Development of nanosized assemblies for biomedical applications

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DEVELOPMENT OF NANOSIZED ASSEMBLIES FOR BIOMEDICAL APPLICATIONS

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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DEVELOPMENT OF NANOSIZED ASSEMBLIES FOR BIOMEDICAL APPLICATIONS

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Abbreviations

ACN	Acetonitrile
Ac4ManNAz	Peracetylated N-azidoacetylmannosamine
BHQ-1	Black hole quencher
CLSM	Confocal laser scanning microscopy
CPT	Camptothecin
DIPEA	N-Ethyldiisopropylamine
DMF	Dimethylformamide
DMEM	Dulbecco's modified Eagle's medium
DCM	Dichloromethane
DLS	Dynamic light scattering
DOX	Doxorubicin
DBT	4,7-di(thiophen-2-yl)-2,1,3-benzothiadiazole
DMEM	Dulbecco's Modified Eagle Medium
5-FAM	5-carboxyfluorescein
FACS	Fluorescence-activated cell sorting
Fmoc	9-fluorenylmethyl chloroformate
FDA	Food and Drug Administration
FBS	fetal bovine serum

HBTU	Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HPLC	High Performance Liquid Chromatography
MeOH	Methanol
MS	Mass spectrometry
MDR	Multidrug resistance
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear magnetic resonance spectroscopy
NBD	4-nitro-2,1,3-benzoxadiazole
PBS	Phosphate buffered saline
PEI	polyethyleneimine
PEG-PLA-PPA	poly(ethylene glycol)-poly(Laspartic acid)-poly(L-phenylalanine)
RPMI 1640	Roswell Park Memorial Institute 1640
rt	Room temperature
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
TEM	Transmission electron microscopy
UV-Vis	ultraviolet-visible spectroscopy

Abstract

Recently, self-assembled nanostructures have received considerable attention due to their potential applications in nanomedicine. Despite the initial success, it is still ongoing challenges to fabricate more effective and specific nanoplatforms for disease treatment and essential physiological processes investigation in living system. Therefore, various artificial and natural self-assemblies are highly desirable, and extensive strategies still need to be further exploited in clinics.

To solve this problem, in this dissertation, we fabricated two different kinds of significant self-assemblies, artificial peptide-based nanoconjugates and natural extracellular vesicles-based nanostructures. The former strategy was designed to overcome a major challenge, multidrug resistance (MDR), which is considered as a huge obstacle for effective cancer treatment in clinics. More importantly, the second approach was specifically constructed to introduce functional proteins from parent cells to defective recipient cells, which could be utilized to precisely manipulate cellular functions for several human diseases related to defects in membrane proteins. The relevant details are shown in the following parts.

Firstly, in chapter 2, smart peptide self-assemblies are designed to reverse MDR effect in chemotherapy. As a well-known mechanism for MDR, overexpression of the drug efflux transporter, P-glycoprotein (P-gp), could significantly pump out the exogenous therapeutic drugs from cancer cells and induce the drug resistance in tumor treatment. Herein, we fabricated a reduction-responsive nanosystem based on peptide assemblies, which including two bioactive molecules (an antitumor drug and a peptide inhibitor of P-gp) for combating MDR of cancer cells. Upon internalization, the nanostructures could disassemble in respond to the intracellular reductive condition,

leading to the controlled release of the anticancer drug and P-gp inhibitor, which synergistically improved the therapeutic efficacy.

In order to further enhance the cancer treatment efficiency, a novel tumor microenvironment-responsive nanoconjugate was developed in chapter 3 based on the pH activatable doxorubicin prodrug and another P-gp inhibitor. This inhibitor-encapsulated nanogel exhibited rapid responsiveness, controlled cargoes release, and effective inhibition of the P-gp activity, thereby significantly enhancing the accumulation of the antitumor drugs in resistant cancer cells. The synergetic effect of the P-gp inhibitor and the chemotherapeutics successfully defeated resistant cancer cells.

Moreover, in addition to modulate the pathological processes via artificial peptide assemblies, we further explored the natural nanosized assemblies for manipulation of cellular functions based on specific membrane proteins. Currently, a number of major human diseases are related to defects in membrane proteins. However, effective strategies to restore the defective cellular functions from parent cells to target cells are highly desirable. Despite genetic engineering and protein transport techniques are commonly used in biomedical studies, some unavoidable limitations still occur in these approaches, including potential gene mutation and complicated protein purification steps. To overcome these drawbacks, in chapter 4, we designed a simple and efficient strategy based on EVs for transferring bioactive proteins to target cells and conferring the cells with specific biological function. To proof the concept, a light-gated membrane channel protein (ChR2) was transported to another recipient cells by using EVs nanovehicles. Upon blue light irradiation, the membrane protein was activated, allowing the calcium ion (Ca^{2+}) influx across the plasma membrane and intracellular Ca^{2+} -dependent transcription factors activation. The results demonstrated

that our strategy provided a novel insight on the manipulation of membrane functions, which is benefit for several relevant human disease treatments in the future.

Furthermore, in chapter 5, in order to investigate this dynamic behavior of EVs, a universal membrane modification strategy based on metabolic glycol-engineering was proposed for effective EVs labeling and investigating the interaction between different types of EVs and cells. Preliminary results demonstrated that the metabolic passway could effectively introduce azide groups to the surface of EVs. Moreover, bioorthogonal click chemistry allowed imaging agents to be covalently conjugated on the surface of modified EVs. Therefore, the present azide-incorporated MVs could be endowed with various imaging agents via bioorthogonal click chemistry for extensive investigation of the biological behaviors of EVs in future.

In summary, these proposed strategies in this dissertation provided a prospective future of these artificially and naturally self-assembled nanostructures for the biomedical applications. We believe the present works will lead to inspiring innovations in this research field and will be developed continuously to benefit human health in the future.

Chapter 1

General Introduction

1.1 Molecular self-assembly

As a universal phenomenon in nature, molecular self-assembly is the significantly spontaneous association of molecules into ordered and stable hierarchical structures through noncovalent interactions, including hydrophobic interactions, hydrogen bonds, van der Waals' bonds, and electrostatic interactions.^[1, 2] Specifically, most structures in biological system are formed by the molecular self-assembly process in a significant accurate manner (Figure 1.1).^[3] For example, the cell membrane is self-assembled by phospholipids and the DNA double helix is composed by individual nucleotide.^[4] These endogenous assemblies are organized by basic biological molecules (*e.g.*, amino acids, lipids, nucleotide, saccharides, *etc*) through weak interactions to precisely exert specific biological functions.

Recently, nanometer-sized structures have attracted major attention owing to their potential for diverse applications ranging from nanotechnology to biotechnology. Many self-assembly nanostructures have been developed from biomolecules including peptides, nucleic acids, phospholipids and carbohydrates. Especially, peptides have become known as immensely useful building blocks for creating self-assembled nanostructures in biomedical applications due to their intrinsic biocompatibility and biodegradability.^[5] In addition to these artificially self-assembled nanostructures, some natural nanostructures, such as extracellular vesicles (EVs), also remain one of the most promising self-assembled nanovehicles in biomedical applications in recent decades. These artificial and natural self-assemblies could potentially achieve a better

understanding of the self-assembly mechanism and utilize them as smart nanoplatforms to transport numerous exogenous cargos for therapeutic applications. In this part, we will briefly introduce the commonly utilized strategies for the construction of self-assembled peptide nanostructures and EVs to achieve various biomedical applications *in vitro* and *in vivo*.

Figure 1.1 Illustration of endogenous assemblies in cells.

1.2 Self-assembled peptide nanostructures

In recent decades, self-assembled peptide nanostructures have drawn considerable attention due to their outstanding properties, including simple structure, relative chemical and physical stability, diversity in sequences and shapes, and the feasibility to synthesize them in large amounts.^[6-8] Amino acids, the building blocks of proteins, are ideal for designing peptide nanomaterials. First, permutation and combination of the 22 natural amino acids as well as the non-natural amino acids offer an enormous space for designing self-assembling peptides.^[3] Second, amino acids side chains exhibit different sizes, charges, and polarity (Figure 1.2).^[9] Variation in the peptide sequence significantly influences the self-assembly process and physicochemical

A Aliphatic hydrophobic residues: provide a hydrophobic environment.

B Aromatic hydrophobic residues: involved in π - π stacking.

C Hydrophilic residues: involved in hydrogen bonding interactions

D Charged residues: involved in charge-charge interactions.

Basic:

Acidic:

E Others:

A target for chemical modification.

Reduces steric hindrance and introduces a high degree of flexibility

Introduces structural rigidity.

Figure 1.2 Structures of amino acids and their properties in the design of self-assembled peptides.^[9] (Reproduced and adapted by permission from Elsevier)

properties of nanostructures. Third, the reactive groups (amino, carboxylic acid, and thiol) of peptides allow conjugation of a range of small molecules to the self-assembling backbones, producing a wide variety of self-assembling molecules. Moreover, one or more functional molecules could be incorporated into the peptide backbones, providing tailored functions.^[10]

1.2.1 Self-assembled peptides

According to the building blocks, there are two categories of self-assembled peptides: peptides and peptide derivatives. The first category employs the natural existing self-assembly peptide motifs present in proteins, such as β -sheets, α -helices and coiled coils. Extensive investigation of protein sequences has gained some basic rules for the formation of these conformational units, therefore it is possible to design an array of peptides based on these rules. For the peptide derivatives, the amino acids are covalently conjugated to other molecules such as an alkyl chain or aromatic groups.

1.2.1.1 β -sheets and β -hairpin

β -sheets, the most common natural peptide motifs for driving the self-assembly of peptide, consist of alternating hydrophilic and hydrophobic amino acids (Figure 1.3a). These alternating residues can assemble into a sheet with hydrophilic and hydrophobic face.^[3] β -sheets could further assemble to form different hierarchical structures including ribbons, tapes, fibrils, and fibers by varying number of packed sheets.^[11]

β -hairpin is composed of two β -sheet sequences in anti-parallel directions linked by a turn peptide sequence (Figure 1.3b). Schneider and Pochan designed a β -hairpin structure (VKVKVKVKV^DPP^TKVKVKV) with a turn sequence (-V^DPP^T-) and flanked peptide sequences.^[12] The peptide is unfolded at low pH in pure water, while increased pH and ionic strength induce the formation of β -hairpin and subsequently

assembles to fibrous structure. The formed hydrogels from this β -hairpin peptide show broad-spectrum antibacterial activity.

Figure 1.3 Schematic illustrations of the molecular self-assembly involved in the formation of different secondary structures peptides: (a) β -sheet, (b) β -hairpin, (c) α -helix, and (d) coiled-coil.^[13] (Reprinted by permission from Royal Society of Chemistry)

1.2.1.2 α -Helices and coiled coils

The α -Helix formed from the polypeptide is a common secondary structure of proteins. In the α -Helix, the polypeptide backbone is winded into a right-handed helix that contains 3.6 amino acids per turn (Figure 1.3c). The internal backbone hydrogen bonds between the amide hydrogen and the carbonyl oxygen in residue i and $i + 4$ stabilize the α -Helical structure.^[14] However, only few self-assembled systems formed from α -Helices due to more amino acids (20-30) required for establishment of stable α -Helix.^[15-17]

The coiled-coil motif is formed by two or more α -Helices. A typical coiled-coil usually consists of seven residue repeats and the residue in each repeat is labeled as a-g (Figure 1.3d). Normally, the position of a and d are hydrophobic residues which

offer the stabilization through the hydrophobic interaction, while the charged residues at position e and g mediate association between helices.^[18] An example of the use of coiled-coil motif for design of self-assembled system was reported by Woolfson et al.^[19] They designed two complementary peptide sequences, which co-assembled into α -Helical coiled-coil dimer with complementary sticky ends, which further promoted the formation of fibers.

1.2.1.3 Diphenylalanine (FF)

Diphenylalanine, the core recognition motif of Alzheimer's β -amyloid polypeptide,^[20] has become a common self-assembling peptide motif for construction of various functional nanostructures including nanotubes, nanofibrils, nanowires, spherical vesicles and ordered chains.^[21-23] These nanostructures are formed by both π - π stacking and hydrogen bonding of phenyl rings. The self-assembled FF-based nanomaterials have found their applications in 3D cell culture, bioimaging, biosensor, drug delivery, nanofabrication as well as guest encapsulation.^[24-27]

1.2. 1.4 Peptide amphiphiles

Peptide amphiphiles are class of molecules that consist of hydrophobic tail and hydrophilic peptides. The most well-known amphiphilic peptide molecules typically have four domains (Figure 1.4): domain I is a hydrophobic moiety, usually alkyl chains with different length and composition. Palmitic acid, a saturated fatty acid with a 16-carbon, is the most widely used alkyl tail. Domain II is β -sheet-forming peptide for determining the morphology of nanostructures. Domain III is comprised of charged amino acids that improve the solubility of PAs. Domain IV consists of biological epitope for introducing desired bioactive function.^[28] The main driving forces of self-assembly of PAs in aqueous solution are the intermolecular hydrogen bonding of β -sheet region and hydrophobic interaction of alkyl chains. Moreover, the morphology

and functionality of resultant nanostructures could be changed by adjusting the structural components of PAs. Based on these principles, Stupp's group pioneered various self-assembling peptide amphiphiles for biomedical applications.^[29-35]

Figure 1.4 (A) Chemical structure and space filling mode of PA molecules; (B) Illustration of a self-assembled PA nanofiber.^[28, 36] (Reprinted with permission from Elsevier)

1.2.1.5 Aromatic short peptide derivatives

The addition of various aromatic groups to short peptides has been demonstrated to facilitate self-assembly through π - π stacking.^[37] For instance, an aromatic group, such as fluorenylmethoxycarbonyl (Fmoc), naphthalene, or carbobenzyloxy, was conjugated to the N-terminus of some short peptides, leading to the formation of stable hydrogels (Figure 1.5).^[38-40] The morphology of the obtained nanostructure depends on the chemical property of the building blocks and the triggered method of self-assembly (e.g., solvent polarity changes, pH adjustment, or enzyme catalysis).^[41-44]

Figure 1.5 Illustration of aromatic peptide self-assembly; (a) Structure of aromatic peptide amphiphile; (b) possible stacking arrangements; (c) self-assembled nanostructures; and (d) a nanofibrous network.^[40] (Reprinted with permission from Royal Society of Chemistry)

One of representative aromatic short peptides is Fmoc-FF peptide, which could self-assemble to form supramolecular hydrogels.^[45] In the proposed mode, the peptide moiety adopts an anti-parallel β -sheet arrangement; the π -stacked pairs are formed by interleaved Fmoc groups from alternate β -sheets; the second sheet is rotated in relation to the first due to the twist of β -sheets, generating a cylindrical structure. In addition to Fmoc-FF peptide, self-assembly systems from aromatic short peptide derivatives are continually being investigated and developed. Moreover, deep understanding of the design rules will push forward the development of new self-assembly systems.

1.2.2 Methods of triggering self-assembly process

In general, a stimulus, such as temperature change, pH adjustment, light irradiation, as well as enzymes is needed to drive self-assembly.

1.2.2.1 Temperature change

The change of temperature is one of the most common methods for constructing self-assembled systems. One way is the heating-cooling cycle. In general, elevated temperature could increase the solubility of compounds and result in homogeneous solution. During the cooling process, molecules undergo rearrangement to form more ordered nanostructures that are thermodynamically stable.^[46, 47] During the past decade, many self-assembled peptide nanostructures that formed through heating-cooling cycle have been developed.^[48-51] For example, Nandi developed a thermoreversible hydrogel composed of riboflavin and melamine, which was formed by cooling a mixture solution from 120 to 30 °C.^[52]

1.2.2.2 pH adjustment

The simple method to control self-assembly process is the change of pH. The pH-triggered self-assembly largely relies on the protonation/deprotonation of basic or acidic groups. A variety of peptide-based building blocks could self-assemble to form nanostructures on the basis of change of pH.^[53, 54] For instance, Schneider and coworkers designed a peptide MAX1 with the sequence VKVKVKVKV^DPPTKVKVKVKV-NH₂. The peptide was unfolded at pH of 5.5, while increased pH (9.0) triggered self-assembly of peptide to form fibrous nanostructures.^[12] This transition process mainly attributes to the protonation of the lysine residue. When the pH is low the pK_a of the lysine side chains, the protonation of the lysine results in charge repulsion between neighboring lysines, hindering the folding of the peptide. Under the basic condition, some of the lysine residues are neutral, which triggers the formation of β -hairpin and subsequent self-assembly.

1.2.2.3 Reductive reaction

Another powerful method for controlling molecular self-assembly is the use of disulfide bond reduction. Rao's group firstly utilized the method of disulfide reduction

to trigger a covalent condensation reaction between 2-cyanobenzothiazole and 1,2-aminothiols for controlled self-assembly of nanostructures in vitro and in living cells.^[55] Nilsson and coworkers also applied this strategy to design a self-assembling peptide (Ac-C(FKFE)₂CG-NH₂), which was cyclized by disulfide bonds (Figure 1.6).^[56] The cyclic conformation restrained the formation of β -sheet structure that was necessary for self-assembly. After reduction of disulfide bonds, the linear peptide adopted β -sheet conformation, and subsequently self-assembled to form fibrillar nanostructures. Yang's group employed disulfide bond as cleavable linker to conjugate a self-assembling peptide motif with a hydrophilic segment. Upon cleavage of disulfide bond, the released self-assembling motif would form molecular hydrogels.^[57-59] All in all, the approach could maintain the homogenous of the self-assembly process, which is crucial for the potential applications in biomedical field.

Figure 1.6 Cyclic to linear peptide conformational switch using a reductive trigger.^[56]

(Reprinted with permission from American Chemical Society)

1.2.2.4 Light irradiation

Light with precise temporal and spatial resolution is also an effective tool to direct molecular self-assembly. Generally, a photosensitive group such as azobenzene, α -carboxy-2-nitrobenzyl (CNB), merocyanine, and tetrazole is incorporated into the building blocks.^[60-63] For example, Schneider and coworkers attached a photocage (α -

carboxy-2-nitrobenzyl) to cysteine of a 20-residue peptide, obtaining the peptide MAX₇CNB. The photocaged peptide was unable to self-assemble in aqueous medium under ambient light. However, the presence of UV irradiation caused decaging to release uncaged peptides that fold to β -hairpin conformation and hence self-assemble to fibrous network.^[64]

1.2.2.5 Enzymatic interaction

Recently, enzymes have been emerging tools to control the self-assembly of peptides under physiological conditions.^[42, 65] In general, a non-self-assembling precursor is converted into a self-assembling building block by an enzyme, therefore initiating the self-assembly process. Until now, several enzymes have been exploited to control self-assembly process.^[66-68] An example of an enzyme initiated self-assembly is phosphatase, which converts hydrophilic precursors into more hydrophobic molecules via cleaving the hydrophilic phosphate group and subsequently triggers self-assembly process. A broad range of precursors have been designed as substrates of phosphatase for construction of nanoscale assemblies.^[69-71] Another typical example is thermolysin. This enzyme could catalyze the conjugation of two building blocks through a covalent bond, thereby triggering the self-assembly.^[41] In fact, due to the mild reaction process, enzyme-instructed self-assembly becomes a promising method for the development of biofunctional materials.

1.2.3 Biomedical applications

1.2.3.1 Drug delivery

The effective delivery of drugs to target disease sites plays vital roles in successful chemotherapy. However, small molecule anticancer drugs suffer from few limitations including poor solubility in water, limited bioavailability, nonspecific selectivity, rapid blood clearance, adverse side effects, and severe multidrug

resistance.^[72] To address these issues, delivery vehicles such as liposomes and inorganic materials have been intensively used over the last decades.^[73] Normally, drugs are encapsulated within these nanovesicles or conjugated to the nanocarriers. Unfortunately, low drug loading capability, metabolic burden of inert vehicles, and side effects are the major drawbacks of these carriers.^[74]

In recent years, peptide-based self-assembled nanostructures have attracted considerable research in the field of drug delivery.^[9] The therapeutic agents can be physically entrapped inside the nanostructures or covalently linked to the self-assembling molecules.

For physical encapsulation, a broad range of designs have been presented.^[75-77] For example, Stupp's group developed a class of self-assembling peptide amphiphile (PA) nanofibers composed of peptide sequences and fatty acids for delivery of anticancer drugs.^[35, 78, 79] Schneider and coworkers reported a localized curcumin delivery system by the encapsulation of curcumin within a self-assembling peptide hydrogel. In vitro studies demonstrated the encapsulated curcumin retained its biological activities. Moreover, the release rate of curcumin and its therapeutic efficacy can be modulated by adjusting the concentration of the peptide.^[80]

Besides physical encapsulation, the exploitation of drug molecules as building units to construct ordered nanostructures of various morphologies has been a growing trend in nanomedicine.^[81-83] This strategy considers the drug molecule itself as a main component of the self-assembly to obtain its own assemblies, which combine the advantages of prodrugs and nanocarriers, and therefore possess high and fixed drug content, self-deliverable property, elimination of leakage, prolonged blood circulation time, and enhancement of tumor accumulation.^[84] Generally, drug molecules are covalently conjugated to a peptide segment with self-assembly property to afford well-

defined nanostructure. In the assemblies, the drug molecules not only retain their bioactivity but also serve as building blocks. While the peptide acts as functional segment for assembly purpose. Moreover, stimuli-responsive linker could be introduced to link the hydrophilic segment and the drug, resulting in controlled release of the drug. It is worth noted that subtle changes of these three segments could significantly influence the system's performance. Therefore, rational design of all the motifs is essential.

