Tumor extracellular microenvironment responsive carbon dots for enhanced cancer theranostics

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2017

Feng, T. (2017). Tumor extracellular microenvironment responsive carbon dots for enhanced cancer theranostics. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/72895>

<https://doi.org/10.32657/10356/72895>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**TUMOR EXTRACELLULAR
MICROENVIRONMENT RESPONSIVE CARBON DOTS
FOR ENHANCED CANCER THERANOSTICS**

FENG TAO
SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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

2017

ACKNOWLEDGEMENT

First, I would like to express my deep appreciation to my supervisor, Assoc. Prof. Zhao Yanli, for his patient guidance, support, and encouragement during my graduate study. He helped me be familiar with the frontiers in biomedicine and trained me be experienced in interdisciplines of chemistry, material science, and biology, which would benefit me a lot in my future career.

Secondly, I want to thank all my group members, Dr. Li Peizhou, Dr. Liu Jia, Dr. Liu Guofeng, Dr. Xing Pengyao, Dr. Chen Hongzhong, Dr. Yang Chaolong, Dr. Zhu Jianjun, Dr. Yin Bin, Dr. Wang Jingui, Dr. Shi Huifang, Dr. Zhao Lingzhi, Dr. Tan Si Yu, Dr. Ang Chung Yen, Dr. Bai Linyi, Dr. Qu Qiuyu, Ms. Tham Huijun Phoebe, Mr. Ong Wee Kong, Ms. Lim Wei Qi, Ms. Phua Soo Zeng Fiona, Mr. Xu Hesheng Victor, Ms. Bindra Anivind Kaur, Mr. Jana Deblin, Mr. Wong Mun Fei Eddy, Ms. Shen Jianhui, Ms. Xiang Huijing, and Mr. Wang Yang, for their generous suggestion and assistance in the past four years.

Thirdly, I would like to appreciate the hard work from summer-research and final-year-project students, Ms. Teo Shu Han, Ms. Lim May Ern Rachel, Ms. Ong Hui Min, Ms. Nur Farhanah Binte Rosli, Ms. Chua Huoy Jing, Ms. Ng Wei Ting, Ms. Ng Yu Hui, and Mr. Wong Zhi Sheng.

Lastly, I am grateful to Mr. Ai Xiangzhao and my family for their endless understanding, support, and encouragement.

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ABSTRACT

Cancer theranostics has been a rising field toward personalized cancer therapy for the benefit of patients through integrating bioimaging diagnostics with cancer treatment. Owing to the rapid advancement of nanotechnology, various theranostic nanoparticles have been developed, among which fluorescent carbon dots (CDs) are extraordinary nanomaterials because of their remarkable optical and biocompatible capabilities. So far, CDs have been extensively utilized as drug nanocarriers for chemotherapy drugs, photosensitizers, and therapeutic genes. However, these CDs often have insensitive surface properties, such as PEGylated, negatively or positively charged, or active targeting ligands attached on the surface, which cannot take full advantage of the difference between normal physiological condition and tumor microenvironment, leading to limited cancer therapeutic efficacy *in vivo*. To solve these problems and further promote the practical applications of CDs, in this thesis, tumor extracellular microenvironment responsive CDs were designed for enhanced cancer theranostics.

Firstly, charge-convertible and anticancer prodrug-loaded CDs were prepared, which could undergo charge reversion from negative charge/PEGylation in physiological environment to positive charge under tumor extracellular condition. Thus, this nanocarrier possessed increased circulation half-life in blood, potent accumulation in the tumor area, facilitated internalization by cancer cells, enhanced escape from endosome, and reduction-triggered drug release, leading to low side effects and high tumor-inhibition efficacy toward carcinoma-bearing mice in contrast to the noncharge-convertible CDs.

Secondly, tumor-triggered active-targeting and anticancer prodrug-loaded CDs were developed, which were PEGylated under physiological condition and exposed

the inner targeting ligands in tumor extracellular microenvironment. Therefore, this nanocarrier could be uptaken efficiently by receptor-overexpressing cancer cells through specific ligand-receptor interaction, resulting in better therapeutic efficiency at tumor extracellular pH than that at physiological pH or against cancer cells with low receptor expression.

Lastly, to better understand the activation of anticancer prodrug used in this thesis, fluorescence resonance energy transfer (FRET)-based CDs were designed. Upon anticancer prodrug activation under reductive conditions, the quenched blue fluorescence of CDs could be recovered while the unquenched green and red fluorescence of CDs was unaltered. Therefore, the gradually enhanced intensity ratio of blue-to-green or blue-to-red fluorescence could indicate the real-time reduction-responsive activation of anticancer prodrug in living cells.

In summary, CDs with tumor extracellular microenvironment triggered charge-conversion or active-targeting capabilities have been successfully prepared for enhanced cancer therapy without appreciable side effects. Moreover, a CD-based ratiometric fluorescent sensor has also been developed to illustrate the intracellular activation mechanism of anticancer prodrug used in above systems. Therefore, these designs can offer promising chances to create safe and effective CD-based theranostic agents for potential anticancer applications.

Chapter 1

Introduction

1.1 Cancer

Cancer is one of the reigning reasons of morbidity and mortality globally, with 8.8 million deaths in 2015, which accounts for approximate 16.7% of total death worldwide.¹ For example, in Singapore, new cancer cases from 2011 to 2015 were 338.5 for female and 330.7 for male in every 100,000 people, and the cancer death in 2015 contributed to 29.7 % of total death; in the United States, it's projected that 1,688,780 new cancer cases and 600,920 cancer deaths will occur in 2017.^{2,3} In particular, five cancers, lung, liver, colorectal, stomach, and breast cancer, lead to the majority of mortality worldwide. Moreover, the reasons for the cancer can be primarily divided into two kinds, exogenous and endogenous. The main exogenous causes are tobacco use, radiation, diet, increased exposure to environmental pollution or industrial carcinogens, and infections from viruses, bacteria, or parasites.⁴ Based on the statistics from World Health Organization, tobacco use contributes to nearly 22% of global cancer deaths and approximately 70% of global lung cancer deaths.¹ The major endogenous factors are polygenetic disorders, deoxyribonucleic acid (DNA) mutations, dysregulation in cell signaling, replication, or transcription, and overexpression of genes, enzymes, or surface receptors.⁴

1.2 Clinical Chemotherapeutic Drugs

Currently, in addition to surgery and radiotherapy, chemotherapy is the most significant modality for cancer treatment. Until now, various chemotherapeutic drugs have been approved by U.S. Food and Drug Administration (FDA), which are summarized in Table 1.⁴ Herein, several typical categories are presented.

Table 1: FDA-approved chemotherapeutic drugs based on different categories.

Categories of chemotherapeutic drugs	Examples
antimetabolites	methotrexate, fluoropyrimidines (e.g. 5-fluorodeoxyuridine, capecitabine), cytarabine, gemcitabine
antimitotic agents	taxanes (e.g. docetaxel), vinca alkaloid (e.g. vincristine)
alkylating agents	carboplatin, cisplatin, oxaliplatin
antitumor antibiotics	anthracyclines (e.g. Doxil, daunorubicin), podophyllotoxins (e.g. etoposide, teniposide), camptothecins (e.g. topotecan, irinotecan)
tyrosine kinase inhibitors	imatinib, gefinitib, dasatinib, sunitinib, afatinib, lapatinib, vismodegib
cyclin dependent kinase inhibitors	alvociclib, palbociclib
poly(ADP-ribose) polymerase inhibitors	olaparib, rucaparib, veliparib
histone deacetylase inhibitors	mocetinostat, belinostat, romidepsin, vorinostat, trichostatin
mitogen-activated protein kinase kinase inhibitors	selumetinib, trametinib, cobimetinib
serine/threonine-protein kinase B-Raf inhibitors	vemurafenib, sorafenib, dabrafenib
mammalian target of rapamycin inhibitors	temsirolimus, everolimus
phosphoinositide 3-kinase inhibitors	idelalisib
ribonucleotide reductase inhibitors	cladribine, tezacitabine, fludarabine
DNA methyltransferase inhibitors	5-azacytidine, 5'-deoxy-azacytidine
retinoids	tretinoin, bexarotene
monoclonal antibodies	ofatumumab, ibritumomab tiuxetan, rituximab

1.2.1 Antimetabolites

Antimetabolites function by inhibiting the utilization of metabolites, resulting in abnormal metabolism and cytotoxicity. For example, 5-fluorouracil is used to treat pancreatic, stomach, oesophageal, and skin cancers by inhibiting thymidylate synthase irreversibly; methotrexate is indicated for trophoblastic neoplasms and osteosarcomas by acting as a competitive antagonist of dihydrofolate reductase during the synthesis of tetrahydrofolate, which is essential to synthesize thymidine and DNA.

1.2.2 Antimitotic Agents

Antimitotic agents act by disrupting microtubules and can be classified into two kinds according to different mechanism of action: vinca alkaloids and taxanes. Vinca alkaloids can hamper tubulin polymerization, while taxanes can inhibit tubulin depolymerization. For instance, docetaxel is employed for head and neck cancer, hormone-resistant prostate cancer, locally advanced or metastatic breast cancer,

non-small cell lung cancer, and gastric cancer by inhibiting mitotic spindle assembly; vincristine is widely used for neuroblastoma, acute lymphoblastic leukaemia, and acute myeloid leukaemia through hampering mitotic spindle disassembly; vinblastine is utilized for the treatment of several hematological and solid tumors by hampering the microtubules formation and glutamic acid metabolism; vinorelbine is approved for relapsed metastatic breast cancer through the similar mechanism of action as vinblastine.

1.2.3 Alkylating Agents

An alkylating agent functions by attaching an alkyl group to DNA. For example, carboplatin, oxaliplatin, and cisplatin can treat various types of cancers by coordinating platinum to the N7 atoms of guanine, which results in inter-strand or intra-strand crosslinks to inhibit the action of DNA and ribonucleic acid (RNA) polymerases; chlorambucil is utilized for chronic lymphoid leukaemia through interfering DNA replication.

1.2.4 Antitumor antibiotics

Antitumor antibiotics act by interfering with DNA, leading to blocking of cell growth. For instance, mitomycin C is indicated for stomach and pancreas adenocarcinomas; bleomycin is used to treat Hodgkin's disease, squamous cell cancer, and germ cell tumors; doxorubicin (DOX) can treat pediatric solid tumors, breast carcinoma, Hodgkin's disease, and ovarian cancer by intercalating between DNA or inhibiting DNA topoisomerase II; teniposide and etoposide are utilized for the therapy of leukemia through the inhibition of DNA topoisomerase II; topotecan and irinotecan are respectively employed for relapsed ovarian carcinoma and refractory metastatic colon cancer by inhibiting DNA topoisomerase I.

1.2.5 Tyrosine Kinase Inhibitors

Tyrosine kinase inhibitors function by inhibiting tyrosine kinases, which are

needed to activate various proteins through signal transduction cascades. For example, afatinib, gefitinib, and erlotinib are widely employed to treat non-small cell lung cancer; imatinib is approved for chronic myeloid leukemia; sunitinib can treat imatinib-resistant gastrointestinal stromal tumor and renal cell carcinoma; vandetanib is used for the therapy of late-stage (metastatic) medullary thyroid cancer by simultaneously inhibiting rearranged during transfection tyrosine kinase and vascular endothelial growth factor receptor.

1.2.6 Cyclin Dependent Kinase Inhibitors

Cyclin dependent kinase inhibitors act by inhibiting cyclin dependent kinase. For instance, palbociclib can treat human epidermal growth factor receptor 2-negative and estrogen receptor-positive breast cancer through serving as cyclin dependent kinase 4 and 6 inhibitor; alvocidib is used for the therapy of acute myeloid leukemia by inhibiting cyclin dependent kinase 9, interfering with RNA polymerase transcription, and halting the cell cycle.

1.3 Nanoparticle-Based Therapeutic Agents

Despite the global application in clinic, chemotherapeutic agents usually suffer from some disadvantages. As most of the drugs are hydrophobic, they don't have enough solubility to pass through the aqueous environment in the body before accumulating inside cells. In addition, the hydrophobic drugs tend to aggregate after intravenous injection, which can cause local toxicity and embolisms.⁵ Moreover, the hydrophilic drugs cannot be uptaken easily by cells due to the hydrophobic nature of cell membranes. On the other hand, they possess low bioavailability and short half-life during blood circulation, resulting from their poor stability by degradation through proteolysis and hydrolysis.^{6,7} Most importantly, anticancer drugs cannot differentiate between cancer cells and normal cells, leading to serious toxic effects to patients. Therefore, it's urgently demanding to develop advanced therapeutic agents to overcome above problems.

Table 2: Nanoparticle-based therapeutic agents in clinic use or under clinical trials.⁸

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Trade name	Formulation	Drug	Company	Application	Phase of development
Abraxane	albumin-bound nanoparticle	paclitaxel	Abraxis Bioscience, Inc.	metastatic breast cancer	approved
Caelyx	PEGylated liposome	doxorubicin	Schering-Plough	metastatic breast and ovarian cancer, Kaposi sarcoma	approved
DaunoXome	liposome	daunorubicin	Galen Ltd	Kaposi sarcoma	approved
DepoCyt	liposome	cytarabine	Pacira Pharmaceuticals, Inc.	lymphoma	approved
Doxil	liposome	doxorubicin	Sequus Pharmaceuticals, Inc.	Kaposi sarcoma	approved
Genexol-PM	polymeric micellar nanoparticle	paclitaxel	Samyang Biopharmaceuticals	breast cancer	approved
Marqibo	liposome	vincristine sulfate	Talon Therapeutics, Inc.	lymphoblastic leukemia	approved
Myocet	liposome	doxorubicin	Zeneus Pharma Ltd	metastatic breast cancer	approved
Oncaspar	PEGylated asparaginase	asparaginase	Enzon Pharmaceuticals, Inc.	acute lymphoblastic leukemia	approved
Zinostatin stimalamer	poly(styrene-co-maleic acid)-conjugated neocarzinostatin	neocarzinostatin	Astellas Pharma, Inc.	hepatocellular carcinoma	approved
NK105	micellar nanoparticle	paclitaxel	Nippon Kayaku Co., Ltd	breast cancer	phase III
BIND-014	polymer matrix	docetaxel	BIND Therapeutics, Inc.	prostate cancer	phase II
Genexol-PM	methoxy PEG-PLA	paclitaxel	Samyang Biopharmaceuticals	ovarian and lung cancer	phase II
CRLX101	cyclodextrin-PEG micelle	camptothecin	Cerulean Pharma, Inc.	ovarian/tubal/peritoneal cancer, rectal cancer	phase I/II
CYT-6091	gold nanoparticle	tumor necrosis factor α	Cytimmune Sciences, Inc.	pancreatic cancer, melanoma, soft-tissue sarcoma, ovarian, and breast cancer	phase I/II
L-Annamycin	liposome	annamycin	Callisto Pharmaceuticals, Inc.	acute lymphocytic leukemia, acute myelogenous leukemia	phase I/II
NL CPT-11	liposome	irinotecan	University of California, San Francisco	solid tumor	phase I/II
Rexin-G	pathotropic nanoparticle	dominant negative cyclin G1 construct	Epeius Biotechnologies	breast cancer, osteosarcoma	phase I/II
Anti-EGFR immunoliposome	liposome	doxorubicin	University Hospital, Switzerland	solid tumor	phase I
AuroLase	gold nanoparticle		Nanospectra Biosciences, Inc.	lung cancer, head and neck cancer	phase I
BikDD nanoparticle	liposome	proapoptotic Bik gene (<i>BikDD</i>) siRNA	National Cancer Institute	pancreatic cancer	phase I
CALAA-01	cyclodextrin-containing polymer		Calando Pharmaceuticals, Inc.	solid tumor	phase I
CRLX301	cyclodextrin-based polymer	docetaxel	Cerulean Pharma, Inc.	solid tumor	phase I
DEP™-Docetaxel	dendrimer	docetaxel	Starpharma Holdings, Ltd	breast, prostate, lung, and ovarian cancer	phase I
Docetaxel-PNP	polymeric nanoparticle	docetaxel	Samyang Biopharmaceuticals	advanced solid malignancies	phase I
TKM-080301	lipid nanoparticle	siRNA	National Institutes of Health Clinical Center	liver cancer	phase I
C-dots	PEG-coated SiO ₂		C-dots Development	melanoma	IND approved

In the past decades, benefited from the advancement of nanotechnology, various nanoparticle-based therapeutic agents have been developed to meet above requirements, including organic, inorganic, or organic-inorganic hybrid nanoparticles.⁴ Until now, varieties of nanotherapeutics have been approved by FDA or under clinical investigations (Table 2), and have demonstrated significantly

improved outcomes for the treatment of cancers, which possess the potential to reform the pharmaceutical industry.⁸ In comparison with the traditional anticancer drugs, the use of nanoparticles as drug carriers has enormous advantageous properties, including increased solubility and prolonged circulation half-life, passive or active tumor targeting, stimuli-responsive drug release, and combination with bioimaging.

1.3.1 Increased Solubility and Prolonged Circulation Half-Life

Figure 1. (a) Scheme of the molecularly self-assembled oligonucleotide nanoparticle for the delivery of siRNA.⁶ Copyright 2012 Nature Publishing Group. (b) The nanopatform based on Meo-PEG-*b*-P(DPA-co-GMA-TEPA-C14) and iRGD-PEG-*b*-PDPA polymers for siRNA delivery and corresponding pH-responsive mechanism for siRNA loading and release.⁷ Copyright 2015 Wiley-VCH.

Using nanoparticles as carriers is a potent method to increase the solubility of hydrophobic drugs in water. For example, Park and co-workers loaded paclitaxel, a drug with poor solubility, into nanosized micelles prepared from amphiphilic block copolymers, and demonstrated that the concentration of paclitaxel in water could be improved by a more than 1000-fold.⁹ Allen and co-workers encapsulated a hydrophobic anticancer drug ML220 (2-(5-bromo-1H-indol-3-yl)-1H-phenanthro [9,10-d] imidazole) into liposome based on poly (ethylene glycol) (PEG) functionalized distearoylphosphatidylethanolamine and egg phosphatidylcholine, and confirmed that the water solubility of ML220 was enhanced with a fold of more than 50000.¹⁰ In addition, using nanoparticles as carriers can protect hydrophilic drugs

from severe conditions in the body, such as the presence of proteases or other enzymes in the blood, and the acidity inside intracellular lysosomes or in the stomach, resulting in prolonged circulation half-life of the drugs. For instance, in Anderson's work (Figure 1a), when naked small interfering RNA (siRNA) was injected into mice through vein tail, it could be cleared from blood rapidly within 6 min. However, when siRNA was self-assembled with short DNA fragments to form molecularly identical oligonucleotide nanoparticles, its circulation half-life could be extended to 24.2 min.⁶ In Farokhzad's work (Figure 1b), naked siRNA had a short blood half-life of less than 30 min upon intravenous injection, while a prolonged half-life of about 3.56 h was achieved by using nanocarriers based on pH-responsive and tumor-penetrating polymers.⁷

1.3.2 Passive Tumor Targeting

Figure 2. Scheme of the transport of small molecules and nanoparticles with different sizes in the (a) normal and (b) tumor tissues.⁸ Copyright 2014 Wiley-VCH.

In normal tissues, the endothelial cells on vascular walls are linked by tight junctions, which allow the passing through of small molecules but prevent the transport of nanoparticles due to their relatively large sizes (Figure 2a).⁸ However, in tumor tissues, large gaps between endothelial cells, in a size range from 100 to 800

nm, are present owing to the rapid angiogenesis and the resulting density decrease of endothelial cells and loss of tight junctions (Figure 2b).⁸ Therefore, nanoparticles with suitable sizes can pass through the leaky vascular walls effectively.¹¹ Moreover, because of the absent or dysfunctional lymphatic system inside a tumor and the resulted insufficient drainage, nanoparticles are preferable to accumulate in the tumor site.¹² This phenomenon has been defined as enhanced permeability and retention (EPR) effect, which is the basic theory for passive tumor targeting. In general, the optimal sizes of nanoparticles are between 30 to 200 nm so as to achieve efficient passive tumor targeting.¹³ In addition, the shape or surface properties of nanoparticles can also be optimized to decrease the absorption by protein and the clearance by mononuclear phagocyte system, which lead to enhanced circulation half-life and effective accumulation in the tumor area.^{14,15}

1.3.3 Active Tumor Targeting

Figure 3. Schematic illustration of (a) active tumor targeting and (b) active vascular targeting.¹⁶ Copyright 2009 American Chemical Society.

