Transition metal chalcogenides based molecular probes in biomedical applications

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Transition Metal Chalcogenides Based Molecular Probes in Biomedical Applications

This thesis is presented as part of the requirements for the award of the Degree of

Doctor of Philosophy

from

University of Wollongong

By

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Institute for Superconducting and Electronic Materials
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March 2017
Certification

I, Shaohua Zhang, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Institute for Superconducting and Electronic Materials (ISEM), University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

Shaohua Zhang
March 20, 2017
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Abstract

Various emerging nanotechnologies possess unprecedented advantages in biomedical applications. Among them, transition metal chalcogenide nanomaterials represent a novel type of material with versatile physicochemical properties that have enabled new horizons for applications in both cancer diagnosis and therapy. Studies have demonstrated that transition metal chalcogenide nanomaterials may be used in diverse aspects, including i) bioimaging for organ visualization or cancer detection due to their high propensity towards tumour markers; ii) molecular imaging for guided tumour therapies; and iii) drug and gene loading, for photothermal and photodynamic cancer therapies. In this doctoral thesis, the construction of three transition metal chalcogenide based molecular probes for biomedical applications are presented.

Firstly, we synthesized a seeded watermelon-like mesoporous nanostructure (mSiO$_2$@CdTe@SiO$_2$, mSQS), composed of a novel dendritic mesoporous silica core, fluorescent CdTe quantum dots (QDs), and a protective solid silica shell, by loading the QDs into dendritic mesoporous silica nanoparticles through electrostatic interaction, and then coating them with a solid silica shell by the modified Stöber method. The shell thickness of mSQS can be tuned from 0 to 32 nm as desired by controlling the reaction parameters, including the amount of silica precursor, tetraethyl orthosilicate, that is introduced, the solvent ratio (H$_2$O: ethanol), and the amount of catalyst (NH$_3$⋅H$_2$O). These fluorescent mSiO$_2$@QDs@SiO$_2$ nanoparticles possess excellent stability and thickness-dependent cytotoxicity, and have been successfully applied in bioimaging.

Secondly, we report ultra-small polyethylene glycol treated (PEGylated) Cu$_{2-x}$Se nanoparticles with strong near-infrared absorption prepared by an ambient aqueous method. The resultant water-soluble and biocompatible nanoparticles are demonstrated to be a novel nanotheranostic agent for effective deep-tissue photoacoustic imaging, computed tomography imaging, single-photon emission computed tomography imaging, and photothermal therapy of cancer.

Finally, on the basis of the Cu$_{2-x}$Se probe, we developed a facile and chelator-free doping method for constructing Fe-doped Cu$_{2-x}$Se nanoparticles. The position and intensity of the near-infrared-localized surface plasmon resonance in the hybrid
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nanostructure can be tuned by altering the feeding amount of Fe$^{3+}$ ion precursors. Owing to their tunable near-infrared absorption and Fe doping, the hybrid nanostructures are demonstrated be a novel nanotheranostic agent for effective deep-tissue photoacoustic imaging, magnetic resonance imaging, and photothermal therapy of cancer.
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## Nomenclature

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<th>Full name</th>
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<tr>
<td>0 D</td>
<td>Zero dimensional</td>
</tr>
<tr>
<td>1 D</td>
<td>One dimensional</td>
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<td>2 D</td>
<td>Two dimensional</td>
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<td>3 D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>4T1 cells</td>
<td>Murine breast cancer cells</td>
</tr>
<tr>
<td>A549 cells</td>
<td>Human lung cancer cells</td>
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<tr>
<td>AAO</td>
<td>Aluminium oxide</td>
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<tr>
<td>a.u.</td>
<td>Arbitrary unit</td>
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<tr>
<td>BET</td>
<td>Brunauer Emmett Teller</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DETA</td>
<td>Diethylenetriamine</td>
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<tr>
<td>DIW</td>
<td>Deionized water</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DOS</td>
<td>Density of states</td>
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<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<td>EDS</td>
<td>Energy dispersive X-ray spectroscopy</td>
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<td>EPR</td>
<td>Enhanced permeability and retention effect</td>
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<tr>
<td>fcc</td>
<td>Face centered cubic</td>
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<tr>
<td>FESEM</td>
<td>Field emission scanning electron microscopy</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier transform</td>
</tr>
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<td>Nomenclature</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectrum</td>
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<td>HAADF</td>
<td>High-angle annular dark field</td>
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<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<td>HT</td>
<td>High temperature</td>
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<td>HRTEM</td>
<td>High-resolution transmission electron microscopy</td>
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<td>ICP-AES</td>
<td>Induced couple plasma-atomic emission microscopy</td>
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<td>ICP-OES</td>
<td>Inductively coupled plasma-optical emission</td>
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<td>spectrometry</td>
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<td>ITO</td>
<td>Indium-tin oxide</td>
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<td>JCPDS</td>
<td>Joint committee on powder diffraction standards</td>
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<tr>
<td>LED</td>
<td>Light emitting diodes</td>
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<td>MCs</td>
<td>Metal chalcogenides</td>
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<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTT</td>
<td>Methyl thiazolyl tetrazolium</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>NIR</td>
<td>Near-infrared</td>
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<td>NP</td>
<td>Nanoparticles</td>
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<td>PA</td>
<td>Photoacoustic</td>
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<td>Photoacoustic imaging</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PEG</td>
<td>Polyethylene glycol</td>
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<td>PET</td>
<td>Positron emission tomography</td>
</tr>
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<tr>
<td>PTT</td>
<td>Photothermal therapy</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
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<td>QD</td>
<td>Quantum dot</td>
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<td>RAW 264.7 cells</td>
<td>Murine macrophage cells</td>
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<td>RES</td>
<td>Reticuloendothelial system</td>
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<td>RS</td>
<td>Rock salt type structure</td>
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<td>RT</td>
<td>Room temperature</td>
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<td>SAED</td>
<td>Selected area electron diffraction</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SPECT</td>
<td>Single-Photon Emission Computed Tomography</td>
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<tr>
<td>STEM</td>
<td>Scanning tunneling electron microscopy</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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<td>TGA</td>
<td>Thermogravimetric analysis</td>
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<td>TMCs</td>
<td>Transition metal chalcogenides</td>
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<td>TMDs</td>
<td>Transition metal dichalcogenides</td>
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<td>TOP</td>
<td>Triocylphosphine</td>
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<td>Triocylphosphine oxide</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<td>UV-vis</td>
<td>Ultraviolet-visible spectroscopy</td>
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<td>UV-Vis-NIR</td>
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<td>XPS</td>
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<td>2θ</td>
<td>Detection angle in XRD</td>
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<td>Extinction coefficient</td>
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<td>Bq</td>
<td>Becquerel</td>
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<td>d</td>
<td>Lattice spacing</td>
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<td>c</td>
<td>Heat capacity</td>
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<td>e</td>
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<td>$\lambda$</td>
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<tr>
<td>$\eta$</td>
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Chapter 1 Introduction

1.1 General background

Nanotechnology (nanotech) involves manipulation of matter on an atomic, molecular, and supramolecular scale. A more generalized definition of nanotechnology is the manipulation of matter with at least one dimension from 1 to 100 nanometers in size. One nanometer (nm) is one billionth, or $10^{-9}$, of a meter. By comparison, typical carbon-carbon bond lengths, or the spacing between these atoms in a molecule, are in the range of 0.12-0.15 nm, and a DNA double-helix has a diameter of around 2 nm (Figure 1.1). Several phenomena exist related to these very small dimensions, including statistical mechanical effects and quantum mechanical effects, for example, the “quantum size effect”, where the electronic properties of solids are altered with great reductions in particle size. The “quantum size effect” does not come into play by going from macro- to microscale dimensions. Quantum effects can become significant, however, when the nanometer size range is reached, typically at lengths of 100 nanometers or less, the so-called quantum realm. Additionally, a number of physical (mechanical, electrical, optical, etc.) properties change when compared to macroscopic systems. One example is the increase in the surface area to volume ratio, altering the mechanical, thermal, and catalytic properties of materials. Materials reduced to the nanoscale can show different properties compared to what they exhibit on the macroscale, enabling unique applications. For instance, opaque substances can become transparent (copper); stable materials can turn combustible (aluminium); and insoluble materials may become soluble (gold).

One of the most promising applications of nanotechnology is in the field of medicine. Indeed, a whole new field of “nanomedicine” is emerging. Nanomedicine ranges from the medical applications of nanomaterials and biological devices, to nanoelectronic biosensors, and even possible future applications of molecular nanotechnology such as biological machines. With sizes comparable to biological molecules and structures, but orders of magnitude smaller than human cells, nanoparticles (NPs) can offer unprecedented interactions with biomolecules, both on the surface of and inside the cells, which may revolutionize disease diagnosis and treatment. Upon incorporation of certain targeting moieties, these NPs can be
employed to interrogate specific molecular and cellular events in living systems. Therefore, nanomaterials can be useful for both *in vivo* and *in vitro* biomedical research and applications. Thus far, the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicles.\[^2\] There are three applications of nanomedicine: 1) nanomedicine for diagnostic purposes, including nanotechnology-based biosensors for *in vitro* and *ex vivo* diagnosis and nanoprobe for *in vivo* imaging; 2) nanomedicine as a potential platform for therapeutic applications; 3) nanotheranostics,\[^3\] which combines diagnostic and therapeutic capabilities into a single nanoagent (Figure 1.2).

![Comparison of sizes of nanomaterials](image)

**Figure 1.1** Comparison of sizes of nanomaterials.\[^1\]
Functional agents such as imaging agents and therapeutic agents serve as molecular probes, which are of significance for developing theranostic platforms. Advanced functional nanomaterials and relevant modification nanotechnologies contribute a great deal to the fabrication of various functional agents. In particular, much attention is being paid to nanostructured transition metal chalcogenide (TMC) materials, including metal sulphides, selenides and tellurides, which include at least one metal cation and one chalcogen anion. TMC nanostructures have an incredible wealth of properties because chalcogenide elements are easy to form into compounds with different kinds of structures, and compositions. These have been widely investigated for diverse applications in the fields of energy and biomedicine, such as solar cells, fuel cells, Li/Na-ion batteries, supercapacitors, thermoelectric devices, and water splitting; bioimaging, including fluorescent labels for fluorescence imaging, photoacoustic imaging, and surface enhanced Raman spectroscopy based imaging; photothermal therapy; and drug delivery. TMC quantum dots (QDs) represent one kind of promising probe for biomedical imaging of living tissue. For example, the development of near-infrared (NIR) quantum dots with maximum emission spectra ranging from 700 to 900 nm may overcome the limitations that endogenous
autofluorescence in the visible spectrum may interfere with signals from labelled probes and may even mask weak labelling signals.\textsuperscript{76} Polydentate phosphine-coated biocompatible NIR CdTe/CdSe QDs were developed to serve as imaging agents to direct lymph node surgery under the guidance of fluorescence imaging. A small dose of injected NIR QDs (400 pmol) enabled real-time imaging of a sentinel lymph node (approximately one centimetre depth under the skin) in both a mouse and a pig.\textsuperscript{77} As another type of TMCs, copper chalcogenide semiconductors are promising candidates as photothermal agents owing to their superior properties, such as low cost, high stability, environmental compatibility, and the intrinsic NIR region absorption derived from the \textit{d-d} energy band transitions.

In 2010, Li and co-workers\textsuperscript{78} first synthesized thioglycolic acid-stabilized CuS nanoparticles via a wet chemistry method and applied it as a new type of agent for photothermal ablation of cancer cells. These uniform CuS nanoparticles have a mean size of approximately 3 nm and show an increased absorption band in the NIR region, with maximum absorption at 900 nm. A further cell photothermal destruction assay of HeLa cells and toxicity assay indicated that the CuS nanoparticles displayed outstanding photothermal ablation efficiency and minimal cytotoxic effects. Layered transition metal dichalcogenides, such as MoS\textsubscript{2}, MoSe\textsubscript{2}, WS\textsubscript{2}, and WSe\textsubscript{2}, also showed great potential as photothermal agents. Chou et al. demonstrated for the first time that MoS\textsubscript{2} nanosheets could serve as a new NIR photothermal agent, with approximately 7.8 times greater NIR absorbance relative to graphene oxide (GO) on a per mass basis.\textsuperscript{79} The extinction coefficient of MoS\textsubscript{2} nanosheets at 800 nm was 29.2 L/g·cm, which was higher than that of gold nanorods (AuNRs; 13.9 L/g·cm) and comparable to that of reduced GO (RGO; 24.6 L/g·cm). In another work, bismuth selenide (Bi\textsubscript{2}Se\textsubscript{3}) nanoplates were used as a theranostic platform for simultaneous cancer imaging and therapy via local administration into tumours.\textsuperscript{80}

1.2 Challenges facing nanomedicine

The advanced properties of nanomaterials (molecular platforms, surface to volume ratio, shape/size control, and inherent optical response) make the field of nanomedicine diverse and thus full of potential. Despite the remarkable progress over the last decades, there are several critical challenges facing nanomedical research for widespread use of TMC-based agents and potential clinical translation.
The first important problem is how to achieve appropriate nanostructured TMCs. The physicochemical characteristics of nanoparticles, such as size, shape, and surface chemistry, are critical for tumor-specific accumulation. Rigid nanoparticles with sizes in the range of 10-100 nm, according to our understanding of enhanced permeability and retention (EPR) effect,\cite{81,82} have prolonged blood circulation times because they are large enough to avoid renal clearance but at the same time small enough to avoid capture by the reticuloendothelial system (RES).\cite{83} Surface chemistry influences both systemic-level processes (such as opsonization) and cellular-level processes (such as cell uptake). Cationic surface charges enhance cellular uptake of nanoparticles due to increased electrostatic interactions with cell membranes, while at the systemic level, positive charges can cause opsonization, i.e., adsorption of serum proteins and subsequent clearance in the RES.\cite{84} Therefore, hydrophilic, i.e. water-soluble, nanoparticles with a size of 10 nm to 100 nm and neutral or low negative charge on their surface are more appropriate and therefore preferred. In general, the synthesis of water-soluble TMCs is mainly achieved through two synthetic routes: 1) indirect organic-phase synthesis followed by a ligand-exchange process; 2) direct aqueous-phase synthesis. Although yielding good-quality NPs with a narrow size distribution and high crystallinity, the organic-phase synthetic route is strongly limited by the high-cost, unfriendly experimental conditions and the laborious ligand-exchange process.\cite{62,69,70} Alternatively, the direct aqueous synthetic route provides a facile and greener approach to acquiring water-soluble TMCs.\cite{5,85,86} Nevertheless, due to the strong inherent dipole-dipole interaction or lack of suitable ligands, the NPs often tend to aggregate.\cite{87-89} Consequently, it is highly desirable to develop an efficient synthetic approach for achieving aqueous TMCs with excellent colloidal stability and size tunability.

Aside from the fabrication of suitable TMCs, there are two important issues for further clinical applications of those TMCs in nanomedicine. One is the potential long-term safety concerns relating to the TMCs, especially those inorganic ones that are not biodegradable and would be retained inside the body for long periods of time after administration. For example, the potential toxicity of QDs is still a major concern, due to their chemical compositions, which include toxic elements such as Cd, Se, Hg, Pb, As, etc. Although a large number of reports have demonstrated that many inorganic nanomaterials, such as gold and carbon nanomaterials, if they have
appropriate surface coatings and sizes, are not noticeably toxic in vitro and in vivo in the tested dose ranges, it could still be extremely difficult for those nanoagents to finally achieve US Food and Drug Administration (FDA) approval for clinical use. The other issue is limited light penetration depth in phototherapy for cancer. For photothermal therapy, NIR absorbing photothermal agents are absolutely preferred over those with visible absorption, considering the reduced light absorbance and scattering in the NIR window. Even if NIR light is used to trigger phototherapy, the effective penetration depth of NIR light is still usually limited to no deeper than 1 cm. For some types of cancers, such as skin cancers, oral cancer, esophageal cancer, and even stomach cancers, light can be induced to locally irradiate the tumors with the help of certain facilities (e.g., gastroscopy, endoscopy). For other types of cancers with tumors located deep inside the body, effective phototherapy would require the appropriate design of medical devices (e.g., with optical fibers) that can deliver light into those deep lesions.

1.3 Objectives of the research and thesis structure

The main goal of this doctoral work is to construct robust transition metal chalcogenide based molecular probes for bioapplication, including bioimaging and therapy. For fluorescent imaging, the traditional CdTe QDs were selected as my first research subject, because their toxicity and stability can be significantly reduced and enhanced, respectively, by coating them with a silica layer. In contrast to the CdTe/SiO₂ probe, multifunctional nanotherapeutic probes (Cu_{2-x}Se nanoparticles) were fabricated, which demonstrated great feasibility for multimodal imaging-guided photothermal therapy of cancer. Based on the Cu_{2-x}Se probes, the influences of Fe-doping of the nanostructures on the properties and application were well studied. The scope of the research in this doctoral thesis is briefly outlined as follows:

Chapter 1 introduces the general background, along with some challenges facing nanomedicine, the objectives of this doctoral research, and an outline of this thesis.

Chapter 2 presents a comprehensive literature review on nanostructured transition metal chalcogenides, including their fabrication approaches, structural features, and related applications.
Chapter 1 Introduction

Chapter 3 introduces the chemicals and materials, and synthesis methods, as well as the generally used characterization techniques and and facilities, including some bioimaging techniques used in the thesis.

Chapter 4 introduces a fluorescent seeded-watermelon-like mesoporous nanostructure (mSiO$_2$@CdTe@SiO$_2$) for bioimaging.

Chapter 5 reports ultra-small PEGylated Cu$_{2-x}$Se nanoparticles as a multifunctional nanotherapeutic probe for multimodal imaging-guided photothermal therapy of cancer.

Chapter 6 develops a facile and chelator-free doping method for constructing Fe-doped Cu$_{2-x}$Se nanoparticles, which were demonstrated be a novel nanotheranostic agent for effective deep-tissue photoacoustic imaging, magnetic resonance imaging, and photothermal therapy of cancer.

Chapter 7 summarizes the work in this doctoral work and provides some prospects for further research work related to both the synthesis and applications of nanostructured transition metal chalcogenide materials.

1.4 References


Chapter 2 Literature Review

2.1 Structures and properties of transition metal chalcogenides

Chalcogens are the chemical elements of group 16 (i.e., group VIA) of the periodic table, including oxygen (O), sulfur (S), selenium (Se), tellurium (Te), and polonium (Po). A transition metal (Group 3 to Group 12) chalcogenide is a chemical compound consisting of at least one chalcogen anion and at least one more electropositive transition metal element. In general, the term chalcogenide is more commonly reserved for sulfides, selenides, and tellurides, rather than oxides and polonium compounds, due to the extremely strong non-metallic properties of oxygen and the strong metallic properties of polonium. There are various kinds of transition metal chalcogenides with different structures and compositions, such as ScS₂, TiS₂, TiSe₂; CrS₂, MnSe₂, FeS₂, Fe₃S₄, FeSe₂; CoSe₂, Cu₁.₉₇S, Cu₂S; CuSe, Cu₂₄Se; CdTe, HgS, etc., owing to the non-metallic properties of chalcogens and the unfilled d orbitals of transition metals. The family of transition metal chalcogenides is huge and diverse, and could be categorized in many different ways. For example, simply in terms of the chalcogen elements, they could be divided into sulfides, selenides, tellurides, and multi-chalcogen chalcogenides; while according to the number of elements, binary, ternary, quaternary, and multi-chalcogenides are defined. Most of the main group metal chalcogenides are stoichiometric compounds, but for transition metals, due to their unfilled d orbitals, it is easy to form off-stoichiometric transition metal chalcogenides (TMCs). Non-stoichiometric binary TMCs can be grouped into “chalcogen rich” phases, such as TaS₂, TiS₂, TiSe₂, and Re₂S₇, and “metal rich” phases, such as Ta₆S₇, Ta₅S₈, Ta₈Te₅, and Gd₂Te. The complexity of metal-chalcogen bonding, as well as excess chalcogen-chalcogen or metal-metal bonding, further increases the diversity of structures for the binary transition chalcogenides. For example, based on the degree of sulphur-sulphur bonding, the transition metal sulfides could be classified as “sulphur-rich” phases featuring S-S bonding (such as FeS₂), usually in the form of persulfide units such as S₂⁻, or compounds such as TaS₃ and TiS₃ containing both per- and monosulfide units. In addition, there is also a wide class of transition metal sulphides, including members with isolated sulfide (S²⁻) centers, e.g., MoS₂ and FeS. On the other hand, one would encounter metal-rich phases (such as Ta₃S₂) exhibiting metal-metal bonding, even to the point that they
should be considered as being metallic alloys. Consequently, atom sizes and valence electron concentrations are of significance for the determination of structural features and types.\textsuperscript{[1]}

Low-dimensional structures and metal clustering are frequently found among TMCs, because compared with the ionic three-dimensional (3D)-type oxides, these compounds tend to form covalent structures, so that the reduced relative charge on the metal favours metal-metal bonding. Thus preferred coordination polyhedra occur for the chalcogen atoms in the compounds containing M-M bonds. The linkage of these polyhedral often results in an arrangement of metal clusters, which are usually condensed by sharing common vertices, edges, or faces, because clusters are rarely isolated in the chalcogenide structures. They also form columns, in which the central metal atoms interact to give chains running in the same direction,\textsuperscript{[2]} while in layered chalcogenides, there are enough $d$-electrons in the transition metal for significant M-M bonding, leading to the formation of a two-dimensional (2D) structure. Moreover, even 3D metal frameworks (i.e., as a metal packing arrangement, such as a cluster network) can be found in certain cases. It should be noted that the occurrence of M-M bonds in TMCs has supported the use of classification schemes based on structural elements rather than oxidation numbers, thus rationalizing the coincidental integer values of the oxidation states of transition metals and consequently, the apparent stoichiometries.\textsuperscript{[3]}

Transition metal chalcogenides occur with various stoichiometries and kinds of structures, among which, however, the most common and most important structures are the chalcogenides with simple stoichiometries, such as 1:1 and 1:2 because most binary compounds belong or are related to the very basic structural types. “Three-dimensional” structures, commonly the cubic NaCl (rock salt; RS) and zinc blende (ZB), or the hexagonal NiAs and wurtzite (W) types, as well as “2D” layered-lattice varieties related to the CdI$_2$ type, are the major structure types observed, as shown in Figure 2.1. The simple compounds formed by selenium and tellurium are generally isomorphous with their sulfide analogues, although there are differences, especially for tellurides.

The electronegativity (or electropositivity, the converse of electronegativity) of TMCs plays a key role in the determination of their structures. In particular, the alkali metal chalcogenides that are derived from the hydrides of the more
electropositive metals (such as Groups 1 and 2) show the antifluorite structure while many other monochalcogenides of rather less basic metals (e.g., the monosulfides of Pb, Mn, La, Ce, Pr, Nd, Sm, Eu, Tb, Ho, Th, U, and Pu) adopt the 6:6 NaCl-type structure. With cations of increasing polarizing power and increasingly readily polarized chalcogenide anions, the ionic RS structure gives way to the ZB and W types. Thus, the increasing covalency of many metals in the later transition-element groups affords chalcogenide structures with lower coordination numbers; for example, the monosulfides of Be, Zn, Cd, and Hg adopt the ZB structure,\textsuperscript{[4-7]} while those of Zn, Cd, and Mn adopt the W one.\textsuperscript{[8-10]} When the bonding becomes more metallic (i.e., decreasing electronegativity), the 6:6 NiAs structure is observed, where the metal is of octahedral coordination. This can be regarded as transitional between the RS structure and the more highly coordinated structures typical of pure metals. Most first row (3d) transition metal monochalcogenides MX (M = Ti, V, Cr, Fe, Co, Ni) come under this case. These compounds often contain vacancies at the metal site, so that the crystal becomes distorted owing to vacancy ordering, which varies with temperature as well as composition. Their magnetic and electrical properties are generally complex and depend on the type of vacancy order.

\textbf{Figure 2.1} Crystal structures for (a) NaCl (RS), (b) zinc blende (ZB), (c) wurtzite (W), and (d) nickel arsenide (NiAs) type.
Chapter 2 Literature Review

Most transition metal elements react with chalcogen atoms to give dichalcogenides (MX₂) with a precise 1:2 stoichiometry, crystallizing in either 2D or 3D structures, which originate from the competition between cationic d levels and anionic sp levels. Two-thirds of the about 60 MX₂ compounds assume layered structures, which found in particular for all the early transition metals of Groups 4-6. Although comprising a structurally and chemically well-defined family, the layered transition metal dichalcogenides demonstrate various remarkable characteristics, including a broad range of homogeneity, order-disorder transitions, strong d-p covalent mixing, and fast ionic diffusion. They actually cover a wide spectrum of electrical properties, ranging from insulators such as HfS₂, through semiconductors such as MoS₂ and semi-metals such as WTe₂ and TcS₂, to true metals such as NbS₂ and VSe₂. Moreover, this class of compounds has played a pivotal role in pioneering investigations on unusual electronic phenomena, such as superconductivity, quantum size effects, and charge density waves (i.e., coupled fluctuations of electronic density and atomic positions along a conducting chain or layer). Most of the non-layered MX₂ compounds formed by the "late" transition metal elements in Group VIII (Mn, Fe, Co, Ni) are composed of infinite 3D networks of metal atoms and discrete X₂ units. Two similar structures exist in this connection: pyrite (e.g., for the disulfides of Fe, Mn, Co, Ni, Cu, Ru, and Os) and marcasite (known only for FeS₂ among the disulfides), as shown in Figure 2.2. Dichalcogenides of this type can be formulated, respectively, as M²⁺(X₂)²⁻ or M⁺(X₂)¹⁻. For example, FeS₂, the most prevalent modification of the disulphide, is pyrite (band gap, E₉ = 0.95 eV), which may be considered as a distorted NaCl structure, where Fe atoms occupy sodium positions and S₂ groups are placed with their centres at the chloride positions. Another modification of FeS₂ is the very similar to pyrite, but somewhat less regular marcasite structure (Figure 2.2b).
Figure 2.2 (a) Pyrite-type crystal structure, shown as FeS$_2$ for example, in which Fe and S are displayed in orange and yellow respectively. (b) Marcasite-type crystal structure.$^{[16]}$

Various stoichiometries and polymorphs are found in the binary copper chalcogenides, which are the well-known IB-VIA semiconductors and exhibit defect phases with large ranges of homogeneity.$^{[17, 18]}$ In the Cu-S system, on the “copper-rich” side lies chalcocite, Cu$_2$S, and at the “copper-deficient” side the pyrite-type CuS$_2$, with such intermediate phases as Cu$_{2-x}$S ($\text{Cu}_{1.96}$S, Cu$_{1.94}$S, Cu$_{1.8}$S), Cu$_7$S$_4$, Cu$_8$S$_8$, and CuS (covellite).$^{[17]}$ Likewise, the Cu-Se system includes Cu$_2$Se, Cu$_{2-x}$Se, Cu$_7$Se$_4$, Cu$_3$Se$_2$, Cu$_5$Se$_4$, CuSe, and CuSe$_2$ stoichiometries, some of which (in one polymorphic form or another) occur as minerals, e.g., berzelianite ($\text{Cu}_{2.3}$Se), umangite ($\text{Cu}_3$Se$_2$), and klockmannite (α-CuSe).$^{[19]}$ Non-stoichiometric phases of the Cu-Te system have also been found for different Cu/Te ratios, including Cu$_2$Te, Cu$_7$Te$_5$ (rickardite), Cu$_3$Te$_4$, CuTe, and CuTe$_2$.$^{[18]}$

Cu$_2$X ($X = S$, Se or Te) compounds are usually dimorphic. Although they have the simple chemical formula Cu$_2$X, they also have quite complex atomic arrangements and show phase transitions.$^{[20-23]}$ For example, low-chalcocite Cu$_2$S (monoclinic phase at a temperature below 377 K) and Cu$_2$Se undergo a α-β transition (at the transition temperature $T_{\text{tr},\alpha-\beta} \sim 410$ K) from the low-temperature monoclinic α-phase to the high-temperature β-phase (face-centred cubic (fcc) structure, Figure 2.3a-b), in which superionic Cu ions are kinetically disordered throughout the structure.$^{[24, 25]}$ The high-temperature modifications of these compounds have defect structures, normally metal-deficient, such as in the (high-digenite) Cu$_{2-x}$S (hexagonal phase at temperature between 377 K and 709 K), where the anions together with half
of the metal ions form a ZB structure, in the interstices of which copper ions are statistically distributed. CuS, as the mineral covellite (also known as coveellite), exhibits a very unusual structure, in which the Cu is partly 3-coordinate and partly 4-coordinate, with two-thirds of the sulfur atoms existing as S₂ groups like those in pyrites (Figure 2.3c). The low-temperature form of CuSe also has a covellite structure, with the high-temperature modification (β-CuSe) being orthorhombic. All CuX₂ compounds assume pyrite-type structures.

II-VI semiconductors are compounds consisting of elements from Groups IIB (Zn, Cd, Hg) and VIA (S, Se, Te) of the periodic table. Various semiconductors are composed of combinations of these elements and include binary, ternary, and quaternary compounds. All the monochalcogenides of the II-VI semiconductors crystallize in the tetrahedral zinc blende (ZB) or wurtzite (W) structures, with the exception of HgS, which also exists in a distorted rock salt (RS) form. Most II-VI semiconductors (except for HgTe and HgSe) have a direct band gap, which consequently endows the II-VI materials with great potential for optical and optoelectronic applications. In Table 2.1 we highlight some major structural and semiconductor properties of the main binary II-VI compounds. The applications of I-VI semiconductors have received in-depth investigation in various fields for various applications, including optical devices, luminescence, photovoltaics, sensors, lasers, biological labels, biological detection, and diagnostics.

Figure 2.3 (a) The β-Cu₂Se crystal structure (blue and green are Cu and Se atoms). (b) Multiple atomic sites for Cu⁺ and Se²⁻ ions, with designations.[23] (c) Covellite hexagonal unit cell of CuS.[26]
Table 2.1 Physical and chemical properties of II-VI semiconductors.

<table>
<thead>
<tr>
<th>Properties</th>
<th>ZnS</th>
<th>ZnSe</th>
<th>ZnTe</th>
<th>CdS</th>
<th>CdSe</th>
<th>CdTe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stable phase at 300 K</td>
<td>Z/W</td>
<td>Z</td>
<td>Z</td>
<td>Z/W</td>
<td>Z/W</td>
<td>Z</td>
</tr>
<tr>
<td>Density (g cm(^{-3})) (Z/W)</td>
<td>4.088/4.087</td>
<td>5.266</td>
<td>5.636</td>
<td>4.82/4.82</td>
<td>5.81/5.81</td>
<td>5.86</td>
</tr>
<tr>
<td>Melting point (K)</td>
<td>1991</td>
<td>1799</td>
<td>1563</td>
<td>1750</td>
<td>1533</td>
<td>1370</td>
</tr>
<tr>
<td>Thermal conductivity at 300 K (W cm(^{-1})K(^{-1}))</td>
<td>0.27 (Z)</td>
<td>0.19</td>
<td>0.18</td>
<td>0.2</td>
<td>0.009</td>
<td>0.001</td>
</tr>
<tr>
<td>Heat capacity (J mol(^{-1}) K(^{-1}))</td>
<td>45</td>
<td>51.88</td>
<td>46.44</td>
<td>53.9</td>
<td>48.46</td>
<td>23.9</td>
</tr>
<tr>
<td>Band gap (eV,300 K) (Z/W)</td>
<td>3.72/3.58</td>
<td>2.64</td>
<td>2.35</td>
<td>2.5/2.482</td>
<td>1.74</td>
<td>1.475</td>
</tr>
<tr>
<td>Absorption coefficient 10.6 µm (cm(^{-1}))</td>
<td>≤0.15</td>
<td>1-2 × 10(^{-3})</td>
<td>-</td>
<td>≤0.007</td>
<td>≤0.0015</td>
<td>≤0.003</td>
</tr>
<tr>
<td>Exciton binding energy (meV)</td>
<td>38</td>
<td>21</td>
<td>10</td>
<td>30.5</td>
<td>15</td>
<td>10.5</td>
</tr>
<tr>
<td>Thermo-optical coefficient (dn/dT) (λ= 10.6 µm)</td>
<td>4.7</td>
<td>6.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11</td>
</tr>
</tbody>
</table>

For example, CdTe is the most attractive semiconductor for photovoltaic application among Si, and the III–V and II–VI compounds.[35, 36] CdTe has a direct band-gap energy of about 1.47 eV, which is very close to the theoretically calculated optimum value for solar cells. CdTe also has a high absorption coefficient (> 10\(^5\) cm\(^{-1}\) at a wavelength of 700 nm), so that approximately 90% of the incident light is absorbed by a layer thickness of only 2 mm (compared with around 10 mm for Si). Furthermore, CdTe can be made from polycrystalline thin films on glass substrates, thus avoiding the need for expensive single-crystal substrates. CdTe is one of the
most promising photovoltaic materials available for use in low-cost, high-efficiency solar cells. The theoretical efficiency for the CdS/CdTe photovoltaic cell is about 30% at air mass 1.5. (The air mass number is 0 for no air mass (i.e. for a satellite solar array), 1 for the sun directly overhead, and 1.5 for the sun at 45° to the horizon.)\(^{[37]}\) It is the first and only thin-film photovoltaic technology to surpass crystalline silicon photovoltaics in cheapness.\(^{[38]}\) The CdTe photovoltaic market is growing rapidly, and CdTe is the second most utilized solar cell material in the world.\(^{[37, 39]}\)

### 2.2 Synthesis approaches for transition metal chalcogenides

The microstructure (composition, size, shape, etc.) of nanomaterials determines the electronic density of states (DOS) of nanomaterials and ultimately determines their electronic, optical, magnetic, and mechanical properties, which are the fundamental to the current excitement and growing applications of nanomaterials. The wide application of TMCs, including in fuel cells, solar cells, sensors, Li/Na batteries, supercapacitors, biological labels, diagnostics, etc. is benefitting from the progress in the synthesis of novel nanostructured materials in different sizes and with new morphologies.\(^{[28-30, 40, 41]}\) New physical and chemical properties emerge when the size of TMCs is reduced to the nanometer scale, owing to the well-known quantum size effect.\(^{[42]}\) In addition, compared with their bulk counterparts, nanostructured TMCs possess a much higher specific area (i.e. more reaction sites). For example, well-studied semiconductor nanocrystals which usually are referred to as quantum dots (QDs) exhibit strong size-dependence of their optical properties when their size is smaller than the Bohr exciton radius.\(^{[43]}\) Their novel properties make these nanomaterials very attractive in diverse applications, ranging from energy conversion and storage to biomedical imaging and diagnostics.