The pioneering work of self-assembling peptide-drug conjugate was reported by Cui and coworkers.^[85] In their work, the hydrophobic therapeutic agent camptothecin (CPT) was linked to a β -sheet forming short peptide through a reducible linker (disulfylbutyrate, buSS) (Figure 1.7). In order to precisely control the drug loading content, branching points were introduced for attachment of one, two or four drug molecules, obtaining drug loading amount of 23, 31, and 38%, respectively. All the drug conjugates assembled into filamentous structure with a peptide- corona and a

Figure 1.7 Schematic illustrations of the designed drug amphiphile and control molecules.^[85] (Reprinted with permission from American Chemical Society)

drug-core, which protected the CPT from the external environment. Interestingly, the drug release could be controlled through the reduction of buSS linker. Upon addition of glutathione to the nanostructures, the slow release of drug was observed for di-CPT or tetra CPT and fast release occurred for mono-CPT. These results were attributed to the different stability of the assembling nanostructures.

Inspired by the concept of self-assembling peptide-drug amphiphiles, other groups also investigated the assemblies formed by peptide-drug amphiphiles. For example, a CPT-conjugated dipeptide that can self-assemble into nanotubes with diameters from 80-120 nm was reported by Parquette group.^[86] These nanodrugs displayed high drug loading and showed exceptionally stable. In vitro studies demonstrated the nanoassemblies exhibited good activity against cancer cells. However, the large size of nanostructures hindered their systemic delivery. Li and coworkers developed self-assembled peptide-platinum prodrug, which could form supramolecular self-assemblies in the presence of phosphatases and release drug under reductive environment. The self-assembled prodrug exhibited improve antitumor efficacy due to the enhanced drug accumulation and sustained release of the drug.^[87]

Interestingly, a special class of peptide-drug amphiphiles could be designed to self-assemble into supramolecular hydrogels in water. Typically, the peptide-drug amphiphiles are able to self-assemble into nanofibers, which further entangle to form hydrogels with a three-dimensional network. Xu's group pioneered the design of PTX-conjugated peptide (NapFFKpY), which can self-assemble into supramolecular hydrogels through enzymatic reaction. A phosphorylated tyrosine residue was introduced for improving hydrophilicity, which can be removed by phosphatase to reduce the solubility of the conjugates and initiated the self-assembly.^[88] Similar designs were further developed by Xu and others to create peptide-drug amphiphiles

based on a broad range of drugs, including anticancer drugs Taxol, curcumin, and gemcitabine.^[89-94]

1.2.3.2 Regulation of cell fate

Cancer therapies have progressed considerably through extensive efforts in the past two decades. Unfortunately, cancer remains a big challenge to public health. Therefore, innovative strategies for cancer treatment are still urgently needed. As mentioned before, the self-assembly of clinically available anticancer drugs through physical encapsulation or chemical modification have shown significantly potentials in improving the therapeutic efficiency.

Recently, a new direction in anticancer therapy has been proposed. The self-assembled nanostructures themselves without drugs were developed as potential therapeutics for regulation of cell fate. Wells and coworkers found that nanofibrils assembled from small molecules could stimulate activation of procaspase-3 to generate active caspase-3, thus further initiating the apoptosis process.^[95] The preliminary results encouraged scientists to extensively investigate the potential and mechanism of self-assembling small molecules in regulating cell fate.^[96-99] For example, the nanofibers formed by enzyme-instructed self-assembly (EISA) approach demonstrated the feasibility of the self-assembly of small molecules for manipulation of cell fate. Short peptide Nap-FF were demonstrated to exhibit selective inhibition of the growth of glioblastoma cancer cells, while being nontoxic to neuronal cells (Figure 1.8).^[100] In vitro experiments revealed that nanofibers disrupted the dynamics of cytoskeletal component (e.g., microtubules) and consequently induced apoptosis of cancer cells. When an ester bond was linked to the C-terminal of Nap-FF, the resulting conjugate could easily enter cells and self-assemble into nanofibers upon hydrolysis by esterase. Interestingly, the nanofibers selectively killed cancer cell (HeLa), while

remained innocuous to NIH 3T3 fibroblast cells. These results attributed to the higher expression level of esterase in HeLa cancer cell than NIH 3T3 fibroblast cells.

Figure 1.8 (a) Chemical structure of Nap-FF; (b) Proposed molecular arrangement of Nap-FF; (c) Possible mechanism for selective killing of glioblastoma cells by nanofibers of Nap-FF peptide.^[100] (Reprinted with permission from Wiley-VCH)

Besides intracellular formation of nanofibers of small molecules for controlling cell behavior, selective formation of pericellular nanofibrils around cells is another new mechanism to regulate the fate of cell. In brief, ectoenzyme overexpressed on specific cancer cells could catalyze designed precursor to self-assembling molecules, which subsequently form fibrous nanonets on the cell membrane. This nanonet normally impedes the cellular mass change and cell communication, thus causing cell apoptosis cascade.^[101, 102] This strategy was firstly achieved by the utilization of phosphatase overexpressed on certain cancer cell surface (Figure 1.9). The secretory phosphatase transformed a small D-peptide (D-1) to a hydrogelator (D-2), which accumulated and self-assembled into nanofibers. These nanofibers impaired cellular uptake and mass exchanges, thus initiating cell apoptosis. Moreover, due to the

overexpression of alkaline phosphatase by cancer cells, this designed D-1 selectively caused cancer cell death.^[103]

Figure 1.9 (a) The formation of nanonets around the cell membrane to induce cell death; (b) Chemical structure of D-1 and D-2.^[103] (Reprinted with permission from Wiley-VCH)

1.2.3.3 Imaging

Fluorescence imaging is a highly sensitive and powerful method to detect biological analytes and elucidate sophisticated biological events. Until now, a number of small molecular probes and nanoprobe have been designed and developed for fluorescence imaging. Recently, nanoprobe based on self-assembling small molecules emerged as promising tools for visualization of biological processes.^[104, 105] In general, self-assembly induced fluorescence turn on and disassembly-driven fluorescence turn on are commonly used mechanisms by self-assembled nanoprobe for bioimaging application.

1.2.3.3.1 Self-assembly induced fluorescence turns on

Conventional fluorophores possess the property of aggregation-caused quenching, which significantly influence the sensitivity. To solve this dilemma, specific

fluorescent dyes including environment sensitive dyes and aggregation-induced emission (AIE) dyes have been developed.

For environment sensitive dyes, they emit negligible fluorescence in hydrophilic conditions because of the charge transfer, while they show strong fluorescence signal in hydrophobic environments due to the reduced charge transfer. Take advantages of this unique feature, the combination of self-assembly and environment sensitive fluorophores was developed for biological imaging. For example, Gao and coworkers have used this method to imaging self-assembly of small molecules in live cells (Figure 1.10).^[106] An environment sensitive dye (NBD) and a phosphatase trigger (tyrosine phosphate) was introduced to generate the precursor (NapFFK(NBD)pY), which entered into cells by diffusion and emitted little fluorescence. After enzymatic catalysis by cellular ALP, the precursor was converted to the hydrogelator which self-assembled to form fibrillar nanostructures. Since the nanofibers provided a hydrophobic environment for the condensed and localized NBD, the resulting nanofibers exhibited remarkably stronger fluorescence than the precursors and the

Figure 1.10 Principle of imaging enzyme-triggered supramolecular self-assembly inside cells.^[106] (Reprinted with permission from Nature Publishing Group)

hydrogelators. This obvious contrast offered a potential for investigating the molecular self-assembly of small molecules inside cells. This strategy was subsequently extended by other researchers to detect the activities of ectophosphatases, the distribution of different self-assembling small molecules and cellular apoptosis.^[107-109]

1.2.3.3.2 Disassembly induced fluorescence turn on

Although conventional fluorophores often suffer from aggregation-induced quenching at high concentration, the Hamachi group smartly turned this notorious effect to their advantages. They introduced a novel mechanism for the development of “turn on” fluorescent probe. The self-assembling probe is nonfluorescent in assembled state due to the aggregation-induced quenching, while recognition-induced disassembly of the probe leads to fluorescence recovery in the presence of target protein. By using this principle, the target proteins of human carbonic anhydrase I, avidin, and trypsin were successfully detected in the test tube setting.^[110] More importantly, they replaced the hydrophobic fluorophore with more hydrophilic dyes by adding a hydrophobic module. The resulting self-assembling nanoprobe were demonstrated to imaging cell membrane proteins such as folate receptor and carbonic anhydrase in live cells.^[111] In a subsequent work, they tried to detect specific intracellular proteins by using similar design. The MTX ligand of intracellular enzyme dihydrofolate reductase (eDHFR) was conjugated to a fluorophore of tetramethylrhodamine through a hydrophobic linker to obtain the probe. The probe-treated HeLa cells that overexpress eDHFR exhibited strong fluorescence inside cells. However, the normal HeLa cells without eDHFR displayed weak fluorescence. Upon addition of inhibitor of eDHFR, the fluorescence of the probes disappeared.^[112] These results suggested the effective imaging of cytosolic enzyme by using disassembly-driven turn on probe.

The Hamachi group made great contributions to detect target protein through the strategy of disassembly-driven signal turn on. In addition to protein imaging, this strategy can also be applied to detection of enzyme activities. Cui and coworkers designed a new self-assembled nanobeacon for imaging of enzymes (Figure 1.11). The self-assembling amphiphilic molecule contained a hydrophilic cell-penetrating peptide, a hydrophobic quencher BHQ1, and a green dye (5-FAM) that is conjugated to the peptide via a Cat B-responsive linker GFLG. The amphiphilic molecule could self-assemble to form micelles in physiological environment, preventing undesirable enzymatic degradation. Upon internalization in lysosome, the nanoprobe would disassociate to monomeric form because of the dilution and/or the acid triggering. The cleavable linker in monomeric form was susceptible to Cat B protease and released fluorophore 5-FAM, thereby generating strong fluorescence signals.^[113]

Figure 1.11 (A) Proposed detection mechanism; (B) Molecular structure of the nanobeacon.^[113] (Reprinted with permission from American Chemical Society)

All in all, the development of imaging tools based on self-assembly opens a new area for visualization of biomarker and biological events. However, the development of the self-assembled nanoprobe is still in initial stage. More research efforts are needed to extend their applications.

1.3 Bioresponsive strategies

1.3.1 pH-sensitive strategy

The pH gradients in specific tissues and intracellular compartments have been extensively used to design stimuli-responsive systems. The tumors exhibit acidic states that pH values range from 5.7 to 7.8. The pH value of the tumor extracellular microenvironment is about 6.5, which is lower than the normal tissues (pH 7.4).^[114] The specific acidity is partly caused by the accumulation of lactic acid in the fast-growing tumor cells which have elevated metabolic rate of glucose.^[115] Beyond that, poor lymphatic drainage and insufficient blood supply also cause low pH values of tumor microenvironment.^[116] Beside tumor tissues, the endosome also showed acidic pH that ranges from 5.0 to 6.5, and the lysosome is even more acidic (pH = 5.0 – 5.5).^[117]

Several strategies are exploited for the design of acid-responsive systems. One strategy is to incorporate ionizable groups such as amines, imidazoles, and carboxylic acids to the system segments. Generally, ionizable moieties could induce the corresponding system to undergo surface charge conversion, solubility and/or conformational changes in response to microenvironment pH variation.^[118-120] For example, Ling developed a pH-responsive self-assembled magnetic nanogrenades (PMNs) composed of iron oxide nanoparticles and pH-sensitive imidazole-containing ligands for bio-imaging and tumor treatment *in vivo* (Figure 1.12). The PMNs were

negative at neutral environment. The acidic tumor environment caused protonation of imidazole group which reversed surface charge from negative to positive, leading to enhanced internalization of PMNs by cancer cells. Upon entering into endosome, the particles exhibited increased ionization level due to the more acidic condition. These ionized groups repel each other resulting in disassembly of the PMNs, thereby recovering MR contrast fluorescence and photoactivity. The designed PMNs were demonstrated to show superior therapeutic efficiency in resistant tumors.^[121]

Figure 1.12 (a) Schematic illustration of pH-sensitive, ligand-directed self-assembly of extremely small iron oxide nanoparticles (ESIONs); (b.c) Schematic illustration of acidic tumor microenvironment-responsive treatment strategy by PMNs.^[121] (Reprinted with permission from American Chemical Society)

The second strategy is the use of acid-labile bonds that connect different components of the carrier or covalently link payloads onto a carrier. The commonly

used acid labile linkers are summarized in Figure 1.13. These pH-sensitive bonds are very stable at neutral condition, but easily cleavable under acidic environment. Until now, various carriers containing different acid- labile linkages have been reported for pH-triggered delivery of therapeutics and imaging agents. For example, Tang et al. developed a type of micelles formed by the self-assembly of PEG- β -polymethacrylates bearing acid-sensitive ortho ester side-chains for acid-triggered delivery of DOX.^[122] Yang's group developed a series of pH-sensitive materials containing acid-labile benzoic imine moiety for biomedical applications.^[123] Wang's group engineered a tumor acidity-responsive delivery system (PEG-Dlink_m-R9-PCL) containing an

Name	Structure	Products
Hydrazone	$\begin{array}{c} \text{R} \\ \diagup \\ \text{C}=\text{N}-\text{N}-\text{R}'' \\ \diagdown \\ \text{R}' \end{array} \longrightarrow \begin{array}{c} \text{R} \\ \diagup \\ \text{C}=\text{O} \\ \diagdown \\ \text{R}' \end{array} + \begin{array}{c} \text{H}_2\text{N} \\ \\ \text{N}-\text{R}'' \\ \\ \text{H} \end{array}$	
Ester	$\begin{array}{c} \text{O} \\ \\ \text{R}-\text{C}-\text{O}-\text{R}' \end{array} \longrightarrow \begin{array}{c} \text{O} \\ \\ \text{R}-\text{C}-\text{OH} \end{array} + \text{HO}-\text{R}'$	
Imine	$\begin{array}{c} \text{R} \cdot \text{N} \cdot \text{R}' \\ \\ \text{C} \\ \\ \text{H} \end{array} \longrightarrow \text{R}-\text{NH}_2 + \begin{array}{c} \text{O} \\ \\ \text{C}-\text{R}' \\ \\ \text{H} \end{array}$	
Orthoester	$\begin{array}{c} \text{O} \\ \\ \text{R}-\text{C}-\text{O}-\text{R}' \\ \\ \text{O} \end{array} \longrightarrow \begin{array}{c} \text{OH} \\ \\ \text{R}-\text{C}-\text{O}-\text{H} \\ \\ \text{O} \end{array} + \text{R}'-\text{OH}$	
Vinylether	$\text{R}-\text{O}-\text{CH}=\text{CH}-\text{R}' \longrightarrow \text{R}-\text{OH} + \text{O}=\text{CH}-\text{CH}-\text{R}'$	
Thiopropionate	$\begin{array}{c} \text{O} \\ \\ \text{R}-\text{C}-\text{CH}_2-\text{CH}_2-\text{S}-\text{R}' \end{array} \longrightarrow \text{R}-\text{OH} + \begin{array}{c} \text{O} \\ \\ \text{HO}-\text{C}-\text{CH}_2-\text{CH}_2-\text{S}-\text{R}' \end{array}$	
Phosphoramidate	$\text{R}-\text{NH}-\text{P}(\text{O})(\text{O}^-)-\text{O}-\text{R} \longrightarrow \text{R}-\text{NH}_3 + \text{H}_2\text{PO}_4 + \text{R}'-\text{OH}$	
Carboxy dimethylmaleic anhydride	$\begin{array}{c} \text{O} \\ \\ \text{R}-\text{N}-\text{C} \\ \\ \text{H} \end{array} \begin{array}{c} \text{O} \\ \\ \text{C} \\ \\ \text{OOC} \end{array} \begin{array}{c} \text{O} \\ \\ \text{C} \\ \\ \text{OOC} \end{array} \begin{array}{c} \text{O} \\ \\ \text{C} \\ \\ \text{OOC} \end{array} \longrightarrow \text{R}-\text{NH}_2 + \begin{array}{c} \text{O} \\ \\ \text{C} \\ \\ \text{O} \end{array} \begin{array}{c} \text{O} \\ \\ \text{C} \\ \\ \text{O} \end{array} \begin{array}{c} \text{O} \\ \\ \text{C} \\ \\ \text{O} \end{array} \begin{array}{c} \text{O} \\ \\ \text{C} \\ \\ \text{O} \end{array}$	

Figure 1.13 The commonly used acid labile linkers.

extracellular pH (pH_e) labile linker (2-propionic-3-methylmaleic anhydride, CDM) for targeted delivery of siRNA (Figure 1.14). This system exhibited prolonged blood circulation due to the protection of PEG. At tumor site, pH_e -sensitive linker CDM was cleaved to induce PEG detachment and R9 exposure, thereby achieving enhanced cellular internalization.^[124]

Besides modification of various carriers with acid-cleavable bonds, the payloads can also be conjugated onto carriers via acid-sensitive linkages. For instance, Lee et al. linked Dox onto the MSN nanochannel walls through hydrazone bonds for controlled release of Dox molecules.^[125] Wang et al. conjugated Dox onto the surface of AuNPs via hydrazone bonds for overcoming drug resistance in cancer cells.^[126] Zhang et al. reported polymer-cisplatin nanoparticles with conjugation of cisplatin drug to the copolymer chains via a hydrazone bond. The resultant nanoparticles exhibited enhanced cytotoxicity due to nanoparticles uptake and subsequent controlled drug release.^[127]

Figure 1.14 Schematic illustration of self-assembly of PEG-Dlink_m-R9-PCL and the nanoparticle's change in response to tumor acidity.^[124] (Reprinted with permission from American Chemical Society)

1.3.2 Redox-responsive strategy

The high redox potential differences exist between the extracellular environment and the intracellular space. Generally, the redox status of biological system is mainly maintained by biological species, such as glutathione (GSH), cysteine, homocysteine, thioredoxins, and NADPH.^[128] Among these reducing agents, GSH is the most abundant biological thiols. Studies reveal that the concentration of GSH in cytoplasm (approximately 2-10 mM) could be 100-1000 fold higher than that in the extracellular fluids (2-20 μ M).^[129] Meanwhile, the intracellular GSH concentration in some cancer cells is much higher than that in normal cells, and is often increased in drug resistant cancer cells.^[130] Disulfide bonds are well known to be responding to various reducing agents and can be reduced to free thiols through redox reactions.

By taking advantage of this feature, a myriad of reduction-responsive prodrugs, fluorescent probes and nanocarriers have been developed.^[131-133] For instance, Kim and coworkers reported a multifunctional prodrug composed of an RGD cyclic peptide as targeting motif, a naphthalimide group as fluorescent indicator, a disulfide bond as reduction-responsive linker, and camptothecin as an anticancer agent. The cleavage of disulfide bond by reducing agents such as GSH resulted in the release of camptothecin and production of a red-shift fluorescence signal.^[134] Therefore, the designed conjugate could serve as theranostic delivery system for real-time monitoring of targeted cellular uptake and intracellular drug release. Zhu et al. described a NIR prodrug by conjugating an anticancer drug (camptothecin) to a NIR fluorophore (dicyanomethylene-4H-pyran derivative) via a disulfide linker. The high GSH concentration in cancer cells caused the cleavage of the disulfide bond, leading to simultaneously drug release and NIR fluorescence turn-on.^[135]

Recently, reactive oxygen species (ROS) also have been explored as biological stimuli for development of oxidation-responsive materials. In general, ROS include hydrogen peroxides (H_2O_2), hydroxyl radicals ($\text{OH}\cdot$), superoxides (O_2^-) and peroxynitrites (ONOO^-), which are generated from insufficient reduction of oxygen in the mitochondria and NADPH oxidase in the cellular membrane.^[136] ROS plays an important role in physiological events, such as cell proliferation, cell signaling, apoptosis and immune responses.^[137, 138] However, excessive amounts of ROS may induce oxidative stress and cause damage of cellular critical components including proteins, DNA, and lipids by oxidation, which have been implicated in many pathological disorders such as inflammation, cancer, infections, diabetes, and atherosclerosis.^[139-141] Taking advantages of this biological feature, ROS-responsive materials are able to be utilized for site-specific delivery for imaging agents and therapeutics. So far, ROS-responsive systems based on various motifs have been devised.^[142] The commonly used ROS-sensitive motifs and their oxidation processes are summarized in figure 1.15.

Figure 1.15 The commonly used ROS-sensitive motifs and their oxidation processes.

