Multifunctional polymer and polymer-hybrid nanoparticles for triggered drug release and potent combined photothermal-chemotherapy

Zhang, Yuanyuan

2016

Zhang, Y. (2016). Multifunctional polymer and polymer-hybrid nanoparticles for triggered drug release and potent combined photothermal-chemotherapy. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/69221>

<https://doi.org/10.32657/10356/69221>

NANYANG
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**Multifunctional Polymer and Polymer-Hybrid
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Potent Combined Photothermal-
Chemotherapy**

Zhang Yuanyuan

School of Physical and Mathematical Sciences

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

2016

Acknowledgements

First of all, I wish to express my sincerest acknowledgement to my supervisor Associate Professor Zhao Yanli in respect to his invaluable guidance and support. It has been a great honor to be supervised by him. I have developed systematic research skills and learnt essential knowledge under his effective academic instructions. Besides, he gave me invaluable encouragement and advice when I was confused in my research, which built my confidence and thus allowed me to complete my research.

Also I'd like to express my deep appreciations to the lab colleagues in Zhao's group for their helpful suggestions, discussions as well as assistance during my graduate study. I really enjoyed my research life when working and collaborating with them. Without their help, I would not be able to effectively carry out my research project. Particularly, I am also grateful to my undergraduate students who participated and helped in my research.

Besides, I would express special gratitude to my family for their understanding and encouragement during the period of my study abroad. Without their consistent support, I would not have been abroad for my four-year study and realized my academic dream.

Last but not least, I'd like to convey my deepest gratitude to School of Physical and Mathematical Sciences for giving me the research scholarship for pursuing my Ph.D. degree and providing the facilities and services for my research work. It is a great privilege for me to study in Nanyang Technological University, which will make me be proud all my life.

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Abstract

Various polymer based nanoparticles have been developed and explored as therapeutic nanovehicles for the treatment of cancer. In an effort to reduce the side effects and minimize the undesirable damage to normal tissues, the drug molecules could be incorporated into these nanocarriers by physical loading or covalent conjugation and successfully delivered into the tumor sites and cancer cells. However, traditional polymeric drug delivery system suffers the slow drug release inside tumor tissues or cancer cells, which might compromise its ultimate therapeutic effects. Thus, tremendous efforts have been put forward by the researchers to develop stimulus-responsive polymeric nanomedicine that achieve burst drug release in response to biological or external triggers.

In view of the above, the primary focus of my research is to design, synthesize and characterize multifunctional stimulus-responsive polymer and polymer-hybrid nanovehicles encapsulation of therapeutic agents including anticancer drug and photothermal dye for the triggered drug release and combined photothermal-chemotherapy.

In Chapter 2, we aim to prepare multifunctional hollow mesoporous silica nanoparticles (HMSNs) with the polymer coating, which achieved the triggered release of the encapsulated drug molecules under three stimuli independently. By modification of HMSNs with initiating group, pH responsive poly(2-(diethylamino)-ethyl methacrylate) (PDEAEMA) was grafted onto the surface with both reduction cleavable disulfide bond and UV-cleavable *o*-nitrobenzyl ester linkage. Doxorubicin (DOX) was easily loaded into the polymer-hybrids and quickly released in response to the triggers such as acidic environment, reducing agent or UV irradiation.

Despite chemotherapy being the leading therapeutic method for the treatment of cancer, the concept of combination therapy has been further put forward to ensure therapeutic efficacy for cancer. Hence, in chapter 3, we utilized the polymeric DOX prodrug coated HMSNs to load the photothermal dye IR825 for the combined photothermal-chemotherapy. DOX was covalently immobilized on the grafted polymer coating via acid-cleavable hydrazone linkage while released efficiently under weak acidic endo/lysosomal environment. Besides, the hollow space of nanohybrids could also retain NIR absorbing dye IR825 at high loading content for the effective photothermal therapy. In vitro cell studies further demonstrated that the nanohybrids were endocytosed by HeLa cancer cells effectively and anticancer activity was significantly improved by NIR irradiation.

Folic acid can bind to numerous types of cancer cells with over-expressed folate receptors and enhance the selectivity and efficiency of drug delivery. In chapter 4, we fabricated the folic acid functionalized polymeric prodrug micelles to load photothermal dye IR825 for the combination therapy. The resulting dye loaded micelles showed excellent colloidal stability and monodispersity in aqueous solutions. Specifically, the conjugated DOX could be efficiently released in response to acidic environment. In addition, the encapsulated dye could convert the NIR irradiation into heat energy with high efficiency, making the self-assembled micelles an effective candidate for photothermal therapy. Significant tumor regression was also observed in the zebrafish liver hyperplasia model after combination therapy.

Thus, these as-prepared polymer and polymer-hybrid nanocarriers may provide new insight in cancer treatment with unprecedented therapeutic outcomes.

List of Acronyms

AIBN	azobisisobutyronitrile
APTS	3-aminopropyltriethoxysilane
ATRP	atom transfer radical polymerization
AuNRs	gold nanorods
BET	Brunauer-Emmett-Teller
BOC	<i>tert</i> -butoxycarbonyl
CPT	camptothecin
CTAB	cetyltrimethylammonium bromide
DAPI	4',6-diamidino-2-phenylindole
CLSM	confocal laser scanning microscopy
DIC	<i>N,N'</i> -diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DLC	drug loading content
DLE	drug loading efficiency
DMAP	4-(dimethylamino)pyridine
DMEM	Dulbecco's modified eagle medium
DMPA	(dodecylthiocarbonothioylthio)-2-methylpropionic acid
DOX	doxorubicin
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPR	enhanced permeability and retention
FBS	fetal bovine serum
FDA	Food and Drug Administration
GSH	glutathione
HMSNs	hollow mesoporous silica nanoparticles
IC50	half-maximal inhibitory concentration
ICG	indocyanine green
MABH	methacrylamide <i>tert</i> -butyl carbazate
MPTS	3-mercaptopropyl-trimethoxysilane
MSNs	mesoporous silica nanoparticles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
MWCO	molecular weight cut-off
NHS	<i>N</i> -hydroxysuccinimide
NIR	near-infrared
NMR	nuclear magnetic resonance
ONB	<i>o</i> -nitrobenzyl ester
P(Asp)	poly(aspartic acid)
PB	phosphate buffer
PBS	phosphate buffered saline

PcBu ₄	<i>tetra-t</i> -butylphthalocyanine
PCL	poly(caprolactone)
PDEAEMA	poly(2-(diethylamino)-ethyl methacrylate)
PEG	polyethylene glycol
PEI	polyethylenimine
PLA	poly(<i>D,L</i> -lactide)
PLGA	poly(lactic- <i>co</i> -glycolic acid)
PMDETA	<i>N,N,N',N'',N'''</i> -pentamethyldiethylenetriamine
RAFT	addition–fragmentation chain-transfer
ROP	ring opening polymerization
SEM	scanning electron microscope
TFA	trifluoroacetic acid
TEA	triethylamine
TGA	thermogravimetric analysis
XPS	X-Ray photoelectric spectroscopy

Chapter 1: Introduction

1.1 Nanoparticles in drug delivery

Transportation of therapeutic compounds to specific pathological site is crucial for the success in treatment of many diseases, since the therapeutic agents only take effect inside the targeted cells.¹⁻³ For the past two decades, various types of nanoparticles have been prepared as promising vehicles to deliver therapeutic agents such as anticancer drug, gene, protein and photothermal dyes for the treatment of cancer. They have shown a great application perspective for clinical use and certain amount of nanomedicine formulations have already been developed and approved for clinical trials or in sale.^{4,5}

1.1.1 Role of nanoparticles in drug delivery

Traditional cancer chemotherapy using drug molecules by direct systemic administration is often accompanied with serious side effects on account of the simple diffusion and free distribution of drug within various tissues. Meanwhile, the lack of drug selectivity, rapid drug clearance by kidney and wide fluctuations of the blood drug levels also undoubtedly compromise the therapeutic efficacy. Thus, to guarantee therapeutic outcomes, high dose of medication is often adopted in the treatment of the intended disease, which further lead to more serious side effects on the body.¹ It is certainly worth developing versatile and intelligent therapeutic formulations that are able to selectively accumulate at the cancerous tissues and efficiently release drug inside pathological sites in response to microenvironment biological or external stimulus.⁴

The nanoscale drug delivery system is not only able to avoid rapid degradation or clearance from body, but also increase the accumulated drug concentration in solid target

tumor tissues and cancer cells.^{5,6} These developed nanocarriers have shown a great application perspective for anticancer drug delivery since they possess several benefits, such as increased drug aqueous solubility, reduced side effects, enhanced blood drug circulation time and the passive targeted accumulation at the tumor sites via the enhanced permeability and retention (EPR) effects.⁷⁻⁹ Prior to the release in the pathological sites, the encapsulated drug can avoid the direct contact with the biological environment with the protection of carriers, reducing their damage to normal tissues or cells during blood circulation. The physicochemical property of the nanovehicles are able to fine-tune the pharmacokinetic profiles and biodistribution of the therapeutic drug.¹⁰ Despite the encapsulation of anticancer drug, other therapeutic or diagnostic agents can also be delivered by the versatile nanocarriers to meet various requirements and ensure therapeutic efficacy for cancer.

Passive tumor targeting is realized by incorporating of drug to the designed nanocarriers by the well-known EPR effects. Generally speaking, normal vasculature has tight junctions between endothelial cells, preventing the extravasation of nanoparticles into normal tissues on account of the relatively larger size of the particles compared with the small molecules. However, in order to acquire more nutrients and metabolites for the rapid and immoderate growth of tumor, solid tumor vasculature tends to be inherently leaky, which facilitate the nanoparticles with the right sizes to transport through permeable vascular wall and selectively accumulate in solid tumor tissue (Figure 1).^{11,12} High permeability of tumor tissues caused by EPR effect have been first noticed by Maeda and co-workers and evidence for passive tumor accumulation by the EPR effect has also been fully approved by using the animal models in the past decades. The intensive studies of the EPR effect in vivo

directly prompt the booming development of nanoscale therapeutic systems for drug delivery. However, in consideration of peculiar heterogeneity of leaky tumor vasculature and individual differences in patients, it is difficult to define the exact size limit for nanoparticles to move through the tumor vascular barriers. It is generally accepted that nanoparticles smaller than 200 nm are particularly effective for localization nanomedicines within tumor tissues via EPR effect. The result of passive tumor targeting ability resulting from EPR effect determines the success or failure of nanomedicine application for cancer therapy.

Figure 1. Diagrammatic representation of appropriate size of nanoparticles selectively passing the leaky tumor vascular wall via the EPR effect, resulting in the passive targeted accumulation of the therapeutic agents inside tumor (right) tissue other than the normal (left) tissue. (Reproduced with permission from reference³, copyright 2015 Nature Publishing Group.)

1.1.2 Composition of drug delivery nanocarriers

The strategy of designing and preparation of drug delivery system is under constant and rapid growth. Up to now, extensive libraries of materials have been used as drug

nanocarriers for the treatment of tumors as shown in Figure 2.¹³⁻¹⁶ According to the material used, these nanocarriers can be classified into two main categories: organic nanoparticles and inorganic nanoparticles.

Figure 2. A summary of anticancer drug nanocarriers. (Reproduced with permission from reference¹⁷, copyright 2015 Elsevier.)

The organic nanoparticles such as quantum dots,¹⁸ nanotubes,¹⁹ graphene,²⁰ nanocrystals,²¹ mesoporous silica nanomaterials²² and iron oxide,²³ have been used as nanocarriers of anticancer drugs for the treatment of tumor. Given the biodegradable and biocompatible merits, organic nanomaterials including liposome and polymer based nanoparticles have shown great clinical translations potential. The organic nanocarriers are now the leading choice for the clinical treatment and a certain number of them have already been developed for clinical trials or in sale. Up to now, increasing number of publications have been reported to use polymer based nanoparticles as the anticancer drug carrier for

cancer treatment. Most of these research is oriented toward the novel polymer structure design, stimulus-responsive behaviors and multifunction of the nanocarriers, which will undoubtedly guide and facilitate the further development of future nanomedicine formulation for more effective cancer therapy.

1.1.2.1 Polymeric micelle nanocarriers

Recently, polymer based nanocarriers such as polymeric nanoparticles,²⁴⁻²⁶ polymeric micelles,^{27,28} polymersomes^{29,30} and dendrimers^{31,32} are especially promising for drug delivery applications, owing to their excellent biodegradability and biocompatibility, large scale production, a wide variety of chemical functionalities and facile postpolymerization modification. Moreover, by varying the macromolecular conformation and introducing the stimuli-responsive groups, polymer based nanocarriers could easily achieve triggered release of the loaded cargoes under the stimuli of different biological or external triggers.

For the construction of the nanoassemblies, block copolymers have been most widely applied due to their facile synthesis and remarkable chemical flexibility.³³ Hydrophilic poly(ethylene glycol) (PEG) is generally incorporated into the commonest amphiphilic block copolymers as the hydrophilic block. These block copolymers could be easily prepared via ring opening polymerization (ROP) or atom transfer radical polymerization (ATRP) of the hydrophobic monomer by using the commercial available PEG as the macro-initiator. As shown in Figure 3, amphiphilic block copolymer could self-assemble to form a range of morphologies,^{34,35} including spherical micelles,^{36,37} cylindrical micelles^{38,39} and polymersomes⁴⁰ when the polymer concentration is above the critical micellar level⁴¹ in aqueous solution, for the purpose of reducing energetically unfavorable interactions with water. The assembled morphology depends on the used materials and relative ratio of the

two building blocks.³⁴ In general, the hydrophilic PEG fraction of copolymers higher than 45-50% is beneficial for the yield of spherical micelles, which is most stable among the three assemblies.

Figure 3. Three self-assembled nanoscale morphologies self-assembled by amphiphilic block copolymers with different composition. (Reproduced with permission from reference³⁵, copyright 2009 Wiley.)

Among the aggregates discussed above, micelles with the highest solubilization component ratio are most widely studied due to their high stability and appropriate size compared with other aggregates. Micellar structure with the size range between 10 and 200 nm are assembled to form hydrophobic core in the interior and the hydrophilic polymer shell exposing to water outside.^{42,43} The hydrophobic drug can be encapsulated within the hydrophobic core by the hydrophobic interaction between hydrophobic block chain and small drug molecule (Figure 4). The enhanced solubilization effect of hydrophobic molecules is crucial for the chemotherapy of cancer. Many widely used anticancer drug are insoluble in aqueous medium and cannot be directly injected for the treatment. Thus, most common injectable drug formulations contain toxic organic solvent to solubilize the drug, which lead to adverse effects on the body. One example is the utilization of Cremophor EL as the solvent to solubilize paclitaxel for the treatment. However, the injection of additional

organic solvent often causes serious hypersensitivity reactions to take place. To avoid introducing excessive organic solvent, micellar drug delivery system has been developed to solubilize and deliver water-insoluble therapeutic agents.^{28,44} Besides, the outer hydrophilic shell confer the excellent colloidal stability and reduce non-specific interactions with proteins during circulation.

Figure 4. Hydrophobic drug loaded process during micellation process of amphiphilic block copolymer.

Since micellar colloids are in dynamic equilibrium in the aqueous solution, part of the physical loaded drugs inside the assemblies tend to leak out during the long-time blood circulation, causing destabilization of the system within body. Recently, increasing efforts have been devoted to prepare stabilized nanomedicine with minimized premature drug release during circulation.⁶ Besides traditional delivery methods of loading the drugs inside polymeric nanoagents, there is also an alternative approach which is to prepare the polymeric prodrug by anchoring the drug molecules on the polymer substrate via cleavable covalent binding.⁴⁵⁻⁴⁷ Most of the reported research relied on the coupling of drugs to polymer substrate to form polymeric prodrug for self-assembly. As shown in Figure 5, the typical model normally consists of five components: (1) the immobilized drug molecules with therapeutic effects; (2) the polymer backbone; (3) degradable spacer that connect the drug and backbone and (4) solubilizing agent that assist in self-assembly in water. In most cases, PEG is often introduced as the solubilizing constituent to construct nanocarriers.^{48,49}

PEG could minimize interactions with blood proteins and reduce its clearance by the mononuclear phagocyte system,⁵⁰ which could significantly stabilize the nanoparticles in blood circulation and improve the drug bioavailability for cancer therapy. In addition, by anchoring the ligands that target tumor cells to the polymeric prodrug, active targeting strategy could also be achieved to improve the accumulation of nanoparticles inside the specific tumor cells.

Figure 5. Schematic illustration of polymeric prodrug for self-assembly.

As compared with conventional drug loading method, polymeric prodrug is particularly advantageous, as it could not only minimize the undesired drug premature release during circulation but also significantly improve drug loading efficiency.⁵¹ The polymeric prodrug nanoagents effectively combine the stability of small molecule prodrug with prolonged circulation time of nanocarriers. Since the drug is connected to the polymer bone via the selectively degradable linkage, the drug molecules would only be released from prodrug nanocarriers in response to the specific external triggers, leading to the cleavage of the covalent bond under these circumstances.

For example, much attention have been paid to the hydrazone bond for the construction of acid-responsive polymeric prodrug because this covalent bond can be efficiently cleaved under the weak acidic endo/lysosomal environment.⁵²⁻⁵⁴ The related studies are mainly

based on DOX, one of the most widely used anticancer drug, of which the conjugation could be easily achieved through formation of hydrazone linkage between the carbonyl group of DOX and hydrazide groups on polymer substrate.⁵⁵ In spite of the weak acidic conditions, the reducing environment inside the cancer cells is also exploited as a trigger for the controlled drug release. As a consequence, the anticancer drug has also been covalently attached on the polymer substrate via disulfide linkage to form the reduction-responsive amphiphilic polymeric prodrug.⁵⁶⁻⁶⁰ A typical example is shown in Figure 6, and polymeric prodrug was synthesized via ROP of phosphoester containing monomer and finally functionalized with camptothecin (CPT) via disulfide linkage.⁶⁰ This as-prepared polymeric prodrug possess advantages of facile preparation, high drug conjugation content, high stability, and triggered release upon exposure to reducing environment.

Figure 6. Schematic illustration of the polymeric CPT prodrug micelles for the triggered CPT release under reduction environment. (Reproduced with permission from reference⁶⁰, copyright 2015 Royal Society of Chemistry)

1.1.2.2 Inorganic nanoparticles for drug delivery

With the rapid development of nanoscience, inorganic nanoparticles are also of growing

interest for drug delivery applications due to controllable size and morphology, high specific surface area, and distinct photonic, electric or magnetic properties for biomedical applications.^{61,62} In principle, nanocarriers for biomedical applications are required to be biocompatible and nontoxic in regard to the biosafety issues. Among the widely studied inorganic materials, more and more attention have been paid to the mesoporous silica nanoparticles (MSNs) on account of its biocompatibility.^{63,64} Furthermore, MSNs possess unique features such as easy surface functionalization, excellent stability, relatively high surface area, and tunable pore size for drug delivery applications.⁶⁵⁻⁶⁸ The tunable mesoporous channels of MSNs could serve as interspace for the effective storage of drug molecules.

The formation of mesopore channels rely on the assembled cationic surfactant cylindrical micelle as the structure directing template.^{69,70} Taking the most representatives silica solids MCM-41 as an example,^{71,72} after the self-assembly of surfactant cetyl trimethylammonium bromide (CTAB) into cylindrical micelles, the silica component condensate at the surface of micellar template by electrostatic interaction and polymerize to form silica wall around the surface of the micelles. After the removal of the template surfactants that are filled inside the mesopores, MCM-41 type MSNs are eventually obtained via calcination or extraction (Figure 7). The MCM-41 nanoparticles with ordered arrangement of uniform hexagonal mesopore channels have a diameter ranging from 1.6 to 10 nm and the specific surface area is higher than 700 m² g⁻¹.⁶⁶

Figure 7. Schematic illustration of the formation mechanism of MCM-41 type MSNs. (Reproduced with permission from reference⁷², copyright 2012 Royal Society of Chemistry.)

Another distinctive advantage of MSNs is that the abundant silanol groups on silica surface could be further used for the modification with other functional groups to improve the aqueous dispersity, introduce functional groups, and work as the “gate” to control the drug loading and release.^{68,73} The surface modification generally utilizes silane coupling agent containing ethoxy or methoxy groups which will efficiently react with silane groups to form Si–O bond on the surface of MSNs. By this means, reaction moieties including amine, carboxyl, chloride and thiol could be easily introduced after silane coupling, facilitating further immobilization of functional moieties on MSNs for specific biomedical applications.

