Part I : Peptide functionalized materials for
gram-negative bacteria targeted PDT and LPS
sensing. Part II : Small peptide and membrane
lipid based self-assembled nanostructures for
controlled drug/gene delivery

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NANYANG TECHNOLOGICAL UNIVERSITY

**Part I: Peptide Functionalized Materials for Gram-negative Bacteria
Targeted PDT and LPS Sensing**

**Part II: Small Peptide and Membrane Lipid Based Self-assembled
Nanostructures for Controlled Drug/Gene Delivery**

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

In

Chemistry

By

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Assoc. Prof. Tianhu Li,

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Abbreviations

ABDA	9, 10-anthracenediyl-bis(methylene) dimalonic acid
AMPs	Antimicrobial peptides
APTES	3-aminopropyl)triethoxysilane
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Cephalin	Phosphatidylethanolamine
CLSM	Confocal Laser Scanning Microscopy
CTAB	Cetrimonium bromide
DCC	Dicyclohexylcarbodiimide
DIPEA	N,N-Diisopropylethylamine
DLS	Dynamic light scattering
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DOX	Doxorubicin
Dp6	Maltohexaose
dsDNA	Double stranded DNA
EDC	N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride
ESI-MS	Electrospray ionization mass spectrometry
FA	Folic acid
FBS	Fetal bovine serum
FDA	U. S. Food and Drug Administration
Fe ₃ O ₄ @ SiO ₂	Fe ₃ O ₄ @SiO ₂ core-shell nanoparticles
Fmoc	9-Fluorenylmethyl chloroformate
FRET	Fluorescence resonance energy transfer
FTIR	Fourier transform infrared spectroscopy
GDP	Guanosine diphosphate
Glu	Glutamate
GSH	Glutathion
HBTU	(1H-Benzotriazol-1-yloxy)(dimethylamino)-N,N-dimethylmethaniminium hexafluorophosphate
HOBt	Hydroxybenzotriazole
HOSu/NHS	N-Hydroxysuccinimide
HPLC	High-performance liquid chromatography
LAA	L-ascorbic acid
LB medium	Lysogeny broth medium
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix-assisted laser desorption/ionization mass spectrometry
MDR	Multiple drug resistance

MH medium	Müller-Hinton medium
MNP	Magnetic nanoparticle
NMR	Nuclear Magnetic Resonance
PACT	Photodynamic antimicrobial chemotherapy
PBS	Phosphate buffered saline
PDI	Perylene diimide
PDT	Photodynamic therapy
PEG	Poly(ethylene glycol)
PpIX	Protoporphyrin IX
RNAi	RNA interference
RPMI 1640	Roswell Park Memorial Institute 1640
siRNA	Small interfering RNA
ssDNA	Single stranded DNA
TBS	Tris-buffered saline
TEA	Triethylamine
TEM	Transmission electron microscopy
TEOS	Tetraethyl orthosilicate
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography

Cell lines

<i>E.coli</i> DH5 α	<i>Escherichia coli</i> . DH5 α (ATCC53868)
<i>E.coli</i> BL21	Ampicillin resistant <i>Escherichia coli</i> BL21
<i>S. enterica</i>	<i>Salmonella enterica</i> (ATCC 14028)
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i> (ATCC 700603)
Jurkat T cell	Human T cell lymphoblast-like cell line
HeLa cell	Human epithelial carcinoma cell line
A549	Human lung adenocarcinoma epithelial cell line
HEK293	Human Embryonic Kidney 293 cells
A2780	Human ovarian carcinoma cells
A2780/ADR	Adriamycin resistant cell line A2780 cell line
RAW 264.7	Mouse leukaemic monocyte macrophage cell line

Abstract

Part I: Peptide Functionalized Materials for Gram-negative Bacteria Targeted PDT and LPS Sensing

Combating infections caused by Gram-negative bacteria is of great importance and remains a big challenge in antimicrobial studies. Generally, Gram-negative bacterial pathogens have additional outer membranes mainly composed of highly conserved and unique lipopolysaccharide (LPS) molecules, which serve as permeability barriers to prevent the cell bodies from toxic antimicrobial molecules¹⁻² including many photosensitizers, which have been successfully used in photoinactivation of Gram-positive strains.³⁻⁴ Furthermore, LPS, a potent inducer to human innate immune systems, hence called endotoxin, is now primarily responsible for sepsis associated with the infections of Gram-negative pathogens.⁵⁻⁶ Therefore, the most promising strategy is use of LPS neutralizing peptide as the binding ligand to deliver antibacterial agents meanwhile minimize the side effects.⁷

The first objective of the research in part I is the design and synthesis of photosensitizer-peptide conjugates for multivalent targeted cellular imaging and photodynamic inactivation (PDI) against Gram-negative bacterial strains. This simple and specific strategy was based on the conjugation of protoporphyrin IX (PpIX), a photosensitizer (PS), with lipopolysaccharide (LPS) binding peptides through a divalent manner. The fluorescent imaging and photodynamic antimicrobial chemotherapy (PACT) studies indicated that the divalent PpIX-peptide conjugate could really serve as a fluorescence probe to image the bacterial cells. Furthermore, those synthetic porphyrin-peptide conjugates indicated promising activities against Gram-negative pathogens even for those with antibiotics resistance upon the exposure to light irradiation.

In part I, the second objective is the development of dual-functional nanosystems for both detection and clearance of bacterial lipopolysaccharide (LPS or endotoxin). The dual-functional nanoparticles were synthesized by integration of core-shell $\text{Fe}_3\text{O}_4@\text{SiO}_2$ magnetic nanoparticles with perylene-diimide (PDI) conjugated

LPS-recognizing peptides. The detection mechanism of our rational design is mainly based on the fact that the specific interactions between LPS and neutralizing peptide could break up the π - π stacking conformation of neighbouring PDI molecules on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticle surfaces, and thus efficiently turn on the self-quenched fluorescence. More importantly, the stable and easily controlled LPS sensing nano-platform can also rapidly remove the toxic LPS from bacterial cell lysates and human serum without obvious loss of total proteins. Using the detoxified serum supplemented medium as macrophage culture medium could greatly decrease the nitric oxide generation of macrophages that was based on the inflammatory stimulation by LPS. This was the first example of a simple and novel magnetic nano-platform to address the challenging requirement for both effective detection and removal of LPS in biological samples.

Part II: Small Peptide and Membrane Lipid Based Self-assembled Nanostructures for Controlled Drug/gene Delivery

The usage of nanoscale materials may greatly facilitate the development of novel therapeutic methods for controllable release of bioactive agents at location of diseases with desired dosage, which has been considered to be of clinical significance to guarantee the curative effect while minimizing the drug resistance and other side effects.⁸⁻¹⁰

Among the available nanocarriers, the self-assembled nanostructures from highly biocompatible materials (e.g. oligo-peptide, membrane lipids, etc) have been extensively studied due to their unique features of high water content, high drug/gene loading efficiency and promising therapeutic effect.¹¹⁻¹³ More importantly, they are naturally biocompatible and biodegradable thus ensuring desirable bio-safety, whereas in terms of inorganic nanoparticle-based delivery systems, additional surface modifications were often required to achieve a good biocompatibility.

In part II, my first project is the development of novel drug-loaded nanogels for pH sensitive drug release, which will be described in chapter 3. In our research, the most commonly used model drug doxorubicin (Dox) was conjugated to an Fmoc group protected short peptide through an acid-labile hydrazone-bond to form a peptide-Dox prodrug. Cellular imaging studies displayed that the peptide nanogels could self-deliver drugs into cells through endocytosis and therefore provided effective antitumor activities toward resistant tumor cells. Moreover, our results also unequivocally indicated that ROS could serve as one non-negligible factor to overcome the endo/lysosomal barriers and to facilitate the fast translocation of caged drugs into nucleus. This study provided a simple strategy to investigate the details regarding the rapid intracellular drug redistribution by overcoming the different biological barriers in resistant cancer cells, which could help to better understand the mechanisms of drug delivery, and it may also facilitate the rational design of new carrier systems towards the enhanced drug activity in cancer therapy.

The second project of the part is the rational design of novel clickable membrane vesicles (MVs) for tumor-targeted drug/gene delivery. The cell derived MVs are of important self-assembled membrane structures, which are found to play critical roles

in antigen presentation, cell to cell communication, cell migration and so on.¹⁴⁻¹⁶ In our research, a novel chemical engineered membrane vesicle was developed by introducing an amphiphilic benzophenone derivative into membranes. Meanwhile upon Ca^{2+} stimulation, a greatly increased production yield of MV from HEK 293 cells was achieved in our research. Our preliminary results indicated that these MVs could be functionalized with additional azido tagged ligands (e.g. Cy5 molecules, oligo His₆, etc) through click chemistry. The subsequent study using such clickable MVs as controlled drug/gene delivery vehicles is still in progress.

Part I

Peptide Functionalized Materials for Gram-negative Bacteria

Targeted PDT and LPS Sensing

Introduction

I-1 Bacterial drug resistance and new antibacterial therapeutics and strategies

Since penicillin was first applied in the 1940s in clinic practice, various antibiotics have been developed in successful treatment of microbial infections. Large amounts of antibiotics have been used during the past few decades in worldwide, which were estimated to be 100,000–200,000 tones every year.¹⁷ Although antibiotics have saved millions of lives since 1940, their usage is a source of major concern today because of the emergence of resistance. Actually, not long after penicillin was first developed for clinical use, mechanisms of resistance developed by bacteria have plagued their therapeutic use (Table I-1).¹⁷ Basically, the extensive use of antibiotics in daily life especially in healthcare and agriculture does provide a potential selective way for bacteria drug resistance and which is often considered to be the main reason for the rise of resistance.¹⁸⁻¹⁹ This selective evolutionary pressure resulted in the spread of resistance through *de novo* gene mutations and even horizontal gene transfer between bacterial cells.²⁰⁻²¹ The research based on analysis of available bacterial genome sequences indicated that there would be more than 20,000 resistance relative genes of hundreds of different types. Fortunately, only a small part of mutants among them were predicted to be the functional determinants in pathogens resistance.²²

Furthermore, to make matters worse, some of bacterial pathogens that may cause infection diseases have developed their multidrug-resistant subgroups subsequent to antibiotic use. Actually, some strains have become resistant to practically all of the commonly used antimicrobial agents. For example, the methicillin resistant *S. aureus* (MRSA) has been found resistant to not only methicillin but also many other antibiotics.²⁴ In the recent study of skin and soft tissue infections in European medical centre, *S. aureus* was found to be the most common pathogens in clinic cases (71% of cases), where 22.5% of the isolates being methicillin resistant.²⁵ Such a serious pathogen was commonly found in hospitals and responsible for large numbers of

Table I-1. List of dates of deployment of commonly used antibiotics and their resistance observed. [Source ²³].

Antibiotics	Year deployed	Resistance observed
Sulfonamides	1930s	1940s
penicillin	1943	1946
streptomycin	1943	1959
chloramphenicol	1947	1959
tetracycline	1948	1953
erythromycin	1952	1988
vancomycin	1956	1988
methicillin	1960	1961
ampicillin	1961	1973
cephalosporin	1960s	late1960s

deaths.²⁶ Some MRSA strains evolved into “Superbug” resistant to vancomycin, a type of glycopeptide antibiotic, which was thought to be the last line of defense against methicillin resistant strains.²⁷ The multi-drug resistant *M. tuberculosis* (TB) for another case, a common pathogen mainly found in developing and industrialized countries, is often acquired in early life of children.²⁸ According to recent data from WHO (2010), every second, one more person in the world is newly infected with *M. tuberculosis* and about 10 percent of these infected people will become sick or infectious later.²⁹

The economic cost caused by antibiotic resistance is already enormous. For example, in 2007, there were about 400,000 people infected by multidrug-resistant bacteria and the infections cause 25,000 deaths in Europe. Treatments of these infections cost over €1.5 billion each year. In USA, antibiotic-resistant infections are responsible for estimated dozens of billions US dollar per year in terms of excess health care costs.¹⁹

Antibiotic resistance will always be a worldwide problem because bacteria can continuously find their ways to avoid or decrease the killing of antimicrobial reagents. Fortunately, the government and scientists have recognized the problem and will attend to address the spread of antibiotic resistance. People are searching approaches

to fight back to resistance by improving prescription strategies and increasing interests in the development of novel antimicrobial strategies.

Figure I-1 New strategies against bacterial infection.

In terms of serious resistance, the development of new antibacterial therapeutics and strategies is very important not only for human health-care use but also for agriculture and stockbreeding. As indicated in Figure I-1, a lot of efforts have been conducted to combat resistance, including the discovery of novel antibacterial targets,³⁰ novel antibacterial agents³¹ (small molecules, hybrid bio-conjugates, nanomaterials, etc), as well as the exploring of new strategies such as the drug combination therapy³² or even some multidisciplinary approaches (e.g. photodynamic therapy, etc).³³ Genomics has guided the discovery of new drug targets for novel-acting inhibitors based on target-based screening. Up to now, several potent antimicrobial agents have been developed toward the newly discovered targets such as the inhibitors against two-component signal transduction systems (TCSTS),³⁴ co-enzyme metabolism,³⁵ protein secretion, cell division, and novel protein in DNA replication.³⁰ However, among them, only inhibitors to peptide deformylase (PDF) have reached Phase II trials so far.³⁶ Except for the searching of novel antibiotics based on the target based screening methods, the development of new strategies and alternative approaches is also very important to combat resistence.³⁷⁻³⁹

I-2 Photodynamic antimicrobial therapy (PACT) against resistance

Figure I-2 Photochemical mechanisms in photosensitized reactions

To address the problem of antibiotic resistance, there has been increased interest in the searching of novel, convenient and non-resistant approaches. One promising multidisciplinary approach for microbiological inactivation is photodynamic antimicrobial chemotherapy (PACT).⁴⁰⁻⁴¹ Although the mechanism based on photodynamic inactivation (PDI) has been widely used in cancer treatment and some cases have been approved by FDA,⁴² the usage of PDI in the treatment of microbial infection is still in its infancy. Such a simple and controllable system consists of three key elements: molecular oxygen, a photosensitizer and a light source with an appropriate wavelength. The term “photodynamic action” here is used to define the photochemical reactions in which singlet oxygen was generated by consuming oxygen.⁴³ Upon excitation, the lowest excited singlet state (S₁) of the sensitizer could be achieved through photon transition. Intersystem crossing (ISC) subsequently generates the triplet state (T₁) of sensitizer (Figure I-2). Meanwhile, the thereby excited photosensitizer in its triplet state generates bio-toxic free radicals (type I mechanism) and singlet oxygen (type II mechanism).⁴⁴⁻⁴⁵

The process of antimicrobial therapy based on generated singlet oxygen was called PACT (photodynamic antimicrobial chemotherapy). Normally, porphyrins or related compounds (e.g. photofrin and foscan) were the most commonly used photosensitizers (PSs) to generate singlet oxygen (¹O₂) when exposed to sunlight. Singlet oxygen is highly reactive due to pairing of two electrons in one π^*2p anti-bonding orbital. Because of its lifetime of approximately 2 μ s in aqueous media, singlet oxygen causes

only highly localized damage to bio-molecules such as membrane lipids, proteins and DNA, thus leading to cell killing.⁴⁶ There are many advantages of PACT compared to standard therapies such as: ⁴⁷(1) bacterial resistance to PACT has not been shown to occur; (2) their controllable properties on photo-inactivation may ensure the less side effects compared to other traditional antimicrobial methods.⁴⁸ Generally, an ideal photosensitizer possesses the favorable characteristics to display low dark toxicity, an ideal absorption band (600-800 nm), a high quantum yield of triplet state as well as a long triplet life time.

Figure I-3 Photochemical and photobiological mechanisms in photodynamic therapy.

Except for its direct cell killing effect, PACT has also indicated promising properties on damaging or inhibiting bacterial biofilm formation.⁴⁹⁻⁵⁰ Biofilms contribute a protected mode of bacterial growth that allows their survival in an antibiotics enriched environment, which were considered to play important roles in bacterial antibiotics resistance.⁵¹ In general, biofilms can tolerate antibiotics at a concentration that is about 10–1,000 times higher than those needed to kill equivalent bacteria in planktonic type.⁵² By using photofrin as the PS and a 630 nm laser as light source, the viability of *S. mutans* in plaque-like biofilms was significantly reduced at 30 J/cm² light dosage.⁵³ In addition, bacterial swarming defined as a rapid multicellular bacterial movement has been shown to play key roles in the initiation of biofilm formation on a surface.⁵⁴⁻⁵⁵ More recently, we and our cooperators have demonstrated that bacterial swarming model (e.g. collective motion) could be dramatically disrupted during PDT process before the cell death.⁵⁶ The result may imply meaningful information on the relationship between PACT and bacterial swarming viability or biofilm formation.

Although photodynamic antimicrobial chemotherapy exhibited many advantages in bacterial inactivation than traditional therapeutic methods, one of the major challenges is specific cellular localization of PS in the bacteria rather than the host tissues to minimize the side effects upon illumination.⁴³ In consideration of the highly negatively charged bacterial cell surface, many efforts have been made on the development of cationic photosensitizers (e.g. phenothiazine or some porphyrin derivatives) for effective photo-inactivation of a broad spectrum of bacteria.⁵⁷⁻⁵⁸ Compared to anionic or neutral PSs, cationic ones exhibit the relatively higher affinity for bacterial cells by attaching to highly negatively charged cell wall components such as lipoteichoic acid and lipopolysaccharide through electrostatic interactions.³³ However, the membranes of mammalian cells consist of various phospholipid species that are also highly negatively charged. It is difficult to achieve bacteria selective inactivation in infection sites by such simple cationically modified photosensitizers.⁵⁹

I-3 Peptide as affinity ligand for bacteria-targeting PACT

In terms of the weak binding affinity and poor selectivity of the most commonly used photosensitizers in photodynamic antimicrobial chemotherapy (PACT), conjugation of additional bacterial affinity ligands to PS molecules was an effective approach to maximize the selective accumulation of PSs at infection sites. Such bacteria-targeting ligands could be certain antibiotic molecules (e.g. beta-lactam antibiotics, vancomycin, etc), antibodies, cationic polymers or peptides.⁶⁰⁻⁶³ Recently, by conjugation of two vancomycin molecules to a PS molecule (porphyrin), a divalent vancomycin-porphyrin-vancomycin conjugate was developed in our lab which has been proved to be effective in photo-inactivation of Gram-positive pathogens including the vancomycin resistant *Enterococci* strains (Van-A and Van-B).⁶¹ Our result indicated that multivalent modification of photosensitizers with bacteria-targeting ligands could greatly increase the bacterial binding affinity via multivalent/polyvalent interactions between the disubstituted affinity ligands and bacterial strains, thus enhance the photo-inactivation effect. It was observed that there were some differences in susceptibility to PACT between Gram-positive and Gram-negative strains. Generally, Gram-positive strains were found to be more

sensitive to the photoinactivation caused by neutral or anionic photosensitizers.⁶⁴ Such difference is mostly attributed to the different structures of cell walls between Gram-negative and Gram-positive strains. The cell wall of Gram-negative bacteria contains an inner membrane and an outer membrane separated by a peptidoglycan layer (Figure I-4). The highly organized bacterial outer membrane contains a highly negatively charged lipid layer on the surface, which would block the effective attachment of PSs to bacterial membranes and thus intercept the toxic $^1\text{O}_2$.⁶⁵ To overcome the problem, additional binding ligands such as cationic polymers and poly-peptides were often employed in recent antibiotics delivery systems against Gram-negative bacteria.⁶⁶⁻⁶⁹

Figure I-4 Schematic of the Gram-negative bacterial cell wall, showing the lipopolysaccharide (LPS) layer out of the cell membrane.

Among all kinds of available affinity ligands, the short cationic peptides have attracted the great interests due to their easy preparation and structural modification. Furthermore, the selective photo-inactivation could be easily achieved by conjugation of the bacteria-targeting peptides to proper photosensitizers. Hamblin and Hasan et al. performed a series of *in vitro* and *in vivo* PACT studies with poly-lysine functionalized chlorin e6 or methylene blue conjugates,⁷⁰⁻⁷¹ in which polylysine served as the target ligand with a wide range of binding toward both Gram-negative pathogens. The cell-penetrating HIV-1 Tat peptide that was usually used in cancer drug delivery was also found to greatly enhance the PACT effect against Gram-negative strains.⁷² Similarly, by conjugation of an antibacterial peptide (KLAKLAK)₂ to PS molecule eosin Y, the greatly increased photo-inactivation effects toward *E. coli* and *S. aureus*

were achieved.⁷³

Although, up to now, several water-soluble polymers and polypeptides have been developed to achieve the improved bacterial localization of PSs and most of them have indicated enhanced photoinactivation effects,⁷³⁻⁷⁶ the development of simple and specific affinity ligand molecules that can selectively deliver PSs to the surface of Gram-negative bacteria pathogens has not been explored yet. Since the lipopolysaccharide (LPS) moiety was indicated to play important roles in preventing bacterial pathogens from the permeability of antimicrobial agents including some PSs, it could also be used as a specific target of PSs delivery systems by imploring a LPS targeting ligand. Besides, learning from the rational design of using multivalent vancomycin-porphyrin complex for effectively inactivating resistant strains, we developed a novel divalent photosensitizer (protoporphyrin IX, PpIX) delivery system by using LPS-recognizing peptides as Gram-negative bacteria selective targeting ligands (Chapter 1). The intracellular fluorescent imaging and photodynamic antimicrobial chemotherapy (PACT) studies supported our hypothesis that multivalent modification of potosensitizers with LPS-recognizing peptides could generate novel effective fluorescence probes and photo-active agents against Gram-negative bacterial pathogens especially for those with antibiotics resistance based on the increased binding of peptide-tagged sensitizer to bacterial surface.

I-4 Bacterial lipopolysaccharide (LPS): structure, detection and clearance

Figure I-5 Chemical structures of bacterial lipopolysaccharide and Lipid A

Bacterial lipopolysaccharide (LPS) is one key component in the outer membranes of most Gram-negative bacterial strains. Typically, one LPS molecule consists of three structural parts: Lipid A, oligosaccharide core structure and O-antigen (Figure I-5). Lipid A is the hydrophobic anchor that also serves as the main toxic part of lipopolysaccharide. The oligosaccharide core structure that covalently attached to the Lipid A can be further divided into two parts, the inner core that often contains unusual sugars and the outer core consists of common sugars. In some wild-type strains, there may be some additional O-antigen sugars extended from core structures (Figure I-5).⁷⁷⁻⁷⁸ Based on its structural properties, the LPS layer on Gram-negative bacterial outer membrane represents a highly negatively charged permeable barrier of bacterial cells from environmental stress factors including various antibacterial agents.⁷⁹

Furthermore, LPS was also well known as bacterial endotoxin, one type of potent inducers that may cause serious septic shock in the intensive cares⁵⁻⁶ and many other health problems such as pyrogenic reaction, diarrhea, hypotension and vascular blood clotting.⁸⁰ Currently, bacterial endotoxin associated septic shocks and related diseases become one of the leading causes of death in the western world and there are estimated 1.5 million cases reported annually.⁸¹ The extreme toxicity of bacterial endotoxin has thus urgently initiated extensive research efforts towards the development of novel and specific strategies to sensitively identify, and more importantly, to rapidly remove such highly toxic bacterial membrane components from biological and pharmaceutical products. One commonly used FDA-approved technique for LPS detection is the enzymatic limulus amoebocyte lysate (LAL) assay. In this system, LPS serves as a coenzyme that was required for the enzymatic reaction in the presence of synthetic color producing substrate. The cleavage of the substrate results in the color change of colorless reaction solution to yellow. However, this assay requires a rather long and complicated testing procedure, moreover, the results from LAL assay are also susceptible to environmental variations, especially in pH and temperature.⁸²⁻⁸³ Recently, an endotoxin-ELISA method was developed for LPS detection using a bacteriophage receptor protein as LPS binding protein in the modified ELISA system. Although this assay was reported to have a good sensitivity in detection of LPS from 0.05 EU/mL to 500 EU/mL, the procedure was rather complex and may be difficult to

control.⁸⁴ Except for the enzyme or binding protein-based techniques, several electrochemical assays have been reported to detect LPS in aqueous solutions. DNA aptamers were often used in such systems as the LPS selective binding ligands.^{80,85-87}

Very recently, several fluorescent or colorimetric methods based on the functionalized small molecules,⁸⁸⁻⁹¹ liposomes,⁹²⁻⁹³ or polymers⁹⁴ have been proposed for bacterial LPS sensing in aqueous solutions. Among them the LPS affinity ligands (e.g peptide, amino acid) functionalized polydiacetylene (PDA) liposomes have been extensively studied.⁹²⁻⁹³ The photo cross-linked polymeric liposomes have a deep blue color in the absence of LPS. Upon the binding of LPS to the embedded affinity agents, the color of liposomes would be changed from blue to red due to the change of the backbone conformation.

