIR-responsive nanomaterials, through which the controlled drug release and photothermal ablation of tumor tissues have been observed both *in vitro* on HeLa cell lines and *in vivo* on live zebrafish models. The studies demonstrated the implementation of NIR light in typical therapeutic nanosystems as well as investigated the possible approaches to enhance the anticancer efficiency. Further studies with these photothermal agents could be helpful for the development of even more advanced therapeutic systems with higher tumor specificity and selectivity.

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List of Publications

1. Menghuan Li, Hong Yan, Cathleen Teh, Vladimir Korzh, Yanli Zhao,*
“NIR triggered drug release from switchable rotaxane-functionalized
silica-covered Au nanorods” *Chem. Commun.* 2014, 50, 9745-9748.
2. Menghuan Li, Cathleen Teh, Chung Yen Ang, Si Yu Tan, Zhong Luo,
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infrared light-absorptive stealth liposomes for localized photothermal
ablation of tumors combined with chemotherapy”, *Adv. Funct. Mater.*
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3. Hui Duan, Menghuan Li, Guang-Hui Zhang, James R. Gallagher,
Zhiliang Huang, Yu Sun, Zhong Luo, Hongzhong Chen, Jeffrey T.
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performance and kinetic investigations” *ACS Catal.* 2015, accepted.
4. Yuanyuan Zhang, Qiuyu Qu, Menghuan Li, Yanli Zhao,* “Intracellular
reduction-responsive sheddable copolymer micelles for targeted
anticancer drug delivery” *Asian J. Org. Chem.* 2015, 4, 226-232.
5. Yang Zhao, Zhong Luo, Menghuan Li, Qiuyu Qu, Xing Ma, Shu-Hong
Yu,* Yanli Zhao,* “A preloaded amorphous calcium carbonate-
doxorubicin@silica nanoreactor for pH-responsive delivery of
anticancer drug” *Angew. Chem. Int. Ed.* 2015, 54, 919-922.
6. Chung Yen Ang, Si Yu Tan, Yunpeng Lu, Linyi Bai, Menghuan Li,
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- “Turn-on fluorescence probe integrated polymer nanoparticles for sensing biological thiol molecules” *Sci. Rep.* 2014, 4, 7057.
7. Zhong Luo, Yan Hu,* Kaiyong Cai, Xingwei Ding, Quan Zhang, Menghuan Li, Xing Ma, Beilu Zhang, Yongfei Zeng, Peizhou Li, Jinghua Li, Junjie Liu, Yanli Zhao,* “Intracellular redox-activated anticancer drug delivery by functionalized hollow mesoporous silica nanoreservoirs with tumor specificity” *Biomaterials* 2014, 35, 7951-7962.
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 9. Liangliang Zhu, Hong Yan, Chung Yen Ang, Kim Truc Nguyen, Menghuan Li, Yanli Zhao,* “Photoswitchable Supramolecular Catalysis by Interparticle Host–Guest Competitive Binding” *Chem. Eur. J.* 2012, 18, 13979-13983.