For example, the sulfides can be oxidized to more hydrophilic sulfoxides or sulfones by ROS. Based on this property, Hubbell and coworkers reported the first example of oxidation-responsive material in the field of drug delivery. In their work, copolymeric amphiphile composed of poly(propylene sulphide) (PPS) and PEG could self-assemble into vesicles in aqueous solution. However, in oxidative environment, the sulfide moieties were oxidized to sulfoxide and ultimately sulfones, thus increasing the hydrophilicity of vesicles and subsequently oxidative destabilization.^[143] Similar to sulfide motif, hydrophobic selenides also become hydrophilic selenoxides or selenones upon oxidation.^[144] Zhang group reported various redox-responsive selenium-containing materials for controlled drug release.^[145-147] Recently, boronic esters gain increasing attention for the design of ROS-responsive materials.^[148, 149] Broaders and coworkers developed ROS-responsive dextran nanocarrier modified with arylboronic ester for vaccine application.^[150] Almutairi et al. reported a new adipic acid-base polymer with arylboronic esters that can be responding to biologically relevant concentrations of H_2O_2 , which is one of the most sensitive ROS-responsive systems.^[151]

1.3.3 Hypoxia-responsive strategy

Hypoxia, a state in which the tissue is lack of sufficient oxygen supply, is a prominent characteristic of various refractory diseases, including solid tumors, ischemic cardiomyopathy, stroke, and rheumatoid arthritis.^[152] In particular, tumor tissues become hypoxic due to rapid cancer cell proliferation and abnormal tumor vasculature.^[153] Studies have shown that the oxygen level of tumor tissues is greatly heterogeneous with majority tissues at low levels (less than 5 mmHg), while the oxygen concentration is about 70 mmHg in normal tissues. Even worse, in some solid tumors, the oxygen pressure decreases to near zero mmHg.^[154] The low oxygen level

of solid tumors is considered to be responsible for tumor invasiveness, increased metastasis, poor prognosis, and resistance to chemotherapy and radiotherapy.^[155] Given the unique characteristics of tumor microenvironment, hypoxia has been considered an endogenous stimulus for design of stimuli-sensitive systems.^[156, 157]

The commonly used hypoxia-responsive moieties include azo linkages, nitroimidazoles, and nitrobenzyl alcohols (Figure 1.16).^[158-160] In general, the hypoxia-sensitive motifs are reduced to hydrophilic groups in response to hypoxia,

Name	Structure	Products
Azo derivatives		
Nitroimidazole		
Nitrobenzyl alcohol		$R-NH_3^+ + CO_2 +$

Figure 1.16 The commonly used hypoxia-responsive moieties.

leading to the change of carrier solubility and size. For example, Gu and coworkers developed a 2-nitroimidazole-grafted conjugated polymer-based nanocarrier for light-activated ROS generation and hypoxia-responsive anticancer drug release (Figure 1.17).^[161] This nanocarrier was able to generate ROS and induce hypoxia under visible/near-infrared light irradiation. In the meanwhile, the hydrophobic 2-nitroimidazole can be reduced to hydrophilic 2-aminoimidazole under hypoxic tumor environment, leading to dissociation of nanocarrier and subsequent release of doxorubicin. The combination of photodynamic therapy and chemotherapy resulted in

enhanced treatment efficacy. Torchilin et al. developed a polymeric nanocarrier by conjugating hypoxia-sensitive azobenzene linker to PEG and PEI-phospholipid polymer for hypoxia-targeted delivery of siRNA. In hypoxic environment, the azobenzene was cleaved, resulting in detachment of PEG from nanocarrier. The remaining positively charged PEI/siRNA complex could be easily taken up by cancer cells. *In vitro* and *in vivo* studies showed enhanced gene silencing efficacy.^[162]

Figure 1.17 Schematic of the light-activated hypoxia-responsive system.^[161]

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1.3.4 Enzyme-responsive strategy

Enzymes, a class of efficient and specific catalysts, play a key role in numerous biological processes. However, dysregulation of enzyme expression or enzymatic activity is associated with various diseases, such as inflammation, infection, obesity, and cancer.^[163] Recently, enzymes have become attractive targets for the development of enzyme-responsive systems.^[164]

Proteases are extensively investigated as endogenous stimuli for the development of bioresponsive systems.^[165] Proteases can hydrolyze specific peptide sequence to

perform their functions. Of these proteases, Cathepsin B, a lysosomal cysteine protease, is one of the most representative cancer-associated proteases. It is localized in lysosomes in normal cells. However, Cathepsin B is highly upregulated in tumors.^[166] Studies showed that Cathepsin B cleaves some short peptide sequences, such as Phe-Lys, Ala-Leu, Phe-Arg, and Ala-Leu-Ala-Leu.^[167] A broad range of cathepsin B-cleavable prodrugs have been developed. PK1, Dox-HPMA conjugate with cleavable peptide Gly-Phe-Leu-Gly, now has entered clinical trials.^[168] Recently, our group developed unique enzyme-responsive rear-earth nanostructures for enhanced upconversion emission and photodynamic therapeutic. In the design, an enzyme-cleavable peptide was conjugated onto the surface of upconversion nanoparticles (UCNPs), which was cleaved by Cat B to expose free cysteine. Subsequently, the cysteine reacted with 2-cyanobenzothiazole on neighbouring UNCP, triggering the localized accumulation of UCNPs in cancer cells.^[169]

Matrix metalloproteinases (MMPs), another family of proteolytic enzymes, are associated with the degradation and remodeling of basement membranes and the extracellular matrix.^[170] In particular, MMP-2 and MMP-9 are the most frequently investigated MMPs and are usually upregulated in extracellular tumor microenvironment, which play a critical role in tumor proliferation, angiogenesis and metastasis.^[171, 172] Therefore, the MMPs specific substrates including peptides have been frequently exploited for the design of MMPs-responsive prodrugs or nanocarriers. For example, an MMP2-reponsive peptide sequence (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln) was used as cleavable linker between the TAT-modified liposomes and the PEG chains. In the tumor microenvironment, the cleavage of the peptide by overexpressed extracellular MMP2 resulted in exposure of TATs and subsequent enhanced intracellular internalization.^[173] In another study, an activatable cell-penetrating

peptide (dtACPP) that responds to tumor microenvironment, acidic pH_e and upregulated MMP2, was developed. In dtACPP, a pH-sensitive masking peptide was conjugated to polycationic CPP via a MMP2-cleavable peptide, thus shielding the internalization function of CPP. When in the tumor microenvironment, the lowered pH eliminated the shielding effect and the highly expressed MMP2 cleaved the linker, leading to the activation of CPP. Using this unique feature, the author further constructed gene nanocarrier (dtACPPD) by conjugating dtACPP to the surface of dendrigraft poly-L-lysine, which was utilized to co-delivery anti-VEGF plasmid (shVEGF) and DOX. After systemic administration, the nanoparticle (dtACPPD/shVEGF-DOX) selectively accumulated in tumor tissues through the EPR effect. Then the unique tumor microenvironment activated the dtACPP to expose the CPP, which facilitated the penetration of nanoparticles into the intratumoral cells. Both *in vitro* and *in vivo* results revealed that the dtACPP-modified nanoparticles successfully achieved enhanced antitumor efficacy by the combination of anti-angiogenesis and cell apoptosis.^[174]

Phosphatases, especially alkaline phosphatases (ALP), catalyze the dephosphorylation of the substrates in Nature. Studies revealed that ALP is highly expressed in the pericellular space of some osteosarcoma cells.^[175-177] Based on this finding, Xu's group developed a variety of peptide-based hydrogelators that self-assemble to form hydrogels in response to ALP.^[65] Recently, they reported ALP-instructed self-assembly of small molecules in the pericellular space of some cancer cells to selectively induce cancer cell death.^[102]

1.4 Extracellular vesicles

Extracellular vesicles (EVs) are nanosized membrane-based assemblies secreted by all cell types that play a pivotal role in intercellular communication.^[179] EVs are enclosed by lipid bilayer membrane, which are similar to the cell membrane. The EVs membrane contains various proteins such as receptors, specific markers as well as major histocompatibility complex molecules.^[180] While the EV lumen contains soluble proteins, DNA, mRNA, miRNA, and other noncoding RNA sequences.^[181-183] There are two major types of EVs on the basis of their membrane properties and biogenesis: exosomes and shedding microvesicles (Figure 1.18). Exosomes are small (30–150 nm) assemblies formed by the fusion between the multivesicular bodies (MVBs) and the cell membrane.^[184] Some specific protein markers (CD9, CD63, TSG 101, Alix) have been demonstrated to be enriched in exosomes.^[185] MVs are assembled and secreted directly from the cytoplasmic membrane via outward budding and their sizes range from 100 to 1000 nm.^[186] The molecular composition of MVs is still poorly understood, but some proteins including integrins, glycoprotein, and matrix metalloproteinases (MMPs) have been shown to be enriched in MVs, depending on the cell type.^[187] Moreover, MVs exhibit high amounts of cholesterol and phosphatidylserine (PS) due to their biogenesis.^[180] Both exosomes and shedding MVs are generated by healthy cells.

1.4.1 Biological roles of EVs

EVs, a mediator of intercellular communication, play a function role in physiological and pathological processes.^[179] Under physiological conditions, EVs involved in homeostasis, blood coagulation, inflammation, tissue repair, immune regulation, as well as stem cell maintenance.^[188] For example, EVs might mediate

immune activation or suppression depending on the status of immune cells.^[189] They can activate immune system by promoting proliferation of haematopoietic stem cells and the stimulation of natural killer (NK) cells, monocytes, and B cells. By contrast, EVs also mediate immune suppression through several mechanisms such as inhibition of dendritic cells (DCs) maturation, suppression of NK cell activity, as well as enhanced function of regulatory T cells. EVs have also been implicated in neurobiological functions via mediating neurotransmitter delivery.

Figure 1.18 Schematic illustration of subtypes of extracellular vesicles (EVs) secreted by a cell.^[186] (Reprinted with permission from Elsevier)

EVs have also been shown to contribute to numerous diseases such as tumor progression, arthritis, Alzheimer, Parkinson, autoimmunity, ischemic stroke.^[190-192] In particular, various studies shown that EVs-mediated molecules transfer could stimulate tumor progression, including angiogenesis, extracellular matrix digestion,

tumor metastasis, evasion from immune surveillance, and drug resistance.^[193] Angiogenesis plays vital roles in tumor survival and growth, accompanying by proliferation of endothelial cells.^[194] Several studies demonstrate that tumor-derived EVs are capable of stimulating the proliferation of endothelial cells to facilitate angiogenesis through the transfer of mRNAs and miRNAs.^[183] Researchers also find that EVs released by tumors contain MMPs, such as MT1-MMP, MMP2, and MMP9, which degrade extracellular matrix and promote tumor invasion and metastasis.^[195, 196] In addition, studies indicate that EVs also facilitate tumor escape from immune surveillance through several mechanisms.^[197] Vesicles shed by some cancer cells could inhibit the formation of antigen-presenting cells from monocytes.^[198] Also, tumor-derived EVs with FasL are shown to initiate apoptosis of activated T cells, thereby eliminating the attack of activated T cells to tumor cells.^[199-201] Finally, it is documented that cancer cells treated with chemotherapeutics release and accumulate the drugs in EVs, indicating the EVs involve in drug resistance.^[187, 202, 203]

1.4.2 Extracellular vesicles as delivery platforms

Considering EVs' natural characteristic in transporting bioactive cargos between cell, EVs have recently attracted significant attention in the field of drug delivery. In principle, EVs harbor multiple advantages over artificially synthesized carriers. First, it has been reported that EVs, especially those derived from autologous cells, are less immunogenic than synthetic vehicles because of their natural compositions.^[204] Second, EVs can potentially across the blood brain barrier and deliver cargos directly to the brain.^[191] Third, the phospholipid bilayers of EVs not only protect the encapsulated cargos from degradation in blood circulation but also improve the cellular internalization of cargos through direct fusion with the plasma membrane of targeted cells.^[205] Fourth, EVs have been reported to possess natural targeting

properties.^[206] These potential advantages make EVs ideal delivery platforms for transporting encapsulated cargos.

Up to now, EVs have been developed to transport various exogenous cargos including miRNA, siRNA, proteins, and chemotherapeutic agents for drug delivery applications.^[207-209] For example, Zhang et al. firstly reported the EV-mediated transfer of exogenous nucleic acids.^[210] In their work, THP-1 macrophages transfected with miR-150 were able to secrete microvesicles loaded with miR-150, which transported miR-150 to human HMEC-1 cells. Moreover, *in vivo* studies demonstrated that miR-150-loaded MVs can effectively reduce the expression level of c-Myb and enhance recipient cell migration. Later, Kuroda showed that exosomes with GE11 peptide specifically transferred let-7a miRNA to EGFR-expressing breast tumors in mice, which greatly suppressed tumor growth.^[211] Recently, Wood and coworkers provided the first example for delivery of exogenous siRNA using EVs. They developed exosomes from self-derived dendritic cells for targeted delivery of siRNA to the brain of mice.^[212] The donor cells were engineered to express exosomal membrane protein (Lamp2b) fused to neuron-specific peptide (RVG), which endowed exosomes with targeting capability. Subsequently, exogenous siRNA was encapsulated into exosomes by electroporation. Upon intravenous injection, siRNA-containing exosomes selectively delivered siRNA to neurons and microglia in the brain, leading to specific gene knockdown. Moreover, this siRNA-exosome system caused negligible immune response and minimal toxicity. Finally, they have demonstrated the potential of siRNA-loaded EVs for silencing Alzheimer's disease-related gene expression. Similarly, Zen et al. utilized cell-derived MVs to deliver transforming growth factor β 1 siRNA to sarcomas cells for cancer treatment. *In vivo* studies shown that MVs containing TGF- β 1siRNA significantly inhibited the TGF- β 1 expression and

downstream signaling in the tumor cells, and thus further suppressed tumor growth as well as lung metastases.^[213]

Besides for delivery of nucleic acid molecules, EVs have also been exploited to transport anticancer drugs. For instance, Huang et al. reported that tumor cell-derived microvesicles can deliver chemotherapeutic agent methotrexate to tumor cells, leading to inhibition of tumor growth.^[214] Later, integrin-targeted exosomes loaded with anticancer drug doxorubicin were shown to specifically inhibit breast tumor growth without obvious toxicity.^[215] Pessina and coworkers demonstrated exosomes derived from mesenchymal stromal cells can effectively deliver Paclitaxel for delaying tumor growth.^[216] Molly group systematically investigated the drug loading efficiency of extracellular vesicles from various different cell types using different loading methods.^[217] Results suggested that active encapsulation method displayed higher loading efficiency than passive methods. Moreover, compare to free drug or drug-loaded liposome, EVs loaded with drug exhibited enhanced cellular uptake of hydrophobic porphyrins and phototoxic effects. In addition to cancer, EVs have also been developed for treatment of neurodegenerative diseases. Evidence documents that Parkinson's disease (PD) is associated with reduced level of some enzymes such as superoxide dismutase, catalase, and other antioxidants.^[218] Therefore, effective brain delivery of antioxidants may improve the therapy of PD. Batrakova et al. recently designed catalase-loaded exosomes for the treatment of PD.^[219] These catalase-contained exosomes were shown to accumulate in microglial cells and neurons in the brain, thus providing considerable neuroprotective effects.

1.5 Research goals

Currently, different kinds of self-assembled nanostructures have been fabricated for biomedical applications. Inspired by the natural nanovehicles, a wide variety of artificial assemblies have been exploited in recent decades, which may potentially have profound impacts on the cellular functions. Therefore, the exploration of artificial or natural nanosized self-assemblies for investigation and modulation of various biological events not only provides novel insights on essential physiological processes, but it will also open up new avenues for personalized medicine.

In chapter 2 and 3, we developed two different kinds of self-assembled peptide nanostructures for effective cancer therapy. It is still challenging that one of the characteristic events in tumor treatment is the presence of multidrug resistance (MDR). The cancer cells with MDR exhibit resistance to diverse therapeutic agents, which significantly hamper cancer therapy. Studies have demonstrated that overexpression of the drug efflux transporter P-glycoprotein (P-gp) greatly contributes to MDR of cancer cells. Therefore, effective control of P-gp function will be of significance in the treatment of cancer. In order to overcoming MDR in cancer, we constructed a reduction-responsive nanosystem assembled from a peptide inhibitor of P-gp and an anticancer drug for simultaneously control of P-gp activity and induce cell apoptosis. In order to further improve the therapeutic efficacy and effectively reverse tumor resistance, in chapter 3, we developed a smart acid-responsive peptide nanogel that co-assembled from peptide-Dox prodrug and another P-gp inhibitor with high affinity for reversing MDR.

Furthermore, apart from the development of artificially self-assembled nanopaltforms, we also exploited the studies in the natural nanosized assemblies. In chapter 4, we developed a simple and efficient strategy based on natural EVs for

transferring bioactive proteins to target cells and conferring the cells with specific biological function. In order to investigate the dynamic behaviors of cell-derived EVs from different cells, in chapter 5, we described a convenient labeling strategy that combined metabolic glycanengineering and bioorthogonal click chemistry to visualize the interaction between EVs of different origins and other cells.

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Chapter 2

Reduction-Responsive Peptide Self-Assemblies for Targeted Cancer Imaging and Therapy

ABSTRACT: Overexpression of drug efflux transporters such as P-glycoprotein (P-gp) is one of the major mechanisms for multidrug resistance (MDR) in cancer cells. Hence, effective inhibition of P-gp activity is of great significance in cancer therapy. In this work, we developed a reduction-responsive peptide-based nanosystem for fighting the multidrug resistance of cancer cells. Basically, a novel nontoxic peptide inhibitor of P-glycoprotein (P-gp) was conjugated with anticancer drug Doxorubicin (Dox) through a thiol-specific cleavable disulfide linker to afford Dox-peptide prodrug with high drug loading. The developed prodrug can effectively self-assemble into nanoparticles, thus improving the cellular uptake of the inhibitor and anticancer drug. After cellular internalization, reduction agents such as GSH caused cleavage of the disulfide linker, releasing Dox and peptide inhibitor, which showed anticancer activity in synergistic effects. These stimulus-responsive peptide self-assemblies with targeted drug release provide significant advances toward the development of drug delivery systems for cancer therapy.

2.1 Introduction

Cancer is the leading cause of death around the world.^[1] Therefore, how to treat cancer is a great challenge for modern medicine. Until now, Surgery, chemotherapy, radiotherapy and immunotherapy are the commonly used strategies for cancer therapy.^[2] Among these approaches, chemotherapy is a major therapeutic approach for the treatment of cancer due to its high efficiency. However, traditional small molecular anticancer drugs usually suffer from several limitations including rapid blood clearance, low accumulation in tumors, nonspecific selectivity, adverse side effects and severe multidrug resistance (MDR).^[3] In particular, as one of the major reasons for the failure of cancer therapy, MDR is resistant to multiple drugs, which can cause 90% chemotherapeutic failure of patients.^[4]

Currently, several mechanisms underlying MDR have been proposed, such as decreased uptake of drugs, increased drug metabolism, and enhanced drug efflux.^[5] Among these mechanisms, upregulated efflux transporters are mainly responsible for drug resistance. The efflux transporters are a family of energy-dependent transmembrane proteins, known as ATP-binding cassette (ABC) transporters, which use the energy of ATP hydrolysis to transport substrates out of the cell.^[6] Tumor cells either inherently express ABC transporters or increase the expression of ABC transporters in response to chemotherapy. The first identified human ABC transporter is P-glycoprotein (P-gp), which is a 170 kDa protein composed of two transmembrane (TM) domains and two nucleotide-binding domains.^[7] The physiological role of P-gp is the extrusion of waste products.^[8] However, upregulated P-gp also extrudes a range of hydrophobic drugs from the cytoplasm, reducing the intracellular concentration of drugs.^[9] Therefore, P-gp is one of the available targets for cancer chemotherapy.

In the past decades, an enormous amount of research effort goes into overcome the MDR of cancer cells. One approach is the development of transporter inhibitors^[10]. Although these inhibitors display high effectiveness, their low specificity limited their applications.^[11] The second approach is the development of prodrugs. A successful prodrug strategy can improve drug availability and reduce toxicity.^[12] However, its efficacy is often inhibited by rapid clearance and premature degradation. Another strategy is the development of drug delivery systems through nanotechnology.^[13-15] The nanoscale delivery systems indicate the great potentials to improve the anticancer indexes because they can accumulate in the tumor vicinity by the enhanced permeability and retention (EPR) effect.^[16] So far, various types of nanocarriers such as polymeric nanoparticles, inorganic particles, and micelles have been developed to overcome the MDR of cancer cells.^[17-21] Despite the encouraging successes, there are still issues in those carriers, such as metabolic burden of inert vehicles, side effects, drug loading capacity. Therefore, new strategies that could combine the advantages of the above developed methods while avoid their disadvantages are urgently needed.

Recently, self-assembling peptide nanostructures as promising materials have attracted considerable attention in biomedical field due to their unique features, such as excellent biocompatibility, biodegradability, manipulated bioactivity and rapid responsiveness.^[22-24] Taking advantages of this promising material, the development of tumor microenvironment-responsive peptide self-assemblies may provide new opportunities for controlled drug release and targeted cancer therapy.

Figure 2.1 Schematic illustration of reduction-responsive peptide self-assemblies for targeted cancer therapy.

In this chapter, we developed a novel tumor microenvironment-responsive self-assembled peptide nanosystem for targeted cancer therapy. The distinct feature of our design is that the developed system integrates the advantages of nanosized material, prodrug and inhibitor into one platform, which can make up their own shortcomings. In the design, two bioactive molecules (an antitumor drug and a peptide inhibitor of P-gp) were linked by bioresponsive moiety to form prodrug, which can self-assemble in aqueous condition into nanostructures and accumulate around cancer cells by EPR effect. Upon internalization by cancer cells, the sensitive linker of nanostructures could be cleaved by specific tumor molecules, leading to the release of the anticancer drug and peptide inhibitor. The peptide inhibitor will interact with P-glycoprotein on the cell membrane and block the function of P-glycoprotein, while the drugs will exert their anticancer activities. They will play a synergistic effect and finally improve the therapeutic efficacy (Figure 2.1).

