Multifunctional plasmonic nanostructures for biomedical application

Wang, Peng

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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**Multifunctional Plasmonic Nanostructures for
Biomedical Application**

WANG PENG

Interdisciplinary Graduate School

2017

Multifunctional Plasmonic Nanostructures for Biomedical Application

WANG PENG

Interdisciplinary Graduate School

Nanyang Environment and Water Research Institute

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List of Abbreviations

Au	Gold
PDA	Polydopamine
IO	Iron oxide
QDs	Quantum dots
IO/PS	Iron oxide@polystyrene
HRP	Horseradish peroxidase
TMB	3,3,5,5-Tetramethylcyclohexanone
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
NHS	N-hydroxysuccinimide
CTAB	Cetyltrimethylammonium bromide
PEG	Poly(ethylene glycol)
CM-PEG-SH	Carboxymethyl-PEG-Thiol
PI	Propidium iodide
TRIS	Tris(hydroxymethyl)aminomethane
PBS	Phosphate-buffered saline
CF	Chloroform
DMF	Dimethylformamide
GO	Graphene oxide
CCK-8	Cell Counting Kit-8
PCR	Polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
Van	Vancomycin
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
HRTEM	High-resolution transmission electron microscopy

XRD	X-ray Diffraction
UV	Ultraviolet
CT	Computed tomography
NIR	Near-infrared
NIR-II	Second near infrared window
LSPR	Localized surface plasmon resonance
SERS	Surface-enhanced Raman scattering
MRI	Magnetic resonance imaging
PAI	Photoacoustic imaging
OCT	Optical coherence tomography
OD	Optical density
MHT	Magnetic hyperthermia
PLASM	Plasmonic heating
PTT	Photothermal therapy
ROS	Reactive oxygen species
PDT	Photodynamic therapy
PMNCs	Polydopamine coated magnetic nanochains
GOx	Glucose oxidase enzyme
MFO	Manganese doped iron oxide
MNP	Magnetic nanoparticle
AuNP	Gold nanoparticle
AuNR	Gold nanorod
AuSF	Au snowflake
C-B	Core-Branched
H&E	Hematoxylin and eosin
ERP	Enhanced permeability and retention
HER2	Human epidermal growth factor receptor 2
EGFP	Epithelial growth factor receptor

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Summary

Multifunctional Plasmonic Nanostructures for Biomedical Application

Student: Peng Wang

Supervisor: Associate Professor Hongwei Duan

Co-supervisor: Associate Professor Teik-Thye Lim

Mentor: Associate Professor Rong Xu

The work in this thesis focuses on developing a new class of multifunctional Au nanomaterials with integrated plasmonic and magnetic properties, for synergistic biosensing, bioimaging and therapeutic applications. Taking advantage of a unique array of properties, such as localized surface plasmon resonance (LSPR), surface-enhanced Raman scattering (SERS), and enzyme-mimicking catalysis, I have explored their applications in optical sensing, imaging, and targeted therapy. My results have demonstrated that the structural integration of the plasmonic and magnetic components in these nanostructures complement each other in the biomedical applications, leading to excellent performance that cannot be achieved using individual building blocks.

First, we prepared glucose oxidase (GOx)-mimicking magnetic nanochains

(MNCs)-supported AuNPs, which can serve as self-mixing nanocatalysts, leading to label-free colorimetric detection of DNA in small volumes of specimens by combining mixing and magnetic separation. The MNCs were prepared by aligning iron oxide/polystyrene nanoparticles (IO/PS) together in the presence of a magnetic field, followed by chemical crosslinking by a rigid polymer coating, *i.e.*, polydopamine (PDA). The PDA can easily induce localized reduction of HAuCl_4 to prepare PMNCs@AuNPs with high-density of small and uniform AuNPs on the surface. These “naked” AuNPs on the surface of PMNCs exhibit GOx-mimicking properties and can be deactivated upon molecule anchoring *via* gold-sulphur chemistry. Built upon these “on-off” enzyme-mimicking properties, I developed nanosensors that allow for sensitive detection of virus DNA sequences down to 10 nM.

Second, plasmonic gold snowflake (AuSF) nanoparticles containing multiple branches, which exhibit broad LSPR and high photothermal conversion efficiency, have been prepared for SERS detection and photothermal killing of *S. aureus* and *E. coli* O157:H7. AuSF nanoparticles were prepared by seeded-growth in hexane-water phases. Because the reducing agent in water phase is separated from Au precursors in hexane, the slow reduction of Au precursors on the hexane-water interface can hinder the self-nucleation of Au ions. Therefore, the growth of Au branches only occurred on the surface of Au seeds to form AuSF nanoparticles. The strong plasmonic coupling between neighboring Au branches leads a broad LSPR, as well as the high

electromagnetic fields, which provides the possibilities to achieve a high SERS signal amplification and photothermal conversion efficiency. Combined with aforementioned immunomagnetic nanochains, a convenient theranostic strategy was developed for SERS detection and NIR-II laser-active photothermal killing of bacteria.

Finally, we produced magnetic core@gold branches magnetoplasmonic nanoparticles (C-B NPs) with small size (40 nm), by an interfacial seeded growth on Janus Au-MFO nanoparticles, and explored their properties for in vivo multifunctional diagnostic and second near-infrared (NIR-II) laser-based photothermal therapeutic applications. The Au branches of C-B NPs were grown from the Au part of Janus seeds to cover the MFO part, forming a unique core-branches nanostructure to protect the magnetic cores from separation. The inter-branches plasmonic coupling leads to broad LSPR peak from visible to NIR-II region (600 to 1350 nm), resulting in SERS and PTT response across a broad spectral range. In addition, the superparamagnetic parts of these C-B NPs serve as T_2 MRI contrast agents. As proved by in vivo mice model, the magnetoplasmonic C-B NPs served as a theranostic platform that integrated MRI for clinical diagnostic, SERS for tumor margin detection and NIR-II PTT for tumor ablation.

Chapter 1 Introduction

1.1 Background

1.1.1 Threat of Clinical Pathogens

Infectious bacteria and viruses remain to be leading causes of illness and death. Pathogenic bacteria in food, water or pharmaceutical products have severe effects on human health even if the amount of consumption is minimal. Harmful pathogens can cause different infectious diseases for humans and animals, such as antibiotic-associated diarrhea, pneumonia, and foodborne diseases (Table 1.1).¹ Therefore, there is a necessity to detect and separate pathogenic bacteria to reduce the bacterial infectious diseases. An effective method to detect infectious pathogens needs to be highly sensitive and rapid, because even little amount of bacteria in food or water would give rise to high fatality rates for human.

Table 1.1 Pathogens, corresponding diseases, toxins, and infection sources.

Bacteria	Disease	Toxin	Infection sources
<i>B. anthracis</i>	Anthrax	Edema factor	Milk or meat
<i>B. melitensis</i>	Brucellosis		Milk or meat
<i>C. jejuni</i>	Diarrhea dysentery		Dairy products, meats, mushrooms
<i>C. botulinum</i>	Botulism	Neurotoxin	Food

<i>C. diphtheriae</i>	Diphtheria	Diphtheria toxin	
<i>E. coli</i>	Gastroenteritis	Enterotoxin	Meats, fish, milk, rice, vegetables
<i>M. tuberculosis</i>	Tuberculosis		
<i>S. paratyphi</i>	Paratyphoid		Fecal contamination, eggs, milk, meats
<i>S. typhi</i>	Typhoid fever		
<i>S. dysenteriae</i>	Bacillary dysentery	Neurotoxin	Fecal contamination
<i>S. aureus</i>	Pneumonia	Enterotoxin	Human carriers
<i>S. pneumoniae</i>	Pneumococcal pneumonia	Erythrogenic toxin	Human carriers
<i>T. pallidum</i>	Syphilis		Infected exudate or blood
<i>V. cholerae</i>	Cholera	Enterotoxin	Fecal contamination
<i>Y. pestis</i>	Bubonic plague	Plague toxin	

The traditional approaches for pathogen detection are time-consuming (up to 72 h), expensive, and it also requires professional training and sufficient experience for the operation (Figure 1.1).² For example, polymerase chain reaction (PCR) is an approach to replicate the nucleic acid for pathogen detection. Although expensive reagents and disposable materials could be used to reduce the risk of contamination, potential false would happen even from a small amount of contamination. Besides, PCR is also not effective when

analyzing large amount of samples.³ Immunological assay like enzyme-linked immunosorbent assay (ELISA) is a simpler and faster method for pathogenic bacteria detection, which is used for the recognition specificity of antibodies and identification of color change of bacteria. Yet, immunological assays suffer from the requirement of many labor-intensive manipulations and hefty fabrication cost of monoclonal antibodies.

Figure 1.1 A flowchart of the processing steps involved and the time taken in detecting a pathogen.

1.1.2 Threat of Cancer

Cancer is a collection of diseases involving the growth of abnormal cell and their diffusion into surrounding tissues. According to the newest report of ministry of health Singapore in 2015, cancer is the major cause of death in Singapore. About 64000 incident cancer cases had been diagnosed during the

period 2011-2015. The colorectal, lung, and prostate were the major cancers for male, and breast, colorectal, and lung were the most common cancers among female population. In USA, according to the cancer statistics in 2016,⁴ there are approximately 4,600 newly-reported cancer patients per day, and lead to about 60,000 deaths annually, which is the second most common causes of death in the USA. Consequently, an annual cost for the treatment of cancer is more than 1 trillion US dollars.

By far, chemotherapy, radiotherapy, and surgery are the most common treatments for cancer patients. Among them, chemotherapy is a cancer therapy method which uses anti-cancer drugs to damage or stress cancer cells resulting in the death of treated cell. However, chemotherapy suffers from the side effects that the anti-cancer drugs cause toxicity to either cancer cells or many normal cells. The most common side effects are alopecia (hair loss), myelosuppression, muscle or stomach pain, nerve or heart damage, and fertility problems. Moreover, the radiotherapy, which uses ionizing radiation to damage the DNA of cancerous tissue, is always synergistic with chemotherapy. In this case, the cancer cells can be killed by high-energy X-rays. However, the normal cells and tissues close to the treatment areas can also be damaged by radiotherapy, which gives rise to side effects, such as skin problems and fatigue. Apart from the chemotherapy and radiotherapy, tumor surgery is the most effective and oldest treatment for the removal of the tumor. However, it is quite hard to completely remove the entire tumor, and the residual tumor cells would be a potential threat

for health, moreover, the attempt to cut more affected tissue may also cause damage to body. Utilization of surgery, chemotherapy, and radiation therapy is to kill the tumor cells though the accompanying side effects mentioned above greatly hold back their applications.

1.1.3 Gold Nanomaterials for Biomedicine

Recently, Au nanomaterials have emerged as one kind of the most promising nanomaterials for biomedical applications.⁵ AuNPs are able to realize the LSPR, which means that the free conduction electrons on the surface of AuNPs would oscillate collectively by applying a specific wavelength of the incident light (Figure 1.2). The strong electric fields, which are generated on the surface of AuNPs, can be used to enhance the optical signals. In addition, the oscillation will decay by absorption and scattering of light, resulting in an enhancement of LSPR wavelength and the corresponding characteristic peaks for different structures of AuNPs. The light scattering of AuNPs is of great value in optical imaging, and the light absorption can be converted to heat for PTT treatment. The LSPR peak of AuNPs can be easily adjusted from visible to NIR region to achieve maximum depth of penetration into bodies and tissues due to the low absorption and scattering from water and blood.⁶

Figure 1.2 Schematic of the LSPR on AuNPs. Copyright 2016 American Chemical Society.

The LSPR peak of Au nanomaterials are determined by the size, shape, and structure.⁵ According to previous reports,^{5,7} tuning the morphologies of Au nanomaterials is more efficient than the size for adjusting the LSPR peak to NIR region. For example, AuNRs with different aspect ratio and Au nanoshell with different thickness have been reported,^{8,9} leading to large shifts in the LSPR peak. In addition, other Au nanomaterials, such as boxes,¹⁰ cages,⁷ or frames hollow structure,¹¹ plates, and polyhedral,¹² also have been studied to show the LSPR peak in NIR region. In addition to the optical properties, Au nanomaterials also have many other advantages for biomedical applications, including (I) facile combination of two or more imaging and therapeutic properties in a single nanostructure, (II) large surface area for drug loading and chemical conjugation, (III) specific targeting for drug delivery and imaging by surface modification, and (IV) enhancement of circulation time in the blood by optimizing size and surface area of nanomaterials.

Figure 1.3 Summary of some biomedical applications enabled by different types of Au nanomaterials. Copyright 2015 American Chemical Society.

In recent years, hybrid nanomaterials with plasmonic, magnetic, and fluorescent signatures have been widely investigated and that make these nanomaterials an attractive platform for biomedical applications.^{5, 13, 14} Multifunctional magnetoplasmonic nanomaterials, which combine both magnetic and plasmonic properties, have demonstrated a synergic efficiency in bioimaging and therapy.¹⁵ Magnetic NPs can be directed to the targeting site by applying an external magnetic field. By using a high-frequency alternating magnetic field, the fluctuations of the magnetic NPs can convert electromagnetic energy into heat. Besides, magnetic NPs can also be taken as contrast enhancing agents for magnetic resonance imaging (MRI). In cooperation with plasmonic NPs, the magnetoplasmonic NPs have shown various properties, such as magnetic enrichment, multi-imaging, and multiple therapeutic modalities. Therefore, the design and synthesis of composite

magnetoplasmonic NPs have been widely investigated, which have been summarized in the next chapter.

Figure 1.4 (a) Schematic scheme of the preparation of IO@Au NPs. TEM images of (b) bacteria incubated with the Van-IO@Au NPs. Pictures of (c) Van-IO@Au NPs and (d) IO@Au NPs incubated with pathogens, and followed by magnetic separation. Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA.

Magnetoplasmonic nanomaterials have been studied for bacteria or cancer cell diagnostic applications. For example, IO@SiO₂@Au NPs (IO@Au NPs)

were synthesized by coating of SiO₂ on IONPs following by growth of a layer of Au shell (Figure 1.4a).¹⁶ The vancomycin-coated IO@Au (van-IO@Au) NPs showed highly specific targeting to eight pathogens, such as *E. coli* O157:H7 and *S. saprophyticus* (Figure 1.4b). These pathogens can rapidly aggregate with van-IO@Au NPs and quickly undergo magnetic separation within 15 min (Figure 1.4c). After that by irradiating with an 808 nm laser, pathogenic bacteria can be selected and killed by the photothermal properties of the Au shells. Moreover, magnetoplasmonic NPs were synthesized and applied also for photothermia combined with magnetic hyperthermia (MHT) for cancer cell therapy (Figure 1.5).¹⁷ The combined heating showed a synergic therapeutic efficiency on tumor cells for in vivo animal model.