To further enhance the targeting efficiency of nanoparticles, ligands including

small molecules, peptides, nucleic acids, and antibodies can be conjugated on the surface of nanoparticles. The specific interaction between the targeting ligands and corresponding receptors on the surface of cancer cells can facilitate the accumulation of nanoparticles inside cancer cells through receptor-mediated endocytosis, which can improve the intracellular drug concentration and enhance the anticancer effects. This phenomenon is described as active tumor targeting (Figure 3a).¹⁶

In addition, active vascular targeting is proved as another promising method for tumor targeting, which eradicates cancer cells by targeting and destroying the endothelial cells of the tumor vessels to terminate the supply of oxygen and nutrients (Figure 3b).¹⁶ So far, various nanoparticles have been fabricated to target different moieties on the vascular walls, such as vascular endothelial growth factors and their receptors, annexin A1, integrins, large tenascin-C isoforms, and fibronectin extradomain B.

1.3.4 Stimuli-Responsive Drug Release

Figure 4. Commonly used linkers for stimuli-responsive drug release. The dotted line means the cleavage site under corresponding stimulus shown in parentheses.⁸

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By introducing stimuli-responsive characteristics to nanocarriers, chemotherapeutic drugs can be released controllably in target tissues and cells, leading to increased delivery and therapeutic efficiency. Such stimulus consist of internal stimulus, including variation in pH, enzymes, or small molecules, and external stimulus, such as light, heating, magnetic field, electric field, or ultrasound (Figure 4).⁸ Herein, some representative stimuli-responsive drug release were summarized.

1.3.4.1 pH-Responsive Drug Release

Figure 5. (a) Schematic illustration of the synthesis and drug release mechanism of DOX-loaded MSNPs with ZnO quantum dots as nanolipids.¹⁹ Copyright 2011 American Chemical Society. (b) Scheme of the synthesis and drug release behavior of DOX-conjugated BaGdF₅:Yb³⁺/Tm³⁺-based UCNPs.²⁰ Copyright 2014 Elsevier.

It's well known that pH in physiological condition is 7.4 and weak acidity with pH of ~6.8 is found in tumor tissues due to the enhanced glycolysis and production of lactic acid by cancer cells.¹⁷ Moreover, a much lower pH from 4.5 to 6.5 is present in endosome and lysosome.¹⁸ Until now, various nanocarriers, capable of releasing drugs by utilizing the specific pH of cancer cells, have been developed. In Zhu's work,

anticancer drug DOX were loaded in the nanopores of mesoporous silica nanoparticles (MSNPs), followed by sealing the nanopores by ZnO quantum dots (Figure 5a).¹⁹ In physiological environment with pH 7.4, little DOX were released, while under acidic condition with pH 5, DOX were fast released because of the dissolution of ZnO nanolids. In Lin's work, DOX were conjugated on BaGdF₅:Yb³⁺/Tm³⁺-based upconversion nanoparticles (UCNPs) through hydrazone bonds (Figure 5b).²⁰ The drug release profiles showed that only 8.9% DOX were released at pH 7.4 and more than 70% DOX were released at pH 5 after 72 h, demonstrating the hydrolysis of hydrazone bonds under acidic condition and the pH-responsive drug release.

1.3.4.2 Reduction-Responsive Drug Release

Figure 6. (a) The self-assembled micelles from PEG-SS-PCL block copolymer for efficient DOX release under intracellular reductive condition.²¹ Copyright 2009 Elsevier. (b) The reduction-responsive DOX release *in vitro* and *in vivo* from multifunctional hollow MSNPs.²² Copyright 2013 American Chemical Society.

Reducing agents, such as glutathione (GSH) and ascorbic acid, are playing significant roles in the maintenance of redox homeostasis in biological systems. In particular, the concentration of GSH inside normal cells is in the range of 2-10 mM, with a much lower concentration in plasma (1-2 μ M) and normal tissues (2-20 μ M).¹⁸ However, the GSH concentration can increase to a higher level in the tumor tissues or

inside cancer cells. Until now, various nanoparticles have been developed for efficient drug release under specific intracellular reductive conditions. For example, in Zhang's work, DOX was encapsulated into biodegradable micelles, which were self-assembled reduction-sensitive PEG-*b*-poly(ϵ -caprolactone) (PEG-SS-PCL) block copolymer (Figure 6a).²¹ When the micelles were treated with reducing dithiothreitol (DTT) at a concentration of 10 mM, almost 100% DOX was released after 12 h, while less than 20% DOX was released after 24 h without the incubation with DTT or for the reduction insensitive PEG-PCL micelles with the treatment with 10 mM DTT. In Zhao's work, DOX was loaded into multifunctional hollow mesoporous silica nanocontainers, with the nanopores sealed by mechanically interlocked [2]rotaxanes through disulfide bonds (Figure 6b).²² In the absence of GSH, only 10% DOX was leaked after 26 h, indicating the stability of nanoparticles. However, when the nanoparticles were incubated with 10 mM GSH for 26 h, over 85% DOX could be released. This was due to the removal of [2]rotaxane from the surface of nanoparticles to uncap the nanopores through the cleavage of disulfide bonds under reductive conditions.

1.3.4.3 Enzyme-Responsive Drug Release

In tumor tissues, various enzymes are over expressed with abnormally high activity, which significantly contribute to the development and invasion of cancers. These enzymes include esterases, glycosidases, phospholipases, matrix metalloproteinases (MMPs) and others.¹⁸ Moreover, by utilizing the specific enzymes in the tumor microenvironment, controlled drug release from nanoparticles can be realized. For instance, Cai and co-workers loaded DOX into nanopores of MSNPs, which were capped by phenylboronic acid functionalized human serum albumin through a polypeptide consisting of MMP-2 responsive peptide and cell penetrating polyarginine (Figure 7a).²³ When the nanoparticles were incubated with MMP-2 enzyme at a concentration of 5 $\mu\text{g mL}^{-1}$, about 73% of DOX was released after 24 h, while only around 15% of DOX was released without treatment with MMP-2 enzyme. Moreover, when the MMP-2 responsive peptide was replaced by scrambled peptide

Figure 7. (a) Scheme of the synthesis of enzyme-triggered MSNPs for DOX delivery.²³ Copyright 2016 Royal Society of Chemistry. (b) Schematic illustration of enzyme-responsive MSNPs for CPT delivery.²⁴ Copyright 2016 Royal Society of Chemistry.

sequence, only appropriate 17% of DOX was released in the presence of MMP-2 enzyme. These results confirmed efficient cleavage of substrate peptide in response to MMP-2 enzyme and removal of capping human serum albumin for DOX release. Zhang and co-workers loaded a hydrophobic drug camptothecin (CPT) into the pores of MSNPs, with surface modification by tumor targeting peptides and pores blocking thorough MMP-2 enzyme responsive peptide (Figure 7b).²⁴ In the absence of MMP-2

enzyme, only about 35% of CPT was leaked from the nanoparticles after 36 h, while over 87% of CPT was released in the presence of MMP-2 enzyme, indicating the enzyme triggered CPT release through removal of MMP-2 responsive peptide to uncap the nanopores.

1.3.4.4 Photo-Responsive Drug Release

Figure 8. (a) Design of photo-induced 5-fluorouracil release from Au nanoparticles.²⁵ Copyright 2009 American Chemical Society. (b) Scheme of photo-activated Pt(IV) prodrug loaded UCNPs for cancer tri-modality imaging and therapy.²⁶ Copyright 2013 American Chemical Society.

In addition to the internal stimulus of tumor microenvironment, light is a

commonly used external stimulus for controlled drug release from nanoparticles in a temporary and spatial manner. For example, in Rotello's work, a caged anticancer drug, 5-fluorouracil, was linked on the surface of Au nanoparticles by a photoresponsive *o*-nitrobenzyl group (Figure 8a).²⁵ Under 365 nm ultraviolet (UV) light irradiation, maximum 5-fluorouracil could be activated after 10 min. In Lin's work, a light activated platinum(IV) prodrugs, *trans,trans,trans*-[Pt(N₃)₂(NH₃)(py)(O₂CCH₂CH₂COOH)₂] was conjugated on the surface of UCNPs, NaYF₄:Yb³⁺/Tm³⁺@NaGdF₄/Yb³⁺, which could convert near-infrared (NIR) light with low-energy photons to UV, visible, and NIR light with high-energy photons through the multiphoton processes (Figure 8b).²⁶ Specifically, the UV emissions at 291, 346, and 363 nm of UCNPs under 980 nm laser illumination could be used to activate platinum(IV) prodrug. When the nanoparticles were placed in a buffer solution of pH 7.4 at 37 °C for 4 h in the dark condition, about 25% platinum was released owing to the ester hydrolysis. Subsequently, if the nanoparticles were irradiated with 980 nm NIR light for 30 min, the released platinum could reach 48.4%, in comparison to 57.3% with the nanoparticles exposed to 365 nm UV light. Most importantly, NIR light is advantageous to UV light due to its deeper tissue penetration and less damage to normal tissues, holding great promise in biomedical fields.

1.3.4.5 Thermo-Responsive Drug Release

Besides light, heat is another commonly used external stimulus to realize controlled drug release. For instance, Zhang and co-workers synthesized polyprodrug by using thermo-sensitive azobis(*N*-(2-carboxyethyl)-2-methylpropionamidine) to conjugate DOX with lipoic acid functionalized biotin-PEG-*b*-poly(*L*-lysine), which were further coated on gold nanorods (GNRs) through the interaction between Au and thiol (Figure 9a).²⁷ When the nanoparticles were treated at 37 °C, little DOX was released after 6 h, while more than 60% DOX was released at 60 °C within 6 h, which was owing to the cleavage of thermo-sensitive linkage at high temperature. Most importantly, taking advantage of the photothermal properties of GNRs, DOX could be

Figure 9. (a) Schematic illustration of loading DOX on GNRs through thermo-sensitive molecule, azobis(*N*-(2-carboxyethyl)-2-methylpropionamidine).²⁷ Copyright 2015 Wiley-VCH. (b) Scheme of loading DOX into thermo-responsive polymer, poly(*N*-isopropylacrylamide-*co*-acrylic acid), decorated silica-coated GNRs.²⁸ Copyright 2014 American Chemical Society.

continuously released when the nanoparticles were irradiated with a periodic 808 nm laser at 1.74 W cm^{-2} . In another work, Chen and co-workers loaded DOX into thermo-responsive poly(*N*-isopropylacrylamide-*co*-acrylic acid) polymer-decorated silica-coated GNRs through electrostatic interaction (Figure 9b).²⁸ The nanoparticles could undergo size shrink at high temperature because of the reversible phase transition of poly(*N*-isopropylacrylamide-*co*-acrylic acid). Therefore, when the water dispersion of nanoparticles was illuminated with a 760 nm laser, the temperature increased significantly, which could trigger the release of DOX.

1.3.5 Cancer Theranostics

Besides above advantages, it's also possible to integrate bioimaging diagnostics with cancer treatment within a single nanoplatform, which is also known as cancer theranostics and is a new field towards personalized nanomedicine to benefit patients.⁴ Up to now, a lot of theranostic nanoparticles have been developed, which combine different imaging modalities, such as magnetic resonance (MR) imaging,

Figure 10. (a) Schematic illustration of $\text{NaYF}_4:\text{Yb,Er}@ \text{Fe}_3\text{O}_4@ \text{Au-PEG}$ nanoparticle for UCL/MR imaging-guided and magnetically targeted PTT.²⁹ Copyright 2012 Elsevier. (b) Design of PEGylated FeS nanoplates for MR imaging-guided PTT.³⁰ Copyright 2015 Elsevier.

photoacoustic imaging, positron emission tomography, X-ray computed tomography, and fluorescence bioimaging, with various therapeutic techniques including radiation therapy, chemotherapy, photodynamic therapy (PDT), and PTT.^{29,30} Herein, two representative examples are introduced. Liu and co-workers fabricated a multifunctional nanoplatform $\text{NaYF}_4:\text{Yb,Er}@ \text{Fe}_3\text{O}_4@ \text{Au-PEG}$ for magnetically targeted PTT under the guidance of upconversion luminescence (UCL)/MR dual-modal imaging (Figure 10a).²⁹ When the tumor was intravenously injected with this nanoparticle and placed nearby a magnet, the UCL intensity enhanced by 7 folds and the T2-weighted MR signals decreased by 62.1%, demonstrating the magnetically targeted accumulation of nanoparticle in the tumor site. Furthermore, when the tumors were only irradiated with a NIR laser of 808 nm, the temperature (about 38 °C)

showed no obvious change, resulting in the continuous growth of tumors. However, when the mice were injected with the nanoparticle under a magnet and illuminated by laser in the tumor area, enhanced surface temperature (about 50 °C) was observed, leading to the complete ablation of tumors. The results demonstrated the capability of multifunctional nanoparticles as cancer theranostic agents. Liu and co-workers also prepared magnetic FeS nanoplates for simultaneous MR imaging and PTT (Figure 10b).³⁰ FeS nanoplates were synthesized according to a simple one-step method and showed high absorbance in the NIR region and strong superparamagnetism after functionalizing with PEG. In comparison with iron oxide nanoparticles or some clinical approved T2-contrast agents, FeS-PEG nanoplates exhibited a much higher transverse relaxivity, r_2 , of 209.8 mM⁻¹ S⁻¹. Moreover, upon intravenous injection, FeS-PEG nanoplates could accumulate efficiently in the tumor area and could also be eliminated from major organs of mice, leading to negligible toxicity towards the treated mice at a high concentration of 100 mg kg⁻¹. To conduct the PTT efficiency *in vivo*, mice were injected with FeS-PEG nanoplates at a dose of 20 mg kg⁻¹, followed by illumination with laser of 808 nm at the tumor site, which resulted in the complete elimination of tumors. These results exhibited the potential of using PEGylated FeS nanoplates as effective theranostic agents to treat cancer in clinic.

1.4 Carbon Dots (CDs)

In recent years, CDs, one class of fluorescent carbonaceous nanomaterials with size less than 10 nm, have attracted great attention in biomedical fields since their first preparation by Scrivens and co-workers in 2004 through electrophoretic refinement of single-wall carbon nanotubes.³¹⁻⁴¹ Herein, to best understand CDs, the synthetic methods, properties, and their applications in bioimaging and cancer treatment were summarized.

1.4.1 Synthetic Methods

Typically, CDs can be prepared through top-down and bottom-up approaches

Table 3. Summary of synthetic methods of CDs.

Synthetic route	Precursor	Synthetic method	Reference
Top-down	Graphite	Laser ablation, HNO ₃ treatment, ZnS doping	32
	Graphite rod	Electro-oxidization	33
	Lampblack	HNO ₃ treatment and NaBH ₄ reduction	34
	Candle soot	HNO ₃ reflux	35
	Single-/multi-walled carbon nanotubes	HNO ₃ /H ₂ SO ₄ reflux	36
Bottom-up	Citric acid and ethylenediamine	Hydrothermal treatment at 200 °C	37
	Glycerol	Pyrolysis	38
	Sucrose	H ₃ PO ₄ treatment at 60 °C	39
	Glucose	Alkali- or acid-assisted ultrasonication	40
	Milk	Microwave-assisted pyrolysis	41

(Table 3). Top-down method is to break down bulk carbon (graphite rod, carbon nanotubes, carbon black, and candle soot) by electrochemical oxidation, laser ablation, nitric acid/sulfuric acid oxidation, or arc discharge.³²⁻³⁶ For example, Liu and co-workers synthesized bright yellow CDs by treating graphite or single-walled/multi-walled carbon nanotubes with a mixed acid (nitric acid and sulfuric acid ratio of 1 to 3) under refluxing at 80 °C for 24 h, followed by purification through filtration and dialysis.³⁶ Bottom-up method is to use hydroxyl, carboxyl, and amine groups functionalized small molecules (e.g. citric acid, amino acid, ascorbic acid, or glycerol) or bio-products (e.g. bovine serum albumin, animal hair, plant extracts, or grass) as precursors through calcination, microwave-assisted pyrolysis, or hydrothermal techniques.³⁷⁻⁴¹ In He's work, CDs could be synthesized in one pot and large scale by treating milk at 175 °C for 25 min through a microwave-assisted method, followed by purification through centrifugation, dialysis, and filtration.⁴¹

1.4.2 The Properties of CDs

1.4.2.1 Composition

Carbon, hydrogen, and oxygen are three basic elements of CDs, with various ratios for different CDs. Moreover, heteroatoms can also be introduced to adjust the position of conduction band or valence band of CDs to endow supplemental functions.

For example, incorporating nitrogen element can increase the quantum yield of CDs and incorporating sulfur element can produce CDs with a wide band gap.^{37,42} In addition, CDs doped with phosphorus atom can exhibit green fluorescence and CDs doped with boron or chlorine atom can also be synthesized by varied raw materials or synthetic methods.^{43,44}

1.4.2.2 Fluorescence

Figure 11. (a, b) The preparation of PEG-passivated CDs and corresponding emission under excitation wavelengths from 400 to 694 nm.⁴⁶ Copyright 2006 American Chemical Society. (c, d) The synthetic route of multi-colored CDs from three isomers of phenylenediamines and corresponding emission under 365 nm excitation.⁴⁷ Copyright 2015 Wiley-VCH.

Due to the variety of CDs, the fluorescence wavelength can range from blue to NIR, which is mainly resulted from two reasons. One reason is the excitation-wavelength dependence that the emission peaks vary with the change of excitation wavelengths, which can be accounted for different emission traps on the surface of CDs or optical selection of distinct sized CDs.⁴⁵ In Sun's work, the prepared PEG-passivated CDs emitted blue, green, and red fluorescence by increasing the excitation wavelengths from 400 to 694 nm (Figures 11a and b).⁴⁶ The other

reason is the different synthetic and purification methods. For instance, by using three isomers of phenylenediamines (*o*-phenylenediamine, *m*-phenylenediamine, and *p*-phenylenediamine) as precursors, Lin and co-workers synthesized different CDs with bright and stable green, blue, and red luminescence through solvothermal methods (Figures 11c and d).⁴⁷

For fluorescent nanomaterials, brightness and photostability are two significant parameters to take into consideration. At first, CDs have a low quantum yield (QY) around 10%. Later, various methods, including inorganic salt doping, surface passivation, and element doping, are used to increase QY of CDs.^{48,49,37} For inorganic salt doping, Sun and co-workers doped CDs with nanosized semiconductors to prepare CDs. The QY was more than 50% or around 45% respectively when CDs were doped with ZnO or ZnS.⁴⁸ For surface passivation, Sun and co-workers used diamine-terminated PEG to passivate non-emissive CDs, resulting in a highest QY of 60% for CDs purified through an aqueous gel column.⁴⁹ For element doping, in Yang's work, nitrogen-doped CDs with a high QY of 80% was obtained by treating citric acid and ethylenediamine with a hydrothermal method, and this QY was similar to that of organic dyes.³⁷

Furthermore, CDs have good photostability, which make them advantageous than organic dyes and quantum dots. This is because organic dyes suffer from photobleaching and quantum dots have the drawback of photoblinking. In Mohapatra's work, CDs were synthesized from orange juice by hydrothermal method. When CDs were irradiated under 360 nm for 3 h, there was no meaningful decrease in fluorescence intensity.⁵⁰ In addition, Huo and co-workers used nitric acid to oxidize commercially activated carbon, followed by passivation with 4,7,10-trioxa-1,13-tridecanediamine to afford multicolor luminescent CDs. These CDs exhibited no obvious deduction in fluorescence intensity upon illumination under 365 nm for 10 h.⁵¹

1.4.2.3 Biocompatibility

Figure 12. (a) The relative body weight and (b) the hematoxylin and eosin (H&E) staining of major organs for the control group without CDs treatment and the CD-injected mice (20 mg kg^{-1}) at different time points over three months.³⁶ Copyright 2012 Wiley-VCH.