I-4.2 Clearance

Although all of these attempts for LPS detection have shown the great promise with their individual detection limit from micromolar to picomolar level,⁹²⁻⁹⁴ none of these assays was capable for effective clearance of LPS from the bio-samples. Given the clinical importance of LPS, a simple and specific system for clearing bacterial endotoxin is very important and remains a big challenge. Traditionally, several conventional techniques including ultrafiltration, two-phase extraction, chromatography and so on have been utilized to remove LPS contaminants from some biological preparations, especially for those target protein samples generated from recombinant microorganisms, mostly based on the individual physical, chemical and biological properties of lipopolysaccharides.⁹⁵⁻⁹⁶ These procedures provided different degrees of success in the separation of LPS from solutions containing biomolecules.⁹⁵ However, these techniques exhibited several potential shortcomings such as relatively long operation time, solvent consumption, complicated coupling of affinity ligands to matrix gel supports, and low protein recovery during removal process.

More recently, the affinity ligands engineered magnetic nanoparticles (MNPs) were chosen as solid platforms because they have demonstrated great promise in biomedical applications including cancer imaging and therapy,⁹⁷⁻⁹⁹ bio-separation¹⁰⁰⁻¹⁰¹, targeted drug or gene delivery,¹⁰²⁻¹⁰⁴ and the sorting of living cells and bio-molecules

such as DNA and proteins.¹⁰⁵⁻¹⁰⁷ Due to the favorable properties, the LPS binding ligand conjugated MNPs can also be used in the development of novel nanoplatfroms for bacterial cell or LPS capture and clearance. Zinc-coordinated bis(dipicolylamine) (bis-Zn-DPA) was recognized as a bacteria selective binding ligand which was often used in bacterial imaging *in vitro* and *in vivo*. A magnetic microfluidic device based on bis-Zn-DPA conjugated MNPs has been reported to effectively remove the bacterial cells and free LPS molecules from the infected blood samples.¹⁰⁶ Similarly, using MNPs as solid phase and gentamicin as bacterial affinity ligands to generate a magnetic nanosystem, which was also successfully applied in bacterial magnetic clearance.¹⁰⁸

Although the MNPs offered us nanoplatfroms enabled the convenient and rapid clearance of LPS, these techniques could not report the specific and sensitive determination of LPS directly. Therefore, the development of a simple and novel strategy with a combination of sensitive detection and efficient removal of LPS will be of great importance in pharmaceutical industry and such highly desirable studies have not been exploited yet. In the chapter 2, I will present a novel nanosystem combining the dual-functions of both detection and clearance of LPS.

Chapter 1

Peptide Conjugated Photosensitizer for Gram-negative Bacteria Targeted Imaging and Photodynamic Inactivation

1.1 Introduction

Generally, two distinct lipid membrane structures are found in Gram-negative bacteria, an inner membrane containing bilayer of glycerophospholipids and an outer membrane covering with a unique lipopolysaccharide (LPS) layer. Bacterial LPS is a type of amphiphilic molecules consisting of a lipid A structure, a lipid fragment with a short fatty-acid-acylated chains, and polysaccharides components with negatively charged phosphates. LPS has been shown as an efficient barrier to prevent bacterial pathogen strains from the permeability of antimicrobial molecules including some photosensitizers (PSs).^{6,109-110} Therefore, the searching of simple and specific affinity molecules that can selectively direct PSs to Gram-negative bacterial pathogens is of great importance and remains a big challenge. Besides the function as a cell wall component, LPS was also well known as an important bacterial signal molecule that may cause serious endotoxic shock or sepsis in intensive care units.^{5,111} One alternative way to eliminate or decrease septic shock will be the rational design of functional LPS neutralizing agents, which would disturb the LPS recognition of its protein receptors. Up to now, antimicrobial peptides have been extensively studied in development of LPS neutralizing agents and Gram-negative bacterial targeting delivery systems due to their simple preparation, easy chemical modification and unique biochemical properties.¹¹²⁻¹¹⁴ In the previous research of our co-workers, a series of LPS recognition peptides have been screened that exhibited promising binding affinity to LPS in micelle systems.⁷

Along with our continuous efforts in selective bacterial imaging and photo-inactivation based on multivalent bacteria-targeting agents, in this research, we performed a simple and rational design by conjugation of LPS binding peptides with a commonly used photosensitizer (protoporphyrin or PpIX) to form a potential Gram-negative bacteria targeting PACT agent. The cationic LPS binding peptide with the sequence of YVLWKRKRKFCFI-amide was chosen as the bacteria-targeting

ligand.

Figure 1.1 Schematic of the peptide-photosensitizer based Gram-negative bacterial LPS targeted photoinactivation.

This new designed divalent peptide-PpIX conjugate may indicate a unique advantage to selectively target to the surface of Gram-negative bacterial strains based on the enhanced multivalent/polyvalent interactions between disubstituted peptides and LPS moieties on bacterial strains, and thus serve as a promising fluorescent probe and potent photosensitizer for specific imaging and photodynamic inactivation of drug resistant Gram-negative bacterial strains.

1.2 Result and discussion

1.2.1 Synthesis and spectroscopic studies

In general, unlike most of Gram-positive organisms, the cell wall of Gram-negative strains contains a layer of lipopolysaccharide (LPS) in the outer membrane, which generally acts as a protective permeability barrier. The LPS molecules increase the negative charges to outer membrane, thus reduce the binding efficiency of many neutral and anion drugs. In the previous work of our co-workers, a series of *de novo* designed antimicrobial and antiendotoxin peptides have been developed. These peptides were designed based on the binding site of a LPS binding protein from Gram-negative strain *E. coli* and had been shown to selectively interact with LPS and adopt a boomerang like structure in LPS containing micelles.⁷

Figure 1.2 Schematic of the specific interaction between peptide and LPS

Figure 1.2 shows the mode of action, these peptides can penetrate into outer membrane of Gram-negative bacterial cells by binding with LPS, thus destabilize the membrane structures. In our study, we chose a well-designed analog of such LPS recognition peptides YI13WF with the sequence of YVLWKRKRKFCFI-amide as binding ligand due to its higher binding affinity to LPS component in the outer membrane of Gram-negative bacteria.

The peptide sequence was obtained through a multistep solid-phase peptide synthetic process based on Fmoc chemistry and 2-Chlorotrityl Chloride Resin. To facilitate the conjugation between porphyrin derivatives and peptides, a unique cysteine residue was introduced into the sequence. The short linker (**3**) was

synthesized through a few steps of process. As illustrated in Scheme 1.1, the maleimide

Scheme 1.1 Synthesis and characterization of the monomeric and dimeric PpIX-peptide conjugates. Reagents and conditions: (a): Boc_2O , Et_3N , EtOH , 0°C ; (b): 1. Maleic anhydride, Et_3N , Et_2O , 0°C ; 2. Ac_2O , Na_2OAc , $60 - 70^\circ\text{C}$; (c): TFA, DCM, from 0°C to rt.; (d) and (e): EDC HCl, HOSu, DMF, 0°C to rt.; (f): DIPEA, DMSO, rt.

tagged porphyrin derivatives were synthesized by conjugation of the short linker (3) with the bared protoporphyrin IX (PpIX). Finally, the aimed monomeric porphyrin-peptide conjugate (6) was obtained by linking porphyrin derivative (4) with a single peptide through the specific reaction between maleimide and the unique cysteine residue in peptide. Similarly, the dimeric conjugate (7) was obtained through the same reaction between porphyrin derivative 5 and excess amount of peptides.

Figure 1.3 HPLC analysis of PpIX-YI13WF dimeric conjugate 7 (A) and monomeric conjugate 6 (B).

The reaction mixture was then added into 10 mL diethyl ether to give a crude product precipitate followed by purification with reverse-phase HPLC. After overnight

lyophilization, the monomeric PpIX-peptide product **6** (yield 56 %) and the dimeric conjugate **7** (yield 48%) were obtained. The purities of the final products **6** and **7** were further analyzed by reverse phase HPLC, and found to be over 95% (Figure 1.3). Besides, the final products as well as the intermediates were also characterized by proton NMR spectra and mass spectrometry (ESI-MS).

Figure 1.4 UV-Vis absorption spectra and fluorescent emission spectra. A): absorption at concentration of 5.0 μM in DMSO; B): emission spectrum excited at 504 nm at the same concentration.

With the pure PpIX-peptide derivatives in hand, their photochemical properties were studied by measurement of the UV-Vis absorption and fluorescence spectra. As shown in Figure 1.4, both the monomeric and dimeric PpIX-conjugates exhibited similar absorption properties as free porphyrin where an intense Soret band near 400 nm and four Q-band peaks between 500 and 620 nm were detected. The fluorescence spectra of

Figure 1.5 Singlet oxygen generation measurement under white light irradiation monitored by ABDA.

the synthetic peptide PpIX-peptide derivatives are also similar to that of free PpIX molecule with no obvious difference observed (Figure 1.4). The results from spectroscopy study indicated that the peptide conjugation did not affect the absorption and emission properties of PpIX moiety. With the favorable results, we thought that whether such peptide modification would affect the generation of reactive oxygen species (e.g. singlet oxygen, etc) from the excited PpIX-peptide conjugates. To reveal this, the efficiency of singlet oxygen generation was determined by 9, 10-anthracenediyl-bis (methylene) dimalonic acid (ABDA) that is a well-established probe for singlet oxygen identification.¹¹⁵ As demonstrated in Figure 1.5, the result indicated that this two PpIX-peptide conjugates generate similar amount of singlet oxygen when excited by white light irradiation.^{61,115}

1.2.2 Anti-bacterial minimum inhibitory concentration (MIC) tests.

Table 1.1 MIC values of PpIX, free peptide YI13WF and PpIX-YI13WF conjugates.

	MIC (μ M)			
	<i>E.coli</i> DH5 α	<i>E.coli</i> BL-21	<i>K. pneumoniae</i>	<i>S.enterica</i>
Conjugate 7	2	4	8	4
Conjugate 6	8	32	32	32
YI13WF	8	32	32	~ 24
PpIX	> 64	> 64	> 64	> 64

The antibacterial studies were then performed by determining the minimum inhibitory concentration (MIC) of PpIX-peptide derivatives compared with free peptide and porphyrin. In our study, four standard Gram-negative bacterial strains including *E.coli* DH5 α (ATCC 53868), *S. enterica*. (ATCC 14028), ampicillin resistant strains *E. coli* BL21 (Amp^r *E. coli*) and *K. pneumoniae* (ATCC 700603) were chosen as model organisms. As expected, the antimicrobial peptide YI13WF exhibited an excellent activity against these tested bacterial strains. That may be mostly attributed to the strong binding ability towards LPS components existing at outer membrane of Gram-negative strains.^{7,116} Compared to the free peptide, monomeric PpIX-peptide conjugate shows the similar trend in the inhibition of bacterial growth (Table 1.1), implying that peptide modification with PpIX would not significantly affect the binding efficiency between the peptide and LPS moieties. Moreover, four to eight

fold-decreased MIC values were found in terms of dimeric PpIX-peptide conjugate against the same bacterial strains, indicating that the multivalent conjugation could greatly improve the LPS neutralizing effect of peptide derivatives. As a control, the antimicrobial activity was also evaluated in terms of free PpIX molecule alone (Table 1.1). The high MIC values (all $>64 \mu\text{M}$) of PpIX toward all tested strains clearly indicated the limited activity in inhibition of bacterial growth, which was consistent with other reported results.^{61,117-118}

1.2.3 Bacterial live cell imaging studies

On the purpose of monitoring the binding effects of PpIX-peptide conjugates towards various bacterial strains, living cell imaging was performed under a confocal laser scanning microscope (CLSM) upon excitation at the wavelength of 488 nm (within the range of Q band absorptions of PpIX). Typically, the bacterial cells were incubated with the tested compounds as described in the experiment part. As indicated in Figure 1.6, after incubation with PpIX itself, nearly no any obvious fluorescence was detected on bacterial cells. However, upon the incubation of the cells with PpIX-peptide conjugates, the fluorescence image indicated a stronger red emission signal from the bacterial cells. Furthermore, the cells stained with the dimeric conjugate were much bright than that stained with monomeric conjugate. The result further suggested a higher binding ability of the divalent peptide conjugate toward the surface of bacterial cells. The different imaging effects were further determined by quantification of fluorescence intensity. The FL intensity from the monomeric conjugate stained cells is about 8 times higher than that from PpIX stained cells. Such difference would even rise over 20 times when staining with the dimeric PpIX-peptides (Figure 1.6B). In contrast, after incubation with porphyrin itself, bacterial cells did not show obvious fluorescence signals, mostly because of the weak binding effect of anionic PpIX molecules with negatively charged bacterial surface. The result from living bacterial imaging and fluorescent intensity quantification clearly confirmed our hypothesis that LPS neutralizing peptide YI13WF may act as a LPS-targeting ligand for effectively directing PpIX onto the cell wall of Gram-negative bacterial strains. Indeed, as indicated in Figure 1.7, under the laser scanning microscope, the cell surfaces exhibit

Figure 1.6 Fluorescent staining of bacterial cells with free PpIX and its peptide derivatives. A) The bacterial cells were incubated with 2 μ M compounds at room temperature for 10 minutes. and fluorescence (excited at 488 nm) photos were taken through a long-pass filter at 650 nm. Scale bar =10 μ m; B) Quantitative analysis of bacterial staining based on the imaging data, mean $\pm$ S.E.; *, $P < 0.05$ versus the monomeric conjugate.

much bright red fluorescence than the central positions, indicating that the divalent PpIX-peptide conjugate could selectively bind to the cell wall components. The multivalent modification of PpIX indeed greatly improved the binding efficiency of PpIX derivatives toward living bacterial cells.¹¹⁸⁻¹²¹

Figure 1.7 White light and fluorescence images show the bacterial cell (*K. pneumoniae*) surface staining with dimeric PpIX-peptide. The bacterial cells were incubated with 2 μ M compounds at room temperature for 10 minutes.

Encouraged by the excellent bacterial imaging results, the most promising dimeric PpIX-peptide was further investigated in selective recognition and imaging of bacterial strains over co-cultured systems with bacterial and mammalian cell together.

Figure 1.8 Fluorescent imaging of Jurkat T cells and two antibiotics resistant Gram-negative bacteria: A) *E.coli* BL21 and B) *K. pneumoniae* incubated with dimeric conjugate 7 (4 μ M). Left: white light images, right: fluorescent images. The fluorescence of PpIX is highlighted in red.

In the process of selective imaging, Jurkat T cells, a mammalian cell line, were chosen to co-culture with Gram-negative bacteria Amp^r *E.coli* BL21 and *K. pneumoniae*

respectively. The dimeric PpIX-peptide conjugate was then added into the mixed solution of Jurkat T cells and bacterial cells. After incubation at 37 °C for a short time (10 min), the living cell images were taken by confocal fluorescence microscope. From Figure 1.8, we learn that in the co-culture mixtures of Jurkat T cells and bacterial cells, only the bacterial cells exhibit a significant red fluorescence while no obvious fluorescence signal was detected in Jurkat T cells (Figure 1.8 and Figure 1.11). Such higher fluorescence detected in bacteria clearly reflected the strong ability of dimeric PpIX-peptide conjugate in selective recognition of bacterial surface over mammalian cells. Compared to the different phospholipids phosphatidylcholine (PL) and phosphatidylserine (PS) in the mammalian cell membranes, the major components of LPS in outer membrane of Gram-negative bacterial cells would be the major reason to greatly contribute to the target selection of dimeric PpIX-peptide toward bacterial cells rather than mammalian cells.¹¹⁴

1.2.4 Photodynamic inactivation of bacteria

Figure 1.9 Bacterial photo-inactivation with PpIX-peptide conjugates. A and B, dose dependent effects in photo-inactivation against bacterial cells in the presence of 0.5 μM PpIX-peptide conjugates 7 and 6. C and D, survival curves of the bacterial cells upon the treatment with various concentrations of 7 and 6 at a certain energy of irradiation (30 J/cm²). *E. coli* DH5a (squares), *E. coli* BL21 (round dots), *S. enterica* (triangle upwards), and *K. pneumoniae* (triangle downwards).

To investigate whether our synthetic PpIX-peptide conjugates are capable in photodynamic inactivation of bacterial pathogens, the PACT treatments were performed by exposure of the tested bacterial strains to white light source in the presence of PpIX-peptide conjugates. After a certain time irradiation, the bacterial viability was evaluated by counting the colony forming units (cfu) after overnight culture on LB agar plates. It was found that both the monomeric and dimeric PpIX-peptide conjugates were effective in photo-inactivation against the bacterial strains including the drug resistance ones (e.g. ampicillin resistant *E.coli* BL21 and *K. pneumoniae*). Compared with the monomeric PpIX-peptide conjugate, the dimeric one demonstrated much higher photo-inactivation ability as more than 99 % bacterial

Figure 1.10 Photodynamic antibacterial activity towards bacterial cells. A): Photodynamic antibacterial activities of free PpIX against four selected bacterial strains: *E.coli* DH5a (squares), *E.coli* BL21 (round dots), *S.enterica* (triangle upwards), and *K. pneumoniae* (triangle downwards). B, C and D) Photodynamic antibacterial activity of Conjugate 7 (squares), Conjugate 6 (round dots), free YI13WF peptide (triangle upwards) and free PpIX (triangle downwards) at 2.0 µM against three Gram-negative bacterial strains compared with control where no compounds was employed.

lethality ($> 2.0 \log_{10}$ reduction) was obtained at only $0.5 \mu\text{M}$ upon 30 J/cm^2 of light irradiation (Figure 1.9 A and C). As expected, the control studies where the cells were not irradiated indicated a limited bacterial killing effect. Under the fixed concentration ($0.5 \mu\text{M}$) of photosensitizers, of all tested compounds, the dimeric conjugate has been shown to have more potency in photo-inactivation of bacteria. This photodynamic bacterial inactivation indicated more potent activity than the dark activity based on MIC values of both monomeric and dimeric PpIX-peptide conjugates against all the tested bacterial strains. Moreover, the efficiency of bacterial photo-inactivation was also determined to be concentration dependent. A significant bacterial killing with over 99.9 % bacterial cells death ($> 3.0 \log_{10}$ reduction) could be easily obtained when the photo-inactivation was conducted in the presence of a relative higher concentration of dimeric PpIX-peptide ($2 \mu\text{M}$) (Figure 1.9 and Figure 1.10). In contrast, the free PpIX alone could not produce any detectable bacterial inactivation when the bacterial strains were irradiated with the same white light at the same dosage (Figure 1.10).

Furthermore, the viability test of Jurkat T cells was conducted by Tox-8 assay upon the white light irradiation in the presence of PpIX-peptide conjugates in cell culture medium. The result indicated that no significant cell viability changed after the treatments under both the light irradiation and dark condition (Figure 1.12). The above results clearly supported our hypothesis that the divalent PpIX-peptide conjugate could offer an effective photodynamic agent for inactivation of Gram-negative pathogens over mammalian cells.

1.3 Conclusion

In this work, we presented a rational design and development of a simple and novel strategy for effective fluorescent imaging and photo-controllable inactivation of Gram-negative bacterial cells by the conjugation of photosensitizer protoporphyrin IX (PpIX) with two lipopolysaccharide (LPS) recognition peptide sequences. By benefiting from the excellent and multivalent binding of the cationic peptide towards the LPS components, the dimeric PpIX-peptide conjugate can effectively direct the photosensitizer PpIX to the outer membrane of Gram-negative bacteria and thus increase the local concentration of PSs. Under illumination of white light, the dimeric

PpIX–peptide conjugate has shown a potent activity against the growth of Gram-negative pathogens including drug resistant bacterial strains. Besides the potential photodynamic antimicrobial application, the dimeric PpIX–peptide conjugate has also displayed the promising properties in real-time fluorescent imaging of living bacterial cells. Most importantly, both imaging and photo-inactivation experiments have also demonstrated that the dimeric PpIX-peptide conjugate could selectively target bacterial cells over mammalian cells and generate less damage to mammalian cells. This rational and novel developed strategy based on the divalent LPS affinity peptide conjugate may probably provide the great possibility for further exploring the biological processes in Gram-negative bacterial strains under *in vitro* or *in vivo* settings. It could also supply a potential alternative method for the effective inactivation of bacterial pathogens especially for those with drug resistant properties.

1.4 Experiment part

1.4.1 HPLC analysis of PpIX-YII3WF conjugates

The purity analysis of the aimed products was conducted on a Shimadzu reverse-phase HPLC system using an Alltima C-18 (250 x 4.6 mm) column. An eluting system consisting of A (water with 0.1% TFA) and B (acetonitrile with 0.1% TFA) was used under a linear gradient to elute the product which was monitored by UV-Vis absorption at 280 nm and 400 nm. The column was washed with 30% solution B for the first 2 minutes followed by a gradient started from 30% to 80% solution B in the next 43 minutes.

1.4.2 Photodynamic antibacterial activity towards bacterial cells

Bacterial cells were grown and washed as described in the section of imaging and MIC tests. From OD₆₀₀ of 0.7 in PBS, the bacterial solution was conducted a dilution to reach 2×10^8 cfu/mL. 60 μ L aliquots of this bacterial suspension were mixed with the tested compounds (in 60 μ L PBS) individually in a well of the 96-well plate under the final concentration of compound (0, 0.25, 0.5, 1.0 or 2.0 μ M). The plate was then incubated in the dark at 37 °C for 20 minutes followed by light irradiation (50 mW/cm²) for 0, 2, 5 and 10 minutes. 20 μ L from the irradiated solutions were taken

out at the desired light exposure time and further underwent a serial dilution to give various ten times concentrations from 10^7 cfu/mL to 10^4 cfu/mL. Then 25 μ L solution from each diluted suspension was subsequently plated onto LB agar plates followed by overnight incubation at 37 °C in the dark. The accounting of colony forming units was conducted after incubation and the data was plotted into a log scale.