Controllable morphology, size, composition, structure, and suitable surface modification of TMC materials play a key role in their practical application. A range of approaches have been utilized for the preparation of TMC nanostructures, and those synthetic routes can be classified as top-down (e.g., mechanical cleavage, chemical exfoliation, sonication-assisted exfoliation, and chemical reactions) and bottom-up approaches (e.g., chemical vapour deposition, direct wet chemical synthesis, and solvothermal reactions), as shown in Figure 2.4.\(^{[44]}\) In the top-down approach, the dimensions of bulk materials are progressively reduced to the nanodomain by using physical and chemical means. Examples involve ball milling
and acid-etching. Several top-down synthesis approaches are mostly based on exfoliating nanosheets from bulk crystals or larger morphologies, whereas bottom-up approaches are conducted through a range of synthesis routes with the addition of diverse kinds of metal and chalcogenide precursors. Conventional solid-state or gaseous-state strategies are often unsatisfactory. For example, the drawbacks of the mechanical ball-milling approach are that it is easy to introduce impurities, the particle distribution is uneven, and the process is time consuming, while the equipment for gaseous-state strategies are often complicated, expensive, and high energy consuming. Comparatively, liquid-based wet chemical methods have been demonstrated to be powerful for obtaining desired nanostructured TMCs with controlled sizes, compositions, and shapes. In a liquid-based synthetic system, the target TMCs and/or surfactants/ligands can be selected based on rational criteria, and the nucleation and growth process of the products can be easily controlled by adjusting the thermodynamic and kinetic parameters of the reaction, such as the temperature, amount and type of ligands or surfactants, stirring speed, and even the feeding sequence of reactants, etc. Thus their composition, size, shape, and surface properties can be exquisitely tailored. Additionally, liquid-based synthetic routes avoid the use

**Figure 2.4** Top-down and bottom-up approaches in nanotechnology.
of drastic conditions (such as high temperatures and high pressures), and the cost is relatively low because they do not need expensive facilities. Various liquid-based wet chemical methods have been established to synthesize high-quality TMCs. In this section, the main and general liquid-based wet chemical methods for controlled synthesis of TMCs are selectively reviewed and summarized.

2.2.1 Hot colloidal chemistry

Various inorganic nanoparticles of varying types and compositions can be synthesized using general hot colloidal chemistry. They include semiconductor quantum dots, magnetic nanoparticles, plasmonic metal or alloyed metal nanoparticles, plasmonic transparent conductor nanoparticles, lanthanide-doped multicolour upconversion nanoparticles, etc. Hot colloidal chemistry allows manipulation of their optical or electronic properties in a rationally defined way. This approach typically utilizes mixing of relevant charged ions in a prefixed ratio at a defined crystallizing temperature in a ligand-regulated solution environment, followed by precipitation of the nanoparticle.\[45\] This approach generally employs three components: (1) a high boiling point solvent, which is required to achieve a temperature high enough for complete solubilization and decomposition of reactants and crystallization of the inorganic nanoparticles; (2) selected ligands (also called surfactants or capping groups, such as trioctyl phosphine (TOP), trioctyl phosphine oxide (TOPO), oleic acid, and oleylamine), with the ligands or surfactants used for adsorption on the surfaces of the nanoparticles, limiting their growth; (3) metal salts or organometallic compounds as precursors. The precursors provide the inorganic elements (e.g., Cd and Se for CdSe QDs), from which the particles are formed.\[46\] Typically, a solution containing surfactants/ligand is heated to 150 to 350 °C for synthesis of the desired nanoparticles,\[42, 47\] employing a typical apparatus, as illustrated in Figure 2.5a.

The typical growth process for inorganic nanoparticles is illustrated in Figure 2.5b. The growth process encompasses four main stages: (1) monomer (precursor) accumulation in solution (phase I); (2) nucleation (phase II); (3) particle growth (phase III); and (4) recrystallization processes. An illustration of the corresponding phase I to phase III is depicted in Figure 2.5b. Phase I illustrates the first stage of accumulation of the monomer, which is produced from raw organometallic materials or by thermolysis of the corresponding precursors in solution. The nucleating process
of phase II does not take place until the accumulated monomer concentration in the solution reaches above the nucleation concentration. When the monomer concentration drops back below the nucleation concentration, the nucleation process will stop, and all the formed nuclei will gradually grow and crystallize into nanocrystals (phase III). When the concentration of the monomer drops to the saturation level, the growth phase is complete, and phase IV of the recrystallization process or Ostwald ripening process will occur. The Ostwald ripening process is unique, as it can cause the particle size to nonuniformity if the size is relatively uniform during the growth phase, while it also can lead the particle size to uniformity if the size deviation of different nanoparticles is large. Based on the preparation conditions, prevention of this process or intended utilization of this process can be selectively effected to produce uniform inorganic nanoparticles.

![Diagram](image)

**Figure 2.5** (a) Typical laboratory-scale apparatus for preparing inorganic nanoparticles using hot colloidal synthesis. (b) Schematic illustration of the typical growth process for inorganic nanoparticles.

There are two ways of loading precursors into the-three neck round bottom flask shown in Fig. 2.5(a): (1) the ‘‘hot-injection method’’;[48] (2) the ‘‘heating-up method’’.[49] In the ‘‘heating-up’’ process, the temperature of the reaction solution containing pre-loaded precursors is elevated from room temperature to a specific high temperature to produce nanoparticles. The ‘‘hot-injection’’ process employs the injection of pertinent precursors into a hot solution containing a high-boiling-point solvent and the surfactants. The ‘‘hot-injection’’ process creates a burst of nucleation to sharply separate the nucleation process from the growth process, which is critical for producing uniform inorganic nanoparticles. The size of nanocrystals can be
Chapter 2 Literature Review

**Figure 2.6** Transmission electron microscope (TEM) images of different morphologies of CdSe. The surfactant ratio was increased from (a) 8 to (b) 20 to (c) 60% hexylphosphonic acid (HPA) in TOPO, with an injection volume of 2.0 mL of stock solution. The injection volumes used were (d) 1.0, (e) 1.5, and (f) 2.0 mL. 20% HPA in TOPO was used, as it was found to provide optimal rod growth conditions. A greater injection volume favours rod growth (d-f).[50]

controlled by adjusting the temperature, the nature of the solvent, the surfactant(s), and the aging time. As the resulting nanoparticles at the end of the growth stage are generally uniform, prevention of the Ostwald-ripening process is necessary by terminating the reaction at a specific time point. Typically, semiconductor QDs are synthesized by the “hot-colloidal injection” method.[46, 48, 50] For example, arrow-, teardrop-, tetrapod-, and branched tetrapod-shaped nanocrystals of CdSe have been achieved by single-injection experiments, as shown in Figure 2.6. In this study,
three fundamental synthesis condition parameters including the ratio of the surfactants (HPA/TOPO); the volume of the initial injection; and the time dependence of the monomer concentration have strong relationships with the shape of the CdSe nanoparticles.\textsuperscript{50} Besides controlling the morphology of the nanocrystals in one reaction system, many other morphologies of high-quality semiconductor nanocrystals have also been synthesized by this method, such as Cu$_2$S nanoparticles, nanoribbons assembled from β-Cu$_2$S nanodisks, chain-like Cu$_2$S nanocrystals, and Cu$_2$S nanodisks, as shown in Figure 2.7.\textsuperscript{51-58}

The second way of loading precursors into the three-neck round-bottom flask is the “heating-up” approach, whereby the temperature of the reaction is elevated from
room temperature to a specific high temperature to produce nanoparticles. This approach generally explores two routes to produce uniform inorganic nanoparticles. One is to abruptly release metal ions from metal complexes at their thermal decomposition point, creating a burst nucleation process, followed by a size-focused growth stage,\(^{49}\) which are based on the same principles as described for the “hot-injection” method. The other route relies on the Ostwald-ripening process to lead nanoparticles of varying sizes produced by mixing related precursors at room temperature, to grow into a uniform critical size. This approach allows the synthesis of alloyed metal nanoparticles (Ag/Au). In contrast, when the hot injection method is used, Ag and Au prefer to nucleate by themselves (forming Ag and Au nanoparticles, rather than the Ag/Au alloyed nanoparticles).\(^{59}\)

The hot colloidal approach can achieve high quality nanocrystals with good crystallinity and narrow size distributions. The drawbacks of this method lie in the following aspects, however: (i) toxic and expensive organics may be used, which can do harm to both humans/ the environment and increase the cost; (ii) there are always capping ligands (such as oleic acid, oleylamine, TOPO, etc.) on the surface of the product, which are generally hydrophobic, so that further surface modification is required to make the nanocrystals dispersible in an aqueous solution or biological buffer, where surface hydrophilicity is a prerequisite; (iii) to prevent oxidation or absorption of water, purging of the protection gas or Schlenk line is needed, which leads to complexity in the operation; (iv) sometimes, in order to obtain monodispersed nanostructures, a low concentration of precursors is needed, meaning low yield.

### 2.2.2 Solvothermal and hydrothermal method

#### 2.2.2.1 Solvothermal method

Along with the development of the hot colloidal chemistry technique, the solvothermal method was introduced to prepare semiconductors and nanocrystalline chalcogenides in 1996\(^{60}\) and developed rapidly. In contrast to the hot colloidal chemistry approach, the solvothermal process uses organic solvents as the reaction medium in a sealed steel pressure vessels with Teflon liners (Figure 2.8), which are then heated to the designated temperature (usually higher than the boiling point of the solvent) to promote the reaction. Because the reactants are placed in a closed
system, the solvent auto-generates pressure upon heating. The pressure in the solvothermal system is much larger than that under ambient conditions, which is helpful to further enhance the solubility, as well as the reactivity, of the reactants, and also improves the crystallinity of the as-prepared nanocrystals. By choosing an appropriate organic solvent, commonly used raw materials (e.g. elemental S, Se, Te, P, As, and Sb) can have enhanced solubility and will be more reactive than under conventional conditions. Under these extreme (high temperature, high pressure) conditions, even in pure solvent, some quite unexpected reactions will take place, accompanied by the formation of nanoscopic morphologies and new phases, which cannot be achieved by traditional reactions. The solvent properties, such as polarity, viscosity, and softness, have strong effects on the solubility and transport behaviour of the precursors in the liquid-based synthesis, which will control the reactivity, shapes, sizes, and phases of the final products. For example, various TMC nanocrystals have been synthesized with elegant control of their size and morphology, and also their crystallinity, including wire-like Fe$_{1-x}$S$\text{(en)}_{0.5}$,$^{61}$ FeS$_2$,$^{62}$ Cu$_2$Te,$^{63}$ and Ag$_2$Te,$^{64}$ belt-like ZnSe,$^{65}$ CoTe nanotubes,$^{66}$ nanoweb-like FeS$_2$,$^{67}$ dendrite-like Cu$_{2-x}$Se,$^{68}$ and Cu$_2$S,$^{69}$ plate-like CuS,$^{70}$ flower-like FeSe$_2$,$^{71}$ and CoS$_{1.097}$,$^{72}$ α-MnSe nanospheres,$^{73}$ etc. (see Figure 2.9).

Figure 2.8 Schematic diagram of solvothermal synthesis setup: (1) stainless steel autoclave, (2) precursor solution, (3, 4) Teflon liner, (5) stainless steel lid.
Figure 2.9  TMC nanocrystals with various morphologies. Scanning electron microscope (SEM) images of (a) FeS$_2$ with wire-like morphology,$^{[62]}$ (b) Cu$_2$Te nanowires/nanoribbons;$^{[66]}$ (c) belt-like ZnSe; $^{[65]}$ TEM images of (d) CoTe nanotubes;$^{[66]}$ (e) FeS$_2$ nanowebs; $^{[67]}$ dendrite-like Cu$_{2-x}$Se$^{[68]}$ (f) and Cu$_2$S$^{[69]}$ (g); (h) plate-like CuS; $^{[70]}$ flower-like FeSe$_2$$^{[71]}$ (i) and CoS$_{1.097}$$^{[72]}$ (j); (k) α-MnSe nanospheres.$^{[73]}$ The insets of (a) and (b) are enlargements of single nanowires, and the inset of (e) is the corresponding electron diffraction pattern.

During these solvothermal synthesis approaches, a reaction temperature was usually adopted in the range of 100-250 °C in order to reach the pressure of vapour saturation. Stabilizing/capping agents, appropriate solvents, and chalcogen sources have been extensively employed as “shape controllers” and are particularly significant for the
final TMC nanocrystals. For examples, single-crystalline, hexagonal covellite (CuS) nanoplatelets (diameter: 26 nm, thickness: 8 nm; Figure 2.9h) were successfully synthesized by the solvothermal method in toluene by using hexadecylamine as the capping ligand. Although the size of the nanoplatelets could be tuned by varying the reaction time and temperature, self-assembled stacking was mostly controlled by the capping agent and van de Waals attraction.\textsuperscript{[70]} Zhang et al. reported the synthesis of CuS nanotubes (Figure 2.10a) by the solvothermal process in an oil/water (O/W) microemulsion system consisting of oleic acid, water, and polyvinylpyrrolidone (PVP).\textsuperscript{[74]} Another interesting case is the successful synthesis of concave-faceted cuboctahedral CuS crystals by a solvothermal reaction in ethylene glycol (EG) solution (Fig. 2.10b).\textsuperscript{[75]} Each concave-faceted cuboctahedron has four identical-hexagonal flakes and contains 14 concave cavities. The usage of EG as a solvent is one of the key points for the formation of these geometric ‘‘stars’’. Wang et al. synthesized non-stoichiometric compositions of copper sulfide (CuS) via the solvothermal method simply by varying the Cu\textsuperscript{2+}/S\textsuperscript{2-} molar ratio in the precursor solutions.\textsuperscript{[76]} The assembly process through the route zero-dimensional (0D) → 2D → 3D was attributed to the oriented-attachment mechanism of copper sulphides (Figure 2.10c). Besides changing the ratio of precursors, adopting different precursors can lead to the formation of different morphologies of TMC nanocrystals. CuS nanocrystals in the form of spherical nanoflowers, doughnut-shaped nanospheres, and dense nanospheres were obtain under the same reaction conditions, but changing the S precursor, Na\textsubscript{2}S, Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}, or CS(NH\textsubscript{2})\textsubscript{2}, led to the formation of Cu complexes with different morphologies (Figure 2.10d-f).\textsuperscript{[77]}
Figure 2.10 SEM images of various morphologies of CuS nanocrystals synthesized by the solvothermal method. (a) CuS nanotubes prepared in water and PVP.\textsuperscript{[74]} (b) Concave-faceted cuboctahedral CuS crystals prepared in EG.\textsuperscript{[75]} (c) Octahedral \(Cu_9S_5\) nanocrystals.\textsuperscript{[76]} (d) CuS nanoflowers prepared with \(Na_2S_2O_3 + CuCl_2\) in ethanediol. (e) CuS doughnut-shaped nanospheres prepared with \(CS(NH_2)_2 + Cu(NO_3)_2\) in ethanediol. (f) CuS dense nanospheres prepared with \(CS(NH_2)_2 + CuSO_4\) in ethanediol.\textsuperscript{[77]}

2.2.2.2 Hydrothermal method

Similar to the solvothermal method, the hydrothermal method uses water as the main medium, which is very low-cost and able to dissolve other water soluble coordinating molecules (such as ethylenediamine, hydrazine hydrate, mercaptoethanol, etc.). The temperature adopted is often higher than 100 °C for the hydrothermal process, so that autogenous pressure will be developed in the closed system. The pressure generated in situ within the reactor not only strongly depends on the reaction temperature, but also relies on other experimental factors, such as the amount of liquid added and any dissolved salts. The hydrothermal and solvothermal processes are relatively low temperature processes and have many advantages such as fast reaction kinetics, short processing times, phase purity, high crystallinity, high yield, homogeneous particle products, easy composite formation, and narrow particle-size distributions. They are also cost-effective, environmentally benign, and easily scalable. Moreover, the hydrothermal and solvothermal processes facilitate different reactions, such as multicomponent reactions, heat treatment reactions to modify or change the composition, phase transformation reactions, ion exchange, crystal growth, dehydration reactions, decomposition reactions, extraction, precipitation, disproportionation reactions, crystallization, and solidification. There are also a few disadvantages to hydrothermal and solvothermal processes, such as (i) the need for expensive stainless-steel autoclaves and Teflon liners, (ii) problems with reproducibility of the product, because many occasional factors such as residual solvent on the wall surface, situation of the vessel sealing, etc., could affect the properties of the final product; (iii) possible safety issues during reaction processes, and (iv) the impossibility of studying the mechanisms of the in-situ reactions due to the closed (or ‘black box’) systems.\textsuperscript{[78]}
As discussed previously in the section on the solvothermal method, the stabilizing/capping agents, and appropriate solvent and chalcogen sources affect the morphologies of the final TMC nanocrystals. The application of “mixed solvents” in the hydrothermal method can be rather effective in some cases in producing TMCs with unique morphologies or new nanoarchitectures.[40, 79-82] The mixed solvents, with the assistance of small organic amine molecules, can play the role of effective structure-directing agents in assisting anisotropic crystal growth.[83-86] Gao et al. reported Fe₇Se₈ polyhedra with high-index facets and Fe₇Se₈ nanorods can be selectively synthesized by a thermal treatment in a mixed solvent of diethylenetriamine (DETA) and deionized water (DIW). It was found that the morphologies of Fe₇Se₈ nanocrystals can be effectively controlled by adjusting the volume ratio of DETA to DIW, suggesting the “magic power” of mixed solvents for shaping nanocrystals with new morphologies and structures.[80] Zhao et al. fabricated CuS nanostructures with the assistance of different organic amines using CuCl and thiourea as the precursors.
Nanostructures, including nanowires, nanotubes, and nanovesicles, can be obtained in the presence of triethylenediamine (TEDA), trimethylenglycine (TEMA), and di-n-butylamine (DBA), respectively. Different interactions between CuCl and amines were the key factor in structural and morphological control.\textsuperscript{[88]} Without the assistance of organic amines, diverse CuS nanostructures can be achieved under hydrothermal treatment, as shown in Figure 2.11, simply by varying the ratios of water: ethylene glycol as solvent.\textsuperscript{[87]} CuS nanotubes consisting of nanoparticles were obtained with an optimum solvent ratio (1:3 of water: EG, Figure 2.11d-e). The authors proposed that the formation of different morphologies can be attributed to the
optimum ratio of water to EG, which affects the solubility, reactivity, and diffusion behaviour of the reagents and intermediates. The reaction medium plays an important role in controlling the shape of the basic CuS building blocks in their self-assembly process. In particular, the higher viscosity of EG increases the steric hindrance in the reaction system causing reduction of the particle size of the product.\[87\]

### 2.2.3 Ion exchange method

The chemical transformation of nanocrystalline solids for inorganic nanostructure synthesis has emerged as a very promising strategy. Among the various chemical transformations, the ion exchange reaction is especially interesting, as it can alter the composition of the existing ionic nanocrystal by replacing the ion within the crystal lattice with a different ion. Ion exchangers include cation exchangers that exchange positively charged ions (cations) and anion exchangers that exchange negatively charged ions (anions) (ie. cation exchange and anion exchange reaction). Typically, the ion exchange reaction is based on the big difference in the solubility product ($K_{sp}$) between the original nanocrystals and the final product. In thermodynamic terms, the solubility product ($K_{sp}$) of an ionic solid is referred to as the equilibrium constant between an ionic solid and its dissolved state. It is related to the free energy change of a solution. Because the free Gibbs energy for insoluble materials ($\Delta G$) can be expressed by Equation (2.1), in which $R$ is the gas constant, $T$ is temperature of the reaction system, $K_{sp}$ is the solubility product of the insoluble MCs (M$_a$X$_b$, M is metal element, $X = S$, Se, Te), and $J_{sp}$ can be calculated from Equation (2.2). $C(M^{b+})$ and $C(X^a)$ are the concentrations of cations and anions in the system, respectively. Thus, the transfer reaction from the higher $K_{sp}$ material to a lower one may happen spontaneously.

$$\Delta G = RT \times \ln \frac{J_{sp}}{K_{sp}} \quad (2.1)$$

$$J_{sp} = C^a(M^{b+}) \times C^b(X^a) \quad (2.2)$$

Although the ion exchange reactions are heavily affected by various factors, such as the activation energy barriers to phase change and surface energy change, the real concentration of ions in the solution, the effect of solvents, etc., we can still roughly make a judgement as to whether the ion exchange reaction can happen or not by using the solubility product.\[89\] The cation exchange reaction is particularly effective
for the transformation of TMCs,\textsuperscript{[90-93]} the crystal structures of which are determined by the framework of the chalcogen anions, and the metal cations are relatively mobile in the structure, leading to an easier replacement of the cations. The exchange reaction was found to rely on the thermodynamic driving force and the kinetics of a transformation (activation barrier). It is also possible that the transformation reaction from lower $K_{sp}$ reactants to a higher $K_{sp}$ product can occur via control of the reaction conditions.\textsuperscript{[90]} The solubility product of the ionic solids in the reaction medium is supposed to determine the thermodynamic direction of the transformation. When the ionic solid product has a lower solubility than a reactant one in the reaction medium, a forward reaction will occur, and the kinetics of the transformation can be controlled by tuning the reaction temperature to overcome the activation barrier. Because a selective increase in the solubility of a product salt can proceed via the cation exchange reaction, selective ligand complexation to the product salt can be an effective approach to turn a thermodynamically unfavourable reaction into a favourable one (such as from lower $K_{sp}$ reactants to a higher $K_{sp}$ product). A milestone was achieved when Alivisatos and coworkers pioneered reversible cation exchange reactions between CdE and Ag$_2$E (E = S, Se, Te) nanocrystals in the form of spheres and tetrapods (Fig. 2.8).\textsuperscript{[90]} The reverse exchange reaction (Ag$_2$E $\rightarrow$ CdE) was realized in the presence of complexing molecules (tributyl phosphate (TBP)/TOP) under ambient conditions. In addition, they also investigated the structural changes in nanocrystals during the cation exchange reaction by comparing their equilibrium and non-equilibrium shapes. Owing to the small dimensions, comparable to the reaction zone ($\sim$5 nm), the reaction was immediately completed in 1 s. The morphological change occurs when the size of the nanocrystal is smaller than the width of the reaction zone. Since the structure of the anion sublattice must be changed during the cation exchange reactions, the structural difference between the reactant and the product can play a vital role in determining the activation barrier. Volume change during the transformation is another factor in the morphology. Son and co-workers have reported that the final morphologies of the product nanomaterials are affected mainly by the volume change during the cation exchanges from CdE to M$_x$E$_y$ (E = S, Se, Te; M = Pd, Pt).\textsuperscript{[94]} The product nanocrystals with large volume reduction can release the lattice stress by generating voids. Transformations with large volume expansion lead to fragmentation of the products.
into nanoparticles. The products experiencing small volume changes readily preserved their original morphology.

**Figure 2.12** (A) Schematic demonstration of the reverse cation exchange between CdSe and Ag₂Se. TEM images of the initial CdSe nanocrystals (B), Ag₂Se derived via a forward cation exchange reaction (C), and CdSe nanocrystals recovered using a reverse cation exchange reaction (D). TEM images of the initial CdTe tetrapods (E), Ag₂Te tetrapods produced from cation exchange of CdTe (F), and CdTe recovered through a reverse cation exchange reaction (G).

By taking advantage of the partial transformation of ionic nanocrystals via cation exchange, heterostructured nanocrystals can be achieved. Typical examples include the CdS-Cu₂S binary nanorods reported by Alivisatos et al.;\(^{95}\) the CdS-PtS, CdSe-PtSe, CdTe-PtTe, CdS-PdS, and CdSe-PdSe nanocrystals reported by Son et al.;\(^{94}\) and CuS-Ag₂S nanorods.\(^{96}\)

Ion exchange reactions also can be employed to prepare hollow structures of various TMC nanomaterials through a Kirkendall effect,\(^{97-101}\) a classical phenomenon in metallurgy, which involves a non-equilibrium mutual diffusion
process at the interface of a diffusion couple, causing the net flow and coalescence of lattice vacancies, and the final formation of voids near the original interface and within the fast-diffusion side.\textsuperscript{[102]} A pioneering work was reported by Yin et al.\textsuperscript{[97]} where it was demonstrated that hollow Co\textsubscript{3}S\textsubscript{4}, Co\textsubscript{9}S\textsubscript{8}, or CoSe nanocrystals can be fabricated by reacting Co nanocrystals with either sulfur or selenium in solution through a Kirkendall-effect-induced method. Each resultant TMC nanocrystal has a single void. The diameter of the final voids is much smaller than that of the original Co nanocrystals, indicating that the void formation is controlled by both the inward transport of sulfur (or selenium) anions and the outward diffusion of Co cations. They also found that the reaction temperature has a pivotal effect on the voids in the nanocrystals.\textsuperscript{[98]} Performing the reaction at high temperature (> 120 °C) can lead to the rapid formation of a single void inside each shell and a porosity/defect structure is observed in the shells, whereas at room temperature, multiple voids are generated due to the strongly temperature-dependent diffusivities for vacancies, which lead to obvious fracturing and open structures (Figure 2.13).\textsuperscript{[98]} Except for hollow nanoparticles, the Kirkendall-effect-induced method is also promising for preparing other hollow nanostructures of various metal chalcogenides (MC) nanomaterials, such as hollow Co\textsubscript{3}S\textsubscript{4}, CoSe\textsubscript{2}, and CoTe chains,\textsuperscript{[103]} hollow Bi\textsubscript{2}Te\textsubscript{3},\textsuperscript{[99]} and RuSe\textsubscript{2}\textsuperscript{[104]} nanotubes.

\textbf{Figure 2.13} Schematic illustration of the temperature dependence of hollow particle evolution at LT (low temperatures) and HT (high temperatures).\textsuperscript{[98]}

\textbf{2.2.4 Liquid exfoliation method}
The development of two-dimensional (2D) materials with designed physical and chemical properties has been experiencing a renaissance since the arrival of graphene (the well-known 2D nanocarbon). In the big family of 2D materials, layered transition metal dichalcogenides (TMDs) are playing increasingly important roles in both fundamental research and technological applications due to their unique crystal structures, wide range of chemical compositions, and various material properties. Originating from the competition between the cationic $d$ levels and anionic $sp$ levels, most transition metal atoms (M) will react with chalcogen atoms (X) to form a $X_{2}$-$M$-$X$ configuration with stoichiometry $MX_{2}$ (Figure 2.13). Transition metals, ranging from group 4 to group 10, have different numbers of $d$-electrons, which fill up the non-bonding $d$ bands to different levels, resulting in varied electronic properties, including insulating (such as HfS$_{2}$, TiSe$_{2}$), semiconducting (such as MoS$_{2}$ and WS$_{2}$), metallic (NbS$_{2}$ and VSe$_{2}$), and superconducting (such as PdTe$_{2}$) properties. Although the bonding within the hexagonal M layer and the two X layers is covalent, adjacent sheets of the sandwich layers are coupled by weak van der Waals interactions to form a 3D crystal, allowing the crystal to be readily cleaved along the layer surfaces. It is assumed that about 40 $MX_{2}$ compounds are layered structures, and they particularly found in the early transition metals of Groups 4-7 (except for manganese), whereas some of the group 8-10 TMDs are commonly found in non-layered structures (Figure 2.13).

Before using the extraordinary capabilities of these layered materials, bulk TMD crystals must be exfoliated into mono- or few-layer nanosheets to meet their full potential. Some single-layer graphene and TMD nanosheets can be harvested by using adhesive tape for mechanical cleavage due to the weak van der Waals interaction forces between neighbouring layers. Thus, nanosheets can be harvested from bulk materials by peeling or exfoliation by insertion of other compounds. Although this solid mechanical exfoliation method can fabricate samples with high quality, it remains a challenge to scale up the process for their real utilization. Inspired by the milestone work on the synthesis of sheets from bulk clay materials in the 1960s, intense studies have been focused on exfoliating sheets of TMCs in solution. Owing to their layered structures, TMD bulk materials can be intercalated by various kinds of intercalation materials, such as organic molecules,
transition metal halides, and lithium ions.\cite{120} The resulting intercalated compounds can be exfoliated to mono- and few-layer 2D TMD nanosheets by simple ultrasonication.\cite{123-127} The ultrasonication method is time-consuming, however, and the degree of lithium insertion is uncontrollable. Therefore, a controllable electrochemical lithiation method to produce high-yield, single-layer TMD nanosheets was developed.\cite{3} The lithium intercalation in bulk materials can be monitored and finely controlled during the discharge process by incorporating the layered TMD bulk material as the cathode in an electrochemical cell. The obtained intercalated compounds were then

\textbf{Figure 2.14} (a) About 40 different layered TMD compounds exist. The transition metals and the three chalcogen elements that predominantly crystallize in those layered structures are highlighted in the periodic table. Partial highlights for Co, Rh, Ir, and Ni indicate that only some of the dichalcogenides form layered structures. (b) Schematic illustrations of 2D TMDs. M represents a transition metal, and X represents a chalcogen.
ultrasonicated and exfoliated in water or ethanol to achieve high quality TMD monolayers on a large scale.\textsuperscript{128} It should also be noted that lithium intercalation chemistry often results in structural deformation in TMDs to alter electronic properties and even leads to the decomposition of TMDs and the formation of metal nanoparticles and Li\textsubscript{2}S.

Besides those factors (such as the kind and amount of intercalation substance and the surface energy of the layered materials) that influence the exfoliation of nanosheets, the surface tensions of the solvents also pay a key role in the liquid exfoliation technique to produce nanosheets, including monolayers. After dispersion and ultrasonication of each inorganic starting material in about 30 common solvents with varying surface tensions, Coleman and co-workers\textsuperscript{129} found that the best solvents have a surface tension close to 40 mJ m\textsuperscript{-2} by using optical absorption spectroscopy to measure the quantity of the dispersed materials. They proposed that successful solvents are those that minimize the energy of exfoliation. For example, N-methylpyrrolidone (NMP) and isopropanol (IPA) are very effective solvents for exfoliating various layered compounds.

2.2.5 Microwave method

Microwave chemistry has been widely applied to prepare various kinds of inorganic nanomaterials in liquid phase owing to its specific advantages, such as high reaction rate (can reduce reaction time often by orders of magnitude), low processing costs, high yields, and side reaction depression.\textsuperscript{130} In the microwave frequency range (usually 0.3 to 2.45 GHz), electromagnetic energy is converted into thermal energy, which induces a chemical reaction leading to the synthesis of nanostructured materials. The microwave dielectric heating mechanism consists of two main processes, namely dipolar polarization and ionic conduction,\textsuperscript{130} which is different from the direct absorption of high energy electromagnetic radiation needed to induce chemical reactions. The microwave stimulation method has been greatly expanded to the synthesis of various MC nanomaterials.\textsuperscript{131-134} Feng et al. reported that carbon-supported Co\textsubscript{3}S\textsubscript{4} and CoSe\textsubscript{2} nanoparticles with small sizes and narrow distributions can be prepared in situ within a short time (3-5 min) by microwave stimulation, while at least several hours were needed for the conventional heating method at a refluxing temperature of about 142 °C.\textsuperscript{131} In another report, non-stoichiometric
copper sulfide (Cu$_2$S) nanorods were prepared by the microwave-assisted decomposition method from a previously formed precursor, which was made from a composite of [Cu(NO$_3$)$_2$·3H$_2$O] - sodium dodecyl sulphate (SDS)-thioacetamide (TAA). Ramanath et al. reported the rapid and scalable microwave-stimulated solvothermal synthesis of sulfurized Sb$_2$Se$_3$ nanowires and nanotubes. The diameter and length of these one-dimensional (1D) nanostructures can be controlled by adjusting the microwave dose (i.e. the microwave power × time). A similar morphology control process was reported by El-Shall and co-workers in the microwave synthesis of semiconductor nanorods and nanowires (Figure 2.15). By simply varying the microwave radiation times from 30-60 s to 1-2 min to > 3 min, the growth of ZnS and ZnSe nanowires from small spherical nuclei to short aligned rods to long assemblies of nanowires was accomplished in a stepwise manner (Figure 2.15).