For drug delivery applications, it is of importance that drug nanocarriers with high drug loading efficacy generally are employed, which could avoid undesirable side effects of the nanocarriers in chemotherapy. Hollow MSNs (HMSNs) have been developed for the more effective drug delivery, since large hollow cavities of HMSNs allows encapsulation of greater amount of therapeutic agents as compared to conventional MSNs.^{74–76} HMSNs

could be easily prepared from soft templates method based on self-assembled polymeric micelles^{77,78} and vesicles⁷⁹. However, since one surfactant is used for the preparation of soft core template while another surfactant is also needed to construct the mesoporous structure, the coexistence of the two templates often leads to the broad particle size distribution and incomplete mesoporous structure. To cope with this challenge, the hard nanoparticles with the uniform size, including polymer beads, silica colloids and metal oxides, have been used as internal template to form core-shell structure (Figure 8). It should be noted that the template should be completely removed without disintegrating the mesoporous silica channels. Thus, polymer beads^{80,81} and solid silica nanoparticles^{82,83} have often been explored to be used as hard template, since polymer core template can be easily removed at high temperature (300-500 °C) while the solid silica core can be selectively etched by alkaline solution.

Figure 8. Schematic illustration of hard template for the preparation of HMSNs using small molecular surfactant or polymer for mesoporous shell construction. (Reproduced with permission from reference⁶⁹, copyright 2013 Royal Society of Chemistry.)

Figure 9. TEM images: (a) solid silica nanoparticle template, (b, c) HMSNs, (d) low-angle X-ray diffraction pattern of solid silica nanoparticle and HMSNs, and (e, f) N₂ sorption isotherms and the pore size distribution of solid silica nanoparticle and HMSNs. (Reproduced with permission from reference⁸², copyright 2011 Royal Society of Chemistry)

Preparation of HMSNs using solid silica core for hard template was developed by Zhen's group (Figure 9).⁸² The oriented mesoporous shell was coated on the surface of the core template via surfactant-template sol-gel process. By treating the nanoparticles in Na₂CO₃ solution, the solid core was selectively etched while the mesoporous shell structure was well maintained with the protection of cationic surfactant (*i.e.* CTAB).

To achieve controlled drug release and improved therapeutic efficacy, the outer surface of MSNs is generally functionalized by various types of capping agents including inorganic nanoparticles, organic compounds, biological compounds or polymer.^{68,73} Among these capping agents, coating MSNs with polymers is of special significance since the grafted polymers can provide tailored properties including better biocompatibility, higher stability, multiple functionality, and excellent controlled release of the loaded cargoes to meet various requirements. The coating of MSNs with polymer have been realized via three

different approaches, including cross-linking, “graft to” and “graft from” method.

For example, it has been reported that the MSNs were easily coated by the biocompatible copolymer with side pyridine disulfide groups and the polymer layer was subsequently cross-linked the dithiothreitol (DTT) (Figure 10).⁸⁴ The as-prepared MSNs could load more drug than the nanoparticles with direct chemical modification, as the modified functionalities may limit encapsulation of large mass molecules. A second method is to first conjugate unsaturated double bond onto the surface on the MSNs,⁸⁵ before polymer is cross-linked onto the surface of MSNs by the radical polymerization.

Figure 10. Schematic illustration of one-pot preparation of the reduction-responsive cross-linked polymer-gatekeeper modified MSN decorated with targeting ligand. (Reproduced with permission from reference⁸⁴, copyright 2015 Wiley.)

The “graft to” approach employs pre-prepared polymer with functional moiety to directly immobilize on the surface of MSNs through the reaction with complementary groups.⁸⁶

This method is easy to be accomplished due to the lack of intricate synthetic procedures.

The major advantage of this approach is that the polymer precursor can be prepared with specific molecular weight and structure by variation of polymerization conditions. The polymer could be thoroughly characterized via various methods prior grafting process. For example, aminated PEG was used to attach the carboxyl functionalized MSNs via the functionalization by amidation.⁸⁷ In addition, trimethoxysilane terminated poly(ϵ -(carbamic acid benzyl ester)-caprolactone)⁸⁸ or poly(2-hydroxyethyl methacrylate)⁸⁴ was firstly prepared by ROP and subsequently attached to the surface of MSNs by the “graft to” method via the silane hydrolysis and condensation. However, despite the convenience of this method, the grafted polymer chains tend to inhibit the diffusing of free polymer for further reaction, resulting in the reduced grafting density.

The “grafting from” technique,⁸⁹ which initiates the polymerization by the attached initiating groups, is intensively studied to prepare the polymer coated MSNs. By functionalize the MSNs with different initiating groups, the hybrid nanoparticles could be prepared by surface initiated ATRP⁹⁰ and addition–fragmentation chain-transfer (RAFT)⁹¹ polymerization. By modifying the surface of MSNs with ATRP initiator, a variety of polymers such as poly(methyl methacrylate),⁹² poly(*N*-isopropylacrylamide),^{93,94} PDEAEMA^{95,96} and poly(2-(methacryloyloxy)ethyl ferrocenecarboxylate)⁹⁷ have been grafted onto MSNs with higher grafting density. In addition, copolymers integrating positive charged quaternary amines and PEG side chains have been modified onto MSNs via surface initiated RAFT polymerization.⁹⁸ It is demonstrated that the hybrid nanoparticles with high positive zeta potential could enhance the EPR effect and accumulation in the tumor sites, leading to the improved therapeutic efficacy in vivo. Besides, by anchoring bi-functional hetero-initiator on the MSNs (Figure 11),⁹⁶ two

different polymers can be grafted independently via RAFT polymerization and subsequent ATRP, providing more choices for the polymer coating.

Figure 11. Synthetic route of pH-responsive polymer modified MSNs by combination of two types of surface initiated controlled living radical polymerization and the schematic illustration of DOX loading and acid-triggered release behavior. (Reproduced with permission from reference⁹⁶, copyright 2012 Willey.)

1.1.3 FDA-approved therapeutic nanoagents

The ideal drug delivery system should enhance the bioavailability of the delivered drug by avoiding the clearance of body and facilitating the selective uptake inside tumor sites. However, in the academic research of the drug nanovehicles, more attention has been given to the materials innovation, versatility and therapeutic efficacy of the nanomedicine, regardless of the potential clinical translation.⁹⁹ Research on nanomedicines for cancer therapy has increased dramatically over the past 10 years. So far, liposome and lipid-based nanocarriers have been the most successful candidates for clinical translation. However, there are only four nanoparticle-based drug delivery platforms (Doxil, DaunoXome, Marqibo and Abraxane) approved by U.S. Food and Drug Administration (FDA).⁶ One

example is Doxil, which is the PEGylated liposomal formulations for the delivery of drug DOX.¹⁰⁰ The PEG introduced on the liposomes could minimize undesired interactions of with proteins as well as uptake by phagocytes in the mononuclear phagocyte system, resulting in the prolonged circulation time. Animal models have been used to demonstrate that PEGylated liposomes could significantly accumulate in the tumor tissue by EPR effect and release drug exclusively at tumor sites. Both DaunoXome¹⁰¹ and Marqibo¹⁰² are not PEGylated liposomes formulations for the delivery of DOX and vincristine, respectively. The non-PEG design prompt the nanomedicine to be phagocytosed by monocytes in circulation, avoiding initial high plasma concentrations and allowing the slow diffusion of the drug out for sustained therapy.

Table 1. Polymeric micelle formulations for anticancer therapy for clinical investigation in U.S.

Polymer	Chemical structure of polymer	Name and drug	Incorporation	Status	Ref.
PEG-PLA		Genexol-PM, paclitaxel	physical loading	II	103
PEG-P(Asp)		NK105, paclitaxel NK911, doxorubicin	physical loading physical loading	II II	104 105
PEG-P(Glu)		cisplatin NC-4016, HACHPt NK012, SN-38	Coordinate bonding Chemical conjugation	I / II I II	106 107 108
Pluronic		SP1049C, doxorubicin	physical loading	III	109

Abbreviation: DACHPt, dichloro-(1, 2-diaminocyclohexane) platinum(II); SN-38, 7-ethyl-10-hydroxy-camptothecin.

Besides, polymeric micelle based drug delivery system is another class of nanomedicines whose application has enjoyed clinical success. To date, various types of micellar

nanomedicines have been developed for sale or in different stages of clinical trials (Table 1).⁴⁴ The primary goals of the micellar nanocarriers in clinical trials are to enhance anticancer drug solubility in blood and achieve the passive tumor accumulation via EPR effect.

For example, paclitaxel is an effective microtubule-stabilizing drug for treating various common tumors including the common lung, breast and ovarian cancers. However, additive organic solvent Cremophor EL is often included in the injection formulation in order to solubilize the hydrophobic drug. Genexol-PM,¹⁰³ a nanomedicine formulation of paclitaxel loaded inside poly(ethylene glycol)-block-poly(D,L-lactide) (PEG-PLA) micelles, has been approved by South Korea for the treatment of refractory cancer in 2007 and is also approved for sale within Europe. In addition, Genexol-PM is also currently in phase II clinical trials within USA. The polymer poly(ethylene glycol)-poly(aspartic acid) (PEG-P(Asp)) modified with 4-phenyl-1-butanol self-assembled micelles have been used to retain paclitaxel to form the nanomedicine formulation NK105, which is now also in phase II clinical trials in USA with encouraging results.

1.2 Design rules for targeted drug delivery nanoplatform

Controlled drug delivery provides the possibilities to decouple physiologically imposed design constraints, allowing improved drug bioavailability and reduced side effects by selectively releasing the drug into tumor sites. Although tremendous amount of research on drug delivery platforms have been published every year, only several anticancer nanomedicines have been approved for sale or clinical trials. The development of new and applicable nanomedicines is still challenging and the structure-property relations should be understood to improve their pharmacokinetics and biodistribution. The advancement of

material chemistry has offered new opportunities for the development of drug delivery platform of the next generation, creating smart nanovehicles that are stable, biodegradable, biocompatible, and responsive to changes in environment and accumulates selectively at desired pathological sites.

1.2.1 Stimuli-responsive drug release

The main goal of the nanocarrier based delivery system is to deliver the nanoagents to the desired pathological sites and then quickly release the encapsulated drug inside. However, traditional drug delivery system suffers from the disadvantage such as the slow and inefficient drug release at the pathological sites and cancer cellular compartments, leading to the undesired and compromised treatment in clinics. As a result of the multi-drug resistance mechanism,¹¹⁰⁻¹¹² the slow-releasing anticancer drug may be pumped out of the tumor cells by the transporter overexpressed proteins and lose their therapeutic efficacy even, when the nanomedicines have been endocytosed by the tumor cells. To overcome this challenge, the successful and burst release of drugs from nanocarriers upon entering the cancer cells is critical to induce the rapid and effective apoptosis of cancer cells.

To address the above issue, researchers have turned to focus on the development of stimuli-responsive nanocarriers. Stimuli-responsive nanocarriers exhibit smart and “on demand” drug release feature after being endocytosed by tumor cells, leading to increased therapeutic efficacy and minimized side effects.¹¹³⁻¹¹⁵ The stimuli-responsive drug delivery systems should recognize the targeted cellular environment with “on-demand” temporal (at right time), spatial (at targeting sites) and dosage control. As compared with conventional non-responsive counterparts, these stimuli-responsive nanomedicine have shown enhanced therapeutic performances both in vitro and in vivo experiments. Generally, biocompatible

functional materials are required in response to the specific trigger and subsequently undergo corresponding protonation, cleavage, rearrangement reaction or supramolecular conformational change. Over the past decade, major advancements have been witnessed in the development of therapeutic nanoagents that are stable under physiological environment (*e.g.* during the blood circulation), while release drug rapidly in response to biological triggers (pH change,¹¹⁶⁻¹¹⁸ redox stimuli,^{119,120} enzymatic-mediated reaction¹²¹) or external triggers (temperature variation,^{122,123} light irradiation^{122,123}) (Figure 12).

Figure 12. Illustration of biosignals existing in the tumor extracellular microenvironments and cancer cells. (Reproduced with permission from reference¹²⁴, copyright 2015 Elsevier.)

Taking advantage of pH variations within different tissues and cellular compartments, the pH-responsive nanocarriers have been widely exploited to transport anticancer drug to tumor tissue or cellular compartments and further release drug inside. For instance, extracellular environment of solid tumors is only slightly acidic (pH 6.5-7.2) in contrast to physiological environment (pH 7.4). In addition, the intracellular microenvironment of cancer cells is even more acidic in lysosomes (pH 4.5-5.0) and endosomes (pH 5.0-6.5),¹²⁵ which facilitate the effective drug release from pH-responsive nanomedicine. Two main strategies are applied to realize the pH-responsive behavior. The foremost method is to

integrate the nanoplatform with the primary, secondary or tertiary amine, which are protonated with solubility changes in response to weakly acidic environment. The other strategy is to introduce the acid-sensitive bonds including acetal, hydrazine and ortho ester that are readily cleaved under the weak acidic environment. For example, the block copolymer PEG-*b*-poly(β -amino ester) consisting of a hydrophobic alkyl chain and a hydrophilic PEG was easily prepared. The formed micelles were stable at physiological pH, whereas the loaded DOX was released quickly under weak acidic environment upon the protonation of the tertiary amine groups.¹²⁶ Immobilizing the pH-responsive polymer containing tertiary amine groups on the surface of MSNs have been reported by several literature.^{95,96,127} Under physiological conditions (pH = 7.4), the mesopore channels were capped by the polymer film coating to retain drug inside. However, in weakly acidic environment, the acid triggered release of the loaded cargoes was achieved because of the extending of the protonated polymer to the aqueous solution and subsequent unblocking of the pores.

Reductive environment is also a fascinating and unique internal trigger for “on-demand” drug release. The disulfide bonds, one of the versatile and bio-cleavable linkages, have been widely used as an intermediate linker to build up reduction-responsive nanocarriers for efficient release of anticancer drug inside cancer cells.^{128,129} The content of reducing agent such as glutathione (GSH) within cancer cells is approximately 10^3 folds higher than that of GSH in extracellular matrix,¹³⁰ thus the disulfide bonds could be readily cleaved in the cytoplasm of cancer cells, causing the release of the encapsulated drug. Up to now, certain types of reduction-responsive polymeric micellar assemblies have been proposed for drug delivery application according to the composition and amount of reduction-cleavable

disulfide linkages, such as reduction-responsive shell-sheddable micelles,¹³¹ reduction-responsive core-degradable micelles¹³² or reduction-responsive cross-linked micelles.¹³³

Figure 13. Synthetic route of reduction-responsive supramolecular mechanized HMSNs with the folic acid targeting ligand. (Reproduced with permission from reference¹³⁶, copyright 2013 American Chemical Society)

Our group reported on the amphiphilic block copolymer with controlled molecular weight and functionalities, which was prepared via the ROP and subsequent ATRP using the multifunctional double-head initiator containing disulfide bond.¹³⁴ The copolymer self-assembled into shell-sheddable micelles in physiological environment while quickly released the loaded drug under reductive environment on account of the cleavage of disulfide linkage and dissociation of the nanocarriers. Besides, our group has also demonstrated several other examples of reduction-responsive supramolecular mechanized MSNs by capping the cyclodextrin onto the pores of the nanoparticles with the disulfide linkage (Figure 13).¹³⁵⁻¹³⁹ The disulfide bonds between the cyclodextrin and MSNs can be

cleaved under reductive environment, and the drug release rate was significantly enhanced followed by opening of the pores.

1.2.2 Biocompatibility and biodegradability

Choice of appropriate material for constructing the nanocarriers is crucial, since biocompatibility and non-toxicity are primary prerequisites for the success and safe drug delivery application.¹⁴⁰ The biocompatibility should integrate well with tissue of human body without eliciting immune response or undesirable effects.¹⁴¹ All the drug delivery nanoagents approved by FDA or in clinical trials are made of the biocompatible constituents.^{142,143} Take the polymeric nanomedicine formulation for clinical trial for example, the hydrophilic constituent is almost PEG, which has widely been used as biomedical hydrophilic material with the approval by U.S. FDA. Moreover, it could efficiently reduce unfavorable interactions with serum proteins during circulation and escape the capture by mononuclear phagocyte system.^{144,145} In addition, the core of the assembled nanocarriers consists of hydrophobic moiety which allows encapsulation of drug through hydrophobic interactions. Among them, the hydrophobic polymer blocks are mostly aliphatic biodegradable polyesters including poly(amino acids), poly(propyl oxide) and poly(D,L-lactide) (PLA).⁴⁴ Aliphatic polyesters exhibit excellent biodegradability and could be degraded completely inside the body in period ranging from days to months.

Evaluation of the biocompatibility of MSNs is challenging, because the biological effects of MSNs in body are complex and vary enormously, on account of its particles size, pore structures, shapes and synthetic routes.^{64,146} Numerous studies have shown increasing evidence that MSNs are more compatible than other nonporous silica colloids, since mesoporous structure could reduce the effective MSNs/membrane contact area. In addition,

MSNs are generally functionalized with biocompatible material on its surface which further reduces the toxicity as demonstrated in in vivo experiment. However, the degradability of MSNs is still a potential concern for clinical translation. To cope with this challenge, our group have prepared the cyclodextrin capped MSNs with different sizes for the targeted anticancer therapy.¹³⁸ The DOX encapsulated MSNs with the size of 48 nm showed superior efficacy in treatment of cancer and could be efficiently excreted from mice after 12 days post injection. Later, our group also fabricated the ultra-small silica-polymer hybrid dots which was utilized for loading of paclitaxel, and the small hybrid dots could be completely cleared for tumor-bearing mice model quickly.¹⁴⁷

1.2.3 Stability, size and targeting

To reduce side effects of the anticancer therapeutic agents, the nanomedicine should exhibit excellent colloidal stability to limit the unexpected drug leakage during the circulation. In addition, the nanocarriers should be chemically inert under physiological environment at 37 °C, so that they would not be degraded or destabilized during transportation of the drug. Furthermore, the nanomedicine should not interact with the proteins in blood and should avoid being directly taken up by the mononuclear phagocyte system.

Size, which is indispensable parameter for study of nanoparticles, affects the property and performance of these nanomaterial, especially in drug delivery application. Generally, to enhance the circulation time, the size of the nanoscale materials should be more than 8 nm, so as to avoid the rapid clearance by the kidney during circulation. Besides, in consideration of the effective transporting through leaky vasculature in tumor due to EPR effect, the size of the nanoparticles should be designed smaller than 200 nm.¹⁴⁸

Figure 14. The active targeting moieties used for anticancer drug delivery. (Reproduced with permission from reference¹⁷, copyright 2015 Elsevier.)

For the successful and effective treatment of tumor, the selective accumulation of therapeutic nanoagents inside the tumor sites and cancer cells is crucial. The concept of “magic bullet” for therapy was brought forward by Paul Ehrlich in 1908.¹⁴⁹ In his point of view, it is important that the nanoagents could intelligently differentiate between the healthy cells and the cancer cells, which allows the selective treatment of cancer cells to reduce side effects. Although passive EPR effect has been an area of intense study, active targeting could be further supplemented by modification of nanoparticles with targeting ligand.^{150,151} The ligand can be recognized by the corresponding over-expressed receptors on the surface of particular cancer cells, promoting efficient endocytosis to increase the concentration of therapeutic agents inside tumor cells.¹⁵²⁻¹⁵⁴ Up to now, a broad range of targeting ligands including small molecules, polysaccharides, peptides and proteins have been used to functionalize the nanocarriers for enhancing the active tumor targeting ability (Figure 14).¹⁵⁵ Among these ligands, small molecules vitamins such as folic acid is most widely studied,^{156,157} since folic acid is readily available with low cost. Most importantly, folic acid can bind to over-expressed folate receptors on many types of cancer tumor cells

and significantly enhance the accumulation inside tumor cells.

1.3 Photothermal therapy

Although chemotherapy is the dominant therapeutic approach for the clinical treatment of cancer, its efficacy is often compromised owing to acquisition of multidrug resistance. The occurrence of multidrug resistance is caused by the insufficient drug transportation to the tumor sites and the continually low dose of drug in cancer cells, proving to be the main obstacle for successful chemotherapy.¹⁵⁸ To overcome the inadequacy of chemotherapy, the medical treatments of phototherapy have been put forward to convert light into other form of energy for the treatment of tumor. So far, photodynamic therapy as well as photothermal therapy are the two main types of light induced therapies under wide investigation for the treatment of cancer.¹⁵⁹⁻¹⁶¹

1.3.1 NIR induced photothermal therapy

The recent extensive study of photothermal therapy have brought out the new insights for the successful treatment of cancer.¹⁶² Cells are sensitive to the temperature and could only survive and reproduce in a specific narrow temperature range. It has been reported that the local hyperthermia with the temperature above 45 °C would cause direct cancer cells death.^{163,164} At excessive high temperature, the cell membranes loose and protein denatures inevitably, leading to the direct cancer cellular damage and ultimate eradication of tumor tissue. Under light irradiation with a specific wavelength, the photo-absorbing agents could efficiently convert the absorbed light into heat energy for the photothermal treatment of cancer. At the same time, photothermal therapy exhibits the noninvasive therapeutic nature. By selective illumination of tumor sites, the localized hyperthermia for the ablation of targeted tumor would not cause additional damage to un-illuminated healthy tissue and

normal cells.¹⁶⁵ For the ideal photothermal therapy and complete ablation of the tumor, photothermal agents should exhibit specific tumor accumulation ability, acceptable photostability, good biocompatibility, and high photothermal efficiency. As a general rule, photothermal agents with strong absorption in the near infrared (NIR, 700-900 nm) region are often employed, since NIR light could penetrate deeply through soft tissue with the minimum absorption. Therefore, maximum amount of light could be utilized by the NIR absorbing agents along with minimum damage to the surrounding tissue and cellular components. In all, to increase the application potential for clinical translation, a great effort has been paid to the design and fabrication of biocompatible NIR absorbing agents with strong absorbance in NIR range as well as high photothermal conversion efficiency.