1.4.3 Bacterial cell selective staining over Jurkat T cells

Live Jurkat T cells alone in PBS (pH 7.2) were incubated with or without 4 μ M of dimeric PpIX-peptide **7** at 37 °C for 10 min. After being harvested by centrifugation for 5 min at 500 g, the cell pellets were suspended in PBS. 8 μ L of this cell solution was dropped onto a poly-L-Lysine coated coverslip glass slide followed by slightly covering for immobilization. Fluorescent images were acquired by using confocal laser scanning microscope (Nikon Eclipse TE2000-E, CFI VC 100 $\times$ oil immersed optics), equipped with a 488 nm laser and a LP emission filter at 650 nm.

Figure 1.11 Fluorescence images of Jurkat T cells alone incubated with 4 μ M of dimeric PpIX-peptide **7** (A) or without any compound (B) at 37 °C for 10 minutes. Left: white light images, right: fluorescent images. The fluorescence of PpIX is highlighted in red.

1.4.4 Photo-toxicity of conjugate 7 against Jurkat T cells

The cell toxicity assay was performed through standard Tox-8 assay (from Sigma

Aldrich). Bioreduction of the dye resazurin by viable cells transfer its oxidized form (blue color) to reduced form (red color),¹²² and meanwhile the fluorescence intensity peak at 590 nm increases. In brief, Jurkat T cells were cultured in a 96-well plate at cell density of 2×10^4 cells/well, and then were incubated with the dimeric PpIX-peptide conjugate (**7**) of various concentrations at 37 °C for 20 minutes. After that, the cells were then irradiated with white light for 10 minutes to reach a dosage of 30 J/cm², and cell was then incubated for further 24 hours in dark. For the dark control, the plate with the cell and conjugate **7** was kept in dark during the whole process. Once removing the culture medium by centrifugation, the cells were subsequently incubated with the Tox-8 reagent 10% in basic RPMI 1640 (from Sigma Aldrich). Two hours later, the absorbance was detected at the wavelength of 600 nm by a Tecan's Infinite M200 microplate reader. The absorbance measurement at the wavelength of 690 nm was set as reference. The data from the well without cells but with Tox-8 reagent were used as reagent controls. Each experiment was performed in five repeats.

Figure 1.12 Photo-toxicity of the dimeric PpIX-peptide conjugate towards Jurkat T cells

The cell viability rate (VR) was calculated based on the following equation: where A is

$$VR = [(A-B)/(A-C)] \times 100\%$$

the absorbance of the Tox-8 control, B is absorbance of experimental groups treated with conjugate **7** with or without irradiation, and C is absorbance of the control group without **7** and irradiation,

1.4.5 Bacterial imaging and fluorescence intensity quantification

The bacterial cells were cultured and treated as described in the experiment section of the manuscript. After incubation with free PpIX and its two conjugates at 2 μ M for 20 min in dark at 37 °C, the cells were collected and washed with PBS by centrifugation to remove the unbound compounds. A small portion of the suspension (8-10 μ L) was dropped onto poly-L-lysine pretreated coverslips followed by immobilizing with another coverslip. Fluorescence images as well as transmitted light images were acquired with Confocal Laser Microscope (Nikon Eclipse TE2000-E, CFI VC 100 $\times$ oil immersed optics), using a laser at wavelength of 488 nm and a LP emission filter centered at 650 nm.

Fluorescence intensity quantification based on the living cell imaging was performed with Image J software 1.44 (NIH) according to its standard manual and the reported method.¹²³ In brief, all the bacterial cells in the images were selected individually using the freehand selection tool of the software. The mean pixel intensity of each bacterial cell on the fluorescence image was measured. In addition, in the same image the background was measured on the area outside the cell covered area, which was later subtracted from the values obtained from the stained cells. Statistic analysis was carried out using Origin 8.0 software. Error bars reflect the standard error of the mean.

Chapter 2

Dual-functional Magnetic Nanoparticles for Effective Detection and Clearance of Bacterial Lipopolysaccharide (LPS)

2.1 Introduction

Bacterial lipopolysaccharide (LPS), one key component in the outer membrane of Gram-negative bacterial cell wall, has been found to help bacteria resist antibiotics as well as the complement system and other environmental stresses.⁵⁻⁶ Furthermore, this plasma membrane permeable molecule was also well known as bacterial endotoxin, one type of potent inducers towards the innate immune system that may cause serious septic shock in the intensive cares.⁵⁻⁶ Besides, LPS was also responsible for many other health problems such as pyrogenic reaction, diarrhea, hypotension and vascular blood clotting.⁸⁰ Therefore detection of LPS contamination is highly necessary in pharmaceutical production and highly relevant in life science and medicine.^{87-88,90,92-94} The enzymatic limulus amoebocyte lysate (LAL) assay based on the clotting of the LAL and endotoxin has been practiced used in clinical for LPS detection. However, it requires a rather long and complicated procedure and the results are susceptible to environmental changes, especially pH and temperature.⁸²⁻⁸³ Recently, some dye functionalized small molecules,⁸⁸⁻⁹⁰ liposomes⁹²⁻⁹³ and polymers⁹⁴ have been developed for fluorescent sensing of LPS. Although some of those reported systems have been demonstrated to be capable for LPS detection, none of these assays indicated the capacity for the effective separation of LPS from the detection mixtures. Traditionally, several conventional techniques including ultrafiltration, two-phase extraction, chromatography, etc have been utilized to remove LPS contaminations from certain biological preparations, especially for those target protein samples generated from recombinant microorganisms, mostly based on the individual physical, chemical and biological properties of lipopolysaccharides.^{95-96,124-125} These procedures provided different degrees of success in the separation of LPS from solutions containing biomolecules. However, several potential shortcomings such as relatively long operation time, solvent consumption, complicated coupling of affinity ligands to

matrix gel supports, and low protein recovery during removal process may still exist, which make the efficient and cost-effective clearance of LPS quite challenging.⁹⁵⁻⁹⁶ Although the reasonable LPS separation from complex liquids has also been proposed recently by using functionalized nanoparticles,^{124,126} these techniques may not report the specific and sensitive determination of LPS directly, and the effective LPS clearance could be potentially affected by environmental changes (e.g. pH).¹²⁴ Therefore, the development of a stable and easily controlled strategy with a combination of sensitive detection and efficient clearance of LPS in complex biological samples such as bacterial cell lysate and human serum will still be of great importance in pharmaceutical industry and such highly desirable studies have not been fully explored yet.

Figure 2.1 LPS neutralizing Peptide functionalized Fe₃O₄@SiO₂ core-shell nanoparticle for fluorescence turn on detection and capture of LPS.

Here we present such a dual functional magnetic nanoparticle for both detection and clearance of bacterial LPS by introduction a dye molecular conjugated LPS binding ligand onto magnetic nanoparticles. Such affinity ligand engineered magnetic nanoparticles (MNPs) have shown promise in applications in bio-imaging,¹²⁷ bio-sensors,¹²⁸ and the sorting of living cells or bio-molecules.^{100,106,108,129} Considering that LPS is an amphiphilic and highly negatively charged bio-molecule, a sensitive and selective binding ligand must have a strong affinity towards LPS through

both electrostatic and hydrophobic/hydrophilic effects. The short synthetic peptides are such promising molecules for LPS binding, since their easy preparation, and moreover, their convenient modulation on the balance of electrostatic and hydrophobic/hydrophilic interactions towards LPS by simply changing the amino acid residues.^{114,130-131} Along with our continuing efforts in the exploration of LPS neutralizing peptide for LPS neutralization,⁷ bacterial selective imaging¹³², we herein employ a novel strategy by conjugating a rational designed LPS neutralizing peptide

Figure 2.2 TEM images of Fe_3O_4 nanoparticles (a) and the $\text{Fe}_3\text{O}_4@\text{SiO}_2$ core-shell nanoparticles

(YVLWKRKRKFCFI-amide) to the surface of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ core-shell nanoparticles to form a peptide functionalized magnetic nanoparticle complex. A short linker with perylene diimide (PDI) moiety was introduced between the nanoparticle and peptide. In the absence of LPS, the fluorescence of the highly hydrophobic perylene diimide molecules undergo self-quenching mostly because of its strong $\pi\cdots\pi$ stacking ability.¹³³⁻¹³⁵ However, in the presence of LPS, the strong interaction between the peptide and LPS would pull the hydrophobic part of LPS into the hydrophobic gap between the perylene diimide molecules, thus enlarging the distance between them and then causing the fluorescence enhancement (Scheme 2.1). Moreover, the LPS captured MNPs can be easily separated by a simple magnet, therefore reducing the endotoxin contents in the LPS contaminated biological solutions.

2.2 Result and discussion

2.2.1 Synthesis and characterization

Scheme 2.1 Synthesis of perylene diimide-peptide conjugate. a) imidazole, 125 °C, 4h; b) Mal-PEG₂-NHS ester, propargyl-NHS ester, DIPEA, DMF, r.t, overnight; c) DIPEA, r.t, overnight.

This short peptide was obtained by the Fmoc-chemistry based solid phase peptide synthesis. To facilitate the conjugation, a single Cys residue was introduced into the sequence.

Figure 2.3 TEM image and size distribution analysis of Fe₃O₄ nanoparticles

The monodispersed Fe_3O_4 nanoparticles with a narrow size distribution of about 9 nanometers were prepared according to the reported procedure (Figure 2.3).¹³⁶ Then, the silica shell with a layer of amide group was introduced according to the reported

Figure 2.4 FTIR spectra of free ligands, amino group functionalized $\text{Fe}_3\text{O}_4@\text{SiO}_2$, and azido group subsequently functionalized $\text{Fe}_3\text{O}_4@\text{SiO}_2$.

microemulsion based procedure¹³⁷ to form the $\text{Fe}_3\text{O}_4@\text{SiO}_2$ core-shell nanoparticles. The azide group with a short PEG linker was then conjugated to the silica surface through the reaction between the NHS-ester and amide group on particles. The presence of azido groups on particle surface was subsequently confirmed by FTIR

Figure 2.5 TEM image and size distribution of monodisperse PDI-peptide conjugated MNPs.

spectra where peaked at about 2110 cm^{-1} (Figure 2.4). The perylene diimide derivative functionalized with a maleimide group at one end and an acetylene group at another

end was chosen as the connection linker between the nanoparticle and LPS binding peptide. The peptide was firstly conjugated to the connection linker through the reaction between the unique cysteine residue and maleimide, and then introduced onto the nanoparticles by “Click-reaction”. TEM image shows that the peptide functionalized

Figure 2.6 UV-Vis absorption spectra of free PDI-peptide (2.0 μM), MNP (200 $\mu\text{g/mL}$), MNP_{PDI} (200 $\mu\text{g/mL}$) and $\text{MNP}_{\text{PDI-peptide}}$ (200 $\mu\text{g/mL}$) in DMSO

Figure 2.7 UV-Vis absorption spectra (right) and fluorescence emission spectra¹³⁸ exited at 450 nm of 200 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$ in different solvents.

magnetic nanoparticles ($\text{MNP}_{\text{PDI-peptide}}$) have a narrow size distribution of about 25 nanometers (Figure 2.5). After getting the $\text{MNP}_{\text{PDI-peptide}}$, the amount of peptide conjugate on particles was determined to be 10 nmol/mg by UV-Vis absorption spectra. Besides, a control particle with PDI moieties but without peptide was prepared followed the same synthetic process. The presence of PDI on particle surface was

confirmed by UV-Vis absorption spectra (Figure 2.6), and then the fluorescent properties of $\text{MNP}_{\text{PDI-peptide}}$ in different solvent were studied. The fluorescence intensity dramatically decreased when water was introduced into the solution (Figure 2.7). In addition, an obvious change was observed on the UV-Vis spectra during this progress. Unlike the strong fluorescence in DMSO, only very weak signals observed in water clearly suggested the significantly different aggregation between the neighboring PDI molecules on the particle surfaces in water and in DMSO. The larger $\pi \cdots \pi$ stacking of PDI in water could thus efficiently self-quench the fluorescence.

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2.2.2 LPS-selective fluorescence turn-on detection by $\text{MNP}_{\text{PDI-peptide}}$

With the favorable self-quenching properties in hand, to prove the concept, we further examined if $\text{MNP}_{\text{PDI-peptide}}$ may respond to LPS endotoxin and induce fluorescence change in buffer solutions. To do this, $\text{MNP}_{\text{PDI-peptide}}$ (100 $\mu\text{g/mL}$) was incubated with 10 μM LPS in TBS buffer (Tris 50 mmol/L, NaCl 150 mmol/L, pH 7.4) at 37°C. The fluorescence ($\text{ex}_\lambda = 450 \text{ nm}$) spectra were recorded every 3 minutes on a fluorescence spectrometer. As demonstrated in Figure 2.8, the fluorescence intensity increased during the first few minutes, but remained nearly constant after 15 minutes incubation. The result implied that the interaction between the peptide and LPS could quickly disrupt the stacking between perylene diimide molecules, thus recovering the fluorescence.

This disruption of PDI-stacking was further confirmed by the results from fluorescence lifetime measurements, in which the fast decay component suggested from some stacked conformation¹⁴¹ disappeared in the presence of LPS (Figure 2.9).

Figure 2.8 Increasing fluorescence intensity of $\text{MNP}_{\text{PDI-peptide}}$ (100 $\mu\text{g/mL}$) in TBS upon addition of 10 μM LPS at different time interval.

As a control, the MNP_{PDI} conjugate without LPS recognition peptide sequence on the surface was also applied to respond to the addition of LPS (Figure 2.10a). No obvious fluorescence change observed clearly confirmed that the LPS neutralizing peptide functioned as an indispensable moiety to specifically recognize LPS endotoxin, which changed the conformation of stacked PDI dye molecules and eventually released the fluorescence from $\text{MNP}_{\text{PDI-peptide}}$. For a further study, $\text{MNP}_{\text{PDI-peptide}}$ (100 $\mu\text{g/mL}$) was incubated with different concentrations of LPS (from 0, 0.1 μmol -25 μmol) in TBS buffer. Figure 2.10-a indicated that the fluorescence intensity enhanced as the concentration of LPS increased. Over 7-fold increases of the FL intensity at 539 nm and 580 nm were observed after 15 min incubation with 10 μM LPS. From the plotting of the normalized fluorescence intensity at 539 nm versus the LPS concentration, a linear-like relationship was observed. The LPS detection limit for 100 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$ in TBS buffer was calculated to be 28 nmol according to the reported methods,⁹⁴ which was comparable to some other reported systems.^{88-89,93}

Figure 2.9 Fluorescent lifetime measurements of 100 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$ in TBS buffer (pH 7.4) after 15 min incubation with (red points) or without (purple points) LPS (10 μmol).

Figure 2.10 a) Fluorescence spectra (excited at 450 nm) of $\text{MNP}_{\text{PDI-peptide}}$ and MNP_{PDI} with or without LPS (10 μM) in TBS; b) Fluorescence spectra of $\text{MNP}_{\text{PDI-peptide}}$ (100 $\mu\text{g/mL}$) after incubation with different concentrations (from 0, 0.1 to 25 μM) of LPS in TBS buffer at 37 $^{\circ}\text{C}$. The inset shows the plot and linear fitting of normalized fluorescence intensity at 539 nm vs the concentration of LPS (0-2.0 μM); c) The selective increase of fluorescence upon addition of LPS against various biologically important species (at a concentration of 10.0 μM). d) The fluorescence properties of 100 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$ in the absence or in the presence of LPS (10 μM) in TBS buffer under different pH values (pH =4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0, 11.0).

Since the fluorescence response of $\text{MNP}_{\text{PDI-peptide}}$ has been shown to be high sensitivity to LPS, a further selective study was performed by incubation of $\text{MNP}_{\text{PDI-peptide}}$ with various small molecular or macromolecular bio-interferes.

Figure 2.11 Competitive binding analysis of $\text{MNP}_{\text{PDI-peptide}}$ (100 $\mu\text{g/mL}$) solution pretreated with ATP, BSA, dsDNA or ssDNA (10 μM) in the absence and presence of LPS (10 μM). The fluorescent intensity was recorded at 539 nm.

Figure 2.10-c indicated that no obvious fluorescence enhancement was observed against all the tested interferes including some popular bio-molecules, such as ATP and DNA. Even the highly negatively charged BSA which was reported to show the great interfere during the LPS detection.⁹² Moreover, the zeta potential of $\text{MNP}_{\text{PDI-peptide}}$ itself was determined to be + 25.50 mV at pH 7.4 (Table 2.1), clearly suggesting that the simple non-specific electrostatic interactions between the negatively charged bio-molecules and the positively charged particle surface may not be sufficient to disrupt the hydrophobic stacking of PDI and to trigger the significant fluorescence recovery. To further confirm this hypothesis, $\text{MNP}_{\text{PDI-peptide}}$ was chosen to individually incubate with the negatively charged ATP, BSA or DNA first. As expected, there was no obvious fluorescence enhancement detected in these solutions. However, upon the addition of LPS into these bio-molecules pre-incubated particle solutions, the fluorescence was greatly recovered within 15 minutes (Figure. 2.11). The competitive binding analysis confirmed that the specific LPS peptide interaction could easily replace the nonspecific electrostatic attraction between the negatively

charged bio-molecules and LPS affinity peptides, and then triggered peptide conformation change.^{7,132}

As mentioned before, the environmental pH stability was considered as an important fact to evaluate the LPS detection system. We tested the LPS responsive properties of $\text{MNP}_{\text{PDI-peptide}}$ under the same buffer systems at different pH values from pH 4- pH 11. As shown (Figure 2.10-d), the intensity enhancement at 539 nm was over 6 times at all tested pH conditions from 7.0-10.0. The result unequivocally suggests that the as-prepared LPS-recognizing peptide functionalized MNPs could serve as a stable and reliable platform towards sensitive LPS detection.

2.2.3 Bacterial staining with $\text{MNP}_{\text{PDI-peptide}}$

In terms of the promising property of $\text{MNP}_{\text{PDI-peptide}}$ for highly sensitive and selective determination of LPS in buffer solutions, we then tested the possibility whether $\text{MNP}_{\text{PDI-peptide}}$ were capable of interacting with LPS on bacterial cell surface. In this study, Gram-negative *E. coli* DH5 α cells (10^8 cfu/mL) were chosen to incubate with 200 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$ at room temperature. After 10 minutes incubation, the bacterial imaging was conducted under confocal laser scanning microscopy (CLSM). As shown in Figure 2.12, bright fluorescence was observed around the bacterial membrane and the fluorescent intensity of the bacterial membrane was stronger than that of nucleus (Figure 2.12-a and b). Similarly, TEM images also displayed the obvious attachment of $\text{MNP}_{\text{PDI-peptide}}$ onto bacterial membrane (Figure 2.12-c and d). These results demonstrated that $\text{MNP}_{\text{PDI-peptide}}$ could interact with LPS components on the surface of Gram-negative bacteria.

Figure 2.12 a) CLSM images of *E. coli* cells after incubation with $\text{MNP}_{\text{PDI-peptide}}$ (200 $\mu\text{g/mL}$) for 10 min at room temperature. b) Plot the fluorescence intensity versus the distance from the left image (red line); c and d) TEM images of the cells after incubation with $\text{MNP}_{\text{PDI-peptide}}$ (200 $\mu\text{g/mL}$) at room temperature for 10 min. The picture (d) shows an enlarged view of the area enclosed by the red square in image (c).

2.2.4 LPS clearance from bacterial cell lysates by $\text{MNP}_{\text{PDI-peptide}}$

More importantly, as proposed above, the peptide was initially designed to have the ability on LPS neutralization. Encouraged by the good performance in LPS detection and bacterial staining of $\text{MNP}_{\text{PDI-peptide}}$, we further studied its potential in LPS capturing and clearance. In this study, *E. coli* DH5 α bacterial cell lysates were prepared as the LPS containing solutions. $\text{MNP}_{\text{PDI-peptide}}$ was then added into cell lysates at 0 μg , 50 μg , 200 μg and 400 $\mu\text{g/mL}$. After 15 minutes incubation, the $\text{MNP}_{\text{PDI-peptide}}$ was removed by a simple magnet (Figure 2.13-a). The LPS concentration in the supernatant was evaluated by LAL assay and the protein concentration was evaluated by standard Bradford testing. As shown in Figure 2.13-b, the LPS clearance efficiency was concentration dependent. Compared with the control

Figure 2.13 a) Schematic illustration of LPS removal from bacterial lysates based on $\text{MNP}_{\text{PDI-peptide}}$, b) Quantified LPS removal and protein recovery from *E. coli* cell lysates with different amounts of nanoparticles; c) Multi-cycles removal of LPS from *E. coli* and *B. Sub.* cell lysates via 200 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$.

Figure 2.14 Protein recoveries after 1-4 cycle treatments with 200 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$. The protein quantification was performed using Bio-Rad protein assay.

experiment in the absence of $\text{MNP}_{\text{PDI-peptide}}$ treatment, 5% of total LPS was removed by

50 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$, and the removal percentage was increased to 85% when 400 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$ was used. Moreover, no obvious loss of the total protein was observed at all tests (Figure 2.14). On the other hand, we studied the clearance efficiency by a multi-cycle removal process using the fixed amount of $\text{MNP}_{\text{PDI-peptide}}$ (200 $\mu\text{g/mL}$) at each cycle. As shown Figure 2.13-c, the LPS in lysates dramatically decreased when the removal cycle increased from 1 to 4. After 4 turns' removal, the LPS left in the lysates was even less than 1 EU/mL. The less amount of LPS means less toxic when our bodies exposed to such bacterial lysates. In contrast, since no LPS in Gram-positive *B.sub* cell lysates, only background level signal was observed in LAL assay before and after treatment with $\text{MNP}_{\text{PDI-peptide}}$ (Figure 2.13-c). The result suggested that the as-prepared $\text{MNP}_{\text{PDI-peptide}}$ could selectively remove LPS endotoxin generated from Gram-negative bacterial membrane structures.

2.2.5 Human serum detoxification by $\text{MNP}_{\text{PDI-peptide}}$

Figure 2.15 a) Schematic illustration of LPS removal from human serum based on $\text{MNP}_{\text{PDI-peptide}}$, b) Quantified LPS removal and protein recovery from serum with different amounts of nanoparticles; c) NO generated from RAW 264.7 cells pre-treated with as prepared serum samples

Most importantly, the $\text{MNP}_{\text{PDI-peptide}}$ was successfully used in detoxification of LPS contaminated human serum samples. Similar to the clearance in bacterial lysates, the efficiency of LPS clearance also strongly depends on the concentrations of $\text{MNP}_{\text{PDI-peptide}}$. The LPS contents were decreased over 89% after a single cycle removal with 2.0 mg/mL $\text{MNP}_{\text{PDI-peptide}}$. It's well known that the endotoxic LPS could serve as a pre-inflammatory stimulus to induce macrophages to generate NO in a concentration dependent way,¹⁴²⁻¹⁴³ which could be estimated by measuring the concentration of nitrite, its stable metabolite released into the medium.¹⁴³ In the process of further evaluation of the detoxification effects by our $\text{MNP}_{\text{PDI-peptide}}$ in serum, the LPS containing serum samples with or without treatment with $\text{MNP}_{\text{PDI-peptide}}$ were added into the basic cell culture medium (serum to medium 1:3) to form full culture mediums. The macrophage RAW 264.7 cells that were reported to generate nitric oxide upon the stimulation of LPS, here, were incubated in those pre-prepared mediums for 24 hours and then the generated nitric oxide due to the response of macrophage to LPS were quantified by Griess reagent. As demonstrated in Figure 2.15, the cells incubated in the medium supplemented with the pre-treated serum generated less nitric oxide, implying less LPS left in serum after clearance with $\text{MNP}_{\text{PDI-peptide}}$ which was consistent with the quantification result by using LAL method.