Figure 1.5 (a) The synthesis of magnetoplasmonic NPs. The nanomaterials are injected into the tumour (T+), (b) photothermal imaging under MHT alone, PTT alone, and MHT + PTT. (c) temperature elevation of the injected (T+) and

non-injected tumors (T-) as a function of time. Copyright 2015 The Royal Society of Chemistry.

1.1.4 Current Challenges

Recently, the vast researches about multifunctional Au nanomaterials have proved their unique features for biomedical applications. There is a significant progress in understanding the mechanisms and controlling the synthesis of Au nanomaterials. Now, scientists continue to push this topic to a new height, where Au nanomaterials with various controllable properties ranging from SERS, photothermal conversion, and catalysis, can be explored. However, some existing challenges must be addressed in order to open up new prospects in the field of bio-applications.

Firstly, some Au nanomaterials are complicated in design and preparation. Multifunctional applications of Au nanomaterials may complicate their synthesis, and uses of active targeting ligands will further add the cost and time for their production and commercialization. So, when it comes to a new Au nanostructure system, researchers may need to seek for a “simple” approach in design. Secondly, Au nanomaterials with LSPR in NIR-II window which is more safe than NIR-I windows for practical applications have rarely been investigated. Several reported NIR-II laser responsive Au nanomaterials are unsuitable for biomedical application because of the big size or high laser power required. Therefore, Au nanomaterials with a high absorption in NIR-II window should be well designed and prepared for clinical applications. Thirdly,

it is a challenge to compact sufficient multifunctionalities into small nanoparticles. The high surface energy of the nanoparticles makes it difficult to control the morphologies and functional properties for in vivo applications. Therefore, effective method is needed for assembling ultrasmall nanomaterials with diverse properties for in vivo biomedical applications.

1.2 Objectives

The overall objective of this thesis is to prepare well-defined Au nanomaterials with multifunctionalities in simple preparation and design for bioapplications, such as DNA detection, SERS detection of bacteria, in vivo cancer imaging, and other biomedical applications.

Firstly, the seed-growth method is used for synthesizing Au nanostructures. It is easy to control the morphologies and properties of Au nanomaterials by adjusting the ratio between Au precursors and seeds. Based on this method, we prepare well-defined PMNCs-supported Au nanomaterials containing both magnetic and plasmonic properties. Here, the dopamine undergoes self-polymerization to form a PDA layer for constructing the MNCs. The PDA can induce localized reduction of Au precursors, leading to easy loading of AuNPs on the surface of PMNCs. Besides, PDA can bridge the AuNPs and MNCs to exhibit combined and improved performance compared to their individual counterpart. The key finding is that these PMNCs-supported AuNPs can self-mix, easily be magnetically separated, and show GOx-mimicking

catalytic activity which can be switched from “active” to “inactive” upon treatment with single-stranded DNA (ssDNA) in solution.

The second objective is to synthesize multifunctional Au nanomaterials with LSPR in NIR-II window. In order to enhance the optical density in NIR-II range, the seed-growth in two-phase system is used for growing long and closely-packed Au branches on Au seeds. For the two-phase system, the reduction of precursors is typically much slower than that in one-phase system, thus leading to slow growth of Au branches on seeds to gain the narrow distribution of nanomaterials. More important, the strong plasmonic coupling of Au branches lead to a broad absorbance in both NIR-I and NIR-II region.

Furthermore, in order to compact sufficient properties into small nanomaterials, the magnetoplasmonic NPs with unique core@branches (C-B NPs) nanostructure have been prepared *via* Janus Au-MFO seed-growth method in two-phase system, integrating both magnetic and plasmonic properties into nanomaterials smaller than 50 nm. The C-B NPs show high NIR-II region absorbance by the inter-coupling of the plasmonic Au branches, which is benefit for NIR-II laser-active PTT of tumor. The small size of the C-B NPs may permeate barriers more rapidly, leading to high uptake by tumor based on EPR effect. Taking advantages of the integrated magnetic core and Au branches, these C-B NPs can provide a theranostic platform to combine multi-imaging and therapy for in vivo tumor resection.

Chapter 2 Review of the Literature

2.1 Synthesis of Gold Nanomaterials

Au nanomaterials are typically prepared by reducing HAuCl_4 to Au (0) atoms, and the Au (0) atoms are precipitated out to act as nuclei for the growth of particles with different structures depending on the reaction condition.⁶ In order to prevent the aggregation of AuNPs, capping ligands are usually added to bind on the Au surface to keep particles separated from each other.¹⁸ The properties of Au nanomaterials are related to their sizes, shapes, and surface properties.⁵ For biomedical applications, Au nanomaterials with LSPR peak in NIR region have been studied for the effective penetration depth in tissues.

2.1.1 Synthesis of Gold Nanomaterials

In 1951, Turkevich and co-workers reported the reduction of HAuCl_4 by sodium citrate in an aqueous solution for the first time,¹⁹ and different sizes of AuNPs were prepared by Turkevich method (Figure 2.1a).²⁰ In this case, it is easy to control the size of AuNPs by adjusting the ratio of HAuCl_4 and sodium citrate. Apart from the AuNPs, Au nanorods (AuNRs) with an anisotropic shape have also attracted numerous investigations in the past decade.^{8, 21} Seed-mediated growth which is a simple synthetic pathway has been widely used for preparing AuNRs (Figure 2.1b).²² Typically, in the absence of Ag^+ , CTAB was chosen as ionic surfactant for specific binding with Au (100) crystal

facets on Au seeds.²³ The newly formed Au (0) atoms were preferentially grown on the Au (111) crystal facets to form AuNRs along the (100) direction. On the other hand, Ag^+ ion can be reduced to Ag atoms on the Au (111) facets, and show strong interaction with Br ions of CTAB.²⁴ As a result, AuNRs can only grow along the (110) direction. Anisotropic Au nanomaterials with branches, arms, stars, flowers, or octopus morphology were widely investigated because of the significantly tunable optical properties (Figure 2.1c-e).²⁵⁻²⁸ The synthetic mechanism of these nanostructures are as following (i) growth on multiple sites on a seed to generate anisotropic branches or (ii) aggregation of many self-nucleated Au seeds followed by Au growth.²⁹ Although many protocols of preparing branches of Au nanomaterials have been reported, it is still a challenge to control the number, shape and length of arms.

Au nanomaterials with hollow core and porous shells, named nanocages or nanoframes, were developed for encapsulation and delivery of drug (Figure 2.1f).^{7, 10, 11} In 2002, Xia and co-workers first reported the synthesis of hollow metal nanostructures by galvanic replacement reaction.³⁰ Au nanocages or nanoboxes with the size ranging from 20 to 200 nm have also been prepared by changing the size of templates.³¹⁻³³ The Au nanoshell is typically synthesized by coating a layer of Au on various cores, such as silica, polymers, IONPs, and QDs.^{34, 35} These hybrid nanomaterials can serve as multi-functional agents because of their LSPR features, photoluminescence or magnetic properties.

Figure 2.1 TEM images of (a) AuNPs, (b) AuNRs, (c) Au nanobranched, (d) Au nanoflowers, (e) Au octopus, and (f) Au nanocages. (a) Copyright 2007 American Chemical Society. (b) Copyright 2012 American Chemical Society. (c) Copyright 2014 American Chemical Society. (d) Copyright 2008 American Chemical Society. (e) Copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA. (f) Copyright 2013 American Chemical Society.

2.1.2 Synthesis of Magnetoplasmonic Nanomaterials

Recently, synthesis of multi-metal NPs containing the unique plasmonic, magnetic, fluorescence properties within a single system has been a hot research area.³⁶ Many metals, such Fe,³⁷⁻³⁹ Ag,^{40, 41} Pt,⁴² and QDs,⁴¹ were linked to or alloyed with Au to form dumbbell shape, core-shell, or nanorod shapes nanostructures. To date, the combination of plasmonic and magnetic properties has been mostly investigated. MNPs which show superparamagnetic phenomenon can be manipulated by external magnet. This superparamagnetic

phenomenon prevents the MNPs from aggregation and well-dispersed in solution. In general, integrating AuNPs and MNPs in one system endows it a great promise for a wide range of research and applications.

The most common measure of preparing magnetoplasmonic Au nanomaterials is the sequential growth of Au or magnetic parts on the seeds.⁴³⁻⁴⁶ Two key points for successful synthesis of magnetoplasmonic nanomaterials are well match of lattice spacing and avoiding the homogeneous nucleation to form separate NPs. Sun and co-workers reported the growth of 4 to 20 nm IONPs on the 2 to 8 nm Au seeds by the decomposition of $\text{Fe}(\text{CO})_5$ (Figure 2.2a-b).³⁹ Because the crystal lattices of these two NPs are almost the same, the dumbbell-like Au-IO NPs were formed through epitaxial growth. Furthermore, Au-IO NPs have been reported for preparing IO-Au-IO NPs by heating in the presence of sulphur (Figure 2.2c).⁴⁷ The AuNPs were fused in high temperature and then connected by sulfur. In addition, magnetoplasmonic NPs have been reported by seed-growth of Au shells on the MNPs.^{48, 49} The surfaces of MNPs were first functionalized with amine terminated ligands, and Au ions are possible to be localized around the MNPs by electrostatic attraction. The Au shells can be formed on the surface of MNPs by reducing agents. According to this method, many groups reported hybrids nanostructures which used IONPs as a core and Au star,⁴⁸ popcorn,⁵⁰ or nanorose⁴⁴ as a shell.

Figure 2.2 TEM images of (a) 3-14 nm, (b) 8-14 nm Au-IO NPs, and (c) IO-Au-IO NPs. (a) and (b) Copyright 2005 American Chemical Society. (c) Copyright 2006 American Chemical Society.

Magnetoplasmonic nanomaterials were also developed by attaching MNPs and AuNPs into a nanostructure by silica or polymers. By this approach, it is simple to vary the contribution of each function. For example, Kotov group reported an $\text{IO@SiO}_2\text{@AuNPs}$ core-shell nanomaterial.⁵¹ The silica-coated IONPs (130 nm) were first capped by amino groups, following by electrostatic adsorption of AuNPs in an acidic aqueous solution. These $\text{IO@SiO}_2\text{@AuNPs}$ can be used for drug loading and apoptosis of cancer cells. In our group, we have prepared a core-shell magnetic/plasmonic nanomaterials with IO/PS as core, PDA as a layer, and Au shell on the surface of PDA.⁵² The PDA can serve as a controllable nanogap and a redox-active agent to promote the Au shell growth. The Raman dye-labeled magnetic/plasmonic nanomaterials show strong SERS signal, and have been successfully applied for SERS detection and efficient photothermal killing of *E. coli* O157:H7.

2.2 Properties of Gold Nanomaterials

2.2.1 Localized Surface Plasmon Resonance

The Basics

The LSPR of AuNPs represents a collective oscillation of free electrons when irradiated by light. For a spherical structure, the calculation model is generally referred to the Mie theory.⁶ In this theory, the extinction of light can be distinguished with scattering and absorption. The absorption of light by AuNPs associated with the conversion of photons to heat, leading to a photothermal effect. Besides, the scattering is a form of radiation without changing the frequency of light. From the Mie theory, the intensities of both absorption and scattering increase with increasing sizes of AuNPs, and the scattering is increased faster than absorption.⁵³ The scattering of AuNPs are stronger than organic dyes by 5 orders, which can be detected by confocal microscopy and dark-field microscopy. However, there are some limitations of the application of Mie theory, for example, only AuNPs smaller than 40 nm can be used.⁵⁴

Figure 2.3 The experimental LSPR of (a) AuNPs with different diameters, (b) AuNRs with different aspect ratios, (c) Au nanostars with different ratio of Au salt concentration to Au seed concentration, and (d) Au nanocages with different volumes of HAuCl_4 solution. (a) Copyright 2015 American Chemical Society. (b) Copyright 2006 American Chemical Society. (c) Copyright 2010 American Chemical Society. (d) Copyright 2007 Nature Publishing Group.

Tuning LSPR into the NIR

In general, turning LSPR peak of Au nanomaterials into NIR region is required for an enhancement of light transparency for biomedical applications.⁵⁵ For sphere-shape AuNPs with sizes ranging from 5 and 70 nm of AuNPs, they only show a red-shift from 520 to 530 nm (Figure 2.3a).⁶ By synthesizing specific morphology of Au nanomaterials, the LSPR peak can shift from UV to NIR region, such as AuNRs,⁵⁶ Au nanostar,²⁹ Au nanocages,⁵⁷ and so on (Figure

2.3b-d).⁵⁸ For AuNRs, the electrons can oscillate along two directions parallel to the short or the long axes of AuNRs. Thus, the LSPR of AuNRs have both transverse and longitudinal mode, and show two peaks. The longitudinal peak can be easily tuned into the NIR region by increasing the aspect ratio (ξ), because the LSPR peak show a relationship with aspect ratio according to an equation reported by EI-Sayed: $\lambda_{\max}=95\xi+420$.⁵⁹ Another method to tune the LSPR peak of Au nanomaterials into NIR region is based on the synthesis of Au nanostar, nanocages, and nanoshells. Halas group reported that the LSPR peak of Au nanoshells can change from 700 to 1000 nm by tuning the thickness of shell from 20 to 5 nm.⁶⁰ The LSPR peak of Au nanomaterials in NIR region is beneficial to the PAI and PPT by NIR light irradiation.