1.3.2 NIR absorbing materials for photothermal therapy

The advancement of nanotechnology has endowed new opportunities for the further development of photothermal therapy. For conversion of NIR light into heat, the performance of NIR absorbing agents is critical to the successful photothermal treatment of cancer. So far, the most widely studied NIR absorbing nanomaterials for photothermal therapy include gold nanostructures,^{166,167} carbon nanomaterials,^{168,169} copper sulfide nanoparticles,¹⁷⁰ and conjugated polymers.¹⁷¹ On account to the nanoscale size, these NIR absorbing nanomaterials not only possess the merit of prolonged blood circulation time, but also passively accumulates in the cancer cells via EPR effect. Upon the selective transportation into tumor sites, these systems can convert the absorbed light energy into heat accompanied by reduced side effects to the normal tissue. To further improve the selective endocytosis by the specific tumor cells, the nanoagents could also be fabricated with targeting ligands which strongly bind to over-expressed with relevant receptors of

cancer cells.

Nevertheless, the nonbiodegradable nature and potential toxicity of inorganic nanomaterials severely inhibit their clinical translation potential.¹⁷² In this regard, NIR-absorbing dyes,¹⁷³ particularly the family of indocyanine derivatives which have excellent biodegradability and photothermal conversion efficiency, are more promising candidates for photothermal therapy.

Traditional NIR fluorescent dyes can strongly absorb the NIR light to reach an excited singlet state, which tend to undergo radiative relaxation to its ground state by emitting the light with a longer wavelength, called fluorescence. It should be noted that their emitted fluorescence generally falls into the range of NIR region, significantly reducing the interference of auto fluorescence with improved tissue penetration.¹⁷⁴ Therefore, the NIR fluorescent dyes with high quantum yield can be employed as diagnostic probe for non-invasive fluorescent tumor imaging in vivo.

Apart from the fluorescence transition way, a portion of energy of the excited singlet state may be relaxed by non-radiative transitions, generating heat instead of fluorescence. Thus, some of these NIR dyes can be used as multifunctional theranostic agents for both photothermal therapy and fluorescence imaging. However, excess loss of fluorescent energy undoubtedly has unfavorable effect on the photothermal therapeutic efficacy. The dyes with high absorption efficiencies and lower levels of quantum yields may provide better photothermal efficiency with the reduced fluorescence energy loss. The competitive energy emission process is schematically shown in Figure 15. With recent progresses of photothermal therapy, NIR absorbing dyes have become the promising therapeutic agents for tumor treatment.

Figure 15. Schematic illustration of energy loss process for the nanoparticles based photothermal therapy. (Reproduced with permission from reference¹⁶², copyright 2014 Royal Society of Chemistry.)

Figure 16 presents some of commonly used NIR absorbing organic photothermal dyes for the photothermal treatment of cancer. The extended conjugation chemical structure help push the absorbed photons into the NIR regions. Besides, a few methyl groups in the dye structure facilitate intramolecular rotation, promoting the internal conversion process to enhance the light to heat conversion efficiency.

Figure 16. Chemical structures of commonly used NIR absorbing dyes for the photothermal treatment of cancer.

All the above mentioned dyes could efficiently convert the light energy into heat. However, due to their poor aqueous stability or solubility, these free dyes could not be

directly used through systematic administration, hindering their biomedical application. In order for the dyes to perform photothermal treatment effectively, the dyes should be transported to at the pathological tumor sites and emit the heat directly there. Thus, these dyes could be encapsulated inside the polymeric nanocarriers,¹⁷⁵ which can significantly adjust the bio-distribution of the dyes and selectively deliver the dyes to the tumor sites via the EPR effects.

1.3.3 Polymer for the delivery of NIR-absorbing dye

Polymer-based nanovehicles including polymeric micelles, polymeric nanoparticles, dendrimers and polymersomes have been employed as the carriers to transport the photothermal agents to the tumor sites. Using polymer with versatile chemical structures as nanocarriers would improve the multi-functionality, photostability, aqueous solubility and biocompatibility of NIR absorbing payloads, thus significantly improving their application potential. Generally, NIR absorbing agents could be incorporated into the polymeric nanocarriers by physical loading, covalent conjugation, coordination binding, or electrostatic interaction.

For the application of photothermal therapy, indocyanine green (ICG) has been widely studied among various organic NIR-absorbing dyes. Due to its good biocompatibility and strong NIR fluorescence, it has been approved by FDA as clinical NIR imaging agent. Besides, as a potential theranostic agent, ICG can also convert part of the absorbed energy into heat. However, the direct use of free ICG for photothermal therapy is severely restricted by its physiochemical limitations, such as nonspecific binding to proteins, concentration-dependent aggregation as well as poor aqueous stability. To overcome these limitations, various polymeric nanocarriers using the polymer such as poly(lactic-*co*-glycolic acid)

(PLGA),¹⁷⁶ PEG-*b*-poly(oxypropylene),¹⁷⁷ poly(styrene-*alt*-maleic anhydride)-*b*-poly(styrene)¹⁷⁸, phospholipid-PEG (DSPE-PEG),¹⁷⁹ and nanoparticle/polymer microcapsules¹⁸⁰ have been employed to encapsulate ICG in aqueous solution. It is demonstrated that ICG could be stabilized in the nanocarriers with the prolonged blood circulation time. Besides, PLGA-*lecithin*-PEG,¹⁸¹ PLGA-stabilized DSPE-PEG,¹⁸² PEG-*b*-poly(L-lysine)-*b*-poly(L-leucine)¹⁸³ as well as dextran-based block copolymer have also been used to construct the nanocarriers to load ICG, achieving the image-guided photothermal therapy of cancer. Besides, cypate, which has the similar structure of ICG, has also been reported to be encapsulated in PEG-*b*-poly(aspartic acid(decylamine)) micelle for long-term NIR fluorescence imaging of cancer.¹⁸⁴

Apart from the ICG, ICG derivative IR780 exhibits the improved photostability which could be used as the robust theranostic agent for the continuous imaging guided photothermal therapy of cancer. For example, the polymeric assemblies of folic acid conjugated natural heparin polysaccharide (Figure 17)¹⁸⁵ and PEG-*b*-poly(caprolactone) (PEG-*b* PCL)¹⁸⁶ have been reported to loaded IR780 for the treatment of cancer, serving as theranostic agents for bioimaging and photothermal therapy both in vitro and in vivo. The strategy of PEGylation has been also been proved as effective delivery approach to improve the aqueous solubility of hydrophobic molecules. Since the meso-chloro group in the cyclohexenyl ring of cyanine derivative could be easily substituted by nucleophile reagents, IR780 was modified with sulfhydryl¹⁸⁷ or amino¹⁸⁸ terminated PEG, and the resultant amphiphilic products could form polymeric micelles which were directly used as photothermal nanoagents.

Figure 17. Synthetic route and self-assembly of IR780-loaded heparin-folic acid nanoparticles for the photothermal therapy. (Reproduced with permission from reference¹⁸⁵, copyright 2013 Elsevier)

Another example of NIR absorbing dye other than cyanine derivatives employed for photothermal therapy is *tetra*-butylphthalocyanine (PcBu₄). As compared with ICG and cypate, PcBu₄ possesses improved photostability. Lim et al.¹⁸⁹ prepared Pluronic-based nanoparticles encapsulating hydrophobic dye PcBu₄, and the therapeutic system exhibited robust photothermal effect superior to ICGvloaded nanoparticles as the control (Figure 18).

It should be noted that all the NIR absorbing dye mentioned above possesses a certain level of fluorescence quantum yield and undesired photostability. To cope with this challenge, Liu and co-workers designed a new version of NIR absorbing heptamethine indocyanine dye IR825, which has high NIR absorption coefficient but low fluorescence quantum yield. Besides, this dye also exhibits excellent photostability upon repeated NIR irradiation, which could improve the photothermal therapeutic efficacy to the maximum extent.¹⁹⁰ In their research, IR825 was solubilized by the PEG grafted amphiphilic polymer, showing the excellent colloidal stability of the resulting micelles. After intravenous

injection of dye loaded micelles, mouse-bearing tumor tissues were completely removed under a low-power laser irradiation.

Figure 18. Schematic illustration of the fabrication of naphthalocyanine loaded theranostic nanoplateform. (Reproduced with permission from reference¹⁸⁹, copyright 2015 Royal Society of Chemistry.)

1.3.4 Combined photothermal-chemotherapy

In most cases, it is difficult for one therapeutic approach alone to achieve the desirable therapeutic efficacy and completely remove the tumor tissue. Thus, the concept of combining chemotherapy, such as combined photothermal-chemotherapy, has been put forward to ensure therapeutic efficacy for cancer. Combined photothermal-chemotherapy is capable of overcoming the limitations of each therapeutic approach alone, which may yield additive or synergistic effects and exhibits unprecedented therapeutic outcomes.

For instance, both ICG and DOX were loaded simultaneously in the monoclonal antibodies modified PLGA nanoparticles for the targeted combined photothermal-chemotherapy.¹⁹¹ Another example was also reported by Cai and co-workers,¹⁹² in which DOX and ICG were co-loaded in PLGA-*lecithin*-PEG polymeric nanoparticles. Besides,

Chen et al.¹⁹³ reported the co-loading of cypate and DOX inside pH/reduction-responsive polymeric vesicles, which demonstrated superiority as a therapeutic platform for synergistic therapy. In another recent study, IR825, the newly developed NIR absorbing dye, was first complexed with cationic polymer polyethylenimine (PEI) and then coated with PEG.¹⁹⁴ The *J*-aggregates was eventually loaded with DOX, and exhibited combined therapeutic effect under the NIR laser irradiation.

As compared with ICG, Cypate is the newly developed dye for photothermal therapy. Although Cypate has similar absorption and emission properties with ICG, Cypate possesses reactive carboxylic acid groups which could be easily conjugated with biologically active agents or functional materials. Ge and co-workers developed polymer conjugated with cypate and cisplatin prodrug via “click” reaction for the combined photothermal-chemotherapy.¹⁹⁴ Other than click reaction, cypate could also be conjugated to various substrates via direct esterification or amidation reaction of the carboxylic acid groups. Li and co-workers reported the direct conjugation between the carboxylic acid group of Cypate and pendant hydroxyl groups on pH-responsive amphiphilic polymer via ester bond formation (Figure 19).¹⁹⁵ This dye conjugated polymer was co-assembled with the amphiphilic polymer with terminated DOX molecules, and the in vivo experiments showed that this system could completely ablate tumor tissue, overcoming the occurrence of multi-drug resistance.

Figure 19. Chemical structures of DOX or dye conjugated amphiphilic polymer, and the schematic illustration self-assembled polymeric micelles for therapy. (Reproduced with permission from reference¹⁹⁵, copyright 2014 Elsevier)

Gold nanorods (AuNRs) have been widely used as photothermal agents. By simply increasing the aspect ratio of the nanorod, the absorption of AuNRs could be easily tuned into the NIR region to increase the light penetration through tissue. Liao et. al. prepared PEG-*b*-PCL based polymersomes to load both DOX and AuNRs simultaneously. Under the NIR laser irradiation, DOX was released efficiently owing to the disassembly of the polymersome nanocarriers caused by the generated heat.¹⁹⁶ Despite of traditional delivery methods by loading drugs inside polymeric nanosystems, an alternative strategy is to convert drugs into stable nanocarrier-drug conjugates through biologically cleavable covalent bonds. By applying this strategy, the undesired leakage of the drug during circulation could be further prevented. Li et. al.¹⁹⁷ firstly used PEG modified PAMAM dendrimers to coat the AuNRs via the thiol-Au coordination. After that, the anticancer drug DOX was conjugated to the polymer using the acid liable hydrazone linkage (Figure 20). The as-prepared nanohybrids are stable under physiological conditions. However, the hydrazone bond was cleaved under acidic endo/lysosomal conditions, resulting in the

enhanced release of DOX. Eventually, as compared to PTT or drug treatment alone, this combined photothermal-chemotherapy exhibited a synergistic therapeutic efficacy, showing a great potential for the cancer treatment.

Figure 20. Schematic illustration of pegylated dendrimeric DOX prodrug coating onto AuNRs for the combination therapy. (Reproduced with permission from reference¹⁹⁷, copyright 2014 Elsevier)

Chapter 2: Polymer-Coated Hollow Mesoporous Silica Nanoparticles for Triple-Responsive Drug Delivery

2.1 Introduction

Over the past decades, MSNs have attracted extensive attention using as effective nanocarriers for the treatment of cancer, as described in the Chapter 1. However, if MSN-based nanocarriers are not modified, their therapeutic effects are often compromised for the therapy, since the physically loaded drug tends to be pre-released during the blood circulation.²² The leaked drug from the nanocarriers during the circulation would cause undesired damage to healthy cells and organs. More seriously, the loss of transported drug would cause the low levels of drug accumulation in the targeted cancer cells, leading to the occurrence of multidrug resistance.

To reduce the unexpected side effects and increase their therapeutic efficacies, various types of stimuli-responsive MSN-based drug delivery systems have been fabricated. These nanocarriers could achieve controlled and “on demand” drug release feature after being endocytosed by tumor cells.^{70,72} MSNs with responsive polymeric coating are of increasing interests on account of the versatility of the polymer structure, multifunctional capability and excellent drug encapsulation capacity.⁸⁸ Specifically, various stimuli-responsive polymer have been utilized to coat the MSNs and control the release of loaded drug. For example, given the lower pH values in the endo/lysosomal compartments in cancer cells, pH-responsive polymers such as PDEAEMA,^{95,96} poly(2-phenyl-1,3-dioxan-5-yl methacrylate),¹⁹⁸ and poly(acrylic acid)¹⁹⁹ were proposed to be anchored on MSNs to achieve acid triggered drug release. Another example is the disulfide bond, which is one of

the bio-cleavable linkages, has been used to cross-link the polymer shell coating on MSNs, controlling the release of guest molecule loaded inside.²⁰⁰ Compared with above biological stimuli which utilize innate properties of cancer cells, external triggers like light illumination are more advantageous due to its superior controllability, as it allows the dose magnitude and timing to be controlled remotely. Thus, in recent years, polymers containing photochromic groups such as coumarin,⁵⁶ *o*-nitrobenzyl ester (ONB),²⁰¹ spiropyran²⁰² and azobenzene²⁰³ were introduced to coat the MSN to achieve the light triggered drug release. These designs greatly expanded the range of functional polymer coating in drug delivery systems, allowing for the development of more versatile stimuli-responsive MSN-based nanocarriers.

In order to meet various requirements under complicated physiological environment, two or more stimulus-responding mechanisms can be practically introduced into one drug delivery system.^{204,205} Notably, these dual and multi-stimuli responsive drug delivery systems possess the benefits belonging to different stimuli at the same time, which exhibit unprecedented control over drug delivery to the pathological sites and the subsequent release.^{114,206} For the controlled drug release, this multi-response design is not only capable of responding to each stimulus individually, but also exhibits enhanced drug release capability when triggered with multiple-stimuli simultaneously. For example, Zhang *et al.*²⁰⁷ utilized a pH and redox dual-responsive polymer to coat on the surface of MSNs. The as-prepared nanohybrids achieved pH/reduction triggered release on account of the acid hydrolyzable benzoic-imine bond and reduction cleavable disulfide linkage introduced by the polymer. Another example of nanohybrids with the same stimuli-responsiveness was developed by Wang *et al.*²⁰⁸ using disulfide cross-linked poly [(*N*-vinylcaprolactam)-*co*-

(methacrylic acid)] as the gate keeper to control the drug release of loaded cargoes inside the MSNs. In an another study, Liu and co-workers²⁰ investigated the release behaviors of the thermo and pH dual-responsive hybrid MSNs coated by sensitive polymer poly[(*N*-isopropylacrylamide)-*co*-(methacrylic acid)]. Furthermore, by an elaborate design, two kinds of cargoes could be trapped into the nanopores of MSN separately, each capped with a specific type of pseudorotaxanes which were either light responsive or acid responsive.²⁰⁹ The two different cargoes could be released selectively via the matching responsive mechanism. However, to the best of our knowledge, MSN-based nanocarriers with more than two responsive release pathways have not been reported. Considering the sophisticated blood circulation environment after the drug carrier entered the blood stream, the integration of multi-stimuli response for the more precise control of the drug release seems to be necessary and important.

Schematic illustration of triggered drug release of HMSNs-PDEAEMA in this study is shown in Scheme 1. The HMSNs were functionalized with ATRP initiating group with three stimuli-cleavable linkages. After the surface initiated ATRP of the pH-responsive monomer DEAEMA, the resulting PDEAEMA coated nanoparticles could retain the encapsulated drug inside in deionized water. Since PDEAEMA polymer chains would be in a collapsed state in neutral or alkaline environments,^{210,211} the polymer coating could effectively inhibit the drug diffusion out of the channels under these conditions. On the contrary, the protonated polymer chains would adopt an extended conformation in acidic condition, allowing the opening of mesopores and rapid release of loaded cargo. Thus, the coated PDEAEMA shell could easily control the drug release by changing the pH values of the suspension. In addition, both photolabile ONB linkage and reduction-cleavable

disulfide bond were used as the linkage to immobilize the pH-sensitive PDEAEMA on the surface of HMSNs. Disulfide bond, one of most widely used linkage for the construction of reduction-responsive nanocarriers, could be readily cleaved under reducing environment.^{134,128} Besides, ONB group is often used as photo-labile linkages. When exposed to UV light, the photolabile ONB group is isomerized rapidly, accompanied with the cleavage of the linkage with the enhanced drug realease.^{212,213}

Scheme 1. Schematic illustration of triggered drug release mechanisms of HMSNs-PDEAEMA in response to acidic environment, reducing agent or UV irradiation.

The distinctive advantages of this therapeutic system are: (1) triple-response to the stimuli of acid environment, reducing agents, as well as external UV irradiation independently; (2) excellent stability during the blood circulation compared to the pure polymeric micelles/vesicles drug delivery system;^{55,37} (3) higher DOX drug loading content owing to the huge hollow interiors of HMSNs, and (4) DOX could be simply loaded and retained inside the nanoparticles simply by changing pH value of the suspension under mild conditions, since the mesopores was reversibly opened and closed by pH responsive

PDEAEMA. Thus, this kind of nanocomposites may have the potential to be promising multifunctional platform for delivery vehicles for “on-demand” drug release.

2.2 Materials and methods

Toluene (Sigma-Aldrich) was dried with CaH_2 and re-distilled under reduced pressure prior to usage. 2-(diethylamino)ethyl methacrylate (DMAEMA, Sigma-Aldrich) was passed through a basic alumina column to remove the inhibitors prior to usage. Copper(I) bromide (CuBr , Sigma-Aldrich) was purified by stirring in acetic acid, washed with methanol, and then dried under vacuum. Azidoacetic acid, copper(II), sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), DTT, DOX HCl, ethylenediaminetetraacetic acid (EDTA), 3-mercaptopropyl-trimethoxysilane (MPTS), *N,N,N',N'',N'''*-pentamethyldiethylenetriamine (PMDETA), and sodium ascorbate were all purchased from Sigma-Aldrich and used without purification. Acetic acid, *N*-hydroxysuccinimide (NHS), and *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) were obtained from Alfa and used as received. The compound 5-propargylether-2-nitrobenzyl bromoisobutyrate and *S*-(2-aminoethylthio)-2-thiopyridine hydrochloride were synthesized according to procedures reported in literatures.^{214,136}

^1H nuclear magnetic resonance (NMR) spectrum was recorded on a Bruker BBFO-400 spectrometer using deuterated chloroform (CDCl_3) as the solvent. Transmission electron microscopy (TEM) images were obtained on JEM-1400 (JEOL) at 100 kV. Transmission electron microscopy (TEM) images were collected on JEM-1400 (JEOL) at 100 kV. Scanning electron microscope (SEM) images were collected on JSM-7100F (JEOL, Japan) operated at 100 kV. The fluorescence intensities were measured by fluorescence spectrophotometer (RF5301PC, Shimadzu, Japan). UV-vis spectra were recorded from

Shimadzu UV-3600 spectrophotometer. Zeta-potential values were determined by a Malvern Instruments Zetasizer Nano-S at 25 °C. X-Ray photoelectric spectroscopy (XPS) was carried out on a Phoibos 100 spectrometer and a monochromatic Mg X-ray radiation source (SPECS, Germany) was used for the wide-range and high resolution S 2p scans at 12.53 kV. The specific surface areas were calculated from the adsorption data in low pressure range using the Brunauer-Emmett-Teller (BET) model and pore size was determined following the Barret-Joyner-Halenda (BJH) method. Thermogravimetric analysis (TGA) was carried out for powder samples using a TGA Q500 recorded from 100 to 1000 °C in an air flow at a heating rate of 10 °C min⁻¹.