![Figure 2.15 TEM of ZnS (a) rods, (b) wires, (c) ZnSe wires, and (d) CdSe rods. Higher resolution images are shown in the insets.](image)

Figure 2.15 TEM of ZnS (a) rods, (b) wires, (c) ZnSe wires, and (d) CdSe rods. Higher resolution images are shown in the insets.
2.2.6 Electrodeposition method

Besides the microwave method, electrochemical deposition or anodization is another simple, low-cost, and high-throughput technique to directly fabricate nanostructures on the substrate. The electrodeposition method is based on the cathodic reduction reaction under stable voltage and current in aqueous or organic solvents. The substrate material, such as indium-tin oxide (ITO), anodized aluminium oxide (AAO), or other materials, can be used as a cathode and is immersed in a solution containing the metal salt to be deposited. The dissolved metallic ions are attracted to the cathode and then reduced to the metallic form. The distinctive advantages of the electrodeposition method lie in the capacity to enable the conformal deposition of structures, and it can accurate control the quality of products, such as the thickness, morphology, and compositions of the deposited products via adjusting the current density, voltage, temperature, surfactants, concentration, selection of electrolyte and deposition substrate, etc. For example, copper sulfide nanostructures were deposited onto Cu substrates or Cu foil acting as anode and Ti metal as cathode in the voltage range of 1.5-8 V in Na$_2$S aqueous solution. Cu$_2$S and CuS nanorod and nanowall arrays were achieved by manipulating the voltage as well as the reaction temperature (Figure 2.16).

![Image](image_url)

**Figure 2.16** Field emission SEM (FESEM) images of the morphology of copper sulfide nanowalls oriented vertically to the substrate formed by anodization of copper.
foil in aqueous Na$_2$S electrolyte at low voltage and/or low temperature: (a) 1.5 V at room temperature (profile view), (b) 3 V at 5 °C (top-view), (c) 1.5 V at room temperature (top-view), and (d) 1.5 V at 5 °C (top-view).

Single-crystal CuTe nanoribbons with thickness of about 50 nm, width of 300-600 nm, and lengths of several micrometers could be uniformly deposited on ITO-coated glass substrate in an aqueous solution of 20 mM TeO$_2$, 0.2 mM CuSO$_4$ and 0.536 M NH$_3$·H$_2$O at pH 13. Polycrystalline BiTe arrays of nanowires and nanotubes were prepared by using commercially available polycarbonate (PC) as the template.

**2.2.7 Photochemical method**

Photochemical synthesis is based on the use of powerful lasers to induce the decomposition of a certain precursor and then promote the initiation of the nucleation and growth of the nanocrystals. Various high energy light sources including light emitting diodes (LEDs, and ultraviolet (UV) and γ-ray sources are often selected for synthesis. The photochemical method has been commonly adopted to fabricate different TMC nanomaterials, such as ZnS nanowires, NiS and PdS nanofilms, BiSe nanoparticles, Bi$_2$Se$_3$ nanospheres and nanorods, Ag$_2$Se nanotubes and dendrites, layer-structured WS$_2$, etc. Mathew et al. reported that copper sulfide (Cu$_x$S) particles had been synthesized via the photochemical method from an aqueous solution containing copper sulfate (CuSO$_4$) and sodium thiosulfate (Na$_2$S$_2$O$_3$) in different compositions. The particles of various compositions were deposited in an acidic medium (pH ~ 3) for 1-2 hours of photoirradiation. The amount of particles formed depended on the irradiation time, and about 75% of the reagents were converted into fine Cu$_x$S particles. The photochemical strategy is environmentally-friendly, low-cost, safe, and can be conducted at low temperature compared with other synthetic methods. It should be noted, however, that this method is often time-consuming and faces the problem of low yields.

**2.3 Biomedical application of transition metal chalcogenide based nanomaterials**

As briefly discussed in the introduction section, TMC nanostructures have been widely investigated for diverse applications due to their wealth of properties. Here, we will review recent progress on transition metal chalcogenide based nanomaterials
in biomedical applications, which include 1) bioimaging, such as fluorescence imaging, photoacoustic imaging, and multimodal imaging; 2) photothermal therapy; and 3) drug and gene delivery.

### 2.3.1 Fluorescence imaging

Similar to the rationale of nanotechnology, when the size of materials is reduced to the nanoscale range, the resultant nanomaterials possess unusual optical, electrical, magnetic, mechanical, and chemical properties, which are distinctly different from those of their bulk analogues. The semiconductor nanocrystals (also referred to as quantum dots (QDs)) exhibit strong size-dependence of their optical and electrical properties when their size is small enough (e.g. smaller than the Bohr radius). Fluorescent QDs include semiconducting nanoparticles from Groups IV (C, Si, and Ge dots),\[^{[149-151]}\] II-VI (CdE and ZnE, E = S, Se, and Te), III-V (InP), and I-III-VI (CuInS\(_2\), CuInSe\(_2\))\[^{[152-154]}\] among which, the TMC II-VI QDs (especially CdSe and CdTe based QDs) have been extensively investigated as prototypes of semiconductor QDs due to their strong quantum confinement effects and high fluorescence quantum yields (QYs).\[^{[155-158]}\] The earliest reported bioapplications of fluorescent QDs can be dated back to as early as 1998 when Bruchez et al. coated core-shell CdSe@CdS QDs with a thin layer of silica and then conjugated them with biotin.\[^{[28]}\] The biotinylated QDs were successfully applied to label 3T3 mouse fibroblast cells. Chan et al. used small-molecule mercaptoaetic acid (TGA) for solubilization and covalent protein attachment to make hydrophobic CdSe@ZnS QDs water-soluble, and then conjugated them with transferrin proteins.\[^{[27]}\] The TGA-modified QDs and transferrin-QD conjugates were incubated with HeLa cells, respectively, and it was found that no QDs could be observed inside the cell in the absence of transferrin, while QDs were internalized into the cells in the presence of transferrin due to the occurrence of receptor-mediated endocytosis.

Inspired by the above pioneering research, intense nonspecific or targeted bio-labelling and imaging have been carried out at different levels, ranging from *in vitro* to *in vivo* models.\[^{[29, 30, 159]}\] *In vitro* nonspecific cellular labelling is often achieved by using the hydrophobic and electrostatic interactions between the surface capping molecules of QDs and biomolecules in the cell membrane. Therefore, their surface ligand properties and the cell type largely determine the nonspecific adsorption and uptake of QDs. In order to overcome nonspecific adsorption,
polyethylene glycol (PEG) and its derivatives have been used to modify the QD surface, as they can effectively minimize and prevent the nonspecific interactions of QDs with biomolecules, cells, and tissues. Similar to the in vitro nonspecific adsorption of cells, non-targeted QDs can accumulate within tumours through the enhanced permeability and retention (EPR) effect. Such passive targeting is attributed to the leakiness of the tumour vasculature and its poor lymphatic drainage, which enables QDs or other nanoparticles to accumulate in tumours. The EPR effect could lead to more than a 50-fold increase in nanoparticle accumulation within tumours, compared with healthy tissues. Generally, the longer the nanoparticle circulation time, the greater the EPR-induced accumulation. It is still a challenge, however, to maximize nanoparticle accumulation through the EPR effect, as this effect varies from tumour to tumour, and strongly depends on the particle size and the surface charge. In addition, the EPR effect is not commonly observed in some types of cancers, including gastric and pancreatic cancer. In other cases, the tumour core may not be well-perfused.

In contrast to passive targeting, active targeting can be achieved by attaching various targeting ligands to QDs to enable recognition of specific cell surface molecules or proteins. A wide variety of targeting moieties, such as small molecules (e.g. folic acid and hyaluronic acid), peptides (e.g. arginine-glycine-aspartic acid (RGD)), and proteins (e.g. antibodies, antibody fragments, transferrin, etc.), have been investigated for in vivo targeted imaging with QDs. For example, In 2004, Gao et al. reported a landmark work on in vivo cancer targeting with QDs. They first encapsulated hydrophobic luminescent CdSe@ZnS core-shell QDs with an ABC triblock copolymer (i.e. polybutylacrylate-polyethylacrylate-poly(methacrylic acid) by using strong hydrophobic interactions between the capping ligands of the QDs (tri-n-octylphosphine oxide (TOPO)) and the hydrophobic segments of the block copolymer (polymer hydrocarbon). Then, they conjugated tumour-targeting ligands and drug-delivery functionalities (such as peptides, antibodies, or small-molecule inhibitors) with the polymethacrylic acid segment. The in vivo imaging results indicated that these QD probes accumulated at tumour sites through passive and active mechanisms, but passive targeting is much slower and less efficient than active targeting.
Fluorescent imaging in the second near-infrared window (NIR-II, 1000 to 1400 mm) has attracted intense attention due to the minimal body autofluorescence and negligible tissue scattering in this region, affording maximal penetration depth (> 5 mm) for deep tissue imaging with high feature fidelity\cite{166,170} and a signal-to-noise ratio of > 100 compared to emission in the visible region (400-700 nm) and the traditional near-infrared window (NIR-I, 700-950 nm). When combined with wavelength-resolved imaging, the QD probes allow sensitive and multicolour imaging of cancer cells in living animals. The use of near-infrared-emitting QDs should improve both the tissue penetration depth and the imaging sensitivity owing to the reduced photon absorption and scattering by tissues. In the past decade, great efforts have been devoted to identifying NIR-II emitting agents for in vivo imaging applications. Although NIR-II emitting CdHgTe,\cite{171} PbS,\cite{172} and PbSe\cite{173} QDs have been successfully developed, the intrinsic toxicity of the heavy metal elements (Pb, Cd, and Hg) prevents their potential use in in vivo imaging in practice. Therefore, NIR-II fluorescent nanoprobeS without these aforementioned toxic elements are highly desirable for biological imaging in this beneficial spectral region. Qiangbin Wang et al. reported a new type of NIR-II QDs, Ag$_2$S QDs, by using thermal decomposition of a single-source precursor.\cite{168,174} The PEGylated-Ag$_2$S QDs mainly accumulate in the reticuloendothelial system (RES) after intravenous administration and can be gradually cleared out, mainly by faecal excretion, demonstrating low toxicity.\cite{175} They also synthesized Ag$_2$Se QDs with their emission spectra centred at 1300 nm (the optimal wavelength for in vivo imaging with the deepest tissue penetration due to the lowest photon absorption and tissue scattering)\cite{176}. After functionalizing the surface of Ag$_2$Se QDs with an amphiphilic anhydride-alt-1-octadecene)-polyethylene glycol (C$_{18}$-PMH-PEG) polymer, the obtained C$_{18}$-PMH-PEG-Ag$_2$Se QDs exhibited bright NIR-II fluorescence, good water solubility, and high colloidal stability and photostability, as well as decent biocompatibility.
Figure 2.17 (A) PEGylated-Ag$_2$S QDs photoluminescence (PL) emission spectrum, with the inset showing an NIR-II PL image of the PEGylated-Ag$_2$S QD suspension under an 808 nm excitation (left), and an NIR-II PL image (right) collected in the 1100-1700 nm region of a tumor-bearing BALB/c mouse treated with a 15 mg/kg dose of PEGylated-Ag$_2$S QDs at 24 h post injection.$^{[175]}$ (B) Optical properties of C$_{18}$-PMH-PEG-Ag$_2$Se QDs, with the inset showing a PL image of the C$_{18}$-PMH-PEG-Ag$_2$Se QDs suspended in water at a concentration of 0.6 mg/mL (left), and in vivo imaging (right) of live mice in supine position after tail injection of C$_{18}$-PMH-PEG-Ag$_2$Se QDs.$^{[176]}$

2.3.2 Photoacoustic imaging

Photoacoustic imaging (PAI) is a newly emerging biomedical modality that provides both high spatial resolution and strong biochemical contrast, benefiting from the combined merits of ultrasound imaging and optical imaging.$^{[177-180]}$ Based on the ultrasonic signal generated from the photoacoustic effect, it is possible to obtain images with high spatial resolution and optical contrast in a region up to 5-6 cm deep in biological tissues, which breaks through the optical diffusion limit.$^{[181, 182]}$ PAI is based on the detection of thermoelastic expansion and ultrasonic signals induced by photothermal expansion of light-absorbing tissues or contrast agents.
under irradiation by short laser pulses. Although PAI facilitates imaging of biological tissue based on endogenous chromophores (oxyhemoglobin and deoxyhemoglobin), its potential applications can be significantly expanded by exogenous contrast agents. One of the most important factors necessary for the fabrication of photoacoustic contrast agents is the ability to convert absorbed light into heat to produce ultrasound waves. Exogenous contrast agents with NIR (> 700 nm) absorption can be utilized to enhance the photoacoustic signal, due to their relatively low absorption coefficient and low scattering coefficient within this wavelength region, thus providing a relatively clear window for imaging with optical techniques. A great many metal/semiconductor materials possess this function, such as silver, gold, carbon, quantum dots, etc. By reconstructing the composition, size, shape, and optical properties of the imaged tissues, these structures have great potential in the detection and imaging of cancerous tissue as distinguished from healthy tissue. Owing to their intrinsic absorptive properties, plasmonic nanomaterials, such as gold nanostructures, are widely applied as potential PAI contrast agents.\textsuperscript{[181, 183-185]} Those nanostructures also have several limitations as contrast agents for PAI, however, including dependence of the optical properties on shape, size, and geometry of the nanoparticles (NPs) and on environmental factors. In addition, these NIR-absorbing nanostructures usually have relatively large sizes (usually > 35 nm TEM size) which can result in rapid capture by organs of the mononuclear phagocyte system (MPS) and slow clearance from the body.\textsuperscript{[186-188]}

Benefiting from the strong localized surface plasmon resonance (LSPR) effect originating from free holes, copper chalcogenide based nanomaterials such as CuS and Cu\textsubscript{2}Se nanoparticles provide an alternative to Au particle-based photoacoustic contrast agent due to their inherent low hole density and high hole effective mass.\textsuperscript{[58, 189-192]} Chun Li et al. initially reported the use of CuS NPs as a novel class of contrast agent for \textit{in vitro} and \textit{in vivo} PAI.\textsuperscript{[193]} The absorption of CuS NPs could be tuned to peak at wavelengths greater than 900 nm. This makes it possible to use a Q-switched neodymium-doped yttrium aluminium garnet (Nd:YAG) laser, which emits laser light at 1064 nm, as the light source for PAI. The Nd:YAG laser at a setting of 1064 nm can provide high pulsed energy with a nanosecond pulse width, which should translate to a stronger PAI signal, a higher signal-to-noise ratio, and a greater field-of-view. Because of the low absorption and scattering coefficient in normal tissue at
1064 nm (the second optical window), the strong light absorbed at this wavelength by CuS NPs allows deep tissue PAI. Successful CuS NP mediated PAI of the lymph nodes and brain has been achieved in mouse models. Figure 2.18 presents PAI imaging of a mouse brain with CuS NPs. A nodule on the left cortex was clearly imaged (Figure 2.18b) 24 h after intracranial injection of 15 mL CuS NPs compared to the photoacoustic tomography (PAT) image acquired before injection (Figure 2.18a). Nevertheless, the brain nodule in Figure 2.18c disappeared 7 days after injection in the photograph of the mouse head shown in Figure 2.18d, probably because the CuS NPs had been cleared from the injection site. These promising findings suggest that CuS NPs may be used for molecular PAI in clinical applications, especially for superficial tumors or lesions, such as for imaging breast lesions up to 5 cm in depth, or possibly other superficial lesions in the skin, lymph nodes, limbs, and head and neck. Swihart et al. synthesized Cu$_2$-xSe nanocrystals (NCs) via a hot injection method. The particle size could be tunable by adjusting the ratio of oleylamine to oleic acid and the reaction time. They found that the PAI detection limit of Cu$_2$-xSe NCs was 400 times higher than that of Au nanocages. Later on, the same group synthesized Au-Cu$_2$-xSe nanodimers using the seeded-growth method and demonstrated their utility for high contrast multimodal imaging in vitro and in vivo. The high photoacoustic imaging depth achieved, up to 17 mm, showed that these novel contrast agents could be clinically relevant.
Figure 2.18 *In vivo* PAT images of a mouse brain. Images were acquired using laser light (A) at a wavelength of 532 nm without CuS injection, (B) at 1064 nm after 24 h and (C) at 1064 nm 7 days after intracranial injection of CuS NP solution. (D) Photograph of the head of the mouse. Laser light was irradiated from the top. The arrows indicate a nodule site on the left cortex.\(^{193}\)

### 2.3.3 Multimodal Imaging

Multimodality and multifunctional imaging has evolved into a fast-growing research field with the goals of detecting and measuring biological processes *in vivo* non-invasively.\(^{195, 196}\) Many traditional medical imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound, have been routinely used to monitor the therapeutic effects of cancer intervention.\(^{195, 197}\) The field of molecular imaging, recently defined by the Society of Nuclear Medicine as “the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living
systems”[198] has flourished over the last decade. In general, molecular imaging modalities include molecular MRI (mMRI), magnetic resonance spectroscopy, optical bioluminescence imaging, optical fluorescence imaging, targeted ultrasound, single-photon emission CT (SPECT), and positron emission tomography (PET).[199] However, monomodal imaging of nanoparticles makes it difficult to satisfy ideal imaging demands such as integrity of spatial resolution and ultrasensitivity, owing to the respective drawbacks of the various methods.[200, 201] For instance, MRI has superb soft tissue contrast and good spatial resolution, but it suffers from poor sensitivity; fluorescent imaging has excellent sensitivity, but it faces the challenge of quantification and deep tissue penetration. Although PET is superior in sensitivity, quantification, and tissue penetration, its resolution is relatively low. The combination of multiple imaging techniques using a single probe can potentially overcome these disadvantages and provide synergistic information.

Many hybrid systems that combine two or more of these imaging modalities are also commercially available, and certain others are under active development.[202-204] In the clinic, integrated examination using PET/CT has gained widespread acceptance as a tool for tumor diagnosis, staging, prognosis, treatment planning, and assessment of treatment response. Similarly, PET/MRI has begun to be used as an alternative hybrid modality for clinical applications. The combination of PET(PAI)/CT or PET(PAI)/MRI may offer new opportunities by providing potentially more comprehensive and accurate information as a result of the complementary features of these technologies. Therefore, attempts have been made to develop plasmonic CuS-based NPs as contrast agents for CT or MRI. The copper ion (II) is regarded as a candidate MRI contrast agent because it possesses unpaired electrons. In aqueous solution, the $T_1$ relaxivity ($r_1$) of Cu$_{2-x}$S NPs was measured to be 0.26 mM$^{-1}$ s$^{-1}$ at 3 T, which was substantially lower than that of gadolinium-diethylenetriamine penta-acetic acid (Gd-DTPA) (∼4.5 mM$^{-1}$ s$^{-1}$). Nevertheless, contrast enhancement with Cu$_{2-x}$S NPs was visualized in vivo either after intravenous (IV) injection or intratumoral injection. In addition, due to their NIR optical absorption, these NPs also served as an efficient contrast agent for enhancing PAI.[205] Recently, Ni-doped CuS NPs were proposed for PAI/MRI dual imaging.[206] The hybrid nanostructures demonstrated NIR absorption and enhanced $T_1$ relaxivity, which is suitable for PAI and MRI, respectively. The relaxivity of the hybrid
nanostructures was about 2.3 mM\(^{-1}\) s\(^{-1}\), which was nearly 8 times higher than that of pegylated multidentate polymer with imidazole pendant groups (PMAH-PEG)-Ni\(^{2+}\). These dual modality Ni-CuS hybrid NPs were successfully used for in vivo lymph node PAI and MRI (Figure 2.19).

![Figure 2.19](image.png)

**Figure 2.19** *In vivo* PAI imaging of a mouse before (a) and after (b) injection with PMAH-PEG-capped Ni-doped CuS NPs; \(T_1\) weighted MRI of the mouse before (c) and after (d) injection of the dual-modal nanoprobe. The red circle indicates the lymph node.\(^{[206]}\)

For a fluorescence/MRI dual-modality imaging nanoprobe, a core/shell nanoprobe was constructed for dual-modality imaging of breast cancer, which was composed of an Fe\(_3\)O\(_4\) NP core and two outside layers of silica shell.\(^{[207]}\) CdSe/ZnS QDs (with emission peak at 600 nm) and NIR fluorescent CdSeTe/CdS QDs (with emission peak at 780 nm) were embedded inside each silica layer to form the dual modality agent, which was denoted as the MQQ-probe. After conjugation with an antibody against human epidermal growth receptor 2 (anti-HER2 antibody), the HER2-MQQ-probe was injected into tumor-bearing mice. NIR fluorescence imaging demonstrated enhanced tumor specific accumulation of the HER2-MQQ-probe as compared to the non-targeted MQQ-probe. Meanwhile, MRI provided the detailed anatomical structure of the tumor.

### 2.3.4 Photothermal therapy
Recently, near-infrared (NIR) (700-3000 nm) laser-induced photothermal therapy (PTT), which can localize elimination of tumour cells without affecting healthy tissue, has emerged as a new effective cancer treatment.\(^{208, 209}\) As blood cells and water barely absorb NIR, NIR can penetrate tissues to reach deeply-placed, optically sensitive nanoparticles. As a result, near-infrared light-responsive nanomaterials which have strong absorption in the NIR region can absorb penetrating NIR to generate local heat for PAI imaging and produce hyperthermia (> 45 °C) to kill tumor cells directly.\(^{210}\) Thus, PTT as a highly promising non-invasive bioimaging technique that has attracted tremendous attention in the past few years owing to its highly specific selectivity towards the targeted sites. The key to PTT is the use of NIR-responsive nanomaterials, owing to their unique physical and chemical properties. It is well known that intravenously administered nanoparticles preferentially accumulate in tumours in a passive process through the enhanced permeability and retention (EPR) effect,\(^{211}\) and also have longer retention times in tumour sites than small molecules, so that they provide a much longer time window for tumour diagnosis and therapy. Various near-infrared light-responsive nanomaterials, including gold nanomaterials,\(^{212-214}\) graphene nanomaterials,\(^{215-217}\) copper chalcogenide nanocrystals,\(^{218, 219}\) and other related materials,\(^{220-222}\) are designed to achieve PTT. Herein, we would like to review the progress on copper chalcogenide nanocrystals, one kind of transition metal chalcogenide used for PTT.

Copper-based nanocrystals (NCs), such as copper sulfide (CuS) and copper selenide (Cu$_2$Se), have emerged as excellent new photothermal agents due to their strong absorbance in the NIR region. In 2010, Li and co-workers first synthesized thioglycolic acid-stabilized CuS nanoparticles via a wet chemistry method and applied it as a new type of agent for photothermal ablation of cancer cells.\(^{218}\) These uniform CuS nanoparticles have a mean size of approximately 3 nm and show an increased absorption band in the NIR region, with maximum absorption at 900 nm. A further cell photothermal destruction assay and toxicity assay indicated that the CuS nanoparticles displayed outstanding photothermal ablation efficiency and minimal cytotoxic effects. The only challenging issue is that for \textit{in vivo} applications, a high powered laser is required, as high as 16 and 24 W/cm$^2$, which is ~48 and 72 times higher, respectively, than the conservative limit (~0.33 W/cm$^2$) for activation due to their relatively weak absorbance and poor photothermal conversion efficiency. In
order to improve the photothermal conversion efficiency, Tian et al. reported hydrophilic flower-like CuS superstructures with a mean size of ~1 µm as an efficient 980 nm laser-driven photothermal agent for ablation of cancer cells, which were synthesized by using a controllable hydrothermal route assisted by poly(vinyl pyrrolidione) (PVP) and were assembled from hexagonal plate-like building blocks (Figure 2.20a).\textsuperscript{219} The uniform and monodispersed three-dimensional (3D) CuS hierarchical structures exhibited enhanced absorption and ~50% increased photothermal conversion efficiency upon irradiation with a 980 nm laser when compared to the nanoplates (Figure 2.20b). It was supposed that the faceted end planes of these crystalline hierarchical structures can serve as excellent laser-cavity mirrors for a 980 nm laser, which led to a great improvement in the reflection and absorption capability of the laser. The efficacy of these agents for photothermal ablation was evaluated both \textit{in vitro} and \textit{in vivo}, and the results showed that even at a low laser power density of < 1 W/cm\textsuperscript{2}, the CuS nanostructures were capable of inducing cell death \textit{in vitro} (Figure 2.20c). Furthermore, histological examination of the tumours treated with CuS hierarchical structures showed degenerative necrotic and karyolytic regions. Nevertheless, although the CuS hierarchical structures showed enhanced photothermal conversion efficiency, the large mean size (~1 µm) is a huge obstacle that prevents this material from being a suitable photothermal agent in biological applications. Unfortunately, the absorption intensity of CuS nanomaterials is greatly affected by the particle size and is reduced dramatically with decreasing size. For instance, the photothermal conversion efficiency of small CuS NCs (~ 3 nm) was too low for them to serve as \textit{in vivo} PTT agents. Therefore, there is an urgent need to develop novel copper chalcogenide semiconductors with small size (< 100 nm) but high photothermal conversion efficiency to meet the demands of biological application. As a result, Tian and colleagues developed hydrophilic Cu\textsubscript{9}S\textsubscript{5} plate-like nanocrystals with a mean size of ~70 nm × 13 nm by a simple thermal decomposition route followed by a ligand exchange process.\textsuperscript{56} This Cu\textsubscript{9}S\textsubscript{5} exhibited superior photothermal conversion efficiency, up to 25.7%, which is higher than that of as-synthesized Au nanorods (23.7%). More importantly, cancer cells can be efficiently killed \textit{in vivo} by the photothermal effect, which can be realized by a very low concentration (40 ppm) aqueous dispersion of the Cu\textsubscript{9}S\textsubscript{5} NCs under irradiation by a 980 nm laser with a low and safe power density of 0.51 W/cm\textsuperscript{2}. Therefore, these
Cu$_9$S$_5$ NCs have great superiority as a new photothermal agent for the NIR-induced photothermal ablation (PTA) of cancer, due to their small size (< 100 nm) and high photothermal conversion efficiency, as well as their low cost and low cytotoxicity.

**Figure 2.20** Photothermal ablation with CuS hierarchical structures. (a) Schematic representation of a CuS hierarchical structure with nanosheets serving as laser-cavity mirrors for a 980 nm laser and its photothermal conversion. (b) The CuS hierarchical structures exhibited superior photothermal properties when compared with the building blocks. (c) CuS hierarchical structures can cause efficient photothermal ablation when excited with a 980 nm laser with power density of < 1 W/cm$^2$. *In vitro*, only dead HeLa cells can be labeled with trypan blue. In PC-3 tumour-bearing mice, obvious tumour damage can be seen in hematoxylin and eosin (H&E) staining after photothermal ablation.$^{[219]}$

Similar to CuS nanomaterials, amphiphilic polymer-coated Cu$_2$S$_x$Se nanocrystals approximately 16 nm in diameter were demonstrated to be an effective photothermal
transduction agent, as shown in Figure 2.21a.[223] The nanocrystals readily disperse in water and possess strong NIR optical absorption with a high molar extinction coefficient at 980 nm. The photothermal conversion efficiency of Cu$_2$-xSe nanocrystals was comparable to those of the Au nanorods (NRs) and Au nanoshells when exposed to an 808 nm laser with a 2 W cm$^{-2}$ power density (Figure 2.21b). In vitro PTT of Cu$_2$-xSe NCs in the presence of human colorectal-cancer cells (HCT-116) caused cell destruction after 5 min of laser irradiation at 30 W cm$^{-2}$, whereas photothermal cell destruction was not observed in either the cells treated with Cu$_2$-xSe alone (without laser) or the cells treated with laser irradiation alone (without Cu$_2$-xSe), as shown in Figure 2.21c. The example discussed above demonstrates the promising application of copper based NCs as an ideal photothermal agent for in vivo PTT of tumor tissues, and surface-modification strategies are currently urgently needed to endow copper based NCs with drug-delivery capabilities for applications in thermo-chemotherapy.

Figure 2.21 Copper selenide nanocrystals, to be used as a photothermal therapy agent. (a) TEM image of copper selenide nanocrystals. The inset shows a photograph of oleylamine-capped Cu$_2$-xSe nanocrystals dispersed in chloroform. (b) Plot of the photothermal transduction efficiencies for the Cu$_2$-xSe nanocrystals, Au nanorods, and Au nanoshells. (c) Comparison of photothermal destruction of cancer cells without (top row, left and right) and with (bottom row, left and right) the addition of Cu$_2$-xSe nanocrystals. Cells irradiated at 30 W cm$^{-2}$ with an 800-nm laser for 5 min (circular spot size of 1 mm) were stained with Trypan blue to visualize cell death.[223]

2.3.5 Drug and gene delivery

During the past several decades, smart and multifunctional drug delivery systems have achieved great developments in terms of targeting, low toxicity, excellent intracellular biostability and biocompatibility, multidrug delivery, and theranostics.[224-226] Diverse drug delivery systems, such as hydrogels,[227]
liposomes,\textsuperscript{228} dendrimers,\textsuperscript{229} and inorganic nanoparticles,\textsuperscript{230-232} have been widely used as carriers for drug delivery. Among these carriers, CuS NPs have demonstrated promising potential as drug carriers. Some reports have shown that CuS nanomaterials, including hollow nanostructures\textsuperscript{[233]} and mesoporous silica nanocomplexes,\textsuperscript{[234]} can be used to control drug delivery. Owing to the mesoporous and hollow structure of CuS NPs, they possess drug delivery capability and sustained drug-release properties. Hollow CuS NPs were used as a vehicle for enhanced transdermal drug delivery through a localized thermal effect on the skin from a nanosecond-pulse laser. Hollow CuS NPs were also coated with chitosan for the delivery of an immunostimulatory agent that enhances immunotherapy (Figure 2.22a).\textsuperscript{[235]} In this study, it was shown that there was PTT-induced release of tumor antigens into the surrounding milieu, while CpG (cytosine triphosphate deoxynucleotide ("C") followed by a guanine triphosphate deoxynucleotide ("G"), with the "p" referring to the phosphodiester link between consecutive nucleotides), potentiated host antitumor immunity. The results demonstrated that combined photothermal immunotherapy is more effective than either immunotherapy or PTT alone against primary (treated) and distant (untreated) tumors. Moreover, these hollow CuS nanoparticles are biodegradable and can be eliminated from the body after laser excitation. Because hollow CuS NPs possess large specific surface areas and numerous mesoporous pores, one could efficiently load them with hydrophobic drug molecules. For example, camptothecin was loaded onto hollow CuS NPs, and its release from the NPs could be remotely controlled using a 980 nm NIR laser.\textsuperscript{[236]} Synergistic antitumor activity was achieved by integrating PTT and chemotherapy, enabled by a single NP system. In another study, CuS nanocages were synthesized using a solid-liquid reaction between Cu$_2$O nanocubes and thiourea.\textsuperscript{[237]} The CuS nanocages displayed exceptionally high drug loading capacity for doxorubicin (DOX) and released the anticancer drug in a pH-dependent fashion. Such strategies to develop multifunctional NPs represent a promising avenue for enhancing antitumor efficacy.
Figure 2.22 (a) Schematic illustration of assembly of HCuSNPs-CpG conjugates, near-infrared light-triggered disintegration of HCuSNPs, and system reassembly. HCuSNPs-Chi: chitosan-coated HCuSNPs. Chi-CpG-NPs: chitosan-CpG nanocomplexes. SCuSNPs: small CuS NPs.\(^{[235]}\) (b) Schematic illustration of the synthetic protocol for Cu\(_9\)S\(_5\) @mSiO\(_2\)-PEG core-shell nanocomposites. Schematic illustration of the nanocomposites as a multifunctional nanoplatform for combining photothermal therapy and chemotherapy with infrared thermal imagining for cancer treatment.\(^{[234]}\)

Mesoporous silica is a form of silica that contains pores that are 2-50 nm in diameter. The large surface area of the pores of mesoporous silica NPs allows the particles to be filled with drugs or imaging agents. Song et al. coated CuS nanocrystals with a mesoporous silica (mSiO\(_2\)) shell to form Cu\(_9\)S\(_5\)@mSiO\(_2\)-PEG core-shell nanostructures for cancer drug delivery (Figure 2.22b).\(^{[234]}\) The mesoporous silica shell is provided as the carrier for loading DOX, and the release of DOX from the nanostructures could be triggered by pH and NIR light. The same
group also conjugated folic acid to CuS@mSiO$_2$-PEG core-shell nanostructures for targeted delivery.\cite{238} In another study, DOX-loaded core-shell Cu$_9$S$_5@mSiO$_2$ NPs were incorporated into poly(ε-caprolactone) and gelatin to form nanofibrous fabrics using an electrospinning process. The resultant composite fibers could be implanted directly in the tumor to achieve combined chemotherapy via the controlled release of DOX from the mesoporous silica and PTT with the imbedded Cu$_9$S$_5$ NPs.\cite{239} Several other CuS NP-mesoporous silica systems based on similar concepts have been developed and tested.\cite{240, 241} In one system, mesoporous silica was conjugated to CuS NPs with two complementary DNA sequences.\cite{240} In this study, CuS NPs acted as both a gatekeeper, preventing premature release of drugs, and as a photothermal conducting agent for ablation of cancer cells.