CDs possess great biocompatibility as well as low toxicity, which have been demonstrated both *in vitro* and *in vivo*. For example, Sun and co-workers synthesized CDs with good biocompatibility by using citric acid and polyene polyamine as precursors and a pyrolysis method. The CD-incubated mouse fibroblast L929 cells could reveal a cell viability of higher than 75% at a series of concentrations up to $500 \mu\text{g mL}^{-1}$ after 48 h when compared with the control group without CDs treatment.⁵² In Zhou's work, CDs were prepared through hydrothermal heating of milk, and they exhibited little toxicity to human brain glioma tumor U87 cells at a high dose of 1 mg mL^{-1} after 24 h.⁵³ Moreover, Liu and co-workers studied biodistribution and

toxicology of CDs in living mice. The results showed that CDs could be cleared through renal and fecal routes, and the mice injected with CDs at a concentration of 20 mg kg^{-1} revealed no obvious body weight change and histological abnormality or lesion over three months, demonstrating the potential application of CDs *in vivo* (Figure 12).³⁶

1.4.3 Biomedical Applications

By now, fluorescent CDs have been used widely in biomedical fields, including biosensors, bioimaging, and cancer therapy.^{54,55}

1.4.3.1 Biosensors

Figure 13. (a) Schematic illustration of CD-based nanoplateform for sensing of Hg^{2+} and biothiols.⁵⁶ Copyright 2012 Royal Society of Chemistry. (b) Design of CD-based sensor for detection of Fe^{3+} and dopamine.⁵⁷ Copyright 2013 Wiley-VCH. (c) Scheme of CD-based sensor for determination of K^+ .⁵⁸ Copyright 2012 Royal Society of Chemistry.

First, CDs can be utilized to fabricate fluorescence sensors. Qu and co-workers synthesized CDs from ethylenediaminetetraacetic acid and designed CD-based nanoplatfrom for sensitive and selective sensing of Hg^{2+} and biothiols (Figure 13a).⁵⁶ The mechanism was that CDs fluorescence could be quenched effectively by Hg^{2+} through electron transfer, while biothiols could recover the CDs fluorescence by removing Hg^{2+} from CDs surface. This method was economic and easy to operate, holding great potential for application in environmental and biological fields. Qu and co-workers also fabricated a label-free method based on CDs for detecting Fe^{3+} and dopamine sensitively and selectively (Figure 13b).⁵⁷ CDs were prepared from dopamine by hydrothermal route, with excitation wavelength-dependent emission from 380 to 525 nm. Due to the existence of catechol groups on the surface of CDs and the ability of Fe^{3+} to oxidize hydroquinone to quinone, Fe^{3+} could quench the fluorescence of CDs, with a detection limit as low as 0.32 μM . Moreover, owing to the competitive reaction of dopamine with Fe^{3+} , dopamine could recover the fluorescence of CDs, with a detection limit of 68 μM . Furthermore, this sensor could be successfully used in Fe^{3+} detection in real water samples and dopamine determination in human serum and urine samples. Qu and co-workers also developed a sensitive sensor for K^+ determination, based on 18-crown-6 ether-modified reduced graphene oxide and alkylammonium-functionalized CDs (Figure 13c).⁵⁸ Because of the strong binding between alkylammonium and 18-crown-6 ether, the fluorescence of CDs was quenched due to the effective FRET from CDs and reduced graphene oxide. By adding K^+ , the FRET process was inhibited owing to the specific binding between K^+ and 18-crown-6 ether. Therefore, the increased fluorescence could be used to determine K^+ with a detection limit of 10 μM and a detection range of 0.05 to 10 mM. Moreover, this sensor exhibited excellent selectivity to K^+ than other biological ions including Li^+ , Na^+ , Mg^{2+} , and Ca^{2+} .

In addition, CDs can also be used to design electrochemiluminescent (ECL) sensors. Qu and co-workers detected cancer cells with a CD-based ECL sensor, which was fabricated by sequential modifying glassy carbon electrode with graphene,

Figure 14. (a) Design of CD-based ECL sensor for cancer cell detection.⁵⁹ Copyright 2013 Royal Society of Chemistry. (b) Schematic illumination of CD-based nanoenzyme sensor for H_2O_2 sensing.⁶⁰ Copyright 2011 Royal Society of Chemistry.

$CDs@Ag$ nanocomposites, folic acid (FA), bovine serum albumin, and $S_2O_8^{2-}$ (Figure 14a).⁵⁹ Decorating graphene could increase electron transfer because of the large surface area and excellent conductivity, and the Ag shell on CDs could enhance the electron transfer between $CDs@Ag$ and graphene. Moreover, conjugating FA could facilitate the binding between cancer cells and electrode. Finally, this electrode was successfully used to sensitively and selectively determine cancer cells, with a detection limit of 10 cells per mL.

Moreover, CDs can be utilized as enzyme mimetics to develop biosensors. Huang and co-workers found CDs had peroxidase-like activity, which was used to oxidize 3,30,5,50-tetramethylbenzidine (TMB) in the presence of H_2O_2 to create a color change (Figure 14b).⁶⁰ This assay could detect H_2O_2 with a detection limit of $0.2 \mu M$ and a detection range of 0.0010 to 0.10 mM. Moreover, by combination with glucose oxidase, this colorimetric method could sense glucose with a detection limit of 0.4

mM and a detection range of 0.0010 to 0.50 mM, which could also be utilized in serum samples.

1.4.3.2 Bioimaging

Figure 15. (a) Fluorescence imaging of L929 cells without or with CDs incubation. The images were recorded by excitation with different wavelengths, 405, 488, and 543 nm.⁶¹ Copyright 2012 Royal Society of Chemistry. (b) Scheme of CD-based sensor for ratiometric detection of NO and corresponding fluorescence imaging in HeLa, L929, and Raw 264.7 cells.⁶² Copyright 2013 Royal Society of Chemistry.

As fluorescent nanomaterials, CDs have been used for bioimaging in living cells. For example, Liu and co-workers incubated L929 cells without or with CDs, followed by imaging under different excitation wavelengths, 405, 488, and 543 nm (Figure 15a).⁶¹ Compared with the little fluorescence for control cells without CDs incubation, the cells treated with CDs exhibited strong blue, green, and red emission. Wu and co-workers synthesized a CD-based nanoplatform for ratiometric sensing of NO in

Figure 16. (a) Time-dependent *in vivo* fluorescence imaging of HeLa tumor-bearing nude mice after intravenous injection of CDs. (b) The corresponding *ex vivo* fluorescence images (from top to bottom) and (c) fluorescence intensity of mice organs: heart, liver, spleen, lung, kidneys, tumor. (d) Time-dependent fluorescence intensity at the tumor site within 24 h.⁶³ Copyright 2015 Wiley-VCH. (e) Time-dependent *in vivo* fluorescence imaging of glioma-bearing nude mice after intravenous injection of CDs. (f) The distribution of CDs in the brain by three-dimensional reconstruction imaging after injection for 20 min. (g) *Ex vivo* imaging of CDs in the brain after injection for 90 min.⁶⁴ Copyright 2015 American Chemical Society.

living cells (Figure 15b).⁶² In this system, CDs were used as FRET donor and the

carrier for NO probe, which acted as FRET acceptor upon reaction with NO. By treating with NO, the fluorescence intensity at 535 nm increased while that at 455 nm decreased, resulting in a color change from blue to green. Therefore, this sensor was able to track exogenous NO in living human cervical carcinoma Hela cells, L929, and mouse macrophage Raw 264.7 cells, as well as endogenous NO in Raw 264.7 cells.

Moreover, CDs have also been used for bioimaging in living animals. Wang and co-workers studied the fluorescence imaging of CDs *in vivo* by using nude mice with HeLa tumor (Figures 16a-d).⁶³ Upon intravenous injection, CDs could accumulate in the tumor site through EPR effect, with fluorescence intensity increasing dramatically within 2 to 5 h, reaching a plateau within 6 to 9 h, decreasing gradually with increasing time. Moreover, upon injection of CDs for 24 h, the major organs of mice, including heart, liver, spleen, lung, kidneys, and tumor, were excised and imaged. The fluorescence intensity of each organs indicated that CDs dominantly accumulated in tumor, liver, lung, and kidneys other than spleen and heart. Sun and co-workers synthesized a new kind of CDs from *D*-glucose and *L*-aspartic acid with pyrolysis method and applied them in diagnosis of brain cancer (Figures 16e-g).⁶⁴ The *in vivo* fluorescence imaging of CDs was recorded over time by using mice with orthotopic C6 glioma upon tail vein injection. The results indicated that CDs could cross blood brain barrier and accumulate in the brain area after injection for 5 min. Moreover, the highest fluorescence intensity was detected at 15 min, and the steadily decreased fluorescence intensity proved the clearance of CDs over time. In addition, both the three dimensional reconstruction imaging upon injection for 20 min and *ex vivo* imaging of the brain upon injection for 90 min confirmed the targeting ability of CDs towards glioma area.

1.4.3.3 Cancer Therapy

In addition to biosensors and bioimaging, CDs can be not only utilized as the nanocarriers to deliver chemotherapeutic drugs, photosensitizers, and genes, but also selected as the photodynamic or photothermal therapeutic agents to treat cancer.

1.4.3.3.1 Carriers for Chemotherapeutic Drugs

Figure 17. (a) The preparation of DOX-loaded CD-based drug nanocarriers and the corresponding mechanism for real-time monitoring of drug release.⁶⁵ Copyright 2013 Wiley-VCH. (b) The synthesis of oxaliplatin prodrug-loaded CD-based drug nanocarriers and their application in theranostics *in vitro* and *in vivo*.⁵² Copyright 2014 Wiley-VCH.

First, CDs can serve as the carriers for chemotherapeutic drugs. Zheng and co-workers constructed a CD-based DOX delivery system relying on FRET (Figure 17a).⁶⁵ CDs were prepared by a modified electrochemical approach and used as both drug nanocarriers and FRET donor. The fluorescent DOX was loaded on the surface of CDs through electrostatic and π - π interactions and used as FRET acceptor. The real-time release of DOX could be monitored by the alteration of FRET signal at varied pH. Moreover, under 810 nm excitation, two-photon imaging could be achieved for this nanocarrier at tumor tissues of 65-300 μ m, proving the capability of CDs for tracking the drug release in deep tumor tissues. Sun and co-workers

developed a multifunctional CD-based theranostic agent by integrating the bioimaging characteristics of CDs and the therapeutic effect of oxaliplatin (Figure 17b).⁵² By loading oxaliplatin on the surface of CDs in its oxidized form, the prepared nanocarriers could be tracked by fluorescence imaging and exhibited efficient anticancer effects *in vitro* and *in vivo*. This design could hold great potential to design CD-based drug delivery platforms with excellent biocompatibility and negligible toxic side effects for cancer therapy.

1.4.3.3.2 Carriers for Photosensitizers

Figure 18. (a) The preparation of Ce6-loaded CDs for PDT *in vivo*.⁶⁶ Copyright 2012 Wiley-VCH. (b) The synthesis of ZnPc-loaded CDs for targeted PDT *in vivo*.⁶⁷ Copyright 2014 Wiley-VCH.

In addition, CDs can be utilized to deliver photosensitizers. Chen and co-workers

developed a CD-based theranostic agent by conjugating photosensitizer chlorin e6 (Ce6) on the surface of CDs (Figure 18a).⁶⁶ Taking advantages of excellent stability, good water solubility, noncytotoxicity, remarkable biocompatibility, increased photosensitizer fluorescence detection, and great photodynamic effects under laser illumination, the synthesized nanocarriers revealed enhanced PDT toward gastric cancer under guidance of fluorescence imaging *in vivo*. This design may hold great promise for treating gastric or other cancers in clinic in the foreseeable future. Kim and co-workers synthesized CDs by thermal decomposition of α -cyclodextrin and loaded PEG, FA, and photosensitizer zinc phthalocyanine (ZnPc) on the surface of CDs to fabricate a multifunctional nanoplatform for simultaneous bioimaging and targeted PDT (Figure 18b).⁶⁷ Under illumination, this nanocarrier revealed targeted PDT toward folate receptor-overexpressing cancer cells *in vitro* and *in vivo*. This design was promising to offer an efficient and facile method for enhanced PDT in future cancer therapy.

1.4.3.3.3 Carriers for Genes

Moreover, CDs can be applied for the delivery of genes. Liu and co-workers synthesized polyethylenimine (PEI) functionalized CDs from glycerol and branched PEI25k through microwave-assisted pyrolysis. CDs had enhanced fluorescence owing to the surface passivation of PEI, which could also serve as polyelectrolyte to condense DNA (Figure 19a).⁶⁸ Upon changing the pyrolysis time, CDs revealed low toxicity and high gene expression of plasmid DNA (pDNA) in human liver cancer HepG2 cells and fibroblast-like COS-7 cells. Moreover, fluorescence signals could also be detected inside cells under excitation with different wavelengths. These results showed the potential applications of CDs in bioimaging and gene delivery. Kim and co-workers assembled CDs, Au nanoparticles, and non-labeled pDNA by electrostatic interaction to prepare nanohybrids for efficient gene delivery (Figure 19b).⁶⁹ This complex could monitor the association of pDNA through the quenched fluorescence of CDs by Au nanoparticles. More importantly, under the condition with high salt concentration, the complex could dissociate to release pDNA, which accompanied

Figure 19. (a) Schematic illustration of the preparation of DNA-loaded CDs for gene delivery.⁶⁸ Copyright 2012 Elsevier. (b) Scheme of the synthesis of CD-PEI/Au-PEI/pDNA nanohybrids for gene delivery.⁶⁹ Copyright 2013 Elsevier.

with the recovery of CDs fluorescence owing to the increased distance between CDs and Au nanoparticles. Therefore, the enhanced fluorescence was used to monitor the dissociation of pDNA under intracellular condition. Moreover, this complex indicated the great potential of CDs/Au nanoparticles as carriers for gene delivery with low cytotoxicity and high efficiency.

1.4.3.3.4 Therapeutic Agents

Furthermore, CDs can be used as photodynamic or photothermal therapeutic agents. Wang and co-workers treated a polythiophene derivative with hydrothermal method to prepare CDs with high photostability and pH stability, excellent biocompatibility, and high singlet oxygen generation yield of above 1.3 (Figure 20).⁷⁰ When CDs were subcutaneously injected into the back of a nude mouse, intense

Figure 20. (a) The synthetic route of polythiophene derivative to prepare CDs. (b) Bright-field and (c) red-fluorescence image of CDs in living mice after subcutaneous injection in different sites. (d) The relative tumor volume change and (e) photographs of the mice on day 1, 9, 17, and 25 after different treatments. C1: CDs without light; C2: saline with light (400-800 nm, 80 mW cm^{-2} , 10 min); PDT: CDs with light (400-800 nm, 80 mW cm^{-2} , 10 min).⁷⁰ Copyright 2014 Nature Publishing Group.

fluorescence could be observed in the injection area, with a signal-to-noise ratio as high as 229.5. Moreover, when the mice with human breast MDA-MB-231 tumor were intratumorally injected with CDs and irradiated with white light, the tumor could be destroyed after 17 days, with no regrowth over 50 days. However, the tumor couldn't be inhibited if the mice were only treated with CDs or light irradiation. This

Figure 21. (a) The synthetic route of CDs. (b) The irradiation time-dependent temperature change and IR thermal imaging at the mice tumor area after intravenous injection of CDs and laser irradiation (671 nm, 2 W cm⁻²). (c) Photographs of the mice on day 0, 6, and 16, and relative tumor volume change after different treatments. C1: CDs without laser; C2: saline with laser (671 nm, 2 W cm⁻², 10 min); PTT: CDs with laser (671 nm, 2 W cm⁻², 10 min).⁶³ Copyright 2015 Wiley-VCH.

work may help to develop new photodynamic therapeutic agents with superior performance based on CDs. Wang and co-workers synthesized novel red-emissive CDs by carbonization of another polythiophene derivative for fluorescence and photoacoustic imaging guided PTT in living mice (Figure 21).⁶³ The prepared CDs had wide absorption in the wavelength range of 400 to 750 nm, fluorescence emission under excitation wavelengths of 350 to 600 nm, high photothermal conversion efficiency of 38.5% and photoacoustic signal under illumination with NIR light. When the mice with Hela tumor were injected intravenously with CDs and irradiated by laser, the temperature could reach 50.4 °C within 3 min and further increase to above 60 °C within 10 min at the tumor site, which led to efficient PTT as irreversible damage to cells and tissues could be caused with temperatures above 50 °C.

Figure 22. (a) The synthetic route of CDs. (b) IR thermal imaging and the irradiation time-dependent temperature change at the mice tumor area after intravenous injection of CDs and laser irradiation. (c) The relative tumor volume change and (d) photographs of the mice on day 0 and 14 after different treatments. C1: CDs without laser; C2: saline with laser (635 nm, 2 W cm⁻², 10 min); PDT: CDs with laser (635 nm, 0.1 W cm⁻², 10 min); PDT/PTT: CDs with laser (635 nm, 2 W cm⁻², 10 min).⁷¹ Copyright 2016 Wiley-VCH.

Therefore, the tumor continued to grow upon the only treatment with CDs or laser illumination, while it was greatly suppressed upon the treatment with CDs and laser illumination. The results confirmed that CDs could serve as potential photothermal theranostic agents. Wang and co-workers also prepared another multifunctional CDs, capable of simultaneous PDT and PTT, by carbonization of polythiophene benzoic acid (Figure 22).⁷¹ The synthesized CDs had broad absorption in the wavelength range of 400 to 700 nm and showed fluorescence peaks from 640 to 680 nm with the excitation wavelengths increasing from 380 to 620 nm. More importantly, CDs

exhibited a singlet oxygen generating efficiency of 27% and a photothermal conversion efficiency of 36.2%, leading to simultaneous PDT and PTT. *In vivo* experiments showed that the temperature at the tumor site could be above 60 °C within 10 min for the PDT/PTT group treated with CDs and laser irradiation (2 W cm⁻²), which were much higher than the temperature for other groups treated with CDs, laser irradiation (2 W cm⁻²), or CDs and laser irradiation (0.1 W cm⁻²). Therefore, complete tumor ablation could be achieved by simultaneous PDT and PTT. To sum up, these studies have significantly broadened the application of CDs in biomedicine.

1.5 Research Objectives

Benefiting from the unique properties including simple synthesis, excitation wavelength-dependent fluorescence, excellent biocompatibility, and facile surface modification, fluorescent CDs have exhibited enormous potentials in biomedical fields, such as biosensors, bioimaging, and cancer therapy. In particular, CDs can be widely utilized as nanocarriers for chemotherapeutic drugs, photosensitizers, or genes, as well as photodynamic or photothermal therapeutic agents to treat cancer. However, these CDs usually possess insensitive surface properties, such as PEGylated, negatively or positively charged, or active targeting ligands attached on the surface, which cannot satisfy the complicated tumor microenvironment. On one hand, PEGylated or negatively charged CDs can extend blood circulation time because of PEG stealthy layer or effective electrostatic repulsion to withstand protein adsorption, but they exhibit decreased cellular uptake due to PEGylation or electrostatic repulsion with negatively charged cell membrane, while positively charged CDs can afford effective internalization by interaction with negatively charged cell membrane and promote endosome escape to avoid drug degradation in lysosomes through “proton sponge” effect, but they are rapidly cleared from blood circulation owing to strong nonspecific interactions with serum components. On the other hand, CDs with active targeting ligands attached on the surface can be uptaken effectively by receptor-overexpressing cancer cells through specific ligand-receptor interaction, but they have short circulation half-life in blood due to the direct or through-opsonin

recognition of targeting ligands by reticular endothelial system and cause serious side effects by interaction with receptors expressed on the cell membrane of normal cells. Therefore, CDs with single surface properties can result in limited therapeutic efficacy for cancer treatment.

To solve above-mentioned problems, in this thesis, we have focused on the development of novel CD-based nanocarriers, which are responsive to mildly acidic tumor extracellular microenvironment to alter the surface characteristics for enhancing cancer therapeutic efficiency. In chapter 2, charge-convertible CDs were developed, which were negatively charged and PEGylated in normal physiological condition and reversed to be positively charged in acidic tumor extracellular microenvironment. This design could endow CDs with prolonged circulation time, facilitated cellular internalization, and promoted endosome escape, resulting in enhanced cancer therapeutic efficiency *in vivo* in comparison to the noncharge-convertible CDs. In chapter 3, tumor-triggered active-targeting CDs were designed, which were PEGylated at normal physiological pH and exposed targeting ligands at tumor extracellular pH for increased cellular internalization. This design could surmount the disadvantages of directly loading targeting ligands on the surface of CDs, including early clearance from blood circulation and potential side effects, leading to better therapeutic efficiency against receptor-overexpressing cancer cells at tumor extracellular pH than that in physiological condition or toward receptor-lowexpressing cancer cells.