2.2.1 Preparation of *o*-nitrobenzyl linkage

The mixture of THF and water (v/v = 8:1, 30 mL) was used to dissolve azidoacetic acid (0.224g, 2.2 mmol) and 5-propargylether-2-nitrobenzyl bromoisobutyrate (0.710g, 2.0 mmol) at first. After stirring for a while, sodium ascorbate (0.079g, 0.20 mmol) and CuSO₄ 5H₂O (0.050g, 0.20 mmol) was added and the solution was further stirred at room temperature for 6 h. After the removal of the solvent THF and water under reduced pressure, the resulting crude product was purified by silica gel column chromatography (CH₂Cl₂:MeOH = 20:1) to give the product HOOC-NBO-*i*BuBr as a brown hard solid. Yield: 94%, mp 114-116 °C. ¹H NMR (CDCl₃, 400 MHz) δ 8.27 (s, 1H, triazole-*H*), 8.19 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 7.32 (s, 1H, Ar-*H*), 7.29 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 5.58 (s, 2H, -CH₂-OCO-), 5.36 (s, 2H, HOOC-CH₂-), 5.30 (s, 6H, triazole-CH₂-Ar), 1.94 (s, 6H, -CH₃). ¹³C NMR (400 MHz, CDCl₃): δ 170.3, 168.5, 162.2, 141.6, 140.1, 134.2, 128.0, 126.4, 114.8, 114.4, 64.2, 61.8, 57.0, 50.6, 30.2. TOF HRMS: Calcd. for C₁₆H₁₇BrN₄O₇ [M + H]⁺, 457.2370; found, 457.0359. IR (KBr) ν/cm⁻¹ 3171.3, 2934.3, 2497.7, 1736.8, 1617.9,

1578.7, 1514.5, 1374.2, 1339.0, 1323.5, 1104.5, 1233.5, 1048.9, 1152.9, 1085.8, 1070.4, 1010.3, 945.6, 870.4, 835.8.

2.2.2 Preparation of PDEAEMA coated HMSNs

First of all, CTAB was used as the surfactant template to prepare the core-shell nanoparticles with mesoporous silica filled with CTAB as the shell, according to the literature report. Then, the solid silica was selectively removed using Na_2CO_3 as the etching reagent.^{47,48} The obtained HMSNs@CTAB (0.8 g) were added into anhydrous toluene (40 mL) and nanoparticles were dispersed assisted by sonication. Then MPTS (0.6 mL) in 40 mL of anhydrous toluene were added and the mixture was stirred gently at 60 °C for 36 h. The resulting sulfhydryl modified HMSNs filled with CTAB in the mesopores were collected by centrifugation and were washed 4 times with absolute ethanol to remove the unreacted MPTS. In order to remove CTAB template inside mesopores, the above modified nanoparticles (0.6 g) were re-suspended in MeOH (150 mL), to which a concentrated hydrochloric acid solution (37%, 10 mL) was added, and the suspension was refluxed at 80 °C for 48 h. The HMSNs-SH with the removal of CTAB were collected by centrifugation, washed thoroughly with ethanol, and eventually dried under vacuum.

Second, the nanoparticles were functionalized with disulfide bond with terminal amino group. Basically, *S*-(2-aminoethylthio)-2-thiopyridine hydrochloride (0.5 g) was added in the suspension of HMSNs-SH (0.5 g) in acetic acid (1.2 mL) and ethanol (30 mL). The mixture was stirred for 48 h at room temperature and resultant disulfide-bond containing nanocarriers HMSNs-NH₂ were collected by centrifugation. After washing exclusively with ethanol, HMSNs-NH₂ were finally dried under vacuum at room temperature for subsequent use.

In the presence of NHS and EDC, HMSNs-NH₂ were modified with HOOC-NBO-*i*BuBr via an amidation reaction. Briefly, EDC (46.0 mg, 0.24 mmol) and NHS (27.6 mg, 0.24 mmol) were added to the stirring solution of HOOC-NBO-*i*BuBr (91.4 mg, 0.20 mmol) in anhydrous DMF (5.0 mL) at 0 °C, and the resulting turbid solution was stirred for 3 more hours at room temperature. After that, suspension of HMSNs-NH₂ (0.3 g) in DMF (30 mL) was added, and the mixture was stirred at room temperature overnight. The products were obtained through centrifugation and denoted as HMSNs-Br. The pellet was washed two times with DMF, four times with ethanol and then dried under vacuum at room temperature.

Briefly, HMSNs-Br (0.10 g) were dispersed into methanol (5 mL) in a Schlenk tube, then the PMDETA (87.0 mg, 0.50 mmol) and DEAEMA (1.38 g, 7.5 mmol) was added and the mixture which was degassed by three freeze–pump–thaw cycles. After that, CuBr (36 mg, 0.25 mmol) was introduced into the mixture under N₂ protection. After stirring at 70 °C for 24 h, the polymerization was stopped by exposing the reaction mixture to air. The PDEAEMA coated nanoparticles were centrifuged and washed 3 times with DMF to remove the ungrafted polymer or free reagents mixed in the product. Then, the residue was washed with 5% EDTA solution twice to remove to copper residue. After additional washing three times with ethanol, the final products were dried under vacuum at room temperature for 24 h and denoted as HMSNs-PDEAEMA.

2.2.3 Preparation of DOX loaded nanoparticles

Briefly, HMSNs-PDEAEMA (5.0 mg) were dispersed into 1.0 mL of deionized water under sonication. Then, DOX HCl (2.0 mg) was added to the above suspension and mixture was stirred for 24 h at room temperature in the dark. After that, DOX loaded nanoparticles were obtained by centrifugation and washed extensively with phosphate buffer (PB, pH 7.4,

10 mM). Finally, the nanoparticles were dried under vacuum and denoted as HMSNs@DOX. To determine the drug loading content, the amount of DOX in the collected supernatant was firstly determined by UV–Vis absorption at 485 nm, and the amount of loaded DOX was further calculated by subtracting amount in supernatant from the amount of original feed DOX.

2.2.4 Drug release behavior

The triggered release of conjugated DOX from HMSNs@DOX was investigated using the dialysis method. The release behavior was studied at 37 °C in response to different stimuli: (i) PBS (100 mM, pH 7.4), (ii) PBS (100 mM, pH 7.4) containing 10 mM DTT, (iii) acetate buffer solution (100 mM, pH 5.0), and (iv) acetate buffer solution (100 mM, pH 5.0) containing 10 mM DTT. Briefly, HMSNs@DOX suspension in PB (pH 7.4, 3.0 mL) was firstly placed into a dialysis bag (molecular weight cut-off (MWCO) = 12,000 Da) and dialyzed against PB medium (pH 7.4, 40 mL) in the dark at 37 °C. At the designed time intervals, release medium (4.0 mL) was taken out and its fluorescence emission intensity at 560 nm under the excitation wavelength of 480 nm was determined by the fluorescence spectrometer, while same amount (4.0 mL) of fresh buffer medium was added back to compensate the volume loss. The concentration of DOX presented in the dialysate was calculated. The releasing experiments were repeated three times independently, and the mean value was obtained as the final result. Besides, the UV triggered release of DOX was also studied in PB at pH 7.4 without the presence of DTT. At designated times, the HMSNs@DOX suspension was taken out and irradiated with UV light (365 nm, 20 mW/cm²) for 10 min, and the concentration of released DOX in dialysate was determined as described above.

2.2.5 Cellular uptake study

HeLa cells were cultured with Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), penicillin (100 U mL^{-1}) and streptomycin ($100 \mu\text{g mL}^{-1}$) under 5% CO_2 atmosphere at 37°C .

In the confocal laser scanning microscopy (CLSM) study, HeLa cells were seeded in a 6-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well. After culturing at 37°C and 5% CO_2 for 24 h, an appropriate amount of free DOX and HMSNs@DOX suspension with an equivalent DOX concentration of $5 \mu\text{g mL}^{-1}$ was added into the cell culture medium. After further incubation for 3 h, the cells were illuminated with UV light (365 nm, 20 mW) for 10 min and incubated for another 3 h. In addition, unirradiated cells were incubated for as a control. Then the culture medium was removed and the cells were washed three times with phosphate buffered saline (PBS). Subsequently, the cells on the slide were fixed with 4% formaldehyde at room temperature for 15 min and stained with 4',6-diamidino-2-phenylindole (DAPI, blue color) for 5 min. After washing three times with PBS, the cells were mounted for observation with CLSM.

The cellular uptake of the nanodrug was also evaluated by flow cytometry quantitatively. Briefly, HeLa cells were also seeded in a 6-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well. After culturing for 24 h, free DOX or HMSNs@DOX suspensions were transferred to the above medium, all of which the equivalent DOX concentrations were $5 \mu\text{g mL}^{-1}$ for every well. After incubation for 3 h, the cells were irradiated by UV light for 10 min and incubated for another 3 h. In addition, cells without UV irradiation were also incubated for comparison. Then the cells were washed with PBS for two times and treated with trypsin (0.5 mL) for 2 min. Subsequently, fresh medium (0.5

mL) was added to each culture well, and the cells were collected via centrifugation at 2000 rpm for 3 min. After one more round of washing with PBS (0.5 mL) and centrifugation, the re-suspended cells in PBS (1 mL) were subjected to the flow cytometry analysis.

2.2.6 Cytotoxicity study

The cytotoxicity of HMSNs-PDEAEMA, HMSNs@DOX and free DOX was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. Briefly, the cells were seeded in 96-well plates (100 μ L medium) and incubated overnight. When the cell confluence reached around 60-70%, the medium was replaced with fresh one (90 μ L). Then the free DOX solutions or HMSNs@DOX suspensions (10 μ L each) with various equivalent concentrations of DOX were added to the medium. The culture medium was replaced after 4 h of incubation, and the cells were irradiated with UV light (365 nm, 20 mW) for 10 min. Meanwhile, the cell groups without any treatment were used as controls. After following incubation for 20 h, 10 μ L of MTT solution (5 mg L⁻¹) was added in the medium. The cells were incubated for another 4 h, and then the medium was replaced by DMSO (150 μ L) to dissolve the resultant purple crystals. Finally, optical densities of the samples were measured using a microplate reader (infinite M200, TECAN) at 490 nm.

2.3 Results and discussion

2.3.1 Preparation of HMSNs-PDEAEMA.

The synthetic route of HMSNs-PDEAEMA was shown in Scheme 2. Firstly, for the preparation of bare HMSNs, dense silica was used as the core while mesoporous silica filled with cetyltrimethylammonium bromide (CTAB) was then coated on the solid silica as the shell. At last, the dense silica core was selectively removed by using Na₂CO₃ as etching

agent, while the mesoporous shell was well maintained with the protection of CTAB during the etching process. HMSNs with CTAB inside mesopores were functionalized with sulfhydryl group in the presence of a thiol-based silane coupling agent MPTS in toluene. The CTAB packed inside the mesopores could prompt silane coupling agent to selectively graft on the exterior surface of HMSNs rather than the internal surface of mesopore channels. Subsequently, by refluxing in methanol solution of hydrochloric acid, the template CTAB inside the mesopores was removed affording HMSNs-SH. Furthermore, HMSNs-NH₂ containing terminal amino group with disulfide bond linkage in between were obtained through the disproportionation reaction between sulfhydryl group on HMSNs-SH and pyridine group of *S*-(2-aminoethylthio)-2-thiopyridine. HMSNs-NH₂ containing terminal amino group with disulfide bond linkage in between were obtained through the disproportionation reaction between sulfhydryl group on HMSNs-SH and pyridine group of *S*-(2-aminoethylthio)-2-thiopyridine.

Scheme 2. Synthetic route of multifunctional HMSNs-PDEAEMA with reduction/UV cleavable linker and acid responsive polymer coating.

The morphology of HMSNs-NH₂ was characterized by SEM and TEM (Figure 1a,c,e). The spherical HMSNs with uniform hollow structure were clearly observed in the TEM images. The average diameter of the nanoparticles were determined to be around 150 nm,

while the average thickness of the mesoporous shell was about 20 nm. The mesopores were homogeneously arranged, endowing efficient channels for the diffusion of the loaded drug in or out.

Figure 1. SEM images of (a) HMSNs-NH₂ and (b) HMSNs-PDEAEMA. TEM images of (c,e) HMSNs-NH₂ and (d,f) HMSNs-PDEAEMA.

Furthermore, the ATRP initiator HOOC-NBO-*i*BuBr with terminal carboxylic group was functionalized onto the surface of HMSNs-NH₂ via an amidation reaction. Herein, the successful immobilization of initiating group was confirmed by XPS and FT-IR spectra. As shown in Figure 2, the *N* 1s peak intensity of HMSNs-Br in the XPS spectra increased a bit, confirming the ATRP initiating group has been successfully anchored. Compared with FT-IR spectrum of HMSNs-NH₂ (Figure 3a), new weak peaks were also observed around 1550 cm⁻¹ and 1730 cm⁻¹, due to the stretching vibration of N-H bending vibration of the formed secondary amine and the newly introduced ester carbonyl group.

Figure 2. XPS spectra of (a) HMSNs-NH₂, (b) HMSNs-Br and (c) HMSNs-PDEAEMA.

Figure 3. FT-IR spectra of (a) HMSNs-NH₂, (b) HMSNs-Br and (c) HMSNs-PDMAEMA.

Surface-initiated ATRP method was employed for the coating of the pH-sensitive polymer PDEAEMA onto the mesoporous shell. TEM images of HMSNs-PDEAEMA (Figure 1d,f) clearly show that the mesopore structure became blurry as compared with the precursor HMSNs-NH₂, on account of the successful polymer coating around the mesoporous shell. In addition, as exhibited in XPS spectrum (Figure 2), both C 1s and N

1s peaks of HMSNs-PDEAEMA (Figure 2c) increased significantly after the coating of the polymer PDMAEMA. Consistently, due to the stretching vibration of ester carbonyl group on the grafted PDEAEMA, FT-IR spectrum of HMSNs-PDEAEMA (Figure 3c) displays a strong absorption peak appeared at 1730 cm^{-1} . Besides, the increased absorption peak also appeared at 2953 cm^{-1} , ascribed to the stretching vibration of the C-H bond on the polymer coating.

Thermogravimetric analysis was employed to roughly assess the grafted amount of the coated PDEAEMA. The silica materials are stable at high temperature while organic functionalities would be decomposed, thus leading to the weight loss of the hybrid nanoparticles upon heating. The results of TGA analysis indicate that the weight losses of HMSNs-NH₂, HMSNs-Br and HMSNs-PDEAEMA were 16.6%, 18.3% and 35.3 % respectively, when the temperature reached 1000 °C. After modification with initiator, the weight loss of HMSNs-Br increased about 1.9% as compared with HMSNs-NH₂. By subtract the weight loss of HMSNs-Br from HMSNs-PDEAEMA, the grafting ratio of PDEAEMA was roughly determined to be 17.0 %. To further characterize the coated polymer structure on the surface of HMSNs, the silica nanoparticles were etched by HF solution, and the cleaved polymer was purified by dialysis against deionized water. As exhibited in Figure 4, all the characteristic proton signals of the coated PDEAEMA polymer could be found in the ¹H NMR spectrum, confirming the successful grafting of PDEAEMA on the HMSNs.

Figure 4. ^1H NMR spectrum of grafted polymer obtained from HMSNs-PDEAEMA in CDCl_3 after etching out silica content with HF solution.

Before the removal of the surfactant CTAB packed inside mesopores, HMSNs- NH_2 were functionalized with the ATRP initiating group. Thus, the surface-initiated ATRP of DEAEMA should only take place on the exterior surface of mesoporous silica shell, due to the protection of CTAB.²¹⁵ Therefore, the porosity of resulted HMSNs would not be compromised upon PDEAEMA coating. The availability of the pore structure of the synthesized nanoparticles was further investigated by the nitrogen adsorption/desorption isotherms. As shown in Figure 5, all the isotherms of the samples exhibited characteristic type *IV* N_2 adsorption/desorption patterns, indicating the presence of mesopore channels in the nanoparticles. The specific surface area and pore volume of HMSNs- NH_2 were calculated to be $1571 \text{ m}^2 \text{ g}^{-1}$ and 1.208 mL g^{-1} respectively (Table 1), with a uniform pore size distribution of about 4.11 nm in diameter. After the modification with the initiator, the specific surface area ($1495 \text{ m}^2 \text{ g}^{-1}$) and pore volume (1.189 mL g^{-1}) of HMSNs-Br decreased slightly. After surface initiated polymerization, the specific surface area of HMSNs-PDEAEMA decreased dramatically to $433 \text{ m}^2 \text{ g}^{-1}$, while the pore volume dropped to 0.599 mL g^{-1} , which are consistent to the pore changes reported in literature.⁹⁶ The pore size was

determined to be 3.92 nm after coating, indicating the grafted PDEAEMA polymers only grafted on the outer surface of the nanoparticles without interfering with the porosity of the mesopores.

Figure 5. N₂ adsorption/desorption isotherms and corresponding pore size distributions (inset) of (a) HMSNs-NH₂, (b) HMSNs-Br and (c) HMSNs-PDEAEMA.

Table 1. The structure parameters of HMSNs-NH₂, HMSNs-Br, and HMSNs-PDEAEMA determined by N₂ adsorption–desorption isotherm.

Samples	BET surface area (m ² g ⁻¹)	Pore volume (mL g ⁻¹)	Peak pore diameter (nm)
HMSNs-NH ₂	1571	1.208	4.11
HMSNs-Br	1495	1.189	4.08
HMSNs-PDEAEMA	433	0.599	3.92

Zeta-potential values (ζ) of HMSNs-PDEAEMA were also evaluated at different pH values. When the pH value of the HMSNs-PDEAEMA suspensions increased, the ζ decreased continuously as displayed in Figure 6. This is because the grafted PDEAEMA was partially or completely protonated under acidic conditions, resulting in a positive charge state. However, when the nanoparticles were transferred to a neutral or alkaline medium, the grafted PDEAEMA was deprotonated and became negatively charged. The

critical pH for the electrostatic equilibrium of HMSNs-PDEAEMA was determined at about 7.2, which means that PDEAEMA should be deprotonated at pH value of blood circulation (pH 7.4). Based on this observation, the immobilized PDEAEMA could serve as smart gatekeeper to control drug loading and release behavior simply by changing pH value of the suspensions.

Figure 6. Apparent zeta-potential values of HMSNs-PDEAEMA at different pH values.

2.3.2 Loading and triggered release of DOX

At acidic conditions, positively charged PDEAEMA polymer would become hydrophilic on account of the protonation of tertiary amine, so that the coated PDEAEMA polymer would tend to stretch into water, resulting in the opening of mesopores for the DOX loading. When the pH value was adjusted to be 7.4, PDEAEMA polymer chains were protonated and returned to being hydrophobic. Thus, in this case, PDEAEMA aggregated on the surface of HMSNs to block the mesopores, thus inhibiting the release of loaded DOX. The reversible pH-responsive behavior of PDEAEMA allows for easy DOX loading and release simply by changing the pH values of the solution. HMSNs-PDEAEMA could trap DOX

effectively, since the DOX loading content of HMSNs-PDEAEMA was $20.5 \pm 3.0\%$ ($n = 3$), which is much higher than that of conventional MSNs (less than 10%).

Figure 7. Stimuli-triggered release of DOX from HMSNs@DOX at 37 °C at different pH values without and with DTT: (a) pH 7.4, (b) pH 7.4 and 10 mM DTT, (c) pH 5.0, and (d) pH 5.0 and 10 mM DTT.

The triggered release of DOX from HMSNs@DOX was investigated. As shown in Figure 7, less than 12% of encapsulated DOX was released from HMSNs@DOX after the continuous incubation at pH 7.4 for 22 h. It indicates that the grafted PDEAEMA polymer coating exhibited good blocking capability to hold DOX inside. However, when HMSNs@DOX were transferred to the medium at pH 5.0, approximately 64% of the loaded DOX was released after continuous incubation for the same period of time. This result indicates that grafted PDEAEMA was protonated in the acidic environment, resulting in the opening of the mesopores for drug diffusing out. Besides, the release of DOX at pH 7.4 could be accelerated following the addition of DTT through the reduction-induced cleavage of disulfide bond, since 52% of loaded DOX was released in 22 h. Furthermore, the synergistic effect under the two stimuli for intracellular drug release was also observed.