Tuning LSPR into the NIR-II

The autofluorescence in biological tissue may cause high noise and low detection sensitivity in the NIR-I regions below 900 nm. The signal-to-noise ratio are greatly improved in the NIR-II window (1000-1400 nm) because a weak autofluorescence of tissue. The Au nanostructures, which show strong LSPR peak at NIR-II window, will be a pivotal requirement for biomedical imaging and therapy. Liz-Marzan group reported a seed-growth of AuNRs in the presence of iodide ions which show LSPR peak at about 1100 nm.⁶¹ The aspect ratio of the AuNRs can be adjusted to 8, and the longitudinal LSPR of AuNRs can exceed 1000 nm. Au nanostar is one of the widely investigated Au

nanostructures for high SERS properties. Tuan group reported a surfactant-free method to prepare biocompatible Au nanostars.⁶² By changing the Ag^+ ion concentration, the branches of Au nanostars showed different aspect ratio, and the products with the densest branches showed a broad LSPR absorbance from 600 to 1200 nm.

2.2.2 Surface-Enhanced Raman Scattering

SERS is an optical phenomenon that the Raman scattering can be enhanced by more than 9 orders in magnitude by molecules deposited on plasmonic metal surface.⁶³ The Raman shift, which shows an inelastic light scattering indicating an energy transfer between the incident photons and chemical bonds in the molecules, was first reported by C. V. Raman in 1928.⁶⁴ The SERS of metal electrode was first reported by Fleischmann in 1974.⁶⁵ So far, Raman detection has been widely used in biomedical application, life sciences and analytical chemistry.⁶⁶ There are some advantages of Raman spectroscopy, such as narrow peak, high sensitive, and stability against photobleaching.^{67, 68} The mechanism of SERS can be divided into chemical and electromagnetic origins. For chemical mechanism, the enhancement is mainly ascribed to a charge transfer between Fermi energy of metal and lowest unoccupied molecular orbital or highest occupied molecular orbital of molecules.⁶⁹ However, the chemical enhancement of SERS is usually lower than 10^2 . The well-accepted mechanism for nanocrystals-based SERS is

electromagnetic enhancement. When nanocrystals are irradiated by light, the free electrons on the surface of nanomaterials show collective oscillations, giving rise to a local electric field enhancement by 10^2 to 10^4 .⁷⁰ Thus, the Raman scattering cross section shows a significant enhancement.

Au nanomaterials-based SERS have been widely studied, and the Raman enhancement efficiency is highly related to the size and shape of Au nanomaterials. As a trend, the SERS intensity is in direct proportion to the sizes of AuNPs because the intensity of the local electromagnetic field is proportional to the size of the nanostructures and the number of excited electrons.⁶⁸ For example, Chen group reported that the SERS signals of 2-naphthalenethiol on AuNPs are increasing with the NPs size.⁷¹ The shape and morphology of Au nanomaterials are another two pivotal parameters to enhance the SERS intensity. Researchers were inclined to synthesize Au nanomaterials with more tips or hot points to increase local electromagnetic fields. For example, Au nanostars with more sharp apexes and tips show much high SERS enhancement.⁷² The coupling of adjacent AuNPs also shows very high electromagnetic field, leading to strong SERS activity. Our group has successfully made Raman dyes labeled vesicles of AuNPs or AuNRs which displayed much higher SERS signals than that of single AuNPs or AuNRs, respectively.^{73, 74} The SERS enhancement efficiency of assembly amphiphilic AuNPs was about 7.2×10^4 , and the AuNRs-based vesicles showed the enhancement factor of 5.7×10^6 .

2.2.3 Enzyme-Mimicking Catalysis

Artificial enzymes are low-cost and highly stable alternatives to natural enzymes. Many materials, such as crystals, proteins, polymers, and nucleic acids, have been utilized as artificial enzymes.⁷⁵ Recently, considerable research has been devoted to utilize AuNPs for enzyme-mimicking activity as well as their further biological applications.

Enzyme-Mimicking Catalysis

Beside the optical properties, Au nanomaterials also show potential candidates for enzyme-mimicking properties. Recently, citrate-coated AuNPs have been reported for exhibiting glucose oxidase (GO_x)-mimicking catalyst to oxidize glucose in the presence of oxygen.⁷⁶ The mechanism is proposed by Rossi group where the glucose was attracted by AuNPs and then reacted with oxygen to generate gluconic acid and H₂O₂.^{77, 78} However, other metal nanomaterials, such as Ag and Pd, do not exhibit oxidase mimicking activity. The GO_x-mimicking catalytic activity of AuNPs follows Michaelis-Menten kinetics, and the Michaelis-Menten constants are higher than GO_x. In addition, as reported by Fan group, AuNPs also exhibited a higher tolerance to pH and a better thermal stability than that of natural enzyme. Inspired by this phenomenon, GO_x-catalytic AuNPs-based colorimetric assay has been developed for DNA detection (Figure 2.4).^{76, 79} When AuNPs are covered by ssDNA, the GO_x-mimicking catalytic activity of AuNPs will be inhibited.

However, if ssDNA hybrids are conjugated with complementary DNA to form dsDNA, the dsDNA binds weakly to the AuNPs which gives “naked” AuNPs. As a result, the GO_x-mimicking catalytic activity of AuNPs is “switched on” and active for colorimetric test or self-growth of AuNPs. Therefore, the different affinities of ss and dsDNA with AuNPs can be used for detection of targeted DNA.

Figure 2.4 Scheme of the GO_x-mimicking catalytic activity of AuNPs for DNA detection, which can be either amplified by colorimetric assay or chemiluminescence variations. Copyright 2011 Wiley-VCH Verlag GmbH & Co. KGaA.

AuNPs with negative or positive surface charges have been developed for peroxidase-mimicking catalysis. The surface modification of AuNPs is important for changing the affinities between the AuNPs and substrates. As reported, AuNPs show peroxidase mimicking activity when coated with CTAB or BSA, which can catalyze H₂O₂ into HO⁻ and O₂⁻/HO₂. Qu group reported that graphene oxide-supported AuNPs (GO-AuNPs) exhibited

peroxidase-mimicking activity.⁸⁰ These nanomaterials show high stability and catalytic activity in harsh solutions. After modification by folic acid, the GO-AuNPs were incubated with MCF-7 or NIH-3T3 cells for 1.5 h and subsequently centrifuged. As a contrast, the folic acid-coated GO-AuNPs can be centrifuged with MCF-7 cells together because that exhibited strong binding to cancer cells. After removal of unattached GO-AuNPs, the precipitate catalyzed a colorimetric reaction in the presence of TMB and HRP, which could be monitored by the appearance of the blue color.

Combined Enzyme-Mimicking Catalysis

Recently, researchers have developed some rational approaches to prepare hybrid Au nanomaterials, which may possess the advantages of dual enzyme-mimicking activity and high stability. AuNPs have been conjugated with many materials, such as graphene, silica, and magnetic nanomaterials, for improving their catalytic activities with advantages of high stability, tenability in catalytic properties, and synthesis in low cost. For example, a mesoporous silica-supported AuNPs have been prepared to show both oxidase and peroxidase catalysis.⁸¹ The AuNPs on the surface of silica are very small (5 nm), which show high enzyme-mimicking activity. More important, the mesoporous silica can hinder the interaction between these AuNPs, resulting in a highly catalytic stable in different conditions. In addition, magnetoplasmonic nanomaterials, such as IO@Au@SiO₂, have been reported with both GOx and

peroxidase mimicking catalysis.⁸² The IO@Au@SiO₂ with a magnetic core can make the enzymatic nanomaterials to be easily recyclable (Figure 2.5). Furthermore, both the IONPs and AuNPs were encapsulated in SiO₂ shells to prevent the self-aggregation under harsh conditions. Besides, graphene sheets-supported “naked” AuNPs have been reported for exhibiting significant peroxidase-mimicking activity.⁸³ These hybrid nanomaterials show a synergetic effect, which is more active than graphene or AuNPs alone. In summary, by using a multifunctional support for such hybrid nanomaterials, an artificial enzyme could be made with high performance that is comparable with natural enzyme, which extends their applications areas.

Figure 2.5 Cascade enzymatic activity of IO@Au@SiO₂. Copyright 2013 Royal Society of Chemistry.

2.3 Gold Nanomaterials for Biomedical Applications

2.3.1 Optical Sensing

Au nanomaterials can be used for colorimetric detection of biomaterials

and metal ions by the change of LSPR, which is highly sensitive to ligands, local refractive index, and particles size.⁸⁴ Rothberg group reported colorimetric detection of DNA by naked eyes depending on the different absorption between AuNPs and ssDNA or dsDNA.⁸⁵ ssDNA can adsorb to the surface of citrate-coated AuNPs to improve the stability of AuNPs in high salt concentration. However, after the addition of complementary targeting ssDNA, the as-formed dsDNA is to be detached from AuNPs, and consequently AuNPs will be aggregated in the presence of high concentration salt, which is evidenced by an obvious color change. In addition, AuNPs can be modified by polymer, aptamer, or antibody through Au-sulfur bond for specific detection. Our group reported polymer-coated AuNPs for sensitive detection of Cd^{2+} (Figure 2.6).⁸⁶ The Cd^{2+} -chelating polymers-coated AuNPs were aggregated in the presence of Cd^{2+} , and the aqueous dispersion of AuNPs changed from red to purple following with the increase of ion concentration. The nanogap between the aggregated AuNPs generated hotspots, and the Raman signal of AuNPs showed a 90-fold enhancement.

Figure 2.6 Schematic illustration showing mechanisms for the colorimetric detection of (a) Cd^{2+} ions and (b) UV-vis spectra of Cd^{2+} -chelating

polymer-AuNPs aqueous solution upon addition of Cd^{2+} ions (inset: the aqueous solutions of AuNPs before and after Cd^{2+} addition). Copyright 2011 American Chemical Society.

2.3.2 Enzyme-Mimicking Activity for Biomedical Applications

AuNPs have been discovered for colorimetric assay of biomolecules based on their enzyme-mimicking activities which are sensitive to their surface modification. Taking the advantage of these properties, AuNPs can be used for sensitive, rapid and highly specific colorimetric detection of DNA or molecules.^{79, 87, 88} In this assay, the catalytic activity of AuNPs can be turned-off by blocking their surface via the adsorption of DNA or aptamer. However, desorption of DNA or aptamer from AuNPs would lead to turning-on of the catalytic activity. For example, Bansal developed a tyrosine-reduced AuNPs with peroxidase-mimicking activity for colorimetric detection of kanamycin.⁸⁹ In this assay, the AuNPs were first modified by aptamer which led to the blocking of the peroxidase activity. However, in the presence of kanamycin, the aptamer can specifically conjugate with kanamycin, thereby resulting in a “naked” AuNPs for oxidation of TMB. Furthermore, in order to apply it in complex biological systems, hybrid Au nanomaterials have been developed for avoiding the aggregation of AuNPs. Qu group reported a silica-supported AuNPs with dual enzyme-mimicking activities and high stability for antibacterial applications.⁸¹ The peroxidase-mimicking activity and oxidase-mimicking activity of nanomaterials can generate $\bullet\text{OH}$ and ROS,

respectively. As a result, the silica-supported AuNPs have exhibited excellent antibacterial properties against bacteria. In addition, graphene-supported AuNPs with peroxidase-mimicking activity over a broad pH range have been developed for cancer cell detection.⁸⁰ After modification with folic acid, these nanomaterials can be used for selective binding to the surface of cancer cells (Figure 2.7). In the presence of H_2O_2 and substrate, the graphene-supported AuNPs conjugated cancer cells can catalyze a color change reaction. Although substrate-supported AuNPs show superior catalytic properties over AuNPs alone, their potential applications are still limited by low catalytic activity and recycling problem. A suitable method of the preparation of recyclable enzyme-mimicking Au nanomaterials with high catalytic activity should be developed for application in harsh environment with ultralow concentration of targeted biomolecules.

Figure 2. 7 (a) Synthesis of folic acid-labeled hybrid AuNPs-graphene and (b) cancer cell colorimetric detection. Copy right 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.3.3 Bioimaging

The elastic scattering Au nanomaterials can be recorded by dark-field optical microscopy for optical imaging. The scattering of nanomaterials, such as AuNPs, AuNRs, Au nanoshells, Au vesicles, Au nanocages, show various optical properties depending on their shapes and sizes.⁹⁰ In our group, Au vesicles which were assembled from amphiphilic AuNPs showed red color under dark-field microscope.⁷³ The antibody-coated Au vesicles bound to the surface of cells, and obviously the distributed red color was shown on the targeting cells. We also reported a core-satellite plasmonic assembly AuNPs by i-motif DNA linkers with a yellow color under dark-field microscope.⁹¹ A pH change from 8.3 to 5.0 led to the disassembly of i-motif DNA linkers, as a result, core-satellite Au nanostructures were disassembled and the dark field images turned from yellow to green. These core-satellite plasmonic NPs can be used for bio-imaging of pH change in the endocytic pathway in live cells.

Raman dye-labeled Au nanomaterials which show higher sensitivity and shorter acquisition time can provide Raman mapping for biomedical detection. Our group reported that Raman dye-encoded core-shell Au nanogaps can be modified by aptamer, and then specific targeting with MCF-7 cells. Raman mapping at 1646 cm^{-1} showed obvious signal of the MCF-7 cells labelled with aptamer-labeled Au nanogaps.⁶³ Raman tags-encoded Au nanomaterials were also used for in vivo Raman imaging. Nie and co-workers reported that the

AuNPs were first modified by antibody and then injected intravenously into tail of mice.⁹² The antibody on the AuNPs can specifically target to the cancer cells, and the SERS imaging shows high accumulation of antibody-labeled AuNPs in tumor at 5 h postinjection. In addition, the characteristic peaks of different Raman dyes-labeled Au nanomaterials bring about multi-Raman imaging. Gambhir group reported diverse Raman dyes-encoded AuNPs with SiO₂ coating and PEGylation.⁹³ The Raman mapping in vivo showed distinguish results by their unique spectral fingerprints to image the accumulation of different NPs in the deep tissue (Figure 2.8). The ultrasensitive signals and spectral signals of different AuNPs can offer a multiple imaging simultaneously to improve disease detection efficiency.

Figure 2.8 (a) Schematic illustration of SERS dyes based on 60 nm AuNPs, whose surfaces were labeled with different Raman dyes, followed by coating with a SiO₂ shell. The name of each SERS NP is shown on the right. (b) SERS spectra of all 10 SERS tags. (c) Multiplexing of 10 different SERS dyes in vivo. Different colors are assigned to each SERS dyes.⁹³ Copyright 2009 National Academy of Sciences, U.S.A.