Moreover, anticancer prodrugs can increase targeting capability and enhance therapeutic efficacy of anticancer agents owing to the pharmacological inactivation and the ability to metabolize into toxic forms under specific intracellular microenvironments, which are also utilized in above designs. So far, small-molecule organic dyes or single-emission intensity variability-based methods have been proposed for illustrating the activation mechanism of anticancer prodrugs. However, these approaches often suffer from poor photostability and chemical stability or unavoidable influence from uncontrolled microenvironment changes in living samples.

To overcome these limitations, in chapter 4, FRET-based CDs were prepared, which showed recovered blue fluorescence and unaltered green or red fluorescence under reductive conditions, providing a real-time ratiometric platform to monitor the activation of anticancer prodrug in living cells.

To sum up, the objectives of this thesis are to develop novel CDs for enhanced theranostics by surmounting the disadvantages of insensitive surface properties through tumor extracellular microenvironment triggered charge-conversion or active-targeting and to propose a CD-based ratiometric fluorescent method for illustrating the intracellular activation mechanism of anticancer prodrug. Thus, these designs can promote the further anticancer applications of CDs.

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Chapter 2

Charge-Convertible Carbon Dots for Imaging-Guided Drug Delivery with Enhanced *In Vivo* Cancer Therapeutic Efficiency

2.1 Introduction

In the past decade, thanks to rapid development of nanotechnology, various nanoparticle-based theranostic agents have been achieved, for example, quantum dots,¹ iron oxide,² silica nanoparticles,³ and upconversion nanoparticles.⁴ However, these therapeutic nanoparticles often have the problems of low water solubility, poor biocompatibility, or non-biodegradation.⁵ Taking advantages of promising properties of CDs, including excitation-dependent emission, high water solubility, excellent photostability, easy surface functionalization, good biocompatibility, and low toxicity,⁶⁻⁸ and high specificity, great contrast, and excellent sensitivity of fluorescent imaging, CDs have been utilized as imaging-guided drug nanocarriers for the delivery of chemotherapeutic drugs, photosensitizers, and therapeutic genes.⁹⁻¹¹

Up to now, CD-based drug nanocarriers possessing single surface property (PEGylated, negatively or positively charged) would influence their therapeutic efficiency for potential applications. The PEGylated or negatively charged drug carriers can extend blood circulation time, because PEGylation serves as a stealthy layer to prevent the drug carriers from rapid clearance by the immune system and negatively charged property can withstand protein adsorption originated from electrostatic repulsion.^{12,13} However, PEGylation can also impede the uptake of drug carriers by cancer cells and negatively charged drug carriers cannot easily enter into cancer cells due to electrostatic repulsion with negatively charged cell membrane, resulting in decreased therapeutic efficiency.^{14,15} On the other hand, positively charged drug carriers can facilitate the interaction with negatively charged cell membrane to

afford effective internalization by cancer cells,¹⁶ as well as promote the escape of drug carriers from endosome *via* “proton sponge” effect to avoid the drug degradation in lysosomes.¹⁷ Nevertheless, positively charged drug carriers have strong nonspecific interactions with serum components so that the carriers can be phagocytized by reticular endothelial system easily, leading to rapid clearance from blood circulation.¹⁸ In addition, positively charged drug carriers may also be endocytosed by normal cells through the interaction with negatively charged cell membrane to cause undesirable side effects.

To solve abovementioned problems, we developed a pH-responsive charge-convertible CD-based drug nanocarrier, which was PEGylated with negative charge at normal physiological condition and could reverse to be positively charged at tumor extracellular microenvironment.^{19,20} It's well known that the pH at normal physiological environment is 7.4, however, due to the enhanced glycolysis and plasma membrane proton-pump activity of tumor cells, more lactic acid is produced and leached out, inducing a pH decrease (pH 6.8) in the neighborhood of cancer cells (Warburg effect).²¹ The charge-convertible character, endowed by the re-exposure of amino groups after hydrolysis of dimethylmaleic acid (DMMA) and PEG functionalized poly(allyamine) (PEG-(PAH/DMMA)) at tumor extracellular condition, makes the best use of the pH difference between tumor extracellular microenvironment and normal physiological condition for controlled drug release. This CDs-Pt(IV)@PEG-(PAH/DMMA) carrier was fabricated through complexing PEG-(PAH/DMMA) with cisplatin(IV) prodrug covalently functionalized CDs (CDs-Pt(IV)) by electrostatic interaction (Figure 1a). The drug nanocarrier had several attributes, including (1) prolonged circulation time with negative charge/PEGylation in blood,²² (2) accumulation at the tumor site through EPR effect,²³ (3) enhanced tumor cell internalization due to the charge-convertible property in tumor extracellular microenvironment when compared to the nanocarrier without charge-convertible property, (4) less side effects in normal physiological condition because of disabling the charge-convertible property, (5) facilitated endosome escape by “proton sponge”

Figure 1. (a) Schematic illustration for the preparation of charge-convertible CD-based drug nanocarrier CDs-Pt(IV)@PEG-(PAH/DMMA). pH_e means tumor extracellular pH. (b) Schematic illustration for the drug delivery process of CDs-Pt(IV)@PEG-(PAH/DMMA): (1) prolonged circulation time with negative charge/PEGylation in blood, (2) accumulation at the tumor site through EPR effect, (3) responsiveness to tumor extracellular pH, (4) effective uptake by cancer cells, (5) facilitated endosome escape by “proton sponge” effect and controlled cisplatin release from cisplatin(IV) prodrug under reductive cytosol, and (6) cisplatin binding with DNA to exhibit cytotoxicity.

effect, and (6) controlled release of cytotoxic anticancer drug cisplatin from cisplatin(IV) prodrug under reductive cytosol of cancer cells (Figure 1b).²⁴ Thus, this

CD-based drug nanocarrier exhibits enhanced tumor-inhibition efficacy as well as low toxic side effects *in vitro* and *in vivo*, and shows significant application potential in the cancer therapy.

1.2 Materials and Methods

Materials. Carboxylic acid functionalized methoxyl polyethylene glycol (mPEG-COOH, 5 kDa), citric acid, dimethylmaleic anhydride, diethylenetriamine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), hydrogen peroxide (30% wt%), *N*-hydroxysuccinimide (NHS), poly(allylamine hydrochloride) (PAH·HCl), *cis*-[PtCl₂(NH₃)₂], succinic anhydride, and triethylamine were obtained from Sigma-Aldrich without further purifications. The dialysis membranes with molecular weight cut-off (MWCO) of 1, 3.5, and 12 kDa, reagents for cell culture, Roswell Park Memorial Institute (RPMI) 1640 medium, and fetal calf serum (FBS) were purchased from Thermo Fisher Scientific Pte Ltd.

Characterization. Nuclear magnetic resonance (NMR) spectra were performed using Bruker AV 300 NMR system, and chemical shifts (δ , ppm) were recorded by using internal reference tetramethylsilane (TMS). Mass spectra were obtained by a Thermo LCQ Deca XP MAX (ESI measurement). Transmission electron microscopy (TEM) images were obtained at 100 kV with an FEI EM208S Transmission Electron Microscope (Philips). Powder X-ray diffraction (XRD) patterns were carried out on a Philips X'Pert powder X-ray diffractometer. Fourier transformed infrared (FTIR) spectra were measured on a Shimadzu IRPrestige-21 FTIR spectrometer. UV-vis absorption spectra were recorded with a Shimadzu UV/vis/NIR spectrometer. Fluorescence spectra were carried out on a Shimadzu RF5301PC spectrometer. Platinum loading percentage was determined by an Agilent 7700 inductively coupled plasma mass spectrometer (ICP-MS) or inductively coupled plasma optical emission spectrometer (ICP-OES). Dynamic light scattering (DLS) and zeta potential measurements were collected by a Malvern equipment. Flow cytometry was carried out using a BD LSR II flow cytometer. The absorbance for

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was measured with a Tecan's Infinite M200 microplate reader at a wavelength of 490 nm.

Synthesis of CDs. Citric acid (2.1 g) and diethylenetriamine (3.5 g) were reacted in nitrogen atmosphere at 170 °C for 3 h. The mixture was cooled down to room temperature before adding a certain amount of water. The solution was dialyzed against water for 48 h by using a dialysis membrane with MWCO of 3.5 kDa followed by freeze-drying.

Synthesis of *c,t,c*-[PtCl₂(OH)₂(NH₃)₂] (Figure 2). *cis*-[PtCl₂(NH₃)₂] (300.0 mg) was suspended in water (7.5 mL), and then hydrogen peroxide (10.5 mL, 30 wt%) was added dropwise at 50 °C. After stirring for 2 h, the mixture was cooled down to room temperature. The solvent was reduced to about 2 mL by rotary evaporation, and the resulting solution was kept at 0 °C overnight. The pale yellow crystals were collected by filtration and washed with cold water and cold diethyl ether, and then dried in vacuo. Yield: 53%.

Figure 2. Synthetic route of cisplatin(IV) prodrug.

Synthesis of *c,c,t*-[PtCl₂(OH)(NH₃)₂(O₂CCH₂CH₂CO₂H)] (Cisplatin(IV) Prodrug, Figure 2). *c,t,c*-[PtCl₂(OH)₂(NH₃)₂] (120.0 mg) and succinic anhydride (36.0 mg) were dissolved in dimethyl sulfoxide (DMSO, 2.0 mL), and the solution was stirred at room temperature for 24 h. The mixture was lyophilized and the obtained solid was washed by cold acetone and diethyl ether to afford *c,c,t*-[PtCl₂(OH)(NH₃)₂(O₂CCH₂CH₂CO₂H)]. Yield: 62%. ¹H NMR (DMSO-*d*₆): 5.91 (m, 6H, NH₃), 2.39 (m, 4H, CH₂). ESI-MS: calcd: 434.13; found 435.01 [M+H]⁺.

PAH·HCl Deprotonation (Figure 3). In H₂O (5 mL), PAH·HCl (374.0 mg) and

sodium hydroxide (NaOH, 200.0 mg) were added. The mixture was dialyzed against water for 24 h by using a dialysis membrane with MWCO of 3.5 kDa. After being lyophilized, the deprotonated PAH was stored at $-4\text{ }^{\circ}\text{C}$ for further use.

Figure 3. Synthetic routes of PAH, PEG-PAH, PEG-(PAH/DMMA), and PEG-(PAH/SA).

Synthesis of PEG-PAH (Figure 3). In DMSO (5 mL), PAH (22.8 mg), mPEG-COOH (200.0 mg, 5 kDa), EDC·HCl (20.0 mg), and triethylamine (15.0 μL) were added. After reacting at room temperature under nitrogen atmosphere for 24 h, the mixture was dialyzed against water for 48 h by using a dialysis membrane with MWCO of 12 kDa. After being lyophilized, the obtained polymer PEG-PAH was stored at $-20\text{ }^{\circ}\text{C}$ for further use. The chemical structure of PEG-PAH was confirmed by ^1H NMR spectrum (Figure 4).

Synthesis of PEG-(PAH/DMMA) or PEG-(PAH/SA) (Figure 3). In DMSO (5 mL), PEG-PAH (100.0 mg), dimethylmaleic anhydride (45.5 mg) or succinic anhydride (36.1 mg) and triethylamine (130.0 μL) were added. After reacting at room temperature under nitrogen atmosphere for 24 h, the mixture was dialyzed against water (pH = 8-9 adjusted by NaOH) for 48 h by using a dialysis membrane with MWCO of 3.5 kDa. After being lyophilized, the obtained polymer

Figure 4. ^1H NMR (300 MHz, D_2O) spectrum of PEG-PAH.

PEG-(PAH/DMMA) or PEG-(PAH/SA) was stored at $-20\text{ }^\circ\text{C}$ for further use. The chemical structure of PEG-(PAH/DMMA) and PEG-(PAH/SA) was confirmed by ^1H NMR spectra (Figure 5).

Loading Cisplatin(IV) Prodrug on CDs. Cisplatin(IV) prodrug (8.7 mg), NHS (2.3 mg), and EDC·HCl (3.8 mg) were stirred in water at room temperature for 30 min. Then, CDs (40.0 mg) were added to the above solution, and the obtained mixture was stirred for another 24 h. The mixture was dialyzed against water for 48 h by using a dialysis membrane with MWCO of 1 kDa to remove unreacted compounds. After being lyophilized, cisplatin(IV) prodrug loaded CDs (CDs-Pt(IV)) were stored at room temperature for further use. The loading percentage of platinum was 6.7%, measured by ICP-MS.

Complexing PEG-(PAH/DMMA) or PEG-(PAH/SA) with CDs-Pt(IV). CDs-Pt(IV) (1.0 mL , 1 mg mL^{-1} in water) was added dropwise to PEG-(PAH/DMMA) or PEG-(PAH/SA) (2.0 mL , 1 mg mL^{-1} in water). The solution was stirred at room

Figure 5. ^1H NMR (300 MHz, D_2O) spectra of (a) PEG-(PAH/DMMA) and (b) PEG-(PAH/SA).

temperature overnight before freeze-drying, and the obtained PEG-(PAH/DMMA) or

PEG-(PAH/SA) complexed CDs-Pt(IV) (CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA)) was stored at -20 °C for further use.

Quantum Yield (QY) Measurements. The QYs of CDs and CDs-Pt(IV) were determined by using quinine sulfate as a reference (54%, 0.1 M H₂SO₄ as solvent). The formula for calculating QY is:

$$QY_x = QY_{ref} (I_x / I_{ref}) (\eta_x^2 / \eta_{ref}^2) (A_{ref} / A_x)$$

where *x* represents the sample, *ref* represents the standard compound quinine sulfate, *I* means integrated fluorescence intensity, *η* means refractive index for the solvent, and *A* indicates the absorbance at the excitation wavelength (less than 0.1 to decrease reabsorption effects).²⁵

***In Vitro* Drug Release from CDs-Pt(IV).** CDs-Pt(IV) (8.0 mg) was dissolved in phosphate buffered saline (PBS, 1.0 mL, 10 mM, pH 7.4) containing 0 or 10 mM GSH, and transferred into a dialysis tube (MWCO 1 kDa), followed by dialysis against PBS buffer (69 mL, containing 0 or 10 mM GSH) at 37 °C in the dark. At each designated time points, a certain volume of the release media was withdrawn and the same amount of new buffer solution was added to the original release media. The collected release media was used for ICP-OES measurements to determine the amount of released platinum.

Stability of CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA) Measurements. CDs-Pt(IV)@PEG-(PAH/DMMA) (0.5 mL, 1.0 mg mL⁻¹) or CDs-Pt(IV)@PEG-(PAH/SA) (0.5 mL, 1.0 mg mL⁻¹) was incubated with PBS (pH 7.4, 0.5 mL), FBS (0.5 mL) or RPMI 1640 medium (0.5 mL). At designated time intervals, the solution of CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA) was used for DLS measurements.

Zeta Potential Measurements. CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA) (1.0 mg mL⁻¹) was incubated in PBS (10 mM, pH 6.8 or 7.4) at 37 °C under constant stirring. At designated time intervals, a certain volume of

solution was withdrawn and used for zeta potential measurements.

Confocal Laser Scanning Microscopy (CLSM). The human ovarian carcinoma A2780 cells (ECACC) were cultured in RPMI 1640 medium with supplement of 10% FBS at 37 °C in a humidified incubator with 5% CO₂. A2780 cells at a density of 1×10^5 were cultured using a μ -dish with diameter of 35 mm and a plastic-bottom (ibidi GmbH, Germany) at 37 °C for 24 h. Then, the medium was replaced by RPMI 1640 medium with pH 6.8 or 7.4 containing CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA) before the maintenance at 37 °C for another 2 h. After being rinsed by PBS twice, A2780 cells were used for CLSM imaging.

Flow Cytometry Analysis. A2780 cells were cultured in 24-well plates with density of 1×10^5 per well at 37 °C in a humidified incubator with 5% CO₂ for 24 h. Then, the medium was replaced by RPMI 1640 medium with pH 6.8 or 7.4 containing CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA), followed by the maintenance at 37 °C for another 2 h. After being washed and trypsinized, A2780 cells were analyzed using flow cytometer in Pacific Blue channel with an excitation wavelength of 405 nm and an emission wavelength range of 450 ± 50 nm.

In Vitro Cytotoxicity. The MTT assay was used to evaluate the cytotoxicity of CDs-Pt(IV)@PEG-(PAH/DMMA) and CDs-Pt(IV)@PEG-(PAH/SA). A2780 cells were cultured in 96-well plates with density of 1×10^4 cells per well within RPMI 1640 medium (100 μ L) at 37°C in a humidified incubator with 5% CO₂ for 24 h. Then, the medium was replaced by RPMI 1640 medium with pH 6.8 or 7.4 containing CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA) at varied platinum concentrations before the maintenance at 37 °C for another 2 h. After being replaced with fresh complete RPMI 1640 medium at pH 7.4, A2780 cells were incubated for another 72 h at 37 °C. Then, A2780 cells were rinsed using PBS, followed by being incubated with 20% MTT in RPMI 1640 medium at 37 °C for 5 h. After discarding the medium, DMSO (100 μ L) was used to lyse A2780 cells. The absorbance at 490 nm was recorded by Tecan's Infinite M200 microplate reader. Every experiment

was carried out thrice, and the cell viability was calculated by referring to the control group without the sample treatment.

Human cervix carcinoma Hela cells were seeded in 96-well plate and then cultured in 5% CO₂ for 12 h at 37 °C to let the cells to attach the bottom of the wells. The ultrasonically treated CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA) were diluted into concentrations of 0.18, 0.36, 0.72, 1.43, 2.85, 5.72, and 11.44 μM, and then were added into the wells containing culture to replace the original culture medium for another 24 h incubation at 37 °C. Then the as-prepared MTT solution (20 μL, 5 mg mL⁻¹) was added to each well. The plate was then incubated at 37 °C for another 4 h. At last, DMSO (150 μL) was added to each well and shaken for 10 min to blend DMSO and the formazan completely. The absorbance at 490 nm was recorded by a microplate reader.

In Vivo Toxicity. Female Kunming mice (20-25 g) were purchased from Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Harbin, China), and all the mouse experiments were performed in compliance with the criterions of The National Regulation of China. Firstly, the tumors were built up by subcutaneous injection of U14 cells (murine cervix carcinoma cell lines) into the left axilla of each female mouse. After growing for one week, the tumors reached the size of about 6-8 mm. Then, the tumor-bearing mice were randomly divided into three groups ($n = 5$, each group) and were treated by intravenous injection with saline, CDs-Pt(IV)@PEG-(PAH/DMMA), and CDs-Pt(IV)@PEG-(PAH/SA), respectively. The injected amount for CDs-Pt(IV)@PEG-(PAH/DMMA) and CDs-Pt(IV)-PEG-(PAH/SA) was 100 μL saline with platinum dose of 1.5 mg/kg body weight, and the dates of treatment were 0, 4, and 10 days. The tumor size and body weights were measured every day after the treatment.

Histology Examination. Histology analysis was carried out at the 14th day after the treatment. Typical heart, liver, spleen, lung, and kidney tissues of the mice in the control group and the best treatment group were isolated. Then, the organs were

dehydrated using buffered formalin, ethanol with different concentrations, and xylene. After that, they were embedded in liquid paraffin. The sliced organs and tumor tissues (3-5 mm) were stained with H&E, and examined on a Leica SP8 microscope.

2.3 Results and Discussion

2.3.1 Preparation and Characterization of CDs

CDs were prepared through a thermal pyrolysis method with citric acid as carbon source and diethylenetriamine as surface passivation agent, and isolated by freeze-drying after dialysis against water to eliminate small molecules.²⁵ TEM characterization (Figure 6a) showed that the size of CDs was distributed in the range from 5 to 8 nm, with an average size of 7.0 nm. Powder XRD pattern (Figure 6b) revealed that three diffraction peaks at 2θ of 22.1° , 29.7° , and 41.3° corresponding to d spacing of 0.40, 0.30, and 0.22 nm respectively were ascribed to disordered carbon atoms and the (002) and (100) graphite lattice. FTIR was measured to indicate the functional groups on the surface of CDs (Figure 6c). The broad vibration bands in the range of $3000\text{--}3500\text{ cm}^{-1}$ were from O-H and N-H stretching vibration, and the vibration bands at 1569 and 1354 cm^{-1} were attributed to bending vibration of N-H and stretching vibration of C-N respectively, indicating the existence of many amino groups on the surface of CDs. The presence of amino groups on the surface of CDs was also confirmed by the appearance of Ruhemann's purple when the CDs were reacted with ninhydrin, and the amount of amino groups was determined to be $1.27\text{ }\mu\text{mol mg}^{-1}$ by measuring the absorbance of Ruhemann's purple at 570 nm with alanine as the reference.