2.2.6 Cytotoxic Evaluation of $\text{MNP}_{\text{PDI-peptide}}$

Figure 2.16 Cell viability rate to the control assay without particle treatment was evaluated by TOX-8 reagent after 24 h incubation in the presence of 100, 200, 400, 600 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$.

However, the $\text{MNP}_{\text{PDI-peptide}}$ itself did not show obvious cytotoxicity even up to 400 $\mu\text{g/mL}$ (Figure 2.16), which means the trace amount of $\text{MNP}_{\text{PDI-peptide}}$ left in serum during treatment is sufficient to induce cell death. This further indicated that our nano-platform has the promising properties for potential applications in clinic treatments.

2.3 Conclusion

In summary, by combining $\text{Fe}_3\text{O}_4@\text{SiO}_2$ core-shell magnetic nanoparticles with specific affinity ligand based on PDI conjugated LPS neutralizing peptide, we developed a simple and novel strategy for effective fluorescent detection of LPS and rapid capturing of such toxic bacterial endotoxin. On the basis of the specific interactions between LPS and the PDI conjugated LPS neutralizing peptides on the surface of MNPs, self-stacking of adjacent dye molecules would be disrupted, which suppressed self-fluorescence quench and thus restored the fluorescence. This peptide functionalized MNPs could sensitively identify LPS with the promising detection limit down to nano-molar level and it is highly selective for LPS when compared to other interfering bio-analytes. More significantly, such simple and effective LPS detection nanoparticle system also indicated potent ability to rapidly remove toxic LPS from bacterial lysates without obvious loss of total protein. Even more, this nanosensor was successfully used to remove endotoxin from human serum, thus greatly decreased the immuno-stimulation effects of LPS towards macrophages in the cell culture system. We envision that this simple and novel development would hold great potential for the future treatment of bacteria associated sepsis with minimum complicated sample pre-treatment, it could likely be extended to other nano-systems or medical devices for highly sensitive and selective sensing, and meanwhile, for capturing of other important bio-molecules in related biomedical applications.

2.4 Experiment part

Materials and General methods:

Materials: Anhydrous solvents for organic synthesis were purchased from Aldrich and stored over activated molecular sieves (4 Å). Fmoc-Amino Acids and 2-Chlorotrityl Chloride Resin were purchased from GL Biochem Ltd (Shanghai). Commercially available reagents were used without further purification, unless noted otherwise. Thin-layer chromatography (TLC) was performed on pre-coated silica gel 60F-254 glass plates. Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was purchased from Sigma Aldrich (cat.L3012).

Instruments: ^1H NMR spectra were recorded using 400 MHz spectrometer. Mass spectra (MS) were measured with a Thermo LCQ Deca XP Max for ESI. Analytical reverse-phase HPLC analysis was performed on a Shimadzu HPLC system using an Alltima C-18 (250 x 10 mm) column at a flow rate of 3.0 mL/ min for preparation and a C-18 (250 x 4.6 mm) column at 1.0 mL/ min for analysis. TEM images were recorded on an FEI EM208S Transmission Electron Microscopy (Philips) operated at 100 kV. Fluorescence emission spectra were performed on a Varian Cary eclipse fluorescence spectrometer. UV absorption spectra were recorded in a 5-mm path quartz cell on a Beckman coulter DU 800 spectrometer. FTIR (infrared spectroscopy) spectra were recorded on a Shimadzu/Prestige-21 spectrometer.

2.4.1 Synthesis and characterization of perylene diimide-peptide conjugate

Peptide synthesis:

The peptide synthesis was performed by Fmoc-chemistry based solid phase synthesis using 2-Chlorotrityl Chloride Resin. The coupling reaction was carried out at room temperature using HBTU/DIPEA as coupling reagent, and the deprotection of Fmoc group was performed in 20% piperidine-DMF solution.

Preparation of Compound 1

Perylene diimide (100 mg, 0.25 mmol), imidazole (3.0 g) and 1,4-Bis (3-aminopropoxy) butane (300 μL , 1.4 mmol) were mixed in a round-bottom flask with a stirring bar, and then heated to 125 $^{\circ}\text{C}$. After stirring at 125 $^{\circ}\text{C}$ for 4h, the reaction mixture was cooled down to room temperature followed by the addition of 30

mL water. The red precipitate in water was collected by centrifugation at 4000 rpm for 5 min and followed by several times washing with water and diethyl ether. After drying under vacuum, the red solid product (181 mg, 95%) was used directly in the next step without further purification. ^1H NMR (400 MHz, DMSO- d_6): δ = 8.51 (s, 4H), 8.27 (s, 4H), 7.67 (s, 4H), 4.11 (t, J =6.4 Hz, 4H), 3.51 (t, J =5.6 Hz, 4H), 3.37-3.40 (m, 12H), 2.83 (t, J = 7.2 Hz 4H), 1.92 (t, J =5.6 Hz 4H), 1.75 (m, 4H), 1.51 (s, 8H); ESI-MS: m/z 764.38 (calcd) for $\text{C}_{44}\text{H}_{52}\text{N}_4\text{O}_8$; 765.37 $[\text{M}+\text{H}]^+$, 1529.05 $[2\text{M}+\text{H}]^+$ (found).

Preparation of Compound 2

Compound **1** (50 mg, 0.065 mmol), Mal-PEG₂-NHS ester (28 mg, 0.075 mmol) and Propargyl-NHS ester (17 mg, 0.075 mmol) were mixed in 4 mL DMF, and then 20 μL triethylamine was added. After stirring at room temperature over night, the reaction mixture was dropped into 30 mL diethyl ether to give a red precipitate. After 2 times washing, the crude products were purified by column chromatography on silica gel with eluent of methanol/ dichloromethane (1:20) to give 27 mg (35 %) product **2**. ^1H NMR (400 MHz, CDCl_3): δ = 8.67 (d, J = 8 Hz, 4H), 8.61 (d, J = 8 Hz, 4H), 6.69 (s, 2H), 4.32 (t, J =6 Hz, 4H), 4.16 (d, J = 2.4 Hz, 1H), 3.84 (t, J = 7.2 Hz, 2H), 3.75-3.80 (m, 4H), 3.30-3.64 (m, 30H), 2.55 (t, J = 7.6 Hz, 2H), 2.41-2.47 (m, 4H), 2.05-2.12 (m, 4H), 1.74 (dd, J = 12.2 Hz, 6.2 Hz, 4H), 1.55-1.63 (m, 8H); ESI-MS: m/z 1184.53 (calcd) for $\text{C}_{64}\text{H}_{76}\text{N}_6\text{O}_{16}$; 1185.31 $[\text{M}+\text{H}]^+$, 1207.57 $[\text{M}+\text{Na}]^+$ (found).

Preparation of perylene diimide-peptide conjugate (3)

Compound **2** (10 mg, 0.0084 mmol) and peptide with sequence (20 mg, 0.011 mmol) were dissolved in 1 mL DMSO, then 5 μL DIPEA was added. The reaction mixture was stirred at room temperature overnight. After precipitation in diethyl ether, the crude products were purified by reverse-phase high performance liquid chromatography (HPLC) with eluting system consisting of A (0.1% TFA water solution) and B (0.1% TFA acetonitrile solution) under a linear gradient, monitored by UV absorbance at 490 nm. The linear gradient stretched over 20 minutes from t = 0 minutes at 20% solution B to t = 20 minutes at 80 % solution B. The reaction yielded the peptide–peryene diimide conjugate (12 mg, 50 %) after lyophilization. HPLC analysis demonstrated that the purity of the conjugate was over 95%. ^1H NMR (400

MHz, DMSO-d₆): δ = 10.75 (s), 9.21 (br s), 8.60 (d, J = 8.0 Hz), 8.51 (d, J = 6.4 Hz), 8.32 (d, J = 7.2 Hz), 7.97-8.15 (m), 7.78 (br s), 7.59-7.68 (m), 7.31 (d, J = 8.0 Hz), 7.11-7.24 (m), 7.05 (t, J = 7.2 Hz), 6.94-7.03 (m), 6.62 (d, J = 8.4 Hz), 4.54-4.65 (m); 4.19-4.35 (m); 4.03-4.12 (m); 3.97 (dd, J = 8.8 Hz, 4.0 Hz); 3.49-3.62 (m); 2.72-3.27 (m); 2.36-2.43 (m); 2.25-2.32 (m); 1.90 (br s); 1.82 (br s); 1.42-1.62 (m); 1.05-1.30 (m); 0.78-0.89 (m); ESI-MS: m/z 2970.55 (calcd) for C₁₅₂H₂₁₁N₂₉O₃₁S; m/z 1486.76, $m/3z$ 991.51 (found).

2.4.2 The preparation of peptide functionalized Fe₃O₄@SiO₂ core-shell nanoparticles

2.4.2.1 The preparation and characterization of Fe₃O₄ magnetic nanoparticles

The synthesis of metal-oleate complex was performed according to the reported process.¹³⁶ The monodisperse Fe₃O₄ nanoparticles were prepared according to the following procedure. Briefly, iron oleate (1.0 g, 1.1 mmol) and oleic acid (177.3 μ L, 0.56 mmol) were dissolved in 7.1 mL 1-octadecene. The mixture was heated to 100 °C and kept at that temperature for 1 hour. Then, the reaction mixture was heated to 290 °C with a constant heating rate of 3.3 °C per min, and kept at that temperature for 2 hours. After cooling to room temperature, the particles were precipitated in 50 mL ethanol. The precipitates were separated by centrifugation followed by 3 times washing with the solvent mixture of hexane/ethanol (1:3).¹⁴⁴ A small portion of Fe₃O₄ nanoparticles was suspended in hexane, and a drop of the solution was then dropped onto a copper grid for TEM imaging (Figure 2.3). The nanoparticle size distribution was analyzed on 100 individual particles by Image J.¹⁴⁵

2.4.2.2 The preparation and characterization of azide group functionalized Fe₃O₄@SiO₂ core-shell magnetic nanoparticles

3.2 mg of synthesized Fe₃O₄ nanoparticle was dispersed in the solvent mixture containing 30 mL cyclohexane, 4.8 mL triton-x100, 2 mL 1-hexanol, 0.7 mL distilled water and 300 μ L NH₃.H₂O. After stirring at 600-700 rpm for half an hour, 26 μ L Tetraethyl orthosilicate (TEOS) in 1 mL cyclohexane was dropped into the reaction mixture. After 4 hours, 4 μ L of (3-aminopropyl) triethoxysilane (APTS) in 1 mL cyclohexane was dropped into the mixture. After overnight stirring at room temperature, 50 mL pre-cooled ethanol was added into the mixture and the pH value

was adjusted to about 7.0 by 1M HCl at 0 °C. The particle pellets were obtained by centrifugation at 10000 rpm for 8 minutes and washed by water and ethanol.^{127,137}

To achieve the surface functionalization with azide group, Fe₃O₄@SiO₂ core-shell nanoparticles (30 mg) were suspended in 2 mL DMF, excess amount of azido-dPEG₄ NHS ester (10 mg, 0.026 mmol) and 10 µL DIPEA was added. The reaction was shaken at room temperature overnight. The nanoparticles were separated by centrifugation at 10000 rpm for 8 minutes and washed with DMF and EtOH.

FTIR (infrared spectroscopy) spectra were recorded on a Shimadzu/Prestige-21 spectrometer equipped with IR solution software. The samples were prepared by KBr pellet method before measurements. For organic sample, the compound was mixed with KBr (2 %, m/m). For nanoparticles, the sample was mixed with KBr (20%, m/m).

2.4.2.3 Preparation and Characterization of PDI-peptide conjugated Fe₃O₄@SiO₂ core-shell nanoparticles

The azide group functionalized core-shell nanoparticle (20 mg) was mixed with perylene diimide- peptide conjugate (**3**, 400 µM) in 5 mL water/t-butanol (1:1) mixture containing CuSO₄ (10 mmol) and ascorbic acid (10 mmol). The click reaction was kept shaking at room temperature for 48 hours. The nanoparticle was separated by centrifugation at 10000 rpm for 8 min, and followed by washing with H₂O, DMF and then EtOH. The final particles were kept in fridge for further use. The amount of PDI-peptide ligand conjugated to particle surface was determined by subtracting the amount of PDI-peptide left in the supernatant from the total amount of ligand added into the reaction mixture. The PDI-peptide amount in the supernatant was determined by absorbance measurement at 539 nm. The absorbance value was then converted to a concentration value based on a concentration-absorbance standard curve of free PDI-peptide.¹⁴⁶ The amount of PDI-peptide ligand on MNPs was calculated to be 10 nmol/mg. A small portion of final nanoparticles were suspended in ethanol, and a drop of the solution was then dropped onto a copper grid for TEM imaging. The nanoparticle size distribution was analyzed on 100 individual particles by using Image J.¹⁴⁵

2.4.2.4 Preparation of Perylene diimide conjugated Fe₃O₄@SiO₂ core-shell

nanoparticles (MNP_{PDI})

The azide group functionalized core-shell nanoparticle (10 mg) was mixed with perylene diimide derivative **2** (400 μ M) in 3 mL DMF. The click reaction was performed as above described. After 24 hours shaking, the nanoparticle was separated and washed as above described. The presence of Perylene diimide on the surface of particle was confirmed by UV-Vis absorption spectra.

2.4.3 Selective fluorescence enhancement of MNP_{PDI-peptide} solutions upon the addition of LPS

2.4.3.1 LPS triggered MNP_{PDI-peptide} fluorescence enhancement.

The incubation of MNP_{PDI-peptide} (100 μ g/mL) and LPS was performed in TBS buffer (Tris 50 mmol/L, NaCl 150 mmol/L, pH 7.4). The emission spectrum MNP_{PDI-peptide} excited at the wavelength of 450 nm was recorded by a Varian Cary eclipse Fluorescence spectrometer after 15 min incubation at 37 °C. The incubation of MNP_{PDI-peptide} with other biomolecular interferes was also performed under the same conditions as above described. Data normalization was based on the follow concept: setting the FL peak intensity value $I_{539\text{ nm}}$ at 25 μ M LPS as I_{max} , the normalized intensity “ I ” was defined as the measured intensity divided by I_{max} .¹⁴⁷

2.4.3.2 Determination of the detection limit

Figure. 2.16 Plotting the normalized FL intensity at 539 nm after 15 min incubation vs the concentrations of LPS (from 0.1-25 μ M). The inset shows the plot and linear fitting of fluorescence intensity at 539 nm vs the concentration of LPS (0-2.0 μ M).

Based on the linear fitting in Figure 2.16, the detection limit ($C_{\min}$) is estimated to be 28 nmol by a reported method.⁹⁴

$$C_{\min} = \frac{3\sigma}{B}$$

where σ is the standard deviation obtained from three individual fluorescence measurements ($I_{539\text{ nm}}$) of $\text{MNP}_{\text{PDI-peptide}}$ (100 $\mu\text{g/mL}$) without any analyte in TBS buffer and B is the slope obtained after linearly fitting the titration curves within certain ranges.

2.4.3.3 Selective response of $\text{MNP}_{\text{PDI-peptide}}$ fluorescence enhancement toward LPS

LPS, various small molecular or macromolecular interferes (all at 10 μM) was individually incubated with $\text{MNP}_{\text{PDI-peptide}}$ (100 $\mu\text{g/mL}$) in TBS buffer (pH 7.4). The emission spectra (excited at 450 nm) were recorded after 15 min incubation at 37 $^{\circ}\text{C}$.

Figure 2.17 100 $\mu\text{g/mL}$ $\text{MNP}_{\text{PDI-peptide}}$ incubated with 10 μM of different kinds of analytes in TBS buffer, the fluorescence was recorded after 15 min incubation. BSA: Bovine serum albumin; ATP: Adenosine triphosphate; GDP: Guanosine diphosphate; LAA: L- Ascorbic acid; Glu: Glutamate; GSH: Glutathion; Dp6: Maltotetraose; Cephalin: Phosphatidylethanolamine. ssDNA: single-stranded DNA; dsDNA: double-stranded DNA; Blank: $\text{MNP}_{\text{PDI-peptide}}$ only.

2.4.3.4 Fluorescence enhancement of $MNP_{PDI-peptide}$ solution to LPS in different pH conditions

TBS buffers with different pH values (4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 11.0) were prepared by adjusting the pH with 1.0 M HCl and 1.0 M NaOH water solutions from the mother solution (Tris 50 mmol/L, NaCl 150 mmol/L). All the buffers were sterile filtrated through a 0.2 μ m filter before use. The emission spectra of (excited at 450 nm) of $MNP_{PDI-peptide}$ (100 μ g/mL) in TBS buffers with different pH values were recorded after 15 min incubation at 37 $^{\circ}$ C in the absence or in the presence of LPS (10 μ M).

2.4.3.5 Zeta-potential tests of $MNP_{PDI-peptide}$ solution

Zeta-potential of $MNP_{PDI-peptide}$ (200 μ g/mL) in TBS buffer (pH 7.4, Tris 50 mmol/L, NaCl 150 mmol/L) was measured by Zeta-PALS Zeta Potential Analyzer 90 Plus (Brookhaven Instrument Corp.) equipped with an electrode assembly for aqueous systems (Brookhaven Instrument Corp.).

Table 2.1 Zeta potential of $MNP_{PDI-peptide}$ (200 μ g/mL) in TBS buffer (pH 7.4).

Run	Mobility	Zeta Potential (mV)	Rel. Residual
1	1.75	22.44	0.0298
2	1.60	20.42	0.0288
3	2.15	27.54	0.0350
4	2.43	31.05	0.0542
5	2.21	28.33	0.0355
6	2.16	27.66	0.0257
7	1.79	22.90	0.0313
8	1.85	23.70	0.0230
9	1.72	21.99	0.0283
10	2.26	28.97	0.0416
Mean	1.99	25.50	0.0333
Std.Error	0.09	1.14	0.0029

2.4.3.6 Competitive binding of $MNP_{PDI-peptide}$ toward LPS over other important biomolecules

$MNP_{PDI-peptide}$ (100 μ g/mL) was incubated with 10 μ M negatively charged ATP, BSA,

ssDNA (5'-GA TCC TGC GGC CGC TAG TAC TGT CGA CG-3'), dsDNA (5'-GA TCC TGC GGC CGC TAG TAC TGT CGA CG-3') in TBS buffer (Tris 50 mmol/L, NaCl 150 mmol/L, pH 7.4) at 37 °C. After 15 min incubation, the fluorescence spectrum excited at 450 nm was recorded followed by the addition of LPS (10 µM). After another 15 min incubation, the fluorescence spectrum was recorded again.

2.4.4 Effective clearance of Bacterial lipopolysaccharide (LPS) from bacterial lysates by MNP_{PDI-peptide}

E.coli DH5α (ATCC 53868) or *B. subtilis* (ATCC 33677) cells 10⁹ cfu/mL were suspended in TBS buffer (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4), and then were lysated under ice bath using an ultrasonic Processor (VCX 130, Sonics & Materials, Inc.). The parameters were set at 90 W power and 5 s pulse applied cycles followed by 5 s pauses for a total of 45 min. After centrifugation at 4000 rpm for 10 min, the supernatant was collected and used directly in LPS removal assay.

For LPS clearance, MNP_{PDI-peptide} was incubated with bacterial lysates in a 2.0 mL centrifuge tube. After 30 min incubation at 37 °C, the LPS captured MNP_{PDI-peptide} was removed by magnetic separation. The supernatant was used for LPS and protein quantification or for the next cycle clearance.

2.4.4.1 LPS quantification upon the clearance by MNP_{PDI-peptide}

LPS quantification was performed using Limulus amebocyte lysate (LAL) assay (Lonza, Switzerland) according to its standard procedure.¹⁴⁸ To match with the range of linearity of LAL assay, ten-fold serial dilutions were prepared from the original cell lysates or human serum samples. Briefly, 25 µL of each tested solution were loaded into 96-well plates and then 25 µL of LAL enzymatic reagent was mixed well with the sample solutions. After 10 minutes incubation at 37 °C, 50 µL of substrates solution was added. After further 6 min incubation, 50 µL of stop solution was added. After that, the absorbance of each reaction well at 410 nm was recorded by a Tecan's Infinite M200 microplate reader. Each experiment contains 3 repeats. The LPS concentration was calculated according to the described methods in the standard protocol of Limulus Amebocyte Lysate (LAL) QCL-1000. According to the reported data, 100 pg of LPS is corresponding to 1.2 endotoxin units [EU].¹⁴⁹

2.4.4.2 Protein quantification upon the efficient clearance of bacterial LPS

The concentrations of total protein in cell lysates were quantified by Bio-Rad Protein Assay (Bio-Rad Laboratories, USA) according to its standard procedure before and after treatment with $\text{MNP}_{\text{PDI-peptide}}$. Briefly, the dye reagent was diluted for 5 times with distilled water before use. 20 μL of the tested sample was mixed with 200 μL of the diluted reagent in 96-well plates. After incubation at room temperature for 5 minutes, the absorbance at 595 nm was recorded by a Tecan's Infinite M200 microplate reader.¹⁵⁰ The protein concentration was determined according to the described methods in the standard protocol.

2.4.5 Efficient clearance of bacterial LPS from human serum sample by using $\text{MNP}_{\text{PDI-peptide}}$

Human serum (from human male AB plasma, Sigma Aldrich) was sterile filtrated through a 0.2 μm filter. Pure LPS water solution was added into human serum to reach 100 ng/mL. Then the LPS containing serum was incubated with different concentrations of $\text{MNP}_{\text{PDI-peptide}}$ (0, 200, 500, 1000, 2000 $\mu\text{g/mL}$) at 37 °C for 30 min. After removal of the LPS captured $\text{MNP}_{\text{PDI-peptide}}$ by magnetic separation, LPS content in serum was quantified by LAL assay as described before.

2.4.6 Detoxification evaluation of contaminated human serum samples by quantifying NO generation in macrophage cultures.

The serum samples after LPS clearance with different concentrations of $\text{MNP}_{\text{PDI-peptide}}$ (0, 200, 500, 1000, 2000 $\mu\text{g/mL}$) were mixed with basic RPMI 1640 respectively (serum to medium 1:3) to form the cell culture mediums. The mouse macrophage RAW 264.7 cells were seeded at a density of 1×10^6 cells/mL in 24-well plates (1.0 mL/well) for 12 h, followed by treatment with the as-prepared cell culture medium for a further 24 h. The amount of NO production in the medium was detected by the Griess reaction (Life Technologies, USA). 75 μL of each supernatant was mixed with 65 μL water and 10 μL Griess reagent in a 96-well plate. The absorbance at 548 nm was recorded after 30 min incubation at rt. NO quantification was processed according to the standard procedure supplied with Griess reagent (Invitrogen).

2.4.7 Cell viability test of MNP_{PDI-peptide}

Briefly, RAW 264.7 cells were seeded in a 24-well plate with a density of 1.0×10^6 cells per well in RPMI 1640 with 10% FBS. After 12 hours incubation at 5% CO₂ and 37 °C, the medium was changed with fresh medium in the presence of MNP_{PDI-peptide} at different concentrations. The cell viability measurements were determined after 24 hours incubation. The particle containing medium was then replaced with fresh RPMI-1640 (phenol red free) containing 10% TOX-8 reagent. After 2 hr incubation, the fluorescence intensity at 590 nm was measured at excitation wavelength of 560 nm by a Tecan's Infinite M200 microplate reader. The cell viability rate (VR) was calculated according the reported methods.¹³² RAW 264.7 cells were purchased from Sigma-Aldrich (USA).