2.4 References

Chapter 2 Literature Review

Chapter 2 Literature Review

Chapter 2 Literature Review

Chapter 3 Experimental Methods

3.1 Chemicals and Materials

The chemicals and materials used in this thesis are listed in Table 3.1.

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</tr>
<tr>
<td>Nitric acid</td>
<td>HNO₃</td>
<td>65</td>
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</tr>
<tr>
<td>Rhodamine 6G</td>
<td>C₂₈H₃₁N₂O₃Cl</td>
<td>99</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Selenium dioxide</td>
<td>SeO₂</td>
<td>99.9</td>
<td>Aladdin</td>
</tr>
<tr>
<td>Selenium powder (-100 mesh)</td>
<td>Se</td>
<td>≥99.5</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium citrate tribasic dihydrate</td>
<td>HOC(COONa)(CH₂COONa)₂·2H₂O</td>
<td>99</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium borohydride</td>
<td>NaBH₄</td>
<td>99</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium tellurite</td>
<td>Na₂TeO₃</td>
<td>99</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>----</td>
<td>---------------</td>
</tr>
<tr>
<td>Tetraethyl orthosilicate</td>
<td>C₉H₂O₃Si</td>
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<tr>
<td>Triethylamine</td>
<td>C₆H₁₅N</td>
<td>99</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Polyvinylpyrrolidone powder</td>
<td>(C₆H₅NO)n</td>
<td>K16-K90</td>
<td>Sinopharm/Aladdin</td>
</tr>
<tr>
<td>α,ω-Dimercapto poly(ethylene glycol)</td>
<td>HS(CH₂CH₂O)nCH₂CH₂SH</td>
<td>Mₘ = 5000</td>
<td>Adamas</td>
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<td>(3-aminopropyl) triethoxysilane</td>
<td>H₂N(CH₂)₃Si(OCH₃)₃</td>
<td>99</td>
<td>Sigma-Aldrich</td>
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<td>(3-mercaptopropyl) trimethoxysilane</td>
<td>HS(CH₂)₃Si(OH₃)₃</td>
<td>99</td>
<td>Sigma-Aldrich</td>
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</table>

### 3.2 Materials preparation

As discussed in the section of literature review, liquid-phase-based wet chemical methods have been witnessed to be powerful for obtaining desired nanostructured TMCs with controlled size, compositions and shapes. For the preparation of nanomaterials with different morphologies, liquid phase syntheses are always preferred. These synthetic reactions proceed at relatively lower temperatures and therefore require lower energies. The main preparation methods used in this thesis are also liquid-phase-based wet chemical methods including the aqueous refluxing or hydrothermal method, biphase stratification approach, aqueous reduction-precipitate reaction and high-temperature coprecipitation approach.

#### 3.2.1 Aqueous refluxing or hydrothermal method

The MSA-capped CdTe QDs in this thesis can be synthesized via refluxing or hydrothermal treatment method. Over the past decades, a variety of synthetic methods have been developed for the fabrication of QDs. Based on the different media used, these methods can be broadly classified into two types: the organometallic route and aqueous synthesis. QDs synthesized in these organic solvents possess nearly perfect crystal structures, and thus high fluorescence QY. Narrow size distribution is another advantage for QDs prepared via this route. On the other hand, the hydrophobic surface of QDs synthesized using this method is a major obstacle for biological applications. In contrast, aqueous synthesis, with the
advantages of improved simplicity/reproducibility and less toxicity, has gradually become a preferred option, despite the lower QY and broader size distribution. For example, hydrophilic thiol-capped QDs are more suitable for biomedical applications because of their higher stability and better compatibility in biological environment compared to those prepared in organic solvent. In this thesis, MSA-capped CdTe QDs were synthesized via refluxing method. Specifically, the cadmium chloride (CdCl₂, 0.04 M, 4 mL) and sodium citrate tribasic dehydrate (400 mg) were diluted to 50 mL in a three-necked flask, and stirred at room temperature for 1 h. Na₂TeO₃ (0.01 M, 4 mL), MSA (100 mg), and sodium borohydride (NaBH₄, 50 mg) were then added under vigorous stirring. When the color of the mixture turned green, the flask was attached to a condenser and refluxed at 100 °C under N₂ flow for 3 h.

3.2.2 Biphasic stratification approach

It is well known that soft-templating approach is one of the current most useful synthetic methods for the ordered mesoporous silica via surfactant molecular self-assembly. It has indicated that the soft-templating approach usually needs a homogeneous reaction system, whether the soft templates are micelles assembled with surfactants or nanoemulsions constituted of surfactants and oil drops. In this doctoral work, we prepared uniform monodispersed mesoporous silica nanospheres with large center-radial pore channels in oil-water biphasic stratification reaction system, which is a heterogeneous reaction occurred in the macroscopic interface. Typically, a mixed solution (the lower aqueous phase) containing 18 mL H₂O, 12 mL CTAC (25 wt% in H₂O), and 45 µL of TEA was added into a 100 mL round bottom flask and stirred for 1 h at 60 °C. Then, 1 mL TEOS (20% v/v in cyclohexane) as upper oil phase was gently dropped in, and the solution flask was attached to a condenser. The reaction was maintained at 60 °C for 8 h in an oil bath with reflux under magnetic stirring (100 rpm). The products were collected after removal of the upper oil layer, and then centrifuged and washed several times with ethanol to remove the residual reactants.

3.2.3 Aqueous reduction-precipitate reaction

In this doctoral work, in order to develop a large scale, low cost and generic route to fabricate nanostructured TMCs with good properties control, MSA was used as a stabilizer and selenium powder, which are cheaper and more stable and easily can be reduced to Se²⁻ by using the common reduce agent of NaBH₄, are selected to be
starting precursors due to that the Se$^{2-}$ ions are not stable in water and air. After further optimizing of this route, ultra-small metal chalcogenide nanostructures can be fabricated by co-precipitation of cationic and anionic precursors in aqueous solution after a reduction procedure under room temperature. The typical procedure is that 39.48 mg (0.5 mmol) Se powder was reduced by 56.75 mg (1.5 mmol) NaBH$_4$ in 50 mL H$_2$O under magnetic stirring at room temperature with the protection of inert gas. 0.17 g (1 mmol) CuCl$_2$·2H$_2$O and 1 g (6.66 mmol) MSA were completely dissolved in 5 mL distilled water and then added into the selenium precursor solution to immediately generate a black solution, which indicated the formation of copper selenide nanoparticles. The black solution was subsequently purified by ultrafiltration at 5000 rpm, using a membrane with a molecular weight cutoff of 30 kDa.

3.3 Physical and chemical characterization

3.3.1 X-ray powder diffraction (XRD)

XRD is a basic and rapid analytical technique primarily used for phase identification and can determine the atomic and molecular structure of a crystal, in which the crystalline atoms cause a beam of incident X-rays to diffract into many specific directions. Prior to the determination of the average bulk composition, the analysed material could be finely ground and, homogenized. A crystallographer can produce a three-dimensional picture of the density of electrons within the crystal by measuring the angles and intensities of diffracted beams. From this electron density, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder and various other information. X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. The interaction of the incident rays with the sample produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law ($n\lambda=2d \sin \theta$). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted. By scanning the sample through a range of 2θ angles, all possible diffraction directions of the lattice should be attained due to the random orientation
of the powdered material. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacings. Typically, this is achieved by comparison of d-spacings with standard reference patterns. X-ray powder diffraction is most widely used for the identification of unknown crystalline materials (e.g. minerals, inorganic compounds). Determination of unknown solids is critical to studies in geology, environmental science, material science, engineering and biology.

3.3.2 Raman spectroscopy

Raman spectroscopy is a widely used spectroscopic technique to investigate vibrational, rotational, and other low-frequency modes in a chemical system in both qualitative and quantitative applications. Laser light from the monochromatic light source, which usually is in the visible, near infrared, or near ultraviolet range, is applied to illuminated the material, leading to both elastic scattering (Rayleigh scattering) and inelastic scattering (Stokes and anti-Stokes Raman scattering). The laser light interacts with molecular vibrations, phonons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about bands between molecular of samples and vibrational modes in the system. The unique position and intensity of peaks in spectrum make Raman spectroscopy able to identify the chemical elements in the qualitative analysis. Besides, compared to infrared bands, Raman bands are inherently sharper, which present for more straightforward quantitative analysis. In this doctoral work, Raman spectra of samples were collected using a Raman spectrometer (Lab RAM HR, Horiba Jobin Yvon SAS) with a laser at 632.8 nm excitation on a 300 lines/mm grating and a chargecoupled detector CCD. A neutral density filter is applied to adjust the laser intensity in the measurement.

3.3.3 Fourier transform Infrared spectrometer (FTIR)

Fourier transform infrared spectroscopy (FTIR) is a kind of molecule vibration spectroscopy which is used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. Infrared spectroscopy probes the molecular vibrations. Functional groups can be associated with characteristic infrared absorption bands, which correspond to the fundamental vibrations of the functional groups. Thereby, it
is most commonly used for the identification of unknown pure organic compounds. In FTIR, infrared radiation of a broad range of wavelengths is passed through an interferometer and a pathlength difference is introduced into one part of the light beam. This IR beam is then passed through the sample, which absorbs light energies corresponding to various bond-vibration and rotation frequencies. The beam is then focused on a detector, and a computer calculates the absorption of the IR frequencies by the sample, identifying compounds present and their concentration in the sample. In this study, FTIR spectra were collected on a Shimadzu IRPresting-21 model Fourier transform infrared spectrometer.

### 3.3.4 X-ray Photoelectron Spectroscopy (XPS)

XPS is a surface-sensitive quantitative spectroscopic technique that measures the elemental composition, empirical formula, chemical state, binding energies and densities and electronic state of the elements that exist within a material in its as-received state, or after some treatment, for example: fracturing, cutting or scraping in air or ultra-high vacuum to expose the bulk chemistry, ion beam etching to clean off some or all of the surface contamination and so on. XPS spectra are obtained by irradiating a sample with a beam of X-rays while simultaneously measuring the kinetic energy and number of electrons that escape from the top 0 to 10 nm of the material being analyzed. XPS detects only those electrons that have actually escaped from the sample into the vacuum of the instrument, and reach the detector. In order to escape from the sample into vacuum, a photoelectron must travel through the sample. Photo-emitted electrons can undergo inelastic collisions, recombination, excitation of the sample, recapture or trapping in various excited states within the material, all of which can reduce the number of escaping photoelectrons. These effects appear as an exponential attenuation function as the depth increases, making the signals detected from analytes at the surface much stronger than the signals detected from analytes deeper below the sample surface. Thus, the signal measured by XPS is an exponentially surface-weighted signal, and this fact can be used to estimate analyte depths in layered materials. A typical XPS spectrum is a plot of the number of electrons detected (sometimes per unit time) (Y-axis, ordinate) versus the binding energy of the electrons detected (X-axis, abscissa). Each element produces a characteristic set of XPS peaks at characteristic binding energy values that directly
identify each element that exists in or on the surface of the material being analyzed. These characteristic spectral peaks correspond to the electron configuration of the electrons within the atoms, e.g., 1s, 2s, 2p, 3s, etc. The number of detected electrons in each of the characteristic peaks is directly related to the amount of element within the XPS sampling volume. XPS experiments in this thesis were carried out on a VG Scientific ESCALAB 2201 XL instrument using aluminum Kα X-ray radiation. All the XPS data was analyzed using the commercial CasaXPS 2.3.15 software package. Before analysis, the spectra were calibrated by C 1s = 284.6 eV.

3.3.5 Transmission Electron Microscopy (TEM)

TEM is a microscopy technique which can display morphology images of samples by using electrons beam transmitted through an ultra-thin specimen. The image is formed from the interaction of the electrons transmitted through the specimen and is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a charge-coupled device. TEM is specially used to reveal morphology, crystal structure, an internal structure in nanoscale specimen and are capable of imaging at a significantly higher resolution than light microscopes, owing to the small de Broglie wavelength of electrons. This enables the instrument's user to examine fine detail, even as small as a single column of atoms, which is thousands of times smaller than the smallest resolvable object in a light microscope. Selected area electron diffraction (SAED) is an effective technique for observing crystal orientation, which can be performed inside the TEM. As a diffraction technique, SAED can be used to identify crystal structures and examine crystal defects. For example, for thin crystalline samples, this produces an image that consists of a pattern of dots in the case of a single crystal, or a series of rings in the case of a polycrystalline material. Scanning transmission electron microscope (STEM) is the associated technique of TEM which can be modified into by the addition of a system that rasters the beam across the sample to form the image, combined with suitable detectors and enables various analysis techniques such as mapping by energy dispersive X-ray spectroscopy (EDS), electron energy loss spectroscopy (EELS), and annular darkfield imaging (ADF). Moreover, these signals can be obtained simultaneously, allowing direct correlation of image and quantitative data. The TEM images in this doctoral work were
collected on a JEOL-2010 and a JEOL ARM-200F microscope operated at 200 kV. Elemental mapping was performed on the X-ray spectrometer attached to the JEOL ARM-200F instrument by using Al grids.

3.3.6 Thermogravimetric analysis (TGA)

TGA is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature (with constant heating rate), or as a function of time (with constant temperature and/or constant mass loss). Such analysis relies on a high degree of precision in three measurements: weight, temperature, and temperature change. As many weight loss curves look similar, the weight loss curve may require transformation before results may be interpreted. A derivative weight loss curve can be used to tell the point at which weight loss is most apparent. Again, interpretation is limited without further modifications and deconvolution of the overlapping peaks may be required. This technique is capable to determine selected characteristics of materials that exhibit either mass loss or gain due to decomposition, oxidation, or loss of volatiles (such as moisture). For instance, TGA is commonly employed in research and testing to determine characteristics of materials such as polymers, to determine degradation temperatures, absorbed moisture content of materials, the level of inorganic and organic components in materials, decomposition points of explosives, and solvent residues. It is also often used to estimate the corrosion kinetics in high temperature oxidation.

Simultaneous TGA-DTA/DSC measures both heat flow and weight changes (TGA) in a material as a function of temperature or time in a controlled atmosphere. Simultaneous measurement of these two material properties not only improves productivity but also simplifies interpretation of the results. The complementary information obtained allows differentiation between endothermic and exothermic events which have no associated weight loss (e.g., melting and crystallization) and those which involve a weight loss (e.g., degradation).

3.3.7 Brunauer-Emmett-Teller (BET)

BET theory aims to explain the physical adsorption of gas molecules on a solid surface and serves as the basis for an important analysis technique for the
measurement of the specific surface area of materials. The BET theory applies to
systems of multilayer adsorption, and usually utilizes probing gases that do not
chemically react with material surfaces as adsorbates to quantify specific surface area.
Nitrogen is the most commonly employed gaseous adsorbate used for surface
probing by BET methods. For this reason, standard BET analysis is most often
conducted at the boiling temperature of N\textsubscript{2} (77 K). Generally, the two key pieces of
information including specific surface area and pore size distribution can be obtained
from the BET measurements. Specific surface area was calculated from the
adsorption data in the relative pressure (P/P\textsubscript{0}) range from 0.05 to 0.35 while the pore
size distribution was calculated by the Barrett-Joyner-Halenda (BJH) method, using
the amount of nitrogen adsorbed at a relative pressure of P/P\textsubscript{0} = 0.99.

3.3.8 Ultraviolet-visible (UV-vis) spectroscopy

UV-vis spectroscopy refers to absorption spectroscopy or reflectance
spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the
visible and adjacent (near-UV and near-infrared) ranges. The absorption or
reflectance in the visible range directly affects the perceived color of the chemicals
involved. Absorption spectroscopy is complementary to fluorescence spectroscopy,
in that fluorescence deals with transitions from the excited state to the ground state,
while absorption measures transitions from the ground state to the excited state. UV-
Vis spectroscopy is routinely used in analytical chemistry for the quantitative
determination of different analytes, such as transition metal ions, highly conjugated
organic compounds, and biological macromolecules. Spectroscopic analysis is
commonly carried out in solutions but solids and gases may also be studied. For a
liquid sample, the UV-vis absorption comes from molecules absorbing light in
different wavelengths range, which can demonstrates a number of absorption bands
corresponding to structural groups within the molecule. In this thesis, all the
measurement of UV-vis spectroscopy is carried out in solution samples.

3.3.9 Dynamic light scattering (DLS)

DLS is a technique that can be used to determine the size distribution profile of
small particles in suspension or polymers in solution. In the scope of DLS, temporal
fluctuations are usually analyzed by means of the intensity or photon auto-correlation
function (also known as photon correlation spectroscopy or quasi-elastic light scattering). When light hits small particles, the light scatters in all directions (Rayleigh scattering) as long as the particles are small compared to the wavelength (below 250 nm). The scattered light signal is collected with one of two detectors, either at a 90 degree or 173 degree scattering angle. The provision of both detectors allows more flexibility in choosing measurement conditions. Particles can be dispersed in a variety of liquids. Only liquid refractive index and viscosity needs to be known for interpreting the measurement results. Even if the light source is a laser, and thus is monochromatic and coherent, the scattering intensity fluctuates over time. This fluctuation is due to small molecules in solutions are undergoing Brownian motion, and so the distance between the scatterers in the solution is constantly changing with time. This scattered light then undergoes either constructive or destructive interference by the surrounding particles, and within this intensity fluctuation, information is contained about the time scale of movement of the scatterers. Sample preparation either by filtration or centrifugation is critical to remove dust and artifacts from the solution. DLS is widely used to characterize size of various particles including proteins, polymers, micelles, carbohydrates, and nanoparticles. If the system is not disperse in size, the mean effective diameter of the particles can be determined. This measurement depends on the size of the particle core, the size of surface structures, particle concentration, and the type of ions in the medium. The DLS measurement (hydrodynamic size for aqueous solution) in this thesis was measured at 25 °C with a Malvern Zetasizer Nano ZS90 equipped with a solid state He-Ne laser (λ = 633 nm).

3.4 Bioimaging techniques

3.4.1 Photoacoustic tomography (PAT)

PAT can create multiscale multicontrast images of living biological structures. This emerging technology overcomes the high degree of scattering of optical photons in biological tissue by making use of the photoacoustic effect, in which non-ionizing laser pulses are delivered into biological tissues. Some of the delivered energy will be absorbed and converted into heat, leading to transient thermoelastic expansion and thus wideband ultrasonic emission. The generated ultrasonic waves are detected by ultrasonic transducers and then analyzed to produce images. The basic principles of
PAI is shown in Figure 3.1a. Two types of PAI systems, photoacoustic/thermoacoustic computed tomography (also known as photoacoustic/thermoacoustic tomography, i.e., PAT/TAT) and photoacoustic microscopy (PAM), have been developed. A typical PAT system (Figure 3.1b) uses an unfocused ultrasound detector to acquire the photoacoustic signals, and the image is reconstructed by inversely solving the photoacoustic equations. A PAM system, on the other hand, uses a spherically focused ultrasound detector with 2D point-by-point scanning, and requires no reconstruction algorithm.

![Figure 3.1](a) Schematic illustration of the basic principles of photoacoustic imaging. (b) Multispectral optoacoustic tomography in Sight/in Vision 256.

### 3.4.2 Single-photon emission computed tomography/computed tomography (SPECT/CT)

SPECT is a nuclear medicine tomographic imaging technique using gamma rays, which is very similar to conventional nuclear medicine planar imaging using a gamma camera. However, it is able to provide true 3D information. This information is typically presented as cross-sectional slices through the patient, but can be freely reformatted or manipulated as required. This technique requires delivery of a gamma-emitting radioisotope (a radionuclide, like $^{99m}$Tc, $^{125}$I) into the patient, normally through injection into the bloodstream. On occasion, the radioisotope is a simple soluble dissolved ion, such as an isotope of gallium(III). Most of the time, a marker radioisotope is attached to a specific ligand to create a radioligand, whose properties bind it to certain types of tissues. SPECT imaging is performed by using a gamma camera to acquire multiple 2-D images from multiple angles. A computer is then used to apply a tomographic reconstruction algorithm to the multiple projections,
yielding a 3-D data set. This data set may then be manipulated to show thin slices along any chosen axis of the body, similar to those obtained from other tomographic techniques, such as magnetic resonance imaging (MRI), X-ray computed tomography (X-ray CT), and positron emission tomography (PET). SPECT is similar to PET in its use of radioactive tracer material and detection of gamma rays. In contrast with PET, however, the tracers used in SPECT emit gamma radiation that is measured directly, whereas PET tracers emit positrons that annihilate with electrons up to a few millimeters away, causing two gamma photons to be emitted in opposite directions. A PET scanner detects these emissions "coincident" in time, which provides more radiation event localization information and, thus, higher spatial resolution images than SPECT (which has about 1 cm resolution). SPECT scans, however, are significantly less expensive than PET scans, in part because they are able to use longer-lived more easily obtained radioisotopes than PET. In some cases a SPECT gamma scanner may be built to operate with a conventional CT scanner, with coregistration of images. This SPECT/CT allows location of tumors or tissues which may be seen on SPECT scintigraphy, but are difficult to locate precisely with regard to other anatomical structures. Such scans are most useful for tissues outside the brain, where location of tissues may be far more variable. For example, SPECT/CT may be used in sestamibi parathyroid scan applications, where the technique is useful in locating ectopic parathyroid adenomas which may not be in their usual locations in the thyroid gland. Figure 3.2 shows the SPECT/CT used in this thesis.

Figure 3.2 Ultra-fast and sensitive SPECT/CT.
3.4.3 Magnetic resonance imaging (MRI)

MRI is a medical imaging technique used in radiology to form pictures of the anatomy and the physiological processes of the body in both health and disease. MRI scanners use strong magnetic fields, radio waves, and field gradients to generate images of the inside of the body. MRI is distinct from computed tomography (CT), or (CAT). Whereas CT uses higher-energy x-rays with known harmful effects, MRI operates in the radio-frequency portion of the spectrum where there is much less evidence of harm. While the hazards of x-rays are now well understood and mitigated in most medical contexts, MRI can still be seen as superior in this regard. MRI can often yield different diagnostic information compared with CT. MRI scans have the disadvantages of being longer in duration, louder, and usually require that the subject be placed in a narrow and relatively long tube. In addition, people with some medical implants or other non-removable metal inside the body may be unable to safely undergo an MRI examination. MRI is based upon the science of nuclear magnetic resonance (NMR). Certain atomic nuclei can absorb and emit radio frequency energy when placed in an external magnetic field. In clinical and research MRI, hydrogen atoms are most-often used to generate a detectable radio-frequency signal that is received by antennas in close proximity to the anatomy being examined.

![Figure 3.3 MR Solution MRS 3000.](image)

While MRI is most prominently used in diagnostic medicine and biomedical research, it can also be used to form images of non-living objects. MRI scans are capable of producing a variety of chemical and physical data, in addition to detailed spatial images. MRI requires a magnetic field that is both strong and uniform. The field
strength of the magnet is measured in teslas and while the majority of systems operate at 1.5 T, commercial systems are available between 0.2-7 T. Most clinical magnets are superconducting magnets, which require liquid helium and lower field strengths can be achieved with permanent magnets. Figure 3.3 demonstrates the micro-MRI that is suitable for small animal.
Chapter 4 Facile Fabrication of Dendritic Mesoporous SiO$_2$@CdTe@SiO$_2$

Fluorescent Nanoparticles for Bioimaging

This chapter reported the synthesis of a seeded watermelon-like mesoporous nanostructure (mSiO$_2$@CdTe@SiO$_2$, mSQS) composed of a novel dendritic mesoporous silica core, fluorescent CdTe quantum dots (QDs), and a protective solid silica shell by loading QDs into dendritic mesoporous silica nanoparticles through electrostatic interaction, and then coating with a solid silica shell by the modified Stöber method. The shell thickness of mSQS can be tuned from 0 to 32 nm as desired by controlling the reaction parameters, including the amount of silica precursor, tetraethyl orthosilicate, that is introduced, the solvent ratio (H$_2$O: ethanol), and the amount of catalyst (NH$_3$·H$_2$O). These fluorescent mSiO$_2$@QDs@SiO$_2$ nanoparticles possess excellent stability and thickness-dependent cytotoxicity, and are successfully applied to bioimaging.
4.1 Introduction

Semiconductor quantum dots (QDs) have attracted intense scientific interest due to their remarkable optoelectronic properties, including size-tunable fluorescence, narrow emission, and excellent solution processability, which have made significant contributions to the development of biological labels and optoelectronic devices.\cite{1-6} Despite they have distinct advantages (e.g. larger Stokes shift, and higher resistance to photobleaching) over conventional organic dyes,\cite{7, 8} the practical bioapplications of QDs are limited, owing to their photo- and colloidal instability in harsh environments and cytotoxicity caused by the release of heavy metal ions from the QDs.\cite{9-14} To overcome these problems, coating QDs with a silica shell is a promising method because of the excellent stability, biocompatibility, and nontoxicity of silica. In addition, diverse functional groups can be easily introduced onto the silica shell and offer more options for conjugation with biomolecules.\cite{14-17}

Among the different silica nanostructures, ordered mesoporous silica has been widely applied for drug delivery, adsorption, and catalysis due to its high surface area and large pore volume, controllable mesopore channels, and high stability with respect to temperature and organic solvents.\cite{18-22} Very recently, Zhao’s group\cite{23} first developed a new oil-water biphasic stratification approach to fabricate three-dimensional dendritic mesoporous silica nanospheres (3D-dendritic MSNSs) with tunable multigenerational and center-radial mesopore channels. This type of innovative 3D-dendritic MSNSs are very useful for a wide range of applications including catalysis, adsorption, bio-separation, biolabelling, and controlled release.\cite{24-27}

The Stöber method and the reverse microemulsion approach are two typical strategies to prepare silica nanostructures.\cite{15, 28-32} The Stöber method is based on sol-gel chemistry and has been used to prepare silica nanoparticles since 1960s.\cite{33} Compared with the Stöber method, reverse microemulsion shows better control over particle size and its distribution. The experimental procedures are more complex and time-consuming than for the Stöber method, however, when these methods are used to coat QDs, they lead to a huge loss of fluorescence in comparison with un-coated QDs.\cite{15, 16, 34-36} Many efforts have been made to improve the photoluminescence (PL) efficiency of the resultant QD-silica nanospheres. For example, Gao’s group reported in 2005 that fluorescent core-shell CdTe@SiO$_2$ nanoparticles (quantum yield of 7%)
with a single CdTe nanocrystal core or multiple CdTe cores can be obtained by reducing the electrostatic repulsion between the QDs and the silica intermediates.\cite{15} Later on, they prepared highly fluorescent CdTe@SiO$_2$ nanoparticles with a quantum yield of 47% by incubating QDs in an alkaline solution to improve the quantum yield of the QDs to 85% prior to silica coating.\cite{31} In contrast to these reports, in-situ growth of QDs on the surface of a solid silica core and then coating with a protective silica shell has been explored to improve the PL efficiency.\cite{32, 37} The highest fluorescence quantum yield of such QD-silica nanoparticles reported is up to ~61% to the best of our knowledge.\cite{32} It is worth noting most studies of QD-silica nanoparticles are focused on coating QDs with solid silica, and there are a few reports on the loading of QDs into mesoporous silica,\cite{38} but no report on encapsulation of QDs into 3D-dendritic MSNSs which could serve as ideal nanocontainers for small QDs due to their tunable large pore sizes and excellent biocompatibility. By combination of the modified Stöber method with the novel oil-water biphasic stratification approach, we successfully prepare seeded watermelon-like, mesoporous dendritic mSiO$_2$@CdTe@SiO$_2$ (mSQS) fluorescent nanoparticles (NPs) for bioimaging. The resultant mSQS NPs possess excellent stability and low cytotoxicity, and show great potential in bioimaging.

4.2 Experimental Section

4.2.1 Chemicals

Cetyltrimethylammonium chloride (CTAC) solution (25 wt% in H$_2$O), triethylamine (TEA, 99%), tetraethylorthosilicate (TEOS, 99%), cyclohexane (99.5%), ammonium nitrate (NH$_4$NO$_3$, 98%), cadmium chloride hemi(pentahydrate) (CdCl$_2$·2.5H$_2$O, 98%), sodium citrate tribasic dihydrate (99.0%), sodium tellurite (Na$_2$TeO$_3$, 99%), sodium borohydride (NaBH$_4$, 99%), (3-aminopropyl)triethoxysilane (APS, 99%), (3-mercaptopropyl)trimethoxysilane (MPS, 99%), mercaptosuccinic acid (MSA, 99%), and ammonium hydroxide solution (28.0-30.0% NH$_3$ basis) were purchased from Sigma-Aldrich. Milli-Q water (> 18 MΩ·cm) was used in the experiments.

4.2.2 Synthesis of negatively charged CdTe QDs

Negatively charged CdTe QDs were synthesized by using MSA as a stabilizer. Typically, cadmium chloride (CdCl$_2$, 0.04 M, 4 mL) and sodium citrate tribasic
dihydrate (400 mg) were diluted to 50 mL in a three-necked flask, and stirred at room temperature for 1 h. Na₂TeO₃ (0.01 M, 4 mL), mercaptosuccinic acid (100 mg), and sodium borohydride (NaBH₄, 50 mg) were then added under vigorous stirring. When the color of the mixture turned green, the flask was attached to a condenser and refluxed at 100 °C under N₂ flow for 3 h. The resultant MSA-capped QDs were used directly without any treatment.

4.2.3 Synthesis of positively charged dendritic mesoporous silica nanospheres

The DMSNs with diameter of about 110 nm were synthesized by using a one-pot biphase stratification approach.[26] Typically, a mixed solution (the lower aqueous phase) containing 18 mL H₂O, 12 mL CTAC (25 wt% in H₂O), and 45 µL of TEA was added into a 100-mL round bottom flask and stirred for 1 h at 60 °C. Then, 1 mL TEOS (20 v/v % in cyclohexane) as upper oil phase was gently dropped in, and the solution flask was attached to a condenser. The reaction was maintained at 60 °C for 8 h in an oil bath with reflux under magnetic stirring (100 rpm). The products were collected after removal of the upper oil layer, and then centrifuged and washed several times with ethanol to remove the residual reactants. The products were extracted twice with a 0.6 wt% ammonium nitrate ethanol solution at 60 °C for 6 h to remove the templates. The extracted DMSNs (~50 mg) were then refluxed at 110 °C for 6 h with 1 mL APS in a toluene solution under stirring to allow the grafting of the -NH₂ groups to form positively charged DMSNs (i.e. mSiO₂-NH₂). The mSiO₂-NH₂ nanoparticles were centrifuged and thoroughly washed with ethanol and water to remove the unreacted chemicals. The resultant positively charged nanoparticles were redispersed in 5 mL water for further use.

4.2.4 Synthesis of mesoporous-SiO₂@CdTe and mesoporous-SiO₂@CdTe@SiO₂

The electrostatic adsorption approach was adopted to prepare mSQ with strong fluorescence. Briefly, 7 mL of the as-prepared MSA-capped CdTe QD solution (0.7 mM) was slowly added dropwise into 1 mL of mSiO₂-NH₂ solution (0.17 M) under magnetic stirring at room temperature. After stirring for several minutes, the negatively charged QDs would be electrostatically adsorbed on the framework of the positively charged mSiO₂-NH₂ to form mesoporous-silica@CdTe (mSQ) nanoparticles, which were separated by centrifugation and washed with water for further use. The supernatant solution was collected to determine the amount of free QDs in solution.
For MPS or APS incubation, 35 µL APS/MPS-ethanol solution (1:100 by volume) was introduced into the mixture of 1 mL mSQ aqueous solution (0.17 M) and 8 mL ethanol and stirred for 6 h. Then, 40 µL TEOS and 80 µL ammonia were added dropwise into the above mixture in succession. The mixture was stirred for 16 h at room temperature to promote the growth of the solid silica shell over the mSQ. The resultant mSQS nanoparticles were separated via centrifugation and washed with ethanol and water several times. The mSQS samples that originated from mSQ incubated with APS and those from mSQ incubated with MPS were denoted as mSQS-1 and mSQS-2, respectively.