2.2 Results and discussions

As a proof of concept, we selected a first-line antitumor drug doxorubicin (Dox) as a model drug. Dox is an antitumor drug that is extensively used to treat a broad range of hematological malignancies and solid tumors. The action mechanism of Dox is to inhibit topoisomerase II, damage nucleic acid, and induce the formation of reactive oxygen species (ROS).^[25] Then, we chose the disulfide bond as reduction-responsive linker based on the fact that cancer cells have much higher intracellular glutathione (GSH) concentration (1-10 mM) than normal cells (1-10 μ M).^[26] Finally, a peptide derivative (D-Trp-Phe-D-Trp-Leu-AMB) that exhibits potential for reversing MDR served as the inhibitor of P-gp.^[27] The proposed Dox release mechanism from the prodrug is a two-step reaction (Figure 2.2). First, the disulfide bond of the prodrug is recognized and cleaved by GSH, liberating the Dox and peptide intermediates; subsequently, the active Dox and peptide are released from the intermediates through the intramolecular cyclization.

Figure 2.2 Proposed Dox and peptide release mechanism from the prodrug by treatment of GSH.

The synthetic route of the prodrug is presented in Figure 2.3. First, the designed peptide (D-Trp-Phe-D-Trp-Leu) was synthesized using standard solid phase peptide synthesis (SPPS) techniques. Then, the peptide reacted with Isopentylamine by using HBTU and DIPEA, subsequently removing the Fmoc group. The peptide derivate was finally conjugated to the amino group of Dox using a cleavable disulfide linker. The final product was purified by HPLC and further characterized by high resolution mass spectroscopy and HPLC (Figure 2.4). The spectroscopic properties of the conjugates were also characterized by ultraviolet-visible spectrophotometer (UV/Vis) and fluorescence spectroscopy. The UV/Vis spectra showed that the absorption maximum of free Dox and Dox-peptide conjugate is 482 nm (Figure 2.5a). The fluorescence spectra of Dox-peptide conjugate exhibited an emission maximum in the 594 nm,

Figure 2.3 Synthetic route of Doxorubicin-peptide conjugate. (a) Solid Phase Peptide Synthesis (SPPS); (b) Isopentylamine, HBTU, DIPEA, 4 h; (c) 5% piperidine in DMF, 1 h; (d) TEA, 24 h; (e) TEA, 24 h; (f) 50% TFA in DCM, 1 h; (g) Dox, TEA, 24 h.

Figure 2.4 HPLC chromatograms of free Dox, peptide derivate, and Dox-peptide conjugate. HPLC conditions: C-18 (250 × 10 mm) column; 480 nm UV detection wavelength; 3.0 mL/min flow rate; the gradient began at 30% of mobile phase A (water) and 70% (acetonitrile) to 5% mobile phase A and 95% mobile phase B at 15 min and held for another 5 min.

consistent with free Dox (Figure 2.5b). However, the fluorescence intensity of the conjugate was lower than that of free Dox. This decrease may be caused by the conjugated peptide. All experimental results confirmed the successful conjugation between Doxorubicin and peptide.

Figure 2.5 (a) UV/Vis spectra of Dox (10 μ M) and Dox-peptide conjugate (10 μ M); (b) Fluorescence emission of Dox (50 μ M) and Dox-peptide conjugate (50 μ M) in DMSO solution.

After successful synthesis of the Dox-peptide prodrug, the self-assembling ability of the prodrug was tested by addition of the prodrug (5 μ L of 1 mM in DMSO solution) into a water solution (1 mL, pH = 7.4). Upon mixing at room temperature, copper grid with sample solution was prepared for TEM imaging. TEM image indicated that the prodrug assembled into sphere-like nanoparticles (Figure 2.6a). Moreover, DLS results showed that the diameter of self-assembled nanoparticles is around 115.6 nm $\pm$ 39.2 nm (Figure 2.6b). These results clearly proved the success of our design.

Figure 2.6 (a) Transmission electron microscopy (TEM) image of self-assembled Dox-peptide nanostructure. (b) Dynamic Light Scattering (DLS) characterization of self-assembled Dox-peptide in water (pH = 7.4).

Such self-assembled behavior was further evaluated by fluorescent spectra. As shown in Figure 2.7 a, at the same concentration, the fluorescent intensity of Dox-peptide nanoparticles was very low in aqueous solution when compared to the fluorescence of free Dox. We assumed that such fluorescence quenching could be mostly caused by self-assembly of the Dox-peptide prodrug or the covalent linkage between the free Dox and disulfide bond modified peptide sequence. In order to further confirm the possible mechanism of the observed fluorescence quenching, we

investigated the fluorescence changes of free Dox and Dox-peptide prodrug in a DMSO/water mixture. As indicated in Figure 2.7 b and c, the fluorescence enhancement was observed as the DMSO fraction increased. Moreover, the

Figure 2.7 (a) Fluorescence spectra of free Dox (5 μ M) and Dox-peptide nanoparticles (5 μ M) in water solution (pH = 7.4). Emission spectra of (b) Dox-peptide nanoparticles and (c) free Dox in DMSO/water solution with ratios of DMSO to water from 0.5 to 100% under 480 nm excitation (d) Fluorescence intensity of free Dox and Dox-peptide nanoparticles in water and DMSO solution, respectively.

fluorescence of Dox-peptide displayed a 29-fold increment in DMSO as compared to when the same prodrug was dissolved in aqueous solution (Figure 2.7d). However, the fluorescence of free Dox in DMSO solution only demonstrated a 2-fold enhancement than it was in water. In addition, there was no self-assembly of Dox-peptide occurred in DMSO solution. These results clearly indicated that the significant fluorescent quenching of Dox-peptide prodrug in water solution was mainly attributed to self-assembly of conjugated prodrug instead of the covalent linkage between the free Dox and disulfide bond modified peptide sequence. The proposed main driven forces of

self-assembly of the prodrug may be the intermolecular π - π stacking. Therefore, the fluorescence change can act as indicator for the assembly state of the conjugate. Most importantly, these results suggested that the designed system possessed environment-sensitive feature, which could undergo disassembly under relative hydrophobic environment. Such supramolecular nanospheres make the self-delivery possible for efficient cell uptake and subsequent drug and peptide release.

Next, we evaluated the in vitro drug release of the assembled nanostructures by using HPLC. For this study, we use GSH (10 mM), a common reducing agent, to mimic intracellular reduction conditions in cancer cells. The drug release was investigated in the presence and absence of 10 mM GSH. Results showed that upon incubation with GSH, a time dependent increase in the percent of released Dox was observed (Figure 2.8a). About 80% dox could be released within 2 h incubation from the prodrug (Figure 2.8b). In contrast, a control sample without GSH treatment showed negligible release of free Dox. These evident demonstrated the reduction-responsive property of the developed assembled system.

Figure 2.8 (a) The drug release profiles of Dox from Dox-peptide nanostructures in the presence and absence of GSH (10 mM); (b) Time dependent cumulative release of Dox from Dox-peptide nanostructures in the presence and absence of GSH at 37 °C.

At the same time, we also investigated the fluorescence activation of the Dox-peptide nanoparticles treated by GSH. In this experiment, Dox-peptide nanoparticles in PBS solution was incubated with a GSH solution (10 mM), and their fluorescence emissions were measured at different time points. As shown in figure 2.9, the fluorescence of nanoparticles gradually increased with prolongation of incubation time. The observed increase in fluorescence may be contributed to the cleavage of the disulfide bond and the disassembly of the nanoparticles. This finding supported the fact that reduction of the disulfide bond by intracellular reducing agents, such as GSH, can restore the fluorescence of the free Dox.

Figure 2.9 Fluorescence spectra of Dox-peptide nanoparticles (5 μ M) in the presence of GSH (10 mM) in PBS solution (pH = 7.4) at 37 $^{\circ}$ C.

Inspired by the promising reduction-responsive characteristic of the self-assembled nanoparticles, the cellular internalization and distribution of Dox-peptide nanoparticles were investigated by monitoring the fluorescence of Dox in cells. The human ovarian carcinoma cell line A2780 cells and Dox resistant A2780/ADR cells were chosen for the following experiments. The two cell lines express considerable

different P-glycoprotein amount on the cellular membrane, which have been verified by flow cytometer (Figure 2.10). A2780 and A2780/ADR cells were incubated with free Dox and nanoparticles at 37 °C for 3 h. As shown in figure 2.11, after incubation with Dox-peptide nanoparticles, there were bright red fluorescence signals observed in

Figure 2.10 Flow cytometry analyses of P-glycoprotein expression levels on the cellular surface of human ovarian A2780 and A2780/ADR cells, respectively.

both A2780 and A2780/ADR cells and co-staining with Lyso traker, which confirmed the nanoparticles entered to cells through endo/lysosome pathway. These results indicated that nanoparticles may bypass the drug resistance mediated by membrane transporters such as P-gp, and significantly enhanced the internalization and accumulation of DOX in MDR tumors. However, upon incubation with free Dox, the bright red fluorescence of free Dox were observed in A2780 cell nucleus, indicating the different uptake pathways between DOX molecules and nanoparticles, and free Dox could quickly concentrate into cell nucleus through the passive diffusion. In contrast, significantly weak fluorescence was detected in the A2780/ADR cells as compared to the signals in A2780 cells. Such limited DOX uptake in the A2780/ADR cells would be the efflux effect of P-gp. Living cell imaging studies revealed that the

self-assembled nanoparticles could effectively self-deliver DOX into drug sensitive and resistant cancer cells.

Figure 2.11 Confocal images of A2780 and A2780/ADR cells incubated with free Dox (5 μ M) and Dox-peptide nanoparticle (5 μ M) for 3 h at 37 $^{\circ}$ C. Scale bar = 20 μ m.

To further confirm the anticancer efficiency of Dox-peptide nanoparticles, we first examined the cytotoxicity of free Dox and Dox-peptide nanoparticles in the presence and absence of 10 mM GSH using A2780 sensitive cell line by MTT assay. Typically, A2780 cells were incubated with free Dox, nanoparticles, and nanoparticles with GSH for 72 h, respectively. As shown in Figure 2.12a, the results indicated that Dox-peptide nanoparticles in the absence of GSH showed lower cytotoxicity than free dox after incubation with A2780 cells. However, Dox-peptide nanoparticles in the presence of GSH displayed higher anticancer activities in A2780 cells when compared to free Dox and nanoparticles, suggesting the Dox-peptide nanoparticles were

activated by GSH and released active Dox and peptide inhibitor. These results demonstrated that the self-assembled dox-peptide nanoparticles are responsive to

Figure 2.12 In vitro cytotoxicity of free Dox and Dox-peptide nanoparticles with GSH treated or untreated against (a) A2780 sensitive cells and (b) A2780/ADR cells.

tumor-relevant reductive condition and exhibited excellent cytotoxicity against cancer cells. Encouraged by the promising therapeutic efficacy of the self-assembled nanoparticles in sensitive cancer cells, their antitumor efficacy in resistant cancer cells was further performed. Similarly, A2780 cells were incubated with free Dox, nanoparticles in the absence and presence of GSH for 72 h. As presented in Figure 2.12b, nanoparticles-incubated resistant cells in the presence of GSH showed relative higher cytotoxicity than cells treated with nanoparticles in the absence of GSH, indicating the stimuli-responsive features of nanoparticles again. Moreover, 10 μM nanoparticles-treated A2780/ADR cells in the presence of GSH caused obvious cytotoxicity with 55% cell viability, revealing their potential for fighting resistant cells. It should be noted that free Dox exhibited higher cytotoxicity against A2780/ADR cells at high concentration as compared to nanoparticles-treated cells even in the presence of GSH. Here, we proposed some reasons for this result. First, the selected A2780/ADR cell line may have limited drug resistance, which could not completely extrude the drugs from cells. Second, the released peptide derivate did not completely

suppress the function of P-gp, resulting in low accumulation of Dox in resistant cells with prolongation of incubation time.

Therefore, we further evaluated the inhibitory efficiency of the peptide derivate against A2780/ADR cells by detecting the accumulation of Dox in A2780/ADR cells. Generally, the P-gp inhibitors can block the efflux function of P-gp and facilitate the accumulation of Dox molecules in resistant cells. Therefore, the fluorescence intensity of cellular Dox could directly reflect the efficacy of P-gp inhibitors. As shown in Figure 2.13, the fluorescence enhancement was observed when the concentration of the peptide derivate was 60 μM , revealing that the efflux function of P-gp was inhibited by peptide molecule. Moreover, as the concentration of the peptide derivate further increased (e.g., 100 μM), a significant increase in the fluorescence intensity of cellular Dox was observed. These results suggested that the peptide derivate could block the P-gp function when their concentration is high. In other words, the selected peptide derivate exhibits low inhibitory efficacy. Therefore, high effective inhibitors of P-gp are needed to be developed.

Figure 2.13 Flow cytometric analysis of the accumulation of Dox in A2780/ADR cells pretreated with different concentrations of peptide derivate for 3 h. E_x : 488 nm, E_m : 575/25 nm.

2.3 Conclusions

We introduced a new redox-responsive self-assembled peptide nanosystem for cancer therapy. The designed peptide-Dox assemblies have high drug loading and could self-deliver two bioactive molecules to cells. As a proof of concept, this work mainly emphasized the strategy that development of microenvironment-responsive small molecular assemblies composed of multiple bioactive agents for treatment of resistant tumors. The efficacy remains to be enhanced by optimizing the components involved. On the one hand, new peptide inhibitors of P-gp with high sensitivity should be developed. On the other hand, other bioresponsive moieties could be exploited to improve the responsiveness of assemblies. We believe this strategy could be applied to design and prepare nanostructures of other therapeutic agents and bioactive peptides. What's more, this controlled self-assembled peptide nanosystem provide new opportunity for cancer therapy.

2.4 Experimental section

2.4.1 Materials and instruments

2-Chlorotriyl Chloride Resin and all Fmoc protected amino acids were provided by GL Biochem (Shanghai, China) Ltd. All other chemical reagents were purchased from Sigma (Singapore). Anhydrous solvents for organic synthesis were obtained from Sigma (Singapore) and stored in activated molecular sieves (4 Å). Calcein green, AM, PRMI-1640 medium and FBS were purchased from Thermo Fisher Scientific. Human ovarian cancer A2780 and drug resistant A2780/ADR cells were purchased from European collection of cell cultures (ECACC). Ultrapure water (18.2 M Ω) was prepared by Millipore Milli-Q Biocel purification system with a 0.22 μm filter.

^1H NMR spectra were recorded using Bruker AV 300 spectrometer (300 MHz). ESI-MS spectrometric analysis was recorded using a Thermo LCQ Deca XP Max instrument. Reverse-phase HPLC measurement was conducted on a Shimadzu HPLC system with a C-18 (250×4.6 mm) column at a flow rate of 1.0 mL/min for analysis and a Kromasil C-18 (250×10 mm) column at 3.0 mL/min for preparation. Transmission Electron Microscopy (Philips, FEI EM208S) was operated at 100 kV. UV-Vis absorption spectra were performed on Cary 60 UV-Vis Spectrophotometer. Fluorescence emission spectra were recorded on Varian Cary Eclipse Fluorescence spectrometer. Flow cytometry (FACS) was carried out using BD LSR II instrument. The cell viability was assessed using MTT under a Tecan's Infinite M200 microplate reader.

2.4.2 Peptide synthesis and purification

Figure 2.14 Illustration of Solid Phase Peptide Synthesis (SPPS).

All designed peptides were synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) SPPS techniques (Figure 2.14). 2-chlorotrityl chloride resin (1 g, 1 mmol) was firstly swelled in dry DCM for 20 min. The first amino acid (1 mmol) dissolved in DCM with DIPEA (2 mmol) was loaded to the C-

terminal of the resin for 2 h. Upon completion, the reaction was tested with the Kaiser ninhydrin assay. The Fmoc group was removed by 20% piperidine in anhydrous DMF for 25 min. The second Fmoc-protected amino acid reacted with the free amino group of the first amino acid in the presence of HBTU and DIPEA. The previous steps were repeated to add other amino acids. After the last step, the resin was washed with DMF for five times and DCM five times. The target peptide was released from the resin using 95% TFA, 2.5% water, and 2.5% TIPS for 30 min. At the same time, the side chain protecting groups were also removed. While the side chain protecting groups were required for further reactions, the peptide was cleaved from the resin using 1% TFA and 2.5% TIPS in DCM for 1 min ($\times 10$). Then the solvents were removed via rotary evaporation, followed by cold diethyl ether precipitation. The crude products were purified by reverse-phase HPLC.

2.4.3 Synthesis and characterization of Dox-peptide conjugate

Preparation of peptide 1: Solid phase synthesis of the peptide 1 was conducted manually on 2-Chlorotrityl Chloride Resin, using the Fmoc strategy. The final peptide was released from the resin using 1% TFA in DCM for 1 minute ($\times 10$). After removing DCM, the crude product was purified with flash column chromatography (DCM: EA = 5:1) to give compound **1** in 87% yield as a white solid. ^1H NMR (300 MHz, CDCl_3) δ 8.11 (m, 3H), 7.71-7.74 (d, $J = 7.5$ Hz, 2H), 7.48-7.67 (m, 6H), 7.18-7.39 (m, 10H), 7.05-7.10 (t, $J = 7.5$, 2H), 6.92-6.97 (t, $J = 7.2$ Hz, 2H), 6.56 (s, 2 H), 4.76-4.84 (m, 2H), 4.64-4.67 (m, 1H), 4.52 (m, 1H), 4.21-4.24 (m, 1H), 4.10-4.15 (t, $J = 7.3$, 1H), 3.9-4.0 (m, 1H), 3.34-3.41 (m, 2H), 3.13-3.15 (m, 2H), 1.55-1.62 (m, 20H), 1.28 (s, 1H), 0.70-0.76 (m, 6H).

MS: m/z : 1073.01 $[\text{M} + \text{H}]^+$

Preparation for compound 2 : Compound **1** (250 mg, 0.23 mmol) and Isopentylamine (29 mg, 0.23 mmol) were dissolved in 5 mL DMF with HBTU (88 mg, 0.23 mmol) and DIPEA (77 μ L, 0.46 mmol). The mixture was stirred at room temperature for 4 h. Then the mixture was extracted with EA twice and the organic phases were washed with water for two times, and then saturated aqueous sodium chloride for three times. After evaporation of the solvents, the residue was purified by flash column chromatography (DCM: MeOH = 50:1) to give compound **2** in 85% yield as a white solid. MS: m/z: 1142.29 [M + H]⁺.

Preparation for compound 3: The Fmoc group of Compound **2** (200 mg, 0.175 mmol) was removed by 5% piperidine in DMF at room temperature for 1 h. The mixture was extracted with EA twice, then washed with water two times and then brine twice. After removing the solvent, the residue was purified by flash column chromatography (DCM: MeOH = 35: 1) to give the compound **3** in 82% yield as a white solid. MS: m/z: 920.42 [M + H]⁺.

Preparation for compound 4: A solution of 2-hydroxyethyl disulfide (1.26 g, 8.18 mmol) in 1.7 ml of pyridine was added with 4-nitrophenyl chloroformate (6.57 g, 32.72 mmol) in 26 ml of acetonitrile. The mixture was kept stirring for 12 h at room temperature and poured into 100 ml of distilled water and extracted with EA. Finally, the organic phase was collected and concentrated under reduced pressure, and the residue was purified by flash column chromatography (hexane: EA = 2:1) to afford yellow oily compound **4**. ¹H NMR (300 MHz, CDCl₃) δ 8.21-8.27 (m, 4 H), 7.34-7.40 (m, 4 H), 4.56 (t, J = 6.3 Hz, 4 H), 3.08 (t, J = 6.3 Hz, 4 H).

Preparation for compound 5: Compound **3** (133 mg, 0.14 mmol) and **4** (279 mg, 0.56 mmol) were dissolved in 2 mL of DMF with TEA (58 μ L, 0.42 mmol) and allowed to stir at room temperature for 24 h. The mixture was extracted with EA twice

and the organic phases were washed with water for two times, then saturated aqueous sodium chloride for three times and dried over anhydrous magnesium sulphate. The solvent was concentrated under reduced pressure and purified with flash column chromatography (hexane: EA = 1:1) to get compound **5** in 61% yield as a white solid. ^1H NMR (300 MHz, CDCl_3) δ 8.24-8.27 (m, 2 H), 8.10-8.15 (t, $J = 7.5$ Hz, 2 H), 7.48-7.55 (m, 2 H), 7.44 (s, 1 H), 7.32-7.39 (m, 5 H), 7.21-7.29 (m, 4 H), 7.15-7.17 (m, 4 H), 6.90 (s, 2 H), 6.54-6.64 (m, 2 H), 4.45-4.54 (m, 4 H), 4.20-4.26 (m, 4 H), 3.0-3.16 (m, 6 H), 2.81-2.95 (m, 6 H), 1.76 (s, 5 H), 1.65-1.66 (m, 19 H), 0.84-0.86 (d, $J = 6$ Hz, 6 H), 0.68-0.73 (t, $J = 7.2$ Hz, 6 H). MS: m/z : 1265.27 $[\text{M} + \text{H}]^+$.