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Part II Small Peptide and Membrane Lipid Based Self-assembled Nanostructures for Controlled Drug/Gene Delivery

Introduction

II-1. Cancer drug resistance and nano-strategies to combat resistance

Generally, majority of the currently used chemotherapeutic compounds have been developed as a result of drug screening for cytotoxic potency *in vitro* against cancer cells or *in vivo* against tumor models.¹ Most of them have been identified to have a good selectivity, but not always restrictively, target rapidly proliferating cells.¹ The available anticancer drugs have distinct mechanisms of action. Disturbing important cellular processes (e.g. mitosis, meiosis, differentiation, senescence, apoptosis and so on) is one of the most commonly recognized mechanisms of anticancer agents used in clinic practice.² There are many different kinds of drugs, including DNA alkylating agents, antimetabolites, intercalating reagents, mitotic inhibitors, etc, reported to target such critical cellular processes (Figure II-1). Based their action mechanisms, intracellular internalization was usually required for these kinds of chemical agents to guarantee the therapeutic effects. The plasma membrane was believed to be the main barrier for drugs accessing their intracellular targets.

Figure II-1 The action mechanisms of commonly used cancer chemotherapy agents.

Chemotherapy strategies used alone or combined with other treatments such as surgery,

radiotherapy and immunotherapy have been proposed to save many people or at least extend their lifespan. However, over the cancer chemotherapy, cancer cells commonly developed many drug resistant ways against the treatment, giving rise to therapy failure and tumor relapses.³ In the process to overcome drug resistance during traditional chemotherapies, several alternative bioactive molecules have been extensively studied for targeted cancer therapy based on the specific interactions between these molecules and their targets in cancer cells.⁴ For example, tamoxifen is such a well-developed therapeutic agent to prevent breast cancer based on its specific targeting to the oestrogen receptor (OER).⁵ Similarly, another case is the EGFR inhibitor gefitinib, which has been approved for the treatment of non-small-cell lung cancer.⁶ Unfortunately, drug resistance has also appeared to such targeted therapies.⁷⁻⁸

Up to now, several types of potential mechanisms have been proposed to explain cancer drug resistance. Principle mechanisms may include the altered membrane transporter of the drug molecules (e.g. p-glycoprotein), enhanced DNA repair, alteration in cellular targets, drug inactivation due to metabolic effects, cancer stem cells and so on.^{1,9-11} Among various mechanisms in drug resistance, one of the most significant mechanisms against the commonly used chemotherapy agents is by the drug efflux action of membrane proteins (Figure II-2). By this, cancer cells prevent the anticancer drugs from effective internalization, thus keeping intracellular drug concentration below a cell-killing dosage.¹²⁻¹⁴ A phenotype referred to such chemotherapy-resistance was first described for cancer cells with overexpression of the drug efflux membrane transporter P-glycoprotein (P-gp).¹⁵⁻¹⁷ P-gp (P-glycoprotein 1 or MDR1) is a well-characterized ABC-transporter (ATP-binding cassette transporter) which transports various substrates across extra- and intracellular membrane. In human tissues, the glycoprotein P-gp is a 170-kD protein product encoded by the *ABCB1* gene,¹⁸ which has been identified to play important roles in drug resistance in several types of cancer such as breast cancer, gastric cancer, pancreatic cancer, ovarian cancer and leukemia.^{13,19-20} This protein comprised of 1280 amino acids that could be divided into two symmetrical halves: the C-terminal transmembrane domain and the N-terminal ATP-binding domain.²¹⁻²² Although in recent years there has been a great improvement in our understanding of the structure of P-gp, the precise molecular

mechanism of drug transport by P-gp is still not fully understood.²³ Basically, it has been always believed that hydrophobic or amphipathic drug molecules were susceptible to be pumped out of the cells by P-gp, thus reducing the cellular accumulation of drugs and leading to failures in anticancer therapies.^{24-25,26-28}

Figure II-2 Schematic illustration of P-glycoprotein mediated drug resistance.

Based on these issues, searching novel strategies to minimize the P-gp mediated drug efflux would be potential solutions to overcome drug resistance. A promising approach is using P-gp inhibitors to suppress P-gp mediated drug efflux. Currently, many kinds of P-gp inhibitors have emerged and a number of clinical trials have been conducted to investigate their potentials to overcome drug resistance. As expected, in many cases, the combined use of P-gp inhibitors and drugs resulted in significantly elevated intracellular concentrations of anticancer drugs. Although the usage of P-gp inhibitors. Although very promising in principle, there are still many obstacles to overcome in using such inhibitors to assist cancer treatment. The most remarkable one is that non-specific toxicity has been always observed associated with the use of P-gp inhibitors due to the important roles of P-gp in some health tissues.^{24,29}

To address the challenge of serious non-specific site effects caused by P-gp inhibition, the development of alternative strategies based on novel mechanisms is necessary. The most potent way is the use of nanoscale drug carriers to alter the uptake pathway and to improve cellular internalization, thus increasing intracellular localization of drug molecules.

Figure II-3 The commonly used nanocarriers for drug/gene delivery

It is known that cancer drug resistance could be overcome by nanoparticle based drug delivery systems due to their favorable properties including the enhanced permeability and retention (EPR) effect, and tunable nanoparticle characteristics for biomedical applications.²⁶⁻²⁸ Figure II-3 demonstrates several types of nano-vehicles currently used for drug delivery, such as polymeric micelles or nanoparticles, liposomes, mesoporous silica nanoparticles and dendrimers. These nanoscale drug carriers preferentially facilitate accumulation of drugs in tumors by protecting them from rapid degradation in extra or intracellular environments or clearance by drug efflux pumps.^{26-27,30-31}

Generally, the properties such as size distributions, surface charges, drug loading and release efficiency are considered as the most important features of nanoparticles which determine their cellular toxicity, targeting property and *in vivo* distribution in therapeutic applications. Extensive studies have indicated that nanoparticles with hydrodynamic diameter of 10–100 nm have optimal therapeutic properties for *in vivo* applications.³² Smaller or larger nanoparticles could be easily removed from the bloodstream thus decreasing its effective accumulation in tumors.^{27-28,32} Except for favorable size distribution (usually <100 nm), the surface properties are considered to be as an important aspect that influences the biocompatibility of nanoparticles. In general, cationic nanoparticles would have advantages in cellular uptake, but may cause more immune responses and cellular toxicity than neutral or anion ones.³³⁻³⁴

Although nanoparticles can achieve their tumor tissue targeting effectively by passive EPR effects, the surface modification with additional targeting ligands has shown a great improvement to their targeted drug release.³⁴⁻³⁷ Besides, high drug-loading capacity and easier loading process are two important aspects in evaluation of novel designed delivery system. Traditionally, most of drug loading can be accomplished by incorporation of adsorption/absorption strategies. Loading efficiency is highly dependent on the physicochemical properties of drug molecules and drug carriers. Taking the model drug doxorubicin (Dox) and the well-studied silica nanocarriers as examples, Dox loading efficiency of the most commonly used non-hollow mesoporous silica MCM-41 and SBS-15 usually varies at low percentage levels (<10%). However, the loading efficiency could be greatly improved by hollow mesoporous silica, where in some cases the efficiency could reach to 100%.³⁸⁻⁴¹ Moreover, controllable drug-release behaviors have been proposed to be desirable properties ensuring the therapy effects meanwhile minimizing the side effects.⁴²⁻⁴³ Now, such “on-demand” drug delivery based on the stimuli responsive systems that recognize the microenvironments of living organisms (e.g. pH, temperature, redox, specific enzymes, etc) has been widely investigated.⁴³

Besides the favorable size distribution, high drug-loading efficiency and controllable drug-release, the nanocarriers used for medical applications have to be biocompatible and non-toxic. Among all the nanomaterials, the self-assembled lipids nanomaterials (e.g. liposomes, membrane vesicles, etc) have promising biocompatibility and low cytotoxicity mostly because of their composition of natural biomolecules. In the following lecture review sections, the issues regarding design and application of self-assembled nano-structures including nanogels and cell derived membrane vesicles (MVs) will be discussed.

II-2 Self-assembled nanostructures for drug delivery

II-2.1 Nanogel particles for drug delivery

Nanogels, known as hydrogels with nanoscopic dimensions, have similar properties of hydrogels with more added values for drug delivery including their favorable size distribution, injectable properties, etc.⁴⁴ Among all the drug nanocarriers, nanogels

have gained much attention due to their unique features of high water content, low surface tension and suitable nanoscale size for biomedical applications.⁴⁵ Nanogels can be produced by chemical cross-linking via covalent bonds or physical cross-linking via non-covalent bonds (e.g. hydrogen bonds, and hydrophobic/hydrophilic interactions).⁴⁶ A new kind of physically cross-linked nanogel formed via self-assembly in aqueous media.⁴⁷ Up to now, self-assembly strategies have been widely used to generate highly ordered nanogels, which have many advantages compared with chemical cross-linked nanoparticles such as their desired capability of drug loading and relatively easier preparation.⁴⁸⁻⁴⁹

II-2.1.1 Materials

Biodegradability of the selected materials is essential to avoid undesirable side effects when used for biomedical application.^{45,50} By far, a wide range of materials either from natural or synthetic products have been extensively studied to generate nanogels for biomedical usage. Among the commonly used synthetic materials, FDA-approved biodegradable poly-D, L lactic acid (PLA), poly-glycolic acid (PGA) as well as their copolymers have been widely used in nanogel-based drug delivery systems.⁵¹ While of all natural polymeric materials, polysaccharides are the more often-used metric to synthesize nanogels. Such materials are natural carbohydrate polymers, which consist of repeating monosaccharide units.⁵² Moreover, the polysaccharides can be easily chemically modified (e.g. cholesterol conjugation, etc) to yield amphiphilic polymers, which can self-assemble into hydrogel nanoparticles in aqueous solutions.⁵²⁻⁵⁴

Another important type of biodegradable materials, polypeptides, has been widely used as a favorable biomaterial in the past decade.⁵⁵⁻⁵⁶ Recently, there are increased interests in the application of self-assembled peptide nanostructures for drug or gene delivery, mostly because of their unique properties including their easy preparation, less immunogenic, denatured effect and so on. More importantly, they are easier to be modulated on the balance of hydrophobic/hydrophilic property by simply changing of amino acid residues.⁵⁷⁻⁵⁸ In general, small synthetic peptides can achieve the self-assembly ability by introducing some hydrophobic residues into the sequence to form distinct polar and non-polar surfaces. Such hydrophobic residues could be

specific amino acids including phenylalanine, or other non-amino acid structures such as Fmoc group, naphthyl group or even the drug molecule itself.^{49,59-61} An ideal design is using the drug molecules conjugated short peptides to form nanogels, which could self-deliver drugs into its targets thus dramatically change the molecular behaviors of drugs in the process of cellular internalization, cellular localization and so on.⁶²

II-2.1.2 pH responsive nanogels

In recent years, large efforts have been focused on the development of environment-sensitive microgels/nanogels. The smart nanogel was defined as the nanoscale gel particle, which responds to the changes of environmental conditions including temperature, pH, redox, etc.⁶³ Among various external stimuli, pH responsiveness has been proven one of the most frequently utilized strategies since pH varies tremendously in different tissues and cellular compartments. It is known that, certain cancer tissues exhibit acidic local pH (around pH 6.5) due to their hypoxia environment,⁶⁴ while the pH of some acidic cellular compartments, like late endosomes or lysosomes, varies from 4-5.⁶⁵⁻⁶⁶ Based on this, the pH responsive nanogels in terms of their pH-dependent swelling behavior, can be used for cancer tissue or endo/lysosome targeted drug release. Currently, pH responsive microgel/nanogel delivery systems have been extensively investigated to successfully deliver cancer chemotherapy drugs into targeted tumor cells *in vitro* and *in vivo*.⁶⁷⁻⁷⁶

Figure II-4 The structures and their reactions of commonly used acidic liable elements in pH responsive drug release systems.⁷⁷

In the development of pH sensitive nanogels, pH-responsive chemical elements are required. Figure II-4 indicates the commonly used acid labile linkers in conjugation chemistry and hydrolysis reaction. Among all the acid labile elements, hydrazone-containing linkers have been extensively studied due to their tunable properties of super stability under neutral conditions but rapid cleavability at acidic conditions. Based on the selective reaction between hydrazine group and aldehyde or ketone group, hydrazine derivatives were often directly conjugated to anti-cancer drugs or their derivatives (e.g. doxorubicin, taxinol, etc) to form the pH sensitive pro-drugs. Using hydrazone bonds as the acid liable elements, a polyphosphoester based therapeutic nanoparticle was obtained and reported capable of reversing its surface charge from negative to positive at pH 6.8 to improve cellular internalization.⁷² By conjugation of Dox to amphiphilic macromolecules (AM) with hydrazone bond, the pH sensitive nanogels with average size of about 30 nanometers have been developed. The *in vitro* study indicated that this gel particle exhibited an enhanced activity than free Dox against MCF-7 cells. However, the nanogel was found not stable under neutral pH conditions (e.g. pH 7.4), which may cause non-specific drug release. Over 50% of Dox release was detected under pH 7.4 after incubation for 24 hours.⁶⁷ Recently, a similar design was also performed by changing amphiphilic macromolecules to a poly (ethylene glycol) PEG polymer. The nanoparticle has an average hydrodynamic diameter of 192 nm in aqueous solutions. Their results demonstrated that this Dox-PEG nanoparticle was also not stable enough under pH 7.4, and an obviously decreased cytotoxicity was detected.⁷⁸

Except for nanogels that only respond to pH changes, very recently, nanogels that are responsive to dual stimulus (e.g. pH and reduction, pH and thermo and so on) have also been reported.⁷⁹⁻⁸³ For example, disulfide bonds were often used in reduction or pH/reduction sensitive systems. A pH and reduction dual-responsive nanoparticle based on di-blocked poly (ethylene glycol)–SS–poly (2-(diethylamino) ethyl methacrylate) (PEG–SS–PDEA) copolymers was found to be efficient and controllable in release of proteins.⁷⁹ Besides, DOX-loaded mPEG-b-PDEA based nanogels were also reported to be stable under physiological conditions, but may undergo quick release of Dox in mild acidic (e.g. pH 6.8) and reductive (e.g. 10.0 mM glutathione)

environment.⁸⁴ Very recently, a pH-thermal dual responsive nanogel was reported for cisplatin (CDDP) delivery. The release of CDDP from the nanogel was greatly enhanced by reducing the pH value or by decreasing the temperature from 37 °C to 25 °C.⁸³

In order to achieve cell targeting properties of nanogel particles, in some cases, conjugation of the particles with certain target ligands (e.g. Folic acid) were required. Recently, a cancer cell targeting nanogel system was reported by conjugating folic acid to Dox through an PEG spacer.⁸⁵ A similar concept has been proposed, where the folic acid molecule was conjugated to Dox through hydrazone bond after conjugation with the hydrophobic moiety D-α-tocopheryl polyethylene glycol succinate (TPGS). The prepared nanoparticle has been proved to be more effective in cancer cell killing *in vitro* study. More recently, a nucleus-targeted nanogel has been developed by using the widely used TAT-peptide as cell penetrating agent. In aqueous solutions, the peptide-Dox conjugate self-assembled into nanogel particles with an average size of about 100 nm. The results indicated that the nanogel could totally reverse the resistance of Dox-resistant MCF-7 cells.⁸⁶

Although self-assembled nanogels have shown many advantages for environment-sensitive release of their payloads, there are still some problems to be overcome such as nonspecific aggregation and less stability under physiological conditions. The application of nanogels to deliver some therapeutic bioactive compounds (e.g. proteins or nucleotide sequences) is still a big challenge.

II-2.2 Cell derived membrane vesicles for drug/gene delivery

The extracellular matrix, out of multicellular organisms, contains the solutions of various metabolites, ions, carbohydrates and proteins. Besides, it's well known that the extracellular matrix also contains many independent self-assembled extracellular membrane vesicles (MVs) including exosomes, microvesicles and apoptotic bodies.⁸⁷ These cell derived MVs have the size distribution various from tens of nanometers to several micrometers (Figure II-5). It is clear that many types of cells including cancer cells, macrophages and some stem cells can secrete membrane vesicles.⁸⁸ Although cell-released membrane vesicles were firstly described about half a century ago,⁸⁹⁻⁹⁰

until the recent decades, the MVs have been proved to play important roles in physiological and pathological conditions including cell migration, cell-cell communication, host defense against microorganisms and so on. However, the mechanism involved has not been fully recovered yet. In general, MVs have the ability to fuse with neighboring cells, thus transfer the caged cytoplasmic contents from the mother cell to others. Indeed, there are *in vitro* and *in vivo* evidences that MVs can serve as nanocarriers to deliver important bio-macromolecules (e.g. RNA, DNA and proteins) into surrounding cells. This special transfection may influence or reprogram neighboring cells.

Figure II-5 Schematic illustration of structures and size distribution of various extracellular membrane vesicles

Since cell derived MVs are capable of transferring various bioactive molecules from one cell to the others, they thus offer excellent nanomaterials for the purpose of drug/gene delivery. Compared with the artificial drug/gene delivery systems, cell derived MVs have their unique advantages such as their viral-like transfection effect, inherent biological function, minimized inflammatory stimulation and so on.⁸⁸ Generally, in the process of MVs isolation, high-speed centrifugation was often required. Recently, a gene delivery system based on exosome like MVs released from HeLa and HTB-177 cell lines was reported. In their procedure, the siRNA which was designed to suppress the expression of mitogen-activated protein kinase-1 (MAPK-1) was encapsulated into the membrane vesicles by electroporation. Their results indicated that the membrane vesicles can effectively deliver the siRNA into the desired cells, resulted in selective gene silencing of MAPK-1.⁹¹ Except for electroporation, we can also obtain the siRNA loaded MVs from the siRNA gene pre-transfected

cells.⁹²⁻⁹³ Besides gene delivery, the cell derived MVs can be also used as carriers for small molecular drug delivery.⁹⁴⁻⁹⁵ In a recently reported work, the drug-loaded MVs were obtained by incubation of MVs with three types of drugs Dox, methotrexate and cisplatin individually followed by centrifugation from the culture medium. Their *in vivo* antitumor result indicated that the cell derived MVs can effectively deliver the tested drugs into tumors thus arrest the growth and adverse of tumor cells.⁹⁶ More recently, a smart magnetic and photo-responsive delivery system based on MVs was reported. This smart MVs enabled efficient tumor therapy by photodynamic way meanwhile enabled the dual-mode imaging combining fluorescence and MRI.⁹⁷ To achieve neuron-specific target MVs, the dendritic cells were first engineered to over-express an exosomal membrane protein, Lamp2b, which was fused with a neuron-specific RVG peptide. Using the exosome-like MVs released from the engineering dendritic cells as gene container, the desired siRNA molecules were successfully delivered into mouse brain.⁹⁸⁻⁹⁹

Although the genetically engineered MVs have shown a good example in the design of targetable membrane vesicles, the chemically engineered MVs have not been explored yet. Moreover, it is also a big obstacle to collect enough MVs from the general cell culture systems for its potential delivery use. Therefore the development of novel strategies introducing affinity ligands onto MVs thus facilitating their cancer cell targeting as well as the strategies to get MVs from mother cells at a relatively higher yield may greatly expand the possibility for biomedical applications.

II-3 Endo/lysosome in nanoparticle based drug delivery systems: the carrier or the barrier?

As well known to us, most of the commercially used anticancer drugs have their primary targets in intracellular locations (e.g. cell nuclei, mitochondria, etc). In terms of this fact, a drug must overcome several physical, chemical, and biological barriers to reach its cellular sites of action. The first barrier for drug internalization is the cell membrane that consists of lipid bilayers. Typically, the amphiphilic property of membrane lipids and the aqueous intracellular and extracellular environments made the plasma membrane to be a structure with a hydrophobic core and two hydrophilic

surfaces. The hydrophobic cores of cell membranes were believed to be the main barrier of drug transporting from extracellular environment into cells. The drug molecules with different physical and chemical properties have been shown to have different membrane penetrating abilities.¹⁰⁰ In general, the amphiphilic drug molecules, such as steroid hormones, are able to diffuse easily across plasma membranes. However, it's more difficult for hydrophobic, hydrophilic and large size drugs to transport through the membranes.¹⁰¹ Besides the natural barriers of cell membranes, overexpression of drug-efflux pumps (e.g. multidrug resistance proteins) at plasma membranes was also found to suppress intracellular accumulations of drugs, thus limit their anticancer effects in tumor therapy.³

II-3.1 *The carrier*

Using nanocarriers as drug containers to deliver drugs across the plasma membrane may facilitate their effective accumulation at target sites. Extensive studies have revealed that the nanoparticles undergo cellular internalization mainly through an endocytotic pathway which is different to the passive diffusion.¹⁰² Endosome and lysosome, two membrane-bounded compartments inside eukaryotic cells, play the most important roles in nanoparticle based drug deliveries (Figure II-6). Actually, endo/lysosome acts as the secondary drug containers or carriers after the internalization of drug loaded nanocarriers. It's known that, drug internalization through endocytotic pathway could bypass the efflux-pump mediated drug resistance.¹⁰³ This may be attributed to the fact that endo/lysosome vesicles prevent the drug molecules from dispersion throughout the cytosol thus decreased the possibility to be pumped out by MDR proteins.

Up to now, there are many drug delivery systems targeted to the endo/lysosomal systems of cancer cells. Some of them were designed to respond to specific enzyme systems in late endosomes or lysosomes. The prominent ones among them are those respond to cathepsin B, a lysosome localized cysteine protease with up-regulated expression in many human cancer cells.¹⁰⁴ Upon the specific cleavage reaction between the enzyme and peptide substrates, the caged drugs were released from the nanocarriers.¹⁰⁵⁻¹⁰⁶ Besides, targeting to the lysosomal acidic pH was the frequently

used concept for the design of drug controllable release system based on nanoparticles. Various materials such as polymers, mesoporous silica nanoparticles, and hybrid nanostructures were designed to respond to acidic environment. In some cases, tunable pH sensitive release of payload drugs was achieved, which implied the promising potentials for cancer therapy.¹⁰⁷⁻¹⁰⁹

Figure II-6 Schematic illustration of endo/lysosomal internalization of nanocarriers and the fate of the released drugs in lysosome.

II-3.2 The barrier

Although the endo/lysosomal system offers an alternative pathway for drug internalization and controlled release, it also becomes a secondary barrier to prevent drugs reaching its cellular targets. Lysosome, which has also been described as the cell's stomach, contains many digestive enzymes in its acidic environment (pH 4-5) that can degrade drug molecules especially when the drug is a biological macromolecule (e.g. proteins, siRNAs, etc).¹¹⁰ Indeed, the researchers have found evidence that the therapeutic molecules such as proteins¹¹¹ and siRNA¹¹²⁻¹¹³ undergoing degradation in lysosome at the end lose their bio-activities. To address the problem of lysosomal degradation, the design to facilitate the lysosomal escape of drug/gene was introduced into the drug/gene delivery. Stimulus-responsive reversal of the surface charges of NPs (from anionic to cationic) in the intracellular acidic later endosomes or lysosomes was proved to be an effective strategy for rapid escape from endo/lysosome into the cytosol.¹¹⁴ Another method to do this is to introduce

photosensitizers (PSs) into the nanocarriers. In these systems, upon the light irradiation, the PSs can transfer the absorbed energy to other molecules (e.g. molecular oxygen), to generate reactive oxygen species (e.g. singlet oxygen), which cause the oxidative damage to lysosomal membranes thus improving the release of lysosome-caged molecules.¹¹⁵⁻¹¹⁷ The hydrogen peroxide, one of the most important ROS in living system, has been documented to be capable in improving molecular escape from endo/lysosome by disrupting the membrane structure.¹¹⁸⁻¹²¹ Therefore, to control the extracellular or intracellular level of H₂O₂ could be an alternative way to control the drug behaviors.