4.2.5 Characterization

Ultraviolet-visible (UV-Vis) absorption spectra were recorded at room temperature with a Shimadzu UV-3600 in the range of 300 to 800 nm. Photoluminescence (PL) spectra were measured in the range of 455 to 800 nm on a Fluorolog FL3-221 (Horiba) under 450 nm light excitation. The PL quantum yields (QYs) of the QDs were calculated from the integrated emission of the QD samples in solution compared with that of rhodamine 6G (PL QYs, 95%) in ethanol at identical density. Transmission electron microscope (TEM) images of the resultant mSiO₂, mSQ, and mSQS NPs were obtained on a JEOL JEM-2010 electron microscope operating at an acceleration voltage of 200 kV. High-angle annular dark field (HAADF) image and scanning transmission electron microscopy-energy dispersive X-ray spectroscopy (STEM-EDS) spectra were collected using a JEOL ARM-200F and Tecnal G2 F20 operating at 200 kV with an EDAX solid-state X-ray detector. Samples were prepared by drop-casting ethanol-dispersed nanostructures onto TEM copper grids. Ultrathin TEM sample was prepared by embedding mSQS nanoparticles into epoxy and then cut into slices with a thickness of 30 nm by an ultramicrotome. Nitrogen adsorption-desorption measurements were conducted at 77 K with a Quantachrome Autosorb iQ (USA). Before measurements, the samples were degassed under vacuum at 180 °C for 6 h. The Brunauer-Emmett-Teller (BET) specific surface area was calculated from the adsorption data at relative pressure (P/P₀) ranging from 0.05 to 0.35. The pore size distribution was calculated from the adsorption branch of the isotherms using the Barrett, Joyner, and Halenda (BJH) formula. For in vivo fluorescence imaging, the BALB/c female mice were anesthetized with 1.5% isoflurane and the 3D tomographic images were recorded.
with the IVIS Spectrum imaging system. Then the mice were dissected and major organs such as liver, spleen, heart etc were removed and used for fluorescence imaging. Images were acquired and analyzed using the Living Image 4.4 software (Caliper life sciences, Hopkinton, MA, USA). The hydrodynamic size was measured at 25 °C with a Malvern Zetasizer Nano ZS90 equipped with a solid-state He-Ne laser (λ = 633 nm).

4.2.6 Chemical stability test

The MSA-capped CdTe QDs were freshly prepared, and the mSQ and mSQS were collected by centrifuge, and then redispersed in solutions with different pH values (1, 3, 5, 7, 9, 11, 13, with the pH value of the solutions adjusted by hydrochloric acid or sodium hydroxide), respectively. The photoluminescence (PL) was measured 30 min later, and the integrated area of each spectrum was calculated. All the integrated area values were divided by that of the sample in solution with pH 7.

4.2.7 Cell culture

A549 human lung cancer cells and RAW 264.7 murine macrophage cells were cultured in a standard cell medium by American type culture collection (ATCC) at 37 °C in 5% CO₂ atmosphere.

4.2.8 Cell viability assay

Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed to assess the metabolic activity of cells in the presence of nanoparticles. Cells were seeded into 96-well plates at a density of 1.0 × 10⁴ (A549) or 2.0 × 10⁴ (RAW 264.7) cells per well in 200 mL of medium incubated with different concentrations of NPs for 24 h. The cells were then incubated in a medium containing 0.5 mg/mL MTT for 2 h at 37 °C. After this, the medium was removed, and 100 mL dimethyl sulfoxide (DMSO) was added to each well, followed by incubation for 0.5 h at 37 °C. A Bio Tek multifunction microplate reader (Synergy 2) was used to measure the absorbance of each well with background subtraction at 490 nm. All of the tests were independently performed three times.

4.2.9 Cellular uptake and intracellular distribution

To detect the internalization of NPs, sample processing could be briefly described as follows. A549 and RAW 264.7 cells were seeded in dishes at a suitable
concentration for 24 h, then incubated with 100 μg/mL mSQS for another 2 h or 24 h, and finally thoroughly rinsed with phosphate-buffered saline (PBS). After being fixed with nucleus staining by Hoechst 33258 for 30 min at 37 °C, the samples were observed via a confocal microscope (PerkinElmer) with the excitation and emission wavelength were 488 nm and 525 nm respectively.

4.3 Results and discussion

As outlined in Figure 4.1, we first prepared monodispersed 3D-dendritic MSNs and then functionalized them with amino groups to form mSiO₂-NH₂. The 3D-dendritic MSNs were synthesized via an oil-water biphasic stratification approach.[23] The upper oil phase contains the silica source (tetraethyl orthosilicate (TEOS)) and a hydrophobic organic solution (cyclohexane), and the lower aqueous phase is a mixed solution of surfactant (cetyltrimethylammonium chloride (CTAC)) and catalyst (triethylamine (TEA)). After extraction by NH₄NO₃-ethanol solution to remove the surfactant templates, the DMSNs were modified with amino groups via refluxing in (3-aminopropyl) triethoxysilane (APS)-toluene solution. The resultant mSiO₂-NH₂ nanoparticles were used as carriers for loading of QDs to form mesoporous-silica@CdTe (mSQ) nanoparticles which were incubated with APS or (3-mercaptopropyl)trimethoxysilane (MPS). Subsequently, another solid silica shell was coated onto the functional mSQ nanoparticles to produce the mSQS nanoparticles, with the aim of protecting the QDs and improving their stability.

![Figure 4.1](image.png)

**Figure 4.1** Preparation procedure for mSiO₂@CdTe@SiO₂ fluorescent nanostructures.
The transmission electron microscope (TEM) image in Figure 4.2a shows the highly uniform spherical morphology of the monodispersed mSiO$_2$-NH$_2$ nanoparticles with radial and dendritic mesopore channels and a mean diameter of 109 ± 7.32 nm (Figure 4.3). The presence of amino group was verified by the X-ray photoelectron spectroscopy (XPS) spectrum of N 1s for mSiO$_2$-NH$_2$, which is shown in Figure 4.2 d (black line) and in the high resolution XPS spectra in Figure 4.4b. The peak with a binding energy of 399.6 eV is consistent with that of N 1s reported elsewhere.$^{[31, 39]}$ The successful grafting of amino groups onto the framework of mesoporous silica can also be confirmed by the zeta potential analysis shown in Figure 4.5. The mSiO$_2$-NH$_2$ shows positive charge with a zeta potential of 14.7 mV. The dendritic mSiO$_2$-NH$_2$ nanoparticles were also characterized by nitrogen adsorption-desorption analysis, and their Brunauer-Emmett-Teller (BET) surface area and total pore volume were measured to be 443 m$^2$ g$^{-1}$ and 1.16 cm$^3$ g$^{-1}$, respectively (Figure 4.6 and Table 4.1). The mesopore size calculated from the adsorption branch using the Barrett-Joyner-Halenda (BJH) method (Figure 4.6, black line) reveals a narrow distribution centered at $\approx 5.0$ nm, which is consistent with that observed in high resolution transmission electron microscope (HRTEM) in Figure 4.3b.
Figure 4.2 TEM images of a) mSiO$_2$-NH$_2$, b) mSiO$_2$@CdTe (inset: magnified TEM image of mSiO$_2$@CdTe), c) mSiO$_2$@CdTe@SiO$_2$, and d) XPS survey spectra of mSiO$_2$-NH$_2$, mSiO$_2$@CdTe, and mSiO$_2$@CdTe@SiO$_2$.

Figure 4.3 (a, b) TEM and (c) SEM images of mSiO$_2$-NH$_2$ nanoparticles [the inset of (b) shows its HRTEM image], and (d) corresponding histogram of size distribution counted from 100 particles in TEM images.

The above positively charged 3D-dendritic MSNs were used to adsorb negatively charged CdTe QDs (average size is 4.10 ± 0.57 nm; Figure 4.7) through electrostatic interactions to generate mSQ (Figure 4.1b). The energy dispersive X-ray spectroscopy (EDS) elemental mapping images in Figure 4.9a indicate the homogenous distribution of QDs on the 3D-dendritic MSNs. The adsorption of negatively charged QDs leads to a drop in the zeta potential to $-29.6$ mV. The mSQ nanoparticles show reduced surface area (269 m$^2$ g$^{-1}$), which is attributable to the anchoring of the QD particles on the mSiO$_2$-NH$_2$, while the pore size shows little change.

In order to homogeneously coat mSQ with a solid silica shell by the modified Stöber method, the use of a functional silane such as APS or MPS is generally
required to modify the mSQ prior to silica coating. Such a functional silane agent can render the mSQ surface vitreophilic and provide nucleation sites for deposition of a silica shell on its surface. In our study, APS and MPS were chosen because their NH$_2$ and SH groups can strongly bind to the QD surface and leave silane groups for hydrolysis and condensation into a very thin silica shell. If the mSQ nanoparticles were not coated with APS or MPS, it would be very difficult to coat them with a solid silica shell because TEOS becomes hydrolyzed in the alcohol-water medium (conditions: Rv = 1:8, 100 µL of NH$_4$OH, 60 µL of TEOS; Figure 4.8) to form a silica network with random necking and fusion between mSQS spheres (Figure 4.8). After precoating the mSQ with APS or MPS, the growth of the silica shell is easier and mainly governed by three primary parameters, e.g., the H$_2$O:EtOH ratio (Rv), the amount of ammonia solution, and the amount of TEOS introduced, which were comprehensively investigated (Table 4.2) and are discussed later.

**Figure 4.4** High resolution XPS spectra of (a-b) Si 2p and N 1s of mSiO$_2$-NH$_2$, and (c-d) Cd 3d and Te 3d of mSiO$_2$@CdTe.
Figure 4.5 Zeta potential of (a) mSiO$_2$-NH$_2$, (b) mSiO$_2$@CdTe, and (c) mSiO$_2$@CdTe@SiO$_2$ in pure water media (pH=7).
Figure 4.6 Nitrogen adsorption-desorption isotherms and pore size distribution of mSiO$_2$-NH$_2$, mSQ and mSQS.
Figure 4.7 TEM image (a) and HRTEM image (b) of MSA-capped CdTe QDs with PL peak at 550 nm (The inset of (a) shows the size distribution histogram of MSA-capped CdTe QDs counted from 100 particles).
Figure 4.8 Different magnification of TEM images of mSQS without incubation with APS or MPS. A network structure by necking and fusion between mSQS spheres is clearly observed.

A typical TEM image of mSQS nanoparticles is shown in Figure 4.2c. These mSQS nanoparticles have a large mean size of $125 \pm 6.3$ nm with a shell thickness of about 7.5 nm. The scanning transmission electron microscopy-high-angle annular dark field (STEM-HAADF) images and EDS-elemental mapping in Figure 4.9b demonstrate the complete encapsulation of mSQ by the solid silica shell. In order to investigate the location of CdTe QDs in the mSQS nanoparticles, mSQS nanoparticles were embedded into epoxy and cut into ultrathin films (30 nm) with an ultramicrotome for TEM analysis. Figure 4.9c and Figure 4.13 clearly show that QDs were densely anchored on the surface of the mSiO$_2$ due to their dendritic structure and a number of QDs were distributed in the channels of the mSiO$_2$.

Figure 4.9 High resolution STEM-HAADF images and EDS-elemental mapping of a) mSiO$_2$@CdTe, b) mSiO$_2$@CdTe@SiO$_2$, and c) ultramicrotomed mSiO
\[ \text{mSiO}_2 \text{CdTe@SiO}_2 \text{ slice.} \]

**Table 4.1** Mean size of pores, BET surface area and total pore volume of mSiO\(_2\)-NH\(_2\), mSQ and mSQS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean size of pores (nm)</th>
<th>BET ( \text{m}^2 \text{g}^{-1} )</th>
<th>Total pore volume ( \text{cm}^3 \text{g}^{-1} )</th>
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<tbody>
<tr>
<td>mSiO(_2)-NH(_2)</td>
<td>5.3</td>
<td>443.1</td>
<td>1.16</td>
</tr>
<tr>
<td>mSiO(_2)@CdTe</td>
<td>5.2</td>
<td>268.7</td>
<td>0.84</td>
</tr>
<tr>
<td>mSiO(_2)@CdTe@SiO(_2)</td>
<td>NA</td>
<td>60.2</td>
<td>0.07</td>
</tr>
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**Table 4.2** Synthesis parameters and properties of dendritic mSQS nanoparticles.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Effect factors</th>
<th>APS / H(_2)O EtOH H(_2)O S</th>
<th>Mean particle size (nm)</th>
<th>Mean shell thickness (nm)</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sa1</td>
<td>Effect of APS</td>
<td>MPS (mL) EtOH (mL) NH(_3) (_2) (µL) TEO (mL)</td>
<td>182.0</td>
<td>36.0</td>
<td>4.8</td>
</tr>
<tr>
<td>Sa2(^b)</td>
<td>APS/MPS</td>
<td>35</td>
<td>1</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>Sr1</td>
<td>35</td>
<td>1</td>
<td>4</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Sr2</td>
<td>35</td>
<td>1</td>
<td>8</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Sr3</td>
<td>35</td>
<td>1</td>
<td>12</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
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<td>16</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Sr5</td>
<td>35</td>
<td>1</td>
<td>4</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Sr6</td>
<td>35</td>
<td>1</td>
<td>8</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Sr7</td>
<td>35</td>
<td>1</td>
<td>12</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Sr8</td>
<td>35</td>
<td>1</td>
<td>16</td>
<td>40</td>
<td>40</td>
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<tr>
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</tr>
<tr>
<td>St2</td>
<td>35</td>
<td>1</td>
<td>8</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>St3</td>
<td>35</td>
<td>1</td>
<td>8</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>St4</td>
<td>35</td>
<td>1</td>
<td>8</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>
The resultant seeded watermelon-like mSQS particles show a negative zeta potential of -28.3 mV due to the surface silicon hydroxyl on the solid silica shell. Their BET surface area and total pore volume decreased dramatically to only 60.2 m$^2$ g$^{-1}$ and 0.07 cm$^3$ g$^{-1}$, respectively, owing to the complete encapsulation by the solid nonporous silica shell. Moreover, the disappearance of the pore size distribution peak further confirms the protective effect of the solid silica shell over the mSQ core (Figure 4.6, blue line). In the XPS survey spectra of mSiO$_2$-NH$_2$, mSQ, and mSQS in Figure 4.2d, the binding energies of Si 2p, Si 2s, Cd 3d$^{5/2}$, Cd 3d$^{3/2}$, O 1s, Te3d$^{5/2}$, and Te 3d$^{3/2}$ were found to be 104.2, 154.1, 405.5, 412.4, 532.8, 572.6, and 583.1 eV, respectively (for high resolution XPS spectra see Figure 4.4), in which the characteristic binding energies of Cd 3d$^5$/2 and Te 3d$^5$/2 are consistent with those of reported CdTe QDs.$^{[32]}$ The absence of signals of Cd, Te, or N in the spectrum of the mSQS sample indicates that the mSQ were completely coated by the solid silica shell.

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<table>
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<tbody>
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<td>35</td>
<td>1</td>
<td>8</td>
<td>100</td>
<td>100</td>
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<td>1</td>
<td>8</td>
<td>60</td>
<td>20</td>
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<td>35</td>
<td>1</td>
<td>8</td>
<td>60</td>
<td>40</td>
<td>132.5</td>
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<td>1</td>
<td>8</td>
<td>60</td>
<td>80</td>
<td>135.0</td>
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<td>1</td>
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<td>60</td>
<td>100</td>
<td>135.1</td>
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<td>8</td>
<td>60</td>
<td>140</td>
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<td>8</td>
<td>60</td>
<td>180</td>
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<td>35</td>
<td>1</td>
<td>8</td>
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<td>1</td>
<td>8</td>
<td>60</td>
<td>260</td>
<td>138.6</td>
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<td>0.2</td>
<td>8</td>
<td>100</td>
<td>10</td>
<td>118.0</td>
</tr>
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<td>St15</td>
<td>35</td>
<td>0.2</td>
<td>8</td>
<td>100</td>
<td>20</td>
<td>123.0</td>
</tr>
<tr>
<td>St16</td>
<td>35</td>
<td>0.2</td>
<td>8</td>
<td>100</td>
<td>40</td>
<td>125.0</td>
</tr>
<tr>
<td>St17</td>
<td>35</td>
<td>0.2</td>
<td>8</td>
<td>100</td>
<td>60</td>
<td>126.0</td>
</tr>
<tr>
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<td>8</td>
<td>100</td>
<td>100</td>
<td>126.0</td>
</tr>
<tr>
<td>St19</td>
<td>35</td>
<td>0.2</td>
<td>8</td>
<td>100</td>
<td>150</td>
<td>127.0</td>
</tr>
</tbody>
</table>

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$^a$Mean particle sizes are calculated by averaging 100 particles from the TEM images. $^b$St2 and St3 are the same sample, but are given different names and are placed at various positions in the table to allow a clear comparison.
4.3.1 Shell thickness control

As mentioned previously, after pre-coating mSQ with APS or MPS, the growth of the solid silica shell can be tuned by manipulating the H$_2$O/EtOH ratio ($R_v$), the amount of ammonia solution, and the amount of TEOS that is introduced. In order to control the thickness of the shell, a series of mSQS nanoparticles were fabricated by optimizing these three parameters. In this paper, we have investigated the impact of each parameter on the synthesis of mSQS by using mSQ nanoparticles with the same diameter (~110 nm) and incubating them with APS or MPS for the same time (6 h) as seeds. Figure 4.10a presents the dependence of shell thickness on the H$_2$O/EtOH ratio obtained with the same amount of TEOS and two different ammonia concentrations (TEM images in Figure 4.11 and 4.12). The results show that H$_2$O/EtOH ratio strongly affected the hydrolysis of TEOS and the thickness of the silica shell. Lower water content favors the nucleation and growth of silica around mSQ seeds in the range of $R_v$ from 1:4 to 1:8. Decreasing the ratio of H$_2$O/EtOH from 1:8 down to 1:16 led to a decrease in the shell thickness of the mSQS, which could be due to the fact that less water restrained the hydrolysis of TEOS. [33] Therefore, H$_2$O/EtOH ratio was optimized to be 1:8. Ammonia was used as a catalyst for TEOS hydrolysis and determined the hydrolysis rate. Under the catalysis with more ammonia, the fast hydrolysis of TEOS gave rise to a thicker silica shell [Figure 4.10a and Figure 4.11, 4.12]. Figure 4.10b demonstrates that the shell thickness can be tuned from 0 to 32 nm by adjusting the amount of TEOS from 20 to 100 µL under the conditions of 100 µL of NH$_4$OH and an $R_v$ of 1:8. Low-magnification TEM images with more particles are presented in Figure 4.14. The shell thickness of mSQS increases with the amount of TEOS introduced. When more than 60 µL of TEOS was introduced, however, and
Figure 4.10 (a) Effect of H$_2$O to EtOH volume ratio (Rv) on the shell thickness control with 40 µL of TEOS; and shell thickness control by adjusting the amount of TEOS with (b) 100 µL of NH$_4$OH, Rv = 1/8, (c) 60 µL of NH$_4$OH, Rv = 1/8, and (d) 100 µL of NH$_4$OH, Rv = 1/40.

100 µL of NH$_4$OH was used (Rv of 1:8), a peanut-like mSQS structure was predominantly observed, which may due to the too fast hydrolysis of TEOS [Figure 4.14(e-f)]. By decreasing the amount of ammonia from 100 to 60 µL, a set of mSQS samples was successfully synthesized with an Rv of 1:8 over a wide range of TEOS amounts from 20 to 260 µL of TEOS (Figure 4.15). The dependence of shell thickness on the amount of TEOS introduced is shown in Fig. 4.10c. The data show that when TEOS amount was more than 80 µL, the shell thickness of the resultant mSQS increased slowly with increasing amounts of TEOS. A similar trend in shell thickness depending on the amount of TEOS was observed under the condition of 100 µL of NH$_4$OH and an Rv of 1:40 (Figure 4.16). This result further proves that slow hydrolysis of TEOS can deposit a uniform silica layer on the surface of mSQ to result in monodispersed mSQS NPs.
Figure 4.11 TEM images of mSQS obtained with 40 µL TEOS, and 80 µL NH₄OH but different H₂O:EtOH ratios (Rᵥ): a) Rᵥ = 1:4, b) Rᵥ = 1:8, c) Rᵥ = 1:12 and d) Rᵥ = 1:16.
Figure 4.12 TEM images of mSQS obtained with 40 µL TEOS, and 40 µL NH₄OH but different H₂O:EtOH ratios (Rᵥ): a) Rᵥ = 1:4, b) Rᵥ = 1:8, c) Rᵥ = 1:12, and d) Rᵥ = 1:16.

Figure 4.13 TEM images of (a) normal mSiO₂@CdTe@SiO₂ sample and (b) mSiO₂@CdTe@SiO₂ ultrathin sectioning sample with a thickness of 30 nm.
**Figure 4.14** TEM images of mSQ and mSQS obtained with different TEOS feeding amount of TEOS (20 ~ 100 µL), $R_v$ of 1/8, and 100 µL NH$_4$OH.
Figure 4.15 TEM images of mSQS obtained with different TEOS feeding amount of TEOS (20 ~ 260 µL), R_v of 1/8, and 60 µL NH_4OH.
Figure 4.16 TEM images of mSQS obtained with different TEOS feeding amount of TEOS (10 ~ 150 µL), Rv of 1/40, and 100 µL NH₄OH.

4.3.2 Fluorescence and stability of mSQS

The loading amount of QDs was determined by measuring the UV-Vis absorption of free QDs in the supernatant as the absorption of the QDs was linear proportion with the concentration and the standard Absorbance-concentration curve and corresponding linear fitting plots were shown in Figure 4.17, suggesting the lambert-beer law is suitable in the our system. After addition of different amounts of QD solution to the aqueous dispersion of mSiO₂-NH₂, the QDs were immediately adsorbed on the framework of mSiO₂-NH₂. The mixture was centrifuged to separate the unloaded QDs from the mSQ nanoparticles [Figure 4.19(a) and (c)]. The results
showed that the optimal molar ratio of MSA-CdTe to mSiO$_2$-NH$_2$ was $2.48 \times 10^{-4}$:1. Further quantitative analysis of the fluorescence of QDs and mSQ in Figure 4.19(b) indicates that 57.1% of the original QD fluorescence is retained after encapsulation into mSiO$_2$-NH$_2$ (The PL QY of CdTe QDs is 72.4% compared with rhodamine 6G in ethanol at identical optical density; see Figure 4.18). In addition, a 15-nm red-shift of the fluorescence peak was observed, probably due to the sharp change in the zeta potential of the solution. Before further coating of mSQ with the outer solid silica shell, APS or MPS was used for pre-coating and incubation with mSQ for 6 hours. The fluorescence of mSQ incubated with APS shows no obviously changes (99.6%), indicating that modification of mSQ particles with APS has no influence on their fluorescence. After coating with the solid silica shell, the fluorescence of the final mSQS sample (mSQS-6h-1) is up to 80.4% of the initial fluorescence [Figure 4.19(e)]. In the case of mSQ modified with MPS, the fluorescence of mSQ was increased to 112.3%, which is expected because MPS has been reported to help protect QDs and lead to enhanced fluorescence. Only 45.0% of the initial fluorescence was retained for mSQS, however, after coating with the solid silica shell (mSQS-6h-2) [Figure 4.19(f)], because the negatively charged intermediates from the hydrolysis of TEOS quench the fluorescence. Figure 4.19(d) shows a comparison of the fluorescence of the control mSQ, mSQS-1, and mSQS-2, which is consistent with their fluorescence spectra in Figure 4.19(e) and (f). The overall fluorescence QYs of mSQS NPs is in the range of 33% to 18%, which depends on the encapsulation process as summarized in Table 4.3.

**Figure 4.17** (a) The UV-Vis absorption spectra of MSA-CdTe QDs at different concentration. (b) Corresponding linear fitting plots of absorbance at 530 nm versus concentration.
Figure 4.18 PL spectra of CdTe and Rhodamine 6G with an identical optical density.
Figure 4.19 (a) UV-Vis absorption spectra of mSiO$_2$@CdTe supernatant at different MSA-CdTe to mSiO$_2$-NH$_2$ mol ratios. (b) PL spectra of QDs and mSQ. (c) Water dispersion of QDs and mSQ under UV light at 365 nm and room light (inset). (d) Photographs of mSQ, mSQS-1, and mSQS-2 under 365-nm UV lamp. (e) PL spectra of mSQ, mSQ-6h-1, and mSQS-1. (f) PL spectra of mSQ, mSQ-6h-2, and mSQS-2.

Table 4.3 Overall particle sizes, silica shell thicknesses and optical properties of QDs, mSQ and mSQS NPs.

<table>
<thead>
<tr>
<th>NPs’ name</th>
<th>Overall NPs’ size (nm)</th>
<th>Silica shell thickness (nm)</th>
<th>Optical properties</th>
<th>Absorption peak (nm)</th>
<th>Emission peak (nm)</th>
<th>FWHM of emission peak (nm)</th>
<th>QY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSA-CdTe</td>
<td>4.1</td>
<td>——</td>
<td></td>
<td>530</td>
<td>550</td>
<td>51</td>
<td>72.4</td>
</tr>
<tr>
<td>mSQ</td>
<td>110</td>
<td>——</td>
<td></td>
<td>530</td>
<td>565</td>
<td>45</td>
<td>41.3</td>
</tr>
<tr>
<td>mSQS-1</td>
<td>125</td>
<td>7.5</td>
<td></td>
<td>530</td>
<td>557</td>
<td>45</td>
<td>33.2</td>
</tr>
<tr>
<td>mSQS-2</td>
<td>125</td>
<td>7.5</td>
<td></td>
<td>530</td>
<td>557</td>
<td>44</td>
<td>18.6</td>
</tr>
<tr>
<td>St1 (mSQS-5)</td>
<td>120</td>
<td>5.0</td>
<td></td>
<td>530</td>
<td>557</td>
<td>45</td>
<td>32.4</td>
</tr>
<tr>
<td>St2 (mSQS-10)</td>
<td>130</td>
<td>10</td>
<td></td>
<td>530</td>
<td>557</td>
<td>45</td>
<td>32.1</td>
</tr>
<tr>
<td>St3 (mSQS-17)</td>
<td>145</td>
<td>17.5</td>
<td></td>
<td>530</td>
<td>557</td>
<td>44</td>
<td>31.5</td>
</tr>
<tr>
<td>St4 (mSQS-27)</td>
<td>164</td>
<td>27</td>
<td></td>
<td>530</td>
<td>557</td>
<td>44</td>
<td>28.2</td>
</tr>
</tbody>
</table>
Figure 4.20 Fluorescence efficiency of MSA-capped CdTe, mSQ, and mSQS in solutions with different pH values (a), TEM images of mSQS used in pH = 7 solution (b) and mSQS treated with pH = 13 solution for 2 hours (c).

The external solid silica shell is expected to provide protection to the internal CdTe QDs. We investigated the chemical stability of mSQS NPs by using mSQS sample with a shell thickness of 7 nm. The fluorescence of the QDs, mSQ, and mSQS NP solutions versus different pH values is shown in Figure 4.20(a). The fluorescence of CdTe QDs is strongly influenced by minor variation of the solution pH. For example, only 40% and 10% of fluorescence were retained when the pH of the CdTe QD solution was changed from 7 to 3 and 13, respectively. In contrast, the mSQ NPs are stable in a solution pH of 3, and their fluorescence can be enhanced under basic conditions. It worth noted that in the pH range of 1 to 5, obvious aggregation of QDs and mSQ NPs occurred as shown in Figure 4.21. By contrast, the fluorescence of mSQS NPs is stable even in a strongly acidic solution (pH = 1) and no obviously aggregation was observed in the pH range of 1 to 13 (Figure 4.21), owing to the protection of outer solid silica shell. Therefore, mSQS shows significantly improved chemical stability compared with the QDs and mSQ NPs. It should be noted that the fluorescence of both mSQ and mSQS NPs markedly increase under basic conditions, which may due to the corrosion of the external silica shell by NaOH, which is verified by the morphology change of mSQS before [Figure 4.20(b)] and after the alkaline solution treatment [Figure 4.20(c)], and the surface passivation of QDs to eliminate surface defects under basic conditions.\[29, 31]
Figure 4.21 The Hydrodynamic size of MSA-capped CdTe, mSQ, and mSQS in solutions with different pH values.

4.3.3 Cytotoxicity assay

The cytotoxicity of mSQ and mSQS in A549 cells and RAW 264.7 cells has been investigated by using the MTT cell viability assay. A series of mSQS nanoparticles with a shell thickness of around 5 nm, 10 nm, 17 nm and 27 nm (named as mSQS-5, mSQS-10, mSQS-17, mSQS-27 respectively, i.e. the samples of St1 ~ St4 in Table 4.2 and 4.3), together with mSQ NPs, were used to investigate their cytotoxicity for A549 cells. Figure 4.22(a) shows the viability of A549 cells labelled with six different concentrations of mSQ and mSQS, ranging from 0 to 200 µg/mL. The cell viability of the blank control (i.e. untreated cells) was set as “100%”, and the absorbance of each sample was compared to that of the blank control. It clearly shows that the cell viability remained almost 100% with a concentration of 50 µg/mL and even more than 80% at the high concentration of 200 µg/mL after 24 h incubation, which could be attributed to the good protection afforded by the solid silica shell. In comparison, the cell viability for mSQ was reduced to about 50% at the concentrations of 200 µg/mL. In addition, the cell viability increased with the increase of shell thickness at high concentration (i.e. 200 µg/mL), which supports that a solid silica shell (>5 nm) can provide good protection for QDs. In the case of the RAW 264.7 cells, The mSQS-10, mSQS-17 and mSQS-27 NPs showed lower cytotoxicity (50.2%, 65.6% and 69.3% cell viability at 200 µg/mL respectively) than mSQS-5 (44.5% cell viability at 200 µg/mL ) and mSQ NPs (less than 30% viability at ≥ 50 µg/mL) as demonstrated in Figure 4.22(b), suggesting a thicker shell (>5 nm)
can provide better protection for QDs. The high cytotoxicity of mSQ NPs may be due to the release of

![Figure 4.22](image)

**Figure 4.22** Cell viability tests performed on (a) A549 cells and (b) RAW 264.7 cells incubated for 24 h with mSQ NPs and mSQS NPs at different concentrations.
Figure 4.23 CLSM images of A549 cells (a) and RAW 264.7 cells (b) after incubation with mSQS for 2 h and 24 h. Scale bar: (a) 15 μm, (b) 6 μm.
CdTe QDs. These results reveal that a solid silica shell can efficiently prevent the release of CdTe QDs and improve their biocompatibility. The difference in viability of RAW 264.7 cells and A549 cells can be attributed to the stronger phagocytosis of RAW 264.7 cells than A549 cells as RAW cells are macrophages.\textsuperscript{[42]}

### 4.3.4 \textit{In vitro} cell bioimaging

The intracellular distribution of SQS NPs was examined using a confocal laser scanning microscope (CLSM). As showed in Figure 4.23, after incubation for 2 h, mSQS-5 NPs were taken up by the RAW 264.7 cells and the A549 cells. The RAW 264.7 cells as macrophages could internalize more mSQS NPs than A549 cells because of their higher phagocytosis capability. The dynamic cell uptake process for mSQS NPs described in the supplementary video also suggests that mSQS NPs can be effectively taken up by macrophages via phagocytosis. For the same cell line, there is no significant difference in cell uptake of mSQS NPs for different incubation times (i.e. from 2 to 24 h). We didn’t find that more NPs were taken up by A549 and RAW 264.7 cells with longer incubation. This means that both the A549 and RAW 264.7 cells could internalize mSQS NPs in a short incubation time and reach the maximum uptake at around 2 h. The time-independent cell uptake after 2 h could be attributed to the balance of endocytosis and exocytosis of cells.

![Figure 4.24](image)

**Figure 4.24** \textit{In vivo} fluorescence images acquired pre- a) and at different time points (b): 3 min, c): 60 min, d): 120 min, e): 120 min (supine position)) after tail intravenous injection of mSQS-5 nanoparticles into the mouse and f) fluorescence images of corresponding dissected organs.
4.3.5 In vivo fluorescence imaging

In order to explore the potential of mSQS NPs for in vivo fluorescence imaging, 200 µL of mSQS-5 samples (2 mg/mL) was injected via the tail vein into the mouse. In vivo fluorescence images were acquired at different time points post injection (3 min, 60 min and 120 min) using the IVIS Spectrum imaging system. As demonstrated in Figure 4.24, strong fluorescence signal were observed after the injection of mSQS-5 NPs in comparison with the pre image (Figure 4.24a). These fluorescent mSQS NPs were rapidly distributed over the whole body within 3 min. With the increase of circulation time, these nanoparticles were gradually accumulated in liver, spleen, lung and heart, of which liver displayed stronger fluorescence after injection 120 min as shown in Figure 4.24d,e. The strong fluorescence of liver revealed the most of nanoparticles were in the liver, which could be due to the large size and negative surface of the mSQS NPs, and the rich macrophages in liver. These results demonstrate the potential of mSQS NPs in bioimaging, and preparation of smaller mSQS NPs with proper surface modification is under the way.