In the UV-vis absorption spectrum of CDs (Figure 6d), the band at 240 nm was originated from the $\pi\text{--}\pi^*$ transition of aromatic ring structure, which was formed after dehydration between the hydroxyl, carboxyl, and amino groups from citric acid and diethylenetriamine and subsequent carbonization process during the preparation of CDs,⁸ and the peak at 343 nm was assigned to the $n\text{--}\pi^*$ transition of C=O group. The fluorescence spectra of CDs (Figure 6e) were typical excitation wavelength-dependent.

Figure 6. (a) TEM image, (b) powder XRD pattern, (c) FTIR spectrum, (d) UV-vis absorption spectrum, and (e) excitation wavelength-dependent fluorescence spectra of CDs. (f) The cell viability of A2780 cells treated with CDs having concentrations from 3.125 to 200 $\mu\text{g mL}^{-1}$. (g) Multicolor fluorescence images of A2780 cells treated with CDs under excitation with 350, 480, and 545 nm, respectively. Scale bar = 10 μm .

Upon increasing the excitation wavelengths from 360 to 480 nm, the emission peak redshifted from 458 to 537 nm. Such shift was resulted from different energy levels

associated with various surface states formed by distinct functional groups on the surface of CDs.²⁶ The fluorescence quantum yield of CDs was calculated to be 25.5% under an excitation wavelength of 360 nm by using quinine sulfate as the reference.

Before CDs were used as drug nanocarriers, their biocompatibility was tested toward A2780 cells by the MTT assay. CDs showed almost no toxicity to A2780 cells with concentrations from 3.125 to 200 $\mu\text{g mL}^{-1}$ (Figure 6f). The results indicate that the prepared CDs have a very low toxicity, making them suitable for the utilization as drug nanocarriers. To investigate the bioimaging characteristics of CDs, A2780 cells were cultured with a medium containing CDs. From the fluorescence imaging results shown in Figure 6g, A2780 cells displayed multicolor (blue, green, and red) imaging under excitation with 350, 480, and 545 nm, respectively, and CDs were mainly internalized in the cytoplasm of A2780 cells. The fluorescence imaging results reveal that CDs can be used as imaging-guided drug nanocarriers.

2.3.2 Preparation and Characterization of CDs-Pt(IV)@PEG-(PAH/DMMA) and CDs-Pt(IV)@PEG-(PAH/SA)

In this work, cisplatin(IV) prodrug was chosen as the model drug, which had low toxic side effects due to its low therapeutic potency. Importantly, the cisplatin(IV) prodrug can be activated to cytotoxic cisplatin specifically in reductive cytosol of cancer cells.²⁴ The cisplatin(IV) prodrug was synthesized through the oxidation by hydrogen peroxide in order to add two axial hydroxyl groups to cisplatin first, and the obtained intermediate was then reacted with succinic anhydride to provide carboxylic acid group for further functionalization. CDs were conjugated with cisplatin(IV) prodrug by a typical EDC/NHS amidation reaction between the amino groups on CDs and the carboxylic acid group of cisplatin(IV) prodrug. The platinum loading of the obtained CDs-Pt(IV) was 6.7 wt%, measured by ICP-MS. After loading cisplatin(IV) prodrug on CDs, the relative ratio between the vibration intensity of C=O and that of N-H in the FTIR spectrum of CDs-Pt(IV) (Figure 7a) increased as compared with the same relative ratio in CDs (Figure 6c). Zeta potential measurements indicated that

Figure 7. (a) FTIR spectrum, (b) UV-vis absorption spectrum, and (c) excitation wavelength-dependent fluorescence spectra of CDs-Pt(IV). (d) Platinum release profile of CDs-Pt(IV) in PBS buffer (10 mM, pH 7.4) with or without GSH (10 mM) at 37 °C for 72 h.

CDs-Pt(IV) was positively charged with a zeta potential of 24.73 mV, which was lower than that (35.71 mV) of CDs, demonstrating that some positively charged amino groups on CDs were coupled with cisplatin(IV) prodrug in CDs-Pt(IV). The UV-vis absorption spectrum (Figure 7b) and fluorescence spectrum (Figure 7c) of CDs-Pt(IV) were similar to those of CDs, and excitation wavelength-dependent emission behavior was also observed from CDs-Pt(IV). The fluorescence QY of CDs-Pt(IV) was calculated to be 17.9%, revealing the feasibility of CDs-Pt(IV) for bioimaging application. In order to investigate the *in vitro* cisplatin release behavior from CDs-Pt(IV), CDs-Pt(IV) was dialyzed without or with GSH (10 mM, the concentration inside cancer cells) at pH 7.4 for 72 h. As shown in Figure 7d, only slight amount of cisplatin(IV) prodrug was released from CDs-Pt(IV) without GSH, which was presumably due to the hydrolysis of the linkage between cisplatin(IV) prodrug and CDs.²⁷ Upon incubation with 10 mM GSH, almost 86% of cisplatin was

released after 72 h, which confirmed the controlled release of cisplatin from CDs-Pt(IV) under reductive condition.

Figure 8. Mechanism for hydrolysis of dimethylmaleic amide at pH 6.8.

As the dimethylmaleic amides undergo rapid hydrolysis under acidic conditions due to intramolecular catalysis benefited from the nearby carboxylic acid group, which is maintained close to the amide bond by the rigid conformation of the neighboring double bond (Figure 8),²⁸ therefore, in order to provide CDs-Pt(IV) with charge-convertible property in mildly acidic tumor extracellular microenvironment, PEG (9.8% of amino group confirmed by NMR) and dimethylmaleic acid (DMMA, 79.3% of amino group) functionalized deprotonated PAH (PEG-(PAH/DMMA)) was synthesized (Figures 3 and 5a), and then the negatively charged PEG-(PAH/DMMA) was complexed with positively charged CDs-Pt(IV) to afford CDs-Pt(IV)@PEG-(PAH/DMMA) by electrostatic interaction. The TEM image of CDs-Pt(IV)@PEG-(PAH/DMMA) showed a spherical morphology with an average size of 125 ± 42 nm ($n = 40$), which was smaller than the DLS result due to the hydrodynamic radius in buffer solution (Figures 9a and b). In addition, CDs-Pt(IV)@PEG-(PAH/DMMA) showed strong C-O stretching vibration of PEG units in the FTIR spectrum when compared with the vibration behavior of CDs-Pt(IV) (Figure 9c). Moreover, CDs-Pt(IV)@PEG-(PAH/DMMA) exhibited excellent stability in various physiological solutions, including PBS (pH 7.4), FBS, and RPMI

Figure 9. (a) TEM, (b) DLS result, (c) FTIR spectrum, (g) changes in the particle size upon incubation with PBS (pH 7.4), FBS, and RPMI 1640 medium for 24 h, and (h) zeta potential changes when incubating at pH 7.4 and 6.8 at 37 °C for 4 h for CDs-Pt(IV)@PEG-(PAH/DMMA).

1640 medium (Figure 9d).

Under mildly acidic tumor extracellular microenvironment with pH 6.8, the negatively charged polymer PEG-(PAH/DMMA) from CDs-Pt(IV)@PEG-(PAH/DMMA) could be hydrolyzed to positively charged polymer, and thus dissociated with positively charged CDs-Pt(IV) due to electrostatic repulsion. This property was confirmed by zeta potential change of CDs-Pt(IV)@PEG-(PAH/DMMA) at tumor extracellular pH 6.8 and normal

Figure 10. (a) TEM, (b) DLS result, (c) FTIR spectrum, (d) changes in the particle size upon incubation with PBS (pH 7.4), FBS, and RPMI 1640 medium for 24 h, and (e) zeta potential changes when incubating at pH 7.4 and 6.8 at 37 °C for 4 h for CDs-Pt(IV)@PEG-(PAH/SA).

physiological pH 7.4 (Figure 9e). At pH 6.8, CDs-Pt(IV)@PEG-(PAH/DMMA) showed obvious charge-convertible property from negative charge to positive one with zeta potential increasing from -16.15 mV to +12.01 mV after incubation at 37 °C for 4 h, while at pH 7.4, CDs-Pt(IV)@PEG-(PAH/DMMA) exhibited non-charge-convertible characteristics with a consistent negative charge after incubation at 37 °C for 4 h. For comparison, due to the absence of double bond in succinic acid (SA), the conformation of succinic amides is more flexible and the

terminal carboxyl group is not maintained in close proximity to the amide group, leading to much slower rates for hydrolysis of succinic amides than dimethylmaleic amides.²⁹ Therefore, we also synthesized PEG (9.8% of amino group) and SA (79.5% of amino group) functionalized deprotonated PAH (PEG-(PAH/SA)) (Figures 3 and 5b). The negatively charged polymer PEG-(PAH/SA) was also complexed with positively charged CDs-Pt(IV) to obtain CDs-Pt(IV)@PEG-(PAH/SA). CDs-Pt(IV)@PEG-(PAH/SA) showed an average diameter of 119 ± 31 nm ($n = 40$) as measured by TEM and DLS (Figures 10a and b) and good stability in different physiological solutions (Figure 10d). The zeta potential change at pH 6.8 and 7.4 was also studied. As revealed in Figure 10e, CDs-Pt(IV)@PEG-(PAH/SA) did not show charge-convertible property at mildly acidic tumor extracellular microenvironment, still having negative charge at pH 6.8 and 7.4 after incubation at 37 °C for 4 h.

2.3.3 Cellular Uptake Measured by CLSM and Flow Cytometry

Due to the charge-convertible property of CDs-Pt(IV)@PEG-(PAH/DMMA) at mildly acidic tumor extracellular microenvironment (pH 6.8), the resulting positively charged CDs-Pt(IV) could easily enter into cancer cells with efficient internalization through the interaction with negatively charged cell membrane. On the other hand, the negatively charged CDs-Pt(IV)@PEG-(PAH/DMMA) in normal physiological condition (pH 7.4) and CDs-Pt(IV)@PEG-(PAH/SA) at tumor extracellular microenvironment and normal physiological condition had low uptake by cancer cells because of the electrostatic repulsion with negatively charged cell membrane. Such phenomena were confirmed by CLSM images of A2780 cells incubated with media containing CDs-Pt(IV)@PEG-(PAH/DMMA) or CDs-Pt(IV)@PEG-(PAH/SA) at pH 6.8 and 7.4 for 2 h (Figure 11). The cells incubated with CDs-Pt(IV)@PEG-(PAH/DMMA) at pH 6.8 showed much stronger blue, green, and red colors under excitation wavelengths of 405, 488, and 543 nm respectively as compared to those incubated with CDs-Pt(IV)@PEG-(PAH/DMMA) at pH 7.4. The cells incubated with CDs-Pt(IV)@PEG-(PAH/SA) displayed a little signal at either pH 6.8 or 7.4 under different excitation wavelengths. The results conclude that

Figure 11. CLSM images of A2780 cells after incubating with (a, b) CD_s-Pt(IV)@PEG-(PAH/DMMA) or (c, d) CD_s-Pt(IV)@PEG-(PAH/SA) at (a, c) pH 7.4 and (b, d) pH 6.8 at 37 °C under excitation wavelengths of 405, 488, and 543 nm. Scale bar = 20 μm.

CD_s-Pt(IV)@PEG-(PAH/DMMA) can be efficiently internalized by cancer cells at tumor extracellular pH 6.8 because of the charge-convertible property from negative charge to positive one.

The internalization of CD_s-Pt(IV)@PEG-(PAH/DMMA) and CD_s-Pt(IV)@PEG-(PAH/SA) by A2780 cancer cells was also verified by flow cytometry (Figure 12). When the cancer cells were cultured with a medium containing CD_s-Pt(IV)@PEG-(PAH/DMMA), the mean fluorescence intensity of CDs was much stronger at pH 6.8 than that at pH 7.4 (Figures 12a and c). On the contrary, very low fluorescence of CDs was observed when the cancer cells were cultured with a medium

Figure 12. (a, b) Flow cytometric results and (c, d) corresponding mean fluorescence intensity of A2780 cells after incubating with (a, c) CD-Pt(IV)@PEG-(PAH/DMMA) or (b, d) CD-Pt(IV)@PEG-(PAH/SA) at pH 7.4 and 6.8 at 37 °C. A2780 cells without any treatment were used as a control.

containing CD-Pt(IV)@PEG-(PAH/SA) at pH 6.8 and 7.4 (Figures 12b and d). The flow cytometric results are in good accordance with the results of confocal imaging, indicating greatly improved cellular uptake of CD-Pt(IV)@PEG-(PAH/DMMA) at tumor extracellular pH 6.8 due to the charge-convertible characteristics from negative charge to positive one.

2.3.4 *In Vitro* Cytotoxicity Study

In vitro toxicity of CD-Pt(IV)@PEG-(PAH/DMMA) and CD-Pt(IV)@PEG-(PAH/SA) to A2780 cells was evaluated using MTT assay (Figures 13a and b). After the drug nanocarriers were internalized by A2780 cells and escaped

Figure 13. Cell viability of (a, b) A2780 and (c, d) HeLa cells after incubating with (a, c) CD_s-Pt(IV)@PEG-(PAH/DMMA) or (b, d) CD_s-Pt(IV)@PEG-(PAH/SA) at pH 7.4 and 6.8 at 37 °C.

from endosome *via* “proton sponge” effect, the cisplatin(IV) prodrug is reduced to cisplatin to show the cytotoxicity under reductive cytosol of cancer cells. CD_s-Pt(IV)@PEG-(PAH/SA) showed almost no toxicity to cancer cells at either pH 6.8 or 7.4 with the platinum concentrations from 0.18 to 11.44 μM. When A2780 cells were treated with CD_s-Pt(IV)@PEG-(PAH/DMMA) at pH 7.4 in the same concentration range, less cytotoxic effect was observed, resulting from minor uptake of CD_s-Pt(IV)@PEG-(PAH/DMMA) by A2780 cells and subsequently less cisplatin release. However, the cell death increased significantly when the cancer cells were incubated with CD_s-Pt(IV)@PEG-(PAH/DMMA) at pH 6.8, due to the enhanced uptake of CD_s-Pt(IV)@PEG-(PAH/DMMA) and efficient cisplatin release under the intracellular reductive condition. These results exhibit the advantage of loading cisplatin into charge-convertible CD_s-Pt(IV)@PEG-(PAH/DMMA) and the ability of this drug nanocarrier to release highly cytotoxic cisplatin under reductive cytosol of cancer cells upon incubation at tumor extracellular pH 6.8 for efficient cancer therapy.

2.3.5 *In Vivo* Tumor Inhibition Ability

Figure 14. *In vivo* anti-tumor efficacy of CDs-Pt(IV)@PEG-(PAH/DMMA) on tumor-bearing female Kunming mice. (a) Changes in body weight and (b) relative tumor volume achieved from mice after intravenous treatments with saline solution as a control, CDs-Pt(IV)@PEG-(PAH/DMMA), and CDs-Pt(IV)@PEG-(PAH/SA), respectively. (c) Photographs of mice and excised tumors from euthanized representative mice, and (d) H&E stained tumor slices after 14 days treatment from different groups. The error bars were based on the standard errors of the mean of five tumors per group. *p* values: ***p < 0.001.

Further experiments were performed to investigate the tumor therapeutic efficacy of CDs-Pt(IV)@PEG-(PAH/DMMA) *in vivo*. As therapeutic efficiency of CDs-Pt(IV)@PEG-(PAH/DMMA) and CDs-Pt(IV)@PEG-(PAH/SA) to Hela cells was similar to that for A2780 cells (Figures 13c and d), the murine cervix cell line U14 was chosen as the xenograft model, and U14 cells were injected subcutaneously to the left axilla of each female Kunming mouse to establish tumors. The tumor-bearing mice were divided into three groups (*n* = 5) randomly and were intravenously injected with

Figure 15. H&E stained images of major organs (heart, liver, spleen, lung, and kidney) for control, CDs-Pt(IV)@PEG-(PAH/DMMA), and CDs-Pt(IV)@PEG-(PAH/SA) groups after 14 days treatment.

saline, CDs-Pt(IV)@PEG-(PAH/SA), and CDs-Pt(IV)@PEG-(PAH/DMMA), respectively. Three doses of identical treatments were given at day 0, 4, and 10. The injected cisplatin(IV) prodrug (CDs-Pt(IV)@PEG-(PAH/SA) and CDs-Pt(IV)@PEG-(PAH/DMMA)) dose in 100 μ L saline was 1.5 mg platinum per kg of body weight, and the group with only saline injection (100 μ L) was used as the control group. Both mouse body weights and tumor sizes were monitored every day after the initiation of the treatments. Figures 14a and b showed the mean body weights and mean relative tumor volumes of each group during the treatment. Compared with

the saline group, the growth of tumors on CDs-Pt(IV)@PEG-(PAH/DMMA) injected mice was greatly inhibited over a course of 14 days treatment, and the growth of tumors on CDs-Pt(IV)@PEG-(PAH/SA) injected mice was slightly inhibited after 14 days treatment, which might result from increased cytotoxicity of nanoparticles to cancer cells with longtime administration after CDs-Pt(IV)@PEG-(PAH/SA) accumulated at the tumor site due to EPR effects (Figure 13c and d). Moreover, the body weight of all groups was not obviously affected over the investigation period, indicating little toxic side effects of CDs-Pt(IV)@PEG-(PAH/DMMA) to animals. The digital photos of representative mice with excised tumors in Figure 14c also confirmed that the tumor size with CDs-Pt(IV)@PEG-(PAH/DMMA) treatment was the smallest, demonstrating better tumor inhibition efficacy of CDs-Pt(IV)@PEG-(PAH/DMMA) than that of CDs-Pt(IV)@PEG-(PAH/SA) and control groups. In addition, H&E stained tumor sections (Figure 14d) showed the highest level of tumor tissue cell damage in CDs-Pt(IV)@PEG-(PAH/DMMA) treated group, revealing a good consistency with the tumor growth data. The pathomorphology analysis of the main organs including heart, liver, spleen, lung, and kidney was shown in Figure 15. It could be observed that there were no apparent damages in the detected organs from CDs-Pt(IV)@PEG-(PAH/DMMA) group, CDs-Pt(IV)@PEG-(PAH/SA) group, and control group, implying that CDs-Pt(IV)@PEG-(PAH/DMMA) had a good biocompatibility *in vivo*. Therefore, these *in vivo* results demonstrate that CDs-Pt(IV)@PEG-(PAH/DMMA) can provide a great therapeutic efficacy without appreciable toxic side effects.

2.4 Conclusion

In summary, charge-convertible cisplatin(IV) prodrug-loaded CDs (CDs-Pt(IV)@PEG-(PAH/DMMA)) capable of responding to tumor extracellular microenvironment have been developed successfully for imaging-guided drug delivery with enhanced cancer therapy. CDs-Pt(IV)@PEG-(PAH/DMMA), capable of changing from PEGylation with negative charge at normal physiological condition (pH 7.4) to positive charge at tumor extracellular microenvironment (pH 6.8), possesses a

number of attributes including multicolor bioimaging, prolonged circulation time in blood, effective accumulation at the tumor site, enhanced internalization by cancer cells, facilitated endosome escape, and controlled intracellular drug release, which not only overcomes the limited tumor therapeutic efficacy of CD-based drug nanocarriers with single surface property, but also leads to enhanced tumor inhibition efficiency with low side effects both *in vitro* and *in vivo*. The design of charge-convertible CDs-Pt(IV)@PEG-(PAH/DMMA) offers a promising strategy to prepare drug nanocarriers with sensitive surface properties for enhanced cancer treatment.