After the treatment with a medium at pH 5.0 together with 10 mM DTT, it exhibited the fastest and most complete release of loaded DOX, confirming that HMSNs@DOX could achieve triggered drug release under acid and/or reductive environment.

Considering the UV-cleavable nature of NBO linkage in between HMSNs and the coated polymer, the HMSNs@DOX were subjected to intermittent treatment with UV irradiation. As shown in Figure 8, only a slight drug release was realized over time without UV irradiation, indicating that the DOX was efficiently retained inside the nanoparticles. After the first round of UV irradiation begun at the 4th hour for 10 min, the cumulative release of DOX was achieved in the enhanced manner. The same trend was again observed when the UV irradiation was repeated at the 10th hour. These can be explained that ONB linkage was quickly cleaved under UV irradiation, leading to detachment of the polymer and enhanced drug release.

HMSNs@DOX are expected to be stable without significant drug leakage during the blood circulation. When the nanoparticles reached the pathological sites or the tumor cells, the loaded DOX would be released quickly for the chemotherapy under weakly acidic and reducing environment. In addition, the UV irradiation could also be employed to boost the drug release inside cancerous cells. In all, these results clearly indicate that HMSNs@DOX show a great potential for triple-responsive delivery of anticancer drug.

Figure 8. Triggered DOX release from HMSNs@DOX at pH 7.4 under UV irradiation. The UV light was irradiated at the points marked by arrows for 10 min.

Figure 9. CLSM images of HeLa cells incubated with (a) free DOX for 3 h, (b) HMSNs@DOX for 6 h, and (c) HMSNs@DOX for 3 h, followed by 10 min UV illumination and further incubation for another 3 h. The scale bar refers to 20 μm. For each panel, images from left to right show the HeLa cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlay of both images.

2.3.3 Cellular uptake and intracellular release

Taking advantage of the intrinsic red fluorescence of DOX, the cellular uptake of HMSNs@DOX nanohybrids and intracellular release of DOX in HeLa cells were evaluated by CLSM and flow cytometry. As observed from Figure 9, almost all of the red fluorescence from DOX was observed in the cytoplasm and nucleic regions after 4 h incubation with free DOX. This result reveals that free DOX could enter into HeLa cells rapidly by passive diffusion process and then strongly interact with DNA inside the nuclei. However, when the cells were incubated with HMSNs@DOX for 6 h, the DOX fluorescence accumulated mostly in the perinuclei regions and partly within the nuclei, indicating the fast internalization of HMSNs@DOX and a partial intracellular release of encapsulated DOX. In addition, when the cells were exposed to UV irradiation, the stronger DOX fluorescence was observed in the nuclei for the same incubating time.

Figure 10. Flow cytometric analysis of the DOX fluorescence intensity in HeLa cells incubated with (a) HMSNs@DOX for 6 h, (b) HMSNs@DOX for 3 h, followed by 10 min UV illumination and further incubation for another 3 h, and (c) free DOX for 6 h incubation.

In addition, the quantitatively intracellular DOX release from endocytosed HMSNs@DOX illuminated with UV light was also evaluated by cytometry studies. As compared to the cells without UV irradiation, HeLa cells incubated with HMSNs@DOX under UV irradiation showed the increased intracellular DOX fluorescence intensity (Figure 10). It was documented that the fluorescence of DOX may be self-quenched when encapsulated in the nanoparticles,^{216,217} thus the released DOX tends to exhibit enhanced fluorescence intensity when compared with its encapsulated state. Therefore, the increased DOX fluorescence intensity under UV irradiation was attributed to the triggered release of DOX molecules from the nanoparticles, which is in line with the intracellular release behavior of DOX confirmed by CLSM.

2.3.4 In vitro antitumor study

MTT assay has been used to evaluate the in vitro cytotoxicity of HMSNs-PDEAEMA against HeLa cells. As observed in Figure 11a, it can be seen that the eventual average viabilities of HeLa cells were still above 80% up to a high concentration of nanocarriers applied (0.2 mg mL^{-1}). Thus, HMSNs-PDEAEMA nanocarriers exhibited negligible cytotoxicity, verifying the biocompatibility of the drug nanocarriers for drug delivery application. Besides, the effect of UV irradiation along with the treatment of HMSNs-PDEAEMA has also been studied. As compared with the cell group with UV irradiation, the cell viability of irradiated group did not show obvious change, indicating the cellular growth of HeLa cells was not affected by UV light irradiation as well as light-cleaved byproducts.

Figure 11. (a) Relative viabilities of HeLa cells after being incubated with various concentrations of HMSNs-PDEAEMA nanocarriers without or with UV irradiation against HeLa cells following 24 h incubation. (b) Relative viabilities of HeLa cells after incubation with different concentrations of free DOX and HMSNs@DOX with or without UV irradiation following 24 h incubation.

Moreover, the anticancer activity of HMSNs@DOX with or without UV irradiation against HeLa cells was investigated, while the free DOX was used as the control. Following 24 h incubation, free DOX showed the highest anticancer activity among all the three groups (Figure 11b), which was consistent with the CLSM observation. It could be explained that small molecule DOX quickly diffused into the nucleus and directly interacted with DNA, inhibiting the cell growth in the treatment of early stage. When the cells were treated with HMSNs@DOX, the cell viabilities with or without UV irradiation did not show any significant difference when with equivalent DOX concentrations were

below $2\ \mu\text{g mL}^{-1}$. However, UV irradiation greatly enhanced the cytotoxicity of HMSNs@DOX against HeLa cells at higher concentrations. It is because when the concentrations of HMSNs@DOX were not high, the absolute difference in the concentration of released DOX with or without UV irradiation was almost negligible due to the limited amount of DOX released. Thus, UV irradiation could not confer significant change to the cell viability.

2.4 Conclusion

In summary, the triple-responsive HMSNs coated with PDEAEMA polymer have been prepared in this study. Anticancer drug could be retained inside HMSNs-PDEAEMA easily with a high loading capacity, and the obtained nanoparticles exhibited triple-stimuli-responsive drug release capabilities. The cellular uptake experiment indicated that the loaded drug can be efficiently released into the cytoplasm in acid/reduction intracellular environment after the endocytosis of nanoparticles inside the tumor cells. Meanwhile, upon external UV illumination, the drug release could be significantly enhanced. Besides, the improved therapeutic efficacy under UV irradiation was also confirmed by the in vitro cytotoxicity assay. This triple-responsive nanosystem holds great application potential for controlling the release behaviors in the complicated blood circulation and pathological environment. There is no doubt that this strategy will enrich the design and preparation of multi-stimuli responsive drug delivery platforms in accordance with the practical and diverse requirements.

Chapter 3: Polymeric Prodrug Grafted Hollow Mesoporous Silica Nanoparticles Encapsulating NIR-Absorbing Dye for Potent Combined Photothermal-Chemotherapy

3.1 Introduction

In the field of chemotherapy research, using MSNs as the carriers for controlled drug release is a hot topic.⁶³ Various gated MSNs have been developed, in which the drugs can be retained within the mesopore channels under physiological conditions, and quickly released upon the exposure to specific stimuli such as pH change, temperature variation, treatment of reducing agents, and light irradiation. Despite numerous conventional delivery methods of physical encapsulating the drugs inside, there is also an alternative tactic, which is to couple drugs with MSNs through biologically cleavable covalent bonds.²¹⁸⁻²²⁰ The covalent conjugation of drug with MSNs could minimize the undesired leakage and reduce side effects during the blood circulation. To date, there were only limited reports showing anticancer drugs were directly anchored onto the surface of MSNs, including DOX,^{221,222} cisplatin derivatives,²²³ methotrexate^{185,224} and chlorambucil.²²⁵ However, this strategy suffers from low drug coupling efficiency because of limited specific surface area of MSNs and steric hindrance between the drug molecules attached.

Among many capping agents, coating MSNs with polymers is of special significance, since the grafted polymers can provide tailored properties including better biocompatibility, stability, multi-functionality and the release control of the loaded cargoes to meet various requirements.^{84,88,226} As compared to other strategies, the introduced polymers offer far

more surface functional groups for the drug conjugation on account of the repeated units in the polymer chain. This design may thus enhance the drug loading capacity along with improved therapeutic efficacy of MSN-based prodrugs. As the polymers only cover the surface of MSNs with availability of mesopore channels, such nanocomposites also allows for encapsulation of a second therapeutic agent into mesopores. To our best knowledge, no anticancer drugs have yet been reported to conjugate to the polymer modified MSNs.

Current studies show increasing evidence that chemotherapy alone is not ideal for successful anticancer treatment due to factors such as the development of drug resistance, severe toxicity to normal cells and individual therapy differences in patients.³ To overcome these issues with conventional chemotherapy, combination therapy has been put forward and proven to be a promising strategy. Photothermal therapy is one of the commonly used anticancer treatments that have been easily employed in combination with chemotherapy, which utilizes photo absorbing agents to convert the absorbed light energy into heat, which leads to prompt cell death and subsequent ablation of tumor tissue.^{162,227} With the rapid development of nanoscience, various NIR absorbing inorganic nanomaterials such as gold nanorods,²²⁸ nanographene,²²⁹ carbon nanotubes,²³⁰ and copper sulfide nanoparticles²³¹ have been prepared and exploited as photothermal agents. Compared with the inorganic materials, NIR absorbing organic dyes are more promising for clinical translation, due to their reduced long-term toxicity and excellent biodegradability. Thus, NIR absorbing organic dyes, including ICG^{181,183}, cypate,¹⁸⁴ IR780^{185,187} and IR825,^{190,232} have already been used to achieve encouraging photothermal therapeutic outcomes. Due to their limited aqueous solubility and stability, these organic dyes are commonly encapsulated in

amphiphilic polymer micelles or liposomes to improve the therapeutic efficiency. However, poor colloidal stability and relatively low loading percentage of these self-assembled nanocarriers result in a limitation for further utilization of photothermal dyes. On the other hand, no study has reported the encapsulation of the photothermal dyes within stable nanocarriers, specifically the MSNs.

The development of novel drug delivery systems with remarkable circulation stability, high loading efficiency, favorable biocompatibility as well as multiple functionalities for combination therapy may be of great importance for future clinical use.⁹⁹ Based on this concept, a multifunctional therapeutic nanoplatform based on hollow MSNs (HMSNs) was successfully developed for both photothermal therapy and chemotherapy (Scheme 1). After the grafting of the DOX-conjugated polymer onto HMSNs, IR825, a newly-developed NIR absorbing dye with excellent photothermal conversion efficiency and high photostability, was encapsulated in the HMSNs to fulfill the combination therapy. In this work, HMSNs are not only used to accommodate the therapeutic agents,^{82,233-235} but also employed as an effective support for the conjugation of different kinds of therapeutic agents on the surface. There are several important features associated with this therapeutic nanoplatform. (1) In contrast with conventional MSNs, the loading capacity of HMSNs is rather high, on account of the central hollow cavities used for extra cargo storage. (2) Unlike previously reported MSN-based prodrug systems where the drug molecules were immobilized onto the surface of MSNs directly, the drug molecules were eventually integrated in the polymer shell coated on HMSNs to improve the drug conjugation amount in this study. Acid-labile hydrazone bond was used to connect DOX with the coating polymer, and the triggered

cleavage of the hydrazone linkage under intracellular acidic conditions would readily initiate the controllable release of the DOX. (3) Organic photothermal dye IR825 was loaded inside HMSNs with good dispersity and stability, which could be used for subsequent photothermal anticancer treatments *in vitro*. By using simultaneous combined photothermal-chemotherapy, an improved therapeutic efficiency was achieved with enhanced cancer treatment.

Scheme 1. Schematic illustration for polymeric prodrug coated HMSNs encapsulating NIR absorbing dye for potent combined photothermal-chemotherapy.

3.2 Materials and methods

The acquisition and purification of OEGMA has been described in Chapter 2. 3-Aminopropyltriethoxysilane (APTS), azobisisobutyronitrile (AIBN), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (DMPA), DOX HCl and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich and used without purification. *N,N'*-Diisopropylcarbodiimide (DIC), and 4-(dimethylamino)pyridine

(DMAP) and diisopropylethylamine (DIPEA) were obtained from Alfa and used as received. All solvents were purchased from Sigma and used directly. Methacrylamide *tert*-butyl carbazate (MABH) was prepared according to literature.²³⁶

The models of instruments in this research were described in detail in chapter 2.

3.2.1 Synthesis of HMSNs-RAFT

HMSNs filled with CTAB inside mesopores were prepared according to the literature.^{47,136} For the amino group functionalization, HMSNs (1.00 g) containing CTAB were redispersed in 100 mL of anhydrous toluene in the presence of 10 mL of APTS, and the suspension was stirred gently at 80 °C for 24 h. Then, the unconjugated APTS were removed by the extensively washing with ethanol. To remove the CTAB surfactant inside the mesopore channels, the resultant hollow nanoparticles (0.8 g) were dispersed in methanol (200 mL), to which a concentrated hydrochloric acid (37%, 16 mL) was added, and the suspension was refluxed at 80 °C for 48 h. CTAB-removed HMSNs-NH₂ were finally obtained by centrifugation, washed thoroughly with methanol, and then dried under vacuum for future use.

RAFT functionalities should be immobilized on surface of HMSNs before polymerization. Typically, the RAFT agent DMPA (0.5 g, 1.38 mmol), DIPEA (0.360 mL, 2.76 mmol) and DIC (0.320 mL, 2.76 mmol) were added into CH₂Cl₂ (3 mL) at 0 °C. The resulting solution was stirred gently for 3 h, and subsequently HMSNs-NH₂ (0.50 g) suspension in DMF (5.0 mL) was added dropwise. After 48 h of reaction at room temperature in the dark, the resultant nanoparticles HMSNs-RAFT were collected through centrifugation, washed thoroughly with methanol and then dried under vacuum.

3.2.2 HMSNs-DOX prodrug

Briefly, HMSNs-RAFT (0.10 g) were redispersed in dioxane (3 mL) with sonication and the formed suspension was then added with OEGMA (0.3 mL), MABH (0.5 g, 2.5 mmol) as well as the initiator AIBN (1.60 mg, 0.010 mmol). The mixture was degassed by freeze-pump-thaw for three cycles. After 48 h stirring at 70 °C under nitrogen atmosphere, the polymerization was stopped upon air exposure. The polymer coated nanoparticles were then centrifuged and washed 4 times with THF to remove the unreacted reagents or ungrafted polymer. After being washed with ethanol for additional 2 times, the product was dried under vacuum and denoted as HMSNs-Polymer.

To remove the protection group of BOC, TFA (3.0 mL) was transferred to the suspension of HMSNs-Polymer (0.1 g) in DCM (3.0 mL). After stirring at room temperature for 3 h, the nanoparticles were collected by centrifugation, washed extensively with methanol, dried under vacuum and denoted as HMSNs-NHNH₂.

DOX HCl (20 mg) was added to anhydrous methanol suspension (6 mL) of HMSNs-NHNH₂ (50 mg) at room temperature. The mixture was added with TFA (2 µL), which was stirred for 48 h in the darkness at room temperature. The resultant prodrug was isolated by centrifugation and washed thoroughly with methanol to remove unconjugated DOX. After drying under vacuum, the product was obtained and denoted as HMSNs-DOX.

To evaluate the DOX conjugation efficiency of the prodrug, HMSNs-DOX (2.0 mg) were treated with hydrochloride acid (1.0 M) at 25 °C for 24 h, and the supernatant was collected after centrifugation. The remaining nanoagents were repeatedly washed with deionized water until the supernatant was nearly colorless. The collected supernatant solution was

combined and the total amount of the hydrolyzed DOX was determined by UV–Vis spectrometer.

3.2.3 Preparation of HMSNs-DOX/IR825

The loading of photothermal organic dye IR825 inside HMSNs-DOX prodrug was achieved through the procedures introduced as follows. Typically, HMSNs-DOX (50 mg) and IR825 (30 mg) were added into methanol (0.6 mL), and the mixture was stirred in the dark overnight at room temperature. The nanoparticles were obtained by centrifugation, washed extensively with 10% (v/v) methanol solution, dried under vacuum and denoted as HMSNs-DOX/IR825.

To determine the loading capacity of IR825 in the therapeutic nanosystem, HMSNs-DOX/IR825 (2.0 mg) were washed with methanol by repeated centrifugation/re-dispersion cycles until the supernatant became colorless. The upper supernatants were combined together and the amount of extracted IR825 was determined by using a UV–Vis–NIR spectrometer at a wavelength of 825 nm.

3.2.4 Drug release, dye loading and photothermal effect

The acid-triggered release of conjugated DOX from prodrug was evaluated when exposed to buffer medium at pH 5.0 (acetate), pH 6.0 (phosphate) and pH 7.4 (phosphate). Briefly, HMSNs-DOX suspensions in PB (2 mL, 0.01M) at pH 7.4 were firstly placed into dialysis bags (molecular weight cut-off (MWCO) = 3,500 Da), and dialyzed against buffer medium (40 mL, 0.05M) at the different pH values of 5.0, 6.0 and 7.0 at 37 °C in the dark. At the preset time intervals, a small portion of medium (2.0 mL) was taken out, and the intensity of its fluorescence emission at 560 nm under the excitation wavelength of 490 nm

was monitored using the fluorescence spectrometer, while the same amount of fresh buffer solutions were added back to compensate the volume loss. The concentration of DOX presented in the dialysate was calculated. After conducting the release experiments for three independent times, the mean value was obtained as the final result.

3.2.5 Cellular uptake study

CLSM was further employed to observe the intracellular release behavior of HMSNs-DOX/IR825 within DOX-sensitive HeLa cells and DOX-resistant A2780/DOX^R cells. HeLa cells were incubated in DMEM, while A2780/DOX^R cells were cultured with RPMI 1640. Cells were seeded in a 6-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well. After culturing at 37 °C in 5% CO₂ an atmosphere of 5% CO₂ for 24 h, an appropriate amount of HMSNs-DOX/IR825 suspension with an equivalent DOX concentration ($5 \mu\text{g mL}^{-1}$) was added into the cell culture medium. After further incubation for 4, 12 and 24 h respectively, the culture medium was removed and the cells on microscope plates were washed three times with PBS. In addition, cells treated with free DOX were incubated for 4 h as a control. Finally, the samples were stained with DAPI (blue color) for 5 min and mounted for observation with CLSM.

In flow cytometry study, HeLa cells were seeded in a 6-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well. After culturing for 24 h, free DOX or HMSNs-DOX/IR825 were added in the culture medium, all of which were maintained at the equivalent DOX concentration of $1 \mu\text{g mL}^{-1}$ for every well. After incubation for 1, 3 or 24 h, the cells were washed with PBS for several times and treated with trypsin (0.5 mL) for 2 min. Then, fresh medium (0.5 mL) was added to each culture well, and the cells were

collected via centrifugation at 2000 rpm for 3 min. After one more round of washing with PBS through centrifugation, the cells were re-suspended in PBS (1 mL), and subjected to the flow cytometry analysis.

3.2.6 In vitro cytotoxicity study

The antitumor efficacy of HMSNs-DOX/IR825 on the proliferations of HeLa cells and A2780/DOX^R cells with or without NIR irradiation was quantitatively investigated by using MTT assay. Briefly, the cells were seeded in 96-well plates (100 μ L medium) and incubated. When the cell confluence reached around 60-70%, the medium was replaced with fresh one (90 μ L), and then HMSNs-DOX/IR825 suspensions (10 μ L each) with various concentrations were added. After 4 h of incubation, the cultural medium was replaced and the cells were illuminated by 808 nm laser at certain power densities for 5 min. After incubation for 24 h, the medium was again replaced with fresh one, followed by the addition of MTT solution (10 μ L, 5 mg L⁻¹). The cells were incubated for another 4 h, and then the media were replaced by DMSO (150 μ L) to dissolve the resultant purple crystals. Finally, optical densities of the samples were measured using a microplate reader (infinite M200, TECAN) at 490 nm.

3.3 Results and discussion

3.3.1 Synthesis and characterization of HMSNs-DOX

The synthetic procedure of polymeric prodrug coated nanocarriers HMSNs-DOX is shown in Scheme 2. Initially, highly dispersed HMSNs filled with CTAB in the mesopores were prepared, and then functionalized with amino groups by silylation with amino silane coupling agent in anhydrous toluene. Subsequently, CTAB was removed by heating the

nanoparticles in a mixture of methanol and hydrochloric acid under reflux. The morphology of resulting surfactant-free HMSNs-NH₂ was characterized by TEM shown in Figure 1a, and uniform hollow structure and well-defined mesoporous shell with an average thickness of 20 nm were displayed. The average size of the nanoparticles was observed to be around 120 nm, which was close to the results determined by DLS (Figure 1c). The mesopores were homogeneously arranged, which could serve as efficient channels for dyes diffusion and loading.