4.4 Conclusions

In summary, a series of size-tunable, core-shell, novel watermelon-like fluorescent mSQS NPs were successfully synthesized. The shell thickness could be tuned from 0 to 32 nm by adjusting the amount of silica precursor, the solvent ratio, and the amount of catalyst. The solid silica shell coating significantly improved the chemical stability of the QDs inside. The resultant mSQS NPs investigated with the MTT assay, CLSM images, dynamic cell uptake and in vivo fluorescence imaging exhibit low toxicity, high stability, and strong fluorescence for bioimaging.

4.5 References

Chapter 5 Ambient Aqueous Synthesis of Ultra-small PEGylated Cu_{2-x}Se Nanoparticles as a Multifunctional Theranostic Agent for Multimodal Imaging Guided Photothermal Therapy of Cancer

This chapter reported a kind of ultrasmall PEGylated Cu_{2-x}Se nanoparticles with strong near-infrared absorption prepared by an ambient aqueous method. The resultant water-soluble and biocompatible nanoparticles are demonstrated to be a novel nanotheranostic agent for effective deep-tissue photoacoustic imaging, computed tomography imaging, single-photon emission computed tomography imaging, and photothermal therapy of cancer.
5.1 Introduction

In recent years, nanoscale copper chalcogenides (Cu$_{2-x}$E, E = S, Se, Te, 0 ≤ x ≤ 1) have attracted considerable attention due to their simple chemical formula with complicated crystal structures and variable compositions, which offer an incredible wealth of properties for diverse applications in thermoelectric conversion,$^{[1-3]}$ lithium-ion batteries,$^{[4]}$ quantum-dot-sensitized solar cells,$^{[5, 6]}$ photoacoustic (PA) imaging,$^{[7]}$ and photothermal therapy (PTT).$^{[8-10]}$ Their applications in PA imaging and PTT are attributable to their strong localized surface plasmon resonances (LSPR) in the near-infrared (NIR) window arising from copper deficiency.$^{[7, 10-19]}$ Compared with conventional photothermal agents such as large gold nanostructures (usually larger than 50 nm in one dimension for producing NIR LSPR), Cu$_{2-x}$E nanostructures could be much smaller. In addition, they are biodegradable and can release vital trace elements copper and chalcogen for maintaining good health, e.g., selenium can reduce the occurrence and fatality of liver, prostate, and lung cancers,$^{[20]}$ and copper deficiency can contribute to the development and deterioration of a number of diseases such as cardiovascular disease and diabetes.$^{[21]}$

It should be noted that most research on in vivo applications of Cu$_{2-x}$E nanostructures is focused on Cu$_{2-x}$S nanoparticles,$^{[9, 22-24]}$ and there have been only a few reports in the literature on nanoscale Cu$_{2-x}$Se,$^{[7, 10, 25]}$ due to the difficulties in the preparation of high-quality water-soluble and biocompatible Cu$_{2-x}$Se nanomaterials. Monodisperse Cu$_{2-x}$Se nanoparticles are usually prepared in organic solvents at high temperature, and laborious surface post-modification is necessary to make them suitable for bioapplications.$^{[7, 10]}$ For example, 16-nm Cu$_{2-x}$Se particles synthesized by a colloidal hot injection method were demonstrated to be an effective in vitro photothermal transduction agent after modification by an amphiphilic polymer.$^{[10]}$ 7.6-nm Cu$_{2-x}$Se nanocrystals synthesized by a similar method were employed as a contrast agent for in vivo PA imaging of a sentinel lymph node after encapsulation by a phospholipid.$^{[7]}$ From the perspective of PA imaging and PTT, ultra-small nanoparticles with high photothermal conversion efficiency are highly desirable, because they are less likely to be rapidly recognized and cleared by phagocytes and the reticuloendothelial system (RES) than larger ones, and they can circulate in the blood for long time and passively accumulate within tumors through the enhanced permeability and retention (EPR) effect.$^{[26-29]}$ In this context, the direct preparation of
biocompatible ultra-small copper selenide (Cu$_{2-x}$Se, $0 \leq x \leq 1$) nanoparticles with strong NIR LSPR in aqueous solution under ambient conditions is highly significant and remains a challenging task.\textsuperscript{[25]}

Apart from their potential in PA imaging and PTT, the large X-ray attenuation coefficients ($\mu$) of both Cu and Se elements for low-energy X-rays (e.g. 33.79 cm$^2$/g and 48.18 cm$^2$/g at 20 keV for Cu and Se, respectively) highlight their potential in X-ray computed tomography (CT) imaging, which has not been investigated for ultra-small Cu$_{2-x}$Se nanoparticles. In this study, a facile aqueous route has been developed to prepare water-soluble and biocompatible ultra-small omnipotent Cu$_{2-x}$Se nanoparticles (3.6 ± 0.3 nm) for PA imaging, CT imaging, single-photon emission computed tomography (SPECT) imaging, and PTT of cancer. To the best of our knowledge, sub-5-nm ultra-small Cu$_{2-x}$Se particles with excellent water solubility and biocompatibility for effective multimodal imaging-guided PTT have not yet been reported.

5.2 Experimental Section

5.2.1 Chemicals

All chemicals and reagents were used as received without any further purification. CuCl$_2$·2H$_2$O (≥ 99%), Se powder (-100 mesh, ≥ 99.5%), sodium borohydride (NaBH$_4$, 99%), and mercaptosuccinic acid (MSA, 99%) were purchased from Sigma-Aldrich. Dimercapto poly(ethylene glycol) (HS-PEG-SH, $M_g = 5000$) was bought from Adamas. Milli-Q water (> 18 MΩ·cm) was used in the experiments.

5.2.2 Synthesis of MSA-capped Cu$_{2-x}$Se nanoparticles and HS-PEGylated Cu$_{2-x}$Se nanoparticles

In a typical synthesis, 39.48 mg (0.5 mmol) Se powder was reduced by 56.75 mg (1.5 mmol) NaBH$_4$ in 50 mL H$_2$O under magnetic stirring at room temperature with the protection of inert gas. 0.17 g (1 mmol) CuCl$_2$·2H$_2$O and 1 g (6.66 mmol) MSA were completely dissolved in 5 mL distilled water and then added into the selenium precursor solution to immediately generate a black solution, which indicates the formation of copper selenide nanoparticles (i.e. Cu$_{2-x}$Se NPs). The black solution was subsequently purified by ultrafiltration at 5000 rpm, using a membrane with a molecular weight cut-off (MWCO) of 30 kDa. After removal of free MSA from the nanoparticle solution, 0.2 g HS-PEG-SH was added to modify the surfaces of the MSA-capped Cu$_{2-x}$Se NPs at room temperature. The obtained PEGylated Cu$_{2-x}$Se
nanoparticles (i.e. Cu$_{2-x}$Se-PEG-SH NPs) were purified by a similar ultrafiltration method and then dialyzed against Milli-Q water to remove free HS-PEG-SH. The final product was stored at 4 °C for further characterization and use.

5.2.3 Characterization

Ultraviolet-visible-near-infrared (UV-Vis-NIR) spectra were recorded with a PerkinElmer Lambda 750 UV-Vis-NIR spectrophotometer. TEM images were captured using a FEI Tecnai F20 transmission electron microscope operating at an acceleration voltage of 200 kV. The crystal structure of the nanoparticles was characterized with a Shimadzu XRD-6000 X-ray diffractometer equipped with Cu Kα1 radiation (λ = 0.15406 nm). X-ray photoelectron spectroscopy (XPS) measurements were carried out on a Thermo Scientific Sigma Probe instrument using Al Kα X-ray radiation and fixed analyzer transmission mode. Thermogravimetric analysis (TGA) was performed to analyze the weights of surface ligands at a heating rate of 10 °C min$^{-1}$ from room temperature to 700 °C under argon atmosphere. The hydrodynamic size was measured at 25 °C with a Malvern Zetasizer Nano ZS90 equipped with a solid state He-Ne laser (λ = 633 nm).

5.2.4 Cytotoxicity assay of the PEGylated Cu$_{2-x}$Se nanoparticles

MTT assays were performed to assess the cytotoxicity of the Cu$_{2-x}$Se NPs. In detail, 4T1 cells were first seeded in 96-well plates at a density of 4000 cells per well and cultured for 24 h in DMEM supplemented with 10% FBS. Then, the cells were washed with PBS and incubated with Cu$_{2-x}$Se NPs at different concentrations at 37 °C for 24 h. After that, the cells were washed twice with PBS and cultured for 48 h. Then, 20 µL MTT with a concentration of 5 mg/mL was added and allowed to react with the cells for 4 h before the addition of 150 µL dimethyl sulfoxide (DMSO) to dissolve the precipitates. The absorption of each solution was measured at 490 nm on a microplate reader (Thermo, Varioskan Flash).

5.2.5 Animal model

The tumor models used were established by subcutaneous injection of 50 µL 4T1 cell suspension (~5 × 10$^6$ cells) into the flank region of the right back of 5-week-old male BALB/c mice (for the PTT treatment) or nude mice (for the PA imaging). The tumor imaging studies were carried out 10 days after the inoculation with tumor cells. All animal experiments were carried out according to the protocols approved by the Soochow University Laboratory Animal Center.
5.2.6 In vivo Photoacoustic Imaging

For in vivo photoacoustic (PA) imaging, nude mice bearing subcutaneous tumors were anesthetized with 1.5% isoflurane delivered via a nose cone, and then Cu$_{2-x}$Se NPs (500 μg/mL of Cu, 200 μL) were injected via the tail vein. Photoacoustic images were acquired at different time points post-injection by a multispectral optoacoustic tomography (MSOT) instrument. 10 slices were obtained at each position and averaged to minimize the influence of animal movement in the images.

5.2.7 CT imaging

The contrast efficacy of Cu$_{2-x}$Se-PEG-SH NPs as a CT contrast agent in vitro was compared with commercial iopromide agent in aqueous solution, with concentrations of 0.5, 1, 2, 4, and 5 mg/mL of Cu$_{2-x}$Se-PEG-SH NPs and iopromide. For in vivo CT imaging, 4T1 tumor-bearing mice were anesthetized with isoflurane and placed in an animal bed. Cu$_{2-x}$Se-PEG-SH NPs were then intratumorally and intravenously injected for CT imaging respectively. The images were scanned in an accurate mode using full angle, 3 frame averaging, a 615 mA tube current, and a 55 kV tube voltage.

5.2.8 $^{99m}$Tc Labeled Cu$_{2-x}$Se-PEG-SH NPs for SPECT Imaging

Technetium-99m ($^{99m}$Tc, purchased from Shanghai GMS Pharmaceutical Co., Ltd) with radioactivity of 3 mCi was added into the Cu$_{2-x}$Se-PEG-SH NPs solution (500 μg/mL, 500 μL) in the presence of 200 μL of stannous chloride (SnCl$_2$, 5 mg/mL in 0.1 M HCl) and then stirred gently for 1 h at room temperature. The obtained $^{99m}$Tc labeled Cu$_{2-x}$Se-PEG-SH solution was purified by ultrafiltration to remove free $^{99m}$Tc. For SPECT imaging, mice bearing 4T1 tumors were intravenous injected with Cu$_{2-x}$Se-PEG-SH-$^{99m}$Tc nanoparticles and imaged by an animal SPECT (MILabs, Utrecht, the Netherlands) imaging system at various time points post injection.

5.2.9 Photothermal Therapy

24 tumor-bearing BALB/c mice with an average tumor volume of 130 mm$^3$ were randomly allocated into 4 groups, which were intravenously injected with the same volume of either saline or Cu$_{2-x}$Se-PEG-SH NPs solution. The dose of Cu$_{2-x}$Se-PEG-SH NPs was 200 μL of solution containing 100 μg Cu. The laser irradiation was performed using an 808-nm NIR laser (Hi-Tech Optoelectronics Co., Ltd. Beijing, China) with a power density of 1.5 W/cm$^2$ for 6 min. The tumor sizes and body weights were measured every two days, and the tumor volume was expressed by $L \times W^2/2$, where $L$ and $W$ represent the length and width of the tumor, respectively.
Relative tumor volumes were obtained by dividing by the initial tumor size before laser treatment. Analysis of Variance (ANOVA) was used to assess statistical significance. *p < 0.5, **p < 0.01, ***p < 0.001. The mice treated with NIR irradiation after injection of Cu$_{2-x}$Se-PEG-SH NPs were sacrificed at day 40 to harvest the major organs for histological analysis.

5.2.10 Biodistribution

21 tumor-bearing mice were respectively intravenously injected with 200 μL of Cu$_{2-x}$Se-PEG-SH NPs solution containing 100 μg Cu. They were divided into seven groups, and the various organs or tissues, such as heart, liver, spleen, lung, kidney, tumor, blood, stomach, intestines, skin, bone, and muscle, were resected from the mice in each group at 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, and 48 h post injection, wet-weighed, and dissolved in the digesting solution (HNO$_3$:H$_2$O$_2$ of 2:1 by volume). The contents of copper in each organ/tissue were determined by inductively coupled plasma – optical emission spectrometry (ICP-OES).

5.2.11 Blood circulation behavior

4 healthy BALB/c mice were intravenously injected with Cu$_{2-x}$Se-PEG-SH NPs, and blood from their retinal vein was collected before and after intravenous injection at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, and 72 h, respectively. The contents of copper in the blood were determined by ICP-OES after the samples were digested with HNO$_3$/H$_2$O$_2$ mixture. The decay curve of the copper content in the blood was fitted with a two-compartmental model to extract the blood half-life.

5.2.12 Histological Analysis

For hematoxylin and eosin (H&E) staining, major organs, including the liver, spleen, kidney, heart, and lung, were harvested, fixed in 10% neutral buffered formalin, processed routinely into paraffin, sectioned into thin slices, and stained with H&E for histological analysis.

5.3 Results and discussion

As illustrated in Figure 5.1a, Se powder was completely reduced by NaBH$_4$ in aqueous solution under magnetic stirring under the protection of nitrogen gas.$^{[30]}$ A mixture of CuCl$_2$·2H$_2$O and mercaptosuccinic acid (MSA)$^{[31, 32]}$ was completely dissolved in distilled water and then immediately added into the selenium precursor solution to form a dark green solution, which indicates the formation of Cu$_{2-x}$Se nanoparticles (denoted as MSA-Cu$_{2-x}$Se NPs). The resultant ultra-small nanoparticles
were ultrafiltrated and then functionalized with dimercapto poly(ethylene glycol) (HS-PEG-SH) to improve their water solubility, colloidal stability, and biocompatibility. The PEGylated nanoparticles were denoted as Cu$_{2-x}$Se-PEG-SH NPs, which not only exhibit long blood circulation time for efficient accumulation in tumors through the EPR effect for PA imaging and PTT treatment, but also provide functional groups (i.e. -SH) for labelling with $^{99m}$Tc for SPECT imaging.

![Figure 5.1](image)

**Figure 5.1** (a) Schematic illustration of the synthesis of multifunctional Cu$_{2-x}$Se PEG-SH nanoparticles and (b) their applications in PA imaging, CT imaging, SPECT imaging, and photothermal therapy of cancer.

The morphology and size of the as-prepared Cu$_{2-x}$Se-PEG-SH NPs were examined by transmission electron microscopy (TEM). As shown in Figure 5.2a, b and Figure 5.3a, b in the Supporting Information, they are spherical particles with a mean size of 3.6 ± 0.3 nm. The well-defined crystalline lattice in the typical high-resolution TEM (HRTEM) image (Figure 5.2b) with a lattice spacing of 0.33 nm
matches well with that of (111) planes of cubic berzelianite (Cu$_{2-x}$Se), which can be further confirmed by the fast Fourier transform (FFT) pattern in the inset of Figure 5.2b.$^{[1-5]}$ The crystal structure of Cu$_{2-x}$Se-PEG-SH NPs was further determined by powder X-ray diffraction (XRD, Figure 5.2c), and all the peaks in the pattern can be assigned to the characteristic lines of cubic berzelianite (Cu$_{2-x}$Se, JCPDS Card No. 06-0680). The atomic ratio of Cu to Se was determined by energy-dispersive X-ray

Figure 5.2 Characterization of as-prepared Cu$_{2-x}$Se-PEG-SH NPs: (a) TEM image, (b) HRTEM image with corresponding FFT pattern (inset), (c) XRD pattern in comparison with the standard peaks of cubic berzelianite (JCPDS Card No. 06-0680), (d) high resolution XPS spectra of Cu, (e) hydrodynamic size, and (f) ultraviolet-
visible (UV-Vis)-NIR absorbance spectra of nanoparticle aqueous solutions with different Cu concentrations.

Figure 5.3 (a) Low magnification TEM image of Cu$_{2-x}$Se-PEG-SH NPs, (b) histogram of size distribution of Cu$_{2-x}$Se-PEG-SH NPs, (c) EDS spectrum of Cu$_{2-x}$Se-PEG-SH NPs, with the inset showing the composition and atomic ratio of Cu, Se, and S, (d) XPS survey spectrum of Cu$_{2-x}$Se-PEG-SH NPs, high resolution XPS spectra of (e) Se and (f) S, respectively.

spectroscopy (EDS, Figure 5.3c) to be 1.74, which is consistent with the value determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) and demonstrates the deficiency of copper in the nanoparticles. From the viewpoint
of the molecular formula, non-stoichiometric Cu$_{2-x}$Se can be considered as a mixture of stoichiometric Cu$_2$Se and CuSe, and the valence states of Cu can be determined by X-ray photoelectron spectroscopy (XPS). The high-resolution XPS spectrum of the Cu 2p orbital (Figure 5.2d) confirms the presence of both Cu$^+$ and Cu$^{2+}$ with a calculated ratio (Cu$^+$ to Cu$^{2+}$) of 4.21. The binding energy of Se 3d at 54.6 eV in the XPS spectrum is attributed to Se$^{2-}$, and the peak at 58.8 eV is due to the oxidation of Se (Figure 5.3e).\[33\] The Cu:Se ratio calculated from the XPS results is 1.78, which again supports the existence of a copper deficiency in nanoparticles.

The resultant non-stoichiometric Cu$_{2-x}$Se-PEG-SH NPs can be well dispersed in aqueous solution, saline solution (0.9 wt% NaCl), phosphate buffered saline (PBS) solution, pure Dulbecco’s Modified Eagle’s Medium (DMEM), and fetal bovine serum (FBS) solution (Figure 5.4a). The hydrodynamic sizes for MSA-Cu$_{2-x}$Se and Cu$_{2-x}$Se-PEG-SH NPs in water were determined to be 6.4 nm and 13.6 nm by the dynamic light scattering (DLS) method, respectively (Figure 5.2e and Figure 5.4c). The stability of PEGylated Cu$_{2-x}$Se nanoparticles in 10% fetal calf serum (FBS) media, which is widely used to mimick the physiological conditions, was monitored by measuring their hydrodynamic size over time. As shown in Figure 5.5, the PEGylated Cu$_{2-x}$Se nanoparticles overall exhibit excellent stability in fetal calf serum as their hydrodynamic size only slightly increased over one week due to the adsorption of proteins, i.e. increased from 17.1 nm at day 1 to 34.0 nm at day 6. To further test their stability in serum, we dialysed PEGylated Cu$_{2-x}$Se nanoparticles against fetal bovine serum for 48 h and determined the contents of Cu in the dialysis solution. Figure 5.6a shows a negligible difference in copper contents during dialysis. The good retention of high NIR absorbance further demonstrates the stability of Cu$_{2-x}$Se nanoparticles (Figure 5.6b), and the slight increase of their absorbance in the visible region could be due to the adsorption of proteins. The zeta potential of Cu$_{2-x}$Se-PEG-SH NPs is -8.1 mV, higher than that of MSA-Cu$_{2-x}$Se NPs (-14.1 mV) (Figure 5.4b).

The PEGylation of ultra-small Cu$_{2-x}$Se NPs is demonstrated by their Fourier transform infrared (FTIR) spectrum (Figure 5.4e), and the content of HS-PEG-SH coated on the surfaces of Cu$_{2-x}$Se NPs was estimated by thermogravimetric analysis
(TGA) to be approximately 13.5 wt% (Figure 5.4f). These results demonstrate the successful modification of Cu$_{2-x}$Se NPs by HS-PEG-SH.

**Figure 5.4** (a) Colloidal stability of Cu$_{2-x}$Se-PEG-SH NPs and MSA-Cu$_{2-x}$Se NPs dissolved in different media at 6 h after high speed centrifugation (10 krpm, 10 min), (b) zeta potentials of Cu$_{2-x}$Se-PEG-SH NPs and MSA-Cu$_{2-x}$Se NPs, (c) DLS measured sizes of MSA-Cu$_{2-x}$Se and Cu$_{2-x}$Se-PEG-SH in different solutions, including H$_2$O, NaCl, PBS, DMEM, and FBS media. (d) linear fitting plots of ultraviolet-visible (UV-Vis)-NIR absorbance versus Cu concentration in aqueous
solutions at 808 nm. The inset shows a photograph of the Cu$_{2-x}$Se-PEG-SH nanoparticle aqueous solutions with different Cu concentrations, labelled in units of µg/mL. (e) FTIR spectra of the MSA, HS-PEG-SH, and Cu$_{2-x}$Se-PEG-SH NPs, and (f) TGA curves of MSA, MSA-Cu$_{2-x}$Se NPs, HS-PEG-SH, and Cu$_{2-x}$Se-PEG-SH NPs.

Figure 5.5 Hydrodynamic size of PEGylated Cu$_{2-x}$Se nanoparticles dispersed in 10% fetal bovine serum (FBS).

Figure 5.6 (a) The stability of PEGylated Cu$_{2-x}$Se nanoparticles after dialysis in FBS for different periods of time as indicated by the Cu contents measured with ICP-OES method. The negligible change of Cu-percentage suggests their excellent stability. (b) UV-Vis-NIR absorbance spectra of PEGylated Cu$_{2-x}$Se nanoparticles in FBS before
and after dialysis. The well retention of strong NIR absorbance further confirmed the stability of PEGylated Cu$_2$Se nanoparticles.

**Figure 5.7** (a, b) Photothermal heating curves and corresponding infrared thermal images of pure water and aqueous dispersions of Cu$_2$Se-PEG-SH NPs at different Cu concentrations under continuous irradiation by a 808 nm laser with a power density of 0.75 W/cm$^2$ for 10 min. (c) *in vitro* relative cell viabilities of 4T1 cells after being incubated with various concentrations of Cu$_2$Se-PEG-SH NPs for 24 h, (d) fluorescence images of 4T1 cells stained with live/dead kit after *in vitro* photothermal ablation under NIR laser irradiation with and without the addition of Cu$_2$Se-PEG-SH NPs solutions with different Cu concentrations.

**Calculation of the photothermal conversion efficiency**

1 mL of 20 µg/mL Cu$_2$Se-PEG-SH NPs solution was loaded into a cuvette and irradiated using a 808-nm laser, followed by natural cooling after the laser was turned off. The monitored temperature profile is shown in Figure 5.8a. The
photothermal conversion efficiency ($\eta$) is calculated according to the following equation.

$$\eta = \frac{m \cdot c \cdot (T_{\text{max}} - T_{\text{max,H2O}})}{I \cdot (1 - 10^{-A}) \cdot \tau_s}$$

where $m$ is the solution mass and equal to 1.0 g in the current study, $c$ is the heat capacity of water and equal to 4.2 J/g, $T_{\text{max}}$ and $T_{\text{max,H2O}}$ are the maximum temperatures reached for the nanoparticle solution and for water, which are 40.58 °C and 22.09 °C, respectively, $I$ is the laser power and equal to 0.75 W/cm$^2$ in the current study, $A$ is the absorbance of the nanoparticle solution at 808 nm and equal to 0.16236, and $\tau_s$ is the system time constant and equal to 512.3 s, according to the linear regression of the cooling profile (Figure 5.8b). The photothermal conversion efficiency is calculated to be 64.8% by using these parameters.

Figure 5.8 (a) The temperature profile of Cu$_{2-x}$Se-PEG-SH NPs solution irradiated with a 808-nm laser, followed by natural cooling after the laser was turned off, (b) determination of the system time constant using linear regression of the cooling profile shown in (a).
Figure 5.9 Temperature changes of MSA-Cu$_{2-x}$Se (lower panel) and Cu$_{2-x}$Se-PEG-SH NPs (upper panel) over five cycles of irradiation/cooling (0.75 W/cm$^2$, 10 min). Insets: corresponding optical images of samples at 1 h after five cycles of photothermal stability testing.

The aqueous solutions of Cu$_{2-x}$Se-PEG-SH NPs (see inset of Figure 5.4d) show strong LSPR in the NIR region (from 600 to 1100 nm), as shown in Figure 5.2f, due to the high density of vacancies arising from copper deficiency.$^{[34, 35]}$ Moreover, the absorption at 808 nm is linearly increased with increasing Cu concentration (Figure 5.4d), which was determined by ICP-OES. According to the Lambert-Beer law ($A/L = aC$, where $A$ is the absorption intensity, $L$ is the length of the cuvette, $C$ is the concentration, and $a$ is the extinction coefficient), the extinction coefficient of Cu$_{2-x}$Se-PEG-SH aqueous solution at 808 nm was calculated to be 8.5 Lg$^{-1}$cm$^{-1}$, which is approximately 2.2 times and 2.4 times larger than that of Au nanorods (3.9 Lg$^{-1}$cm$^{-1}$)$^{[36]}$ and graphene oxide nanosheets (3.6 Lg$^{-1}$cm$^{-1}$)$^{[37]}$ both of which have been intensively applied in photothermal therapy.

The large extinction coefficient of Cu$_{2-x}$Se-PEG-SH NPs suggests that they have a pronounced photothermal conversion capability. Therefore, different concentrations of Cu$_{2-x}$Se-PEG-SH NPs in aqueous solutions were exposed to an 808
nm NIR laser with a power density of 0.75 W/cm² for 10 min. Figure 5.7a, b clearly demonstrates the concentration-dependent photothermal conversion of Cu₂₃Se-PEG-SH NPs, and their solution temperature can be precisely controlled between 25 °C and 75 °C by varying the Cu concentration from 0 to 100 μg/mL. In comparison, the temperature increase for pure water is less than 3 °C under the same experimental conditions. The photothermal conversion efficiency is calculated to be 64.8% (Figure 5.8), which is approximately 3 times of previously reported Cu₂₃Se nanocrystals (22%) synthesized by the colloidal hot injection method.[10]

Photothermal stability is of key importance for photothermal agents during PA imaging and PTT treatment. To assess the photothermal stability of Cu₂₃Se-PEG-SH NPs solution, for comparison purposes, the temperature profiles of two solutions containing the same amount of Cu₂₃Se-PEG-SH NPs and MSA-Cu₂₃Se NPs were recorded for five successive cycles of heating/cooling processes (Figure 5.9). Both MSA and HS-PEG-SH capped Cu₂₃Se NPs exhibited stable photothermal conversion capability during five cycles of testing. The MSA-Cu₂₃Se NPs were aggregated 1 hour after the test was finished, however, while the Cu₂₃Se-PEG-SH NP solution remained stable at all times, as demonstrated by the insets of Figure 5.9. The improved stability of Cu₂₃Se-PEG-SH NPs over MSA-Cu₂₃Se NPs is due to the excellent water-solubility and steric hindrance of the PEG molecules.

The remarkable photothermal conversion efficiency and photothermal stability of Cu₂₃Se-PEG-SH NPs suggest that they could serve as excellent agents for PA imaging and PTT of cancer if they have good biocompatibility. Therefore, the potential cytotoxicity of Cu₂₃Se-PEG-SH NPs towards 4T1 cells (murine breast cancer cells) was investigated by a standard methyl thiazolyl tetrazolium (MTT) assay (Figure 5.7c). The results show almost no cytotoxicity to 4T1 cells for Cu₂₃Se-PEG-SH NPs in solutions with copper concentrations below 50 μg/mL, and the cell viability remained 84% when the copper concentration was 100 μg/mL.

Based on the excellent biocompatibility of Cu₂₃Se-PEG-SH NPs, their capability of in vitro photothermal ablation of cancer cells (e.g. 4T1 cells) in different concentrations was investigated with an 808 nm NIR laser at power density of 0.5, 0.75, and 1.5 W/cm². The living cells and dead cells were respectively stained green and red with a Live-Dead Cell Staining Kit after laser irradiation. As shown in Figure 5.7d, 4T1 cells could not be killed when they were treated only with Cu₂₃Se-
PEG-SH NPs or only with laser irradiation (0.5, 0.75, 1.5 W/cm²). In contrast, 4T1 cells treated with a combination of Cu₂₋ₓSe-PEG-SH NPs and laser irradiation died with increasing particle concentration and/or laser power intensity. The results demonstrate that Cu₂₋ₓSe-PEG-SH NPs can effectively kill cancer cells through hyperthermia induced upon by NIR laser irradiation.

The feasibility of Cu₂₋ₓSe-PEG-SH NPs for in vitro photothermal ablation of cancer cells suggests that these nanoparticles could be also used for PA imaging, as they both utilize the heat converted from the NIR laser. Therefore, the in vitro PA imaging performance of the as-prepared Cu₂₋ₓSe NPs was investigated. As expected, the photoacoustic signal was significantly enhanced with the assistance of Cu₂₋ₓSe NPs, and the signal intensity was linearly increased with increasing copper concentration (Figure 5.10a, b), suggesting that Cu₂₋ₓSe NPs could be a promising agent for in vivo PA imaging. Prior to the in vivo imaging experiment, it is important to know the blood circulation kinetics of Cu₂₋ₓSe-PEG-SH NPs. Blood samples were collected at different time points after intravenous injection of nanoparticles into 4T1 tumor-bearing BALB/C mice, and then digested with HNO₃/H₂O₂ solution (HNO₃:H₂O₂ = 2:1 by volume) to determine their copper concentration by ICP-OES measurements. As shown in Figure 5.12, Cu₂₋ₓSe-PEG-SH NPs exhibit a relatively long blood circulation with a half-life of 8.14 h due to their ultra-small size and their surface PEG molecules, which can effectively reduce the phagocytosis of macrophages and the adsorption of proteins.³⁸,³⁹
Figure 5.10 (a) *In vitro* PA images of Cu$_{2-x}$Se NPs in different concentrations (concentration unit: µg/mL of Cu), (b) *in vitro* PA signals of Cu$_{2-x}$Se-PEG-SH NPs as a function of Cu concentration, (c) *in vitro* CT images of Cu$_{2-x}$Se-PEG-SH NPs in comparison with commercially used iopromide in different concentrations (concentration unit: mg/mL), (d) *in vitro* CT signals of Cu$_{2-x}$Se-PEG-SH NPs and iopromide as functions of their corresponding concentrations, (e) *in vivo* PA intensity of tumor determined at different time points after intravenous injection of Cu$_{2-x}$Se-
PEG-SH NPs, (f) tumor uptake of nanoparticles at different time points after intravenous injection of Cu$_{2.3}$Se-PEG-$^{99m}$Tc NPs, (g) photograph of 4T1-tumor-bearing mouse (tumor indicated by red circle) taken at 12 h post intravenous injection of Cu$_{2.3}$Se-PEG-$^{99m}$Tc, and (h) distribution of Cu$_{2.3}$Se-PEG-$^{99m}$Tc determined by counting gamma rays in the tissues and organs of a mouse sacrificed at 24 h post-injection.

**Figure 5.11** In vivo CT images of a mouse taken before injection (Pre), and 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 24 h post intravenous injection of Cu$_{2.3}$Se-PEG-SH NPs (2.5 mg/mL, 200 µL). The tumor is indicated by the circles.

The long blood circulation time highlights that the as-prepared ultra-small nanoparticles could be used for in vivo tumor imaging. Therefore, Cu$_{2.3}$Se-PEG-SH NPs were intravenously injected into 4T1 tumor-bearing BALB/c nude mice, and then a set of PA images of the tumor region were acquired before and after injection. Figure 5.12a and Figure 5.10e show the PA images and the signals from the tumor
site obtained at different times. The results clearly demonstrate that only a weak PA signal in the tumor area could be detected before the injection of Cu$_{2.3}$Se-PEG-SH NPs, and the signal gradually increased with the circulation of nanoparticles after their intravenous injection, due to their gradual accumulation in the tumor through the EPR effect. The PA signal reached its maximum at 12 h post-injection and then decreased until 24 h post-injection. The maximal signal is 2.1 times higher than that of the pre-contrast image.