Multimodal Bioimaging

Optical imaging of cancer cells using a single method often does not provide satisfactory sensitivity and spatial resolution. As a solution, combined imaging techniques, which involves two or more types of properties simultaneously, have been developed to improve the imaging efficiency, such as a combination of Raman mapping, PAI, MRI, CT, or fluorescence imaging.^{5, 94} For example, Gambhir group reported that Raman dye labeled AuNPs@Si modified with Gd^{3+} ions can be used for tumor triple-imaging by MRI, PAI, and Raman modalities (Figure 2.9).⁹⁵ The Gd^{3+} ions can shorten the T_2 relaxation time of surrounding water protons, and can be used as T_2 negative contrast agents for MRI. The MRI which is a powerful noninvasive imaging method can be applied for preoperatively guide tumor resection, and PAI which can convert light into heat serve as a contrast agent overcomes the depth limits of imaging. Combined SERS with highly specific detection, these multifunctional magnetoplasmonic nanomaterials allow for accurate tumor imaging and resection. In addition, IO@Au stars with core@shell nanostructure have been developed for MR, CT, and PA imaging of tumor.⁴⁸ Besides MRI and PAI, the CT imaging provide high spatial and density resolution, yet, the limitation of CT for in vivo applications is the low contrast of CT imaging of soft tissue. The integration of these imaging modalities can overcome some restrictions encountered by each. Although multimodal nanomaterials have drawn a lot of attention owing to their synergetic features, a barrier that limit the clinical use

of combined imaging lies in the big size of nanomaterials. As we know, the size, surface modification, and morphologies of nanomaterials play a pivotal role in tumor targeting. Multifunctional nanomaterials with a suitable size smaller than the gap between the endothelial cells can show a high uptake by tumor cells. In addition, the small nanomaterials are more likely to be cleared from the blood and body to reduce the potential side effects after treatment. Therefore, it needs to integrate multifunctional imaging properties into one nanomaterial with a smaller hydrodynamic diameter or easily be biodegradable.

Figure 2.9 Triple-modality imaging of magnetoplasmonic nanomaterials. Copyright 2012 Nature Publishing Group.

2.3.4 Therapy

Antibacterial Applications

Au nanomaterials have also been widely used in antibacterial treatment. Vancomycin-capped AuNPs (AuNPs@Van) have been reported for their antibacterial properties.⁹⁶ The vancomycin can act as a polyvalent inhibitor to bacteria, and AuNPs@Van which maintains a stable shape and size in solution provides the inhibition against Gram-negative and vancomycin-resistant enterococci bacteria. In addition, Au nanomaterials can be used for killing sterilization by a strong photothermal effect.⁹⁷ Popcorn-shaped IO@Au NPs were reported for SERS detection and photothermal killing of bacteria.⁹⁷ These IO@Au NPs showed strong SERS signal and high photothermal conversion efficiency. After surface modification with antibody, NPs can selectively target to *Salmonella* DT104. Upon irradiation by 670 nm laser for 10 min, most bacteria showed irreparable cellular-damage. Bimetallic Au-metal nanomaterials have been reported as antibacterial agents for their uniquely synergistic properties. Jiang group reported Au-Pt bimetallic NPs with excellent antibacterial activity.⁹⁸ The Au-Pt NPs show no cytotoxicity for human cells, but they can kill the bacteria by a disruption of their cell membrane. The antibacterial activity of Au-Pt NPs probably originated from a rupture in the inner membrane that is associated with an increase of intracellular adenosine triphosphate levels.

Tumor Therapy

Au nanomaterials have also been developed as cancer therapeutic agents by the properties of PTT, PDT, and X-ray radiotherapy. Au nanomaterials can absorb electromagnetic energy and further convert it to heat through photothermal effect, leading to a rapid increase of temperature nearby the targeted sites. A high temperature within the range of 42 to 50 °C can damage cancer cells by denaturing of proteins or destruction of cell membranes in 10 min.⁹⁹ Compared with traditional PTT dyes, Au nanomaterials have some advantages, such as higher photothermal stability, better targeting through surface modification, higher photothermal conversion efficiency, and greater absorption cross section.¹⁰⁰ Based on these properties, Au nanomaterials have attracted wide investigations as a PTT agent. Different Au nanostructures, such as nanorods, nanoshells, nanostars, nanovesicles, and nanocages, have been reported for PTT by NIR laser irradiation.⁶ PEGylated Au nanoshells were reported by Halas group for in vitro and vivo models of photothermal tumor therapy (Figure 2.10).¹⁰¹ With the decoration of thiol-terminated PEG, Au nanoshells are able to achieve a longer circulation time in nude mouse due to the better stability. Likewise, PEG-decorated NPs also showed an enhanced tumor uptake because of an improved enhanced permeability and retention (EPR) effect.^{102, 103} Therefore, low power of lasers at desired site can lead to selective killing of tumors, and minimize the potential damage to healthy tissues. In order to achieve targeted killing of cancer cells, some groups

reported surface modification of Au nanomaterials by targeting ligands, such as antibody, peptides, and specific molecules. Drezek group performed that anti-HER2-conjugated AuNRs can be used for target PTT of breast carcinoma cells.¹⁰² Mostafa group reported that anti-EGFR-conjugated AuNPs showed photothermal malignant cells therapy.¹⁰⁴ In our group, anti-HER2-conjugated Au vesicles have been reported for SKBR-3 cells targeting and PTT.⁷³ In addition, the NIR-II laser based photothermal cancer therapy of Au nanomaterials also has been investigated. Yeh group reported an Au rod-in-shell NPs with absorbance covering both NIR-I and NIR-II windows.¹⁰⁵ The broad LSPR peak covers from 300 to 1350 nm of Au rod-in-shell NPs enabling the photothermal cancer therapy using both by NIR-I and NIR-II lasers. After an irradiation by 3 W/cm² of 1064 nm laser for 7 min, the NPs exhibited an effective anticancer efficiency for in vivo mice model.

Figure 2.10 (a) SEM image of nanomatrixoshkas and photograph of tumor-bearing mouse under photothermal treatment. (b) The mice injected with nanomatrixoshkas or nanoshells and treated with laser. Copyright 2014 American Chemical Society.

Synergetic Therapy

The PDT damages cancer cells by Au nanomaterials through the generation of singlet oxygen upon light irradiation, leading to a localized apoptosis and necrosis of cancer cell where photosensitizers are located.¹⁰⁶ The applications of traditional photosensitizers are limited by the poor hydrophilicity. Yet, PDT photosensitizers can conjugate with Au nanomaterials for in vivo applications. Huang group reported a silicon 2,3-naphthalocyanine dihydroxide-conjugated magnetoplasmonic nanostructures (IO-Au NPs) for PDT and PTT cancer therapy.¹⁰⁷ The photosensitizer was loaded and stabilized on the surface of IO-Au NPs by 11-mercaptopundecanoic acid-PEG. Because of the coincident absorption of photosensitizer and IO-Au NPs in NIR region, the photosensitizer labeled IO-Au NPs showed PDT and PTT for SKBR-3 cells by an 808 nm laser. Recently, Au nanomaterials have been reported for production of $^1\text{O}_2$ by themselves under irradiation by laser.¹⁰⁸ The mechanism is based on the energy transfers from the electrons at excited states of Au nanomaterials to molecular oxygen under excitation by light. The advantages of using Au nanomaterials as photosensitizers are their stability over traditional dyes and the elimination of side effects coming from organic photosensitizers. Hwang group reported that AuNRs,¹⁰⁹ Au nanoshells¹¹⁰ and Au nanoechinus¹¹¹ can be used as PDT agents. Au nanoechinus with strong absorbance in NIR-II region have been reported for NIR-II-laser active therapy.¹¹¹ In vivo experiments, the Au nanoechinus have been demonstrated excellent PDT and PTT agents for

deep-tumor treatments. Other types of Au nanomaterials, such as Au nanocages¹¹² and AuNPs¹⁰⁸, have also been used as photosensitizers for cancer therapy. At current stage, the synthesis of multifunctional Au nanomaterials shows a low uptake value in cancer cells, and the distribution of nanomaterials in tumor is not homogeneous. In the future, multifunctional Au nanomaterials with suitable design, such as surface modification for tumor targeting, good biodistribution, and low toxicity, are needed for improving the cancer therapeutic efficiency.

Chapter 3 Glucose Oxidase-Mimicking, Magnetic Nanochain-Supported Gold Nanoparticles for Colorimetric Biosensing

3.1 Introduction

Natural enzymes with high substrate specificity and catalytic efficiency at mild conditions play a pivotal role in biosensors, biocatalysis, and food industry. However, the limited stability, time-consuming preparation and purification, and associated high cost of enzymes remain major barriers for their broad applications,^{75, 113} creating a need for enzyme-mimicking alternatives. A diverse range of nanostructures of Au, cerium oxide,^{75, 114} IO,¹¹⁵ and carbon nanomaterials have demonstrated inherent enzyme-mimicking activities,⁷⁵ which show high tolerance to harsh environment such as high temperature. Among such nanomaterials, AuNPs-based artificial enzymes are widely studied because of the attractive optical and electronic properties of AuNPs, their low toxicity, and easy surface modification by functional groups through Au-S bonds reaction.⁷⁵ The GOx and peroxidase-mimicking activities of AuNPs have enabled the development of biosensors for DNA, microRNA, xanthine, glucose, and mercury ions detection.^{79, 87, 116, 117} However, such nanomaterials are often difficult to separate and recycle after use and their high surface energy often endow them with poor colloidal stability. In this respect, AuNPs-supported on colloidal/bulk substrates, which show dual enzyme activities and stability over

a broad pH range, have extended the application of enzyme mimicking systems in various biomedical fields.^{80, 82, 118-122} IO@small AuNPs supported on mesostructured SiO₂ showed both GOx and peroxidase mimicking catalytic activities and could be used for artificial cascade reaction.⁸² Mesoporous silica-supported AuNPs also possessed dual enzyme activities, and showed antibacterial properties.¹¹⁸ Graphene sheets-supported AuNPs showed a synergistic peroxidase mimicking activity which was better than graphene or AuNPs alone, and could be applied in DNA detection.⁸³ However, we believe that using a multifunctional support for such nanomaterials will further improve their performance and widen up their application areas much desired for high performance artificial enzymes.

One-dimensional chain-like nanomaterials (nanochains) have been largely investigated and applied in optoelectronics and nanomedicine.¹²³⁻¹²⁷ Among them, magnetic nanochains (MNCs) which were formed in presence of an external magnetic field and stabilized by polymer or silica, showed excellent applications in the field of nanomedicine, optoelectronics, separation and transport. These MNCs can undertake localized rotation when placed in a spinning magnetic field, making it possible for the MNCs to serve as nanostir bar to promote the samples mixing. For example, metal NPs-loaded magnetic nanochains are self-mixing, magnetically recyclable and efficiently used as nanocatalysts, whereas, the magnetic nanochains modified by aptamer can be used as nanomedicine for cancer therapy.¹²⁵ In addition, MNCs exhibited

efficient self-mixing in extremely small volume benefitting their use in low volume chemical or biological reaction or mixing solutions in tiny channels, especially in the field of lab-on-chip, microfluidics and microliter bioassay.¹²⁷ Therefore, a novel nanomaterial possessing the synergistic properties of MNCs and AuNPs will have great potential for extending the application of AuNPs-based artificial enzymes in small volume.

Here, we report GOx-mimicking MNCs-supported AuNPs which can self-mix in small reaction volume and easily be separated and recycled by using high saturation magnetization. Firstly, IO/PS-based MNCs were prepared by aligning them together in presence of an external magnetic field, and covalently linking the closely IO/PS NPs by self-polymerization of dopamine to form the PDA coated MNCs (PMNCs).¹²⁵ We further exploit the reducing ability offered by abundant catechol groups of PDA to integrate AuNPs on PMNCs.¹²⁸ The PMNCs reduce HAuCl₄ locally to prepare small and uniform AuNPs loaded on the surface of PMNCs (PMNCs@AuNPs). The naked AuNPs on the surface of PMNCs exhibit GOx-mimicking catalytic activity which can be controlled by tuning the surface modification of AuNPs. The catalytic activity remains switch-off when biomolecules conjugate with AuNPs via gold-sulphur chemistry passivating the surface of NPs and making it inaccessible for catalysis.⁷⁶ However, in absence of any inhibition-biomolecules, the surface remains free and GOx mimicking catalytic activity of AuNPs remains switch-on. Therefore, PMNCs@AuNPs are able to catalyze the oxidation of glucose in

presence of oxygen producing gluconate and hydrogen peroxide (H_2O_2), which are interrogated by HRP and TMB-based colorimetric assay.⁷⁶ Moreover, both PMNCs and PMNCs@AuNPs could readily undergo magnetic separation, hence easily recyclable, and also can self-mix under a spinning magnetic field. Therefore, employing these properties, we design a platform based on GOx-mimicking PMNCs@AuNPs biosensor for biomolecular recognition, magnetic enrichment, and label-free colorimetric detection in ultrasmall volume.

Figure 3.1 Schematic illustration of the DNA capture and separation based on PMNCs modified probe DNA and colorimetric detection by PMNCs@AuNPs. 1) Capturing the target DNA 2) hybridization with test sample containing target DNA 3) Mixing with inhibition DNA 4) Magnetic separation 5) PMNCs@AuNPs are added into residual free inhibition DNA solution 6) Colorimetric assay.

The label-free colorimetric detection of DNA based on catalytic magnetic nanochains, capture magnetic nanochains and inhibition DNA is illustrated in Figure 3.1. We first modify PMNCs with HS-ssDNA (probe DNA) to prepare capture PMNCs-a through covalent bonding via Michael addition and/or Schiff base reactions between quinone groups of PDA and thiol groups of probe DNA.¹²⁹ The capture PMNCs-a first self-mix and hybridize with target DNA to prepare the capture PMNCs-b. The over-hanging region of the target DNA is then recognized by the inhibition DNA. In presence of the target DNA, the inhibition DNA is hybridized to form PMNCs-c leaving almost no residual free inhibition DNA in the test sample. The PMNCs-c are then magnetically separated and PMNCs@AuNPs are added to the test sample and self-mixed for 1 hour. After magnetic separation and enrichment of PMNCs@AuNPs, TMB and HRP are added for colorimetric assay. If capture PMNCs-b successfully separate most of the inhibition DNA, the surface of PMNCs@AuNPs remain active for catalysis and produce blue color changes in relation to target amounts. Such color signals can easily and directly be traced with naked eye without the need of any analytical instruments. However, in absence of target DNA, the inhibition DNA cannot be recognized and separated, leading to the binding with PMNCs@AuNPs. As a result, all the inhibition DNA passivates the surface of AuNPs and almost completely blocks the GOx-mimicking activity of AuNPs on the PMNCs@AuNPs. Therefore, no color change is observed in final solution.