Preparation for compound 6: The Boc group of compound **5** (100 mg, 0.079 mmol) was removed by 50% TFA in DCM for 1 h. After removing the solvent, the residue was precipitated in diethyl ether to give compound **6** in 80% yields as a white solid. ^1H NMR (300 MHz, CDCl_3) δ 10.80 (s, 1 H), 10.71 (s, 1 H), 8.12-8.33 (m, 5 H), 7.52-7.77 (m, 5 H), 7.18-7.31 (m, 4 H), 6.93-7.06 (m, 10 H), 4.49-4.58 (m, 2 H), 4.34-4.42 (m, 2 H), 4.07-4.19 (m, 4 H), 2.94-3.13 (m, 6 H), 2.72-2.87 (m, 6 H), 1.23-1.36 (m, 6 H), 0.68-0.85 (m, 12 H). MS: m/z : 1065.49 $[\text{M} + \text{H}]^+$.

Preparation for compound 7: TEA (16 μL , 0.11 mmol) was added to a solution of compound **6** (30 mg, 0.028 mmol) and doxorubicin hydrochloride (25 mg, 0.042 mmol) in dry DMF (1 mL). The mixture was stirred at room temperature for 24 h. The final product was purified by reverse-phase HPLC with mobile phases consisting of A (pure water) and B (acetonitrile), monitored by UV absorbance at 254 and 480 nm. Dox-SS-peptide conjugate was obtained after lyophilization in 46% yield as a red solid. HR-MS: calcd $\text{M}^+ = 1468.56$, obsvd. $(\text{M} + \text{H})^+ = 1469.5679$.

2.4.4 Drug loading calculation

The drug loading of the Dox-peptide conjugate is given as the Dox weight as a percentage of the total molecular weight: Drug loading (%) = $(543/1468) \times 100\% = 37\%$

2.4.5 Transmission electron microscopy (TEM)

Dox-peptide conjugate was prepared in a stock solution at a concentration of 1 mM in DMSO solution. Then the stock solution was diluted by water (pH 7.4) to give a final concentration of 5 μ M. For TEM imaging, 5 μ L of the solution was dropped onto the carbon-coated copper grid. The excess solution was removed by piece of filter paper. Then the sample grid was placed to dry at room temperature before the measurement. The morphology was characterized by FEI EM208S Transmission Electron Microscopy (Philips) operated at 100 kV.

2.4.6 Dynamic light scattering

The size distribution of the prepared nanoparticles was characterized by Zetasizer Nano ZSP (Malvern Instruments, NK) equipped with a laser (633 nm), at a fixed angle of 173° and temperature of 25 °C.

2.4.7 Fluorescence Spectroscopy

Fluorescence spectra of free Dox and Dox-peptide conjugate was recorded on Varian Cary Eclipse Fluorescence spectrometer in a 10-mm path length quartz corvette. Samples were excited at 480 nm. The emission wavelengths were from 490 nm to 800 nm with a slit width of 10 nm.

2.4.8 The drug release study

The drug release of the Dox-peptide nanoparticles in the presence or absence of glutathione (GSH) was evaluated by reverse-phase HPLC. Briefly, 10 μ M of prodrug solution in water was freshly prepared before diluted to 5 μ M with deionized water (pH 7.4) with or without GSH (20 mM). The solutions were incubated at 37 °C, and

the analysis of Dox release from the nanoparticles was performed by RT-HPLC at 0.5, 1, and 2 h. The eluting conditions: 280 and 480 nm detection wavelength; 3.0 mL/min flow rate; the gradient began at 30% of mobile phase A (water) and 70% (acetonitrile) to 5% mobile phase A and 95% mobile phase B at 15 min and held for another 5 min.

2.4.9 Cell culture

The human ovarian A2780 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% of antibiotics. A2780/ADR (Doxorubicin resistance) cells were grown in the same medium supplemented with 1 μ M of doxorubicin to maintain its multidrug resistance. Doxorubicin needs to be added to the media every 2-3 passages. Both cell types were incubated at 37 °C with 5% CO₂ in a humidified incubator.

2.4.10 Cell imaging

The living A2780 cells or A2780/ADR cells were cultured at 200, 000 cell/dish in 35 mm diameter μ -dish with plastic-bot-tomed (ibidi GmbH, Germany) for 24 h. Then the medium was changed to fresh PRMI 1640 medium with 5 μ M free Dox nanoparticle or Dox-peptide. After 2.5 h incubation time at 5% CO₂ and 37 °C, LysoTracker® Green DND-26 was added to cell culture medium for additional 0.5 h incubation. The cells were washed twice with PBS. Cell imaging was taken under a confocal miscroscope (Nikon). Dox (E_x: 488 nm, E_m: 590/50 nm), Lyso tracker (Ex: 488 nm, E_m: 515/30 nm).

2.4.11 *In Vitro* Cytotoxicity Assay

The cell viability measurement was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, A2780 and A2780/ADR cells were seeded in 96-well plates with a density of 8.0×10^3 cells in 100 μ L of RPMI 1640 with 10% FBS and incubated at 37°C and 5% CO₂ for 24 h. Then the medium in

each well was changed to fresh medium containing different concentrations of free Dox, Dox-peptide nanoparticles or Dox-SS-peptide in the presence of GSH (10 mM). After 72 h incubation, the culture medium was removed and cells were washed with PBS, and then added with fresh culture medium containing 10% MTT. The cells were further incubated for 4 h. Subsequently, the medium was removed and 100 μ L DMSO was added to each well for dissolving the formazan crystals. Finally, the absorbance was measured at 570 nm with a Tecan's Infinite M200 microplate reader. The wells without cells but with DMSO were set as blank controls. Each experiment was repeated three times.

2.4.12 Flow cytometric assay

Resistant A2780/ADR cells were grown in 6-well plate at a density 9×10^5 cells/well for 1 day. The medium was replaced with fresh PRMI 1640 medium containing various concentrations of peptide derivate (WFWL-AMB). After being incubated for 3 h at 37°C, the Dox molecules were added to a final concentration of 5 μ M, and the cells were further incubated for another 1 h. The cells were washed with PBS for three times to remove the free peptide and the Dox. The cells were trypsinized and centrifuged at 1200 rpm for 3 min, and resuspended in 500 μ L of ice-cold PBS. A BD LSR II FACS with a 488 nm argon laser was used to monitor fluorescence of cellular Dox. 10,000 cells were investigated. (E_m : 575/25 nm).

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Chapter 3

Stimulus-Responsive Short Peptide Nanogels for Controlled Intracellular Drug Release and for Overcoming Tumor Resistance

ABSTRACT: Multidrug resistance (MDR) significantly impedes the success of cancer treatment. As one vital factor contributing to MDR, upregulated P-glycoprotein (P-gp) remarkably reduces the intracellular concentration of chemotherapeutic agents. Therefore, the ability to successfully inhibit the function of P-gp and to accumulate the anticancer drugs in tumor cells is of profound significance in clinical practice. Here, we designed intelligent stimulus-responsive peptide nano-assemblies, termed as PD/VER nanogels, which integrated the acid-responsive prodrug with the P-gp antagonist for overcoming tumor resistance. Systematic investigations showed that the developed inhibitor-loaded nanogels dramatically increased the accumulation of antitumor drug doxorubicin (Dox) in resistance cancer cell, thereby exhibiting evidently higher antitumor activity compared to free drugs. The work revealed the great potential of nanodrugs assembled from bioactive agents with synergistic effects for reversing cancer drug resistance.

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3.1 Introduction

Multidrug resistance (MDR) is considered a huge obstacle for effective cancer treatment. In general, tumors with MDR display multiple resistances to a broad range of chemotherapeutics with different structures and action mechanisms.^[1] It has been documented that there are many mechanisms contributing to MDR.^[2] Of these factors, overexpression of the efflux pump P-glycoprotein (P-gp), an ATP-dependent transmembrane protein, is the primary cause of cancer MDR.^[3] P-gp could significantly pump the exogenous therapeutic agents from cells, thus reducing the intracellular concentration of these agents to sub-lethal dose.

Therefore, strategies to effectively defeat MDR are of vital importance for successful cancer therapy. By far, various strategies have been reported to reverse the MDR. One promising strategy is the development of unique and simple small molecules that effectively block the function of the efflux transporters, thereby increasing the drug concentration inside cancer cells.^[4, 5] Up to now, some inhibitors have shown promising effects in early stages of clinical trials.^[6, 7] Unfortunately, their extensive clinical applications were impeded by their relatively limited specificity.^[8]

The advancement of nanomedicine has opened unprecedented opportunities for cancer treatment.^[9-12] The nanoscale delivery systems have been reported to exhibit prolonged blood circulation, improved pharmacokinetics, and enhanced tumor accumulation through the enhanced permeability and retention (EPR) effect.^[13-17] Moreover, nanomaterials could also evade the drug efflux pumps via different cellular uptake pathways, such as endocytosis,^[18-20] which will protect the encapsulated molecules from extrusion by efflux transporters.

Of these nanosystems, self-assembled peptide nanogels have recently emerged as promising biomaterials in biomedical research.^[21-23] Evidence documents that peptide-

based assemblies possess definite chemical composition, tunable morphology and size, high water content as well as varied bioactivity. Most importantly, they exhibit inherent responsiveness to numerous environmental stimuli^[24], which makes them attractive nanomaterials for controlled drug release, regenerative medicine, and detection of analyte.^[25-34]

In this chapter, we developed an acid-responsive nanogel system assembled from peptide-Dox (PD) conjugate and a P-gp inhibitor for controlled drug release to reverse multidrug resistance. Typically, an anticancer drug (Dox) was linked to short peptide Fmoc-GFLFF via an acid-cleavable linkage to obtain peptide-Dox prodrug (PD) (Figure 3.1). Then, the PD prodrug was co-assembled with verapamil (VER), one of the effective inhibitors of P-gp, to produce PD/VER nanogels in aqueous condition. Upon cellular uptake, the designed nanogels were localized in endo/lysosomes, where the acidic conditions will cause the disassembly of nanogels and the cleavage of acid-sensitive linker in the conjugate to release the VER molecules and Dox drugs. Subsequently, the free inhibitors could block the activity of the efflux pump, thus preventing the therapeutics from being expelled. Finally, high accumulation of free Dox molecules in cancer cells was achieved, which could effectively combat the MDR and improve the therapeutic efficacy.

Figure 3.1 Schematic illustration of acid-responsive nanogel assembled from peptide-Dox and P-gp inhibitor for controlled drug release to overcome MDR.

3.2 Results and discussion

3.2.1 Preparation of Peptide-Dox prodrug

The peptide sequence Fmoc-GFLGG was manually synthesized by well established Fmoc solid-phase peptide synthesis (SPPS) by using 2-chlorotrityl chloride resin. The hydrazine compound was chosen as a pH-responsive moiety and conjugated to the designed peptide sequence **8**. The Dox molecules were further reacted with the compound **10** to afford the Peptide-Dox prodrug **11**(Figure 3.2). The obtained crude products were separated and purified by reverse-phase HPLC (Figure 3.3).

Figure 3.2 Synthetic steps of Peptide-Dox prodrug. i) HBTU, Tert-butyl carbazate, DIPEA, RT, 4 h; ii) 50% TFA in DCM, 1 hr; iii) methanol, 40°C, 48 h.

Figure 3.3 Chromatogram of Peptide-Dox conjugate detected by HPLC at the detection wavelength of 480 nm.

3.2.2 Characterization of Peptide-Dox prodrug

The purified Peptide-Dox conjugates were characterized by mass spectrometry and NMR, which demonstrated the success of the synthesis. Next, the photophysical properties of the prodrug were investigated through spectroscopic analysis. As shown in Figure 3.4 a, the Dox molecules and peptide-Dox prodrug displayed similar UV-vis absorption spectra from 300 to 600 nm, while their absorption maximum was 482 nm.

Figure 3.4 (a) UV-Vis absorption spectra of free Dox molecules, peptide Fmoc-GFLGG, and Peptide-Dox compound (50 μ M). (b) Fluorescence spectra of free Dox molecules and Peptide-Dox conjugate in DMSO solution. (The concentration is 50 μ M, E_x : 480 nm).

Similarly, there is no obvious difference in the fluorescence emission between the Dox molecules and the designed peptide-Dox conjugates (Figure 3.4 b). These results suggested the conjugation of peptide resulted in negligible effect on the spectroscopic characteristics of free Dox drugs.

3.2.3 Preparation and characterization of Peptide-Dox (PD) nanogels and PD/VER nanogels

The possibility of peptide-Dox prodrug self-assembling into nanostructures was explored. To this end, Peptide-Dox molecule was prepared as stock solution in DMSO solvent. After addition of the stock solution into pre-warmed water at 60°C (pH 7.4), the resulted mixture was stirred for 5 min. Subsequently, the clear solution was cooled to room temperature (25°C). The morphology of the conjugate in water solution was studied by transmission electron microscopy (TEM). The spherical nanoparticles were observed in TEM image (Figure 3.5 a), suggesting the designed peptide-Dox conjugate could self-assemble into nanogels in water solution. Moreover, the average hydrodynamic diameter of the obtained nanogels was determined to be about 198.9 ± 7.0 nm by dynamic light scattering (DLS). More important, the TEM images exhibited

Figure 3.5 TEM images and DLS measurement of (a) PD nanogels and (b) PD/VER nanogels.

that the addition of VER inhibitor into the solution of Peptide-Dox resulted in monodisperse nanoparticles (PD/VER) (Figure 3.5 b), clearly indicating the self-assembly property of Peptide-Dox conjugate was not obviously influenced by the addition of VER molecules. Furthermore, the buffer of PD/VER in pH 7.4 displayed an obvious Tyndall effect (Figure 3.6), confirming the formation of nanogels in buffer solution. In addition, the critical micelle concentration (CMC) of the peptide-Dox prodrug and peptide-Dox prodrug with VER inhibitor determined by DLS was 10.0 and 9.1 μM , respectively (Figure 3.7), revealing they have similar assembled behaviors.

Figure 3.6 Tyndall effect of PD/VER nanogels in PBS buffer solution at pH 7.4 (1) and PD/VER nanogels in acid solution at pH 5.0 (2).

Figure 3.7 Critical micelle concentration (CMC) of (a) peptide-Dox conjugate and (b) peptide-Dox conjugate with VER molecules in water solution measured by dynamic light scattering.

The loading amount of Dox drugs and VER agents in PD/VER nanogels was further examined by HPLC analysis. Firstly, the loading content of Dox molecules was quantified to be 44.8 % (w/w), clearly suggesting an excellent drug loading amount in the self-assembled nanogel. At the same time, the loading efficiency of VER in the assembled PD/VER nanogels determined by HPLC was about 3.6 % (w/w), which was enough to perform an inhibitory function on efflux P-gp protein located on the cellular membrane upon its release from PD/VER nanogels.

Furthermore, the self-assembling properties of PD/VER nanogels were evaluated through spectroscopic studies. In general, we measured the fluorescence of the assembled PD/VER nanogels in different pH buffer solutions by employing a fluorescence spectrometer. As presented in Figure 3.8, the PD/VER nanogels showed negligible fluorescence in buffer solution with the pH value of 7.4 at excitation wavelength of 480 nm, and there was no obvious fluorescent enhancement detected with increasing incubation time. However, at the same condition, the free Dox molecules exhibited very strong fluorescence emission with the wavelength of maximum emission at 592 nm. Moreover, the fluorescence of Dox molecules within

Figure 3.8 (a) Fluorescence spectra of free Dox molecules and PD/VER nanogels at the same concentration (50 μ M in buffer solution with the pH of 7.4) under excitation wavelength of 480 nm. Fluorescence changes of PD/VER nanogels dispersed in pH 5.0 (b) and pH 7.4 buffer solution (c) at different time intervals at excitation wavelength of 480 nm.

PD/VER nanogels were gradually recovered in buffer solution with pH 5.0 as the incubation time prolonged. These results indicated that the assembly of Peptide-Dox conjugate resulted in fluorescence quenching of Dox molecules in PD/VER nanogels, probably due to the intermolecular π - π stacking. Moreover, there was no significant Tyndall effect observed when the PD/VER nanogels were suspended in the pH of 5.0 solutions (Figure 3.6), which further reflected that our nanogels could be disassembled in acidic condition.

3.2.4 Controlled drug release of PD/VER nanogels in buffer solution

The prepared PD/VER nanogels are assumed to release free Dox agents and VER molecules under acidic environment. To quantitatively evaluate the release profiles of both bioactive agents from PD/VER nanogels, free Dox drugs and VER molecules in buffer solution with pH 7.4 and 5.0 were monitored by HPLC, respectively. As presented in Figure 3.9b, approximate 88.2 % of VER inhibitors were released in 2 h from PD/VER nanogels at pH 5.0 in contrast to only 22.1 % of VER released at pH 7.4, implying the effective pH-responsive ability of our designed nanogels. As compared with the release behavior of VER, PD/VER nanogels showed a relatively

Figure 3.9 (a) HPLC chromatograms of Dox and Peptide-Dox conjugate upon incubation of designed PD/VER nanogels for different times at pH 5.0. (b) The cumulative release percentage of VER inhibitors from PD/VER nanogels at pH 7.4 PBS solution and in acetate buffer with pH 5.0. (c) The release profiles of free Dox molecules from PD/VER nanogels at pH 5.0 and 7.4.

slow release rate for Dox molecules. The HPLC chromatogram displayed a new peak with the retention time of 7.5 min upon the nanogels dissolved in buffer solution with pH 5.0 (Figure 3.9a). Such new component was analyzed to be free Dox molecule by ESI-MS (Figure 3.10). The release content of Dox drugs gradually increased with prolongation of the incubation time. In addition, we also quantitatively measured the cumulative release amount of Dox molecules at pH 5.0 and 7.4 (Figure 3.9c). There was about 15.7 % Dox drug release observed within 2 h in pH 5.0 solution, and

Figure 3.10 ESI-MS spectrum of Dox molecule released from PD/VER nanogels.

41.3 % of drugs was detected after 24 h incubation. Subsequently, approximate 61.7 % of Dox was released from the nanogels upon incubation for 96 h. For control group, in buffer solution with the pH of 7.4, only 17.1 % of Dox was detected even after 96 h incubation. In a word, the above results demonstrated that PD/VER nanogels were greatly stable in neutral condition while quickly disassembled in the acidic environment. Such promising environment-responsive nano-assemblies could remarkably respond to pH change, which provide us opportunity to control drug release for cancer therapy.

3.2.5 Intracellular uptake and localization

Encouraged by the excellent stimuli-responsive properties of the prepared nanogels, the cellular internalization and localization of PD/VER nanogels were investigated by monitoring the fluorescence signal of Dox molecules in A2780 cells and Dox-resistant A2780/ADR cells. The two cell lines express considerable different P-glycoprotein amount on the cellular membrane, which have been verified by flow cytometer. Both cells were cultured with PD/VER nanogels for 4 h and imaging studies was carried out by confocal microscopy. As shown in Figure 3.11, the red fluorescence signals of Dox were observed in both A2780/ADR and A2780 cells, which co-localized well with the green fluorescence from commercial LysoTracker green DND-26, confirming that the fluorescence from Dox was located in lysosomal organelle. The confocal imaging results suggested that the designed system could be internalized by both of the targeted cells. It was noted that more red fluorescence was accumulated in nuclei than in the cytoplasm after prolonged incubate time (Figure 3.12), revealing the dynamic transport process of the PD/VER nano-system in A2780/ADR cells. As controls, cellular imaging analysis was also performed with free Dox drugs. An obvious fluorescence in the nucleus and significant weak fluorescence

in the lysosome was detected upon the incubation of Dox with A2780 cells for 4 h. However, no obvious fluorescence was observed in A2780/ADR cells. These imaging studies apparently demonstrated that PD/VER nanogels could act as effective nano-platform to facilitate the delivery of small molecular therapeutic agents into drug-resistant cancer cells. More importantly, higher cellular uptake of drugs could probably contribute to overcoming the drug resistance of cancer cells and therefore remarkably improve chemotherapy efficacy.

Figure 3.11 Confocal images of A2780 cells and A2780/ADR cells. (a, b) Non-resistant A2780 cells incubated with Dox molecules (10 μ M) and PD/VER nanogels (10 μ M) for 4 h at 37°C, respectively; (c, d) Dox-resistant A2780/ADR cells treated with Dox (10 μ M) and PD/VER nanogel (10 μ M) for 4 h at 37°C, respectively. Scale bar: 10 μ m. Green color: Lyso Tracker green red color: Dox.

Figure 3.12 Confocal microscopic images of A2780/ADR cells treated with PD/VER nanogels (10 μ M) for different time points at 37°C. Scale bar: 10 μ m. Blue color: Hoechst 33258, red color: Dox.