Scheme 2. Synthetic route of HMSNs-DOX/IR825.

Figure 1. (a,b) TEM images of (a) HMSNs-NH₂ and (b) HMSNs-DOX. (c) Size distributions of HMSNs-NH₂ and HMSNs-DOX in PB (0.01 M, pH = 7.4) determined by DLS.

Prior to the coating of polymer on the nanoparticles by surface-initiated RAFT polymerization, RAFT agent should be first anchored on the surface of HMSNs.⁹⁸ After the attachment of RAFT agent by amidation to form HMSNs-RAFT, a new peak of HMSNs-RAFT around 1535 cm⁻¹ appeared in the FT-IR spectra (Figure 2a), ascribing to the N-H bending vibration of the formed secondary amine on HMSNs-RAFT. Consistently, the increased absorption peak intensities at 2926 and 2855 cm⁻¹ were also observed, which could be explained by the stretching vibration of additional C-H from the introduced RAFT agent. In addition, it was observed that the zeta potential at pH 7.4 decreased significantly (Figure 2b), suggesting that some of the amino groups were consumed during the reaction. Besides, XPS spectra were employed to verify the success in modification step. As compared with XPS spectrum of HMSNs-NH₂ (Figure 3a), the S 2s peak of HMSNs-RAFT appeared, confirming the attachment of RAFT agent.

Figure 2. (a) FT-IR spectra of HMSNs-NH₂, HMSNs-RAFT, HMSNs-Polymer and HMSNs-NHNH₂. (b) Zeta-potential measurements of HMSNs-NH₂, HMSNs-RAFT, HMSNs-NHNH₂ and HMSNs-DOX dispersed in PB (pH 7.4, 10 mM) for the measurements. (c) UV-Vis-NIR absorption spectra of HMSNs-NHNH₂, HMSNs-DOX and HMSNs-DOX/IR825 dispersed in PB (0.01M, pH = 7.4). Besides, the absorption spectra of free DOX (in DI water) and free IR825 (in methanol) were presented as the contrast. (d) TGA curves of HMSNs-NH₂, HMSNs-RAFT, HMSNs-NHNH₂ and HMSNs-DOX. (e) N₂ adsorption-desorption isotherms and corresponding BJH pore size distribution (inset) of HMSNs-NH₂, HMSNs-RAFT, HMSNs-NHNH₂ and HMSNs-DOX.

Figure 3. (a) XPS spectra of HMSNs-NH₂, HMSNs-RAFT, HMSNs-NHNH₂ and HMSNs-DOX. (b) ¹H NMR spectrum of grafted polymer on HMSNs-NHNH₂ in DMSO-*d*₆ after etching out the silica nanoparticles with HF solution.

Two monomers were chosen for the RAFT copolymerization on the surface of HMSNs. One was the macromonomer OEGMA, of which the hydrophilic oligo(ethylene glycol) side chain could improve the water dispersibility and biocompatibility of polymer-coated nanocarriers, whereas the other monomer methacrylamide *tert*-butyl carbazate can provide the acylhydrazine groups for further DOX conjugation after the deprotection. As shown in the FT-IR spectrum of polymer-coated nanocarriers HMSNs-Polymer (Figure 2a), the appearance of the strong absorption peak around 1722 cm^{-1} was assigned to the C=O stretching vibration from the BOC protection group, indicating the formation of the grafted polymer shell.

After the as-prepared polymer coated HMSNs were treated with trifluoroacetic acid in dichloromethane solution, it was observed that the stretching vibration peak of C=O at 1720 cm^{-1} disappeared, which could be explained by the fact that the BOC protection group was cleaved and thus the HMSNs-NHNH₂ were formed. Consistently, after the deprotection of BOC group, the zeta potential of HMSNs-NHNH₂ increased significantly (Figure 2b), indicating the formation of positively charged hydrazide. To further confirm chemical composition of the copolymer shell immobilized on the HMSNs, the silica content of the nanocomposites was etched by hydrofluoric acid and the typical proton signals of both hydrazide group and oligo(ethylene glycol) chain could be found in the ¹H NMR spectrum of the extracted polymer (Figure 3b), again verifying the successful immobilization of the polymer on the surface of HMSNs.

DOX, one of the most widely used chemotherapeutic anticancer drugs, contains the carbonyl group, which allows for easy conjugation of this drug molecule with other

substrates through hydrazone linkage.²³⁷ In this step, the deprotected HMSNs-NHNH₂ were treated with free DOX in anhydrous methanol in the presence of trace trifluoroacetic acid. Initially, the methanol suspension of HMSNs-NHNH₂ was opaque white in color. After conjugation of DOX, it turned light red after the conjugation of DOX. Consistently, the zeta potential increased from -14 mV of HMSNs-NHNH₂ to 13 mV of HMSNs-DOX after the conjugation. In addition, the presence of conjugated DOX was further confirmed by UV-Vis-NIR absorption spectrum. As compared with the absorption of HMSNs-NHNH₂ shown in Figure 2c, HMSNs-DOX had a wide absorption peak in the range of 600-400 nm, assigning to the conjugated DOX.

As it is well known that the silica structure is stable and could endure high temperature, while the organic component would decompose thermally to result in the weight loss of sample. TGA was used to study the relative ratio of different components in the nanocomposites. The results shown in Figure 2d reveal that the weight losses of HMSNs-NH₂, HMSNs-RAFT, HMSNs-NHNH₂ and HMSNs-DOX were 14.1, 24.3, 32.6, and 40.0% respectively, when the temperature increased eventually to 1000 °C. The upward trend of weight loss could be explained by the increasing content of organic component in the nanocomposites as the modification proceeded step by step. All the characterizations above verify the successful grafting of polymeric drug on HMSNs.

Since the introduction of amino groups was taken place before the removal of the surfactant filled in the mesopores, the grafted polymer prepared via RAFT polymerization should not occupy the mesopore channels and thus would not compromise the drug loading and release.^{95,215} To investigate the mesopore structure parameters, the nitrogen

adsorption/desorption measurements were performed and the results are summarized in Figure 2e. As shown in Figure 2e, the isotherms of the samples afforded characteristic type *IV* adsorption/desorption patterns, indicating the presence of cylindrical channel-like mesopores in the nanoparticles. After the grafting of DOX conjugated polymer via multi-step modifications, pore volume of nanoparticles decreased from $0.917 \text{ cm}^3 \text{ g}^{-1}$ to $0.490 \text{ cm}^3 \text{ g}^{-1}$ (Table 1), while the surface area dropped from $1004 \text{ m}^2 \text{ g}^{-1}$ to $305 \text{ m}^2 \text{ g}^{-1}$, suggesting that the mesopore channel entrances were partially blocked by the grafted polymeric DOX prodrug during the drying process before measurement. On the other hand, the relevant pore size did not changed obviously after the surface modifications, confirming that only negligible amount of the polymer was attached onto the internal surface of mesopore channels. Therefore, the as-prepared HMSNs-DOX with the vacant mesoporous nanostructure are capable of subsequent loading of photothermal agents.

Table 1. Structure parameters of HMSNs-NH₂, HMSNs-RAFT, HMSNs-NHNH₂ and HMSNs-DOX.

Samples	BET surface area ($\text{m}^2 \text{ g}^{-1}$)	Pore volume (mL g^{-1})	Peak pore diameter (nm)
HMSNs-NH ₂	1267	0.917	3.99
HMSNs-RAFT	756	0.628	3.99
HMSNs-NHNH ₂	657	0.595	3.92
HMSNs-DOX	514	0.490	3.91

3.3.2 Drug release, dye loading and photothermal effects

Acid-cleavable hydrazone bond was used to connect the drug DOX with grafted polymer. DOX released quickly through cleavage of hydrazone bond under endo/lysosomal acid environment. The DOX conjugation capacity was determined by UV–Vis–NIR absorption measurements after the treatment with 1.0 M HCl. The result reveals the DOX conjugation

content of HMSNs-DOX with polymeric prodrug coating was 12 wt%, higher than conventional direct-attached MSN prodrugs where the DOX conjugation amount was less than 5%. The increased drug conjugation content could improve the therapeutic efficacy as well as reduce the side effects, since the high content of drug could scale down to the necessary amount of therapeutic nanoagents used for the treatment.

The acid-triggered release of DOX from the HMSNs-DOX prodrug in vitro was investigated at 37 °C in buffer media at different pH values. As shown in Figure 4a, only 18% of conjugated DOX was released from the prodrug after continuous incubation at pH 7.4 for 58 h, suggesting that most DOX molecules were still covalently conjugated on the nanoparticles. In contrast, the released amount of DOX drastically increased to 69% and 76% at pH 6.0 and 5.0 during the same incubation time, respectively. These results indicate that HMSNs-DOX have good stability under physiological conditions, and the release of DOX would be significantly boosted upon decreasing pH values. Thus, it was demonstrated that HMSNs-DOX are of great potential for the intracellular delivery of anticancer drug DOX in weak acidic endo/lysosomal environment.

Figure 4. Drug release and photothermal effects. (a) pH-dependent DOX release from the HMSNs-DOX prodrug at 37 °C. (b) Heating curves of PB solution (pH = 7.4, 10 mmol), HMSNs-DOX (1 mg mL⁻¹), and different concentrations of HMSNs-DOX/IR825 (0.2, 0.5 and 1 mg mL⁻¹) suspended in PB under 808 nm laser irradiation at a power density of 0.8 W cm⁻². (c) Temperature increments of HMSNs-DOX/IR825 (1.0 mg mL⁻¹) suspensions under the NIR laser irradiation at the power density of 0.8 W cm⁻² for four cycles (5 min of irradiation for each cycle).

The hollow cavity of HMSNs could be used for the accommodation of hydrophobic photothermal dye IR825. The successful loading of the dye was implied by the color change of nanoparticles from red to dark brown after dye loading. In addition, the resultant HMSNs-DOX/IR825 suspension displayed a new strong absorption band from 700 nm to 900 nm with a sharp absorption peak at 828 nm (Figure 2c), assigning to the loaded dye. Besides, the HMSNs-DOX/IR825 also demonstrated good dispersion in aqueous medium without obvious aggregation, which could be attributed by the modified amphiphilic polymer shell. The loading content of IR825 was determined to be 13 wt%. The high dye loading capacity was ascribed to the hollow cavity inside HMSNs that provide additional room for the storage. Due to its hydrophobicity, the IR825 is expected to be retained very well inside HMSNs without obvious release during the blood circulation, thus the dye loaded nanoparticles should be stable enough for the photothermal agent delivery.

The relatively high IR825 loading capacity enabled the possible use of the nanocomposites as photothermal agents. To explore the photothermal heating capability of IR825 integrated nanocomposites, HMSNs-DOX/IR825 with different concentrations were illuminated by 808 nm NIR laser at a power density of 0.8 W cm^{-1} for 5 min, respectively. As shown in Figure 4b, more rapid heating rate as well as greater temperature increment was observed upon increasing the concentration of HMSNs-DOX/IR825 under the same laser irradiation. By contrast, no obvious temperature fluctuations were observed for HMSNs-DOX suspension or the PB solution under the same experiment conditions. To evaluate the photostability of HMSNs-DOX/IR825, it was irradiated by 808 nm laser for four on/off cycles (Figure 4c). In each cycle, the sample was firstly illuminated by NIR

laser for 5 min, and then cooled down to room temperature. It was found that a temperature increment of 37 °C was achieved in the first cycle of irradiation, and no significant loss of the photothermal capability was observed in the following cycles. Thus, the HMSNs-DOX/IR825 system could be implemented as a robust photothermal heater due to its excellent photostability and photothermal capability, which is preferable for successful photothermal therapy.

3.3.3 Cellular uptake and intracellular release

Motivated by the successful DOX release in weak-acidic condition, we further investigated the cellular uptake of HMSNs-DOX/IR825 and DOX release inside cancer cells using CLSM. As seen from Figure 5a, the fluorescence of DOX (shown in red) and cell-labeling dye DAPI (shown in blue) from the cells was observed at different incubation times. For HeLa cell samples that were incubated with free DOX for 4 h, nearly all the red fluorescence focused in the cell nucleus, suggesting that free DOX could easily diffuse into HeLa cells and rapidly interact with the DNA in the nuclei. However, when HeLa cells were incubated with HMSNs-DOX/IR825, only slight red fluorescence was observed in the perinucleus region of cells following 4 h incubation. This may be due to that uptake of nanocomposites is relatively slow as compared with free drug and the fluorescence of DOX could be self-quenched in the aggregated state when combined into the delivery system.²¹⁷ The intracellular microenvironment of cancer cells was slightly acidic in the endosomal (pH 5.0-6.0) and lysosomal (pH 4.0-5.0) compartments.¹²⁵ After the prolonged incubations of 24 h, strong DOX fluorescence was observed in the cell nuclei region, which was attributed to the sustained intracellular acid-induced DOX release after the internalization

of HMSNs-DOX/IR825 by HeLa cells.

Figure 5. (a) CLSM images of HeLa and A2780/DOX^R cancer cells incubated with free DOX and HMSNs-DOX/IR825 for different period of time. The images in each panel show cell nuclei stained by DAPI with the color blue, DOX fluorescence in cells with the color red, and overlays of the two images (from left to right). Scale bars correspond to 20 μ m in all images. (b,c) Flow cytometric profiles against HeLa cells incubated with (b) free DOX and (c) HMSNs-DOX/IR825 prodrug for 1 h, 3 h and 24 h, and the DOX intensity was recorded through the propidium iodide (PI) channel.

The drug-resistant cancer cells are often associated with reduced cellular drug accumulation due to drug efflux or activation of coordinately regulated detoxifying systems.²³⁸ It was observed that DOX resistant A2780/DOX^R cells exhibited negligible intracellular DOX fluorescence after incubation with free DOX for 4 h. In contrast, the

DOX fluorescence was stronger in the A2780/DOX^R cells incubated with HMSNs-DOX/IR825 with equivalent DOX amount for the same incubation time, indicating that HMSNs-DOX/IR825 could be more easily endocytosed by the cancer cells as compared with the free drug. The DOX fluorescence intensity in the case of HMSNs-DOX/IR825 was more pronounced with the increasing incubation time. This fact not only confirms that HMSNs-DOX/IR825 could enhance the DOX uptake by drug resistant cells, but also indicates that the loaded DOX is persistently released inside these cancer cells.

The quantitative measurement of cellular uptake by flow cytometry showed that the uptake of free DOX and HMSNs-DOX/IR825 were all enhanced evidently over time. After 1 h incubation (Figure 5b and 5c), the DOX fluorescence intensity in the case of free DOX was stronger than that of the prodrug nanoparticles. However, this trend was reversed when the incubation time was prolonged to 24 h, and the DOX fluorescence intensity of prodrug exceeded free DOX. The rapidly increased fluorescence intensity in the case of the HMSNs-DOX/IR825 under longer incubation time could be explained by the fact that the conjugated DOX was persistently released from the HMSNs-DOX/IR825 prodrug after cellular uptake, which is consistent with the intracellular release of DOX evident by CLSM.

3.3.4 In vitro antitumor study

Encouraged by the above results, the cancer cell killing efficiency of HMSNs-DOX/IR825 in vitro was evaluated. The cytotoxicity of HMSNs-NH₂ nanocarriers without DOX conjugation and dye loading were first investigated against HeLa cells and A2780/DOX^R cells by using MTT assay (Figure 6a). It was found that HMSNs-NH₂ nanocarriers have favorable biocompatibility, since the viabilities of two types of cells were

above 80% even when the concentration of HMSNs-NHNH₂ increased to 0.2 mg mL⁻¹ after 24 h incubation, indicating that the nanocarriers were biocompatible for anticancer therapy.

Figure 6. In vitro antitumor activity study. (a) Relative viabilities of HeLa and A2780/DOX^R cells after being incubated with various concentrations of HMSNs-NHNH₂ nanocarriers for 24 h. (b) Relative viabilities of HeLa cells incubated with HMSNs-NHNH₂/IR825 (0.1 mg mL⁻¹) for 24 h after 5 min of 808 nm laser irradiation at different power densities. (c) Fluorescence images of calcein AM/PI co-stained HeLa cells with HMSNs-NHNH₂/IR825 (0.1 mg mL⁻¹) incubation for 24 h with the 808 nm laser irradiation for 5 min at different power densities. Live and dead cells were stained by calcein AM and PI, which displayed green and red colors, respectively. (d,e) Relative viabilities of HeLa and A2780/DOXR cells after incubation with different concentrations of free DOX and HMSNs-DOX/IR825 for 24 h with or without laser irradiation (808 nm irradiation, 2 W cm⁻², 5 min).

To eliminate interference of conjugated DOX during the investigation of the photothermal ablation efficiency, IR825 directly loaded HMNS-NHNH₂ nanoparticles (HMSNs-NHNH₂/IR825) were used as the photothermal agent in the study. Enhanced cancer cell killing rate was observed upon increasing the laser power densities. Notably, when the laser power density was above 1.2 W cm⁻², the light irradiation conferred significant photothermal damage against HeLa as well as A2780/DOX^R cells. It demonstrated that the laser power was the crucial factor for efficient photothermal therapy using this therapeutic system. In order to visualize the cancer cell killing efficiency of the photothermal treatment, the HeLa cells were co-stained by calcein AM and PI (Figure 6c). As expected, the fluorescent images confirm that HeLa cells treated with HMSNs-NHNH₂/IR825 could exhibit enhanced cell death rate upon increasing the laser power density. The HeLa cells were completely eradicated when the laser power density increased to 2 W cm⁻². These data collectively demonstrate the excellent photothermal therapeutic efficacy of the IR825 loaded nanocarriers in vitro.

As shown in Figure 6d and 6e, the in vitro antitumor study revealed a concentration-dependent cytotoxicity of DOX and HMSNs-DOX/IR825. For the treatment of HeLa cell, however, it was shown that the chemotherapeutic efficacy of HMSNs-DOX/IR825 prodrug was lower than equivalent concentrations of free DOX. A possible reason might lie in the fact that the time/pH-dependent drug release characteristics of HMSNs-DOX/IR825 resulted in a delay of therapeutic efficacy, leading to lower cytotoxicity as compared to free DOX. The anticancer efficacy of HMSNs-DOX/IR825 drastically enhanced after 5 min NIR irradiation (2 W cm⁻²), and the cancer cells were almost completely destroyed at high

concentrations of HMSNs-DOX/IR825 (equivalent DOX concentrations were above 5 $\mu\text{g mL}^{-1}$). Under irradiation of NIR laser, the half-maximal inhibitory concentration (IC₅₀) of HMSNs-DOX/IR825 for HeLa cells under combination therapy was 2.8 $\mu\text{g mL}^{-1}$, while the IC₅₀ for the chemotherapy alone was 8.4 $\mu\text{g mL}^{-1}$. In a parallel experiment for the treatment of A2780/DOX^R cells, DOX and HMSNs-DOX/IR825 showed much lower cytotoxicity without NIR irradiation and their IC₅₀ could not be determined under the experimental conditions. On the other hand, the IC₅₀ of HMSNs-DOX/IR825 for the A2780/DOX^R cells under NIR irradiation was determined to be 2.0 $\mu\text{g mL}^{-1}$, lower than the value for HeLa cells under irradiation. These results suggest that DOX resistant A2780/DOX^R cells are more vulnerable to the temperature enhancement. By combining the photothermal treatment with DOX prodrug, the therapeutic outcome was further improved against the drug-resistant cancer cells. Therefore, HMSNs-DOX/IR825 with both grafted polymeric drug and loaded photothermal dye may provide a promising strategy towards improved cancer treatment.

Since the nanocarriers could accumulate at tumor sites via the EPR effect, the present therapeutic system is expected to reach tumor to express cancer cell-killing efficiency by remote-controlled light irradiation while causing minimal side effects to normal tissue. To endow its selectivity and specificity toward tumor, the targeting ligands could be introduced to the nanoparticle system, which will be our follow-up work.

3.4 Conclusion

In summary, a surface-initiated RAFT polymerization strategy has been applied to graft the HMSNs with a polymer layer. By the conjugation of DOX to the polymer layer via an

acid-cleavable hydrazone linkage, the obtained HMSNs-DOX were used to load photothermal dye IR825 with excellent stability and dispersibility. The conjugated DOX from HMSNs-DOX could be released at weak acidic conditions for chemotherapy, while the loaded IR825 dye could convert the NIR irradiation into heat energy for photothermal therapy. By combining these two treatments, the HMSNs-DOX/IR825 showed higher therapeutic efficacy on both DOX-sensitive HeLa cells and DOX-resistant A2780/DOX^R cells. Overall, this HMSNs-DOX/IR825 system might provide a new pathway for efficient anticancer applications.