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Chapter 3

Dual-Responsive Carbon Dots for Tumor Extracellular Microenvironment Triggered Targeting and Enhanced Anticancer Drug Delivery

3.1 Introduction

For a drug delivery system, active targeting can enhance cellular internalization of drug carriers by utilizing specific interactions between targeting ligands and corresponding receptors overexpressed on the surface of cancer cells, resulting in efficient cancer therapy.¹⁻⁵ Commonly used targeting ligands are small molecules (e.g., folic acid), peptides (e.g., Arg-Gly-Asp (RGD) and Tyr-Gly-Arg-(Lys)₂-(Arg)₂-Gln-(Arg)₃ (TAT), antibodies, and aptamers.⁶ The targeting ligands are usually attached on the surface of drug carriers. Such approach has two main disadvantages.^{7,8} The first one is that reticular endothelial system can recognize the targeting ligands directly or through opsonins, enhancing early clearance of the carriers from blood circulation.⁷ The second one is that corresponding receptors to the targeting ligands are not only overexpressed by cancer cells, but also expressed on the surface of normal cells. Therefore, the drug carriers with targeting ligands can cause side effects to the normal cells.⁸ To solve the issues, tumor extracellular microenvironment (e.g., slightly acidic pH or overexpressed enzyme) triggered targeting strategy is proposed, in which targeting ligands are hidden inside the stealth layer of the carriers during blood circulation. Upon the accumulation in tumor tissue through EPR effect, the targeting ligands present on the surface of drug carriers to facilitate the interaction with receptors on the surface of cancer cells.⁹⁻¹³ Although several active targeting drug delivery platforms utilizing fluorescent CDs have been developed,^{14,15} there is still no report on fabricating CD-based drug carriers

Figure 1. (a) Schematic illustration for the preparation of CD-based drug nanocarrier CDs-RGD-Pt(IV)-PEG with tumor triggered targeting property. (b) Schematic illustration of the drug delivery process using CDs-RGD-Pt(IV)-PEG: (1) tracking drug nanocarriers by multicolor fluorescence of CDs with PEGylation in normal physiological condition, (2) tumor-triggered targeting ligands exposed at tumor extracellular pH 6.8, (3) effective uptake by cancer cells through ligand-receptor interaction, (4) cisplatin release from cisplatin(IV) prodrug under reductive cytosol, and (5) cisplatin binding with DNA to exhibit the cytotoxicity.

with tumor extracellular microenvironment triggered targeting characteristics.

Herein, imaging-guided and pH/redox dual-responsive CDs (CDs-RGD-Pt(IV)-PEG) containing targeting ligand RGD peptide were developed (Figure 1), which were PEGylated at normal physiological condition (pH 7.4). The RGD peptide could be exposed at tumor extracellular microenvironment (pH 6.5-6.8). CDs-RGD-Pt(IV)-PEG could be tracked by multicolor fluorescence of CDs, and the tumor-triggered targeting property was offered by the hydrolysis of benzoic-imine¹⁶⁻¹⁹ bond at pH 6.8 to deshield the RGD peptide, leading to effective ligand-receptor interaction and enhanced uptake by cancer cells overexpressing integrin $\alpha_v\beta_3$ on the cellular membrane. Upon the internalization, the loaded cisplatin(IV) prodrug in CDs-RGD-Pt(IV)-PEG was reduced to cisplatin in reductive cytosol of cancer cells to exhibit the cytotoxicity.²⁰⁻²³ The *in vitro* results indicate that CDs-RGD-Pt(IV)-PEG is a promising candidate in the cancer treatment.

3.2 Materials and Methods

Materials. Chemicals including 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N,N'*-dicyclohexylcarbodiimide (DCC), *N,N*-diisopropylethylamine (DIPEA), 4-dimethylaminopyridine (DMAP), 4-formylbenzoic acid, methoxy poly(ethylene glycol) (mPEG), piperidine, trifluoroacetic acid (TFA), and triisopropylsilane (TPS) were obtained from Sigma-Aldrich without further purifications. Reagents for cell culture, Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium Eagle (MEM), and FBS were purchased from Thermo Fisher Scientific Pte Ltd. CDs and cisplatin(IV) prodrug were same to those in chapter 2.

Synthesis of Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH (Figure 2). The peptide Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH was prepared by employing Fmoc chemistry-based solid-phase peptide synthesis with 2-chlorotrityl chloride resin. The coupling reaction was performed with HBTU/DIPEA as coupling reagents at room

temperature, and deprotection of Fmoc group was performed in 20% (v/v) piperidine/*N,N*-dimethylformamide (DMF) for 20 min. After the completion of coupling the last peptide Fmoc-Gly-OH, 2% TFA, and 2.5% TPS in dichloromethane (CH_2Cl_2) were used to cleave the peptide Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH from resin for several times. The cleavage mixture was collected and concentrated by rotary evaporation, followed by precipitation in cold diethyl ether. The peptide Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH was obtained by filtration, dried under vacuum, and stored at $-20\text{ }^\circ\text{C}$ for further use. The molecular weight was determined by ESI-MS (Figure 3), calcd: 1305.45; found: 1305.44 (M^+).

Figure 2. Synthetic route of Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH.

Synthesis of mPEG-CHO (Figure 4). 4-Formylbenzoic acid (112.5 mg) was dissolved in DMF (0.5 mL) and CH_2Cl_2 (5 mL), followed by the addition of mPEG (300.0 mg), DCC (307.5 mg), and DMAP (45.0 mg). The mixture was stirred at room temperature for 24 h. Then, the solvent was removed using a rotary evaporator to afford white solid. The white solid was dissolved in CH_2Cl_2 and filtered through Buchner funnel to discard the precipitate dicyclohexylurea. CH_2Cl_2 was removed *via* rotary evaporator, and the product was then recrystallized using 2-propanol several times to give white solid of mPEG-CHO, which was stored at room temperature for

Figure 3. ESI-MS spectrum of Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH.

further use. ¹H NMR (300 MHz, CDCl₃): δ 10.10 (s, 1H, CHO), 8.20 (d, 2H, benzene-H), 7.94 (d, 2H, benzene-H), 4.51 (t, 2H, CH₂), 3.85 (t, 3H, CH₃), 3.64 ppm (s, 107H, CH₂).

Figure 4. Synthetic route of mPEG-CHO.

Synthesis of CDs-RGD(Pbf) (Figure 5).

Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH (3.3 mg), NHS (0.3 mg), and EDC·HCl (0.5 mg) were stirred in DMSO (0.5 mL) at room temperature for 30 min. Then, CDs (9.8 mg) in DMSO (1 mL) was added into the above solution. The mixture was stirred at room temperature for another 24 h and then dialyzed against water for 24 h by using a dialysis membrane with MWCO of 3.5 kDa to remove the impurity. CDs-RGD(Pbf) was obtained after freeze-drying and stored at −20 °C for further use.

Figure 5. Scheme of the synthesis of CDs-RGD(Pbf).

Synthesis of CDs-RGD(Fmoc) (Figure 6). The CDs-RGD(Pbf) obtained from the above synthesis was dissolved in solvent (1 mL) containing 95% TFA, 2.5% water, and 2.5% TPS. The solution was left for stirring at room temperature for 2 h. Solvent was removed *via* rotary evaporator, and the resultant was then dialyzed against water for 24 h by using a dialysis membrane with MWCO of 1 kDa to remove the impurity. CDs-RGD(Fmoc) was obtained after freeze-drying and stored at $-20\text{ }^{\circ}\text{C}$ for further use.

Figure 6. Scheme of the synthesis of CDs-RGD(Fmoc).

Synthesis of CDs-RGD(Fmoc)-Pt(IV) (Figure 7). Cisplatin(IV) prodrug (4.3 mg), EDC·HCl (1.9 mg), and NHS (1.1 mg) were dissolved in DMF (1 mL) before stirring at room temperature in dark condition for 12 h. The mixture was added into diethyl ether (40 mL), and the obtained solution was placed in a 4 °C refrigerator for 4 h. The solution was then centrifuged at 9000 rpm for 5 min to obtain the precipitate cisplatin(IV)-NHS. The precipitate was washed with diethyl ether three times.

The obtained CDs-RGD(Fmoc) and cisplatin(IV)-NHS were dissolved in DMSO (1 mL) containing DIPEA (3.4 μ L). The mixture was stirred at room temperature in dark condition for another 24 h and then dialyzed against water for 24 h by using a dialysis membrane with MWCO of 1 kDa to remove the impurity. CDs-RGD(Fmoc)-Pt(IV) was obtained after freeze-drying and stored at -20 °C for further use.

Figure 7. Scheme of the synthesis of CDs-RGD(Fmoc)-Pt(IV).

Synthesis of CDs-RGD-Pt(IV) (Figure 8). The obtained CDs-RGD(Fmoc)-Pt(IV) was dissolved in solvent (1 mL) containing 80% DMF and 20% piperidine. The mixture was stirred at room temperature in dark condition for 20 min and then dialyzed against water for 24 h by using a dialysis membrane with MWCO of 1 kDa to remove the impurity. CDs-RGD-Pt(IV) was obtained after freeze-drying and stored at -20 °C for further use.

CLSM. The human breast adenocarcinoma epithelial cell line MDA-MB-231 (ECACC) was cultured in DMEM with the addition of 10% FBS at 37 °C in a humidified incubator having 5% CO₂. The human breast adenocarcinoma epithelial cell line MCF-7 (ECACC) was cultured in MEM with supplementation of 10% FBS at 37 °C in a humidified atmosphere having 5% CO₂.

MDA-MB-231 or MCF-7 cells at a density of 1×10^5 were cultured using a μ -dish with diameter of 35 mm and plastic bottom (ibidi GmbH, Germany) at 37 °C in a humidified incubator with 5% CO₂ for 24 h. Then, the medium was replaced by DMEM or MEM with pH 6.8 or 7.4 containing CDs-RGD-Pt(IV)-PEG before the maintenance at 37 °C for another 2 h. After they were rinsed by PBS twice, MDA-MB-231 or MCF-7 cells were used for CLSM imaging, in which different measurement parameters were used under different excitation wavelengths (405, 488, and 543 nm) to show the CLSM images clearly and same measurement parameters were utilized under the same excitation wavelength for comparison.

Flow Cytometry Analysis. MDA-MB-231 or MCF-7 cells were cultured in six-well plates with density of 5×10^5 per well at 37 °C in a humidified incubator with 5% CO₂ for 24 h. Then, the medium was replaced by DMEM or MEM with pH 6.8 or 7.4 containing CDs-RGD-Pt(IV)-PEG at 37 °C for designated time. After they were washed and trypsinized, MDA-MB-231 or MCF-7 cells were analyzed using flow cytometer in Indo 1 violet channel with an excitation wavelength of 355 nm and an emission wavelength range of 450 ± 50 nm.

Intracellular Platinum Levels Determination. MDA-MB-231 or MCF-7 cells were seeded in 12-well plates with density of 3×10^5 per well at 37 °C in a humidified incubator with 5% CO₂ for 24 h. Then, the medium was replaced by DMEM or MEM with pH 6.8 or 7.4 containing CDs-RGD-Pt(IV)-PEG for 2 h. After the cells were washed, trypsinized and digested, intracellular platinum levels were determined by ICP-OES.

***In Vitro* Cytotoxicity.** The MTT assay was used to evaluate the cytotoxicity of CDs-RGD-Pt(IV)-PEG. MDA-MB-231 or MCF-7 cells were cultured in 96-well plates with density of 4×10^3 cells per well within DMEM or MEM (100 μ L) at 37 °C in a humidified incubator with 5% CO₂ for 24 h. Then, the medium was replaced by DMEM or MEM with pH 6.8 or 7.4 containing CDs-RGD-Pt(IV)-PEG at varied concentrations before the maintenance at 37 °C for another 2 h. After they were substituted with fresh DMEM or MEM, MDA-MB-231 or MCF-7 cells were incubated at 37 °C for another 72 h. Then, MDA-MB-231 or MCF-7 cells were rinsed using PBS, followed by being incubated with 20% MTT in DMEM or MEM at 37 °C for 5 h. After the medium was discarded, DMSO (100 μ L) was used to lyse MDA-MB-231 or MCF-7 cells. The absorbance at 490 nm was recorded by Tecan's Infinite M200 microplate reader. Every experiment was performed thrice, and the cell viability was calculated by referring to the control group without the sample treatment.

3.3 Results and Discussion

3.3.1 Preparation and Characterization

To prepare CDs-RGD-Pt(IV)-PEG, CDs and cisplatin(IV) prodrug were first synthesized according to the same methods in chapter 2. To endow the CDs with active targeting property, the peptide sequence Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH with protection groups was synthesized manually using Fmoc-based solid-phase synthesis method. The molecular mass of this peptide was calculated to be 1305.45, and the measured value was 1305.44 by ESI-MS, confirming successful synthesis (Figure 3). By processing Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH with TFA/H₂O/TPS, the protection groups except Fmoc group were deprotected to produce Fmoc-GRGDSGGGG-OH, (Figures 10 and 11a). The RGD peptide with NH₂-GRGDSGGGG-OH sequence could finally be obtained through deprotection of Fmoc group using DMF/piperidine, and its structure was also verified by ESI-MS (Figure 11b). The mPEG-CHO polymer

Figure 10. The deprotection route of Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH.

The designed drug nanocarrier CDs-RGD-Pt(IV)-PEG was prepared by first conjugating the Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH peptide with CDs through a typical EDC/NHS amidation reaction between the carboxylic acid group of Fmoc-GR(Pbf)GD(O^tBu)S(^tBu)GGGG-OH and a portion of amino groups on CDs. After the protection groups except Fmoc group were deprotected, the cisplatin(IV) prodrug was loaded using the remaining amino groups on CDs. Following the deprotection of Fmoc group to expose the amino groups, mPEG-CHO was introduced to protect the RGD peptide by forming a benzoic-imine bond to produce CDs-RGD-Pt(IV)-PEG. As shown in Figure 12a, CDs-RGD-Pt(IV)-PEG displayed two special peaks of amide bands I and II at 1654 and 1558 cm⁻¹, proving the

Figure 11. ESI-MS spectra of (a) Fmoc-GRGDSGGGG-OH and (b) NH₂-GRGDSGGGG-OH.

presence of RGD peptide on the surface. The strong absorption peak at 1121 cm⁻¹ was originated from the C-O stretching vibration of PEG, confirming the functionalization of the mPEG-CHO polymer. The platinum loading percentage of CDs-RGD-Pt(IV)-PEG was determined to be 4.1 wt% by ICP-MS. DLS data (Figure

Figure 12. (a) FTIR spectra of CDs, RGD (NH₂-GRGDSGGGG-OH), cisplatin(IV) prodrug, mPEG-CHO, and CDs-RGD-Pt(IV)-PEG. (b) DLS result, (c) UV-vis absorption spectrum, (d) fluorescence spectra with excitation wavelengths from 360 to 500 nm, and (e) normalized multi-color fluorescence spectra under excitation wavelengths of 405, 488, and 543 nm of CDs-RGD-Pt(IV)-PEG.

12b) of CDs-RGD-Pt(IV)-PEG presented relatively larger diameter of 31 nm as compared with the size of CDs, further supporting the successful preparation of the drug nanocarrier. The UV-vis absorption spectrum of CDs-RGD-Pt(IV)-PEG (Figure 12c) showed two absorption bands at 250 and 351 nm for the π - π^* transition of the aromatic ring structure and the n - π^* transition of the C=O group. The fluorescence

spectra of CDs-RGD-Pt(IV)-PEG (Figure 12d) were also excitation-dependent, with red-shifted emission peaks from 461 to 534 nm under increasing excitation wavelengths from 360 to 480 nm. As compared with the spectral properties of CDs, the absorption bands and the emission peaks of CDs-RGD-Pt(IV)-PEG showed some shifts or relative-intensity changes, which were due to the varied surface states after functionalization of RGD peptide, cisplatin(IV) prodrug, and mPEG-CHO on CDs.

3.3.2 Cellular Uptake Measured by CLSM and Flow Cytometry

Figure 13. CLSM images of (a, b) MDA-MB-231 and (c, d) MCF-7 cells upon the treatment with CDs-RGD-Pt(IV)-PEG at (a, c) pH 7.4 and (b, d) pH 6.8 at 37 °C under excitation wavelengths of 405, 488, and 543 nm. Scale bar = 20 μ m.

To explore the tumor extracellular acidic condition triggered targeting ability of the nanocarriers, CDs-RGD-Pt(IV)-PEG was incubated with MDA-MB-231 and

MCF-7 breast cancer cells, followed by CLSM imaging for the multi-color fluorescence characteristics of CDs-RGD-Pt(IV)-PEG under different excitation wavelengths (405, 488, and 543 nm) (Figure 12e). Owing to the targeting interaction between RGD-containing peptide and integrin $\alpha_v\beta_3$, MDA-MB-231 cells were used as integrin-positive cancer cells for the overexpression of integrin $\alpha_v\beta_3$ on the cellular membrane, while MCF-7 cells were chosen as the negative control for low expressed integrin $\alpha_v\beta_3$. As shown in CLSM imaging results (Figure 13a), MDA-MB-231 cells showed faint blue, green, and red fluorescence under excitation wavelengths of 405, 488, and 543 nm in physiological condition (pH 7.4), respectively. This observation indicated that only few CDs-RGD-Pt(IV)-PEG were endocytosed by MDA-MB-231 cells, because the benzoic-imine bond was not hydrolyzed at pH 7.4, and RGD peptide was still shielded by the PEG polymer. While at tumor extracellular environment (pH 6.8), MDA-MB-231 cells exhibited stronger blue, green, and red fluorescence under different excitation wavelengths (Figure 13b). The enhanced endocytosis of nanocarriers was achieved by the interaction between the RGD peptide and integrin $\alpha_v\beta_3$ due to the exposure of RGD peptide after the hydrolysis of the benzoic-imine bond at pH 6.8. In contrast, MCF-7 cells displayed weak blue, green and red fluorescence both at pH 7.4 and 6.8 (Figures 13c and d). The low uptake of nanocarriers at pH 7.4 by MCF-7 cells was resulted from the shielding of the RGD peptide by the PEG polymer, whereas at pH 6.8, the exposed RGD peptide could not function effectively due to the low level of integrin $\alpha_v\beta_3$ expression on the cellular membrane of MCF-7 cells.

Flow cytometry analysis was also performed to quantitatively investigate the cellular uptake behavior of CDs-RGD-Pt(IV)-PEG by MDA-MB-231 and MCF-7 cancer cells. The fluorescence of CDs-RGD-Pt(IV)-PEG was recorded in Indo 1 violet channel with an excitation wavelength of 355 nm and an emission wavelength range of 450 ± 50 nm. No obvious difference in the fluorescence intensity of MDA-MB-231 or MCF-7 cells was observed between pH 7.4 and 6.8 (Figures 14a and d). When MDA-MB-231 cells were incubated with CDs-RGD-Pt(IV)-PEG, the

Figure 14. (a, b, d, e) Flow cytometry results and (c, f) corresponding mean fluorescence intensity of (a-c) MDA-MB-231 and (d-f) MCF-7 cells upon the incubation without or with CDs-RGD-Pt(IV)-PEG at pH 7.4 and 6.8.

cellular uptake was apparently higher at pH 6.8 than that at pH 7.4 (Figure 14b). The mean fluorescence intensity of CDs-RGD-Pt(IV)-PEG in MDA-MB-231 cells at pH

Figure 15. Intracellular platinum levels of MDA-MB-231 and MCF-7 cells upon the incubation with CDs-RGD-Pt(IV)-PEG at pH 7.4 and 6.8.

6.8 was 1.9-fold higher than that at pH 7.4 (Figure 14c). While for MCF-7 cells, less uptake of CDs-RGD-Pt(IV)-PEG was observed (Figure 14e), and the mean fluorescence intensity had no significant difference between pH 7.4 and 6.8 (Figure 14f). In addition, increased intracellular platinum levels were also detected for MDA-MB-231 cells treated with CDs-RGD-Pt(IV)-PEG at pH 6.8 (Figure 15). The flow cytometry and intracellular platinum levels results were consistent with the CLSM imaging observations, demonstrating that CDs-RGD-Pt(IV)-PEG could be internalized effectively by integrin $\alpha_v\beta_3$ overexpressed MDA-MB-231 cancer cells with tumor extracellular microenvironment of pH 6.8.