3.2 Experimental Section

3.2.1 Materials and Characterization

Materials. Dopamine, iron (II) chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), ammonium hydroxide, divinylbenzene (DVB), tetradecane, horseradish peroxidase, iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), potassium peroxydisulfate (KPS), octane, sodium dodecyl sulfate (SDS), styrene, oleic acid, 3,3',5,5'-tetramethylbenzidine (TMB), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and methoxy-poly(ethylene glycol)-thiol were purchased from Sigma-Aldrich. Hydrogen tetrachloroaurate(III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), and bovine serum albumin (BSA) were purchased from Alfa Aesar. Sodium chloride was purchased from Merck. DNA strands were purchased from Shanghai Sangon Biotechnology Incorporation.

Table 3.1 Sequence information of ssDNA used in this study

ssDNA	Sequence
Zaire Ebola target DNA	5'-ATT ACA TTA TGC TTT TAT AAC TTA CCT ACT AGC CTG CCC A -3'-CY5
Zaire Ebola probe DNA	5'-TT ATA AAA GCA TAA TGT AAT TTTT-3'-SH
Zaire Ebola inhibition DNA	SH-5'-TTTTT TGG GCA GGC TAG TAG GTA AG-3'-CY5
Hepatitis B virus	5'-TTC ATT TAC AGG AGG ACA TTA TTA ATA GAT GTC

target DNA	AAC AAT ATG TGG -3'-CY5
Hepatitis B virus probe DNA	5'-AA TGT CCT CCT GTA AAT GAA TTTT-3'-SH
Hepatitis B virus inhibition DNA	SH-5'-TTTTT CCA CAT ATT GTT GAC ATC TA-3'-CY5
Influenza A virus target DNA	5'-TTG AAG ACA ACG CCA CGC CCT CTC AGA TTA CCT GAT GGG CCT CCT-3'-CY5
Influenza A virus probe DNA	5'-GG GCG TGG CGT TGT CTT CAA-TTTTT-3'-SH
Influenza A virus inhibition DNA	SH-5'-TTTTT AGG AGG CCC ATC AGG TAA TC-3'-CY5
Mis-match DNA 1	5'-CCA GAA CGT TGT TTT CAG GA-3'-HEX200
Mis-match DNA 2	5'-CTT GTC CTT ACC AGA ACG TTG TTT TCA GGA-3'-HEX200
Mis-match DNA 3	5'-CAG CCC AAC CCT TGT CCT TAC CAG AAC GTT GTT TTC AGG A-3'-HEX200

Characterization. SEM images were tested on a FESEM (JSM-6700F, Japan). TEM observations were obtained by a Jeol JEM 2010 electron microscope at an acceleration voltage of 300 kV. UV-vis absorption spectra and fluorescence spectra were collected on a Shimadzu UV-2501 spectrophotometer

and a Fluoromax-3 spectrometer, respectively.

3.2.2 Synthesis of IO/PS NPs

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (2.4 g) and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.982 g) were firstly dissolved in 10 mL of water under nitrogen gas. Then, ammonium hydroxide (5 mL) was added into the solution at 85 °C. After 1 h, oleic acid (0.4 mL) was added, and the mixture was kept reacting at 85 °C for another 2 h. The obtained IONPs were washed with water and ethanol until pH became neutral followed by dispersing the IONPs in octane for further usage at 65% w/w. As-prepared ferrofluid (2 g) and 40 mg of SDS were added into 48 mL of water. The mixture was treated with ultrasound probe for 20 min to obtain magnetic miniemulsion. A styrene emulsion was prepared using 2.5 mg of styrene, 0.2 mg DVB, 50 mg of SDS, 40 mL of water, and 0.033 mL of tetradecane. Miniemulsion of magnetite nanoparticle, styrene emulsion and 5 mg of KPS were added to a three-neck flask and stirred for 30 min at 500 rpm in a nitrogen atmosphere. Afterward, the flask was placed in a 70 °C bath and maintained for 24 h to obtain magnetic nanoparticles. After cooling down to room temperature, the as-fabricated IO/PS nanoparticles were collected with an external magnet and redispersed in H_2O , and the collection-redispersion cycle was repeated two times before dispersing the IO/PS nanoparticles in 40 mL H_2O for further usage.

3.2.3 Synthesis of PMNCs

0.05 mL of IO/PS NPs were dispersed in 10 mL of tris-HCl buffer (10 mM,

pH = 8.5), followed by addition of 2.5 mg or 5 mg of dopamine. The reaction solution was placed in a magnetic field for 15 min and then incubated for 24 h without disturbing. After 24 h, the brown nanochains were purified by magnetic separation for three times and dispersed in 1 mL of H₂O.

3.2.4 Synthesis of PMNCs@AuNPs

0.2 mL of PMNCs solution was injected into 50 mL of H₂O at 85 °C under vigorous stirring. After 10 min, 4 mL of 1 wt %, 2 wt % or 3 wt % HAuCl₄ was rapidly added into the PMNCs solution. The reaction was kept stirring for 30 min at 85 °C. The color of the solution finally turned red. PMNCs@AuNPs were purified by a magnet, and the obtained nanochains were stored in 2 mL of H₂O.

3.2.5 Glucose Oxidation Reaction

Different amount of PMNCs@AuNPs were incubated with 30 μ L of glucose (100 mg/mL) with or without self-mixing for 40 min in 0.2 mL water. To induce the self-mixing, the reaction solution was placed on a magnetic stir place. Then the produced H₂O₂ from the reaction was tested with a colorimetric reaction involving HRP (0.5 μ g/mL) and TMB (0.4 mM) after removal of PMNCs@AuNPs by magnetic separation. The resulting solution was further incubated for 5 min at room temperature before measurement of absorbance at 652 nm for the oxidized TMB. For catalytic stability test, 10 pM of GOx or 0.5

μg of PMNCs@AuNPs were separately incubated with glucose at different temperatures (22, 30, 40, 50, 60, and 70 °C) and followed by colorimetric test. For recyclable catalytic activity, PMNCs@AuNPs were separated by an external magnet and followed by GOx-mimicking reaction for several consecutive cycles.

3.2.6 Catalytic Activity Inhibition Assay

About 5 μg of PMNCs@AuNPs were first mixed with different concentrations of inhibition DNA in 1 mL tris-HCl buffer. After 1 h of self-mixing, 30 μL of glucose (100 mg/mL) was added and incubated for 40 min. After removal of PMNCs@AuNPs by magnetic separation, the produced H_2O_2 from the reaction was tested with a colorimetric reaction involving HRP (0.5 $\mu\text{g}/\text{mL}$) and TMB (0.4 mM). In order to detect lower concentration of inhibition DNA, PMNCs@AuNPs enrichment is needed by magnetic separation. About 1 μg of PMNCs@AuNPs were first mixed with different concentrations of inhibition DNA in tris-HCl buffer (1 mL). After 1 h self-mixing reaction, the PMNCs@AuNPs were magnetic separation, and supernatant was removed. 30 μL of glucose (100 mg/mL) and 20 μL of DI water was added and incubated for 40 min. Then, PMNCs@AuNPs were removed by magnetic separation, and 5 μL of HRP and 5 μL of TMB were added to the mixtures for colorimetric measurement final concentrations of 0.5 $\mu\text{g}/\text{mL}$ and 0.4 mM, respectively. All the reaction solution was placed on a magnetic stir plate.

3.2.7 DNA Functionalization of Nanochains

TCEP was added into DNA solution to cleave disulfide bond to activate the thiolated DNA. 1 nmol of SH-DNA-cy5 was added into 2 mg of PMNCs with vigorous stirring in 10 mL of tris-HCl buffer (pH = 8.5; 80 mM NaCl) overnight. The remaining surface of the nanochains was then blocked by HS-PEG (2 kDa), followed by magnetic separation. The mixture was tested by fluorescent spectrum, and the SH-DNA-cy5 was almost completely reacted with PMNCs. The product was dispersed in water for further use.

3.2.8 DNA Detection by Colorimetric Assay

The 10 μ g DNA-modified PMNCs (capture PMNCs-a) with about 0.05 nmol DNA on the surface of PDA were incubated with the different amount of target DNA (0.01, 0.02, 0.04, 0.06 0.08 nmol) or mis-match ssDNA in 1 mL of hybridization buffer (10 mM tris-HCl buffer; pH = 8.5; 80 mM NaCl) for 2 h. Then PMNCs-b were separated by magnet and the residual unbound target DNA in supernatant was tested by Fluorescence spectroscopy. After removal of supernatant, 100 μ L of tris-HCl buffer with 0.01 nmol inhibition DNA was added into the capture PMNCs-b, and then followed by self-mixing reaction on a magnetic stir plate for 2 h. After remove of capture PMNCs-c, 1 μ g of PMNCs@AuNPs were added into inhibition DNA samples allowing for 1 h self-mixing reaction. Then, the PMNCs@AuNPs were collected by magnetic separation, and supernatant was removed. 30 μ L of glucose (100 mg/mL) and

20 μL of DI water was added into the PMNCs@AuNPs and incubated for 40 min. After removal of PMNCs@AuNPs, 5 μL of HRP and 5 μL of TMB were added to the supernatant solutions for colorimetric measurement with the final concentrations of 0.5 $\mu\text{g/mL}$ and 0.4 mM, respectively, and measurement of absorbance at 652 nm. For detection of influenza DNA and hepatitis B virus DNA, the PMNCs-a reacted with 0.08 nmol of target DNA, 0.08 nmol of mis-match DNA or no DNA, and followed by same process. The capture PMNCs-b mixed with 0.01 nmol inhibition DNA in 100 μL for 2 h. After remove of capture PMNCs-c, 1 μg of PMNCs@AuNPs were added into inhibition DNA samples allowing for 1 h self-mixing. Then, the PMNCs@AuNPs were collected by magnetic separation, supernatant was removed, and then followed by same colorimetric assay.

3.3 Results and Discussion

3.3.1 Synthesis of Magnetic Nanoparticles

Magnetic nanoparticles with a cluster of IO nanocrystals embedded in a polystyrene matrix (Figure 3.2a) were synthesized by miniemulsion polymerization, and were used as building blocks of the nanochain. Magnetic measurement (Figure 3.2b) shows that the nanoparticles of 90 nm in sizes were superparamagnetic with a high saturation magnetization of 34 emu/g at 300 K.

Figure 3.2 TEM images of (a) IO/PS NPs, and (b) Magnetization curves at 300 K of as-synthesized nanoparticles.

3.3.2 Synthesis of Nanochains

To prepare the PDA-coated IO/PS NPs, the IO/PS NPs dispersed in a dopamine solution (0.15 mg/mL) in Tris buffer, and the solution was stirred for 8 h leading to the deposition of a conformal layer of PDA on the IO/PS NPs. After purification by magnetic separation, the products were tested by TEM. As

shown in Figure 3.3a, the sizes of NPs changed from about 80 nm (Figure 3.2a) to 150 nm (Figure 3.3a), suggestive of forming a layers of PDA on the surface of IO/PS NPs. To prepare the nanochain, the reaction mixture was first exposed to a magnetic field for 15 min and then left undisturbed for 4 h during PDA formation. Separation of the product by magnetic field became much more efficient than the nanoparticles, suggestive of forming larger nanoparticle ensembles. TEM observation (Figure 3.3b) indeed reveals nanochains with a uniform diameter of interconnected nanoparticles rather than individual nanoparticles. Apparently, exposure to the magnetic field aligned the superparamagnetic nanoparticles and deposition of PDA eventually crosslinked them to afford the nanochains. Controlling the amount of the dopamine in the reaction solution allowed for tailoring the thickness of the PDA layers of the nanochains. For 2.5 or 10 mg dopamine, the thickness of the PDA layers increased from 20 nm to 50 nm, and the diameter of nanochains were 105 nm and 200 nm, respectively. In addition, we found that the preparation of more thickness layer of PDA need to be subjected to repeated PDA deposition. The average length of the nanochians is about 20 μm as shown in the SEM image (Figure 3.3 b-c). Notably, the nanochains with higher diameter can provide more surface areas for loading of AuNPs. Therefore, the nanochains with 200 nm diameter were chose in this project.

Figure 3.3 TEM images of (a) PDA-coated IO/PS NPs, PMNCs with diameter with (b) 105 nm (c) and 200 nm (inset, SEM of PMNCs with diameter with 105 and 200 nm, respectively).

3.3.3 Synthesis of AuNPs-Loaded Nanochains

PMNCs which have a uniform diameter of 200 nm were used for synthesis of AuNPs-loaded nanochains (Figure 3.4a). The PMNCs@AuNPs was synthesized by localized reduction of HAuCl_4 by catechol groups of PDA. The different amount of HAuCl_4 solution was injected into the PMNCs at 85 °C under vigorous stirring for loading different AuNPs on the surfaces of PMNCs. After 15 min, PMNCs@AuNPs was purified by an external magnet. TEM image of PMNCs@AuNPs (Figure 3.4b) shows that “naked AuNPs” are uniformly anchored on the PMNCs surface with a uniform size about 15 nm. Previous study indicates that the catalytic activity of AuNPs increases with decreasing the of diameter of AuNPs as more Au atoms can be accommodated with same surface area when they are small as compared to larger NPs.⁷⁶ Therefore, small AuNPs on the surfaces of PMNCs@AuNPs are found to exhibit good GOx-mimicking activity as compared to their larger size.

Figure 3.4 TEM images of (a) PMNCs, (b) PMNCs@AuNPs. (c) SEM images of PMNCs@AuNPs, (inset, at high magnification). (d) UV-vis spectra of PMNCs@AuNPs, PMNCs, and IO/PS NPs, (inset, PMNCs and PMNCs@AuNPs before and after magnetic separation).