Chapter 4: Acid-sensitive Polymeric Doxorubicin Prodrug Micelles Encapsulating Near-Infrared Dye for Targeted Combined Photothermal-Chemotherapy

4.1 Introduction

As compared with conventional drug loading method, polymeric prodrug is particularly advantageous in some respects, since it could significantly improve relative drug loading content with the minimized premature release of drug during circulation. The polymeric prodrug nanoagents effectively combine the stability of prodrug with passive tumor targeting of nanocarriers. From a theoretical perspective for effective therapy, the drug molecules would only be released from prodrug nanocarriers in responsive of environmental stimuli such as lowered pH,^{2,3,239} redox stimuli,^{58,240} enzyme^{241,242} as well as light irradiation, due to the cleavage of the covalent bond under these circumstances. In particular, taking advantages of the pH conditions in the lysosomes (pH 4.5–5.0) and endosomes (pH 5.0–6.5) of cancer cells,¹²⁵ which is much more acidic than the physiological pH of 7.4, much attention has been paid to the hydrazone bond because it can be efficiently cleaved under the acidic endo/lysosomal environment. The related studies are mainly based on DOX, of which the conjugation could be easily achieved through formation of hydrazone linkage between the carbonyl group of DOX and hydrazide groups on polymer substrate.²⁴³ More importantly, the drug conjugated amphiphilic block copolymer could self-assemble in aqueous solutions to form micelles, which could facilitate the co-delivery of a second therapeutic agent into the tumor site by encapsulating it in the

hydrophobic cores.

NIR light mediated photothermal therapy is another emerging modality for anticancer treatment which utilized heat converted from absorbed NIR light to ablate cancer cells efficiently.^{159,162,227} Currently, the inorganic nanoparticles have been most extensively explored for photothermal therapy. Nevertheless, the nonbiodegradable property and potential biotoxicity of these materials severely inhibit their clinical translation potential.¹⁷² In this regard, NIR absorbing dyes, particularly the family of indocyanine derivatives which have excellent biodegradability and photothermal conversion efficiency, are more promising candidates for photothermal therapy. It should be noted that the direct systematic administration of these dyes including ICG,^{181,183,184} cypate,^{195,244} IR780^{185,187} and IR825^{190,245} for photothermal therapy are strictly restricted, due to the poor aqueous stability of ICG or low aqueous solubility of other dyes. To address these issues, these dyes are often incorporated into polymeric nanocarriers to improve the aqueous solubility as well as stability, prolong their blood circulation and facilitate their tumor-specific delivery.^{246,247}

Despite chemotherapy's being the dominant approach in cancer clinical treatment, the approach of combining chemotherapy with other therapeutic modalities, such as photothermal therapy, has been put forward to ensure therapeutic efficacy for cancer. Combined photothermal-chemotherapy is capable of overcoming the limitations of each therapeutic approach alone, which may yield additive or synergistic effects and lead to improve therapeutic outcomes. For example, PLGA nanoparticles have been used to entrap ICG and DOX simultaneously.^{191,192} Chen et al. reported the co-loading of cypate and DOX inside pH/reduction vesicles demonstrated superiority as a therapeutic platform for

synergistic therapy.¹⁹³ In another recent study, IR825, a newly developed NIR absorbing dye with improved photostability and excellent photothermal conversion efficiency, was first complexed with cationic polymer PEI and then coated with PEG.¹⁹⁴ The *J*-aggregates was eventually loaded with DOX, and exhibited combined therapeutic effect under the NIR laser irradiation. Nevertheless, current combinational therapeutic systems are achieved by simply loading anticancer drug and dyes inside polymeric nanoparticles either simultaneously or separately, adding excessive uncertainty to the loading amount of therapeutic agents. Ge and co-workers developed polymer conjugated with cypate and cisplatin prodrug with uncleavable bond which would undoubtedly compromise drug release behaviors.²⁴⁴ As a result, there is urgent need to fabricate the multifunctional polymeric prodrug for the encapsulation of photothermal dyes, since it not only simplifies the drug loading process but also reduces the premature drug release during circulation. However, to the best of our knowledge, no environmental stimuli polymeric prodrug has yet been reported as nanocarriers to transport the NIR absorbing dye inside the tumor sites.

Scheme 1. Schematic illustration of IR825 loaded polymeric prodrug micelles for targeted combined photothermal-chemotherapy.

Based on the above considerations, we first prepared amphiphilic polymeric prodrug (PDOX) via RAFT polymerization,⁹¹ followed by the functionalization of targeting ligands, removal of the protection groups and eventually conjugation of DOX molecules (Scheme 1). Then, dye IR825 was loaded into the self-assembled polymeric prodrug micelles with high loading efficiency. The multifunctional therapeutic system combines the moieties of targeting ligand, DOX prodrug and photothermal agent delivery into one nanocarrier. Other important features of this therapeutic system are: (1) DOX was immobilized onto the polymer substrate via the acid labile hydrazone bond and would only be released in the weakly acidic cellular environment. (2) The NIR absorbing dye IR825 which has high light-to-heat conversion efficiency was loaded into the polymeric DOX prodrug micelles. (3) Folic acid was functionalized on the polymer as the targeting ligand, and the as-prepared polymeric micelles could effectively accumulate in the solid tumors with overexpressed folate receptors. The therapeutic effect of this multifunctional therapeutic system was evaluated by cancer cells in vitro and further validated in the zebrafish liver hyperplasia model in vivo. The results suggest that these NIR absorbing dye loaded prodrug micelles are a promising multifunctional nanoplatform for combined photothermal-chemotherapy.

4.2 Materials and methods

Aminated folic acid (FA-NH₂) was synthesized according to the literature,²⁴⁸ all other reagents or solvents refer to Chapter 3. All the instrument models in this research refer to Chapter 2.

4.2.1 Synthesis of block copolymer

DMPA (72.9 mg, 0.2 mmol), OEGMA (2.77 mL, 6 mmol) in dioxane (12.0 mL) were

transferred into Schlenk tube at first. Then AIBN (4.11 mg, 0.026 mmol) was added to the above solution to give a [OEGMA]:[DMPA]:[AIBN] = 30:1:0.125. The resulting light yellow solution was deoxidized by purging with nitrogen for approximately 20 min and the tube was sealed under nitrogen atmosphere. Polymerization was carried out under nitrogen atmosphere at 70 °C for 6 h. The polymerization was stopped upon air exposure and the polymer was purified by precipitation three times into excess cold diethyl ether. The resultant viscous oil was dried under vacuum (yield 70%) and denoted as POEGMA (2.17 g, yield: 70 %).

The diblock copolymer was polymerized by RAFT polymerization of MABH using POEGMA as the macroRAFT agent. Typically, monomer MABH (0.954 g, 4.8 mmol), POEGMA (0.858 g, 0.06 mmol), AIBN (1.9 mg, 0.012 mmol) in 4 ml of 1,4-dioxane were transferred into the Schlenk tube and the resulting solution was degassed with nitrogen purging for approximately 20 min. After polymerization under nitrogen atmosphere at 70 °C for 12 h, the copolymer was precipitated two times into excess cold diethyl. The resultant block copolymer was dried and denoted as PMABH-*b*-POEGMA (1.22 g, yield: 38 %).

4.2.2 Folic acid modification and deprotection

Initially, the α -terminal carboxylic group of PMABH-*b*-POEGMA was activated using carbodiimide coupling. Typically, PMABH-*b*-POEGMA (1.0 g, 0.05 mmol), NHS (0.0575 g, 0.5 mmol), DIC (0.077 mL, 0.5 mmol), and triethylamine (TEA, 0.035 mL, 0.25 mmol) were dissolved in 15 mL of anhydrous DMF and the resulting solution was allowed to stir at room temperature for 48 h. The NHS activated PMABH-*b*-POEGMA was collected by the precipitation in cold diethyl ether and dried under vacuum.

For the folic acid modification, the above activated PMABH-*b*-POEGMA (0.50 g, 0.025 mmol), FA-NH₂ (0.0966 g, 0.2 mmol) and a catalytic amount of TEA were dissolved in 8 mL of anhydrous DMF and the reaction proceeded at room temperature for 48 h. After the removal of the solvent under vacuum, the obtained product was re-dissolved in DMSO and extensively dialyzed (MWCO = 3500 Da) against fresh DMSO for two days. Subsequently, the mixture was further dialyzed against deionized water for another two days. Finally, folic acid modified polymer PMABH-*b*-POEGMA-FA was collected after lyophilization. The presence of the conjugated folic ligand was confirmed using UV–Vis spectrum at 355 nm.

To remove the protection groups of BOC, trifluoroacetic acid (4.0 mL) was added to the solution of PMABH-*b*-POEGMA-FA (0.2 g) in DCM (4.0 mL), and the reaction mixture was stirred for 3 h at room temperature. After the removal of solvent under vacuum, the product was re-dissolved in THF and precipitated into excess hexane. The resulting deprotected copolymer was dried under vacuum and denoted as PNHNH₂-*b*-POEGMA-FA (1.22 g, yield: 38 %).

4.2.3 Synthesis of DOX prodrug

For the DOX conjugation, PNHNH₂-*b*-POEGMA-FA (50 mg) and DOX (25 mg) were dissolved in 3 mL of anhydrous methanol, followed by the addition of catalytic amount of TFA (2 μ L). After stirring in the darkness at room temperature for 36 h, the resultant prodrug was purified by extensive dialysis against methanol (MWCO 3500 Da) for one day. After removal of the solvent, polymeric prodrug denoted as PDOX (45 mg, yield: 60%) was obtained as dark red powder. To determine DOX conjugating content, 2 mg of PDOX was treated with 1.0 M HCl at room temperature overnight. After dilution with deionized

water, the amount of conjugated DOX was determined using fluorescence spectrum.

4.2.4 Formation of IR825 loaded PDOX micelles

At first, IR825 (3 mg), triethylamine (3 μ L) and as-synthesized PDOX (10 mg) was dissolved in methanol (1.2 mL). After stirring at room temperature for 4 h, 4 mL of PB (pH 7.4) was added dropwise. Then the resulting dispersion was transferred into a dialysis tube (MWCO = 3500 Da), dialyzed against PB (pH = 7.4) for 24 h, yielding IR825 loaded micelles which were denoted as PDOX/IR825. To determine the loading content of IR825, aliquots of the PDOX/IR825 suspension was treated with lyophilization to remove the water. Finally, the obtained solid product was re-dissolved in CH₃OH and analyzed with UV–Vis–NIR spectrum (absorption at 825 nm). The pure PDOX micelles without encapsulated IR825 were prepared using similar method except that no dye was added.

4.2.5 In vitro drug release

The triggered release of conjugated DOX from PDOX prodrug micelles under acidic environment was investigated. Briefly, PDOX suspension in 3.0 mL PB at pH 7.4 were firstly placed into dialysis bag (MWCO = 3500) and dialyzed against 40 mL of buffer medium at pH 5.0, 6.0 or 7.4 at 37 °C in the dark. At the designed time intervals, 2.0 mL of release medium was taken out and its fluorescence emission intensity at 560 nm under the excitation wavelength of 490 nm was determined by the fluorescence spectrometer, while the 2.0 mL of fresh buffer medium was added back to compensate the volume loss. The concentration of DOX presented in the dialysate was calculated. The releasing experiments were repeated three times independently, and the mean value was obtained as the final result.

4.2.6 Cellular uptake study

In the CLSM study, HeLa cells were seeded in a 6-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well. After culturing for 24 h at 37 °C and 5% CO₂, an appropriate amount of PDOX micellar suspension with an equivalent DOX concentration of $5 \mu\text{g mL}^{-1}$ were added into the cell culture medium. After further incubation for 1, 4 and 24 h respectively, the culture medium was removed and the cells were washed three times with PBS. In addition, cells treated with free DOX were incubated for 4 h as a control. Finally, the cells were stained alive with Hoechst33342 (nucleus staining, blue) and LysoTracker Green (lysosome staining, green) and mounted for observation with CLSM.

For the flow cytometric study, HeLa cells were also seeded in tissue culture plates the same as the CLSM study. After culturing for 24 h, free DOX or PDOX micelles were added in the culture medium, all of which the equivalent DOX concentrations were maintained at $1 \mu\text{g mL}^{-1}$ for every well. After incubation for 1, 4 or 24 h, the cells were washed with PBS for two times and treated with 0.5 ml of trypsin for 2 min. Subsequently, 0.5 mL of fresh medium was added to each culture well, and the cells were collected via centrifugation at 2000 rpm for 3 min. Finally, after one more round of washing with of PBS and centrifugation, the cells were re-suspended in 1 ml of PBS, and subjected to the flow cytometry analysis.

4.2.7 In vitro cytotoxicity study

In this study, DOX-sensitive HeLa cells were incubated in DMEM described previously while DOX-resistant A2780/DOX^R cells were seeded in RPMI 1640 supplemented with

the same content of FBS and antibiotics. The antitumor efficacy of PDOX/IR825 micelles with or without irradiated NIR irradiation on the proliferations of two types of cancer cells were quantitatively investigated by using MTT assay. Briefly, the cells were seeded in 96-well plates (100 μ L tertiary amine) and incubated overnight. When the cell confluence reached around 60-70%, the medium was replaced with fresh one (90 μ L) and sample solutions/suspensions (10 μ L) with specific concentrations were added to the each well. After 4 h of incubation, the cultural medium was replaced with the fresh medium. Subsequently, the cells were irradiated with 808 nm laser, while the untreated cell groups were used as a control. After further incubation for 20 h, 10 μ L of 5 mg L⁻¹ MTT solution was added. The cells were incubated for another 4 h, and then the MTT containing medium was completely removed. After that, DMSO (160 μ L) was added in each well to dissolve the resultant purple crystals. Finally, microplate reader (infinite M200, TECAN) was employed to measure the optical densities of the samples in each well at 490 nm.

4.2.8 In vivo antitumor efficacy studies

Mifepristone was used to treat Transgenic zebrafish embryos expressing EGFP-kras^{v12} oncogene to induce liver hyperplasia as reported in literature.²⁴⁸ Briefly, after 4 days of induction with 1 μ M mifepristone, liver tumors bearing zebrafish larvae were selected for use in antitumor efficacy studies. Liver tumor-bearing larvae were fixed in 1% low melting agarose in the dish. It should be noted that the heart and liver should face upward, so that the samples could be directly injected into the liver. Confocal microscopic instrument setup according to the literature our group reported.²⁴⁵ All mounted zebrafish larvae were imaged immediately after the injection of the freshly prepared therapeutic agents for the treatment

with or without laser irradiation, while the larvae injected with water were used as a control. The same larvae were imaged again 1 day later. All images were obtained under the same acquisition settings. Quantification of liver tumor volume was evaluated using the Volumest plugin in Image J.

Scheme 2. Synthesis of folic acid functionalized amphiphilic polymeric prodrug via RAFT polymerization and post-polymerization modification.

4.3 Results and discussion

4.3.1 Synthesis and characterization

The amphiphilic polymeric DOX prodrug was obtained via a multiple synthetic process (Scheme 2). Firstly, the hydrophilic macroRAFT agent POEGMA was prepared by RAFT polymerization of OEGMA with DDMAT as the chain transfer agent. A typical ¹H NMR spectrum of POEGMA is shown in Figure 1a, and all the characteristic peaks including δ 3.37 ppm (terminal methoxy protons) and δ 3.37 ppm (PEG ethylene protons) can be

observed.

The RAFT block copolymerization of MABH was carried out using POEGMA as the macroRAFT agent. The ^1H NMR spectrum of resultant POEGMA-*b*-PMABH copolymer contains a series of peak at δ 3.39, 3.66 and 4.23 attributable to PEG protons as well as those peaks at δ 7.44, 7.27 and 1.41–1.50 attributable to the *tert*-butyl carbazate proton on MABH (Figure 1a). Compared with FT-IR spectrum of POEGMA (Figure 2a), a new peak of strong intensity at around 1674 cm^{-1} was observed which was attributed to the stretching vibration of newly introduced C=O of hydrazide. The results indicate the successful copolymerization of MABH during the formation of the amphiphilic copolymer POEGMA-*b*-PMABH.

Figure 1. (a) ^1H NMR spectra of POEGMA in CDCl_3 , POEGMA-PMABH in CDCl_3 , FA-POEGMA-PNHNH₂ in $\text{DMSO}-d_6$ and PDOX in MeOD. (b) UV-Vis absorption spectra of PMABH-*b*-POEGMA and PMABH-*b*-POEGMA-FA in DMSO. (c) UV-Vis absorption spectra of PNHNH₂-*b*-POEGMA-FA and PDOX in methanol.

To enhance the targeting selectivity of therapeutic agents, the aminated folic acid was conjugated to the terminal carboxylic group of POEGMA-*b*-PMABH via an amidation reaction. However, since the content of terminal conjugated ligand is relatively low, the characteristic proton signal of folic acid could not be identified in the ^1H NMR spectrum of resultant POEGMA-*b*-PMABH-FA. The successful conjugation of folic acid was further confirmed by UV–Vis absorption spectrum (Figure 1b). As compared with the absorption of its precursor, the absorption spectrum of POEGMA-*b*-PMABH-FA displayed a new peak around 355 nm, assigning to the conjugated folic acid. When treated with trifluoroacetic acid, the FT-IR spectrum of PNHNH₂-*b*-POEGMA-FA revealed that that the stretching vibration of C=O of ester at 1726 cm^{-1} reduced significantly (Figure 2a), moreover, the stretching vibration of $-\text{CH}_3$ at 2920 cm^{-1} also decreased, indicating the cleavage of the BOC protecting group. Consistently, ^1H NMR spectrum showed complete disappearance of signals at δ 1.46 attributable to *tert*-butyl protons of BOC group (Figure 1a). Hence, hydrazide containing amphiphilic copolymer had been obtained.

Finally, using trace amount of trifluoroacetic acid as the catalyst, acid-labile PDOX prodrug was prepared by treating the deprotected PNHNH₂-*b*-POEGMA-FA with DOX HCl in anhydrous methanol. After conjugation, the color of the polymer turned dark red and a broad absorption peak appeared in the ranging from 600 to 400 nm for the methanol solution of the polymer (Figure 1c), which was consistent with the UV absorption characteristics of free DOX. The successful attachment of DOX was also verified by ^1H NMR (Figure 1a), in which the signals of the DOX aromatic group protons were clearly observed at δ 8.02, 7.88 and 7.63 ppm. The conjugation level of DOX was measured using

fluorescence spectroscopy followed by the treatment with HCl solution to completely cleave the hydrazone linkage. It was found that the conjugated DOX content in the prodrug was as high as 20 wt%. High drug content is highly beneficial for minimize the total dose of the therapeutic agents required for effective treatment, which could potentially reduce its undesired side effects.⁹⁹

Figure 2. (a) FT-IR spectra of POEGMA, PMABH-POEGMA-FA and PNHNH₂-POEGMA-FA. (b) UV-Vis-NIR spectra of IR825 in methanol, PDOX and PDOX/IR825 micellar suspensions in PB (pH = 7.4). Inset: the images from left to right show the photos of suspended PDOX and PDOX/IR825 micelles. (c) Size distribution of PDOX and PDOX/IR825 micelles determined by DLS. (d) TEM image of dye loaded PDOX/IR825 micelles. Scale bar: 200 nm. (e) The changes of the particle size of the PDOX/IR825 micelles with time in pH 7.4 buffer at 25 °C, monitored by DLS.

4.3.2 Self-assembly behavior

Since the PDOX prodrug consists of a hydrophilic POEGMA and a hydrophobic DOX-containing block, the obtained acid-labile PDOX prodrug could self-assemble to afford micelles in PB (pH 7.4) by solvent exchange. The micellar suspension of the prodrug exhibited red color with the peak around 490 nm, which was attributed to the DOX content

(Figure 2b). The morphology and size of the self-assembled prodrug micelles were investigated by TEM and DLS. As shown in Figure 3a, the TEM image of PDOX micelles revealed a spherical morphology with monodispersed size distribution at around 40 nm, which was in line with the DLS analysis (Figure 2c). The IR825 dye was encapsulated into the hydrophobic domains of prodrug micelles by hydrophobic interaction. After encapsulation of IR825 inside the core, the average size of PDOX/IR825 micelles increased to 150 nm (Figure 2d). Consistently, as revealed in Figure 2c, the size of PDOX/IR825 micelles determined by DLS right-shifted after the dye loading, while the monodispersity was not affected.

As shown in Figure 2b, the successful encapsulation of IR825 also resulted in a change of color in the micellar suspensions from red to brown. The absorption spectrum of free IR825 in methanol showed a sharp absorption peak at 826 nm. On the other hand, IR825 loaded micelles in PB exhibited a broadened and red shifted absorption peak at 842 nm. To determine the loading content of the photothermal dye, PDOX/IR825 suspensions were lyophilized, and analyzed with UV–Vis–NIR spectroscopy in MeOH solution. The loading content of IR825 was found to be 18 wt%, which was essential for the effective photothermal therapy.