3.3.4 *In Vitro* Cytotoxicity Study

The anticancer efficiency of CDs-RGD-Pt(IV)-PEG was evaluated by MTT assay using MDA-MB-231 and MCF-7 cancer cells. The CDs alone showed negligible toxicity toward MDA-MB-231 and MCF-7 cells even at a high nanoparticle concentration of 200 $\mu\text{g mL}^{-1}$ (Figures 16a and c). When MDA-MB-231 cells were incubated with CDs-RGD-Pt(IV)-PEG at pH 6.8, much higher cytotoxicity was observed than that at pH 7.4 under the same nanocarrier concentration (Figure 16b). On the contrary, there was no obvious difference in cytotoxicity for MCF-7 cells incubating with CDs-RGD-Pt(IV)-PEG at pH 7.4 and 6.8 (Figure 16d). The enhanced therapeutic efficiency of CDs-RGD-Pt(IV)-PEG to integrin $\alpha_v\beta_3$ overexpressed

Figure 16. Cytotoxicity assay of (a, c) CDs and (b, d) CDs-RGD-Pt(IV)-PEG at pH 7.4 and 6.8 under different concentrations on (a, b) MDA-MB-231 and (c, d) MCF-7 cells at 37 °C.

MDA-MB-231 cancer cells under tumor extracellular condition was due to its increased uptake through receptor-mediated endocytosis with the aid of the deprotection of PEG polymer and the exposure of RGD targeting ligand at pH 6.8.

3.4 Conclusion

In summary, multicolor imaging-guided and pH/redox dual-responsive drug nanocarrier, CDs-RGD-Pt(IV)-PEG, has been successfully developed for tumor extracellular microenvironment triggered targeting toward integrin $\alpha_v\beta_3$ overexpressed cancer cells. On the one hand, the PEG protection of RGD targeting ligand *via* a benzoic-imine bond enables CDs-RGD-Pt(IV)-PEG to present increased internalization and enhanced therapeutic efficiency at tumor extracellular pH, as well as lowered uptake and less cytotoxicity in physiological condition for integrin $\alpha_v\beta_3$ overexpressed cancer cells. On the other hand, the nanocarrier shows less cytotoxicity

to cells with low integrin $\alpha_v\beta_3$ expression level. The CDs-RGD-Pt(IV)-PEG drug nanocarrier provides a new alternative to design effective active-targeting anticancer therapeutics.

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Chapter 4

Reduction-Responsive Carbon Dots for Real-Time Ratiometric Monitoring of Anticancer Prodrug Activation in Living Cells

4.1 Introduction

In the past decades, chemotherapy has emerged as a dominant modality to treat cancer.¹ However, commonly used chemotherapeutic agents suffer from unavoidable toxicity to healthy tissues due to the nonspecific distribution throughout the body, which seriously limits their clinical applications.² To overcome these drawbacks, a promising strategy based on anticancer prodrugs, which are pharmacologically inactive but can be metabolized into toxic forms under specific intracellular microenvironments, such as pH changes,³ enzyme actions,⁴ and thiol treatments,⁵ is proposed to increase the targeting capability and thus enhance the therapeutic efficacy of anticancer agents.^{6,7} However, it still remains a challenge to demonstrate how and when anticancer prodrugs are specifically activated in living cells.

Benefiting from high sensitivity, excellent contrast, and great specificity of fluorescence imaging,⁸ various fluorescent sensors have been constructed to monitor the activation of anticancer prodrugs in real time.⁹⁻¹¹ However, these probes are mainly based on small-molecule organic dyes or single emission intensity variability, which have the shortcomings of poor photostability and chemical stability or the interference from uncontrolled microenvironment changes in living samples. Therefore, it is urgently required to develop a novel platform to surmount above disadvantages. Taking advantage of the promising properties of CDs, including excellent water solubility, high biocompatibility, excitation-dependent emission, and good photostability,^{12,13} they possess a great potential to be utilized to fabricate

rational systems in order to meet the above-mentioned requirements.

Figure 1. Schematic illustration of FRET-based CDs for real-time ratiometric monitoring of cisplatin(IV) prodrug activation in living cells.

In this work, we reported a ratiometric FRET-based sensor for real-time monitoring of anticancer prodrug activation in living cells, by utilizing cisplatin(IV) prodrug as the model drug and CDs as the drug nanocarriers, which could emit red, green, and blue fluorescence under different excitation wavelengths (Figure 1). By loading the Dabsyl quencher^{14,15} on the surface of CDs with cisplatin(IV) prodrug as the linker (CDs-Pt(IV)-Dabsyl), the blue fluorescence of CDs was effectively quenched on account of the FRET effect. Upon the releases of active Pt(II) species and the Dabsyl unit under reductive conditions, the blue fluorescence of CDs was recovered over time, while the intensity of green or red fluorescence was almost unaltered. Therefore, the gradual increase of fluorescence intensity ratio from the blue-to-green or blue-to-red luminescence could illustrate the activation of the cisplatin(IV) prodrug in real time. More importantly, with green or red emission of CDs as an internal standard, this ratiometric method was capable of monitoring anticancer prodrug activation in complex biological microenvironments.

4.2 Materials and Methods

Materials. The chemicals used were purchased from Sigma-Aldrich without further purification. LysoTracker Red DND-99 and the reagents for cell culture were

obtained from Thermo Fisher Scientific Pte Ltd.

Synthesis of *c,c,t*-[PtCl₂(NH₃)₂(O₂CCH₂CH₂CO₂H)₂] (Pt(IV)-2COOH, Figure 2). *c,t,c*-[PtCl₂(OH)₂(NH₃)₂] (Pt(IV)-2OH) was synthesized according to a previously reported method.¹³ Pt(IV)-2OH (100.0 mg), succinic anhydride (120.0 mg), and DMSO (0.5 mL) were stirred at 70 °C in the dark for 12 h. The mixture was cooled down to room temperature before freeze-drying. The obtained solid was rinsed with cold acetone and diethyl ether to afford Pt(IV)-2COOH. ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.02 (s, 2H, COOH), 6.44 (s, 6H, NH₃), 2.46 (m, 4H, CH₂), 2.33 ppm (m, 4H, CH₂). ESI-MS: calcd: 534.21; found 557.01 [M+Na]⁺.

Figure 2. Synthetic route of Pt(IV)-2COOH.

Synthesis of *N*-(2-Amino-ethyl)-4-(4-dimethylamino-phenylazo)-benzenesulfonamide (Dabsyl-NH₂, Figure 3). 4-(4-Dimethylaminophenylazo)benzenesulfonyl chloride (Dabsyl chloride, 100 mg) was dissolved in CH₂Cl₂ (10 mL), and dropwise added to CH₂Cl₂ (10 mL) containing ethylenediamine (300 μL). The solution was reacted at room temperature in the dark for 24 h, followed by extraction with aqueous sodium bicarbonate twice and water once. The organic layer was dried with anhydrous sodium sulfate. After the filtration, the solvent was reduced under pressure to afford Dabsyl-NH₂. ¹H NMR (300 MHz, CDCl₃): δ 7.92 (m, 6H, benzene-H), 6.78 (d, 2H, benzene-H), 3.12 (s, 6H, NH₃), 3.00 (t, 2H, CH₂), 2.80 ppm (t, 2H, CH₂). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 154.46, 153.10, 142.61, 140.41, 127.75, 125.37, 122.19, 111.59, 46.09, 41.30, 39.82 ppm. ESI-MS: calcd: 347.44; found 348.20 [M+H]⁺.

Figure 3. Synthetic route of Dabsyl-NH₂.

Synthesis **of**

***c,c,t*-[PtCl₂(NH₃)₂(O₂CCH₂CH₂CO₂H)(O₂CCH₂CH₂CONH-Dabsyl)]**
(Pt(IV)-Dabsyl, Figure 4). Pt(IV)-2COOH (34.6 mg) and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 37.4 mg) were dissolved in DMF (1.814 mL) and reacted at room temperature in the dark for 10 min. DMF (1.728 mL) containing Dabsyl-NH₂ (18 mg) and DIPEA (13.0 μL) was then added dropwise to the above solution. The resultant solution was stirred at room temperature in the dark for 24 h, followed by precipitation in diethyl ether and washing with water for several times to afford Pt(IV)-Dabsyl. ¹H NMR (300 MHz, CDCl₃): δ 12.03 (s, 1H, COOH), 7.91 (m, 6H, benzene-H), 6.88 (d, 2H, benzene-H), 6.46 (s, 6H, NH₃), 3.09 (s, 6H, NH₃), 3.06 (m, 2H, CH₂), 2.82 (m, 2H, CH₂), 2.37 (m, 4H, CH₂), 2.23 ppm (m, 4H, CH₂). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 179.90, 179.54, 173.74, 171.58, 154.53, 153.13, 142.62, 140.16, 127.78, 125.40, 122.24, 111.60, 41.98, 39.83, 38.56, 31.37, 31.17, 30.49, 29.82 ppm. ESI-MS: calcd: 863.63; found 863.90 [M]⁺.

Figure 4. Synthetic route of Pt(IV)-Dabsyl.

Synthesis

of

***N*-{2-[4-(4-Dimethylamino-phenylazo)-benzenesulfonylamino]-ethyl}-succinamic acid (Dabsyl-COOH, Figure 5).** Dabsyl-NH₂ (20.0 mg), succinic anhydride (22.9 mg), and triethylamine (82.5 μL) were dissolved in DMF (2.0 mL), and the solution was reacted at room temperature in the dark for 24 h, followed by the addition of water and extraction with CH₂Cl₂. The organic layer was washed with water twice and dried with anhydrous sodium sulfate. After the filtration, the solvent was reduced under pressure to afford Dabsyl-COOH. ¹H NMR (300 MHz, CDCl₃): δ 7.91 (m, 6H, benzene-H), 6.78 (d, 2H, benzene-H), 3.39 (t, 2H, CH₂), 3.15 (t, 2H, CH₂), 3.13 (s, 6H, NH₃), 2.71 (t, 2H, CH₂), 2.46 ppm (t, 2H, CH₂). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 174.01, 171.44, 154.63, 153.24, 142.76, 140.19, 127.79, 125.40, 122.24, 111.61, 42.00, 39.84, 38.51, 30.07, 29.20 ppm. ESI-MS: calcd: 447.51; found 448.20 [M+H]⁺.

Figure 5. Synthetic route of Dabsyl-COOH.

Reactivity with Sodium Diethyldithiocarbamate (DDTC). Solutions with cisplatin (3.3 mM, 50 μL H₂O, and 50 μL DMSO), Pt(IV)-Dabsyl (1.8 mM, 50 μL H₂O, and 50 μL DMSO), or Pt(IV)-Dabsyl (1.8 mM, 50 μL 11.3 mM ascorbic acid, and 50 μL DMSO) were incubated with DDTC (5% in 0.2 M NaOH, 250 μL) at 37 °C in the dark for 8 h. After the extraction with ethyl acetate (2 mL) and the removal of the solvent, the remaining residue was dissolved in acetonitrile (1.5 mL) and subjected to high performance liquid chromatography (HPLC) and ESI-MS analysis.

Loading Pt(IV)-Dabsyl on CDs (CDs-Pt(IV)-Dabsyl, Figure 6). CDs were prepared following a method described in literature.^{16,17} Pt(IV)-Dabsyl (12.6 mg) and HATU (9.2 mg) were dissolved in DMSO (0.445 mL) and reacted at room

temperature in the dark for 10 min. DMSO (0.424 mL) containing CDs (10.0 mg) and DIPEA (3.2 μ L) was then added dropwise to the above solution. The resultant solution was stirred at room temperature in the dark for 24 h, followed by dialysis against water with a dialysis membrane (MWCO = 1 kDa). CDs-Pt(IV)-Dabsyl was obtained after freeze-drying and stored in the dark for further use.

Figure 6. Schematic illustration of the synthesis of CDs-Pt(IV)-Dabsyl by loading Pt(IV)-Dabsyl on CDs.

Loading Dabsyl-COOH on CDs (CDs-Dabsyl, Figure 7). Dabsyl-COOH (6.5 mg) and HATU (9.2 mg) were dissolved in DMSO (0.445 mL) and reacted at room temperature in the dark for 10 min. DMSO (0.424 mL) containing CDs (10.0 mg) and DIPEA (3.2 μ L) was then added dropwise to the above solution. The resultant solution was stirred at room temperature in the dark for 24 h, followed by dialysis against water with a dialysis membrane (MWCO = 1 kDa). CDs-Dabsyl was obtained after freeze-drying and stored in the dark for further use.

Figure 7. Schematic illustration of the synthesis of CDs-Dabsyl by loading Dabsyl-COOH on CDs.

Optimization of Reaction Time. CDs-Pt(IV)-Dabsyl (platinum concentration: 1.44 μM) and ascorbic acid (2.825 mM) were dissolved in PBS (pH 7.4) containing 1% DMSO (v/v), with a total volume of 2 mL. The solution was stirred at room temperature for different time points in the dark, and then subjected to fluorescence spectral measurements.

Optimization of DMSO Ratio. CDs-Pt(IV)-Dabsyl (platinum concentration: 1.44 μM) and ascorbic acid (2.825 mM) were dissolved in PBS (pH 7.4) containing different ratios of DMSO (v/v), with a total volume of 2 mL. The solution was stirred at room temperature in the dark for 4 h, and then subjected to fluorescence spectral measurements.

Optimization of Ascorbic Acid Concentration. CDs-Pt(IV)-Dabsyl (platinum concentration: 1.44 μM) and different concentrations of ascorbic acid were dissolved in PBS (pH 7.4) containing 1% DMSO (v/v), with a total volume of 2 mL. The solution was stirred at room temperature in the dark for 4 h, and then subjected to fluorescence spectral measurements.

Selectivity. CDs-Pt(IV)-Dabsyl (platinum concentration: 1.44 μM) was dissolved in PBS (pH 7.4) containing 1% DMSO (v/v) with a total volume of 2 mL, or in the presence of folic acid, alanine, glycine, glutamic acid, glutamine, or GSH (2.825 mM). The solution was stirred at room temperature in the dark for 4 h, and then subjected to fluorescence spectral measurements.

Fluorescence Response of CDs-Dabsyl toward GSH or Ascorbic Acid. CDs-Dabsyl (dabsyl concentration: 1.44 μM) and ascorbic acid (0 or 2.825 mM) or GSH (2.825 mM) were dissolved in PBS (pH 7.4) containing 1% DMSO (v/v), with a total volume of 2 mL. The solution was stirred at room temperature in the dark for 4 h, and then subjected to fluorescence spectral measurements.

Cell Culture. A2780 cells were cultured in RPMI 1640 medium containing 10% FBS at 37 °C in a humidified incubator with 5% CO_2 .

Preparation of Cell Lysate. 2×10^7 A2780 cells were harvested and washed with PBS (2 mL) twice by centrifugation at 2000 rpm for 5 min, and dispersed in PBS (2 mL). After freezing at $-80\text{ }^{\circ}\text{C}$, thawing at $37\text{ }^{\circ}\text{C}$ for three times and centrifugation at 12000 rpm at $4\text{ }^{\circ}\text{C}$ for 20 min, the supernatant cell lysate was collected and stored at $-80\text{ }^{\circ}\text{C}$ for further use.

Fluorescence Response in the Presence of Cell Lysate. CDs-Pt(IV)-Dabsyl (platinum concentration: $1.44\text{ }\mu\text{M}$) or CDs-Dabsyl (dabsyl concentration: $1.44\text{ }\mu\text{M}$) was dissolved in PBS (pH 7.4) containing cell lysate ($500\text{ }\mu\text{L}$) and 1% DMSO (v/v), with a total volume of 2 mL. The solution was stirred at room temperature in the dark for 4 h, and then subjected to fluorescence spectral measurements.

Fluorescence Imaging. A2780 cells with a density of 1×10^5 per dish were seeded in confocal dishes (ibidi GmbH, Germany) at $37\text{ }^{\circ}\text{C}$ for 24 h, followed by the incubation with CDs, CDs-Pt(IV)-Dabsyl, or CDs-Dabsyl at $37\text{ }^{\circ}\text{C}$ for designated time. Then, A2780 cells were washed with PBS twice and used for fluorescence imaging.

Confocal Imaging. A2780 cells with a density of 1×10^5 per dish were seeded in confocal dishes at $37\text{ }^{\circ}\text{C}$ for 24 h. The cells were incubated with CDs-Pt(IV)-Dabsyl at $37\text{ }^{\circ}\text{C}$ for 6 h, and then treated with LysoTracker Red DND-99 at $37\text{ }^{\circ}\text{C}$ for 0.5 h, followed by using for confocal imaging on a Carl Zeiss LSM 800 confocal laser scanning microscope (Germany). A2780 cells only incubated with CDs-Pt(IV)-Dabsyl at $37\text{ }^{\circ}\text{C}$ for 6 h or LysoTracker Red DND-99 at $37\text{ }^{\circ}\text{C}$ for 0.5 h were used as control groups.

In Vitro Cytotoxicity. The cytotoxicity was studied according to a standard MTT assay. A2780 cells were seeded in 96-well plates with a density of 6×10^3 cells per well at $37\text{ }^{\circ}\text{C}$ for 24 h, followed by the incubation with cisplatin, CDs-Dabsyl, or CDs-Pt(IV)-Dabsyl at different concentrations at $37\text{ }^{\circ}\text{C}$ for 6 h and culturing in fresh RPMI 1640 medium at $37\text{ }^{\circ}\text{C}$ for another 66 h. Then, the medium was removed, and A2780 cells were washed by PBS and cultured in RPMI 1640 medium containing 20%

MTT at 37 °C for 5 h. After replacing the medium by DMSO (100 μ L) to lyse A2780 cells, the absorbance at 490 nm was recorded. Each experiment was conducted three times, and the cell viability was calculated with reference to the control group with no sample treatment.

4.3 Results and Discussion

4.3.1 Preparation and Characterization

To construct CD-based fluorescent sensors for real-time monitoring of cisplatin(IV) prodrug activation, CDs and Dabsyl quencher-functionalized cisplatin(IV) prodrug (Pt(IV)-Dabsyl) were synthesized first. CDs were prepared through a thermolysis approach with citric acid as the carbon source and diethylenetriamine as the surface passivation agent,^{16,17} having a uniform size of 6.3 nm as confirmed by a TEM image (Figure 8a). Moreover, two absorption peaks at 243 and 338 nm, corresponding to the π - π^* transition of the aromatic ring structure and the n - π^* transition of the carbonyl groups, were observed in the UV-vis spectrum of CDs (Figure 8b). Meanwhile, CDs exhibited excitation wavelength-dependent fluorescence (Figure 8c), with the emission peaks red-shifted from 454 to 525 nm under the excitation wavelengths from 360 to 480 nm, which was due to different band gaps from various surface states.¹⁸ At the optimal excitation wavelength of 360 nm, CDs had a QY of 27.7% (Figure 8e). In addition, CDs possessed many amino groups on the surface, at a concentration of 1.43 μ mol per milligram determined by ninhydrin colorimetric method. The presence of these amino groups was further proven by vibration bands at 1309, 1563, and 3000-3500 cm^{-1} for the stretching, bending, and stretching vibration of C-N, N-H, and O-H/N-H in the FTIR spectrum (Figure 8f), respectively.

Pt(IV)-Dabsyl was synthesized through amide condensation from the carboxylic group-functionalized cisplatin(IV) prodrug (Pt(IV)-2COOH) and amino group-functionalized Dabsyl (Dabsyl-NH₂) (Figures 2-4),¹⁹ and the successful

Figure 8. (a) TEM image of CDs. (b) Normalized UV-vis absorption spectra of CDs, Pt(IV)-Dabsyl, and CDs-Pt(IV)-Dabsyl. (c) Normalized fluorescence spectra of CDs under excitation wavelengths from 360 to 545 nm. (d) Normalized fluorescence spectrum of CDs-Pt(IV)-Dabsyl under excitation wavelength of 360 nm. (e) QYs of CDs and CDs-Pt(IV)-Dabsyl. (d) FTIR spectra of CDs, Pt(IV)-Dabsyl, and CDs-Pt(IV)-Dabsyl.

synthesis was characterized by ^1H NMR and ESI-MS spectra. The Dabsyl unit was selected on account of the spectral overlap between the absorption of the Dabsyl unit and the emission of CDs under 360 nm excitation for an effective FRET effect. Then, a CD-based fluorescent sensor CDs-Pt(IV)-Dabsyl was prepared by loading Pt(IV)-Dabsyl on the surface of CDs through the amide condensation (Figure 6). The

platinum loading percentage was determined to be 8.4 wt% by ICP-OES, corresponding to 36.6 wt% for Pt(IV)-Dabsyl, which was comparable with other nanocarriers such as mesoporous nanoparticles.²⁰ To investigate the optical properties of CDs-Pt(IV)-Dabsyl, UV-vis and fluorescence spectra were measured. As shown in Figure 8b, obvious absorption peaks at 380 and 465 nm assigned to Pt(IV)-Dabsyl were observed in CDs-Pt(IV)-Dabsyl, and its fluorescence was almost completely quenched under 360 nm excitation (Figures 8d and e), with a much lower QY of 0.6% than that of CDs. In the FTIR spectra, CDs-Pt(IV)-Dabsyl exhibited obvious vibration peaks at 1604 and 1651 cm^{-1} for Pt(IV)-Dabsyl, corresponding to bending vibration of N-H and a stretching vibration of C=O respectively, indicating the existence of Pt(IV)-Dabsyl on CDs-Pt(IV)-Dabsyl (Figure 8f). These results demonstrate that Pt(IV)-Dabsyl was successfully loaded on CDs and that the Dabsyl unit can effectively quench the blue fluorescence of CDs under 360 nm excitation.