In addition, PMNCs@AuNPs remain one-dimensional, 10-30 μm long chain-like structure even after the growth of AuNPs with 4 mL of 3 wt % of HAuCl₄ solutions as shown in SEM images (Figure 3.4c). UV-vis spectra of PMNCs@AuNPs reveal that a new broad peak around 600 nm has emerged due to the loading of AuNPs on PMNCs (Figure 3.4d). This peak corresponds to the coupling of 15 nm AuNPs leading to significant red shift in the LSPR. Moreover, both PMNCs and PMNCs@AuNPs contain high saturation

magnetization, and can easily undergo magnetic separation (3.4d inset). The color of the dispersion of PMNCs and PMNCs@AuNPs are grey and pink, respectively, which will turn completely colorless due to separation when an external magnet is placed for 5 mins.

3.3.4 Synthesis of Different Amount of AuNPs Loaded Nanochains

To optimize the GOx-mimicking catalytic activity of PMNCs@AuNPs, the amount of AuNPs on the surface of PMNCs is controlled by adding different amount (4 mL of 1 wt %, 2 wt %, 3 wt %, 4 wt %, and 5 wt %) of HAuCl₄ to 0.2 mL of PMNCs solution. When 4 mL of 1 wt %, 2 wt %, and 3 wt % of HAuCl₄ solutions were added to PMNCs solution at 85 °C, the color of the reaction solutions changed red within 15 min, which indicated that the AuNPs were anchored on the PMNCs. However, when 4 wt % or 5 wt % of HAuCl₄ solutions were added in PMNCs solution, the PMNCs aggregated very soon. For the reaction with 1 wt %, 2 wt %, and 3 wt % of HAuCl₄ solutions, the obtained PMNCs@AuNPs were characterized by UV-vis spectra and TEM after purification by a magnet. Figure 3.5a-c shows the TEM images of PMNCs@AuNPs-1, PMNCs@AuNPs-2, and PMNCs@AuNPs-3 loading with different amount of AuNPs, corresponding to 0.2 mL of PMNCs solution reaction with 4 mL of 1 wt %, 2 wt % and 3 wt % HAuCl₄, respectively. Obviously, more AuNPs are loaded on the surface of PMNCs@AuNPs-3 than that on the surfaces of PMNCs@AuNPs-1 and PMNCs@AuNPs-2.

Figure 3.5 TEM images of (a) PMNCs@AuNPs-1, (b) PMNCs@AuNPs-2, and (c) PMNCs@AuNPs-3. (d) UV-vis spectra of PMNCs@AuNPs loaded with different amount of AuNPs (inset, color change corresponding to the same concentration of PMNCs@AuNPs-1, PMNCs@AuNPs-2, and PMNCs@AuNPs-3).

UV-vis spectra exhibit that a new peak at about 600 nm after the loading of AuNPs as shown in Figure 3.5d. In addition, the peak shows a slight red-shift relative to the increasing amount of HAuCl₄ solution. It is evident that more AuNPs packing on the surface of PMNCs@AuNPs by increasing the HAuCl₄

from 1 wt % to 3 wt %. To investigate the GOx catalytic activity, the same nanochain concentration of PMNCs@AuNPs-1, PMNCs@AuNPs-2, and PMNCs@AuNPs-3 were mixed with glucose and the resulting solution was interrogated with H₂O₂-specific colorimetric assay. When HRP and TMB were added, the solution turned blue, confirming the GOx-mimicking activity of AuNPs. As Figure 3.5d inset shows that PMNCs@AuNPs-3 loaded with more AuNPs possess better catalytic activity compared to the PMNCs@AuNPs-1 and PMNCs@AuNPs-2, we use PMNCs@AuNPs-3 (named as PMNCs@AuNPs) for our further studies.

3.3.5 GOx-Mimicking Activity of PMNCs@AuNPs

The catalytic activity is dependent on the concentration of PMNCs@AuNPs and on the external spinning magnetic field. Figure 3.6a shows that the absorbance peak at 652 nm becomes stronger with increasing concentration of PMNCs@AuNPs. Moreover, catalytic activity with stirring in the presence of a spinning magnetic field led to about 40% increase in the reaction efficiency (Figure 3.6a). This is very obvious because constant rotation of the magnetic nanochains promotes efficient mixing and mass transfer to speed up the catalytic reaction. We notice that PMNCs@AuNPs is stable and super-paramagnetic in water, which allow for excellent capability of self-mixing in water, and can easily be separated within 5 mins using an external magnet for recycled applications. Recyclable catalytic activity of

PMNCs@AuNPs via magnetic separation after each reaction cycle is shown in Figure 3.6b. After 5 cycles, the PMNCs@AuNPs still contained a high GOx-mimicking catalytic activity, and have about 90% catalytic activity compared with that in the first time.

Figure 3.6 (a) The concentration-dependent catalytic activity of PMNCs@AuNPs with or without stirring (inset, color change corresponding to different concentrations of PMNCs@AuNPs). (b) Recyclable catalytic activity of PMNCs@AuNPs via magnetic separation.

Figure 3.7 Catalytic stability of PMNCs@AuNPs and GOx as a function of incubation temperature.

To study the catalytic stability of artificial as well as natural enzyme at

higher temperatures (22-70 °C), PMNCs@AuNPs and GOx were mixed with glucose at different temperature followed by H₂O₂-specific colorimetric assay. The catalytic activity of PMNCs@AuNPs increases with increasing temperature, in contrast to natural enzyme GOx whose catalytic activity decreases sharply after 40 °C as shown in Figure 3.7. Therefore, PMNCs@AuNPs shows catalytic stability at higher temperature compared to GOx.

3.3.6 Inhibiting GOx-Mimicking Activity of PMNCs@AuNPs by DNA

PMNCs@AuNPs are used to react and separate with oligonucleotides containing disulfide bond groups (inhibition DNA) by Au-S reaction. Because the GOx-mimicking catalytic activity of PMNCs@AuNPs is extremely sensitive to the surface passivation, inhibition DNA interacts with PMNCs@AuNPs resulting in blocking the GOx-mimicking catalytic activity. Followed by HRP-based colorimetric assay, the color change was largely attenuated. Thus, the color change is highly sensitive with the concentration of inhibition DNA. As a result, we can amplify the Au-S reaction based on the catalytic PMNCs@AuNPs, and develop a colorimetric probe for DNA detection. Figure 3.8a clearly demonstrate that the GOx-mimicking catalytic activity of PMNCs@AuNPs decreases with increasing inhibition DNA concentration due to increased surface passivation of AuNPs. This evidence indicates that the inhibition effect can be related to the steric hindrance effect due to the

formation of the PMNCs@AuNPs-DNA conjugates. Compared with inhibition DNA, ssDNA without any disulfide groups can hardly hinder the GOx-mimicking activity of PMNCs@AuNPs as shown in Figure 3.8a. We believe that the conjugation of PMNCs@AuNPs with inhibition DNA via Au-S bonds has higher efficiency than the electrostatic adherence between ssDNA and AuNPs on the surface of PMNCs@AuNPs. Moreover, when the concentrations of inhibition DNA are lower than 20 nM, it becomes difficult to detect the biomolecules due to very weak color signal coming from low amount of PMNCs@AuNPs-DNA conjugates present in large sample volume. We also find that isolating PMNCs@AuNPs by using an external magnet is much more efficient than that of AuNPs. Therefore, it is possible to isolate and transfer the PMNCs@AuNPs-DNA conjugates by an external magnet to prepare a highly enriched solution of the conjugates in order to intensify the color signals as well as the sensitivity of biomedical detection. First, 1 μg of PMNCs@AuNPs were mixed with less than 20 nM of inhibition DNA in 1 mL of sample solution. After the formation of PMNCs@AuNPs-DNA conjugates, the nanochains were isolated and transferred to a 50 μL sample solution to enrich the concentration of PMNCs@AuNPs-DNA conjugates followed by colorimetric test. As shown in Figure 3.8b, this nanochain enrichment intensifies the color change even for the presence of lower content of inhibition DNA and makes the colorimetric detection easier. Furthermore, low concentrations of PMNCs@AuNPs have weak catalytic activity for glucose oxidation. However, higher enrichment of

nanochains *via* magnetic isolation amplifies the detection sensitivity to about 20 times providing a simple step of enrichment for subsequent analysis.

Figure 3.8 (a) UV-vis absorbance changes of colorimetric assay reaction as function of concentration inhibition DNA (black) and ssDNA (red). Color change corresponding to different concentration of inhibition DNA (inset). (b) Reaction of lower concentration of inhibition DNA with lower concentration of PMNCs@AuNPs, followed by magnetic enrichment of PMNCs@AuNPs to a higher concentration and the colorimetric test. Color change corresponding to different concentration of inhibition DNA (inset).

3.3.7 Colorimetric Detection of Targeted DNA Based on PMNCs@AuNPs

Figure 3.9 (a) Fluorescent intensity of Cy5 obtained by different reaction time of HS-DNA-Cy5 with PMNCs. (b) Fluorescence intensity of target DNA with dye before and after separation of capture PMNCs-b.

Next, we combine all our findings from this study to demonstrate selective capture and separation of target- and inhibition DNA by PMNCs-a and their detection from biological samples by using PMNCs@AuNPs. cyanine dye (Cy5)-modified SH-ssDNA (probe DNA) was used to quantify DNA conjugation efficiency. Because the PDA is the efficient fluorescence quenchers, it is expected that Cy-5 fluorescence would be quenched upon attachment on PMNCs. Fluorescence spectra of the reaction of Cy5-modified probe DNA and PMNCs show that the probe DNA successfully binds to the surface of PMNCs within 4 hours (Figure 3.9a). The PMNCs-a with about 0.05 nmol probe DNA were used for selective capture and separation of target DNA from samples. The percentage separation efficiency of capture PMNCs-a are determined over a concentration range of target DNA from 10 to 80 nM. The residual target DNA is tested by fluorescent spectroscopy after removal of capture PMNCs-b by an external magnet as shown in Figure 3.9b. However, when we use PMNCs-a to capture and separate mis-match DNA as shown in Table 3.1, almost no mis-match DNA is separated as shown in Figure 3.10.

Figure 3.10 The fluorescent intensity of target and mis-match DNA 1, 2 and 3 as shown in Table 3.1 before (black) and after (red) separation by DNA-modified PMNCs.

We then show the hybridizing efficiency of PMNCs by calculating how much target DNA (in nmol) is hybridized and separated by capture PMNCs-a. Figure 3.11a shows that PMNCs-a capture progressively higher amount of target DNA with increasing concentration of target DNA from 10 to 80 nM. About 35% and 42% target DNA was successfully separated by capture PMNCs-a from 1 ml of 80 and 60 nM target DNA solution. It shows that 0.03 nmol target DNA was hybridized with probe DNA on PMNCs-a, whereas only about 0.025, 0.01, and 0.005 nmol target DNA was hybridized with probe DNA on PMNCs-a from 40, 20, and 10 nM target DNA solution, respectively. After the first hybridization with target DNA, capture PMNCs-b were separated by an external magnet, followed by the addition of the 0.01 nmol inhibition DNA for capture via second hybridization and separation. The residual free inhibition DNA due to unsuccessful capture and separation by target DNA is tested by

fluorescent spectroscopy as shown in Figure 3.11b. Different amount of inhibition DNA is captured and separated by capture PMNCs-b depending on the target DNA content on the capture PMNCs-b. When 0.03 nmol target DNA is present on the capture PMNCs-b, about 90% inhibition DNA is hybridized and separated by capture PMNCs-b (see curve for label 5 in Figure 3.11b). After magnetic separation of PMNCs-inhibition DNA conjugates (PMNCs-c), colorimetric test is carried out to monitor the residual inhibition-ssDNA. 1 μ g of PMNCs@AuNPs were added into the large volume of residual inhibition DNA test samples (label 1 to 5) and allowed to react with them for 1 hour with self-mixing, and then the PMNCs@AuNPs-inhibition DNA conjugates were magnetically isolated and transferred into 50 μ l volume for their enrichment. Then, the glucose was added to monitor the GOx catalytic reaction in the presence of O₂. After 40 mins, PMNCs@AuNPs-inhibition DNA conjugates were separated by an external magnet, followed by the addition of TMB and HRP for colorimetric test. As shown in Figure 3.11c, the color change is more obvious and increases with increasing the concentration of target DNA leading to more inhibition DNA separation. Taking the advantages of covalent coupling, PMNCs can further be modified with antibody and other captured DNA having different functional moieties containing thiol and amino groups. Therefore, the application of PMNCs can be expanded to multiplexed detection of diverse targeted bodies in different biological systems. As shown in Figure 3.11d, the assay could detect 0.08 nmol of Influenza DNA and Hepatitis B virus DNA in 1

mL sample solution by modifying special HS-DNA. Firstly, the PMNCs were modified by probe-Influenza ssDNA or probe-Hepatitis B virus ssDNA, via Michael addition and/or Schiff base reactions between PDA and DNA similarly to provide capture PMNCs-a. Then the capture PMNCs-a were added to the test samples (1) control solution (no target DNA), (2) mismatch DNA, and (3) target DNA and were subsequently removed by an external magnet after hybridization. Finally, PMNCs@AuNPs were added to the test samples 1, 2 and 3 to detect the respective residual biomolecules. The colorimetric GOx catalytic activity test is performed by adding TMB and HRP assay in the test samples similarly as mentioned above in case of inhibition DNA. The high selectivity of the target DNA detection illustrated in Figure 3.11d (inset) displays the blue color response due to successful capture of the target DNA. However, there was no color change corresponding to test sample 1 (no target DNA) and 2 (mismatch DNA) due to unsuccessful capture/separation stems from unhybridized capture PMNCs-a. Therefore, PMNCs and PMNCs@AuNPs could be easily modified for diverse biomolecules detection.

Figure 3.11 (a) Hybridization efficiency (the ratio of target DNA was captured by PMNCs-a from different concentrations of target DNA/the amount of target DNA). (b) The fluorescent intensity of the residual free inhibition DNA in supernatant. The intensity decreases from 1 to 5 with increasing target DNA content on capture PMNCs-b. The control means 0.01 nmol inhibition DNA. (c) Colorimetric detection of unbound inhibition DNA by PMNCs@AuNPs. Color change corresponding to different target DNA concentration (inset). (d) Colorimetric detection of (A) Influenza ssDNA and (B) Hepatitis B virus ssDNA by PMNCs@AuNPs. Color change corresponding to (1) no target DNA, (2) mis-match DNA, and (3) target DNA, (inset image corresponding to detection of two different ssDNA of different conditions).