Colloidal stability of the dye loaded micelles has also been studied. After storing at 25 °C for 72 h in PB at pH 7.4, the PDOX/IR825 micelle suspension remained clear without obvious precipitation. Besides, as shown in Figure 2e, there is no significant size variation after storage, indicating its favorable colloidal stability at physiological condition. However, as observed from Figure 3b, when acetate buffer at pH 5.0 was added, the size distribution

curve displayed a bimodal distribution with a small peak around 60 nm and a large peak around 120 nm in 4 h. After incubation for 48 h, it is notable that the size of degraded nanoparticles further increased to over 600 nm. The size change of the nanoparticles may be initiated by the cleavage of acid-labile hydrazone bonds in the PDOX/IR825 micelles when exposed to the acidic environment, resulting in the dissociation of nanostructure. To break down the hydrazone linkages completely, PDOX/IR825 micelles were treated with 0.1 M HCl overnight at 25 °C. As shown in Figure 3c, the loaded IR825 precipitated due to the loss of solubilization effect after the totally dissociation of nanocarriers.

Figure 3. (a) TEM image of PDOX prodrug micelles. Scale bar represents 100 nm. (b) Size change of dye loaded prodrug micelles PDOX/IR825 in phosphate buffer (pH 7.4) measured by DLS. (c) Photos of PDOX/IR825 micelle treated with 0.1 M HCl overnight.

4.3.3 Drug release and photothermal effects

Considering the acid-labile nature of hydrazone linkage, the acidic tumor microenvironment could readily initiate the release of conjugated DOX. Thus, PDOX prodrug micelles were incubated at 37 °C in acidic buffers of pH 5.0 and 6.0, respectively, where the physiological condition of pH 7.4 was used as the control. The release results are shown in Figure 4a. Under mild acidic condition of pH 6.0 and 5.0, the corresponding percentages of DOX release after continuous incubation for 60 h were approximately 67 and 60%, respectively. In contrast, less than 10 % of DOX was released at pH 7.4 after the

same incubation time. These results indicate that PDOX prodrug micelles were stable under physiological conditions and also capable of releasing DOX in an accelerated manner when exposed to weak acidic environment, which are preferable for the transportation of anticancer drug to the slightly acidic intracellular microenvironment of cancer cells.

The relatively high IR825 loading capacity facilitates the utilization of the PDOX/IR825 for effective photothermal therapy. The photostability of the PDOX/IR825 was first investigated, while ICG, an extensively studied photothermal dye, was used as the control. After 5 min of 808 nm laser irradiation (0.8 W cm^{-2}), the green color of ICG in PB (pH 7.4) turned into nearly colorless, on the contrary, no significant change was observed for the color of PDOX/IR825 (Figure 4b). Consistently, the variation of the absorption spectrum of PDOX/IR after laser exposure was much smaller in comparison to ICG under the same illumination conditions, for the latter of which the NIR absorbance had been turned down to almost zero. To assess the photothermal heating efficiency, the micellar suspensions of PDOX/IR825 at various concentrations were exposed to 808 nm laser irradiation at a power density of 0.8 W cm^{-2} , meanwhile, the temperature changes of the suspensions with time were monitored using thermal imager. As displayed in Figure 4c, the temperature elevations in PB solution and PDOX micellar suspension under the NIR irradiation were almost negligible, while the PDOX/IR825 micellar suspension exhibited an evident concentration-dependent temperature increase. The fastest and greatest temperature increase was obtained at the highest concentration of PDOX/IR825 (0.25 mg mL^{-1}), of which the temperature increased by 38°C after 5 min irradiation. This rapid temperatures increase can confer irreversible damage to cancer cells and thermally ablate the tumor tissues. In addition, no

significant loss of the photothermal capability was observed after five cycles of repeated laser-induced photothermal heating (Figure 4d). On account of the excellent photothermal stability of PDOX/IR825, it could be used as a robust photothermal heater for the continuous and repetitive photothermal treatment of tumors.

Figure 4. Drug release and photothermal effects. (a) Acid triggered release of conjugated DOX from the PDOX micelles at 37 °C. (b) UV–Vis–NIR absorbance spectra of PDOX/IR825 micellar suspensions and ICG solutions before and after 5 min of photothermal heating (0.8 W cm^{-2}). Inserted images are the photos of ICG solutions and PDOX/IR825 micelle suspensions before and after laser irradiation. (c) Heating curves of PB (pH = 7.4), PDOX (1 mg mL^{-1}) and different concentrations of PDOX/IR825 micelles suspended in PB (pH = 7.4) under 808 nm laser irradiation at a power density of 0.8 W cm^{-2} . (d) Temperature increments of PDOX/IR825 (0.25 mg mL^{-1}) suspension under NIR laser irradiation at the power density of 0.8 W cm^{-2} for 5 cycles (5 min of irradiation for each cycle).

4.3.4 Intracellular uptake

Efficient intracellular delivery of therapeutic agents into the cytoplasm or nucleus is crucial for the successful treatment of cancer. Taking advantage of the intrinsic red

fluorescence of DOX, the cellular uptake of PDOX micelles and intracellular DOX release inside HeLa cells at different incubation times were evaluated by CLSM and flow cytometry. In the CLSM study, three types of fluorescence were detected and collected, which were the blue color of Hoechst33342 (used for the staining cell nucleus), the red color of DOX and the green color of Lysotracker Green (used for the staining of lysosomes). As shown in Figure 5a, the free DOX was absorbed quickly by HeLa cells, and strong red fluorescence was observed in the cytoplasm and nucleic regions after 4 h incubation. It is attributed that free DOX molecules could be transported through the cell membrane via passive diffusion and interact rapidly with the DNA in the nuclei. In contrast, the DOX fluorescence in those cells treated with PDOX micelles for 4 h was significantly weaker and overlapped largely with the lysosomes (green). On account of the slight acidity in lysosomal compartments (pH 4.5–5.0), the DOX prodrug therein could be released gradually inside. The cellular uptake of PDOX and DOX release after internalization are found to be time dependent, from which the accumulated DOX fluorescence in cytoplasm and nuclei eventually reached a very high level when the incubation time reached 24 h. The significant DOX fluorescence located in the cell nucleus confirmed that DOX has been continuously released from the acid labile PDOX prodrug micelles into the cytoplasm and then interacted with the DNA in the nucleus, since the PDOX micelles themselves could not pass the nuclear pore complex due to the large size of micelle.

Figure 5. Intracellular uptake of free DOX and PDOX micelles. (a) CLSM images of HeLa cells incubated with free DOX and PDOX prodrug micelles for different period of time at $4 \mu\text{g mL}^{-1}$ equivalent DOX concentration. Images from left panel to right present cell nuclei stained by Hoechst 33342 (blue), DOX fluorescence in cells (red), lysosomes stained by LysoTracker Green (green) and overlay of the three images, respectively. All images are acquired in the same scale bar ($20 \mu\text{m}$). (b) Flow cytometry analysis of the DOX fluorescence intensity in HeLa cells incubated with free DOX and PDOX prodrug micelles for different period of time at $1 \mu\text{g mL}^{-1}$ equivalent DOX concentration. Control group refers to the cells without any treatment.

To quantify the cellular uptake behaviors, flow cytometric analysis was then performed on HeLa cells, which were incubated with PDOX prodrug micelles and free DOX at different incubation time. It demonstrated that the free DOX entered the HeLa cells very quickly and DOX fluorescence in those cells after 1 h incubation was much stronger than that of those cells treated with PDOX micelles (Figure 5b). However, as the incubation time increased, the fluorescence of those cells treated with PDOX micelles began to increase even faster than the free DOX treating cell group, and at 24 h they eventually reached a comparable level, which was consistent with the CLSM results. Collectively, the CLSM and flow cytometry results demonstrate that the PDOX micelles were efficiently

endocytosed by HeLa cells and capable of releasing DOX continuously inside cells.

Figure 6. In vitro antitumor activity study. (a) Relative viabilities of HeLa and A2780/DOX^R cells incubated with PDOX/IR825 micelles ($30 \mu\text{g mL}^{-1}$, equivalent DOX concentration $5 \mu\text{g mL}^{-1}$) under 808 nm laser irradiation at different power densities for 5 min. All of the relative viability values were normalized to the untreated cells. (b) Relative viabilities of HeLa and A2780/DOX^R cells after the incubation of PDOX/IR825 micelles ($30 \mu\text{g mL}^{-1}$) under different irradiation durations (power density: 1.2 W cm^{-2}). (c,d) Relative viabilities of HeLa (c) and A2780/DOX^R (d) cells after incubation of free DOX, PDOX/IR825 with or without laser irradiation at different concentration with the power density of 1.2 W cm^{-2} .

4.3.5 In vitro antitumor study

To study the therapeutic efficacy of PDOX/IR825 on cancer cells in vitro, HeLa and A2780/DOX^R cells were incubated with PDOX/IR825 micellar suspensions under different laser irradiation conditions for 5 min. The relative viabilities of two types of cancer cells were assessed by the MTT assay. Laser power and illumination duration are two critical parameters that influence the efficacy of photothermal therapy. After the incubation with

micellar suspension for 4 h, an increase in laser power density improves ablation of treated cancer cells (Figure 6a). With irradiation time fixed at 5 min, most HeLa and A2780/DOX^R cells were destroyed when the power density exceeded 1.2 W cm⁻². Likewise, duration of irradiation is critical to success of cancer cell ablation. When power density was fixed at 1.2 W cm⁻², irradiation duration at 5 minutes or longer significantly enhanced ablation of treated cancer cells (Figure 6b).

Anticancer effect of free DOX, PDOX/IR825 and combinational therapeutic capability were then investigated using cancer cells. Both free DOX and PDOX/IR825 showed concentration-dependent cytotoxicity against tested cancer cells. For the treatment of HeLa cells, free DOX showed better antitumor effects than PDOX/IR825 at the same DOX dose level when the NIR irradiation was excluded. As shown in Table 1, the IC₅₀ for PDOX/IR825 against HeLa cells without irradiation was 6.7 µg mL⁻¹ (equivalent DOX concentration, all subsequent experiments below were performed with the same setting), while IC₅₀ for free DOX was 3.9 µg mL⁻¹. These observations may be explained by the sustained acid triggered release of DOX from prodrug micelles, which resulted in the delayed and abated therapeutic efficacy. Under the irradiation of NIR laser, IC₅₀ value of PDOX/IR825 decreased to 2.0 µg mL⁻¹, which was attributed to additional photothermal damage. Due to the DOX resistance of the cancer cells used in this experiment, the treatment of A2789/DOX^R cells with free DOX or PDOX/IR825 all showed low therapeutic efficacy. The IC₅₀ of PDOX/IR825 was 12.6 µg mL⁻¹, while IC₅₀ of free DOX already exceeded the concentration range. Encouragingly, under the laser irradiation, IC₅₀ of PDOX/IR825 for the A2780/DOX^R cells decreased to 1.7 µg mL⁻¹, which was even lower

than that ($2.0 \mu\text{g mL}^{-1}$) on HeLa cells. These results demonstrate that A2780/DOX^R cells were more susceptible to the temperature increase as compared with HeLa cells. Combined photothermal-chemotherapy provided by PDOX/IR825 is highly effective to both DOX-sensitive HeLa cells and DOX-resistant A2780/DOX^R cells.

Table 1. IC₅₀ values for free DOX and PDOX/IR825 without or with 808 nm laser irradiation against DOX-sensitive HeLa and DOX-resistant A2780/DOX^R cells.

Samples	IC ₅₀ ($\mu\text{g mL}^{-1}$)	
	HeLa	A2780/DOX ^R
Free DOX	3.9	--
PDOX/IR825 without irradiation	6.7	12.6
PDOX/IR825 with irradiation	2.0	1.7

4.3.6 In vivo combinational therapeutic efficacy

Encouraged by the excellent therapeutic effects of PDOX/IR825 in vitro, we further studied their anti-tumor performance on solid tumors using transgenic zebrafish based liver tumor model. The scheme of the combined photothermal-chemotherapy on liver tumor bearing transgenic zebrafish larva was shown in Figure 7a. Strong expression of EGFP tagged oncoprotein, (EGFP)-kras^{v12} in the liver tumor of transgenic zebrafish enables non-invasive, live imaging of the developing tumor,^{248,248} a straightforward approach to evaluate antitumor efficacy of therapeutic agents. In this study, liver tumor-bearing larvae were divided into four groups. Group 1 was injected with water as the control. Group 2 was injected with free DOX, while Group 3 and 4 were injected with PDOX/IR825 micelles without or with NIR laser illumination. Imaging of tumors was acquired by confocal imaging and the volume of each tumor was then assessed before and after 24 h treatment. Representative 3D fluorescent images of each group are shown in Figure 7b. It can be observed that a reduction in tumor size was detected in all groups injected with therapeutic

agents. Similar to in vitro antitumor study, the most efficient regression of tumor volume was realized in the combination treatment group of PDOX/IR825 under 808 nm laser irradiation where reduction in tumor volume was accompanied by decrease in GFP fluorescent intensity. The latter is a result of NIR induced local hyperthermia that damaged cell membranes and degraded cellular proteins (including GFP) during tumor ablation.

Figure 7. In vivo antitumor activity study on solid tumors using transgenic zebrafish based liver tumor models. (a) Scheme of the combined photothermal-chemotherapy of live tumor bearing transgenic zebrafish larva with PDOX/IR825 micellar suspensions under 808 nm laser irradiation. (b) Representative 3D fluorescent images of four liver tumor groups. Liver tumor bearing zebrafish larvae were injected with H₂O, free DOX, PDOX/IR825 without or with irradiation. The irradiated groups were exposed to 808 nm laser irradiation for 5 min (power density: 1.2 W cm^{-2}). (c) Tumor relative volume changes in each sample group, measured at 24 h post-treatment.

To quantify the therapeutic efficacy of each treatment group, the percentage of relative

tumor size change in each sample group was normalized to their initial size. As shown in Figure 7c, tumor growth occurred in H₂O injected control group, while tumor regression was observed in all treatment groups. Free DOX group showed better curative effects than PDOX/IR825 without irradiation after treatment, which was in good agreement with *in vitro* MTT results. Moreover, tumor regression from the NIR-irradiated PDOX/IR825 injected group was the most efficient, where tumor volume decreased by 50% over 1 day. These results collectively demonstrated that combination of prodrug chemotherapy with photothermal therapy significantly improve the overall anticancer efficacy on zebrafish liver hyperplasia model.

4.4 Conclusion

We have developed a multifunctional nanoplatform based on polymeric prodrug micelles encapsulating dye IR825 for the combined photothermal-chemotherapy. It has been found that the dye loaded prodrug micelles PDOX/IR825 were stable under normal physiological pH, while achieved fast DOX release under mild acidic condition. Besides, the encapsulated IR825 dye could convert NIR irradiation into heat energy efficiently upon NIR irradiation, making the self-assembled nanoplatform capable of repeatable photothermal therapy. *In vitro* study confirmed that PDOX/IR825 showed higher therapeutic efficacy on HeLa and A2780/DOX^R cells under NIR laser irradiation, through the combination of chemotherapy with photothermal therapy. Moreover, PDOX/IR825 micelles under NIR irradiation showed superior therapeutic efficacy on zebrafish liver hyperplasia models *in vivo*. Taken together, the reported results might provide a new promising approach for safe and effective site-specific combination anticancer therapy.

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Conclusion and Outlook

This thesis introduced my research progress on multifunctional polymer and HMSNs/polymer hybrid nanoparticles for drug delivery and potent combined photothermal-chemotherapy. In recent years, MSNs have been extensively explored as nanocarriers for the drug delivery and biomedical applications. To reduce the undesired side effects for clinical use, it is advantageous to employ lower amount of nanocarriers with improved drug loading capability. Thus, HMSNs have been further developed with increasing attention, since large hollow cavities inside nanoparticles can be used to store more cargos as compared to the conventional MSNs.

In an effort to perfect the drug release profile under complicated physiological environment, two or more stimuli-responsive behaviors could be combined to construct the versatile MSNs delivery system. The pH-responsive polymer PDEAEMA was grafted on the HMSNs via surface initiated ATRP. The HMSN/polymer hybrid contained one reduction-cleavable disulfide bond as well as one photo-cleavable *o*-nitrobenzyl methyl ester group as the linkage between the silica core and pH-responsive polymer shell. DOX was retained into the hybrid nanoparticles with a high loading content and the polymer coating served as the cap to block and control the release of the encapsulated drug. In response to the stimulus of reducing agent, acid environment or UV irradiation, the loaded DOX was released quickly as compared to the negative control, showing the promising potential for anticancer drug delivery.

Besides, the hollow cavity could also encapsulate other therapeutic agents. Recently, the new indocyanine dye IR825 has been developed as NIR-absorbing agent for photothermal therapy. IR825, with enhanced photostability with excellent photothermal conversion efficiency, can serve as the robust NIR-induced heater for the continuous photothermal therapy. Amphiphilic polymeric DOX prodrug was first grafted on to the HMSNs via surface initiated RAFT polymerization and post-polymerization modification. Unlike the other conventional MSNs prodrug system where the drug was attached to nanoparticles directly, polymeric coating on MSNs could significantly enhanced the drug loading capacity, since the integrated polymer substrate provide far more surface functional groups on the polymer chain for the drug conjugation. In addition, the hollow cavity and mesoporous pore channels could be used to store the hydrophobic photothermal dye IR825, showing excellent aqueous dispersity and stability. By combining the two treatments, the conjugated DOX could be released under weakly acidic conditions, while the loaded dye could convert the photons into thermal energy, resulting in efficient combination therapy.

By loading the dye inside the silica nanocarriers, the silica may work as heat insulation material, resulting in reduced photothermal performance. In the following study, a DOX-conjugated amphiphilic block copolymer with terminal folic acid moiety was prepared and allowed to self-assemble into micelles, encapsulating IR825 for combined photothermal-chemotherapy. The folic acid targeting ligand assists in the effective endocytosis of the micelles into solid tumor cells with overexpressed folate receptors. The therapeutic effect of this multifunctional system was evaluated by

cancer cells in vitro and further validated in the zebrafish liver hyperplasia model in vivo, which demonstrated the superior combined therapeutic efficacy as compared the previous HMSNs/polymer hybrid system.

In conclusion, during my postgraduate study, I have successfully prepared a series of HMSN/polymer hybrid and polymer nanocarriers for drug delivery and potent combined photothermal-chemotherapy. All of these studies demonstrated significant antitumor activity both in vitro and in vivo. In particular, the NIR-induced photothermal therapy together with chemotherapy could overcome the multi-drug resistance.

Up till now, nearly all of the photothermal dyes have been simply introduced to the nanocarriers by physical loading for photothermal application. Future work will be conducted to modify the indocyanine dye with other functional groups including adamantane and PEG, while the new functionalized dye could be used to construct a nanocarrier with β -cyclodextrin containing polymer via the host-guest interaction. By using the functionalized photothermal dye, it is expected to simplify the preparation process of such therapeutic system. Moreover, by functionalizing the NIR-absorbing dye with PEG, the dye may self-construct to organic nanocarriers directly. The biocompatible, biodegradable, and multi-functional dye will show the great promise for clinical uses.

List of Publications

1. **Yuanyuan Zhang**, Cathleen Teh, Menghuan Li, Chung Yen Ang, Si Yu Tan, Qiuyu Qu, Vladimir Korzh, and Yanli Zhao*, Acid-responsive polymeric doxorubicin prodrug nanoparticles encapsulating a near-infrared dye for combined photothermal-chemotherapy. *Chem. Mater.* 2016, 28, 7039–7050.
2. **Yuanyuan Zhang**, Chung Yen Ang, Menghuan Li, Si Yu Tan, Qiuyu Qu, Yanli Zhao,* Polymeric prodrug grafted hollow mesoporous silica nanoparticles encapsulating NIR absorbing dye for potent combined photothermal-chemotherapy. *ACS Appl. Mater. Interfaces* 2016, 8, 6869–6879.
3. **Yuanyuan Zhang**, Chung Yen Ang, Yanli Zhao,* Polymeric nanocarriers incorporating near-infrared absorption agents for potent photothermal therapy of cancer. *Polym. J.* 2015, 48, 58–603.
4. **Yuanyuan Zhang**, Chung Yen Ang, Menghuan Li, Si Yu Tan, Qiuyu Qu, Zhong Luo, Yanli Zhao,* Polymer coated hollow mesoporous silica nanoparticles for triple-responsive drug delivery. *ACS Appl. Mater. Interfaces* 2015, 7, 18179–18187.
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6. Menghuan Li, Cathleen Teh, Chung Yen Ang, Si Yu Tan, Zhong Luo, Qiuyu Qu, **Yuanyuan Zhang**, Vladimir Korzh, Yanli Zhao,* Near-infrared light-absorptive stealth liposomes for localized photothermal ablation of tumors combined with chemotherapy. *Adv. Funct. Mater.* 2015, 25, 5602–5610.