As described, although the chemical-biological methods to overcome the lysosomal membrane barriers have drawn much attention in research of lysosomal escape of drug molecules, the highly toxic ROS involved is difficult to control thus may cause some non-desired side effects. One promising method to destabilize lysosomal membrane is to use in situ formed mechanical forces such as the controllable aggregation, crystal growth or self-assembled nanofibers. However, it's still a big challenge.

Chapter 3

pH Responsive Small Molecule Peptide Nanogels: Strategies toward Overcoming Tumor Resistance and Controlled Intracellular Drug Redistribution

3.1 Introduction

Controlled release of therapeutic agents at location of cancers with desired dosage will be of clinical significance to guarantee the therapeutic effect while minimizing the drug resistance and other side effects in the process of the cancer treatment. Currently, several types of nanoscale delivery vehicles including silica nanoparticles, liposomes, polymeric nanoparticles, self-assembled micelles, etc have been developed to achieve the precise and safe drug release by means of the enhanced permeability and retention (EPR) effect.¹²²⁻¹²⁹ More recently, the self-assembled nanogels have been extensively studied as a novel kind of nanocarriers for drug delivery mostly because of their unique features of high water content and low surface tension. What's more, their flexible morphologies and high loading capacities also make them well adapted to various environmental stimuli, such as pH, light, temperature, ionic strength, enzyme or oligonucleotides treatment, and thus easily trigger the controlled release of payload molecules.^{45,48,59,130-135} Of various external stimuli, pH responsiveness has been proven to be one of the most frequently utilized strategies since pH varies tremendously in different tissues and cellular compartments. Currently, pH responsive microgel/nanogel delivery systems have been widely investigated to successfully deliver cancer chemotherapy drugs into targeted tumour cells *in vitro* and *in vivo*.⁶⁷⁻⁷⁶

Although most of these pH-sensitive nanogels have been well explored to respond to changes in pH, which enable drug release into the acidic organelles (endo/lysosome) through endocytic pathway, very little work has been done on the study of the intracellular redistribution of drug molecules from endo/lysosomes to their targets. Actually, if the entrapped drug molecules could not effectively escape from endo/lysosomes they may face potential degradation or suffer from less access to the target in the cytoplasm or nucleus, thus greatly compromise their therapeutic efficacy.^{122,136-137}

Figure 3.1 Schematic illustration of self-assembled peptide-Dox nanogels for dual-controlled drug release. Stage I, peptide-Dox conjugates self-assembled into nanogels; Stage II, cellular uptake and Dox release in endo/lysosomes; Stage III, lysosome escape and Intracellular translocation of Dox induced by ROS.

Therefore, use of endo/lysosome as natural intracellular drug-loaded vesicles may offer secondary controllable platforms over the enhanced drug behaviors within the targeted cells. Systematic investigations on the intracellular redistribution of the drug molecules after endo/lysosomal release will be very critical to better understand the details about the fate of tested nanocarriers with respect to their different uptake pathways and sub-cellular trafficking against biological barriers. Meanwhile, such significant studies will also provide highly useful insights into the intrinsic mechanisms of controlled drug release, which may greatly facilitate the rational design of new types of drug carriers with high therapeutic efficiency and minimum resistant properties in clinical studies. So far, such relevant investigations have not been fully exploited yet.

In this chapter, our objective was mainly focused on the development of a novel self-delivery nanogel system for controlled drug release in the tumor cells especially for those with drug resistance (Figure 3.1). In this design, a first-line antitumor drug, doxorubicin (Dox) was conjugated to a short Fmoc-protected GFLGG sequence through an acid-labile hydrazone-bond to afford a peptide-Dox prodrug with a high weight loading percentage up to 44.9 %. This peptide-Dox prodrug could easily form stable self-assembled nanogels in aqueous solutions (I), mostly driven by supramolecular interactions including π - π stacking and hydrogen bonding.¹³⁸⁻¹⁴⁰ Such drug conjugated nanogels were assumed to quantitatively self-direct Dox into drug

susceptible and resistant cancer cells through endo/lysosomal pathways. The pH-sensitive hydrazone bonds in peptide sequence could be hydrolyzed in the acidic environment within cellular endosomes and lysosomes to release free Dox molecules (II). Under the environment without any external stimulation, the released Dox can be caged and mainly accumulated in endo/lysosomes with limited membrane permeation for a relatively long time. However, upon exposure to an oxidative stress induced by reactive oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2), the endo/lysosome membrane would be disrupted from oxidative damage. Free Dox could then quickly escape from the disturbed vesicles to reach its intra-nuclear targets (III)¹⁴¹, and therefore exert the effective antitumor activities against the targeted cancer cells.

3.2 Result and discussion

3.2.1 Synthesis and characterization of peptide-Dox conjugate

Scheme 3.2 Synthetic processes of peptide-Dox prodrug. a) EDC.HCl, N-Hydroxysuccinimide, DMAP, rt, 4 hr; b) Tert-butyl hydrazinecarboxylate, rt, 4 hr; c) 20% piperidine in DMF, rt, 30 min; d) HBTU, DIPEA, rt, 4 hr; e) 20% TFA in DCM, rt, 1 hr; f) Doxorubicin, MeOH, 40 °C, 24 hr.

The Fmoc protected peptide was obtained through solid-phase based peptide synthesis, which was based on the Fmoc chemistry and 2-Chlorotrityl Chloride Resin. The intermediate linker (**3**) was then synthesized by a few-step of liquid phase synthesis (Figure 3.2). This simple Fmoc-protected peptide sequence was conjugated with Dox moiety with an acid sensitive hydrazone linker through a multiple synthetic process (Scheme 3.2.1). The final product peptide–Dox conjugate was obtained by purification via HPLC and was further characterized with NMR and mass spectra.

3.2.2 *In vitro* pH responsive study

Figure 3.3 a) Time dependent cumulative release of Dox from peptide-Dox prodrug in acetate buffers with pH of 3.0, 5.0, 6.8 and 7.2 at 37 °C; b) pH (5.0) responsive drug release monitored by HPLC.

After successfully obtaining the Fmoc-peptide-Dox prodrug, the *in vitro* pH responsive drug release was monitored by HPLC (Figure 3.3). Typically, the peptide-Dox prodrug was incubated in aqueous solutions under different pH values at 37 °C. The HPLC results demonstrated that the peptide-Dox conjugate was quite stable under neutral pH conditions and there were nearly no free drug molecules in the solutions. As expected, the sequence could be rapidly cleaved within the acidic environment. About 50% Dox molecules could be released within 24 hours from the peptide-Dox prodrug under pH 5.0 (Figure 3.3) and more significant drug release (e.g. > 90%) could be observed within 1 hour when the pH of environment continuously increased to 3.0.

3.2.3 Self-assembly properties of peptide-Dox conjugate

Figure 3.4 a) TEM image of self-assembled peptide-Dox nanogels and b) the self-assembled nanogels after the treatment with acetate buffer (pH 3.0).

The self-assembly of the peptide-Dox prodrug was evaluated by addition of substrate (3.0 mg) into a water solution (5 mL, pH=7.0). Upon mixing at room temperature, peptide-Dox conjugate was observed to easily self-assemble into a transparent nanogel. Transmission electron microscopy (TEM) images indicated that the self-assembled nanogel particle has a sphere-like morphology with relatively a narrow size distribution of ~ 100 nm (Figure 3.4), which was further confirmed by dynamic light scattering (DLS) measurements (Figure 3.5).

Figure 3.5 Dynamic Light Scattering (DLS) characterization of self-assembled nanogel particles in water.

This nano-spherical gel structure was stable under neutral pH at room temperature for more than one month without obvious change. However, treatment of the peptide-Dox nanospheres with acetate buffer (pH = 3.0) at 37 °C for 1 hour would induce the rapid collapse of nanogels and thus lead to the free drug release into a viscous solution. After acidic treatment, TEM analysis revealed that the nano-spherical structures disappeared and highly ordered coiled nano-fibers were observed in this acidic solution, mostly attributing to the reassembly of cleaved Fmoc-peptide sequence as reported previously (Figure 3.4b).^{131,138-139,142-144} These results suggested that self-assembled peptide-Dox nanogels were acidic sensitive. Such stable but pH responsive supramolecular nano-spheres provided the great feasibility to enable a quantitative and high drug loading in the gel structures without additional carriers, making the self-delivery possible for efficient intracellular drug release and controlled redistribution.

3.2.4 Fluorescence imaging study of cell internalization and intracellular localization of nanogels

Encouraged by the favorable pH sensitive properties of the self-assembled nanogels, the cellular uptake and intracellular trafficking were investigated by monitoring the fluorescence of Dox in live cells. Typically, two cancer cells including one non-resistant A2780 cell and one Dox resistant ovarian A2780/ADR cell were selected as our main targets to incubate with nanogels at 37 °C for 2 hr. As negative controls,

Figure 3.6 Intracellular uptake and localization of peptide-Dox nanogel and free Dox (10 μ M) after 2 hr incubation. The cell nucleus was stained with DAPI. A2780: a Dox sensitive human ovarian cancer cell line; A2780/ADR: the relative Dox resistant cell line

the cells were also incubated with free Dox molecules, respectively. As demonstrated in Figure 3.6, after incubation with nanogels, there were bright red fluorescence signals observed in both A2780 and A2780/ADR cells and co-staining with DAPI molecules confirmed that the effective imaging signals were mainly accumulated in the cytoplasm, but not in the cell nucleus, mostly attributed to the involvement of endocytic pathway during the cellular uptake of nanogels. Control experiments by incubation of Dox with A2780 cells displayed the strong fluorescence in the cell nucleus and the relatively weak fluorescence in the cytoplasm, clearly indicating the different uptake pathways between peptide-Dox nanogels and Dox molecules, and free Dox itself could quickly concentrate into cell nucleus through the passive diffusion. Similar imaging experiments were also carried out by incubation of Dox with resistant A2780/ADR cells and significantly weak fluorescence was detected in this cell as compared to the signals in non-resistant A2780 cells. Such limited Dox uptake in

A2780/ADR cells would be one of the principal mechanisms contributing to the drug resistance in the cancer therapy. Live cell imaging studies indicated that peptide-Dox nanogels could effectively self-deliver Dox into drug resistant tumor cells and the higher cellular uptake of anti-tumor drug would probably circumvent the resistant effect and therefore enhance the efficacy of cancer chemotherapy.

3.2.5 *In vitro* antitumor activity of peptide-Dox nanogels

Next, we examined the *in vitro* antitumor activities of peptide-Dox nanogels as well as that of free Dox in both resistant and non-resistant cell lines. Cell viability was determined by standard Tox-8 assay (Figure 3.7) and the relevant resistant properties were investigated by the resistance indices (RIs) of nanogel and free drug (RI, defined as the IC_{50} value of a given agent in resistant cells, divided by its IC_{50} value in native

Figure 3.7 *In vitro* cytotoxicity of Dox and peptide-Dox nanogel against A) A2780 cells and B) A2780/ADR cells

cells).¹⁴⁵ Typically, both A2780 and A2780/ADR cells were incubated with Dox and peptide-Dox nanogel at various concentrations. As demonstrated in Figure 3.7 and table 3.1, incubation of free Dox with A2780 will lead to obvious cell toxicity ($IC_{50} = 0.135 \mu M$). Whereas relatively lower cytotoxicity ($IC_{50} = 0.64 \mu M$) was detected in the same cells when the nanogels were incubated, suggesting the rapid diffusion of free Dox into non-resistant cancer cells which inactivated tumor cells by interaction with nuclear DNA.^{74-75,146} Unlike the potent activity observed in A2780 cells, free Dox indicated limited cytotoxicity to resistant A2780/ADR cells ($IC_{50} = 4.71$), mainly attributed to the less drug accumulation caused by the multidrug resistance mechanisms in A2780/ADR cells.¹⁴⁷⁻¹⁴⁹ Similar cell viabilities ($IC_{50} = 6.45 \mu M$) were also observed for nanogels when they incubated with resistant A2780/ADR cells. The

RI value of peptide-Dox nanogel was determined to be 10.1, which was 3.5- fold lower than that of free Dox (Table S1, Supporting Information). The lower RI value of nanogel demonstrated the great feasibility to effectively overcome the drug resistance through their slow but steady accumulation of drug molecules into resistant tumor cells.¹⁵⁰⁻¹⁵¹ This is in accordance with the results observed in live cell fluorescent imaging measurements.

3.2.6 Intracellular translocation of Dox

Figure 3.8 Intracellular localization of peptide-Dox nanogels and free Dox (5 μ M) after 6 hr incubation in HeLa cells. A) Lysosome tracked with LAMP1-GFP fusion protein and cell nucleus stained with DAPI; B) Fluorescence images of drug translocation from lysosome to nucleus induced by H₂O₂.

Moreover, we further extended the systematic fluorescent imaging to investigate the hypothesis regarding the controlled release of drug molecules from endo/lysosomal compartments and their subsequent redistribution from cytoplasm to nucleus. Towards these ends, we chose standard HeLa cells, which can be effectively tracked by

fluorescence microscope,¹⁵² to incubate with free Dox and peptide-Dox nanogels. Typically, HeLa cells were incubated with free Dox and peptide-Dox nanogels. The cellular uptake and intracellular localization of Dox moieties were then studied by monitoring the red fluorescence under microscope. Figure 3.8 showed that after 6 hours incubation with Dox, obvious red fluorescence was found mainly in the nucleus of HeLa cells. In terms of nanogels, strong red cellular imaging was found to be co-localized with the fluorescence signal from green fluorescent protein (GFP) fused lysosome-associated membrane protein 1 (LAMP1, one commonly applied lysosome tracker), which confirmed that peptide-Dox nanogels predominantly self-delivered Dox into endo/lysosomal vesicles through an endocytosis pathway (Figure 3.8). Even after 48 hours of incubation, obvious fluorescence was still detected at endo/lysosomes (Figure 3.11), suggesting that the internalization of peptide nanogels by endo/lysosomes may represent an extra diffusion barrier to decrease the translocation of Dox to nucleus targets. One possible mechanism to overcome the endo/lysosomal membrane barrier and to facilitate rapid drug escape from endo/lysosomes would involve the generation of reactive oxygen species (ROS).¹¹⁸⁻¹²¹ As one of the most important ROS in living system, hydrogen peroxide (H_2O_2) has been documented to associate with various disease developments including cardiovascular disease or inflammation.^{121,153-154} As a proof-of-concept, H_2O_2 was chosen as an extracellular stimulus to control the intracellular translocation of drug molecules by disrupting the endo/lysosomal membranes. Typically, HeLa cells were pre-incubated with peptide nanogels (5 μM) for 24 hours followed by further treatment with H_2O_2 (5 mM). Unlike

Figure 3.9 The cell death rate after treatment with H_2O_2 (1.0 mM) and peptide-Dox nanogels (5 μM).

the imaging observed in Hela cells without H_2O_2 , treatment with H_2O_2 for 1 hour would dramatically enhance fluorescence signals in the nucleus, clearly indicating the translocation of internalized Dox from endo/lysosomes to nucleus. In contrast, there was no such translocation observed in HeLa cells when the H_2O_2 containing medium was pre-treated with catalase, one enzyme to efficiently decompose H_2O_2 (Figure 3.8).¹⁵⁵⁻¹⁵⁶ Moreover, cell toxicity of nanogels was also evaluated by incubation of HeLa cells with peptide nanogels (5 μM) in the absence or presence of H_2O_2 (1.0 mM). Cellular treatment with H_2O_2 or nanogels alone could only lead to a relatively small decrease of cell viability (12.5% and 10.6%, respectively). However, a significant cell death (31.4%) was detected when the cells were incubated with both H_2O_2 and nanogels, which was greater than the sum of the independent treatments (Figure 3.9). Although the potential toxicity of H_2O_2 was hard to completely avoid, the higher activity caused by synergistic effect implied the great possibility that ROS oxidation stress could disrupt acidic endo/lysosomal vesicles, and promoted the rapid escape of caged antitumor drugs from endo/lysosomes into nucleus targets, and may therefore increase the drug activities towards the tumor inactivation.

3.3 Conclusion

In summary, this chapter presents the design principle of novel pH sensitive peptide-Dox nanogels for controlled drug release in the resistant tumor cells. This self-assembled nanogel exhibits a narrow size distribution of 100 nm, which is sufficient stable in aqueous solutions under neutral pH, but indicates fast hydrolysis and intracellular release of drugs upon response to endo/lysosomal pH environment. Cellular imaging studies displayed that the peptide nanogels could self-deliver drugs into cells through endocytosis, therefore provide favorable antitumor effects toward resistant tumor cells. Moreover, the fluorescent imaging and *in vitro* cytotoxicity analysis also unequivocally supported the hypothesis that ROS could serve as one non-negligible factor to overcome the endo/lysosomal barriers and to facilitate the fast translocation of caged drugs into nucleus. Our initial exploration would therefore provide a useful strategy to investigate the details regarding the rapid intracellular drug redistribution by overcoming the different biological barriers in live cells, which could help better understand the mechanism of controlled drug release, and it could also facilitate the rational design of new carrier systems towards the enhanced drug activity in cancer therapy.

3.4 Experiment part

Materials and General methods:

Materials: Anhydrous solvents for organic synthesis were stored over activated molecular sieves before use (4 Å). Fmoc-Amino Acids and 2-Chlorotrityl Chloride Resin were purchased from GL Biochem Ltd (Shanghai). Commercially available reagents were used without further purification, unless noted otherwise. Thin-layer chromatography (TLC) analysis was performed on silica gel 60F-254 glass plates.

Instruments: ^1H NMR spectra were recorded using 400 MHz spectrometer. Mass spectra (MS) were measured with a Thermo LCQ Deca XP Max for ESI. Analytical reverse-phase HPLC analysis was performed on a Shimadzu HPLC system using an Alltima C-18 (250 x 10 mm) column at a flow rate of 3.0 mL/ min for preparation and a C-18 (250 x 4.6 mm) column at 1.0 mL/ min for analysis. TEM images were recorded on an FEI EM208S Transmission Electron Microscopy (Philips) operated at 100 kV. The size distribution of the self-assembled nanoparticle was performed using Dynamic Light Scattering (DLS, Brookhaven Instruments Corporation). The cell viability tests were performed using TOX8-assay (Sigma Aldrich) under a Tecan's Infinite M200 microplate reader.

3.4.1 Synthesis and characterization of Peptide-Dox conjugate

Preparation of Compound 2: Fmoc-Gly-OH (500 mg, 1.7 mmol), *N*-Hydroxysuccinimide (230 mg, 2.0 mmol) were dissolved in 8 mL DCM, followed by EDC.HCl (380 mg, 2.0 mmol) and DMAP (10 mg). The reaction mixture was stirred at room temperature for 4 hours. Tert-butyl hydrazinecarboxylate (277 mg, 2.1 mmol) in 2 mL DCM was then slowly added into the reaction mixture. The reaction was continued at room temperature for a further 4 h. The solvent was removed under vacuum and the residue was extracted with EA and washed with saturated aqueous sodium chloride. After drying over MgSO_4 and concentration under vacuum, the residue was purified by silica gel column chromatography with eluent EA/hexane (1:10) to give the compound **2** as a white solid (580 mg, 80%). ^1H NMR (400 MHz, CDCl_3): δ = 7.71 (d, J = 7.6 Hz, 2H), 7.53 (t, J = 7.6 Hz, 2H), 7.34 (d, J = 7.6 Hz, 2H), 7.25 (m, 2H), 7.00 (s, 1H), 4.32 (dd, J = 13.4, 6.8 Hz, 2H), 4.12 (m, 1H), 3.93 (d,

$J=7.8$ Hz, 2H), 1.42 (s, 9H); MS (ESI): m/z $[M+H]^+$ calcd: 412.18, found: 412.22.

Preparation of Compound 3: Compound **2** (500 mg, 1.17 mmol) was dissolved in 10 mL dichloromethane (DCM) containing 20% piperidine and stirred at room temperature for 30 minutes. The mixture was concentrated under vacuum and the residue was poured into 20 mL diethyl ether to give a white precipitate. This crude product was collected by centrifugation and purified with silica gel column chromatography with eluent Methanol/DCM (1:3) to give 196 mg (82%) of **3**.

Preparation of tetrapeptide Fmoc-GFLG-OH (4). The solid-phase peptide synthesis was based on the Fmoc chemistry with 2-Chlorotrityl Chloride Resin (500 mg, 0.5 mmol). The coupling reaction was performed at room temperature for 2-3 hours using HBTU/DIPEA (0.75 mmol) mixture. The deprotection of the Fmoc group was performed within 20% piperidine in dimethylformamide (DMF) at room temperature for 20 minutes. The final peptide product was released from resin using 1% TFA in DCM for 10 minutes. After removing DCM, the residue was reprecipitated in diethyl ether to give a white solid **4** (276 mg, 90% to resin). The crude product was used in the next step without further purification. MS (ESI): m/z $[M+H]^+$ calcd: 615.27, found: 615.05

Preparation of Compound 6. Compound **4** (100 mg, 0.16 mmol) and **3** (37 mg, 0.18 mmol) were dissolved in 5 mL DMF followed by addition of HBTU (74 mg, 0.19 mmol), DIPEA 100 μ L. The mixture was stirred at room temperature for 4 hr. The mixture was extracted with EA twice and the organic phases were washed with water for 2 times and then saturated aqueous sodium chloride for 2 times. After drying and concentration the residue was purified by column chromatography on silica gel with eluent methanol/DCM (1:10) to give 108 mg (85%) of Boc-protected product **5** as white solid. ^1H NMR (400 MHz, MeOD- d_4): δ = 7.75 (d, $J=7.5$ Hz, 2H), 7.59 (m, 2H), 7.37 (t, $J = 7.5$ Hz, 2H), 7.29 (m, 2H), 7.16 (m, 5H), 4.59 (dd, $J=12.8, 6.0$ Hz, 1H), 4.30 (m, 3H), 4.20 (m, 2H), 3.90 (m, 3H), 3.74 (m, 3H), 3.11 (dd, $J=14, 5.8$ Hz, 1H), 2.98 (m, 1H), 1.60 (t, $J=6.0$ Hz, 2H), 1.41 (m, 9 H), 0.85 (t, $J=6.0$ Hz, 6H); MS (ESI):

m/z $[M+H]^+$ calcd: 786.37, found: 786.07.