3.2.6 Inhibitory assay

VER molecules were reported to inhibit the efflux function of P-glycoprotein on the cell membrane. To assess the inhibitory ability of VER agents against P-gp, the frequently used calcein AM was selected as an indicator to evaluate the P-gp activity. In general, nonfluorescent calcein AM could easily enter into cell and convert into impermeable fluorescent calcein molecule by intracellular esterase. However, Calcein AM could be actively excreted from cells expressing transporter P-gp. Therefore, suppression of P-gp activity could enhance calcein AM molecules accumulation

Figure 3.13 The accumulation of fluorescent calcein in A2780/ADR cells pretreated with different concentrations of free VER agents (b) 0 μ M, (c) 0.5 μ M, (d) 1 μ M, (e) 5 μ M. Cells, (a) control group: cell treated with fresh medium.

within cells, which subsequently result in remarkable fluorescence enhancement upon intracellular esterase cleavage. In this assay, intracellular fluorescence of calcein was measured by flow cytometer for assessment of the VER effectiveness. Typically, Dox-resistant A2780/ADR cells were incubated with free VER molecules for 2 h, followed by treatment with commercial calcein AM for 20 min. There was increase in fluorescence detected in VER-treated A2780/ADR as compared to that of A2780/ADR without VER pretreatment (Figure 3.13), revealing that the efflux function of P-gp was definitely inhibited by VER molecules. Moreover, it was found that VER performed its inhibitory function on P-gp in a dose-dependent manner. Furthermore, the effectiveness of VER agents from PD/VER nanogels was also examined by similar assay. The results presented in Figure 3.14 showed that PD nanogels-treated resistant cells lead to no evident fluorescent change, implying that PD nanogels without VER molecules could not increase the accumulation of fluorescent calcein in resistant cells.

Figure 3.14 Flow cytometric quantification of intracellular concentration of calcein in A2780/ADR cells after incubation with PD (10 μ M) and PD/VER nanogels (10 μ M) for 2 h, respectively, followed by treatment with calcein AM for 20 min. Target cells cultured with fresh medium and calcein AM were control group.

In contrast, apparent enhancement in fluorescence was observed in resistant cells pretreated with PD/VER nanogels, indicating that VER within PD/VER nanogels enhanced the intracellular concentration of calcein in A2780/ADR cells. These results suggested that VER from PD/VER nanogels indeed suppressed efflux function the P-gp on the cell membrane of resistant cells.

Encouraged by the promising effect of VER in designed PD/VER nanogel, the fluorescence of Dox from PD/VER nanogels was further monitored by flow cytometry to quantify the accumulation of Dox molecules in the cells. To this end, A2780 and A2780/ADR cells were incubated with free Dox, PD nanogels and PD/VER nanogels at different time, respectively. As shown in Figure 3.15, there was marked fluorescence signal detected in PD/VER nanogels-treated A2780 and A2780/ADR

Figure 3.15 Quantitative analysis of mean fluorescence intensity (MFI) of Dox in (a) A2780/ADR cells and (b) A2780 cells after incubation with Dox, PD nanogels and PD/VER nanogels for various time durations (1, 2, 4, and 8 h). An equivalent Dox dosage of 10 μ M was applied.

cells. Moreover, a considerable increased fluorescence was observed with prolongation of incubation time because of the enhanced release and accumulation of free Dox molecules inside cells. In comparison, incubation of PD nanogels with both targeted cells lead to relative low fluorescence intensity. This phenomenon was mainly because of the inhibitory effect of released VER from PD/VER system on P-gp function, which improved the concentration of released Dox in cells. What is more, dramatically weaker fluorescence intensity was measured in A2780/ADR cells treated with free Dox as comparison to PD/VER nanogels-incubated cells, even after prolonged incubation. These results further verified that PD/VER nano-platform could efficaciously raise the accumulation of Dox drugs in resistant cancer cells.

3.2.7 Cytotoxicity studies

To evaluate the antitumor activity of our designed PD/VER nanogels, the cell viability of sensitive A2780 and P-gp high expressing A2780/ADR cells incubated with free Dox, PD nanogels and PD/VER nanogels for 48 h was examined by Tox-8 assay method. As shown in Figure 3.16 a and b, the cell viability of A2780/ADR cells

and A2780 cells treated with 10 μM PD/VER nanogels were similar, decreasing to approximately 48 % and 43 %. Moreover, higher concentrations of PD/ VER nanogels (e.g., 20 μM) further dropped the viability of A2780/ADR cells and A2780 cells to about 42 % and 16 %, respectively. As compared to free Dox and PD nanogels, PD/VER nanogels with the same concentration of Dox exhibited higher cytotoxicity against P-gp high expressing A2780/ADR cells. As negative controls, the peptide (Fmoc-GFLGG-NH₂) and VER agents caused negligibly cytotoxicity against A2780 and A2780/ADR cells, even at high concentrations (Figure 3.16 c and d). The above results greatly indicated that in drug resistant cancer cell, the prepared system of PD/ VER nanogels was more efficacious than that of Dox molecules. To quantitatively analyze the cytotoxicity of PD/VER nanogels and free Dox, the IC₅₀ values of both samples in targeted cells were calculated. For sensitive cells, the IC₅₀ of free Dox was about 1.9 μM , while a much higher IC₅₀ value (5.5 μM) was calculated upon incubation with PD/VER system. However, the IC₅₀ value was as high as 23.3 μM in free Dox incubated-A2780/ADR cells, while only 10.2 μM was determined for PD/VER nanogels, indicating that PD/VER nanogels showed a higher anticancer activity than Dox against resistant A2780/ADR cells. Most importantly, the resistance indices (RI, defined as the IC₅₀ value of a given compound in resistant cells, divided by its IC₅₀ value in sensitive cells) were investigated for demonstration of effectiveness of the nanogels to reverse drug resistance. The RI value of free Dox was calculated to be 12.3 (23.3 μM / 1.9 μM), which was approximate 6.8-fold higher than that of PD/VER nanogels (1.8 = 10.2 μM / 5.5 μM), revealing that the designed system of PD/VER have the capability to strongly reverse the cancer drug resistance. These data implied that the designed PD/VER nanogels provided a promising therapeutic platform for enhancement of drug efficiency in cancer cells with drug resistance.

Figure 3.16 *In vitro* Cell viability assay of (a) resistant A2780/ADR cells and (b) sensitive A2780 cells after treatment with free Dox, PD nanogels, and PD/VER nanogels for 48 h. Cell toxicity assay of A2780/ADR cells and A2780 cells incubated with various concentrations of (c) peptide compound and (d) VER agent for 48 h.

3.3 Conclusions

We presented smart and novel pH-responsive small molecular nanoassemblies with integration of anticancer drug and drug transporter inhibitor for controlled drug release in cancer cells with drug resistance. The self-assembled nano-platform displayed multiple attractive properties, such as high drug loading, excellent biocompatibility, enhanced cellular uptake, controlled drug release and synergistic effect of Dox molecules and VER agents. Moreover, the designed PD/VER nanogels exhibited higher anti-drug resistance effect against resistant A2780/ADR cells as compared to free Dox alone. This novel system offered a promising strategy to reverse drug resistance for effective chemotherapy.

3.4 Experimental Section

3.4.1 Materials and Instruments

Lyso Tracker[®] Green DND-26, Cell Trace[™] Calcein Green, AM, fetal bovine serum and PRMI-1640 medium were obtained from Thermo Fisher Scientific. Anti-P glycoprotein conjugated FITC antibody [UIC2] was obtained from Abcam Company. All Fmoc-amino acids and resin (2-Chlorotriyl chloride) were from GL Biochem (Shanghai) Ltd. All other chemicals were purchased from Sigma Aldrich Singapore unless otherwise noted.

ESI-MS spectrometric analysis was conducted with a Thermo LCQ Deca XP Max instrument. ¹H NMR spectra were determined by a Bruker AV400 spectrometer (400 MHz). Reverse-phase HPLC analysis was carried out by Shimadzu HPLC system including a C-18 (250 x 4.6 mm) column at a flow rate of 1.0 mL/min for analysis and a Kromasil C-18 (250 x 10 mm) column at 1.0 mL/min for preparation. UV-Vis absorption spectra were recorded on a Cary 100 UV-Vis Spectrophotometer. Transmission electron microscopy (TEM) images were taken on an FEI EM208S transmission electron microscope (Philips) operated at 100 kV. The size distribution of the assembled nanogels was examined by dynamic light scattering (DLS, Brookhaven Instruments). Fluorescence emission spectra were measured on a Varian Cary Eclipse Fluorescence spectrometer. Flow cytometry (FACS) was conducted with a Fortessa x 20 instrument (BD Company). Confocal laser scanning microscopy (CLSM) imaging was recorded on a LSM 510 instrument (Carl Zeiss Inc.). The cell viability was tested using standard TOX-8 assay with a Tecan's Infinite M200 micro-plate reader.

3.4.2 Preparation of Peptide-Dox prodrug

Synthesis of peptide sequence Fmoc-GFLGG 8: peptide 8 was manually synthesized through solid phase peptide synthesis (SPPS) with the resin of 2-

chlorotriyl chloride. Two amino acids were coupled by coupling reagents of HBTU and DIPEA at room temperature for 2 h. The protected group of Fmoc was removed by 20 % piperidine in DMF for 30 min. The target peptide was removed from the 2-chlorotriyl chloride resin by using 1% TFA and 2.5% TIPS in DCM for 1 min (10 times). The DCM and TFA were removed by rotary evaporator, and then the product was precipitated with cold diethyl ether. The crude peptide was used in the next step without further purification; Yield: 87 %. ^1H NMR (400 MHz, DMSO- d_6): δ 8.18 (d, J = 7.6 Hz, 1H), 8.06 (m, 2H), 7.89 (d, J = 7.6 Hz, 2H), 7.81 (t, J = 5.2 Hz, 1H), 7.70 (d, J = 7.6 Hz, 2H), 7.55 (t, J = 6.0 Hz, 1H), 7.41 (t, J = 7.4 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 7.18 (m, 5H), 4.55 (m, 1H), 4.25 (m, 5H), 3.70 (m, 2H), 3.62 (m, 2H), 3.51 (dd, J = 16.6, 6.0 Hz, 2H), 3.02 (dd, J = 13.8, 4.4 Hz, 1H), 2.78 (m, 1H), 1.58 (m, 1H), 1.49 (t, J = 7.2, 2H), 0.87 (d, J = 6.4 Hz, 3H), 0.83 (d, J = 6.4 Hz, 3H). MS: m/z : 672.08 $[\text{M}+\text{H}]^+$.

Synthesis of Fmoc-GFLGG-NHNH-Boc 9: tert-butyl carbazate (39.6 mg, 0.3 mmol) reacted with peptide 8 (200 mg, 0.3 mmol) with the coupling agents of HBTU (113.7 mg, 0.3 mmol) and DIPEA (100 mL, 0.6 mmol). The reaction was allowed to process at room temperature for 4 h, followed by extraction with EA twice. The organic phases were washed twice with water and further washed with saturated aqueous sodium chloride for three times and dried over anhydrous magnesium sulfate. After removal of the solvent of EA, the residue was subjected to flash column (DCM/MeOH = 10:1) to obtain compound as a white solid 9 (85 %). ^1H NMR (400 MHz, DMSO- d_6): δ 8.16 (d, J = 7.1 Hz, 1 H), 8.11–7.98 (m, 2 H), 7.89 (d, J = 7.5 Hz, 2 H), 7.70 (t, J = 9.7 Hz, 2 H), 7.52 (s, 1 H), 7.42 (t, J = 7.4 Hz, 2 H), 7.33 (t, J = 7.3 Hz, 2 H), 7.23 (d, J = 4.1 Hz, 3 H), 7.19–7.12 (m, 1 H), 4.36–4.18 (m, 3 H), 3.76 (s, 3 H), 3.69–3.59 (m, 1 H), 3.54 (d, J = 16.2 Hz, 1 H), 3.42–3.34 (m, 6 H), 3.04 (d, J =

9.0 Hz, 1 H), 2.82 (dd, $J = 25.4, 15.7$ Hz, 1 H), 1.49 (t, $J = 12.1$ Hz, 2 H), 1.45–1.33 (m, 9 H), 1.26 (dd, $J = 10.7, 4.7$ Hz, 3 H), 0.86 (dd, $J = 16.5, 6.3$ Hz, 6 H) ; MS: m/z : 808.30 $[M + Na^+]$.

Synthesis of Fmoc-GFLGG-NHNH₂ 10: 50 % TFA in DCM was prepared to remove the protected group of the above peptide. Upon removing the solvent, the residue was added with cold diethyl ether to obtain the product **10** in 90 % yields as a white solid. The white solid was directly used for the next step. ¹H NMR (400 MHz, DMSO- d_6): δ 8.25 (t, $J = 6$ Hz, 1H), 8.13 (d, $J = 8.0$ Hz, 1H), 8.03 (m, 2H), 7.89 (d, $J = 7.6$ Hz, 2H), 7.70 (d, $J = 7.6$ Hz, 2H), 7.52 (t, $J = 6.0$ Hz, 1H), 7.42 (t, $J = 7.2$ Hz, 2H), 7.32 (t, $J = 7.2$ Hz, 2H), 7.22 (m, 5H), 4.55 (m, 1H), 4.26 (m, 5H), 3.82 (m, 3H), 3.74 (d, $J = 5.6$ Hz, 3H), 3.64 (dd, $J = 16.8, 6.4$ Hz, 2H), 3.53 (m, 2H), 3.02 (dd, $J = 13.6, 4$ Hz, 1H), 2.77 (m, 1H), 1.58 (m, 1H), 1.49 (t, $J = 8$ Hz, 2H), 0.87 (d, $J = 6.4$ Hz, 3H), 0.83 (d, $J = 6.4, 3H$). MS: m/z : 686.30 $[M+H]^+$.

Synthesis of Fmoc-GFLGG-Dox 11: Peptide **10** (60 mg, 0.087 mmol) was reacted with doxorubicin hydrochloride (58 mg, 0.10 mmol) in 5 mL anhydrous methanol. The mixture was stirred in the dark at 40°C for 24 h. The final compound was purified by HPLC with the mobile phases of A (50 mM ammonium acetate aqueous solution) and B (ACN), observed by UV absorbance at the wavelength of 280 nm and 480 nm. The linear gradient was from 0 min at 30 % ACN to 20 min at 90 % ACN. The peptide-Dox prodrug was received as a red solid after lyophilization in 46 % yield. ¹H NMR (400 MHz, DMSO- d_6): δ 11.13 (s, 1 H), 10.57 (s, 1 H), 8.14 (d, $J = 8.1$ Hz, 1 H), 8.06 (d, $J = 8.0$ Hz, 2 H), 7.98 (s, 1 H), 7.95–7.83 (m, 5 H), 7.67 (dd, $J = 16.7, 6.8$ Hz, 3 H), 7.53 (t, $J = 5.9$ Hz, 1 H), 7.39 (dd, $J = 13.4, 6.7$ Hz, 2 H), 7.33–7.27 (m, 2 H), 7.24–7.12 (m, 5 H), 5.75 (s, 1 H), 5.39 (d, $J = 5.7$ Hz, 1 H), 5.31 (s, 2 H), 4.98 (s, 1 H), 4.54 (dd, $J = 12.8, 8.6$ Hz, 1 H), 4.45 (d, $J = 14.4$ Hz, 2 H), 4.33–4.17

(m, 4 H), 4.15–4.08 (m, 1 H), 3.96 (d, $J = 18.8$ Hz, 4 H), 3.76 (d, $J = 21.1$ Hz, 2 H), 3.65 (d, $J = 6.7$ Hz, 2 H), 3.61 (d, $J = 6.0$ Hz, 1 H), 3.51 (dd, $J = 16.5, 5.8$ Hz, 2 H), 3.18 (d, $J = 17.2$ Hz, 1 H), 3.02 (dd, $J = 14.1, 4.4$ Hz, 2 H), 2.89 (d, $J = 17.7$ Hz, 1 H), 2.78 (dd, $J = 13.9, 9.4$ Hz, 1 H), 2.42–2.32 (m, 1 H), 2.23 (d, $J = 8.1$ Hz, 1 H), 1.93–1.79 (m, 2 H), 1.69 (d, $J = 8.2$ Hz, 1 H), 1.62–1.53 (m, 1 H), 1.53–1.44 (m, 2 H), 1.17 (d, $J = 6.5$ Hz, 3 H), 0.85 (dd, $J = 16.5, 6.4$ Hz, 6 H) ; MS: m/z : 1211.33 $[M+H]^+$.

3.4.3 Preparation of PD nanogels and PD/VER nanogels

Peptide-Dox conjugate and VER agents were prepared to be stock solutions (50 mM) in DMSO solvent, respectively. The mixture of peptide-Dox solution (4 μ L) and VER stock solution (4 μ L) was slowly added to pre-warmed water solution (1 ml, 60°C, pH 7.4) under vigorous stir. The solution was continually agitated at 60°C for 5 min and cooled to room temperature (25°C). Free VER molecules were removed by centrifugation at 5000 rpm for 10 min and washed twice with water. The preparation method for PD nanogels was similar to PD/VER nanogels, which did not include VER agents.

3.4.4 Critical micelle concentration (CMC) determination

The CMC values of synthesized peptide-Dox prodrug with and without of the VER agents were measured at 25°C by dynamic light scattering (DLS) with a laser light scattering spectrometer at 632.8 nm. Different concentrations of peptide-Dox conjugates and VER inhibitors were prepared and tested, while light scattering intensity of each sample was recorded.

3.4.5 Characterizations of PD nanogels and PD/VER nanogels

The morphology of PD nanogels and PD/VER nanogels were measured by TEM. Briefly, 5 μ L of nanogel solution was added onto a copper grid coated with carbon. The excess solution was filtered by a piece of filter paper. The prepared grid was

stayed to dry for investigation. Size distributions of nanogels were determined by DLS. The amount of encapsulated VER agents was analyzed by HPLC after dissolution of PD/VER nanogels in DMSO solvent. The dissociated solution was injected into the HPLC column, and the concentration of VER was calculated based on the standard calibration curve of pure VER agents. The loading percentage of Dox in peptide-Dox conjugate was calculated according to the following equation: Drug loading (%) = $W_{\text{Dox}} / W_{\text{total}} \times 100 \%$. The VER loading efficiency (DL) was calculated using the following formula: $DL = W_{\text{VER}} / W_{\text{total}} \times 100 \%$, where W_{VER} is the weight of VER and W_{total} is the total weight of nanogels.

3.4.6 Drug release from nanogels in buffer solution

The release behavior of Dox and VER agents from nanogels was assessed in 0.1 M acetate buffer solution (ABS, pH 5.0) and 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 37°C. In brief, 20 μM PD/VER nanogel in either ABS or PBS was incubated for different times at 37°C. At predetermined time point, aliquots (100 μL) were examined by HPLC. The released Dox molecules and VER agents were monitored by UV spectrometer with the absorption wavelength of 480 nm for Dox molecules and 280 nm for VER agents. The hydrolysis degree was calculated according to the standard calibration curve of free Dox and VER.

3.4.7 Cell culture

Human ovarian cancer A2780 cells and Dox drug resistant A2780/ADR cells was purchased from European collection of cell cultures (ECACC) and cultured at 37°C and 5 % CO_2 in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 100 U/ mL penicillin and 100 mg/ mL streptomycin. Resistant A2780/ADR cells were periodically kept in Dox contained medium to retain high drug-resistant cell

population. Resistant A2780/ADR cells were treated in medium without Dox for 5 days before experiments.

3.4.8 P-glycoprotein expression assay

Sensitive A2780 cells and A2780/ADR cells were grown in plates with 6 wells until 80% confluence. Cells were trypsinized and centrifuged to obtain pellet. The cell pellet was resolved in PBS solution to get a density of $3 \times 10^6 \text{ mL}^{-1}$. The cell suspension was cultured with 5 μL of FITC-conjugated anti P-glycoprotein antibody for 1 h on ice based on the manufacture's suggestion. After the incubation, cells were centrifuged and washed with ice-cold PBS for two times and tested by flow cytometry. Untreated cells were used as controls.

3.4.9 Calcein AM assay

Sensitive A2780 cells and resistant A2780/ADR cells were grown in 12-well plate with density of 2×10^5 cells/well for 1 day. The medium was replaced with fresh PRMI 1640 medium containing various concentrations of VER, 10 μM PD nanogel or PD/VER nanogel (10 μM). After being incubated for 2 h at 37°C, commercial calcein-AM was added to a final concentration of 0.5 μM , and the cells were further incubated for another 20 min. The cells were washed with PBS for three times to remove the free VER, free nanogels and the free calcein AM. Then the cells were trypsinized and centrifuged at 1200 rpm for 5 min and resuspended in 300 μL of ice-cold PBS. A BD LSR Fortessa™ x 20 FACS flow cytometer with a 488 nm argon laser was used to monitor green fluorescence of calcein. 10,000 cells were investigated.

3.4.10 Flow cytometric analysis

Sensitive A2780 cells or resistant A2780/ADR cells were grown in 12-well plates at a density of $2 \times 10^5 \text{ mL}^{-1}$ for 24 h. The cells were cultured in serum-free medium for 1 h and then fresh culture medium, free Dox in medium (10 μM), PD nanogels in

medium (equivalent Dox concentration), PD/VER nanogels in culture medium (equivalent Dox concentration) were added to plates. After different time durations (i.e., 1, 2, 4 and 8 h), the cells were washed with ice-cold PBS for three times and collected by centrifugation, and finally suspended in 500 μ L of ice-cold PBS. The intracellular fluorescence intensity of Dox was detected using a flow cytometer. 10,000 cells were investigated.