Figure 5.12 (a) In vivo PA images of tumor (highlighted by red circles) from a 4T1 tumor-bearing mouse collected before and after tail vein injection of Cu$_{2.3}$Se-PEG-SH NPs solution (500 µg/mL, 200 µL) at different time points, (b) 3D in vivo CT images of a mouse bearing a tumor (indicated by the red circles) before (left) and after (right) intratumoral injection of Cu$_{2.3}$Se-PEG-SH NPs (2.5 mg/mL, 200 µL), (c) in vivo SPECT images of a mouse bearing a tumor (indicated by the red circles) after intravenous injection of Cu$_{2.3}$Se-PEG-$^{99m}$Tc solution, (d) blood circulation and (e) biodistribution of Cu$_{2.3}$Se-PEG-SH NPs after intravenous injection into 4T1 tumor-
bearing mice, as determined by measuring Cu concentrations in tissue lysates with ICP-OES.

In addition to PA imaging, Cu$_{2,x}$Se-PEG-SH NPs exhibit very interesting potential in computed tomography (CT) imaging. As shown in Figure 5.12b and Figure 5.10c, d, the CT signals produced by Cu$_{2,x}$Se NPs and commercial clinically used iopromide are dependent on their concentration, and the Hounsfield units (HU) value of Cu$_{2,x}$Se NPs (~120 HU L/g) is approximately 1.6 times higher than that of iopromide (73.53 HU L/g) at the same concentration. Motivated by the excellent X-ray attenuation capability of the Cu$_{2,x}$Se-PEG-SH NPs in vitro, we intratumorally injected the Cu$_{2,x}$Se-PEG-SH NPs (2.5 mg/mL, 200 μL) into an anesthetized 4T1 tumor-bearing BALB/c mouse and conducted imaging with an animal micro-CT system. As demonstrated by the three-dimensional (3D) CT images in Figure 5.12, the contrast of tumor image can be greatly enhanced by Cu$_{2,x}$Se-PEG-SH NPs. The average CT values for the tumor site were dramatically increased from 78.2 HU (Pre) to 763.2 HU (Post) after injection of Cu$_{2,x}$Se-PEG-SH NPs.

Based on the above results, a solution of Cu$_{2,x}$Se-PEG-SH NPs (2.5 mg/mL, 200 μL) was intravenously injected into another tumor-bearing BALB/c mouse, and CT images were collected at different time points post-injection (Figure 5.11). The images clearly show the contrast enhancement of the tumor due to its passive accumulation of NPs. The mean HU value was gradually increased from 96.8 HU before injection to 108.0 HU at 4 h, 126.7 HU at 8 h, 150.3 HU at 10 h, and 102.8 HU at 24 h after intravenous injection.

Both in vitro and in vivo CT imaging results provide evidence that ultra-small Cu$_{2,x}$Se-PEG-SH NPs can serve as a promising CT contrast agent. Their excellent performance is attributed to the large X-ray attenuation coefficients (μ) of both Cu and Se elements, which drastically decrease with increasing X-ray energy, e.g., Cu and Se have μ values of 33.79 cm$^2$/g and 48.18 cm$^2$/g at 20 keV, respectively, which are decreased to 1.59 and 2.34 cm$^2$/g at 60 keV, and 0.46 and 0.63 cm$^2$/g at 100 keV.$^{[40, 41]}$ Due to the small energy of Cu-Se bonds compared with the X-ray energy, the attenuation of X-rays in Cu$_{2,x}$Se can be treated as attenuation in Cu and Se, respectively, and the attenuation coefficient of Cu$_{2,x}$Se ($x = 0.26$ in our case) can be expressed by Equation (2):
\[
\mu_{\text{Cu}_{1.74}\text{Se}} = \mu_{\text{Cu}} \times \frac{1.74 \times 63.5}{1.74 \times 63.5 + 78.9} + \mu_{\text{Se}} \times \frac{78.9}{1.74 \times 63.5 + 78.9} \tag{2}
\]

Where \(\mu_{\text{Cu}}\) and \(\mu_{\text{Se}}\) are the attenuation coefficients of Cu and Se, respectively. The effective atomic number of \(\text{Cu}_{1.74}\text{Se}\) is calculated to be 43.7 through Equation (3):

\[
Z = \sqrt[2.94]{1.74 \times Z_{\text{Cu}}^{2.94} + Z_{\text{Se}}^{2.94}} \tag{3}
\]

Where \(Z_{\text{Cu}}\) and \(Z_{\text{Se}}\) are the atomic numbers of Cu and Se, respectively. Both the large attenuation coefficients and the high effective atomic number demonstrate the potential of \(\text{Cu}_{2-x}\text{Se}\) in CT imaging.

![Graph](image)

**Figure 5.13** The Cu concentration in the excreted urine at different intervals of post-injection of the \(\text{Cu}_{2-x}\text{Se}-\text{PEG-SH}\) NPs solution and PBS solution (control group), respectively.

Besides their excellent performance in PA imaging and CT imaging for qualitative diagnosis, an advantage of our ultra-small \(\text{Cu}_{2-x}\text{Se}-\text{PEG-SH}\) NPs is their multifunctional groups (–COOH and –SH), which provide more options for quantitative analysis by multimodal imaging. Here, the clinically used radioisotope technetium-99m (\(^{99}\text{mTc}\), half-life, \(t_{1/2} = 6.02\) h, gamma-ray energy, \(E_r = 140\) keV) was selected to functionalize these nanoparticles for SPECT imaging due to its strong gamma-ray emission, short half-life, and low cost.\(^{42}\) \(\text{Cu}_{2-x}\text{Se}-\text{PEG-SH}\) NPs
labeled with $^{99m}$Tc (i.e. Cu$_2$Se-PEG-$^{99m}$Tc) were intravenously (i.v.) injected into a 4T1 tumor-bearing mouse, which was imaged by a U-SPECT instrument at different time points post-injection. Obvious tumor uptake of Cu$_2$Se-PEG-$^{99m}$Tc appeared after injection, and the tumor became obviously black after 12 h (Figure 5.10g), which demonstrates the passive accumulation of Cu$_2$Se-PEG-$^{99m}$Tc in the tumor through the EPR effect. As a consequence, a remarkable emission of gamma-rays from Cu$_2$Se -PEG-$^{99m}$Tc NPs accumulated in the tumor was detected (Figure 5.12c).

The time-dependent accumulation of nanoparticles in tumor were quantified by counting gamma-rays to be 1.27, 1.37, 1.53, 1.83 and 0.84 %ID/g (percentage of injected dose per gram tissue) at 2 h, 4 h, 6 h, 12 h, and 24 h, respectively (Figure 5.10f). The tumor-homing effect observed from the SPECT images is well consistent with the PA results. In addition, strong signals of gamma rays in the liver and the kidney were also observed due to the capture of Cu$_2$Se-PEG-$^{99m}$Tc NPs by the reticuloendothelial system (Figure 5.12). The intense signal in the bladder detected at 2 – 12 h after injection and the weak signal in the bladder at 24 h suggest that these ultra-small Cu$_2$Se-PEG-$^{99m}$Tc NPs might be cleared out through renal excretion. To further confirm the renal excretion of the ultra-small nanoparticles, the mouse was sacrificed, and the major organs were harvested for quantification of gamma-rays (Figure 5.10h). The results show that the kidney has the strongest signal, followed by the liver, spleen, and lung. The strong signal of kidney supports the renal excretion of of ultra-small Cu$_2$Se NPs which could minimize their potential side effects. In addition, Urine solutions of mice were collected at different intervals for ICP-OES analysis. As shown in Figure 5.13, the amount of Cu per gram of urine significantly increased to the maximum at 1 h post injection and then declined to a constant at 8 h post injection of PEGylated Cu$_2$Se nanoparticles, in comparison with that of mice injected with PBS solution. This result demonstrates that a fraction of PEGylated Cu$_2$Se nanoparticles can be excreted by urine due to their ultrasmall size. The excretion of ultrasmall PEGylated Cu$_2$Se nanoparticles through urine could significantly reduce their toxicity or side effects.

All the imaging results (PA imaging, CT imaging, and SPECT imaging) demonstrate that Cu$_2$Se-PEG-SH NPs could be passively accumulated in the tumor by the EPR effect. In order to further quantify their biodistribution, Cu$_2$Se-PEG-SH NPs were intravenously injected into a group of 4T1 tumor-bearing BALB/C mice,
which were sacrificed at 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, and 48 h, and their major organs were harvested to determine the copper contents by ICP-OES measurements. The results in Figure 5.12e reveal that the Cu$_2$Se-PEG-SH NPs are mainly distributed in reticuloendothelial organs such as the liver, spleen, and lung. The high distribution in the kidney again supports the proposition that the ultra-small Cu$_2$Se-PEG-SH NPs can be excreted through renal clearance. The copper content in the tumor reached the maximum value of 4.4 %ID/g at 12 h post injection, which is consistent with the results of PA imaging and SPECT imaging.

The highly efficient photothermal conversion efficiency of Cu$_2$Se-PEG-SH NPs and their outstanding passive accumulation in tumors encouraged us to further investigate the feasibility of in vivo photothermal ablation of cancer cells under the guidance of multimodal imaging. 24 BALB/c mice bearing 4T1 tumors with a volume of around 130 mm$^3$ were divided into 4 groups with 6 mice per group to respectively receive intravenous administration of either PBS solution (200 µL) or Cu$_2$Se-PEG-SH NPs solution (500 µg/mL, 200 µL), and then irradiated for 6 min by an 808-nm NIR laser with a power density of 1.5 W/cm$^2$ at 12 h post injection. The four groups of mice are referred to as (a) mice injected with PBS only (group 1, PBS); (b) mice injected with Cu$_2$Se-PEG-SH NPs only (group 2, Cu$_2$Se); (c) mice irradiated with the NIR laser after injection of PBS (group 3, PBS+NIR); and (d) mice irradiated with the NIR laser after injection of Cu$_2$Se-PEG-SH NPs (group 4, Cu$_2$Se+NIR). The temperature of the tumor area under NIR irradiation was monitored by an infrared thermal camera. The tumor volume and appearance, and the body weights of mice were measured every two days. The negligible differences in the weights of the four groups of mice (Figure 5.15) indicate that the current dose of ultra-small Cu$_2$Se-PEG-SH NPs did not induced acute toxicity or noticeable systemic toxicity. As shown in Figure 5.14a and b, the tumor temperature of the mouse injected with Cu$_2$Se-PEG-SH NPs increased dramatically to 57.6 °C under NIR irradiation, which is sufficient to ablate the cancer cells and eradicate their malignant proliferation. In huge contrast, the tumor temperature of mice injected with PBS solution only increased to approximately 44 °C. Hematoxylin and eosin (H&E) staining of tumor slices collected at day 3 after treatment was further carried out to reveal the therapeutic efficacy. As shown in Figure 5.14c, no obvious malignant necrosis was observed in the mice from control groups 1-3, while apparent
extensive karyopyknosis and necrosis were found in the mice intravenously injected with Cu$_{2-x}$Se and then irradiated with the NIR laser (i.e. group 4), which demonstrates the excellent therapeutic efficacy of ultra-small Cu$_{2-x}$Se-PEG-SH NPs.

The tumors treated with NIR irradiation with the aid of accumulated Cu$_{2-x}$Se NPs shrank and became black scars at day 3 (Figure 5.16), and were completely eliminated within 16 days. No recurrence was observed during the subsequent feeding (Figure 5.14d and 5.16). The tumors of the mice from the PBS+NIR group kept growing until the mice died, which is similar to the cases of mice injected with PBS or Cu$_{2-x}$Se NPs only. In addition, the average lifespans of the mice in these groups are obviously shorter than that of mice treated with NIR irradiation after injection of Cu$_{2-x}$Se (Figure 5.14e). The results demonstrate that the use of PBS, Cu$_{2-x}$Se NPs, or NIR irradiation alone could not prevent tumors from growing. All these results provide strong evidence that ultra-small Cu$_{2-x}$Se-PEG-SH NPs could be a promising biocompatible PTT agent for *in vivo* photothermal therapy of tumor.
Figure 5.14 *In vivo* photothermal therapy of tumors with Cu$_{2-x}$Se-PEG-SH NPs: (a) infrared thermal images and (b) temperature curves of 4T1 tumor-bearing mice intravenously injected with Cu$_{2-x}$Se-PEG-SH NPs solution (500 µg/mL, 200 µL) and then irradiated with an 808 nm NIR laser at a power density of 1.5 W/cm$^2$ for 6 min, in comparison with injection of PBS solution followed by NIR irradiation, (c) H&E staining of tumor sections from different groups of mice sacrificed at day 3 of
treatment, (d) relative tumor volumes normalized to their initial volumes (Analysis of Variance (ANOVA) was used to assess statistical significance. *p < 0.5, **p < 0.01, ***p < 0.001.) and (e) survival curves of different groups of mice after various treatments.

![Graph showing body weight changes over time for different treatments](image)

**Figure 5.15** Body weights of mice in different groups after various treatments (n = 5).

![Representative photographs of mice bearing 4T1 tumors](image)

**Figure 5.16** Representative photographs of mice bearing 4T1 tumors taken at different times after treatment.
Figure 5.17 H&E stained images of major organs collected from mice intravenously injected with PBS and then irradiated with a NIR laser (top row), from healthy mouse (middle row), and from mice intravenously injected with Cu$_2$-Se-PEG-SH NPs and then irradiated with a NIR laser (bottom row, images collected at 40 days after photothermal therapy). Red circles represent tumor metastasis regions.

To further determine that there was no recurrence of tumors or metastasis of the cancer, the major organs, such as the liver, lung, kidney, spleen, and heart, were harvested at day 40 after treatment and stained with H&E. As displayed in Figure 5.17, there are obvious metastases and inflammatory lesions in the livers and lungs of mice treated with PBS, Cu$_2$-Se NPs, or NIR irradiation after administration of PBS. In contrast, no evident inflammation or damage is observed in any of the major organs of the mice treated with NIR irradiation after injection of Cu$_2$-Se-PEG-SH NPs, and the H&E images of these organs are the same as for a healthy mouse, further confirming that there are no side effects or toxicity associated with the Cu$_2$-Se-PEG-SH NPs.

5.4 Conclusions

In summary, a novel multimodal imaging-guided PTT nanotheranostic agent based on ultra-small PEGylated Cu$_2$-Se NPs was successfully fabricated via a facile aqueous route. The Cu$_2$-Se NPs possess a strong, significant NIR LSPR due to the high copper deficiency. The resultant NPs exhibit high stability and biocompatibility.
both *in vitro* and *in vivo*. More importantly, they show highly efficient photothermal conversion for PA imaging and PTT *in vitro* and *in vivo*, and large X-ray attenuation coefficients for CT imaging, as well as multifunctional groups for coordinating with radioisotopes for SPECT imaging. Both the *in vitro* and the *in vivo* results demonstrate that ultra-small PEGylated Cu$_{2-x}$Se NPs could serve as an ideal multifunctional platform for multimodal imaging-guided photothermal therapy of cancer.

### 5.5 References


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Chapter 6 Vacancy Engineering for Tuning the Localized Surface Plasmon Resonances of Fe-doped Cu$_{2-x}$Se Nanoparticles for Dual-modal Photoacoustic/MR Imaging Guided Photothermal Therapy of Cancer

Designing a multifunctional nanotheranostic for integration of precise diagnosis and effective treatment of tumors is desirable but remains a great challenge. In this chapter, we developed a facile and chelator-free doping method for constructing Fe-doped Cu$_{2-x}$Se nanoparticles. The position and intensity in near-infrared localized surface plasmon resonance in the alloyed nanostructure can be tuned by altering the feeding amount of the Fe$^{3+}$ ions precursors. Owing to their tunable near-infrared absorption and Fe doping, the hybrid nanostructure was demonstrated be a novel nanotheranostic agent for effective deep-tissue photoacoustic imaging, magnetic resonance imaging, and photothermal therapy of cancer.
Chapter 6 Fe doped Cu$_{2-x}$Se

6.1 Introduction

Localized surface plasmon resonances (LSPRs) typically arise from the collective oscillation of free charge carriers (i.e., electrons or holes) in resonance with the driving electromagnetic field of incident light.$^{[1-7]}$ Owing to these LSPRs, the nanoparticles show enhanced light absorption and scattering with potential applications in fields ranging from photoelectrical$^{[8-10]}$ to biomedical, such as bio-imaging and photothermal therapy.$^{[2, 11-17]}$ LSPRs can be achieved not only in nanostructures of noble metals, but also in semiconductor nanocrystals (NCs) with appreciable free carrier concentrations.$^{[1, 4]}$ The most common plasmonic noble metals are Ag, Au, and Cu, which have free electron densities in the range of $10^{22}$ - $10^{23}$ cm$^{-1}$ with corresponding LSPRs in the visible spectrum.$^{[18]}$ In contrast to LSPRs in metals, which are attributable to oscillation of free electrons, the LSPRs in the near-infrared (NIR) in heavily doped copper chalcogenide (Cu$_{2-x}$E, E = S, Se, Te) nanoparticles (NPs) arise from free holes, owing to the large amount of hole carriers with high mobility due to their copper deficiency.$^{[19, 20]}$ Both the semiconductor nanocrystals and the noble metals exhibit size- and shape-tunability of LSPRs. The NIR LSPRs of semiconductors can be easily engineered, however, because their free carrier concentrations can be tuned by doping, temperature, and/or phase transitions. In contrast, the LSPR response of metal particles, once they are engineered by the choice of nanostructure parameters (such as shape, size, or metal), is permanently locked in and cannot be dynamically controlled.$^{[1]}$

The berzelianite Cu$_{2-x}$Se is an important $p$-doped copper chalcogenide semiconductor with a relatively high hole concentration and exhibits strong free carrier absorption, which results in LSPRs. Due to the dynamic tunability of LSPRs by simple doping, doped copper chalcogenide nanocrystals have been widely used for photoacoustic (PA) imaging contrast and photothermal therapy (PTT) transducers in biomedical application due to their strong absorption in the NIR.$^{[16, 21, 22]}$ Moreover, introducing various types of foreign functional ions into the vacancies of NPs by doping gives them additional properties and functions, and this has become a promising method to design multifunctional nanoparticles.$^{[23-27]}$ It is well known that multimodal imaging can offer more accurate and reliable biomedical information to improve the efficacy and sensitivity of clinical imaging diagnostics.$^{[16]}$ Magnetic resonance imaging (MRI) has evolved into a routinely used tool in noninvasive
diagnostic imaging thanks to its high spatiotemporal resolution, excellent soft tissue contrast, and non-ionizing radiation.\textsuperscript{[28-30]} PA imaging as an emerging non-invasive hybrid imaging technique possesses the unprecedented advantages of high contrast optical imaging as well as high resolution acoustic imaging at the centimeter penetration depth.\textsuperscript{[31-33]} Therefore, a new imaging technique that integrates MRI with PA imaging, which not only images subsurface tissue structures, but also offers higher resolution and deeper tissue penetration, will lead to better diagnosis of many diseases. Intense efforts have been made to fabricate various kinds of multimodal contrast agents (such as for positron emission tomography/ computed tomography (PET/CT),\textsuperscript{[34]} PET/ fluoroscopy (FL),\textsuperscript{[35]} MRI/FL,\textsuperscript{[36]} MRI/ upconversion luminescence (UCL)\textsuperscript{[37]}) via doping. There are only a few reports in the literature on creating PA/MRI imaging contrast agents by the doping method,\textsuperscript{[27]} let alone detailed investigation on the mechanism of the doping process.

Herein, we have for the first time developed a facile doping method by making use of the oxidation-reduction reaction in the Fe-Cu$_2$-Se system to construct a Fe-doped Cu$_2$-Se therapeutic agent, which was non-covalently functionalized dimercapto poly(ethylene glycol) (HS-PEG-SH) for applications in tumor dual-modal imaging and PTT treatment. It should be noted that this doping method does not involve any surfactant or chelator, and it was performed at room temperature and atmospheric pressure; therefore, no special or complex apparatus is required. Moreover, the possible doping and phase transfer process has been carefully investigated, which surely will further promote the potential exploration of Cu$_2$-Se-based nanomaterials for cancer diagnosis and therapies.

6.2 Experimental Section
6.2.1 Materials

All chemicals and reagents were used as received without any further purification. Copper sulfate (CuSO$_4$·5H$_2$O), polyvinylpyrrolidone powder (PVP) and Iron(III) chloride hexahydrate (FeCl$_3$·6H$_2$O) were purchased from Sinopharm Chemical Reagent. Selenium dioxide (SeO$_2$) was obtained from Aladdin Chemistry, and vitamin C (Vc) was purchased from Alfa Aesar. Dimercapto poly(ethylene glycol) (HS-PEG-SH, MW = 5000) was bought from Adamas. Milli-Q water (> 18 M\textOmega cm) was used in the experiments.
6.2.2 Synthesis of Cu$_{2-x}$Se nanoparticles and PEGylated Fe-doped nanoparticles

Cu$_{2-x}$Se NPs were synthesized according to a modified protocol reported elsewhere.$^{38}$ Briefly, 55 mL Milli-Q water and 16 mL 10 mg/mL PVP-K30 (i.e. 160 mg PVP) were added to a round-bottomed flask, and then 1 mL 0.2 M SeO$_2$ and 3 mL 0.4 M Vc were added successively. After 15 min, a red Se solution was formed. A mixed solution of 1 mL 0.4 M CuSO$_4$·5H$_2$O and 4 mL 0.4 M Vc, in which Vc reduced the Cu$^{2+}$ ions to Cu$^+$, was added into the red Se solution under vigorous stirring. The resultant mixture was allowed to react under vigorous stirring at room temperature until a dark green solution was obtained in 30 h, indicating that PVP-stabilized Cu$_{2-x}$Se NPs had been prepared. The dark green solution was subsequently purified twice by centrifugation with water at 10000 rpm to remove the excess PVP and Vc. After that, 3 mL 0.1 M FeCl$_3$·6H$_2$O aqueous solution (i.e. 0.3 mol Fe$^{3+}$, molar ratio: Cu$_{2-x}$Se: Fe$^{3+}$ = 1: 1.5) was added into the redispersed Cu$_{2-x}$Se NP solution where the color rapidly changed from green to a dark color. After 6 h, the products were centrifuged, washed several times with water, and then redispersed in aqueous solution. 0.2 g HS-PEG-SH was added to modify the surfaces of the CuFeSe NPs at room temperature. The obtained PEGylated nanoparticles were purified by centrifugation at 10000 rpm and the final product was stored at 4 °C for further characterization and use.

6.2.3 Characterization

Ultraviolet-visible-near-infrared (UV-Vis-NIR) spectra were recorded with a PerkinElmer Lambda 750 UV-Vis-NIR spectrophotometer. TEM images were captured using a FEI Tecnai F20 transmission electron microscope operating at an acceleration voltage of 200 kV. HAADF images and STEM-EDS spectra were collected using a JEOL ARM-200F operating at 200 kV with an EDAX solid-state X-ray detector. Samples were prepared by drop-casting ethanol-dispersed nanostructures onto TEM aluminium grids. The crystal structure of the nanoparticles was characterized with a Shimadzu XRD-6000 X-ray diffractometer equipped with Cu Ka1 radiation ($\lambda = 0.15406$ nm). X-ray photoelectron spectroscopy (XPS) measurements were carried out on a Thermo Scientific Sigma Probe instrument using Al Ka X-ray radiation and fixed analyzer transmission mode. Thermogravimetric analysis (TGA) was performed to analyze the weights of surface ligands at a heating rate of 10 °C min$^{-1}$ from room temperature to 900 °C under argon atmosphere.
hydrodynamic size and zeta potential were measured at 25 °C with a Malvern Zetasizer Nano ZS90 equipped with a solid state He-Ne laser (λ = 633 nm). Fourier transform infrared (FTIR) spectra were recorded on a Magna-560 spectrometer (Nicolet, Madison, WI, USA). Raman spectra were collected with a Jobin Yvon HR800 Raman spectrometer with a 10 mW He-Ne laser.

6.2.4 Cytotoxicity assay of the PEGylated CuFeSe nanoparticles

MTT assays were performed to assess the cytotoxicity of the CuFeSe-PEG NPs. In detail, 4T1 cells were first seeded into 96-well plates at a density of 4000 cells per well and cultured for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Then, the cells were washed with PBS and incubated with CuFeSe-PEG NPs at different concentrations at 37 °C for 24 h. After that, the cells were washed twice with PBS and cultured for 48 h. Then, 20 μL MTT was added at a concentration of 5 mg/mL and allowed to react with the cells for 4 h before the addition of 150 μL dimethyl sulfoxide (DMSO) to dissolve the precipitates. The absorption of each solution was measured at 490 nm on a microplate reader (Thermo, Varioskan Flash).

6.2.5 Animal model

The tumor models used were established by subcutaneous injection of 50 μL 4T1 cell suspension (~5 × 10^6 cells) into the flank region of the right back of 5-week-old male BALB/c mice (for the PTT treatment) or nude mice (for the PA imaging). The tumor imaging studies were carried out 10 days after the inoculation with tumor cells. All animal experiments were carried out according to the protocols approved by the Soochow University Laboratory Animal Center.

6.2.6 In vivo Photoacoustic Imaging

Nude mice bearing subcutaneous tumors were anesthetized with 1.5% isoflurane delivered via a nose cone, and then CuFeSe-PEG NPs (dose = 7 mg/kg) were injected via the tail vein. Photoacoustic images were acquired at different time points post-injection by a multispectral optoacoustic tomography (MSOT) instrument. 10 slices were obtained at each position and averaged to minimize the influence of animal movement in the images.

6.2.7 MRI Measurements

MRI was performed on a clinical 3.0 T MRI scanner equipped with a special coil designed for small animals. In-vitro T2-weighted MRI images of nanoparticle
solutions with different Fe concentrations were measured. For in vivo imaging, the mouse was anesthetized with isoflurane (1.5%) at 1 L/min flow throughout the experiment. Prior to imaging, CuFeSe-PEG NPs (50 μL, 2 mg/mL) were administered by intratumoral injection. T2-weighted MRI images were acquired using the T2-RARE sequence (RARE = rapid acquisition with relaxation enhancement) with the following parameters: repetition time (TR) = 3.5 s, echo time (TE) = 76 ms, field of view = 40 mm × 40 mm, matrix size = 512 × 512, number of slices = 7, slice thickness/gap = 2.0 mm/0.4 mm, flip angle = 150°, and number of excitations (NEX) = 4.

6.2.8 Photothermal Therapy

24 tumor-bearing BALB/c mice with an average tumor volume of 130 mm³ were randomly allocated into 4 groups, which were intravenously injected with the same volume of either saline or CuFeSe-PEG NPs solution (7 mg/kg). The laser irradiation was performed using an 808-nm NIR laser (Hi-Tech Optoelectronics Co., Ltd., Beijing, China) with a power density of 1.5 W/cm² for 5 min. The tumor sizes and body weights were measured every two days, and the tumor volume was calculated by \( a \times b^2/2 \), where \( a \) and \( b \) represent the length and width of the tumor, respectively. Relative tumor volumes were obtained by dividing by the initial tumor size before laser treatment. The mice treated with NIR irradiation after injection of CuFeSe-PEG NPs were sacrificed at day 30 to harvest the major organs for histological analysis.

6.2.9 Histological Analysis

For hematoxylin and eosin (H&E) staining, major organs, including the heart, liver, spleen, kidney, and lung, were harvested, fixed in 10% neutral buffered formalin, processed routinely into paraffin, sectioned into thin slices, and stained with H&E for histological analysis.

6.3 Results and Discussion

As demonstrated in Figure 6.1, monodispersed Cu₂Se NPs with polyvinylpyrrolidone (PVP) as a stabilizer were prepared via a modified template-directed synthesis method reported elsewhere (step 1 in Figure 6.1). More specifically, nano-Se NPs were firstly prepared as templates by using ascorbic acid (Vc) to reduce SeO₂ in the presence of PVP. Second, Cu₂Se NPs were formed with
the addition of Cu\(^+\) (mixed solution of CuSO\(_4\) and Vc) to the Se NPs, since Cu\(^+\) can catalyze Se\(^0\) into Se\(^2-\) and Se\(^{4+}\). After that, the as-formed Cu\(_2\)Se NPs can be gradually transformed into Cu\(_{2-x}\)Se with a color change to deep green through a phase transformation process promoted by air exposure and the unreacted oxidants (step 2 in Figure 6.1). The nano-Se NP templates were characterized by transmission electron microscopy (TEM), X-ray diffraction (XRD), and ultraviolet (UV) spectroscopy, as shown in Figure 6.2. Those nano-Se particles are quasi-spherical NPs with a mean diameter of 86.34 ± 10.07 nm. The XRD pattern in Figure 6.2c confirmed the crystal structure of Se NPs, suggesting their good crystallinity. It was noted that there was almost no absorption for Se NPs at wavelengths > 700 nm, as shown in Figure 6.2d.

**Figure 6.1** Schematic illustration of the synthesis of Fe-doped Cu\(_{2-x}\)Se nanoparticles.
The reaction route from the Se template to Cu$_{2-x}$Se was composed of steps 1 and 2, as shown in Figure 6.1, and the whole reaction process was investigated by monitoring the evolution of UV-visible-near-infrared (UV-vis-NIR) spectra of the samples after different reaction times (Figure 6.3a). The Cu$_2$Se nanocrystals were formed 3 hours after the Cu$^+$ ions were mixed with nano-Se templates, which showed elevated absorption in the NIR region. With increasing reaction time, the absorption was gradually enhanced, and the spectrum showed a peak centred at 1256 nm after 15 hours. On further extending the reaction time, a blue shift was observed in the absorption, indicating increasing copper deficiency and thus that the copper deficiency is controllable. After about 24 hours of reaction time, the absorption remained almost stable with only 40 nm of blue shift, as the reaction time went from 24 h (peak centred at 1130 nm) to 30 h (peak centred at 1170 nm). Along with the monitoring of the UV-vis-NIR absorption spectra, their corresponding photothermal conversion capability was evaluated, as shown in Figure 6.3b. The same
concentrations of Cu$_{2-x}$Se sample solutions were exposed to an 808 nm NIR laser with a power density of 0.75 W cm$^{-2}$ for 10 min. Figure 6.3b clearly demonstrates the absorption intensity-dependent photothermal conversion of Cu$_{2-x}$Se NPs, indicating the excellent photothermal conversion capability of the Cu$_{2-x}$Se samples which have high copper deficiency.

Figure 6.3 (a) Ultraviolet-visible-near-infrared (UV-vis-NIR) absorption spectra of the formation and evolution of Cu$_{2-x}$Se sample. (b) Corresponding photothermal heating curves of Cu$_{2-x}$Se sample.

As shown in the TEM images in Figure 6.4, the as-made Cu$_{2-x}$Se NPs coated by different kinds of PVP surfactants were quite uniform and monodispersed, and their size could be slightly adjusted from 72.57 nm to 87.28 nm by changing the molecular weight (MW) of PVP. It is believed that with the increasing K value of PVP, the viscosity increases, and therefore the mobility and reactivity of the surfactant-ion system are decreased, resulting in less nuclei and bigger growth. In addition, we also investigated the concentration effect on the particle size by feeding different amounts of the same kind of PVP-K30 (Figure 6.5). Those TEM images clearly demonstrate the size-tunability of the particles by simple changing the PVP concentration. The possible reason may be that the greater the amount of PVP molecules in the SeO$_2$-Vc system, the smaller the template Se NPs that are formed due to more anchoring groups and more heavily steric hindrance of PVP. In order to better understand those reactions, the reaction procedures for step 1 and step 2 in Figure 1 can be proposed by Equations (1)-(2).

\[
\text{Step 1: } \quad 3\text{Se}^0 \rightarrow 2\text{Se}^{2-} + \text{Se}^{4+}; \quad \text{Se}^{2-} + 2\text{Cu}^+ \rightarrow \text{Cu}_2\text{Se} \quad (1)
\]
Step 1: \[ \text{Cu}_2\text{Se} \rightarrow \text{Cu}_{2-x}\text{Se} + x\text{Cu}^+ + xe^- \] (2)

Based on this optimization of the experimental conditions, we chose the Cu$_{2-x}$Se NP samples obtained by using 160 mg PVP-K30 as stabilizer to perform the following Fe doping experiments.

In the third step in Figure 6.1, during which Fe$^{3+}$ reacts with the Cu$_{2-x}$Se NPs, we

**Figure 6.4** TEM images of Cu$_{2-x}$Se NPs stabilized by (a) PVP-K12 (MW = 3500), (b) PVP-K16 (MW = 8000), (c) PVP-K30 (MW = 40,000), (d) PVP-K60 (MW = 160,000), (e) PVP-K90 (MW = 360,000); and (f) their size obtained by counting 100 particles of each sample.