3.4 Conclusion

Here, by utilizing the reducing activity of abundant catechol groups of PDA, we can obtain 'naked' AuNPs on PMNCs to form PMNCs@AuNPs that have demonstrated for the first time the use of PMNCs and PMNCs@AuNPs for multiplex DNA detection. The change in interfacial catalytic activity is accompanied by PMNCs@AuNPs interacting with inhibition DNA, which provides a self-mixing, high saturation magnetization material for novel nanochains-based multiplex colorimetric biosensors. Moreover, the PMNCs which are easily functionalized by diverse DNA, target DNA can easily be extracted from the complicated biomedical sample, which is the critical step for diagnostic applications. What is more advantageous is that, both the PMNCs and PMNCs@AuNPs contain high saturation magnetization. So it is possible to enrich the target to a high concentration which is important to improve accuracy and sensitivity of detection. Finally, the combination of capturing as well as separation and detection of these two nanochains provide a label-free, colorimetric, fast, simple and sensitive measurement for detecting biological targets. We envision that the GOx-mimicking nanochains have potential application in the development of biocatalysis, bioassays, and smart devices in the future.

Chapter 4 Biphasic Synthesis of Gold Snowflake Nanoparticles as NIR-II-Window Light Mediated Theranostics

4.1 Introduction

Biomedicine based on plasmonic nanoparticles with LSPR peaks in the NIR (750-1400 nm) region has attracted enormous investigation and tremendous potential for diagnostics and therapeutics.⁶ Among a wide spectrum of plasmonic nanoparticles, the NIR-II-response nanomaterials have attracted enormous attention for the properties of depth penetration in tissues, low scattering and autofluorescence, making the NIR-II-based plasmonic nanoparticles potentially suitable for clinically practical application.¹³⁰ For example, NIR-II light with wavelength between 1000 and 1350 nm can pass through blood because of less absorbance in this wavelength of hemoglobin and low scattering of biological tissues.¹³¹ Thus, many research groups have reported various nanomaterials, such as carbon nanotubes,^{132, 133} QDs,^{134, 135} IO@CuS,¹³¹ Au-Cu₂O nanobars,¹³⁶ Au/Ag alloyed nanocages,¹³⁷ Au rod-in-shell nanoparticles,¹⁰⁵ Au nanoechinus,¹¹¹ as theranostic agents by the NIR-II laser (Figure 4.1). Among those nanomaterials, Au nanomaterials, which have low cytotoxicity and biocompatibility, provide a suitable platform for biomedical application.¹³⁰ It is easy to tune the absorption of Au nanomaterials from the visible to the NIR-II region by controlling their size and shape. For

example, AuNR@Au/Ag nanoshell show wide absorbance from up to 1350 nm wavelength by tuning the inter-gap between the AuNR and the Au/Ag nanoshell, and have been used in NIR-II photothermal cancer therapy.¹⁰⁵ However, only Au nanomaterials with LSPR in NIR-II region have rarely been studied. It is still a challenge for preparing Au nanomaterials with small size, easy synthesis, and biocompatibility for biomedical applications in the NIR-II window.

Figure 4. 1 TEM images of (a) QDs, (b) IO@CuS, (c) Au-Cu₂O nanobars, (d) Au/Ag alloyed nanocages, (e) Au rod-in-shell NPs, (f) Au nanoechinus. (a) Copyright 2012 American Chemical Society. (b) Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA. (c) Copyright 2016 The Royal Society of Chemistry. (d) Copyright 2006 Elsevier. (e) Copyright 2013 The Royal Society of Chemistry. (f) Copyright 2014 WILEY-VCH Verlag GmbH & Co. KGaA.

Anisotropic Au nanostructures containing multi-branches similar to star,²⁹ flower,¹³⁸ echinus¹³⁹, and octopus¹⁴⁰ have been widely studied due to the

distinct optical properties. Enormous research about anisotropic Au biomedical diagnostics and PTT have been reported.¹⁴¹ However, the NIR-II-window-related properties of Au multi-branches nanoparticles for biomedical applications have been less studied. The strong absorbance in NIR region due to Au branches coupling and scattering enhancement leading to SERS motivate us to investigate their bio-applications by NIR-II laser.¹⁴²⁻¹⁴⁴ Here, a snowflake-like AuSF nanomaterial with an absorbance from about 550 to 1350 nm was synthesized by two-phase method. It can act as amplified optical probe for SERS, and provides new opportunities for both 808 nm (NIR-I) and 1064 nm (NIR-II) PPT. Results have shown that AuSF nanoparticles have high photothermal conversion efficiency at both 808 and 1064 nm, and show effectively photothermal killing of *S. aureus* and *E. coli* O157:H7 by exposing to 1 W/cm² of 1064 nm laser. Besides, antibody-encoded plasmonic AuSF nanomaterials combined with antibody-encoded magnetic nanochains led to efficiently magnetic separation and ultrasensitive SERS detection of targeted pathogens.

The synthesis route of AuSF is illustrated as shown in Figure 4.2, Au precursor in hexane were dropped into a mixture with hydrophobic 10 nm AuNPs in hexane and NH₂OH·HCl in water under violently stirring. Because Au precursor are separated with reduction agency in different phase, the slow reduction of Au precursor by NH₂OH·HCl makes Au(0) atom deposit on alkyl chain-capped Au seed rather than self-nucleation.¹⁴⁵ By controlling the amount

of Au precursor, the length of Au branches of AuSF nanomaterials can be easily controlled to extend absorbance from NIR-I to NIR-II windows. We have found that the TEM images of resulting AuSF nanoparticles are similar to the snowflake, as shown in the picture taken in winter. So, this AuSF nanomaterials with many Au branches have strong plasmonic coupling toward broadly tunable LSPR across the visible, NIR-I, and NIR-II range.

Figure 4.2 Scheme of the synthesis of AuSF nanoparticles in two-phase system.

4.2 Experimental Section

4.2.1 Materials and Characterization

Materials. $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was purchased from Alfa Aesar. Oleic acid, oleylamine 90%, dimethylformamide (DMF), chloroform (CF), dichloromethane ethanol (DCM), ethanol, hexane, hydroxylammonium chloride ($\text{HONH}_2 \cdot \text{HCl}$), 4-nitrothiophenol, and 4-Bromothiophenol were purchased from Sigma-Aldrich. Carboxymethyl-PEG-thiol (CM-PEG-SH, $M_n = 3400$), Methoxypoly(ethylene glycol)-thiol (MPEG-SH) (PEG-SH, $M_n = 5000$) were received from Laysan Bio, Inc. $10\times$ TAE buffer pH 8.0 and agarose were purchased from 1st base (Singapore). $10\times$ TAE buffer pH 8.3 and agarose were purchased from Promega (Madison, USA). Sodium chloride was purchased from Merck (Darmstadt, Germany).

Characterization. TEM images and UV-vis-NIR absorbance spectra were obtained reference with chapter 3. A RENISHAW Raman microscope with WIRE 2.0 software and air-cooled He–Ne laser were applied for SERS measurements. CW diode lasers of 808 and 1064 nm were used for the photothermal experiments.

4.2.2 Synthesis of 10 nm AuNPs

1.6 mL of 50 mg/mL HAuCl_4 in acetonitrile was added to a mixture of 1 mL of 1-octadecene, 4 mL of oleylamine, and 0.4 mL of oleic acid. The mixture

solution was raised to 100 °C to remove low boiling point compounds under reduced pressure. Next, the mixture was elevated to 135 °C under nitrogen flow. After reaction at 135 °C for 15 min, the solution was cooled to room temperature. The red colored product was centrifuged at 3000 rpm for 5 min after the addition of ethanol, and the AuNPs were stored in 20 mL of hexane at 4 °C for further use.

4.2.3 Synthesis of AuSF Nanoparticle

A hexane-water two-phase interface system was employed to prepare the AuSF nanoparticles. Firstly, Au precursor was prepared by mixture of 0.2 mL of oleylamine, 0.16 mL of 50 mg/mL HAuCl₄ in acetonitrile, and 4 mL of hexane.

In a typical synthesis, 20 µL of above AuNPs solution was added to 2 mL of hexane, and then mixed with 0.3 mL of 0.4 mM NH₄OH·HCl aqueous solution under vigorous stirring. 0.2, 0.5, 1, 2, and 3 mL of Au precursor solution was added dropwise into the two-phase solution with a slow speed. After vigorous stirring for another 15 min, the AuSF nanoparticles were then collected by adding 1 mL of ethanol into the reaction solution, followed by removing the water in the bottom of solution, and centrifugation at 6000, 5000, 4000, 3000, and 2500 rcf for 10 min, respectively. Afterward, 2 mL of CF solutions with 2 mg CM-PEG-SH were mixed with AuSF nanoparticles, and the mixture was treated with ultrasound for 10 min to obtain PEGylated AuSF nanoparticles.

AuSF nanoparticles with different addition speeds of Au precursor solution were synthesized by same process. 2 mL of Au precursor solution was added dropwise into the two-phase solution with a speed of 0.2, 0.5, and 0.7 mL/min, respectively. Another control experiment was to change aqueous solution with CTAB aqueous solution. The Au precursor solution was added dropwise into the 0.2 M of CTAB aqueous solution and hexane two-phase solution.

4.2.4 Hemolysis Assay

Fresh human blood was centrifuged (1000 rpm, 10 min) to separate the erythrocytes, and then washed five times with PBS buffer before diluting to a concentration of 5% (v/v). AuSF nanoparticles in PBS solution (0.1 mL) at different concentrations were mixed with erythrocytes stock (0.1 mL) and then shaken at 37 °C. After 1 h, the mixtures were centrifuged at 1000 rpm for 10 min to remove erythrocytes, and the supernatant was then centrifuged at 3000 rcf for 10 min to remove AuSF nanoparticles. The supernatant (0.1 mL) was then added to a 96-well microplate and diluted with PBS buffer. The absorbance of the solution was tested by a microplate spectrophotometer at 540 nm. The control experiments were taken by water and PBS buffer.

4.2.5 Synthesis of Magnetic Nanochains

Firstly, the magnetic IONPs were synthesized according to previous report.¹²⁵ Briefly, 0.54 g of FeCl₃ and 0.2 g of trisodium citrate were first added

into 20 mL of ethylene glycol, and then 1.2 g of sodium acetate was added into mixture with stirring. The mixture was sealed in a Teflon-lined stainless-steel autoclave and then heated to 200 °C. After 10 h reaction, the black products were separated and washed with ethanol and DI water for 3 times.

The magnetic nanochains were synthesized according to previous report. 1 mg of magnetic nanoparticles and 8 mg of dopamine were dispersed in 18 mL of tris buffer (pH=8.5, 10 mM). The mixture was immediately placed in a uniform magnetic field for 15 min without any shaking. After removal of magnetic field, the mixture was reacted for another 12 h. The magnetic nanochains were then separated by an external magnet and dispersed in water for further use.

For bioconjugation of magnetic nanochains, 20 µL of nanochains was dispersed in 5 mL of Tris buffer under stirring, and then 50 µL of antibody solution (1 mg/mL) was added into the dispersion, following by adding NaCl solution (1 M) to adjust the salt concentration to 80 mM. After 12 h, 0.1 mL of HS-PEG (1 mg/mL) was added in the solution. After another 1 h, the antibody-conjugated nanochains were purified by magnetic separation and stored in 4 °C for further use.

4.2.6 Synthesis of Raman Dye-Tagged AuSF Nanoparticles

Typically, AuSF nanoparticles were dispersed in CF, and Raman dye was

added under continuous stirring. CM-PEG-SH and MPEG-SH (1:1) were added after 5 min. After reacting for 24 h, AuSF nanoparticles were collected by centrifuge and re-dispersed in water. The supernatant of CF solution was tested by UV to calculate the number of 4-nitrothiophenol in the nanoparticles. The enhancement factor of AuSF nanoparticles was determined by computing the ratio of SERS to normal Raman scattering of 4-nitrothiophenol using the following equation: $EF = (I_{SERS} \times C_{Normal}) / (I_{Normal} \times C_{SERS})$, where I_{SERS} and I_{Normal} are the Raman intensities at 1341 cm^{-1} for AuSF nanoparticles and pure 4-nitrothiophenol solution, and C_{SERS} and C_{Normal} the concentrations of 4-nitrothiophenol on nanoparticles and in pure DCM solution.⁵²

4.2.7 Finite-Element Calculations

FEM calculations were performed in the RF module of COMSOL Multiphysics 5.0. The AuSF nanoparticle was modeled as a sphere (diameter = 20 nm) with 9 rod-shaped tips (diameter ranging from 6 to 8 nm, length ranging from 10 to 20 nm). The refractive index of the medium was set to be 1.33, and the dielectric function of gold was adopted from Johnson and Christy. For calculating the electric field profiles, the scattered field was solved with a background field describing a linearly polarized electric field. Several polarization directions as well as field propagation directions were chosen for the calculation. For better visualization, the electric field on the surface of the nanoparticle was plotted.

4.2.8 NIR Laser-Induced Heat Conversion

The aqueous solution of PEGylated AuSF nanoparticles with different ODs (1, 0.75, 0.5) were irradiated by a 808 and 1064 nm laser (1 W/cm²). Water was taken as a negative control. The temperature was monitored during heating (laser on). After the temperature reached a stable stage, the laser was powered off and the cooling stage was monitored. Real-time thermal imaging of the samples was recorded using a FLIR thermal camera. The photothermal conversion efficiency (η) of PEGylated AuSF nanoparticles under 1064 and 808 nm was calculated according to the following equation.^{107, 146}

$$\eta = \frac{hS(T_{\max} - T_{\text{amb}}) - Q_{\text{dis}}}{I(1 - 10^{-A})}$$

where $T_{\max}$ is the maximum equilibrium temperature, T_{amb} is the ambient temperature of the surroundings, Q_{dis} is a parameter expressing the laser-induced heat input by the container, I is the laser power, and A is the absorbance of the NPs at 1064 or 808 nm. The term hS was calculated based on

$$hS = \frac{\sum_i m_i C_{p,i}}{\tau_s}$$

where τ_s is the sample system time constant which is calculated according to the equation of $t = -\tau_s \ln \theta$.