This intermediate product **5** was deprotected by 20% TFA in DCM at room temperature for 1 hour. After removing the solvent, the residue was reprecipitated in diethyl ether to give **6** as a white solid (103 mg, 94%). ^1H NMR (400 MHz, DMSO- d_6): δ = 9.76 (s, 1H), 8.17 (m, 2H), 8.06 (m, 2H), 7.89 (d, J =7.5 Hz, 2H), 7.70 (d, J =7.6 Hz, 2H), 7.51 (t, J =6.0 Hz, 2H), 7.41 (t, J =7.4 Hz, 2H), 7.32 (t, J =7.4 Hz, 2H), 7.19 (m, 5H), 4.55 (m, 1H), 4.26 (m, 5H), 3.73 (m, 4H), 3.63 (dd, J =16.8, 9.2 Hz, 1H), 3.51 (dd, J =16.6, 6.0 Hz, 1H), 3.02 (dd, J =13.8, 4.0 Hz, 1H), 2.77 (dd, J =13.6, 9.6 Hz, 1H), 1.58 (dd, J =12.5, 6.0 Hz, 1H), 1.49 (t, J =6.8 Hz, 2H), 0.87 (d, J =10.4 Hz, 3H), 0.83 (d, J =8.0 Hz, 3H); MS (ESI): m/z $[M+H]^+$ calcd: 686.32, found: 686.13.

Preparation of Peptide-Dox conjugate

Compound **6** (50 mg, 0.062 mmol), doxorubicin hydrochloride (43 mg, 0.075 mmol) was dissolved in 5 mL Methanol. The reaction mixture was stirred at 40 °C for 24 hr. The final product was purified by reverse-phase high performance liquid chromatography (HPLC) with eluting system consisting of A (50 mM ammonium acetate aqueous solution) and B (pure acetonitrile) under a linear gradient, monitored by UV absorbance at 280 and 495 nm. The linear gradient stretched over 20 minutes from $t = 0$ minutes at 30% solution B to $t = 20$ minutes at 90 % solution B. The reaction yielded the peptide–Dox conjugate as a red solid (36.3 mg, 50 %) after lyophilization. ^1H NMR (400 MHz, DMSO- d_6): δ = 11.13 (s, 1H), 10.57 (s, 1H), 8.14 (dd, J =8.0 Hz, 1H), 8.06 (d, J =8.0 Hz, 2H), 7.98 (m, 1H), 7.89 (m, 5H), 7.67 (m, 3H), 7.53 (m, 1H), 7.39 (dd, J =13.7, 6.9 Hz, 2H), 7.30 (dd, J =14.0, 7.0 Hz, 2H), 7.18 (m, 5H), 5.73 (s, 1H), 5.39 (d, J =5.6 Hz, 1H), 4.98 (s, 1H), 4.54 (m, 1H), 4.45 (m, 2H), 4.24 (m, 4H), 4.11 (m, 1H), 3.94 (m, 4H), 3.76 (m, 1H), 3.63 (m, 2H), 3.51 (m, 2H), 3.18 (dd, J =17.2, 1H), 3.02 (m, 1H), 2.89 (d, J =17.6, 1H), 2.78 (dd, J =13.6, 9.6 Hz, 1H), 2.36 (m, 1H), 2.21 (m, 1H), 1.88 (m, 1H), 1.69 (d, J =8.1 Hz, 1H), 1.59 (m, 1H), 1.49 (t, J =6.8 Hz, 2H), 1.17 (d, J =6.4 Hz, 3H), 0.87 (d, J =6.4 Hz, 3H), 0.83 (d, J =6.4 Hz, 3H); MS (ESI): m/z $[M+H]^+$ calcd: 1211.49, found: 1211.23.

3.4.1 HPLC analysis of Dox release from peptide-Dox prodrug

The prodrug nanogel solution (500 μ M) was diluted with acetate buffer of different pH values to reach a final concentration of 200 μ M. These solutions were incubated at 37 $^{\circ}$ C, and the analysis of Dox release from nanogel solutions were performed by reverse-phase high performance liquid chromatography (HPLC) with eluting system consisting of A (50 mM, ammonium acetate aqueous solution) and B (pure acetonitrile) under a linear gradient, monitored by UV-Vis absorbance at 280 and 495 nm. The linear gradient stretched over 20 minutes from t = 0 minutes at 30% solution B to t = 20 minutes at 90 % solution B, with a flow rate of 1.0 mL/min.

3.4.2 The preparation, TEM imaging and DLS analysis of nanogel particles

To produce the nanogels, 3.0 mg peptide-Dox prodrug in 50 μ L DMSO was added into 5 mL pure water. The nanogel particles formed within 5 minutes after mixing at room temperature. For TEM imaging, a portion from the nanogel solution was resuspended in water to give a final concentration of 200 μ M, the diluted solution was then dropped onto the copper grid. The nano-structures were characterized by FEI EM208S Transmission Electron Microscopy (Philips) operated at 100 kV. The measurement of the size distribution of nanogel particles was performed using Dynamic Light Scattering (DLS, Plus 90, Brookhaven Instruments Corporation).

3.4.3 General methods for cell culture, live cell imaging and cell viability assays.

A2780 cells and A2780/ADR (Doxorubicin resistance) cells were maintained in RPMI 1640 medium (HyClone) containing 10% FBS (HyClone) at 5% CO₂ and 37 $^{\circ}$ C. HeLa cells (ATCC NO. CCL-2) were maintained in DMEM medium (Invitrogen) containing 10% FBS (HyClone) at 5% CO₂ and 37 $^{\circ}$ C.

Live cell imaging was performed using 35-mm diameter μ -dish plastic-bottom (ibidi GmbH, Germany). Live cell imaging was performed under a fluorescence microscope (Nikon, Eclipse TE2000-E). The plasmid DNA encoding LAMP1-GFP

fusion protein, here used as lysosomal marker, was friendly presented from Prof. Lu Lei's group in School of Biological Science of NTU.

The cell viability measurements were performed on the basis of Tox-8 assay¹⁵⁷ (resazurin-based reagent, from Sigma Aldrich). Briefly, cancer cells were seeded in a 96-wells plate in RPMI 1640/DMEM with 10% FBS. After 24 hr incubation at 5% CO₂ and 37 °C, the medium was changed to fresh medium containing different substrates. After the desired incubation time, the medium was replaced with fresh RPMI-1640 (phenol red free) containing 10% TOX-8 reagent. After 2 hr incubation with TOX-8 reagent, the fluorescence intensity at 590 nm was measured at excitation wavelength of 560 nm by a Tecan's Infinite M200 microplate reader. The wells without cells but with Tox-8 reagent were set as blank controls. Each experiment was performed in three or four repeats. The cell viability rate (VR) was calculated according to the following equation:

$$VR = [(A-C)/^{158}] \times 100\%$$

where A is the emission intensity from the wells of experimental groups, B is the emission intensity of the control assays with cells and Tox-8 but without any treatment, and C is the emission intensity of Tox-8 loaded wells without cells.

3.4.4 IC₅₀ calculation and Resistance indices (RIs) determination

Briefly, A2780 and A2780/ADR cells were seeded in a 96-wells plate with a density of 6.0×10^3 cells per well in RPMI 1640 with 10% FBS. After 24 hours' incubation at 5% CO₂ and 37 °C, the medium was changed with fresh medium in the presence of free Dox or peptide-Dox nanogels at different concentrations. The cell viability measurements were determined after 72 hours' incubation as above described. Based on the results from cell viability assays, IC₅₀ values (toxicant concentration eliciting a 50 % inhibitory effect) on A2780 and A2780/ADR cells were further calculated by modified Kou-type method.¹⁵⁹ Resistance indices (RIs) were calculated by dividing the IC₅₀ in multidrug-resistant cells by the IC₅₀ in parental cells as described in lecture.¹⁴⁵

Table 3.1 The IC₅₀ values on A2780 cells and A2780/ADR cells, and the calculation of Resistance indices.

	IC ₅₀ (μM)	
	Free Dox	Peptide-Dox Nanogel
A2780	0.135	0.64
A2780/ADR	4.71	6.45
RI	34.9	10.1

RI = IC₅₀ against A2780/ADR cell /IC₅₀ against A2780 cell

3.4.5 *Imaging study of cellular uptake and intracellular localization of peptide-Dox nanogel and free Dox*

For general imaging, HeLa cells, A2780 cells or A2780/ADR cells were seeded at 300,000 cell/dish in 35-mm diameter μ-dish with plastic-bottom (ibidi GmbH, Germany). After overnight culture, the medium was changed to fresh medium in the presence of Dox or peptide-Dox nanogels (5 μM for HeLa cells or 10 μM for A2780 cells and A2780/ADR cells). For analysis of cellular localization of Dox and peptide-Dox nanogels, HeLa cells were transfected with the plasmid DNA encoding LAMP1-GFP fusion protein through a standard procedure. Briefly, HeLa cells were seeded in the μ-dish (35-mm diameter) for 24 hr to give about 80% confluence, the culture medium was replaced with Opti-MEM medium (Invitrogen) containing 0.6 μg LAMP1-GFP plasmid and 2 μL Lipo2K. After 6 hours, the medium was changed to fresh DMEM with 10 % FBS. After 24 hr incubation at 5% CO₂ and 37 °C, the medium was replaced with fresh medium containing 5 μM peptide-Dox nanogel or free Dox. The images were taken after a further 24-hour under fluorescence microscope, with 460/40 nm excitation and 535/50 nm emission filters for GFP protein, 360/40 nm excitation and 460/40 nm emission filters for DAPI, 535/20 nm excitation and 585 /20 nm emission filters for Dox.

For the imaging study of Dox translocation, HeLa cells were seeded in the μ-dish (35-mm diameter). After 24 hr incubation, the medium was replaced and the cells were incubated in the presence of 5 μM peptide-Dox nanogel or free Dox. After a further

24-hour incubation, the cells were washed and treated with H_2O_2 (5 mM) in phenol red free DMEM for 60 minutes. To degrade H_2O_2 , the medium containing H_2O_2 (5 mM) was pre-incubated with catalase 20 mg/L for 1 hr. The concentration and incubation time, here, were considered to be enough for catalase to fully degrade 5 mM H_2O_2 in aqueous solutions.¹⁵⁵

3.4.6 Suppressed cellular uptake at low temperature

HeLa cells were seeded into the μ -dish and cultured overnight before use. The medium was replaced with FBS free DMEM containing 10 μM peptide-Dox nanogel. After 30 min incubation at 4 °C or 37 °C, the cells were washed, and then were imaged under fluorescence microscope. From Figure 3.10, we learn that low temperature incubation significantly suppressed cellular uptake of nanogels. The result indicated that this peptide-Dox nanogel could self-deliver doxorubicin into HeLa cells through endocytosis, one well-established energy dependent pathway of internalization.

Figure 3.10 Live cell imaging of HeLa cells treated with peptide-Dox nanogel (10 μM) in FBS free medium at 37 °C and 4 °C for 30 minutes, respectively.

Time-lapse live cell imaging

HeLa cells were cultured in μ -dish with complete culture medium in the presence of 5 μM peptide-Dox nanogel or free Dox. The fluorescence images were taken at 3, 12, 24 and 48 hr of incubation without pre-washing.

Figure 3.11 Live cell imaging of HeLa cells after incubation with peptide-Dox nanogel or free Dox (5 μ M) for 3 h, 12 h, 24 h and 48 h.

3.4.7 H_2O_2 treatment enhanced the cell toxicity of peptide-Dox nanogel against HeLa cells

For the H_2O_2 treatment experiment, HeLa cells were seeded in a 96-wells plates with a density of $1-1.2 \times 10^4$ cells per well. After 24 hours incubation, the medium was replaced and the cells were incubated in the presence of 5 μ M peptide-Dox nanogel or Dox. After further 24-hour incubation the cells were washed and treated with or without H_2O_2 (1mM) in the phenol red and FBS free DMEM for 30 minutes. After 2 times washing, the cells were incubated in fresh medium for 24 hours before testing with Tox-8.

Chapter 4

Clickable Membrane Vesicles for Drug/Gene Delivery

4.1 Introduction

The cell derived membrane vesicles (MVs), a kind of specific membrane lipid based self-assembled nanostructures, which play key roles in cell–cell communication, antigen presentation, the spread of infectious agents, etc by sharing their genes and proteins.^{87,160-161} More recently, such MVs have been extensively investigated as promising natural materials in the drug/gene delivery,^{94-95,97-98} mainly attributed to their unique advantages over current delivery systems such as their viral-like transfection efficiency, inherent biological function and minimized inflammatory stimulation.⁸⁸ However, there are still some problems to overcome when using such naturally generated MVs for drug/gene delivery especially for cancer cell targeted chemotherapy. The biggest challenge among them is that the MVs freshly collected from the culture medium of mother cells often lack specific targeting ligands that could guide them to the aimed cells. Moreover, it is also a big obstacle to collect enough MVs from the general cell culture systems for its potential delivery use. Therefore the development of novel strategies to introduce affinity ligands onto MVs thus facilitate cancer cell targeting, as well as the strategies to obtain MVs from mother cells at a relatively higher yield could greatly expand the possibility for biomedical applications.

The research in this chapter aims to address the problems in the use of cell derived membrane microvesicles (MVs) for cancer cell targeted drug/gene delivery. Briefly, we aim to develop a simple strategy to introduce a clickable ligand to the membrane surface of MVs and establish an effective method to improve the production yield of MVs from the mother cells

As indicated in Figure 4.1, to achieve the functional MVs, a multifunctional conjugate was required that serves as a membrane tracker and meanwhile a clickable reagent. Learning from the structural properties of some commercial membrane trackers, a hydrophobic aliphatic chain and a positively charged polar head are introduced into the aimed compounds. Moreover, this benzophenone derivative would undergo cross-linking with membrane lipids upon UV irradiation, thus ensuring its

immobilization in membranes.¹⁶² The azide tagged specific target ligands (e.g. folic acid, RGD peptide, aptamer, etc) could be conjugated to MVs through click chemistry, which was well established on cell surface labeling.¹⁶³⁻¹⁶⁴ Such modification can also be achieved by the trace amount of bearing reactive groups such as amines and thiols on the membranes through the reaction with NHS-ester and maleimide. However, complications can arise due to the lack of selectivity inherent, which may result in non-specific labeling on some other biomolecules even on the payload drugs or genes and thus damage their bioactivities. In addition, it is known that the increased Ca^{2+} ions could stimulate several kinds of cells on shedding of membrane vesicles.¹⁶⁵⁻¹⁶⁶ Based on this fact, certain amounts of calcium ions (Ca^{2+} , from 5-20 mM) were used in our study as extracellular stimuli to improve the production yield of MVs.

Based on the rational design, we have successfully obtained the desired MVs. Our preliminary data indicated that click reaction could be easily performed on this chemical engineered MVs. This novel chemical engineering strategy on such cell derived membrane structures may greatly expand their applications in drug/gene delivery studies.

Figure 4.1 Schematic illustration of the concept on clickable cell derived membrane vesicles for drug/gene delivery

4.2 Result and discussion

4.2.1 Synthesis and characterization

4.2.1.1 Synthesis of the clickable benzophenone derivative

Scheme 4.1 The synthesis route of a novel clickable benzophenone derivative. Reagents and conditions: a) K_2CO_3 , DMF, 80 °C, 3h; b) HBTU/DIPEA, DMF, rt., 2h; c) K_2CO_3 , ACN, 55 °C, 8h.

As shown in Figure 4.1, a multifunctional conjugate consists of a benzophenone core structure, a hydrophobic tail and an alkyne-containing polar head (Figure 4.1) was designed to serve as not only a membrane tracker but also a clickable reagent in our study. Basically, benzophenone-4,4'-dicarboxylic was selected as the core structure of the clickable membrane anchor, mostly because of its desired aromatic and hydrophobic properties. Moreover, it is very easy to conduct the synthesis on the carboxylic groups of this benzophenone derivative. Most importantly, this benzophenone moiety would undergo cross-linking with membrane lipids upon UV irradiation, thus ensuring its immobilization in membranes.¹⁶² Learning from the structural properties of some commercial membrane trackers, a hydrophobic aliphatic chain and a positively charged polar head are introduced into the aimed compounds, ensuring a long term membrane tracking. The acetylene group was introduced to the aimed clickable product (3) through a sample coupling reaction.

4.2.1.2 Synthesis of the azide tagged fluorescence dye

Scheme 4.2 The synthesis route of the azide tagged cy5 derivative. Reagents and conditions: a) Triethylamine, DCM, 0 °C-rt., 4h; b) DMF, 50 °C, overnight; c) TFA 20% in DCM, rt., 30 min; d) DMSO, DIPEA, 2h.

To visualize the membrane selective localization of the designed clickable agent, an azido group functionalized fluorescence dye was synthesized through a simple process (Scheme 4.2). In order to minimize the interferences from the auto fluorescence (e.g. green or red signals) of the tested cell itself, a near infrared dye Cy5 was selected here. To get the aimed product, an azide tagged short linker was first prepared followed by the conjugation with the commercial compound Cy5-NHS-ester.

4.2.1.3 Synthesis of the azide tagged His6 chain

Scheme 4.3 The synthesis route of the azide tagged His6 chain. Reagents and conditions: a) 20% piperidine-DMF, rt., 20 min; b) DIPEA, DMF, rt., 4 h; c) TFA 95 %, 2.5% H₂O, 2.5% TIPS, rt., 30 min.

Actually, it is very easy to visualize the cellular localization of the clickable agent (**3**) by using the azide tagged Cy5 derivative (**7**). However, it would be more difficult to visualize its surface localization on membrane vesicles (MVs) due to the much smaller size of MVs than cells. To facilitate the evaluation of the clickable effects of our system, an azide tagged histidine oligomer (His6) was synthesized (Scheme 4.3). The option of His6 is mainly based on the concept that His6 has been shown to have great binding affinity to Ni-NTA beads, which were widely used in His-tagged protein purification. The commercial agarose Ni-NTA beads with the size various from 50 to 200 milliliters could greatly facilitate the observation under microscopy. The synthesis starts from the solid-phase based peptide synthesis through 5 times repeated coupling reactions. After removing the terminal Fmoc group, the azido linker was conjugated to the free amino group of His6 chain on resin (Scheme 4.3). The peptide with azide conjugation was then obtained through the reaction catalyzed by 95% TFA in DCM and finally purified via HPLC.

4.2.1.4 Synthesis of the azide tagged folic acid (FA)

Scheme 4.4 The synthesis route of the azide tagged folic acid. Reagents and conditions: a) EDC, HOSu, DMAP, DMF, rt., 5 h then 1,4-Bis (3-aminopropoxy) butane and TEA, rt., overnight; b) DIPEA, DMF, rt., 6 h.

It's known that folic acid and its derivatives have been widely used as cancer-targeting ligands in drug delivery systems. Their targeting effect is presumed to have originated from the specific interactions between FA and their receptors on plasma membranes that were usually highly expressed in many types of tumor cells. In our system, the folic acid derivative was chosen as the cancer cell-targeting agent. An azido group was introduced to the folic acid to ensure the click conjugation between FA and membrane anchored benzophenone derivative **3**. The synthesis was conducted in liquid phase

through a simple two-step process (Scheme 4.4). The final product with an azido group and a short linker between azide and FA was purified by HPLC and then characterized to be correct by NMR and Mass spectra.

4.2.2 Membrane vesicle (MVs) induction and collection

Figure 4.2 Schematic illustration of the generation (a) and isolation (b) of cell derived membrane vesicles.

Many types of cell lines indicated the properties of membrane vesicle release including some cancer cells, blood platelets, macrophages, dendritic cells and so on.^{167,168} Basically, membrane vesicles (MVs) could be naturally released from resting cells, but the production yield is very low that may greatly limit their biomedical applications.¹⁶⁶ Some additional stimuli such as nutrient starvation would increase the release of MVs from their mother cells in *in vitro* culture systems.¹⁶⁹ Up to now, in many biological and biochemical studies, the nutrient starvation based method has been widely used to stimulate the cell in release of MVs. Recently, this strategy was also applied in some recently developed MV-based drug/gene delivery systems.^{96,98-99,170} Ca^{2+} ions, a very important type of signaling molecules, were found to induce increased shedding responses (Figure 4.2-a).¹⁷¹⁻¹⁷² The released MVs in culture medium can be collected by two-step of centrifugation. After first step at low speed (e.g. 3000 g/min) for 15 min, the pellets consist of cells and cell fragments were discarded and MVs containing supernatant was collected for the next high-speed centrifugation (e.g. 30,000 g/min, 60 min) to give the pellets of MVs (Figure 4.2-b).

In our system, the cell line HEK 293 was selected mostly because of their easy culture as well as their high efficiency in gene transfection and rapid growth. After getting the MVs released from HEK 293 cells upon two types of stimulation (I) nutrient starvation and (II) extracellular Ca^{2+} (Figure 4.2-a), the MV generation efficiency was determined by quantifying the concentration of their protein contents. As known to us, protein quantification was a well-established method to evaluate MV productivity.^{170,173}

Figure 4.3 Schematic illustration of the generation (a) and isolation (b) of cell derived membrane vesicles.

As indicated in Figure 4.3, the PBS solution contains the MVs released from about 5×10^6 cells with Ca^{2+} stimulation is much cloudy than that from starvation treatment at the same cell number (Figure 4.3). The result clearly implied that there were probably more MVs released from the cells upon Ca^{2+} stimulation. Indeed, the protein quantification data indicated that upon the stimulation of Ca^{2+} , 1.56 milligram of MVs was released from per million cells which were found to be 17 folds higher than that from starvation treatment (0.088 mg per million cells).

The preliminary results indicated that extracellular stimulation with Ca^{2+} would be an effective way to generate enough amounts of MVs for further drug/gene delivery studies.

4.2.3 Cell plasma membrane tracking by the synthetic clickable agent

With the clickable agent (3) in hand, we first studied its membrane tracking ability at

the cellular level. Briefly, after pre-incubation with compound **3**, HT-29 cells were washed and then fixed with formaldehyde (10% in PBS buffer). After click chemistry

Figure 4.4 Fluorescence images of HT-29 cells after incubation with compound **3** and the click reaction with azide tagged Cy5. Images were taken under a wide field fluorescence microscopy. The fluorescence of Cy5 (610-650 nm excitation, 670-720 nm emission) is highlighted in red. Cell nuclei were stained with DAPI (blue FL).

reaction with azide tagged Cy5, cell images were acquired through a wide field fluorescence microscopy. As indicated in Figure 4.4, the red fluorescence corresponding to Cy5 emission under the excitation at 650 nm was mainly detected on the cell membrane. This was further confirmed by the images under confocal laser scanning microscopy (CLSM). Furthermore, some shedding of large sizes (low micrometers) from cell membrane were observed as indicated by white arrows in Figure 4.5. Those membrane shedding would be the result of unexpected environmental stresses such as PBS buffer washing and formaldehyde fixation during the process of sample preparation. As a control, the HT-29 cells without pre-incubation with compound **3** were conducted with click reaction under the same conditions. As expected, no fluorescence signal was observed on the cell membrane of those cells mostly because of the absence of acetylene groups on cell surface (Figure 4.9). The imaging data clearly indicated that the benzophenone derivative **3** could specifically insert into membrane structures. More importantly, click reaction could be performed

on the acetylene group functionalized plasma membranes including the membrane vesicles of large size.

Figure 4.5 Fluorescence images of HT-29 cells after incubation with compound **3** and the click reaction with azide tagged Cy5. Images were taken under a confocal laser scanning microscopy. The fluorescence of Cy5 is highlighted in red.

4.2.4 Imaging of membrane vesicles by microbead tracking assay

Figure 4.6 Schematic illustration of microbead based tracking analysis.