**Figure 6.5** TEM images of Cu$_{2-x}$Se NPs stabilized by PVP-K30 in different concentrations: (a) 80 mg, (b) 160 mg, (c) 320 mg, (d) 800 mg, (e) 1600 mg, and (f) 3200 mg. (g) Statistical size of each sample.

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tailored the precursor Cu$_{2-x}$Se: Fe$^{3+}$ molar ratio to monitor the evolution of the properties of the products, including their NIR plasmon absorbance, crystal structures, morphologies, compositions, etc.

Figure 6.6 Evolution of (a) UV-vis-NIR absorbance spectra and (b) XRD patterns of products obtained with different precursor Cu$_{2-x}$Se: Fe$^{3+}$ molar ratios at room temperature (RT) without Vc and PVP.
As shown in Figure 6.6a, the NIR absorbance intensity of the NPs was continuously decreased and the LSPR absorbance peak was blue shifted with increasing amounts of Fe$^{3+}$. The characteristic absorption peak disappeared with the addition of Fe$^{3+}$ at a 1:3 Cu$_{2-x}$Se : Fe$^{3+}$ molar ratio. According to the XRD analysis in Figure 6.6b, there was a progressive shift of the peak of the NPs, as the initial berzelianite (Cu$_{2-x}$Se, JCPDS 06-0680) phase evolved to the copper iron selenide phase (CuFeSe$_2$, JCPDS 81-1959). In addition, a broad XRD feature in the range of 16-36º (region marked by red rectangle in Figure 6.6b) was observed in the Fe-doped NPs indicating the presence of amorphous phase.

**Figure 6.7** TEM images and corresponding size distributions of the nanostructures obtained with different Cu$_{2-x}$Se:Fe$^{3+}$ molar ratios: (a and a’) 1: 0.5, (b and b’) 1: 1, (c and c’) 1: 1.5, (d and d’) 1: 2, (e and e’) 1: 3, and (f and f’) 1: 6, at RT without Vc and PVP.
A collection of TEM images of Fe-doped NPs prepared by reacting Cu$_{2-x}$Se NPs with Fe$^{3+}$ at different molar ratios at room temperature (RT) in the absence of Vc and PVP is shown in Figure 6.7. These Fe-doped NPs seems were corroded to different degrees, with rough surfaces that were different from that of the initial Cu$_{2-x}$Se NP sample. A mean size reduction from 75 nm to 66 nm was found, as demonstrated in Figure 6.7(a'-f'), which could be attributed to the corrosion effect induced by the oxidizability of the Fe$^{3+}$ ion.

![TEM images](image-url)

**Figure 6.8** TEM and HRTEM images and the corresponding FFT patterns (insets) of the nanostructures obtained with different Cu$_{2-x}$Se:Fe$^{3+}$ molar ratios: (a$_1$ and a$_2$) 1: 0.5; (b$_1$ and b$_2$) 1: 1, inset of b$_1$: corresponding scanning transmission electron microscopy (STEM) image; (c$_1$, c$_2$, c$_3$, and c$_4$) 1: 1.5; (d$_1$ and d$_2$) 1: 2; (e$_1$ and e$_2$) 1: 3; (f$_1$ and f$_2$) 1: 5, inset of f$_2$: corresponding selected-area electron diffraction (SAED) pattern; and (g$_1$ and g$_2$) 1: 6.
In order to investigate the evolution of morphology and the crystal transition of the Fe-doped NPs more clearly, a series of high magnification TEM images and high-resolution TEM (HRTEM) images were obtained and are presented in Figure 6.8. As Figure 6.8a2 shows, the as-formed Fe-doped NPs have a lattice spacing of 0.328 nm, corresponding to the (111) planes of the cubic berzelianite Cu$_{2-x}$Se structure, JCPDS 06-0680, which can be further supported by the fast Fourier transform (FFT) pattern in the inset of Figure 6.8a2. On increasing the amount of Fe$^{3+}$ to 1: 1, the well-defined crystalline lattice in Figure 6.8b2 has a lattice spacing of 0.318 nm that matches well with that of the (112) planes of CuFeSe$_2$, JCPDS 81-1959. The FFT pattern in the inset of Figure 6.8b2 also supports the transition of the crystal structure after the Fe doping reactions. The HRTEM image in Figure 6.8c2 displays continuous lattice fringes with spacing of 0.322 nm, which could be indexed to the (112) planes of CuFeSe$_2$ as well. In addition, 0.326 nm lattice spacing was found after carefully measurement of the 1: 1.5 sample in Figure 6.8c4, which can be referred to the (111) planes of Cu$_{2-x}$Se and confirmed by its corresponding FFT pattern. Meanwhile, another series of lattice fringes possessing a 0.301 nm lattice spacing was found in the same particle from the 1: 1.5 sample, which can be attributed to the (103) planes of CuFeSe$_2$, suggesting hybrid phases in this sample, i.e. the coexistence of Cu$_{2-x}$Se and CuFeSe$_2$ in this sample. It was considered that the amount of the Cu$_{2-x}$Se was so small (< 5%) that it was unable to be detected by XRD.

On further increasing the amount of Fe$^{3+}$, the Fe-doped NPs became smaller and tended to form a network with necking and fusion between NPs (Figure 6.8e1,f1,g1). Those HRTEM images and the corresponding FFT patterns and selected-area electron diffraction (SAED) patterns for the 1: 2, 1: 3, 1: 5, and 1: 6 samples all display the detected CuFeSe$_2$ phase features, which are consistent with their XRD results.

The composition and element distribution for Fe-doped NPs can be determined by high-angle annular dark field-scanning transmission electron microscopy (HAADF-STEM) with energy dispersive X-ray spectroscopy (EDS), conducted on a JOEL ARM-200F. Figure 6.9 shows the EDS elemental mapping and line scan results for representative Fe-doped NP samples. These results reveal the coexistence of Cu, Fe, and Se throughout the whole NPs (Figures 6.9 and 6.10), and the molar ratio of the three elements Cu, Fe, and Se for each sample was determined by EDS, as shown in
Table 6.1, which was highly consistent with the composition analysis of Fe-doped NPs supported by inductively coupled plasma – optical emission spectroscopy (ICP-OES).

Figure 6.9 HAADF-STEM EDS elemental mapping images and corresponding line scan results using carbon-coated aluminium grids for different Fe-doped NP samples: (a) 1: 1, (b) 1: 1.5, (c) 1: 3, (d) 1:6.

Figure 6.10 EDS spectra for different Fe-doped NP samples using carbon-coated aluminium grids: (a) 1: 1, (b) 1: 1.5, (c) 1: 3, (d) 1:6.

In addition, the EDS line scan results further confirmed the declining Cu content with increasing Se content on the addition of Fe$^{3+}$. It should also be noted that the 1:3 sample possesses the highest content of Fe (with a normalized formula:
Cu$_{0.205}$Fe$_{0.0658}$Se), which was mainly distributed on the surfaces of the NPs, as displayed in Figure 6.9c and Figure 6.10c. On the basis of the XRD results, no crystal Se signal was detected, but there was a rise in the broad background in the range of 16-36°, and from the elemental analysis by EDS and ICP-OES, there is reason to believe that amorphous Se was produced during the Fe$^{3+}$ oxidation procedure. The evolution of X-ray photoelectron spectroscopy (XPS) measurements for different samples was investigated as shown in Table 6.1.

**Table 6.1** Elemental analysis by energy dispersive X-ray spectroscopy (EDS) and inductively coupled plasma-optical emission spectroscopy (ICP-OES).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chemical quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: 0</td>
<td>Cu$_{1.91}$Se</td>
</tr>
<tr>
<td>1: 1</td>
<td>Cu$<em>{1.09}$Fe$</em>{0.0257}$Se</td>
</tr>
<tr>
<td>1: 1.5</td>
<td>Cu$<em>{0.925}$Fe$</em>{0.0289}$Se</td>
</tr>
<tr>
<td>1: 2</td>
<td>Cu$<em>{0.501}$Fe$</em>{0.0211}$Se</td>
</tr>
<tr>
<td>1: 3</td>
<td>Cu$<em>{0.205}$Fe$</em>{0.0658}$Se</td>
</tr>
<tr>
<td>1: 6</td>
<td>Cu$<em>{0.157}$Fe$</em>{0.0218}$Se</td>
</tr>
</tbody>
</table>

Figure 6.11. It should be noted that there were no noticeable differences in the XPS spectra in Cu 2p region and Fe 2p region for all the corresponding samples, suggesting that the valence states of Cu and Fe are +1 and +3, respectively. The XPS spectra of Se 3d obtained from the 1: 0 sample (i.e. the pure Cu$_{2-x}$Se sample) and the elemental Se sample can be treated as the standard spectra, with Se$^{2-}$ in Cu$_{2-x}$Se and Se$^0$ in Se. Figure 6.11c clearly shows that there is a tendency towards a shift from Se$^{2-}$ toward Se$^0$ with the addition of Fe$^{3+}$, further supporting the formation of Se in coexistence with Se$^{2-}$. In order to determine the structural properties of the Se (i.e. crystalline Se or amorphous Se) and their relative contents, Raman spectroscopy characterization and thermal gravimetric analysis were performed, as depicted in Figure 6.12. It has been pointed out in the literature that the resonance peak of crystalline Se (trigonal) is located at ~236.8 cm$^{-1}$ and that of amorphous selenium is centred at around 250 cm$^{-1}$, which are attributed to the stretching vibrations of Se$_n$ helical chains.$^{39,40}$ In our study, the Raman spectrum of the as-prepared template Se NPs exhibits a peak at 236 cm$^{-1}$, which demonstrates that the product is crystalline Se.
which matches well with its XRD pattern (Figure 6.2d). The Raman spectrum of pure Cu$_{2-x}$Se (i.e. the 1:0 sample) shows two peaks at around 236 and 255 cm$^{-1}$, owing to the characteristic vibration of the Cu-Se bond,$^{[41]}$ which overlaps the reported peaks for crystalline Se and amorphous Se, respectively.

After the Fe doping, the Fe-doped NPs demonstrate a stronger and sharper peak at 255 cm$^{-1}$ than that of the pure Cu$_{2-x}$Se sample, possibly supporting the generation of amorphous Se during the Fe-doping procedure. Thermogravimetric analysis (TGA)

![Figure 6.11](image1.png)

**Figure 6.11** High resolution XPS spectra of (a) Cu 2p, (b) Fe 2p, and (c) Se 3d from the different as-synthesized Fe-doped NP samples.

![Figure 6.12](image2.png)

**Figure 6.12** (a) Raman spectra and (b) thermogravimetric analysis (TGA) curves (under argon atmosphere) of different samples.

was performed to determine the content of amorphous Se in the Fe-doped NPs. The TGA curve of the Se in Figure 6.12b shows two Se evaporation stages in the ranges of 380 to 560 °C and 560 to 780 °C, because the melting point and boiling point of
Se are 217.0 and 684.9 °C, respectively. Compared with the weight loss of the pure Cu$_2$Se sample (1: 0 sample), more weight loss of Se was observed for the Fe-doped samples. These results roughly suggest that there is ~14.94% (i.e. 89.4%-74.46%), ~28.13%, 41.01%, 64.24%, and 68.76% of amorphous Se in these composite 1: 1, 1: 1.5, 1: 2, 1: 3, and 1: 6 samples, respectively. On the basis of the above results and explanations, the reactions taking place in step 3 and step 5 can be proposed as follows:

Step 3: 2Cu$_2$Se + 4FeCl$_3$ → CuFeSe$_2$ + 3CuCl$_2$ + 3FeCl$_2$ (3)
Step 5: CuFeSe$_2$ + 4FeCl$_3$ → CuCl$_2$ + 5FeCl$_2$ + 2Se (4)

Figure 6.13 (a) The evolution of UV-vis-NIR absorbance spectra of samples reacted with Fe$^{3+}$. (b) TEM image of the final sample after the second addition of Fe$^{3+}$ (molar ratio of Cu$_2$Se : total amount of Fe$^{3+}$ added = 1: 2).

Therefore, the composition of Fe-doped NPs can be adjusted by feeding different amount of Fe$^{3+}$ to tune their LSPRs. It is worth mentioning that Fe$^{3+}$ can also be used to replace O$_2$ as the oxygenant in step 2 to react with Cu$_2$Se directly. Figure 6.13a demonstrates the evolution of the UV-vis-NIR absorbance spectra for samples reacted with Fe$^{3+}$. After the addition of a trace of Fe$^{3+}$ solution, the PVP-K30-Cu$_2$Se (the 1 h sample after mixing Cu$^+$ with the template Se NPs ) shows a significant enhancement of LSPRs with the colour changing from brown to green. Adding more Fe$^{3+}$ leads to the damping of the LSPRs with the colour changing to black, which was the same phenomenon as for the sample where Cu$_{2.3}$Se was reacted with Fe$^{3+}$. Figure 6.13b also confirms the same corrosion effect on the surfaces of NPs induced
by Fe$^{3+}$ ions. Thereby, Fe$^{3+}$ in the step 4 can rapidly oxidize the Cu$_2$Se and then modify Cu$_{2-x}$Se NPs.

The introduction of Fe ions into the vacancies of Cu$_{2-x}$Se and the integration of the amorphous Se would endow the hybrid products with different properties and functions. The magnetic properties of these samples were investigated, as shown in Figure 6.14 and Table 6.2. The pure Se NPs show strong diamagnetic behavior at both 300 K [i.e. room temperature (RT)] and 5 K [i.e. low temperature (LT)], while the pure Cu$_{2-x}$Se sample (1: 0 sample) displays paramagnetic properties at RT and weak ferromagnetic properties at LT. The magnetization, with a value of 0.0193 emu/g at RT for the Cu$_{2-x}$Se sample, increased to 0.0615 emu/g and 0.133 emu/g at RT for the 1: 0.5 sample and the 1: 1.5 sample, respectively, suggesting the successful introduction of Fe ions into the Fe-doped samples. On further increasing the amount of Fe$^{3+}$ in the precursor, the 1: 2 sample started to demonstrate weak diamagnetism at RT and paramagnetism at LT because the hybrid included some paramagnetic phase and some diamagnetic phase. As expected, the 1: 3 and 1: 6 samples displayed strong diamagnetic signals as the generated amorphous Se dominated these samples.

For further biological applications, surface modification for NPs was necessary to improve their water solubility and biocompatibility. The as-prepared 1: 1.5 sample was chosen to perform the following experiments due to their adequate absorption which supports their excellent photothermal conversion capability and their potential ability to be MRI contrast agents due to the presence of Fe. The surfaces of the hydrophilic Fe-doped sample (1: 1.5 sample) was modified by HS-PEG-SH polymer at room temperature. The PEGylated 1: 1.5 sample can be denoted as Cu$_{0.91}$Fe$_{0.031}$Se-PEG NPs (CuFeSe-PEG, for short) according to the ICP-OES results in Table 1. After the non-covalent functionalization, the PEGylated NPs were well dispersed with a hydrodynamic size of around 78.8 nm, and there were no precipitates or aggregations of the water-soluble NPs, even after 3 months of storage at 4 ºC in a refrigerator, as shown in Figure
Chapter 6 Fe doped Cu$_{2+x}$Se
Figure 6.14 Magnetization isotherms of NPs recorded at 5 and 300 K, and temperature dependence of the magnetization of NPs measured in a field of 100 Oe for different samples, respectively: (a1 and a2) Se sample; (b1 and b2) Cu$_{2-x}$Se sample; (c1 and c2) 1: 0.5 sample; (d1 and d2) 1: 1.5 sample; (e1 and e2) 1: 2 sample; (f1 and f2) 1: 3 sample; (g1 and g2) 1: 6 sample.

Table 6.2 Magnetic properties of different samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>300 K</th>
<th>5 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se</td>
<td>diamagnetic</td>
<td>diamagnetic</td>
</tr>
<tr>
<td>1: 0</td>
<td>paramagnetic</td>
<td>weak ferromagnetic</td>
</tr>
<tr>
<td>1: 0.5</td>
<td>weak ferromagnetic</td>
<td>weak ferromagnetic + paramagnetic</td>
</tr>
<tr>
<td>1: 1.5</td>
<td>paramagnetic</td>
<td>weak ferromagnetic</td>
</tr>
<tr>
<td>1: 2</td>
<td>weak diamagnetic</td>
<td>paramagnetic</td>
</tr>
<tr>
<td>1: 3</td>
<td>diamagnetic +</td>
<td>diamagnetic +</td>
</tr>
<tr>
<td></td>
<td>paramagnetic</td>
<td>ferromagnetic</td>
</tr>
<tr>
<td>1: 6</td>
<td>diamagnetic +</td>
<td>diamagnetic +</td>
</tr>
<tr>
<td></td>
<td>paramagnetic</td>
<td>ferromagnetic</td>
</tr>
</tbody>
</table>

6.15a, suggesting their excellent stability, which is critical for their further biological applications. The aqueous solutions of the CuFeSe-PEG NPs exhibit well-retained absorbance in the NIR region (500-1100 nm), as shown in Figure 6.15b, and their absorbances at 808 nm linearly increase with the concentration (Figure 6.15c), so that the molar extinction coefficient is calculated to be 9.8 L g$^{-1}$ cm$^{-1}$ according to the Lambert-Beer law, which is about 3.4 times higher than that of Cu$_{2-x}$Se NCs (2.9 L g$^{-1}$ cm$^{-1}$) at the same concentration.$^{[16]}$ The zeta potential of the CuFeSe-PEG NPs was measured to be a negative, nearly -22 mV, as displayed in Figure 6.15d. The PEGylation of the CuFeSe NPs was confirmed by their Fourier transform infrared spectrum (FTIR, Figure 6.16a), and the content of HS-PEG-SH coated on the surfaces of the CuFeSe NPs was estimated by TGA to be approximately 8.21 wt% (Figure 6.16b). These results demonstrate the successful modification of CuFeSe NPs by HS-PEG-SH.
Figure 6.15 Characterization of as-prepared CuFeSe-PEG NPs: (a) hydrodynamic size at different times, (b) UV-vis-NIR absorbance spectra of nanoparticle solutions with different concentrations, (c) corresponding linear fitting plots of UV-vis-NIR absorbance versus CuFeSe-PEG concentration in aqueous solutions at 808 nm, and (d) zeta potential of CuFeSe-PEG NPs.

Figure 6.16 (a) FTIR spectra of the HS-PEG-SH and CuFeSe-PEG NPs, and (b) TGA curves of CuFeSe NPs samples recorded before and after PEG modification.
Prior to bioapplications in vitro and in vivo of the NPs, their potential cytotoxicity toward 4T1 cells (murine breast cancer cells) was first investigated by a standard methyl thiazolyl tetrazolium (MTT) assay (Figure 6.17a). The CuFeSe-PEG NPs demonstrated almost no cytotoxicity towards 4T1 cells with concentrations below 60 μg mL⁻¹, and the cell viability remained 82% when the concentration was 120 μg mL⁻¹. The broad NIR absorbance and the large extinction coefficient of CuFeSe-PEG NPs reveal their excellent photothermal conversion capability. Therefore, the temperatures of solutions with different particle concentrations were monitored when irradiated with an 808 nm laser (0.75 W/cm²) for 600 s. Figure 6.17b clearly shows the concentration-dependent photothermal conversion of CuFeSe-PEG NPs, and the temperature increments can be tuned easily as desired by simply changing their concentration. It is well known that photoacoustic (PA) imaging is based on photothermal conversion efficiency, and therefore, the remarkable photothermal conversion capability of the CuFeSe-PEG NPs highlights their great potential in PA imaging. The in vitro PA imaging performance of the as-prepared CuFeSe-PEG NPs was investigated, as shown in Figure 6.17c. As expected, the PA signal was significantly enhanced with the assistance of CuFeSe-PEG NPs, and the signal intensity was linearly increased with increasing concentration, suggesting that CuFeSe-PEG NPs could be a brilliant photothermal transducer for in vivo PA imaging. Owing to Fe
Figure 6.17 (a) Relative cell viabilities of 4T1 cells after being incubated with various concentrations of CuFeSe-PEG NPs for 24 h. (b) Photothermal heating curves of pure water and aqueous dispersions of CuFeSe-PEG NPs at different concentrations under irradiation by an 808 nm laser with a power density of 0.75 W cm\(^{-2}\) for 600 s. (c) In vitro photoacoustic signal intensity linearly fit to the concentration of Cu for CuFeSe-PEG NPs aqueous solutions; inset: the corresponding PA images. Inset: the corresponding PA images. (d) In vitro T\(_2\)-weighted magnetic resonance (MR) imaging using different concentrations of CuFeSe-PEG NPs. The transverse (r\(_2\)) relaxivities of CuFeSe\(_2\) NCs per Fe ion and per Cu\(_{0.91}\)Fe\(_{0.03}\)Se NP were determined to be 0.925 mM\(^{-1}\) S\(^{-1}\) and 0.0286 mM\(^{-1}\) S\(^{-1}\), respectively. Inset: the corresponding MR images.

doping, the magnetic resonance (MR) imaging capabilities of the NPs dissolved in water were also measured. T\(_2\)-weighted magnetic resonance (MR) images of CuFeSe-PEG NP solutions were acquired on a 3.0 T clinical MR scanner equipped with a small animal imaging coil, revealing the concentration-dependent darkening effect. The transverse (r\(_2\)) relaxivity was determined from the slope of the linear plot of 1/T\(_2\) versus Fe concentration (Figure 6.17d). From the plot, the r\(_2\) value was
measured to be 0.925 mM$^{-1}$ S$^{-1}$ per Fe ion or 0.0286 mM$^{-1}$ S$^{-1}$ for per Cu$_{0.91}$Fe$_{0.031}$Se NP. To further demonstrate

**Figure 6.18** (a) *In vivo* tumour PA images from a 4T1 tumour-bearing mouse, acquired before and after intravenous injection of CuFeSe-PEG NP solution (dose = 7 mg/kg) at different time points, and (b) corresponding time-dependent PA signal intensity of the tumour. (c) *In vivo* MR images of a mouse bearing a tumour (highlighted by the red circles) before and after intratumoral injection of CuFeSe-PEG NPs.

the *in vivo* MR imaging of CuFeSe-PEG NPs, a mouse bearing a 4T1 tumor was intratumorally injected with CuFeSe-PEG NPs (dose = 10 mg/kg). After 1 hour, the tumor in the mouse was imaged using the 3.0 T clinical MR scanner, and the image showed darkening effects when compared with the images before injection (Figure
6.18c), suggesting that the CuFeSe-PEG NPs can serve as an MR contrast agent. Besides the MR imaging, the \textit{in vivo} PA imaging capability of CuFeSe-PEG NPs was evaluated, as shown in Figure 6.18a, b. Mice bearing 4T1 tumors were intravenously injected with 200 μL CuFeSe-PEG NPs solution (7 mg/kg for the dose), and the PA images at different times post-injection are shown in Figure 6.18a. The tumor contrast was gradually enhanced with time after injection and reached its maximum at 8 h post-injection, due to the accumulation of CuFeSe-PEG NPs in the tumor through the enhanced permeability and retention (EPR) effect.\cite{42, 43} The signal intensity is further quantified in Figure 6.18b, which clearly shows the gradual increase to the maximum, followed by a decrease.

\textbf{Figure 6.19} \textit{In vivo} photothermal therapy for tumor. (a) Infrared thermal images and (b) the tumor heating curves of mice bearing 4T1 tumors after intravenous injection of CuFeSe-PEG NPs (7 mg/kg). (c) H&E staining of tumor sections from different groups of mice sacrificed at day 3 of treatment. (d) The relative tumor volumes
normalized to their initial volumes, and (e) survival curves and (f) body weights of different groups of mice after various treatments.

Encouraged by the PA imaging, we further investigate the feasibility of *in vivo* photothermal ablation of cancer cells by using the CuFeSe-PEG NPs as a photothermal agent. Mice bearing 4T1 tumors were intravenously injected with CuFeSe-PEG NPs (7 mg/kg) and then irradiated for 5 minutes by an 808 nm NIR laser with a power density of 1.5 W·cm⁻² at 8 h post-injection. The temperature change in the tumor area under NIR irradiation was monitored using an IR thermal camera. Figure 6.19a and b show the time-dependent thermographic images and temperature curves, respectively, for mice treated with CuFeSe-PEG NPs or phosphate buffered saline (PBS). Obviously, the CuFeSe-PEG NPs-mediated group exhibited a much higher hyperthermia effect, and the maximum temperature after 5 min of irradiation reached 64 °C, which is sufficient to induce irreversible damage to the cancer tissue. It has been reported that even just a short-time exposure (about 5 min) of cancer tissue to hyperthermia above 48 °C can induce massive cancer destruction.[44] To confirm the phototherapeutic effect, tumor tissues were collected and sliced for hematoxylin and eosin (H&E) staining (Figure 6.19c). It was found that the tumor cells of mice treated with CuFeSe-PEG injection were severely damaged when compared with the control group. The *in vivo* therapeutic efficacy of CuFeSe-PEG induced PTT was then investigated. Tumor-bearing nude mice were randomly divided into five groups (n = 6, each group). For the treatment group (CuFeSe+Laser group), mice bearing 4T1 tumors were intravenously injected with CuFeSe-PEG (dose = 7 mg/kg) for 8 h circulation and then irradiated using an 808 nm laser at a power density of 1.5 W·cm⁻² for 5 min. The other three control groups included: (1) mice irradiated with the NIR laser after injection of PBS (PBS+Laser group); (2) mice injected with CuFeSe-PEG NPs only (CuFeSe group); and (3) mice injected with PBS only (PBS group). The tumor sizes and appearance, and the body weights of the mice were measured every two days. The tumors in all three control groups exhibited a similar growth speed, indicating that neither CuFeSe-PEG injection nor laser irradiation of the tumors alone would affect the tumor growth (Figure 6.19d and Figure 6.20). In contrast, tumors in mice treated with both CuFeSe-PEG and laser irradiation were completely killed after the PTT treatment,
without tumor re-growth within 30 days (Figure 6.19e and Figure 6.20). Moreover, the body weights of mice with different treatments demonstrated no obvious change during our experiments, suggesting the biosafety of CuFeSe-PEG at the current dose (Figure 6.19f).

**Figure 6.20** Representative images of mice taken at different times post-treatment.

**Figure 6.21** Representative H&E stained images of major organs from mice treated with intravenously injected CuFeSe-PEG NPs (top row) and control mice (bottom row), taken 30 days after photothermal therapy. Yellow circles represent tumor metastasis regions.

To further confirm the successful treatment of tumors without metastasis, the major organs, including the heart, liver, spleen, lung, and kidney, were harvested at day 30 after treatment and stained with H&E. As displayed in Figure 6.21, there are obvious metastases and inflammatory lesions in the livers and lungs of mice (yellow circles regions) from the control group (mice treated with PBS, CuFeSe, or laser irradiation after administration of PBS). In contrast, no evident inflammation or damage is
observed in any of the major organs of the mice treated with laser irradiation after injection of CuFeSe-PEG NPs, confirming the great feasibility of photothermal therapy for ablation of tumors by using CuFeSe-PEG NPs as a therapeutic agent.

6.4 Conclusions

In summary, a facile ambient aqueous method was developed to prepare Fe-doped Cu$_{2-x}$Se NPs with tunable LSPRs. The intensity of the LSPR absorption exhibited Fe$^{3+}$-dependence, and the composition of the homogeneous alloyed NPs can be tuned simply, by varying the amount of the doped Fe$^{3+}$ ion precursor. PEGylated Fe-doped NPs have been proved to be the excellent contrast agents, not only for PA imaging due to their NIR LSPRs absorption, but more importantly the NPs were also used successfully for MRI. In addition, CuFeSe-PEG NPs can be used as an excellent therapeutic agent for in vivo PTT of tumors. Therefore, our functional metal ion doping approach developed here could be extended to the fabrication of other alloyed nanostructures, which would make it feasible to engineer multifunctional nanostructures.

6.5 References


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7.1 Conclusions

In this doctoral work, different fabrication routes are demonstrated for constructing three transition metal chalcogenides based molecular probes. The influence of different synthetic parameters on the quality of final probe is comprehensively investigated. In addition, their applications on biomedicine including bioimaging, photothermal therapy are evaluated. The general conclusions are given as following:

(1) A series of size-tunable, core-shell, novel watermelon-like fluorescent mSQS NPs were successfully synthesized. The shell thickness could be tuned from 0 to 32 nm by adjusting the amount of silica precursor, the solvent ratio, and the amount of catalyst. The solid silica shell coating significantly improved the chemical stability of the QDs inside. The fluorescence of CdTe QDs is strongly influenced by minor variation of the solution pH. Only 40% and 10% of fluorescence were retained when the pH of the CdTe QD solution was changed from 7 to 3 and 13, respectively. By contrast, the fluorescence of mSQS NPs is stable even in a strongly acidic solution (pH = 1) and no obviously aggregation was observed in the pH range of 1 to 13, owing to the protection of outer solid silica shell. Moreover, the resultant mSQS NPs investigated with the MTT assay, CLSM images, dynamic cell uptake and in vivo fluorescence imaging exhibit low toxicity, high stability, and strong fluorescence for bioimaging.

(2) The novel multimodal imaging-guided PTT nanotheranostic agent based on ultra-small PEGylated Cu$_{2-x}$Se NPs was successfully fabricated via a facile aqueous route. The Cu$_{2-x}$Se NPs possess a strong, significant NIR LSPR due to the high copper deficiency. The resultant NPs exhibit high stability and biocompatibility both in vitro and in vivo. More importantly, they show highly efficient photothermal conversion for PA imaging and PTT in vitro and in vivo, and large X-ray attenuation coefficients for CT imaging, as well as multifunctional groups for coordinating with radioisotopes for SPECT imaging. Both the in vitro and the in vivo results demonstrate that ultra-small PEGylated Cu$_{2-x}$Se NPs could serve as an ideal multifunctional platform for multimodal imaging-guided photothermal therapy of cancer.
(3) Based on the understanding of the above studies for Cu$_{2-x}$Se, we developed a facile and chelator-free doping method for constructing Fe-doped Cu$_{2-x}$Se nanoparticles. The position and intensity in near-infrared localized surface plasmon resonance in the alloyed nanostructure can be tuned by altering the feeding amount of the Fe$^{3+}$ ions precursors. Owing to their tunable near-infrared absorption and Fe doping, the hybrid nanostructure was demonstrated be a novel nanotheranostic agent for effective deep-tissue photoacoustic imaging, magnetic resonance imaging, and photothermal therapy of cancer.

7.2 Perspectives

In this thesis, we have discussed in detail the synthesis and application of transition metal based probe, especially for Cu$_{2-x}$Se NPs, for in vivo cancer imaging, PTT, image-guided therapy. Compared to other inorganic nanomaterials, such as gold nanoshells, gold nanorods, carbon nanotubes and CuS NPs, Cu$_{2-x}$Se NPs have not been studied extensively. However, the number of publications dealing with Cu$_{2-x}$Se NPs in biomedical applications is growing. Because of their multifunctional characteristics, Cu$_{2-x}$Se NPs are ideal materials for theranostic applications: they can serve as nuclear tracers or contrast agents for various imaging and diagnostic techniques; they can destroy cancer cells through photothermal effects; they also can release drugs in a controlled manner in response to an external stimulus. Significantly, all these features can be readily integrated in a single system to fulfill both diagnostic and therapeutic requirements.

Despite many positive results in vitro and in vivo, much more effort should be focused on the in vivo properties and disposition of copper based NPs, including their pharmacodynamics, pharmacokinetics, and potential long-term toxicity before appropriated imaging and therapeutic strategies are tested in cancer patients. With regard to the toxicology of transition metal based probe, the data available to date are limited and fragmentary; more in vivo experiments are needed for a comprehensive understanding and assessment of their toxicity. Moreover, it would be interesting and important to compare the different transition metal based probes side-by-side, to evaluate the advantages and limitations of each probe, as well as identify the most promising ones with high photothermal conversion efficiency, optimal in vivo pharmacology, and manageable long-term toxicity.
Appendix A: List of Publications


11. C. Han, Y. Bai, Q. Sun, S. H. Zhang, Z. Li,* L. Z. Wang,* S. X. Dou, Ambient Aqueous Growth of Cu₂Te Nanostructures with Excellent Electrocatalytic Activity toward Sulfide Redox Shuttles. **Advanced Science** 2016, 1500350. (IF: 6.00)


Appendix B: Conferences


2. 4th Symposium on Innovative Polymers for Controlled Delivery (SIPCD 2016), Suzhou, China, September 23-26, 2016, poster presentation.

3. 3rd International Symposium on Molecular Imaging and Nanomedicine (ISMIN), Suzhou, China, April 25-29, 2015.


Appendix C: Scholarships

1. International Postgraduate Tuition Award (IPTA), 2013-2016, University of Wollongong.


3. Student Top-Up Award, 2015, University of Wollongong.

4. AIIM HDR Student Conference and International Travel Grants, 2016, University of Wollongong.

5. ISEM 2016 Postgraduate Student Best Paper Award, University of Wollongong.