3.4.11 Confocal imaging

The A2780 cells or A2780/ADR cells were seeded in a 35 mm diameter dish at a density 2×10^5 cell/dish and incubated for 24 h at 37°C. Serum starvation was conducted for 1 h, and the medium was replaced with fresh culture medium containing free Dox (10 μ M) or PD/VER nanogels (equivalent concentration of Dox and 1.5 μ M VER). After 3.5 h incubation, Lyso Tracker Green was added to the plates and being further incubated for 0.5 h in the dark. The cells were washed with PBS three times to remove the free nanogels and the free Dox. Nuclei were stained with 5 μ g mL⁻¹ Hoechst 33258 for 20 min. The images were obtained using a confocal laser scanning microscope (Hoechst 33258 E_x = 405 nm, E_m = 410-485 nm); Dox E_x = 488 nm, E_m = 560-620 nm; Lyso Tracker Green E_x = 488 nm, E_m = 500–545 nm; Carl Zeiss LSM 800).

3.4.12 In vitro cytotoxicity

100 μ L of A2780 or A2780/ADR cells were seeded in 96-well plates with a density of 6×10^3 cells per well and incubated at 37°C for 24 h. Then a series of concentrations of Dox, PD nanogels or PD/VER nanogels were added to each well. After incubation for 48 h, the medium was aspirated and replenished with fresh RPMI-1640 (phenol red-free) which contained 10 % TOX-8 reagent for 2 h incubation. The fluorescence emission at a wavelength of 590 nm with excitation at 560 nm was

measured using a Tecan's Infinite M200 microplate reader. The wells without cells but with Tox-8 reagent were blank controls. The cell viability was determined as the (fluorescence of treated cells)/ (fluorescence of control cells) x 100 %.

3.4.13 Statistical analysis

All experiments were conducted in triplicate and repeated three times. Results are presented as mean $\pm$ standard error. All statistical analyses were performed using Student's t-test. Differences were considered statistically significant at a level of $p < 0.05$.

3.5 References

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Chapter 4

Extracellular Vesicles-Mediated Exogenous Protein for Precise Manipulation of Biological Events

ABSTRACT: Efficient transfer of bioactive proteins to target cells is considered to be a potential way for protein therapy and also a powerful way for conferring cells with new biological functions. Current protein intracellular transfer methods commonly suffer from some limitations. Herein, we developed a simple and efficient strategy based on extracellular vesicles (EVs) for transferring bioactive proteins to target cells and conferring the cells with specific biological function. This design was inspired by the functions of natural EVs. EVs are natural assemblies secreted by all cell lines, which are able to transfer various bioactive molecules inherited from parent cells to recipient cells. To demonstrate the concept, a light-controllable membrane channel protein Channelrhodopsin 2 (ChR2) was chosen as a mode. Studies have shown that cell-derived EVs can mediate anchor of ChR2 on the membrane of recipient cells. Upon blue light irradiation, the ion channel was opened, allowing the calcium ion influx across the plasma membrane of recipient cells. Moreover, the increase of intracellular Ca^{2+} also effectively activated Ca^{2+} -dependent transcription factors, thus achieving control of calcium signaling. This strategy can be extended to modulate other cellular behaviors and provides a novel insight on the manipulation of biological functions.

4.1 Introduction

Cell is a highly complicated and collaborative assembled system. It has evolved a wide range of mechanisms to regulate basic physiological processes including proliferation, differentiation and metabolism.^[1-3] However, dysregulation or loss of cellular functions has been implicated in numerous diseases, such as autoimmunity, cancer, diabetes, neurodegenerative disorders, or cardiovascular disease.^[4-6] Therefore, effective strategies to restore the missing or defective cellular functions or confer specific function to target cells are highly desirable, which not only contribute to comprehensive elucidation of essential physiological processes but also open up novel therapeutic modalities.

Up to now, a myriad of strategies have been developed to confer specific function to target cells. For instance, genetic engineering is one of the commonly used methods in biomedical research.^[7] In this method, a specific gene sequence obtained from artificial synthesis or molecular cloning normally needs a vector system such as viruses or polymers to enter target cells.^[8-10] Although promising, the exogenous gene has the potential to cause permanent disruption of endogenous genes. Another popular approach is introduction of target cells with bioactive proteins. In general, proteins with a specific biological function are directly transported to living cells through an artificial carrier.^[11-13] Comparing with genetic engineering, direct protein conferring is more easily to control and possesses lower risk of permanent cell damage. Despite this, this method generally requires purified proteins which are obtained through a series of complicated protein preparation steps, including protein expression, extraction, and purification.^[14] The proteins under this complex purifying process can be inactivated. In addition, the proteins may be further degraded by the endosomal protease in the transfer process. Therefore, a simple and innovative strategy that allow introduction of

specific functional proteins, and more importantly, precise manipulation of their functions remains a big challenge.

EVs are natural nano-assemblies secreted by all cell lines which contain many cytoplasmic contents and membrane constituents.^[15-17] Recent studies have shown that EVs have the capability to transfer secreted bioactive molecules and proteins from parent cells to recipient cells by membrane fusion, thus altering some cellular functions of target cells.^[18] Mounting evidence suggests that EVs participate in many biological processes.^[19-22] For example, EVs have been demonstrated to be involved in homeostasis, blood coagulation, immune regulation, and cancer metastasis.^[23, 24]

By taking advantage of vesicles' natural characteristic in transmitting bioactive molecules between cells, in this chapter, we developed a simple and effective strategy based on EVs to selectively endow specific function to target cells. In this design, EVs both act as the bioactive protein source and transfer carrier. In principle, this strategy exhibits multiple advantages for protein transfer. First, EVs can directly transport original bioactive proteins with intact form from donor cell to recipient cells, which avoids complicated protein extraction and purification. Second, the natural phospholipid bilayers of EVs not only protect the loaded proteins from degradation in blood circulation but also improve the cellular internalization of proteins through direct fusion with the plasma membrane of targeted cells.^[25] Third, EVs, especially those derived from autologous cells, have been reported to be less immunogenic than synthetic vehicles due to their natural compositions^[26]. Taken together, these potential advantages make EVs promising for precise modulation of various biological events.

As a proof of concept, we investigated EVs-instructed anchor of exogenous ion channels on the plasma membrane of target cells for noninvasively regulation of ions influx across the plasma membrane. Ion channels are important membrane proteins,

which allow the flow of specific ions and involve in a large number of cellular processes.^[27] In this study, a light-gated ion channel ChR2 is chosen as model protein to investigate the feasibility of this novel method. As shown in Figure 4.1, natural photoreceptive ion channels expressed in donor cells are supposed to be naturally located on the surface of the secreted EVs owing to the cellular originality of EVs. The membrane fusion between EVs and recipient cells may allow direct attachment of these exogenous photoswitchable ion channels on the plasma membrane of recipient cells. Therefore, a new functional protein is endowed to targeted cells. Upon blue light irradiation, light-sensitive ion channels could be activated, thus allowing noninvasive regulation of ions (e.g., Ca^{2+}) influx across the plasma membrane in living cells.

Figure 4.1 Schematic illustration of EV-mediated localization of intact exogenous ion channel on the cell membrane to achieve remote manipulation of biological events.

4.2 Results and discussion

To investigate the feasibility of the EVs-mediate exogenous proteins in regulating cell functions, we designed an EVs harboring Channelrhodopsin 2 (EV-ChR2) system, in which ChR2 as model protein is a type of natural light-responsive ion channel that control cation ions influx.^[28] First, the recombination protein ChR2-Venus was expressed on the surface of donor HEK293T

Figure 4.2 (A) Confocal imaging of transfected HEK293T cells stained with cell membrane dye. Untreated cells were control group. Green: ChR2-Venus (Ex: 488 nm, E_m : 500 – 550 nm), red: CMSK-Cy5 (Ex: 640 nm, E_m : 650 – 700 nm). Scar bar: 5 μ m. (B) Quantitative analysis of Mean fluorescence intensity (MFI) of HEK293T cells expressing ChR2-Venus by flow cytometry, untreated cells as control (Ex: 488 nm, E_m : 575/25 nm). (C) Quantitative flow cytometric analysis of transfection efficiency of pCAGGS-ChR2-Venus plasmid in HEK293T cells, untreated cells as control.

cells through gene transfection. After 3 days, the expression of ChR2-Venus proteins on the plasma membrane of donor cells were examined by monitoring the fluorescence of Venus tag using confocal microscopy and flow cytometry. As shown in Figure 4.2, after transfection for 3 days, the obvious green fluorescence from Venus reporter was

visualized on the donor cell membrane, which co-localized well with the red fluorescence from commercial cell membrane stain (CMSK-Cy5). The control cells displayed no detected fluorescence on the plasma membrane, demonstrating the successful expression of ChR2-Venus on the HEK 293T cell membrane (Figure 4.2 A). Moreover, flow cytometry analysis (Figure 4.2 B, C) further confirmed that both the Venus mean fluorescence intensity and the percentage of ChR2-Venus-containing cells were significantly higher than those untreated cells, suggesting the high ChR2-Venus protein expression level in transfected HEK 293T cells.

To obtain EVs containing ChR2-Venus proteins (EV-ChR2), the transfected donor cells were cultured in serum-free DMEM medium for EV-ChR2 release. Subsequently, the EVs loaded with ChR2 (EV-ChR2) were isolated from the conditioned supernatants of the transfected HEK 293T cells through differential centrifugation. As controls, EVs without ChR2 proteins were collected from the

Figure 4.3 (A) TEM images of EVs and EV-ChR2. Scar bar: 100 nm. (B) Size distribution of EVs and EV-ChR2.

supernatants of untreated HEK293T cells. TEM images revealed the vesicular structures for both EVs and EV-ChR2 (Figure 4.3 A). DLS analysis suggested that the size distribution of EVs or EV-ChR2 is from 100 nm to 1000 nm (Figure 4.3 B). In addition, the presence of ChR2-Venus proteins on EVs secreted from transfected HEK293T cells was confirmed by monitoring the fluorescence signals of Venus reporter using confocal microscope and flow cytometry. As presented in Figure 4.5 A, there was remarkable green fluorescence observed in EV-ChR2.

Figure 4.4 (A) Confocal images of EVs and EV-ChR2. (E_x : 488 nm, E_m : 525–630 nm). (B) Flow cytometry analysis of EVs and EV-ChR2. (E_x : 488 nm, E_m : 575/25 nm). (C) Quantitative analysis of ChR2-Venus membrane protein loading efficiency and mean fluorescence intensity (MFI) of EVs and EV-ChR2. (E_x : 488 nm, E_m : 575/25 nm).

Moreover, the fluorescence signals were located in the surface of the EVs. However, the EVs isolated from normal HEK293T cells displayed negligible fluorescence, indicating that the ChR2-Venus proteins were loaded on the surface of EV-ChR2 during the production of EVs. Furthermore, the flow cytometry analysis demonstrated that EV-ChR2 vesicles exhibited higher fluorescence intensity than the control group (EVs) (Figure 4.4 B, C). All these results revealed that the ion channel protein (ChR2-Venus) could be effectively shifted to the EVs from donor cells.

To investigate the possibility that exogenous ion channel could be anchored in recipient cells through EVs, we used confocal microscopy to monitor the location of ChR2-Venus in A549 cells incubated with EV-ChR2 for 4 h at 37 °C. As shown in Figure 4.5, confocal images showed that the obvious green fluorescence was visualized on the surface of recipient cells. In contrast, there were negligible

Figure 4.5 Confocal images of A549 cells incubated with (top) or without (bottom) EV-ChR2 vesicles for 4 h. Green: ChR2-Venus (E_x : 488 nm, E_m : 525 – 600 nm). Scar bar: 10 μ m.

signals observed in A549 cells without treatment. These evidences clearly suggested that exogenous ion channel proteins could be incorporated into the target cell membrane by natural vesicles.

To further evaluate the function of anchored exogenous ion channel in recipient cells, blue light was utilized to irradiate light-switched ChR2 membrane channel, which will be opened for the influx of cation ions such as Ca^{2+} . A calcium indicator, Rhod-3 AM, was used to measure the intracellular Ca^{2+} signaling. As presented in Figure 4.6 a, significant red fluorescence enhancement was observed in the cytoplasm of A549 cells incubated with EV-ChR2 under blue light irradiation. As a control, there

Figure 4.6 Cellular Ca^{2+} imaging in EV-ChR2-treated A549 cells with blue light irradiation (~ 480 nm, 10 min) (a) and without blue light irradiation (b). Cellular Ca^{2+} imaging in EVs-treated A549 cells with blue light irradiation (~ 480 nm, 10 min) (c) and without blue light irradiation (d). E_x : 561 nm, E_m : 576–700 nm. Scar bar: 10 μm .

was no obvious fluorescence change in EV-treated cells (Figure 4.6 c and d) and EV-ChR2-incubated cells without light irradiation (Figure 4.6 b). Confocal images clearly demonstrated exogenous ChR2 channel could effectively regulate calcium ions influx from extracellular space under blue light irradiation, which is one of important biological processes.

Calcium ions (Ca^{2+}), as a second message, play a vital role in many diverse physiological processes including cell migration, proliferation, differentiation, and gene transcription.^[29] To this end, we further investigated the ability of anchored exogenous ion channel to mediate Ca^{2+} -dependent NFAT (nuclear factor of activated T cells) translocation. Typically, NFAT factors with phosphorylated form reside in the cytoplasm of resting cells; following an increase in intracellular Ca^{2+} level, they are dephosphorylated by phosphatase calcineurin. Then, NFAT proteins translocate to the nucleus and initiate a variety of gene transcription.^[30-32] Therefore, NFAT possesses a remarkable capability to sense the oscillatory changes of intracellular Ca^{2+} concentration and offer a link between calcium signaling and gene transcription. We examined the Ca^{2+} -dependent nuclear translocation of NFAT by performing anti-NFAT2 immunostaining after different treatments. As shown in Figure 4.7, in the absence of blue light irradiation, red fluorescence was observed in the cytoplasm of cells incubated with EV-ChR2 (Figure 4.7 b) or EVs (Figure 4.7 d), revealing that NFAT2 was located in the cytoplasm. Upon light illumination, EV-ChR2-treated cells presented an increase in red fluorescence in the nuclear region, indicating NFAT2 underwent dephosphorylation and accumulated in the nucleus (Figure 4.7 a). In contrast, there was no apparent fluorescence enhancement visualized in the nucleus of EV-incubated cells under light irradiation (Figure 4.7 c). These results further

demonstrated that EV-transferred exogenous ion channel can act as effective platform to remotely regulate calcium signaling.

Figure 4.7 Immunofluorescence imaging of NFAT2 in EV-ChR2-treated A549 cells with blue light irradiation (~480 nm, 20 min) (a) and without blue light irradiation (b). Immunofluorescence imaging of NFAT2 in EV-treated A549 cells with blue light irradiation (~480 nm, 20 min) (c) and without blue light irradiation (d). Red: anti-NFAT2 (E_x : 640 nm, E_m : 576–700 nm), blue: Hoechst 33258 (E_x : 405 nm, E_m : 410–470 nm). Scar bar: 10 μ m.

4.3 Conclusions

We have exploited a novel strategy based on natural EVs to selectively confer specific biological function to target cells. The constructed EV-ChR2 system could

effectively anchor the exogenous light activated ion channel on the recipient cell membrane. Upon blue light irradiation, the ion channel can be activated, which allowed calcium ion influx. Moreover, the intracellular calcium signaling of modified recipient cells can be invasively regulated. This strategy provided a new insight on precise regulation of target cells and hold great potential for biomedical research.

4.4 Experimental Section

4.4.1 Materials and instruments

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), antibiotic-antimycotic (100×), trypsin-EDTA, Opti-MEM, PureLink™ HiPure plasmid DNA purification kit Lipofectamine® 3000 transfection kit, Cell Mask™ deep red plasma membrane stain, Rhod-3 calcium imaging kit were purchased from Thermo Fisher Scientific. Hoechst 33258 was obtained from Sigma. Anti-NFAT2 antibody and Goat Anti-Mouse IgG H&L (Alexa Fluor® 647) were purchased from Abcam.

The centrifugation was performed on Sorvall™ LYNX™ 6000 superspeed centrifuge. Transmission electron microscopy (TEM) images were taken on an FEI EM208S transmission electron microscope (Philips) operated at 100 kV. The size distribution of the EVs was examined by Zetasizer Nano ZSP (Malvern Instruments, NK). Confocal laser scanning microscopy (CLSM) imaging was recorded Carl Zeiss LSM 800 (Germany). Flow cytometry (FACS) was conducted with a Fortessa x 20 instrument (BD Company).

4.4.2 Cell culture

Human embryonic kidney 293T (HEK 293T) cells and human lung carcinoma A549 cell lines were purchased from American Type Culture Collection (ATCC). All cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented

with 100 µg /mL streptomycin, 100 units/ mL penicillin and 10% FBS. Both cells were cultured in an incubator with 5% CO₂ at 37 °C.

4.4.3 Transient transfection

The plasmid (pCAGGS-ChR2-Venus) encoding Channelrhodopsin2-Venus was obtained from Addgene. For transient transfection, HEK 293T cells were seeded in a 75 cm² flask or 3.5 cm dish until 80% confluence. The culture medium was replaced with fresh culture medium without antibiotics. The commercial Lipofectamine® 3000 (invitrogen) was used for plasmid transfection by following the manufacture's protocol. After 24 h, the growth medium with transfection reagent was replaced with fresh cell culture medium and the cells were continually cultured for another 48 h. The ChR2-Venus expression was confirmed by confocal laser scanning microscopy and flow cytometry. For confocal imaging, the cells in 3.5 cm dish were washed with DMEM medium for two times and stained with CellMask™ Deep Red Plasma Membrane Stain (5 µM) for 10 min. The images were taken on Carl Zeiss LSM 800 confocal microscope. (ChR2-Venus: E_x= 488 nm, E_m=500–550 nm), cell membrane stain: E_x= 640 nm, E_m= 650–700 nm). For quantification of ChR2-Venus on the cell membrane, the collected transfected cells were re-suspended in 0.5 ml cold PBS. The suspensions were analyzed with BD LSR Fortessa™ x 20 flow cytometer (E_x: 488 nm, E_m: 575/25 nm). The mean fluorescence was measured by counting 10,000 cells.

4.4.4 EVs isolation

MVs were isolated and purified from the supernatants of transfected cells or untreated cells by differential centrifugation, according to previous studies. In brief, the cells expressing ChR2-Venus or untreated cells were cultured in serum-free medium with 1 mM calcium for 24 h to allow EVs release. Then, the supernatants were collected and centrifuged for 20 min at 2000 g to get rid of cells and cell debris,

followed by centrifugation at 8000 g for 5 min to remove apoptotic bodies. Subsequently, the EVs pellets were formed by centrifugation of supernatants at 60000 g for 60 min. The obtained MVs were re-dispersed in sterile PBS and centrifuged again for 60 min at 60,000 g. Finally, the purified MVs were dissolved in PBS and used freshly or stored at -80°C refrigerator until further use. All the purification procedures were conducted at 4°C . To quantify the amount of MVs, the total protein content was measured by using a BCA protein assay kit (Bio-Rad).

4.4.5 Characterizations of EVs

The size distribution of the EVs were characterized by Zetasizer Nano ZSP (Malvern Instruments, UK) equipped with a laser (633 nm), at a fixed angle of 173° and temperature of 25°C . The morphology of EVs was characterized by TEM. Briefly, the EVs pellets were fixed with 4% paraformaldehyde in PBS at room temperature for 2 h. After washing with water, the sample solutions were dropped onto a copper grid coated with carbon and stained with 1% Phosphotungstic acid. Samples were performed on an FEI EM208S transmission electron microscope (Philips) at 100 kV. To confirm the expression of the ChR2-Venus proteins on the engineered EVs membrane, confocal images were taken by Carl Zeiss LSM 800 microscope. The fluorescent protein Venus was used as reporter. ($E_x = 488\text{ nm}$, $E_m = 525\text{--}630\text{ nm}$). To quantify the loading efficiency of ChR2-Venus proteins on the membrane of MVs, suspensions containing EV-ChR2 were analyzed with BD LSR Fortessa™ x 20 flow cytometer ($E_x: 488\text{ nm}$, $E_m: 575/25\text{ nm}$). EVs without ChR2 were use as control.

4.4.6 Engineered EVs-mediated localization of ChR2-Venus protein

For Confocal imaging studies, A549 cells were seeded in 3.5 cm ibidi dish at a density of 10^4 cells per well. After 24 h, the cells were washed with PBS and treated with 20 μg of EVs or EV-ChR2. After 4 h incubation at 37°C , confocal images for

each group were captured using Carl Zeiss LSM 800 microscope ($E_x = 488$ nm, $E_m = 525$ – 600 nm).

4.4.7 Calcium influx assay

Intracellular calcium signaling was measured using a Rhod 3 calcium imaging kit (Life Technologies) according to the manufacturer's protocol with some modification. In brief, A549 cells (5×10^4 cells) were seeded in ibidi dish for 24 h, followed by incubation with 20 μ g of EVs or EV-ChR2 for 4 h. Subsequently, the cells were washed with DMEM medium for three times and incubated with Rhod-3 AM (10 μ M) in the dark at 37 °C. After 1 h, the cells were washed with DMEM medium for two times and irradiated with a blue light (~ 480 nm) for 10 min. The cytosolic calcium signals were captured with Carl Zeiss LSM 800 microscope ($E_x = 561$ nm, $E_m = 576$ – 700 nm).