Nano/micro particle tracking analysis was developed recently aiming to facilitate the study on visualization, separation and sizing of biological nanostructures including intracellular organelles and membrane vesicles such as exosomes.¹⁷⁴⁻¹⁷⁵ As described before, the size in diameter of many cell derived MVs is often less than 200 nm. In order to achieve effective visualization of MVs, an agarose microbead based tracking assay was developed. The micro beads with tens of micrometers in diameter were functionalized with a layer of Nickel NTA conjugates, which were designed for

binding of His6. As illustrated in Figure 4.6, HEK 293 cells were first cultured in medium (RPMI 1640 contains 10% FBS) in the presence of CaCl_2 (5 mM) and compound **3** (100 μM). Compound **3** was supposed to insert into membrane structures

Figure 4.7 Fluorescence images of microbeads after incubation with MVs with different treatments. The fluorescence of Cy5 is highlighted in red.

including the released MVs upon calcium stimulation. The petri-dish was then exposed to UV light where compound **3** would undergo photo cross-linking with the adjacent lipid molecules, thus immobilizing itself on the membranes. The chemical engineered MVs were obtained after centrifugation as described before. To achieve the promising bio-compatibility, an effective Cu(I) stabilizing ligand THPTA tris(3-hydroxypropyltriazolylmethyl)amine) (500 μM) was used to perform the click reaction in the presence of CuSO_4 (100 μM),¹⁷⁶ (L-ascorbic acid (1.0 mM), azide tagged Cy5 (**7**, 100 μM) and azide His6 (**11**, 100 μM). With the clickable MVs in hand, the MVs were then immobilized onto the Ni-NTA beads followed by observation under microscopy. As proposed in Figure 4.6, supposedly, if the clickable agent (**3**)

really localized on the membranes and the click reaction is successful, the fluorescence from Cy5 moiety should be detected on the surface of agarose beads.

Fortunately, we indeed observed the fluorescence under confocal laser scanning microscopy. In contrast, no fluorescence was observed on the beads after incubation with MVs without treatment by click chemistry. More importantly, the beads captured by MVs that underwent the click reaction in the absence of CuSO_4 only indicated very weak fluorescence mostly due to the non-specific binding of Cy5 moieties and His6 derivatives to cellular contents (Figure 4.7). The result from microbead tracking clearly indicated that we have successfully achieved the desired clickable MVs.

4.2.5 Membrane vesicle based drug/gene delivery (still in progress)

Figure 4.8 Schematic illustration of surface functionalization of clickable MVs and FA/FA receptor directed drug/gene delivery.

Up to now, a novel clickable cell derived membrane vesicle (MV) has been developed in our lab. Through a simple click reaction, the aimed functional agents were successfully conjugated to the surface of MVs. With the favorable clickable properties, the smart MV may serve as a promising nanocarrier for cell line specific targeting delivery of genes and drugs. Folic acid (FA)/FA receptor pair based strategy has been widely used in the development of cancer cell-targeting delivery systems. For proof of concept, we also adopt the (FA)/FA receptor specific interaction mechanism in our system (Figure 4.8). Up to now, the azide tagged folic acid conjugate has been synthesized and well characterized with NMR and mass spectra. With the clickable MVs and the azide tagged folic acid in hand, the next step is to get the drug/gene loaded cancer cell-targeting MVs.

4.3 Conclusion

In this part, we presented a novel designed strategy using cell derived membrane vesicle to construct targetable drug/gene delivery. The clickable property was introduced to the MVs through a rational designed benzophenone derivative. This multi-functional agent not only serves as a membrane tracker, but also a clickable ligand. Moreover, upon UV light irradiation, the benzophenone core structure may undergo cross-linking with adjacent lipid molecules, thus immobilizing itself in membrane structures. Our imaging data and microbead tracking analysis indicated that click reaction could be performed on the chemical engineered MVs. This chemical engineering strategy on such self-assembled membrane structures may greatly expand the usage of cell derived membrane vesicles. The study on the application of the novel developed MVs in drug/gene delivery systems is still in progress.

4.3 Experiment part

Materials and General methods:

Materials: Anhydrous solvents for organic synthesis were stored over activated molecular sieves (4 Å) before use. Fmoc-Amino Acids and 2-Chlorotriyl Chloride Resin were purchased from GL Biochem Ltd (Shanghai). Commercially available reagents were used without further purification, unless noted otherwise. Thin-layer chromatography (TLC) was performed on precoated silica gel 60F-254 glass plates.

Instruments: ^1H NMR spectra were recorded using 400 MHz spectrometer. Mass spectra (MS) were measured with a Thermo LCQ Deca XP Max for ESI. Analytical reverse-phase HPLC analysis was performed on a Shimadzu HPLC system using an Alltima C-18 (250 x 10 mm) column at a flow rate of 3.0 mL/ min for preparation and a C-18 (250 x 4.6 mm) column at 1.0 mL/ min for analysis.

4.3.1 Synthesis and characterization

4.3.1.1 Synthesis of clickable benzophenone conjugates

Synthesis of compound 1

500 mg (1.85 mmol) benzophenone-4,4'-dicarboxylic was dissolved in 3 mL DMF

followed by addition of 1-bromohexadecane (320 μ L, 1.0 mmol) and K_2CO_3 (255 mg, 1.85 mmol). The reaction mixture was heated to 80 $^{\circ}C$ and keeping stirring for 3 hours. When cooling to room temperature, 20 mL water was added to stop the reaction followed by extraction with 20 mL ethyl acetate (EA). The organic layer was washed against water and was then concentrated by removing the solvent. The crude product was purified via silica gel chromatography (2 % to 4% methanol in DCM) to give compound **1** as a white solid (148 mg, 30%). 1H NMR (400 MHz, DMSO- d_6): δ = 8.10 (m, 3H), 7.84 (m, 4H), 7.53 (m, 1H), 4.32 (t, J =6.4 Hz, 2H), 1.73 (t, J =7.2 Hz, 2H), 1.39 (m, 2H), 1.22 (m, 24H), 1.22 (m, 3H).

Synthesis of compound 2

Compound **1** (50 mg, 0.1 mmol) and N,N-Dimethyl-1,3-propanediamine (12.2 μ L, 0.12 mmol) were mixed in 5 mL DMF. HBTU (45 mg, 0.12 mmol) and DIPEA 20 μ L (>2 eq) were then added into the reaction mixture followed by stirring at room temperature for 2 h. The reaction mixture was extracted with 20 mL EA against 20 mL water and was washed with saturated sodium chloride solution. The crude product **2** (48 mg, 83%) without further purification was directly used in the next step. ESI-MS: m/z 578.41 (calcd) for $C_{36}H_{54}N_2O_4$; 579.59 $[M+H]^+$ (found).

Synthesis of clickable compound 3

Compound **2** 30 mg (0.05 mmol) was mixed with excess amount of propargyl bromide (30 μ L) in 5 mL acetonitrile (ACN) in the presence of 7 mg (0.05 mmol) of K_2CO_3 . The reaction was stirred in the dark at 55 $^{\circ}C$ for 8 hours. The reaction mixture was then extracted with 10 mL EA for three times and the EA layer was washed with saturated sodium chloride solutions. Once removing the solvent, the crude products were purified by silica gel chromatography (10 % to 20 % methanol in DCM) to give the pure product **3** (19 mg, 64%). 1H NMR (400 MHz, MeOD- d_4): δ = 8.20 (d, J = 8.4 Hz, 2H), 8.05 (d, J = 8.4 Hz, 2H), 7.90 (dd, J = 8.4 Hz, 2.0 Hz, 4H), 4.41 (m, 2H), 3.59 (m, 7H), 3.25 (s, 6H), 2.19 (m, 2H), 1.83 (m, 2H), 1.49 (m, 2H), 1.38 (m, 24H), 0.91 (t, 3H); ESI-MS: m/z 617.43 (calcd) for $C_{39}H_{57}N_2O_4^+$; 617.34 $[M]^+$ (found).

4.3.1.2 Synthesis of clickable cy5 derivative

Synthesis of compound 4

3-Bromopropylamine hydrobromide (1.5 g, 6.8 mmol) was dissolved in 20 mL DCM followed by addition of Di-tert-butyl dicarbonate (3.0 g, 13.6 mmol). After cooling to 0 °C under ice bath, 1.0 mL was added into the reaction mixture dropwise over 5 minutes. The reaction mixture was naturally warmed to room temperature and was further stirred for 4 hours. 20 mL water was added to stop the reaction. After a further 10 min stirring, the mixture was extracted with EA and then washed with water and saturated sodium chloride solution. After drying, the product was purified via silica gel chromatography (7 % to 10 % EA in hexanes) to give the product **4** as colorless oil (1.3 g, 80 %). ¹H NMR (400 MHz, CDCl₃): δ= 4.74 (s, 1H), 3.41 (t, *J*=8.8 Hz, 2H), 3.24 (dd, *J*=16.8 Hz, 8.8 Hz, 2H), 2.02 (m, 2H), 1.39 (s, 9H).

Synthesis of compound 5

Compound **4** 200 mg (0.84 mmol) and Sodium azide (109 mg, 1.68 mmol) were dissolved in 5 mL DMF. The reaction mixture was stirred at 50 °C overnight. The reaction mixture was extracted with EA against water. Once washing and drying, the crude product was then purified via silica gel chromatography (15 % EA in hexanes) to give **5** (142 mg, 85%). ¹H NMR (400 MHz, CDCl₃): δ= 4.67 (s, 1H), 3.35 (t, *J*=6.4 Hz, 2H), 3.20 (dd, *J*=12.4 Hz, 6.4 Hz, 2H), 1.76 (m, 2H), 1.43 (s, 9H); ¹³C NMR (100 MHz, CDCl₃): δ= 155.9, 79.4, 49.1, 38.0, 29.3, 28.4.

Synthesis of compound 6

100 mg of compound **5** (0.5 mmol) was dissolved in 8 mL DCM. After cooling under ice bath, 2 mL of trifluoroacetic acid (TFA) was added dropwise into the mixture. The reaction mixture was stirred at room temperature for 30 min. after removing TFA and DCM, the product **6** (about 90 mg) without a further purification was used directly in the next step.

Synthesis of the azide tagged cy5

Excess amount of compound **6** was mixed with 0.5 mg Cy5 NHS ester in 500 μL

DMSO. After addition of 5 μ L N,N-Diisopropylethylamine (DIPEA), the reaction was kept in dark under stirring for 2 hours. The product was purified via reverse phase HPLC. After overnight lyophilization, the product **7** was redissolved in DMSO to reach 10 mM. The azide tagged Cy5 solution was kept at 4 $^{\circ}$ C before use. ESI-MS: m/z 737.28 (calcd) for C₃₆H₄₅N₆O₇S₂⁻; 739.24 [M+2H]⁺ (found).

4.3.1.3 *Synthesis of azide tagged His6 chain*

Solid phase based synthesis His6 chain

The synthesis of peptide His6 was performed by Fmoc-chemistry based solid phase synthesis using 2-Chlorotrityl Chloride Resin. The coupling reaction was carried out at room temperature using HBTU/DIPEA as coupling reagent. The reaction was allowed to last for 2 hours. The deprotection of Fmoc group was performed in 20% piperidine-DMF solution for 20 min. After the removing the terminal Fmoc group from the peptide chain **8**, the resin bonded product **9** could be used directly for the next coupling reaction with NHS ester.

Synthesis amide compound 11

The resin bonded compound **9** (100 mg) was suspended in 4 mL DMF followed by addition of excess amount of azido-dPEG₄ NHS ester (20 mg, 0.052 mmol) and 20 μ L DIPEA. The synthesis tube was gently shaken for 4 hours. After removing the solvent and washing with DMF for 5 times, the peptide chain **10** was released from resin using 2% TFA /DCM solution. The crude product was purified by reverse-phase high performance liquid chromatography (HPLC) with eluting system consisting of A (0.1% TFA water solution) and B (0.1% TFA acetonitrile solution) under a linear gradient, monitored by UV absorbance at 254 nm. After overnight lyophilization, the product **11** was redissolved in DMSO to reach 10 mM. The azide tagged His6 solution was kept at 4 $^{\circ}$ C before use. ESI-MS: m/z 1113.50 (calcd) for C₄₇H₆₃N₂₁O₁₂; 1114.42 [M+H]⁺, 558.14 [M+2H]²⁺, 372.46 [M+3H]³⁺ (found).

4.3.1.4 *Synthesis of azide tagged folic acid derivative*

Synthesis compound 12

Folic acid 100 mg (0.22 mmol) was dissolved in 5 mL under ultrasound sonication. DCC (45 mg, 0.22 mmol), HOSu (20 mg, 0.18 mmol) and catalytic amount of 4-Dimethylaminopyridine (DMAP) were added into the mixture. The reaction was performed by stirring under room temperature for 5 hours. After that, 1,4-Bis(3-aminopropoxy) butane (130 μ L, 0.6 mmol) together with 50 μ L Triethylamine (TEA) were added into the reaction mixture. After a further overnight stirring, the reaction mixture was added into 30 mL diethyl ether to give a light yellow precipitate. The crude product was purified by using reverse phase HPLC followed by overnight lyophilization to give **12** as light yellow powder (30 mg, 22 %). ESI-MS: m/z 627.31 (calcd) for C₂₉H₄₁N₉O₇; 628.31 [M+H]⁺, 314.89 [M+2H]²⁺ (found).

Synthesis of azide tagged folic acid derivative 13

Compound **12** (8 mg, 0.013 mmol) was mixed with azido-dPEG₄ NHS ester (6 mg, 0.016 mmol) and 8 μ L DIPEA in 2 mL DMF. The reaction mixture turned cloudy after addition of DIPEA. After 2h stirring, the mixture turned clear. The stirring was lasted for a further 4 hours. The reaction mixture was added into 10 mL diethyl ether to give a light yellow precipitate. The crude product was purified by reverse-phase high performance liquid chromatography (HPLC) with eluting system consisting of A (0.1% TFA water solution) and B (0.1% TFA acetonitrile solution) under a linear gradient, monitored by UV absorbance at 320 nm. The linear gradient stretched over 20 minutes from $t = 0$ minutes at 100% solution A to $t = 20$ minutes at 40 % solution A. The reaction yielded the azide tagged folic acid derivative **13** (10 mg, 86 %) after lyophilization. ¹H NMR (400 MHz, DMSO-d₆): δ = 8.65 (s, 1H), 8.19 (d, J =7.6 Hz, 1H), 7.80 (m, 2H), 7.65 (d, J =8.8 Hz, 2H), 6.92 (s, 1H), 6.63 (d, J =8.8 Hz, 2H), 4.48 (s, 1H), 4.26 (m, 1H), 3.50-3.60 (m, 19 H), 3.31 (m, 10 H), 3.06 (m, 4 H), 2.28 (t, J =6.4 Hz, 2H), 2.17 (t, J =8.0 Hz, 2H), 2.02 (m, 1H), 1.90 (m, 1H), 1.59 (m, 4H), 1.48 (m, 4H); ¹³C NMR (100 MHz, DMSO-d₆): δ = 174.3, 171.9, 170.4, 166.8, 154.2, 151.2, 149.1, 149.0, 129.4, 128.4, 121.8, 111.7, 70.3, 70.3, 70.2, 70.1, 70.0, 69.7, 68.1, 67.3, 52.7, 50.4, 46.4, 36.6, 36.3, 36.3, 32.5, 29.8, 27.0, 26.5; ESI-MS: m/z 900.45 (calcd) for C₄₀H₆₀N₁₂O₁₂; 901.35 [M+H]⁺ (found).

4.3.2 Membrane vesicle (MV) induction and isolation

Before the cell culture, the exosome like particles in the commercial fetal bovine serum (FBS) were removed by centrifugation at 30,000 g for 2 hours followed by sterilization through a 220 nm membrane filter. HEK 293 cells were seeded at a density of about 5×10^6 per dish in a 10 mm petri-dish. After overnight growth in RPMI 1640 medium supplied with 10% FBS, the medium was changed with freshly prepared RPMI 1640 (without phenol red) supplied with exosome-free FBS. For MV induction, 5 mM CaCl_2 was added into the culture medium. After 24 hours incubation, the culture medium became cloudy that means a large amount of MVs released. To generate the clickable MVs, benzophenone derivative (**3**) (100 μM) was added into the culture medium 3 hours before the addition of calcium. The MV containing medium was collected for centrifugation. After first step centrifugation at 3000 g for 15 min, the pellets were discarded; the supernatant was then centrifuged again at 30,000 g for 60 min. The supernatant was gently removed, and MV pellets was resuspended in PBS (pH 7.4) and then kept at 4 $^\circ\text{C}$ until further use.

Figure 4.9 Fluorescence images of HT-29 cells without pre-incubation with compound **3** but with the click reaction in the presence of azide tagged Cy5. Images were taken under a wide field fluorescence microscopy. The fluorescence of Cy5 (610-650 nm excitation, 670-720 nm emission) is highlighted in red. Cell nuclei were stained with DAPI (blue FL).

4.3.3 Click reaction on cell membranes

HT-29 cells were seeded in 35 mm petri-dish for imaging at 300,000 cell/dish. After

overnight growth, benzophenone derivative (**3**) (50 μ M) was added into the medium. After further 3 hours incubation, the medium was removed. The cells were washed with PBS for two times followed by fixation with 10% formaldehyde for 10 minutes at rt. The formaldehyde solution was then removed followed by two times washing with ammonia chloride solution (50 mM) and the two times washing with PBS (pH 7.4). The click reaction was performed in PBS buffer following the details:

In a 2 mL eppendorf tube, add the MVs and reagents in the following order:

1. add the azide tagged target ligand (e.g. Cy5-azide or His-azide) into MVs solution to reach 50 μ M.
2. add the premixed CuSO_4 and THPTA solution into the MVs solution to reach 100 μ M and 500 μ M respectively.
3. add sodium ascorbate (1.0 mM) into the reaction mixture.

The reaction was lasted for 30 min under room temperature by gentle shaking. Copper ions and ligands were removed by centrifugation and the MVs were then washed once with PBS buffer before observation under microscopy.

4.3.4 Microbead tracking assays

After obtaining the clickable reagent (**3**) functionalized MVs, the click reaction on MVs was conducted as following described:

In a 2 mL eppendorf tube, add the MVs and reagents in the following order:

1. 500 μ L of PBS buffer (pH 7) containing the GFP gene loaded MVs ($\text{OD}_{600} \approx 0.5$).
2. add the azide tagged target ligand (e.g. folic acid) into MVs solution to reach 100 μ M.
3. add the premixed CuSO_4 and THPTA solution into the MVs solution to reach 100 μ M and 500 μ M respectively.
4. add sodium ascorbate (1.0 mM) in to the reaction mixture.

The reaction was performed for 60 min under 4 $^{\circ}\text{C}$ by gentle shaking. Copper ions and ligands were removed by centrifugation and the MVs were then washed once with PBS buffer. The collected MVs were resuspended in PBS and stored at 4 $^{\circ}\text{C}$ before use.

4.3.5 General method for fluorescence imaging:

Cell imaging was performed using 35-mm diameter μ -dish plastic-bottom (ibidi GmbH, Germany). For MV particle imaging, a portion of MVs solution with different treatment was dropped onto a coverslip (22×22 mm) followed by immobilization with another one covering. Wide field images were taken under a fluorescence microscope (Nikon, Eclipse TE2000-E). The confocal laser scanning images were acquired with confocal Laser scanning microscope (Nikon Eclipse TE2000-E, CFI VC 60× oil immersed optics).

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Chapter 5

Conclusion and future works

Drug resistance is a growing problem in antibacterial therapies and cancer treatments, which represents a major threat to public health. To address the worsening problem there is an urgent need to develop new strategies against bacterial pathogens and cancers. The research works in this dissertation mainly focused on these important health-care issues, which consist of two parts of contents: (1) Design and synthesis of peptide functionalized materials for Gram-negative bacteria targeted photodynamic inactivation and LPS sensing (chapter 1 and chapter 2); (2) Rational design and development of self-assembled nanostructures for controlled drug/gene delivery against cancers (chapter 3 and chapter 4). In this chapter, we draw the conclusions and future works based on the above two parts of work.

5.1 LPS-recognizing peptide: further applications in bacterial detection and antibacterial therapy.

Although bacterial lipopolysaccharide (LPS), a main component of Gram-negative bacterial outer membrane, serves as a type of permeability barrier for antibacterial molecules, it also offers us a favorable cellular target for the development of Gram-negative bacterial target PACT or chemotherapy. The part I of this dissertation aims to develop novel bacterial-affinity ligand based bioconjugates for targetable therapy and sensing toward Gram-negative bacteria. By conjugation of photosensitizers with LPS-recognizing peptides, we developed a novel divalent photosensitizer delivery system (Chapter 1). Our result indicated that the divalent porphyrin-peptide conjugate has promising properties in real-time fluorescent imaging and photoinactivation of Gram-negative bacterial pathogens especially for those with drug resistant properties. Furthermore, by combining $\text{Fe}_3\text{O}_4@\text{SiO}_2$ core-shell magnetic nanoparticles with perylene diimide (PDI) conjugated LPS-recognizing peptides, we developed a simple and novel strategy for effective fluorescent detection and rapid clearance of such toxic bacterial endotoxin (Chapter 2).

Although LPS-recognizing peptides, as described in this dissertation, have shown the great potent to serve as promising Gram-negative bacteria targeting agents, their

applications in biomedical practices is still in its fancy. In the future works, we will continue to search novel LPS-recognition peptides and further expand the applications of these peptides in LPS detection and controlled antimicrobial therapy. For example, quenching of the sensitizer excited state as a means to control singlet oxygen production is an approach that has been extensively studied.¹⁷⁷

Figure 5.1 Schematic of a singlet oxygen switch-on system based on specific interaction between peptide and LPS. PS=photosensitizers, Q=quencher, LPS=lipopolysaccharide

As indicated in Figure 5.1, to achieve the functional conjugate, we can introduce a photosensitizer to a quencher using the LPS-recognizing peptide as linker. In the absence of binding ligand (LPS), the peptide linker could fold to make the PS and the quencher close enough, thus ensuring an effective energy or electron transfer. For this case, only weak fluorescence and no singlet oxygen could be detected upon irradiation. However, upon interaction between the peptide and LPS or Gram-negative bacterial cell wall, the peptide would stretch and singlet oxygen photoproduction would be switched on.

5.2 Cell derived membrane vesicles (MVs) for drug/gene delivery: the challenges for practical applications.

Along with our efforts in the development of self-assembled nanostructures including the nanogels from short peptides (Chapter 3) and membrane vesicles from membrane lipids (Chapter 4), the development of novel drug/gene delivery systems based on the clickable MVs will be focused in our future works. Although our previous results have indicated that surface modification could be easily performed on MVs (Chapter 4), there still are some challenges ahead of us to push the concept into therapeutic applications in the real world. One of the most challenged problems is to get MVs with

high loading of drugs or genes. Basically, there are two approaches to achieve the drug/gene loaded MVs:

1: Drug/gene can be loaded into MVs by electroporation pretreatment. It is well known that electroporation is an established method to facilitate gene transfection toward bacterial cells and mammalian cells. Although the gene loaded MVs could be obtained by this method, the efficiency is limited and the operation process is relatively complicated.

2: The drug/gene loaded MVs can be achieved by using gene pre-transfected or drug pre-loaded cells as mother cells for generation of MVs. This is a new strategy to get the therapeutic MVs and only few works have been proposed by far.

Improving drug/gene loading efficiency by optimizing the current methods or developing novel loading strategies would become the key objectives in the applications of MV based drug/gene delivery systems.

Publications

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