4.3.2 Real-Time Monitoring of Cisplatin(IV) Prodrug Activation in Solution

Figure 9. Schematic illustration upon the treatment of Pt(IV)-Dabsyl with ascorbic acid and DDTC.

It is well known that Pt(II) complexes can react with DDTC to produce Pt(DDTC)₂, while the stable Pt(IV) complexes cannot.²¹ Therefore, to study the

Figure 10. (a) HPLC chromatograms for: (1, 2) Pt(IV)-Dabsyl reacted with ascorbic acid and DDTC, (3) cisplatin reacted with DDTC, and (4) Dabsyl-COOH alone. The measurement was recorded with an absorption wavelength of 290 nm for Pt(DDTC)₂ or 500 nm for Dabsyl-COOH, respectively. (b) ESI-MS spectrum upon the treatment of Pt(IV)-Dabsyl with ascorbic acid and DDTC. (c) ESI-MS spectrum upon the treatment of cisplatin with DDTC.

possible release of Pt(II) species from the Pt(IV)-Dabsyl prodrug under reductive conditions (Figure 9), Pt(IV)-Dabsyl was treated with DDTC in the presence of a reducing agent, ascorbic acid, followed by product characterization *via* HPLC and ESI-MS. As indicated in Figure 10, Dabsyl-COOH and Pt(DDTC)₂ were detected with retention time of 11.3 and 18.2 min, as well as mass-to-charge ratios (*m/z*) of 448.29 ([*M*+*H*]⁺) and 490.82 ([*M*]⁺), respectively, while only Pt(DDTC)₂ was detected with retention time of 18.2 min and *m/z* of 490.81 ([*M*]⁺) when cisplatin was reacted with DDTC. The results prove that the Pt(IV)-Dabsyl prodrug can be effectively activated to Pt(II) species by the simultaneous removal of axial moieties

upon the treatment with reducing agents.

Figure 11. (a) Fluorescence spectra under excitation wavelength of 360 nm at 0 and 6 h, (b-d) time-dependent fluorescence intensity at 460, 535, and 610 nm under excitation wavelengths of 360, 480, and 545 nm, and (e, f) corresponding F_{460}/F_{535} and F_{460}/F_{610} ratio for CDs-Pt(IV)-Dabsyl (platinum concentration of $1.44 \mu\text{M}$) treated with ascorbic acid (2.825 mM) in DMSO/PBS ($v/v = 1/99$).

In order to investigate the real-time activation of the cisplatin(IV) prodrug in a reductive environment, CDs-Pt(IV)-Dabsyl was incubated with ascorbic acid in DMSO/PBS ($v/v = 1/99$), and the fluorescence spectra under different excitation wavelengths of 360, 480, and 545 nm were recorded at different time points. As

shown in Figures 11a and b, the fluorescence intensity of CDs-Pt(IV)-Dabsyl at 460 nm under an excitation wavelength of 360 nm increased significantly over time and reached a plateau after 4 h. The blue fluorescence recovery was owing to the release of Pt(II) species and the removal of the Dabsyl unit from the surface of CDs to terminate the FRET effect. However, the fluorescence intensity of CDs-Pt(IV)-Dabsyl at 535 and 610 nm under corresponding excitation wavelengths of 480 and 545 nm remained almost the same over time (Figures 11c and d). The results prove that the

Figure 12. (a) F_{460}/F_{610} ratio for CDs-Pt(IV)-Dabsyl (platinum concentration of 1.44 μ M) treated with different concentrations of ascorbic acid for 4 h in DMSO/PBS (v/v = 1/99). (b) F_{460}/F_{610} ratio for CDs-Pt(IV)-Dabsyl (platinum concentration of 1.44 μ M) treated with ascorbic acid (2.825 mM) for 4 h in DMSO/PBS with different DMSO ratio (v/v). (c) F_{460}/F_{610} ratios of CDs-Pt(IV)-Dabsyl (platinum concentration of 1.44 μ M) incubated with different biomolecules (2.825 mM) for 4 h in DMSO/PBS (v/v = 1/99). (d) Linear relationship between fluorescence intensity at 460 nm and platinum concentration of CDs-Pt(IV)-Dabsyl upon the treatment with ascorbic acid (2.825 mM) for 4 h in DMSO/PBS (v/v = 1/99).

Dabsyl unit can only quench the blue fluorescence of CDs and that constant green or red fluorescence can be used as an internal reference for ratiometric monitoring of cisplatin(IV) prodrug reduction. In view of the higher fluorescence intensity ratio between 460 and 610 nm (F_{460}/F_{610}) than that between 460 and 535 nm (F_{460}/F_{535}), the F_{460}/F_{610} ratio under 360 and 545 nm excitation was used in the following studies (Figures 11e and f). Moreover, to obtain the optimal condition for F_{460}/F_{610} ratio recovery, several parameters including ascorbic acid concentration and DMSO ratio were optimized. The optimal ascorbic acid concentration and DMSO ratio were 2.825 mM and 1% (v/v) for CDs-Pt(IV)-Dabsyl with a platinum concentration of 1.44 μM (Figures 12a and b). More importantly, in the presence of other bioacids, the F_{460}/F_{610} ratio exhibited a negligible change, confirming the high stability of

Figure 13. (a) UV-vis spectra of CDs, Dabsyl-COOH, and CDs-Dabsyl. (b) Normalized fluorescence spectrum of CDs-Dabsyl. (c) F_{460}/F_{610} ratio for CDs-Dabsyl-Pt(IV) (platinum concentration of 1.44 μM) or CDs-Dabsyl (Dabsyl concentration of 1.44 μM) treated without or with GSH (2.825 mM), ascorbic acid (2.825 mM), or A2780 cell lysate for 4 h in DMSO/PBS (v/v = 1/99). p values: *** p < 0.001.

CDs-Pt(IV)-Dabsyl (Figure 12c). Only by the treatment with reducing agents such as ascorbic acid or GSH, the F_{460}/F_{610} ratio revealed a significant increase, proving the activation of cisplatin(IV) prodrug under reductive conditions. For comparison, reduction-nonresponsive CDs-Dabsyl, with the Dabsyl unit loaded directly on the surface of CDs through the amide bond formation, was also prepared (Figures 13a and b). Much lower F_{460}/F_{610} ratio was determined when CDs-Dabsyl was incubated with GSH or ascorbic acid for 4 h (Figure 13c), further confirming the reduction-responsive property of the cisplatin(IV) prodrug.

In order to access the possibility to quantify the activated Pt(II) species, CDs-Pt(IV)-Dabsyl with different platinum concentrations was incubated with ascorbic acid, and the fluorescence spectra under 360 nm excitation were recorded. As shown in Figure 12d, there was a good linear relationship between the fluorescence intensity at 460 nm and the platinum concentration, and the increased fluorescence intensity was owing to the enhanced release of Pt(II) species and the Dabsyl unit. The results indicate that the activation of the cisplatin(IV) prodrug can be easily monitored based on the alteration of fluorescence intensity at 460 nm.

It is well known that various reducing agents are present inside the cells, such as GSH with a concentration of 2-10 mM that can increase to a higher value when cells become cancerous.²² Therefore, in order to demonstrate the activation of the cisplatin(IV) prodrug under intracellular conditions, CDs-Pt(IV)-Dabsyl was treated with A2780 cell lysate. After 4 h incubation, the F_{460}/F_{610} ratio was similar to that in the presence of ascorbic acid (Figure 13c). However, when reduction-nonresponsive CDs-Dabsyl was incubated with A2780 cell lysate, the F_{460}/F_{610} ratio was relatively low (Figure 13c). These results confirm that the cisplatin(IV) prodrug can be successfully activated inside living cells.

4.3.3 Monitoring Cisplatin(IV) Prodrug Activation in Living Cells

To investigate the capability of CDs-Pt(IV)-Dabsyl for monitoring cisplatin(IV)

Figure 14. Fluorescence images of A2780 cells upon the incubation with (a-c) CDs-Pt(IV)-Dabsyl, (d-f) CDs-Dabsyl, or (g-i) for different time points: (a, d, g) 1 h, (b, e, h) 4 h, and (c, f, i) 6 h, and (j) the corresponding ratio between blue channel and red channel ($F_{\text{Blue}}/F_{\text{Red}}$). p values: ** $p < 0.01$, *** $p < 0.001$. The blue fluorescence was measured with an excitation wavelength of 350 nm and an emission wavelength range of 460 ± 25 nm, and the red fluorescence was measured with an excitation wavelength of 545 nm and an emission wavelength range of 610 ± 37.5 nm. Scale bar = 20 μm .

prodrug activation in living cells, CDs-Pt(IV)-Dabsyl was incubated with A2780 cells for varied times of 1, 4, and 6 h, followed by fluorescence imaging under different excitation wavelengths of 350, 480, and 545 nm. As revealed in Figures 14a-c, and 15a-c, A2780 cells showed intensive green and red fluorescence under 480 and 545 nm excitation, and the fluorescence intensity had no significant change upon

Figure 15. Fluorescence images of A2780 cells upon the incubation with (a-c) CDs-Pt(IV)-Dabsyl, (d-f) CDs-Dabsyl, or (g-i) for different time points: (a, d, g) 1 h, (b, e, h) 4 h, and (c, f, i) 6 h. The blue fluorescence was measured with an excitation wavelength of 350 nm and an emission wavelength range of 460 ± 25 nm, and the green fluorescence was measured with an excitation wavelength of 480 nm and an emission wavelength range of 535 ± 25 nm. Scale bar = 20 μ m.

increasing the incubation time, confirming the rapid uptake of CDs by cells. Meanwhile, the intracellular blue fluorescence under 350 nm excitation increased over time, proving successful reduction of the cisplatin(IV) prodrug and the removal of the Dabsyl quencher from the CD surface to terminate FRET effect inside living cells. Using the red fluorescence as an internal reference, the time-dependent enhancing ratio between blue and red fluorescence could also demonstrate the gradual release of Pt(II) species in intracellular reductive environment (Figure 14j). In parallel

experiments, A2780 cells incubated with reduction-nonresponsive CDs-Dabsyl exhibited strong green and red fluorescence, while blue fluorescence could not be recovered upon time (Figures 14d-f and 15d-f), and A2780 cells incubated with CDs revealed constant blue, green, and red fluorescence over time (Figures 14g-i and 15g-i). Most importantly, the ratio between blue and red fluorescence of CDs-Pt(IV)-Dabsyl at 6 h showed a significant difference with a *p* value less than 0.001 in comparison to the corresponding ratio of CDs-Dabsyl (Figure 14j). These results prove that CDs-Pt(IV)-Dabsyl can be employed for real-time monitoring of cisplatin(IV) prodrug activation in living cells by ratiometric fluorescence imaging.

Figure 16. Confocal imaging of A2780 cells upon the treatment with (a) CDs-Pt(IV)-Dabsyl for 6 h, (b) LysoTracker Red DND-99 for 0.5 h, or (c) CDs-Pt(IV)-Dabsyl for 6 h and LysoTracker Red DND-99 for 0.5 h. The blue fluorescence was measured with an excitation wavelength of 405 nm and an emission wavelength range of 460 ± 40 nm, and the red fluorescence was measured with an excitation wavelength of 561 nm and an emission wavelength range of 590 ± 20 nm. Scale bar = 5 μ m.

In order to visualize cellular compartments that were responsive for redox-based processes, A2780 cells were first incubated with CDs-Pt(IV)-Dabsyl for 6 h, followed by labeling with a lysosome-specific dye, LysoTracker Red DND-99, for 0.5 h (Figure 16). Confocal imaging showed that the recovered blue fluorescence of

CDs-Pt(IV)-Dabsyl overlapped well with the red fluorescence of lysosome tracker, while only blue or red fluorescence was recorded for the control groups that were only treated with CDs-Pt(IV)-Dabsyl or LysoTracker Red DND-99. These results further demonstrate that CDs-Pt(IV)-Dabsyl is reduced in lysosomes to release Pt(II) species and that the Pt(II) species presumably diffuse into the cell nucleus to crosslink with DNA to exhibit cytotoxicity.

4.3.4 Evaluation of Cytotoxicity

Figure 17. Cell viability of A2780 cells treated with (a) CDs-Pt(IV)-Dabsyl or cisplatin at different platinum concentrations (μM) and (b) CDs-Pt(IV)-Dabsyl or CDs-Dabsyl at different nanoparticle concentrations ($\mu\text{g/mL}$).

To evaluate the cytotoxicity of CDs-Pt(IV)-Dabsyl, MTT assay was studied against A2780 cells. As shown in Figure 17, reduction-nonresponsive CDs-Dabsyl showed no obvious cytotoxicity to A2780 cells, with an half maximal inhibitory concentration (IC_{50}) value as high as $688.55 \mu\text{g mL}^{-1}$ for nanoparticles. In another parallel experiment, cisplatin showed dose-dependent cytotoxicity, with an IC_{50} value of $8.02 \mu\text{M}$ for platinum. However, CDs-Pt(IV)-Dabsyl revealed an IC_{50} value of $1.72 \mu\text{M}$ for platinum, corresponding to nanoparticle concentration of $3.99 \mu\text{g mL}^{-1}$, demonstrating the successful release of Pt(II) species under intracellular reductive conditions to exhibit cytotoxicity. In comparison to the IC_{50} value of cisplatin, the decreased IC_{50} concentration of CDs-Pt(IV)-Dabsyl should result from the enhanced platinum uptake by the utilization of CDs as nanocarriers.²³ These results show that

CDs-Pt(IV)-Dabsyl can also be used as an effective system for cancer treatment.

4.4 Conclusion

In summary, an excitation-wavelength-dependent and full-color-emissive CD-based ratiometric fluorescent sensor has been successfully developed for real-time monitoring of cisplatin(IV) prodrug activation in living cells. Thanks to the unaltered green or red fluorescence and enhanced blue fluorescence of CDs under intracellular reductive conditions, the increased intensity ratio of blue-to-green or blue-to-red fluorescence can be indicative of the release of active Pt(II) species for cancer treatment. The present ratiometric design, using green or red emission as the internal standard, provides new insights into the activation of cisplatin(IV) prodrug in sophisticated biological microenvironments and thus holds great potential to promote the application of anticancer prodrugs in cancer theranostics.

4.5 References

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Chapter 5

Conclusion and Outlook

5.1 Conclusion

In this thesis, tumor extracellular microenvironment responsive charge-convertible or active-targeting CDs were successfully developed for enhanced cancer theranostics with little side effects.

Firstly, charge-convertible CDs, capable of conversion from negative charge/PEGylation to positive charge upon hydrolysis of dimethylmaleic amide at mildly acidic tumor extracellular pH, were prepared, which could overcome the limitations of single surface properties of CDs, such as the prolonged circulation time but decreased cellular uptake for negatively charged or PEGylated CDs, or rapid clearance from blood circulation but facilitated cellular internalization and endosome escape for positively charged CDs. Therefore, by making best use of the advantages of negative charge, PEGylation, and positive charge, charge-convertible CDs could significantly inhibit tumor growth and possess low side effects toward carcinoma-bearing mice when compared with noncharge-convertible ones.

Secondly, active-targeting CDs, with the ability to expose the inner targeting ligand by hydrolysis of monomethoxy-PEG-functionalized benzoic-imine under tumor extracellular condition, were constructed, which could surmount the disadvantages of directly loading targeting ligands on the surface of CDs, including early clearance from blood circulation and potential side effects. Thus, active-targeting CDs demonstrated better therapeutic outcome against cancer cells with high receptor expression at tumor extracellular pH, in comparison to that in physiological condition or toward cancer cells with low receptor expression.

In addition, CD-based ratiometric fluorescent method was also proposed to illustrate the intracellular activation mechanism of anticancer prodrug used in above systems. By using green or red fluorescence of CDs as an internal reference, the gradually enhanced intensity ratio of blue-to-green or blue-to-red fluorescence could avoid the influence from uncontrolled microenvironment changes and indicate the real-time reduction of anticancer prodrug in solution or in living cells.

To sum up, these designs can provide great opportunities to take full advantages of each surface properties and the difference between tumor extracellular microenvironment and normal physiological condition for developing safe and effective CDs for potential cancer theranostics.

5.2 Outlook

In order to further advance the biomedical application of CDs, the following researches are recommended. Firstly, although CDs have exhibited little toxicity both *in vitro* and *in vivo*, long-term toxicity, pharmacokinetics, or immunogenic response of CDs still need to be demonstrated, which are of great significance for clinical use. Secondly, CDs often exhibit absorption or emission in the visible region, which suffer from the utilization of high-energy excitation wavelength with low penetration depth and potential tissue damage for *in vivo* experiments. Therefore, CDs with upconversion luminescence or strong emission in the NIR-I (750-900 nm) or NIR-II (1000-1700 nm) regions need to be prepared to achieve deep tissue penetration and low background fluorescence. Thirdly, despite the success of CDs as single-modality therapeutic agents under the guidance of fluorescence imaging, CDs with multimodality imaging and intrinsic therapeutic effects are recommended to be synthesized for enhanced cancer theranostics.

Moreover, besides CDs, numerous nanocarriers have been designed to treat cancer, however, very limited nanotherapeutics are approved for utilization in clinic. For the future development of nanocarriers, researches can be focused on the following

directions. Firstly, conventional or biomimetic nanocarriers with excellent biocompatibility, prolonged blood circulation half-life, and easy body clearance are recommended to be prepared. In addition, the potential influence of size, shape, and surface properties of nanoparticles to the therapeutic efficacy should be taken into consideration. Secondly, multifunctional nanocarriers can be designed for effective cancer theranostics by integrating different bioimaging modalities with various therapeutic methods. Thirdly, specific nanocarriers loading with small-molecule immunotherapy agent can also be developed for combined cancer chemo/photodynamic/photothermal/radio-therapy and immunotherapy. Thus, more breakthroughs are expected for translating nanocarrier-based therapeutics into clinical uses in the near future.

List of Publications

1. **Tao Feng**, Xiangzhao Ai, Guanghui An,* Piaoping Yang,* Yanli Zhao.* Charge-Convertible Carbon Dots for Imaging-Guided Drug Delivery with Enhanced In Vivo Cancer Therapeutic Efficiency. *ACS Nano* **2016**, *10*, 4410-4420.
2. **Tao Feng**, Xiangzhao Ai, Huimin Ong, Yanli Zhao.* Dual-Responsive Carbon Dots for Tumor Extracellular Microenvironment Triggered Targeting and Enhanced Anticancer Drug Delivery. *ACS Appl. Mater. Interfaces* **2016**, *8*, 18732-18740.
3. **Tao Feng**, Huoy Jing Chua, Yanli Zhao.* Reduction-Responsive Carbon Dots for Real-Time Ratiometric Monitoring of Anticancer Prodrug Activation in Living Cells. *ACS Biomater. Sci. Eng.* **2017**, *3*, 1535-1541.
4. **Tao Feng**, Yanli Zhao.* Nanomaterial-Based Drug Delivery Carriers for Cancer Therapy. In *Springer Briefs in Applied Sciences and Technology · Nanotheranostics*. Singapore: Springer, **2017**. ISBN: 978-981-10-3297-4 (Print), 978-981-10-3299-8 (Online).
5. **Tao Feng**, Yanli Zhao.* Responsive Carbon Dots for Anticancer Drug Delivery. Book chapter in *Basic Protocols in Pharmaceutical Nanotechnology*. New York: Humana Press, **2017**.