4.4.8 Immunocytochemistry

A549 cells were seeded in 3.5 cm ibidi dish at the concentration of 5×10^4 cells/mL for 24 h. Cells were incubated with 20 μ g of EVs or EV-ChR2 at 37 °C for 4 h. Then, the cells were washed with medium for three times and irradiated with a blue light (~ 480 nm) for 20 min (5 min break after 5 min irradiation). Subsequently, the cells were washed with ice-cold PBS and fixed with ice-cold methanol for 10 min. After incubation with 0.1% Triton X-100 for 5 min, cells were blocked with 5% BSA (bovine serum albumin) in PBS at 37 °C for 30 min. Cells were incubated with anti-NFAT2 antibody at 4 °C for 12 h and then stained with Alexa Fluor 647- conjugated anti-mouse IgG antibody. The cell nucleus was stained with Hoechst 33258. The subcellular localization of NFAT was visualized with Carl Zeiss LSM 800 microscope. (Hoechst: $E_x = 405$ nm, $E_m = 410$ – 470 nm; Alexa Fluor 647: $E_x = 640$ nm, $E_m = 656$ – 700 nm)

4.5 Future work

The exogenous ion channel has been demonstrated to be localized on the cell surface of recipient cells through natural EVs. The attachment efficiency of exogenous channels on cell membrane still needs to be improved. In addition, the feasibility of the developed systems in living animals is being performed. Moreover, the potential of EVs in controlling other physiological processes or even pathological processes will be extensively exploited.

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Chapter 5

Membrane Modification of Extracellular Vesicles for Visualization of their Dynamic Behaviors

ABSTRACT: Extracellular vesicles (EVs), which are natural nanosized assemblies that transfer a variety of biological information between cells, have recently emerged as potential diagnostic markers and natural therapeutic platforms. Yet, much remains obscure about the dynamic behaviors of EVs such as the ways EVs interact with other cells, which hamper the further application of EVs. In order to investigate this dynamic behavior of EVs, a reliable and convenient visualization method is highly desirable. Herein, a universal membrane modification strategy based on metabolic glycoengineering was presented for labeling EVs and investigating interaction between different types of EVs and other cells. Preliminary results showed metabolic strategy can effectively introduce azide groups to the surface of EVs and biorthogonal click chemistry allowed imaging agents to be covalently conjugated on the surface of modified EVs. The dynamic behaviors of different types of EVs will be investigated by utilizing the designed visualization technique.

5.1 Introduction

Extracellular vesicles (EVs) including microvesicles (MVs) and exosomes are natural nanosized assemblies that are released endogenously by all cell lines.^[1] Originally considered as wastes, EVs are gaining recognition as vital transmitter of biological information. Mounting evidences have shown that these cell-derived vesicles mediate intercellular communication through transporting selected bioactive molecules including mRNA, microRNA, cytoplasmic contents and membrane constituents from parent cells to neighboring or even remote cells.^[2-4] They have been increasingly validated to implicate a variety of physiological and pathological processes, such as cell homeostasis, proliferation, tissue repair, pregnancy, inflammation, angiogenesis and cancer.^[5-8] In addition, evidence has shown that EVs released from some cancer cells could propagate oncogenic activity to other distant normal cells and facilitate tumor metastasis.^[6] Moreover, recent studies have indicated that EVs are potential biomarkers in early diagnosis of diseases and promising therapeutic vehicles.^[9-11] Compared to their synthetic counterpart, liposomes, natural EVs are able to overcome biological barriers including the blood brain barrier and exhibit low immunogenicity.^[12, 13] Undoubtedly, natural EVs attract increasing research interest.

Although the biological function and mechanism of EVs are gradually discovered, a great deal of aspects remains obscure, such as the ways EVs interact with other cells. This deficient knowledge has seriously hindered better understanding and clinic application of EVs. Therefore, systematic investigation of the interaction between EVs of different origins and other cells is highly desirable.

In addition, in order to clearly visualize this dynamic behavior of different types of EVs, a versatile and reliable imaging strategy is required. Current visualization

techniques for EVs have some limitations. One of the commonly used approaches relies on fluorescent dyes, such as lipophilic dyes (e.g., PKH26, PKH67, DiO, or DiR), Calcein AM, and carboxyfluorescein diacetate succinimidyl ester (CFSE).^[14-16] They are either encapsulated into the EVs or inserted into the membrane of EVs. However, these processes are non-specific staining and fluorescent dyes can easily diffuse, which hamper the long-term tracking of EVs. Moreover, lipophilic dyes-loaded EVs may be degraded or recycled while the lipid dyes themselves could be retained in other lipid matters for long time, which may not reveal the exact behavior of EVs and generate inaccurate information with regard to the tracking of EVs. Another approach utilized fluorescent proteins linked to proteins existed in EVs, where complicated gene engineering is required.^[17, 18] Therefore, it is highly desirable to develop a reliable and versatile strategy to label different cell-derived EVs for extensive investigation of the interaction between EVs and other cells.

In this chapter, we designed a versatile and convenient strategy for labeling cell-derived EVs to investigate the dynamic behaviors of different types of EVs. This developed visualization strategy is based on metabolic glycoengineering of cells. Metabolic glycoengineering can noninvasively introduce unnatural glycans containing chemical groups (e.g., azide groups) to cells by employing intrinsic glycan metabolism^[19-21]. In general, the cells utilized these unnatural biosynthetic precursors of glycan as building blocks for glycan metabolism.^[22, 23] Therefore, these precursors with various chemical groups will be generated on the cell membrane and become the membrane constituents.^[24] In the design, we selected the molecule Ac4ManNAz as glycan precursor to introduce azide group to the membrane of donor cells. Studies have shown that EVs can inherit their parent cells' membrane contents including glycoproteins and glycolipids.^[25] By utilizing this fantastic characteristic of EVs, the

azide groups on the plasma membrane of donor cells will be incorporated into EVs surface. Further bioorthogonal click chemistry enables various imaging agents to be covalently conjugated on the surface of modified EVs. Therefore, this strategy may cause minimum interference with the function of EVs, which will favor the investigation of EVs behaviors. Moreover, imaging agents are covalently conjugated onto the surface of EVs, avoiding non-specific staining and enabling the long-term tracking of EVs.

Figure 5.1 Schematic illustration of fluorescent labeling of cell-derived EVs through glycan metabolism and bioorthogonal click chemistry, and its applications in real-time tracking of the dynamic behaviors of EVs.

5.2 Results and discussion

Membrane modification of cell-derived EVs was first performed using metabolic glycoengineering. In this study, human lung cancer cell line A549 cell was selected as model donor cell and the unnatural precursor Ac₄ManNAz was chose for metabolic biosynthesis of sialic acid on the cell membrane. To verify the metabolic incorporation of azides into the donor cell membrane, the A549 cells were incubated with different concentrations of Ac₄ManNAz for three days, and then the azide groups were reacted with DBCO-Cy3 through copper-free click chemistry. The metabolic efficiency was analyzed by monitoring the fluorescence of Cy3 using confocal microscope and flow cytometry. As shown in Figure 5.2, the fluorescence gradually increased along with the concentrations of Ac₄ManNAz, indicating that the azides were metabolically incorporated into cell membrane in a dose-dependent manner. Flow cytometry analysis also shown that both the fluorescence percentage and mean fluorescence intensity enhanced with increasing concentrations of Ac₄ManNAz (Figure 5.3). This concentration-dependent metabolism is very useful for artificially tuning of the amounts of azides on cell-surface.

The time-dependent metabolic efficiency of Ac₄ManNAz was investigated by flow cytometry. The results demonstrated that Ac₄ManNAz could be effectively incorporated into parent cells, yielding 28.2%, 36.5%, and 99.3% positive A549 cells after incubation for 24 h, 48 h, and 72 h, respectively. And the mean fluorescence intensity increased with prolongation of incubation time (Figure 5.4). This excellent membrane modification efficiency was also examined by confocal microscopy observations. As indicated in Figure 5.5, confocal images showed that there was dim fluorescence observed after 24 h incubation. Subsequently, the fluorescence signals increased with the prolonged time and reached the maximum after 72 h incubation,

Figure 5.2 Confocal images of azide groups on the membrane of A549 cells pretreated with different concentrations of Ac₄ManNAz for three days. Scare bar: 10 μ m.

Figure 5.3 Dose-dependent metabolic glycoengineering in A549 cells measured by flow cytometry analysis. Both the percentage of azide-contained cells (A) and mean fluorescence intensity (B) were analyzed.

Figure 5.4 Time-dependent metabolic glycoengineering in A549 cells measured by flow cytometry analysis. Both the percentage of azide-contained cells (A) and mean fluorescence intensity (B) were analyzed.

Figure 5.5 Confocal images of azide groups on the membrane of A549 cells incubated with 50 μ M Ac₄ManNAz for different time durations. Scare bar: 10 μ m.

further confirming the effective membrane engineering.

The location of azide groups in cells through metabolic engineering was further confirmed by co-staining with cell membrane tracker (CMSK-Cy5). As presented in Figure 5.6, Ac₄ManNAz-treated cells displayed obvious red fluorescence, which co-localized with the fluorescence from cell membrane tracker. As a control, there is no fluorescence detected in cells without Ac₄ManNAz treatment. Moreover, there is negligible fluorescence observed in cells pretreated with tris (2-carboxyethyl)-phosphine (TCEP) that chemically quenches azide groups. These results suggested that azide groups indeed located on the membrane after metabolic engineering and the high specificity between DBCO and azide groups.

Figure 5.6 Confocal images of A549 cells pretreated with Ac₄ManNAz (50 μ M) in the presence or absence of azide quencher TCEP. Untreated cells were control. Scare bar: 10 μ m.

The biocompatibility of metabolic engineering was further evaluated by monitoring the cell viability of donor cells incubated with Ac₄ManNAz. The donor A549 cells were incubated with Ac₄ManNAz at concentration ranging from 1 to 50 μ M for different incubation durations. The cell viability was tested by Tox-8 assay method. As shown in Figure 5.7, there is negligible cytotoxicity observed during the metabolic process, confirming that the metabolic glycoengineering process is biocompatibility, causing no any damage to donor cells.

Figure 5.7 Cell viability of A549 cells after incubation with indicated concentrations of Ac₄ManNAz (0-50 μ M) for different times.

To investigate whether the azide groups on the plasma membrane of donor cells can be translocated to the surface of EVs during the formation of EVs, the donor cells incubated with Ac₄ManNAz (50 μ M) were stimulated to release of EVs. Subsequently, EVs were isolated from the medium through the differential centrifugation. The isolated EVs were incubated with DBCO-Cy3 for 30 min, followed by removing the free DBCO-Cy3 via ultracentrifugation. The expression of azides in EVs was analyzed by flow cytometry and visualized using a confocal microscope. As shown in Figure

Figure 5.8 Quantification of azide groups on the membrane of unmodified EVs, Ac₄ManNAz-modified EVs (EVs-N3) was analyzed by flow cytometry.

5.8, the azides were efficaciously integrated to the EVs, yielding 91.6% positive EVs (EVs-N3), which was also demonstrated by visualization using confocal microscope. As presented in Figure 5.9, significant red fluorescence from Cy3 was observed, revealing the attachment of azides on the EVs.

Figure 5.9 Confocal image of EVs-N₃ stained with DBCO-Cy3. Scare bar: 5 μm.

The influence of azide modification on the physical properties of EVs was further investigated. Dynamic light scattering analysis confirmed the hydrodynamic diameter of EVs-N₃ was similar to the unmodified EVs. These results indicated that introduction of azide group cause negligible influence on the size of EVs.

Figure 5.10 The hydrodynamic diameter of unmodified EV and EVs-N₃ were measured by DLS.

5.3 Conclusions

In this Chapter, we have presented a versatile and convenient labeling strategy for visualization of biological behaviors of EVs. The preliminary results have shown that reactive azide groups can be introduced into the surface of EVs. Further bioorthogonal click chemistry allowed imaging agents to be covalently conjugated into the EVs. The developed strategy can be further used to label other types of EVs and track the interaction between EVs and other cells.

5.4 Future work

In this chapter, we utilized glycan metabolic strategy to perform membrane modification for EVs. The imaging agents can be covalently conjugated on the surface of EVs by click chemistry, thus achieving effective labeling of cell-released EVs. After confirming the feasibility of the proposed visualization strategy, we will use this strategy to label EVs of different origins, including normal cell-derived EVs, disease-

associated cell-derived EVs, and immune cell-released EVs. Then, we will examine how these EVs interact with other cells and whether EVs from different types of cells have similar or specific recipient cells. Given that tumor cells are known to release a large number of EVs and could propagate specific contents to EVs, cancer cell-released EVs may display different dynamic behaviors from their normal cells. These studies could provide guidance to other basic research and biomedical applications.

5.5 Experimental Section

5.5.1 Cell culture

Human lung carcinoma A549 cell line was provided from American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 μg /mL streptomycin, 100 units/ mL penicillin and 10% FBS, and cultured in an incubator with 5% CO_2 at 37 $^\circ\text{C}$.

5.5.2 Cell membrane labeling of azido glycans on living A549 cells

A549 cells were seeded at a density of 2×10^4 cells/mL onto a 35 mm ibidi dish and grown in DMEM medium with no Ac₄ManNAz or different concentrations of Ac₄ManNAz for different time durations. The medium was gently removed and washed with PBS for three times, followed by incubation with culture medium containing DBCO-Cy3 (10 μM) for 25 min. Then, the cells were rinsed with PBS, stained with Hoechst 33258 (10 μg /mL, 30 min) and CellMask™ Deep Red Plasma Membrane Stain (CMSK-Cy5, 5 μM , 10 min), respectively. For quenched control, cells were culture with TCEP (10 mM) for 20 min and then stained with different dyes. The cells were washed with PBS for three times and imaged by a confocal microscope (Carl Zeiss LSM 800, Germany). (Hoechst: $E_x = 405$ nm, $E_m = 410 - 500$ nm; Cy3: $E_x = 561$ nm, $E_m = 565 - 617$ nm; Cy5: $E_x = 640$ nm, $E_m = 656 - 700$ nm).

5.5.3 Flow cytometry analysis of cell-surface azides

A549 cells were cultured in 12-well plates with 10^5 cells/well and grown in culture medium with no Ac₄ManNAz or different concentrations of Ac₄ManNAz for different time durations. The medium was gently removed and washed with PBS for three times, followed by incubation with culture medium containing DBCO-Cy3 (10 μ M) for 25 min. The cells were washed with PBS, collected and subjected to flow cytometry analysis using BD LSR Fortessa™ x 20 (E_x = 561 nm, E_m = 575/25 nm). The mean fluorescence was measured by counting 10,000 cells.

5.5.4 Cell viability and cell morphology measurement

For cell viability assay, A549 cells were seeded in 96-well plates with a density of 5×10^3 cells per well and incubated at 37°C with increased concentrations of Ac₄ManNAz (0-50 μ M) for different time durations. The culture medium was aspirated and replenished with fresh DMEM (phenol red-free) containing TOX-8 reagent (0.03 mg/mL) for 2 h incubation. The fluorescence emission at a wavelength of 590 nm with excitation at 560 nm was measured using a Tecan's Infinite M200 microplate reader. The wells without cells but with Tox-8 reagent were blank controls. The cell viability was determined as the (fluorescence of treated cells)/ (fluorescence of control cells) x 100 %.

For evaluation of cell morphology, A549 cells were seeded at a density of 2×10^4 cells/mL onto a 35 mm ibidi dish and grown in DMEM medium with 50 μ M Ac₄ManNAz for 3 days. The cells were washed with PBS and imaged by a microscope (Olympus, CKX41).

5.5.5 EV isolation

EVs were isolated and purified from the supernatants of cells with or without membrane azides according to previously described with some modifications. In brief, the cells were seeded in 75 cm² flask and grown in culture medium with no

Ac₄ManNAz or Ac₄ManNAz (50 μ M) for three days, followed by incubation with serum-free DMEM medium for 24 h to allow EVs release. Then, the supernatants were collected and centrifuged for 15 min at 2000 g to get rid of cells and cell debris, followed by centrifugation at 8000 g for 5 min to remove apoptotic bodies. Subsequently, the EVs pellets were formed by centrifugation of supernatants at 60000 g for 60 min. The obtained EVs were re-dispersed in sterile PBS and centrifuged again for 60 min at 60,000 g. Finally, the purified EVs were dissolved in PBS and used freshly or stored at -80°C refrigerator until further use. All the purification procedures were conducted at 4°C .

5.5.6 Characterizations of EVs

The size distribution of the EVs or EVs-N3 were characterized by Zetasizer Nano ZSP (Malvern Instruments, UK) equipped with a laser (633 nm), at a fixed angle of 173° and temperature of 25°C . The morphology of EVs was characterized by TEM. Briefly, the EVs pellets were fixed with 4% paraformaldehyde in PBS at room temperature for 2 h. After washing with water, the sample solutions were dropped onto a copper grid coated with carbon and stained with 1% Phosphotungstic acid. Samples were performed on an FEI EM208S transmission electron microscope (Philips) at 100 kV. To detect the azide groups on membrane of EVs, EVs were incubated with DBCO-Cy3 (1 μ M) at room temperature for 30 min. The excess unbound DBCO-Cy3 dyes were removed by ultracentrifugation (55000 g, 60 min). Confocal images were taken by Carl Zeiss LSM 800 microscope ($E_x = 561\text{ nm}$, $E_m = 565 - 617\text{ nm}$). To quantify the loading efficiency of azide groups on the membrane of EVs, suspensions containing EVs-N3 were analyzed with BD LSR FortessaTM x 20 flow cytometer (E_x : 561 nm, E_m : 575/25 nm). EVs without azide were used as control.

5.6 References

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Summary and Perspectives

In this dissertation, the author presented the development of various nanosized assemblies for investigation and precise manipulation of biological functions. In chapter 2 and chapter 3, tumor microenvironment-responsive self-assembled nanostructures were developed for reversing tumor resistance. In chapter 4, natural EVs assembled from cell membrane were exploited for optical control of ion influx across the plasma membrane. In chapter 5, a versatile and convenient strategy based on glycan metabolism was developed for labeling EVs and investigation of dynamic behaviors of EVs.

Chapter 2 developed a reduction-responsive peptide nanosystem assembled from the anticancer drug doxorubicin and the peptide inhibitor of P-gp for targeted cancer imaging and therapy. The two bioactive molecules can self-deliver themselves to cancer cells and modulate different biological functions that the P-gp activity was modulated by the peptide inhibitor and the cell apoptosis was induced by the doxorubicin. This strategy may provide new opportunities for overcoming cancer resistance.

Chapter 3 described tumor acidic microenvironment-triggered short peptide assemblies that integrated the acid-responsive prodrug with the P-gp antagonist for reversing multidrug resistance. In this study, the controlled drug release and effective inhibition of P-gp activity by the antagonist significantly enhanced the accumulation of the anticancer in resistant cancer cells, thereby successfully overcoming tumor resistance. This promising strategy opened a new venue for control of cancer cells with drug resistance.

Chapter 4 presented a novel strategy for insertion of natural proteins in the cell of interest for regulation of cellular functions. This study utilized cell-derived EVs as mediators to anchor the light-activated ion channel ChR2 on the surface of recipient cells, allowing optical control of calcium signaling. This strategy exhibited great biocompatibility without requiring genetic modification in target cells as well as complicated protein isolation and purification, which provided new insights on spatiotemporal regulation of biological events.

Chapter 5 presented a convenient labeling strategy for visualization of EVs-mediated communication between cells. In this study, exogenous azide groups can be effectively integrated on the surface of EVs through metabolic strategy. The effective membrane modification of EVs allowed conjugation of imaging agents on the membrane of EVs via bioorthogonal click chemistry. This approach will facilitate investigation of dynamic process of EVs.

Although we have constructed several nanosystems through molecular self-assembly for regulation of biological functions, more efforts are needed to exploit their potential in living animals and clinical practice. Regarding the applications of the self-assembled peptide nanostructures in living system, the enhanced targeting ability is required to improve therapeutic efficacy and avoid side effects. In future, we will attempt to develop self-assembling peptide with targeting property for control of specific biological events. In addition, the stability of peptide assemblies *in vivo* is also required to be improved. Current developed peptide assemblies often undergo disassembly after introduction into plasma. Therefore, the future works will focus on rational design of small molecules to guarantee structural integrity of self-assembled systems following systemic administration.

For the practical applications of natural assemblies, several hurdles need to be overcome. First, the production yield and efficiency of EVs should be improved. Although some physical or chemical stimulation have been used to increase EVs production, the effects of these stimuli on donor cells and vesicles remain unknown. Second, it is still a challenge to determine and control the exact composition of natural vesicles. It is also difficult to understand their complex interactions in living animals. Therefore, both target effects and off-target effects should be systematically investigated. In addition, more efforts will be taken to increase the loading efficiency of exogenous components in natural vesicles for various applications.

Publications

1. **L. N. Lyu**, F. Liu, X. Y. Wang, M. Hu, J. Mu, H. L. Cheong, G. Liu, and B. G. Xing. *Chem. Asian. J.*, 2017, 12, 744-752. Featured as “Front Cover” and “VIP paper”.
2. X. Z. Ai⁺, **L. N. Lyu**⁺, Y. Zhang, Y. Tang, J. Mu, F. Liu, Y. Zhou, Z. H. Zuo, G. Liu, B. G. Xing. *Angew. Chem. Int. Ed.* 2017, 56, 3031 –3035. Featured in “Back Cover”. **(+ equal contribution)**
3. X. Z. Ai, **L. N. Lyu**, J. Mu, M. Hu, Z. M. Wang, B. G. Xing. *J. Vis. Exp.*, 2017, e56416, doi:10.3791/56416.