4.2.9 Surface Modification of AuSF Nanoparticles by Antibody

The AuSF nanoparticles were collected by centrifuge and dispersed in 2

mL of 2-(N-morpholino)ethanesulfonic acid buffer (MES buffer) (pH=5.5). To activate the carboxylic acid group on the surface of these particles, 0.2 mL of EDC (5 mg/mL) and sulfo-NHS (5 mg/mL) were added to the solution and incubated for 30 min. After centrifuging to remove excess EDC and sulfo-NHS, the antibody (50 µg/mL) in borate saline buffer (pH 8.0) was quickly added to the activated particles with gentle stirring for 12 h at room temperature. Next, 0.1 mL of BSA solution (5 mg/mL) was added to the mixture to block the unreacted sites for 1 h. The free reactants were removed by centrifuge. The nanoparticles were stored at 4 °C before use.

4.2.10 Bacterial Culture

Escherichia coli (*E. coli*, O157:H7 ATCC 43888), *Staphylococcus aureus* (*S. aureus*, ATCC 6538), and other bacteria were cultured in Luria–Bertani (LB) medium for 24 h at 37 °C (shaking at 200 rpm). After centrifugation at 6000 rpm for 10 min and washed with PBS solution for 3 times, the number of viable cells was determined by plate count. For Raman detection, the bacteria were treated with 0.3% formaldehyde for 24 h to kill all bacteria. The inactivated bacteria were collected by centrifugation at 4000 rpm and resuspended in 0.01 M PBS buffer. Finally, these bacteria were serially diluted to the desired concentrations with 0.01 M PBS for further use.

4.2.11 Photothermal Killing of *E. coli* O157:H7 and *S. aureus*

Different amount of antibody-conjugated AuSF nanoparticles was added to 0.5 mL of bacteria (10^7 CFU/mL). The mixture was shaken for 1 h and exposure to a 1064 and 808 nm laser ($1\text{W}/\text{cm}^2$) for 15 min. After that, the bacteria were transferred to tryptic agar plate and incubated for 24 h at 37 °C. The colony number for each countable plate was counted.

4.2.12 SERS Detection of *E. coli* O157:H7 and *S. aureus*

A 50 μL of antibody-conjugated AuSF nanoparticles (0.2 mg/mL) and 50 μL of antibody-conjugated magnetic nanochains (0.5 mg/mL) were added to 1 mL samples containing 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , and 10^8 CFU/mL of *E. coli* O157:H7, *S. aureus* or other bacteria. The mixture was gently shaken for 1 h and separated by an external magnet for 2 min. The complex of bacteria and nanoparticles was then washed three times with 0.01 M PBS. Finally, the complex of bacteria and nanoparticles in about 20 μL 0.01 M PBS was dropped into a device with same size hole (0.1 cm^2). The samples were placed under Raman microscopy for spectral collection using 50 mW of laser power. The calibration curve was plotted using the peak intensities of 4-nitrothiophenol at 1341 cm^{-1} vs the concentration of *E. coli* O157:H7 or *S. aureus* (10^2 - 10^8 CFU/mL).

4.3 Results and Discussion

4.3.1 Synthesis of AuSF Nanoparticles

AuSF nanoparticles with multi-branches were synthesized by dropping Au precursors solution into a two-phase system with hydrophobic 10 nm AuNPs as seed and $\text{NH}_2\text{OH}\cdot\text{HCl}$ as reducing agent. At the end of the reaction, 1 mL of ethanol was added to the mixture and the products aggregated and precipitated on the interface of hexane and water. After removal of both water and hexane, HS-PEG in CF was mixed with products to re-disperse nanoparticles. The morphologies of the products with different amount of Au precursors were characterized by TEM (Figure 4.3a-f). The sizes of AuSF nanoparticles gradually grow bigger during the adding of Au precursor. Those monodispersed AuSF nanoparticles with multi-branches of about 0, 5, 10, 20, and 30 nm were produced after 15 min reaction in two-phase system with the 0.2, 0.5, 1, 2, and 3 mL of Au precursor (named AuSF-1, AuSF-2, AuSF-3, AuSF-4, and AuSF-5, respectively). The high-resolution TEM (HRTEM) images reveal that the fringe spacing of the Au seeds (Figure 4.3g) is 0.24 nm, which matches the interplanar distance between the (111) planes of Au.⁴¹ The Au branches was confined to the (111) lattice as shown in the HRTEM image (Figure 4.3h), which may because that new formed Au(0) epitaxially grow from the defect-free regions on the Au seed.¹⁴⁷

Figure 4.3 TEM images of (a) Au seeds and (b) AuSF-1, (c) AuSF-2, (d) AuSF-3, (e) AuSF-4, and (f) AuSF-5 nanoparticles at different stages (from b to f refer to adding 0.2, 0.5, 1.0, 2.0, and 3.0 mL of Au precursors, and HRTEM images of (g) Au seeds and (h) a branch of AuSF-3 nanoparticles depicting (111) lattice.

The AuSF nanoparticles were prepared in a novel hexane-water system, which is different from previous approaches, such as seed-mediated method in one phase with ion surfactant.^{50, 148, 149} To investigate the mechanism of AuSF nanoparticles, we first compared nanoparticles at different growth steps. After adding 0.2 mL of Au precursors, the diameter of these AuNPs was larger than Au seeds and some irregularly shaped nanoparticles started to appear (Figure 4.3a and Figure 4.3b). This might be that Au precursors were reduced to Au(0) to form some Au clusters on the seeds. Next, Au(0) were elongated from those irregular part to form several long branches.

The control experiments show that different adding speeds of Au precursor affected growth of Au branches. Quick dropping of Au precursors is preferred to grow thinner and longer branches on seeds, which means that more Au clusters were formed on the seeds for growth of Au branches. UV-vis spectra of AuSF nanoparticles with different drop speeds reveal that 1 min/mL of adding speed cased uniform structure (Figure 4.4) and the most red-shift of LSPR (Figure 4.5) after adding same amount of Au precursors. Secondly, we characterized the synthesis of AuSF nanoparticles by reducing the interface tension of hexane-water system. Same concentration of $\text{NH}_2\text{OH}\cdot\text{HCl}$ was first dispersed in 0.2 M of cetyltrimethylammonium bromide (CTAB) solution, and then mixed with Au seeds in hexane. After adding Au precursors in hexane, the products were transferred from hexane to water phase. After purification, the products were characterized by UV-vis spectrum and TEM. The LSPR peak of

the products was red-shifted from 519 to 580 nm, indicating that the AuNPs grow big rather than forming Au branches (Figure 4.6a). TEM images also show that the nanoparticles had bigger size and fewer short protruding parts after adding the same amount of Au precursors (Figure 4.6b). So it is easy to form Au(0) in the hexane-CTAB-water phase, leading to isotropic growth of Au on the seed.

Figure 4.4 TEM images of AuSF nanoparticles synthesized with different speeding to add Au precursors (a) 0.2, (b) 0.5, (c) 0.7, and (d) 1.0 mL/min.

Figure 4.5 UV-vis spectra of AuSF nanoparticles with different drop speed of Au precursor.

Figure 4.6 (a) UV-vis spectra and (b) TEM images of AuSF nanoparticles synthesized in hexane-CTAB solution.

4.3.2 Optical Study

The LSPR of plasmonic AuSF nanoparticles is highly sensitive to the length and coupling of Au branches, which lead to a significant red-shift in the LSPR. As shown in Figure 4.7, AuNPs of 10 nm with an original LSPR centered at 519 nm experienced a red-shift to 530 nm after adding 0.2 mL of Au

precursor. With continuous growth, a new resonance peak around 700 nm appeared and then became dominant in AuSF nanoparticles with 1 mL of Au precursor because of the short Au branches. The LSPR peak in NIR reign shows a red-shift from 700 to 850 nm after adding 2 mL of Au precursors, finally to more than 1200 nm after adding 3 mL of Au precursors to cover the whole NIR-I and NIR-II range due to the inter-branches LSPR coupling¹³⁹. The associated characteristic color of Au seed and different sizes of AuSF nanoparticles changed from red to purple, blue and final to black (inset of Figure 4.7). In this article, AuSF-5 nanoparticles were chosen for all related studies, and named as AuSF nanoparticles due to their excellent optical properties.

Figure 4.7 UV-vis-NIR spectra and a photograph (inset: from left to right) of Au seeds, and AuSF nanoparticles at different stages.

4.3.3 SERS Study

The electromagnetic field surrounding the roughened metal surface and the

coupling of Au branches plays a pivotal role for enhancing the SERS intensity.^{150, 151} Here, FEM calculations were performed to estimate the electric field intensity on the AuSF nanoparticle surface when excited by a 785 nm laser (Figure 4.8a). The calculated electric field is most intense at the tips of the nanoparticle and the gaps between adjacent branches. The highest $|E/E_0|$ enhancement can be well above 60. In addition, the coupling between plasmonic Au branches gap leads to a dramatically amplified local field, representing an efficient SERS hot-spot. Raman dyes carrying a thiol group were easily modified with AuSF nanoparticles to form SERS-active plasmonic nanoparticles, and the number of thiol-Raman dye anchored can be controlled by the feeding ratio of molecules and the AuSF nanoparticles. The Figure 4.8b shows the Raman spectra of AuSF nanoparticles which were modified by two different Raman dye under the excitation of a 785 nm laser. The excellent SERS activity of the AuSF nanoparticles modification by 4-nitrothiophenol was confirmed by an enhancement factor of 4.6×10^6 .

Figure 4.8 (a) Theoretical analysis of electric field intensity of AuSF nanoparticles. (b) SERS spectra of AuSF nanoparticles modification with different Raman dye.

4.3.4 Photothermal Study

We conducted a comparison to reveal the photothermal conversion efficiency of PEGylated AuSF nanoparticles irradiation by 1064 and 808 nm lasers in NIR-I and NIR-II range, respectively. Aqueous solutions of PEGylated AuSF nanoparticles at different OD_{1064} (1.0, 0.75, 0.5) were first exposed to the 1064 nm laser (1 W/cm^2), and a concentration-dependent temperature increase was shown in Figure 4.9a. After 14 min, the temperature reached a stable state as high as 68.1, 64.1, 60.2 °C for $OD_{1064} = 1, 0.75$, and 0.5, respectively. Meanwhile, PEGylated AuSF nanoparticles at different OD_{808} (1, 0.75, 0.5) were then exposed to the 808 nm laser (1 W/cm^2). After 16 min, the temperature also reached a stable state, and the temperature was 68.7, 66.4, and 59 °C for $OD_{808} = 1, 0.75$, and 0.5, respectively (Figure 4.9b). In comparison, no obvious temperature increasing under both 1064 and 808 nm laser was shown for pure water. The η value of the PEGylated AuSF nanoparticles under 1064 and 808 nm laser excitation were determined to about 52.7% and 53%, respectively. Therefore, the PEGylated AuSF nanoparticles can be developed as PTT agents in both NIR-I and NIR-II region.

Figure 4.9 Temperature elevation profile under irradiation of (a) 1064 nm and (b) 808 nm laser at 1 W/cm² for PEGylated AuSF nanoparticles in H₂O, and corresponding plot of the cooling time vs $-\ln(\theta)$ of (c) 1064 nm and (d) 808 nm.

4.3.5 Cytotoxicity of AuSF Nanoparticles

The cytotoxicity of AuSF nanoparticles were tested by hemolysis, which is important for biomedical application. Figure 4.10 show the hemolytic behaviors of PEGylated AuSF nanoparticles at different concentrations (0.01 to 1.4 mg/mL). It is apparent that AuSF nanoparticles at less than 1.0 mg/mL have low hemolysis, exhibiting less than 0.2% hemolytic activity. Therefore, the excellent cytocompatibility may enable AuSF nanoparticles to be used for biomedical application.

Figure 4.10 Hemolytic activity of PEGylated AuSF nanoparticles.

4.3.6 Antibacterial Study

The high η value and low cytotoxicity of the AuSF nanoparticles motivated us to apply AuSF nanoparticles for biomedical application. AuSF nanoparticles excited by NIR-II laser can be used for photothermal killing food-borne bacterial pathogen *S. aureus* and *E. coli* O157:H7. The *S. aureus* antibodies were conjugated with carboxyl-PEGylated AuSF nanoparticles by EDC and Sulfo-NHS reaction. Zeta potential measurements were employed to confirm the surface charge of AuSF nanoparticles before (-19 mV) and after (-26 mV) antibody conjugation. In the assay, *S. aureus* antibody-conjugated AuSF nanoparticles (anti-AuSF nanoparticles) with different concentration (0.01-0.5 nM) were first introduced in the 0.5 mL of samples containing 10^7 CFU/mL of *S. aureus* for 1 h, respectively. Next, the samples were exposed to 1064 nm laser at 1 W/cm^2 for 15 min, and the photothermal effect of anti-AuSF nanoparticles causes sufficient heat for the killing of bacteria. After

photothermal killing, the amount of live bacteria was confirmed by colony plating.⁹⁷ Figure 4.11a shows that the number of *S. aureus* after 15 min to 1064 nm laser is reducing with increasing the concentration of anti-AuSF nanoparticles. When the bacteria mixed with 0.01 and 0.05 nM anti-AuSF nanoparticles, almost 35%, 50% of *S. aureus* have been irreparably cellular-damaged during NIR-II irradiation, respectively. However, PEGylated AuSF nanoparticles mixed with *S. aureus* and then exposed to laser, only 8% and 10% of *S. aureus* were cellular-damaged (Figure 4.11a and c). Therefore, the antibody-encoded AuSF nanoparticles show 5 times higher PTT efficiency than PEGylated AuSF nanoparticles. When the concentration of anti-AuSF nanoparticles reached 0.3 and 0.5 nM, almost 100% *S. aureus* were cellular-damaged. It is because the temperature of whole samples was higher than 55 °C. Besides, *E. coli* O157:H7 antibody-encoded AuSF nanoparticles show high PTT efficiency of killing *E. coli* O157:H7 (Figure 4.11b and d). Similarly, 60% and 80% of *E. coli* O157:H7 were cellular-damaged for 0.05 and 0.1 nM of *E. coli* O157:H7 antibody-encoded AuSF nanoparticles, and almost all cellular-damaged for 0.3 nM of nanoparticles. Therefore, bioconjugated AuSF nanoparticles could be used in the potential application in targeting and photothermal killing of food-borne bacterial pathogen.

Figure 4.11 Plot demonstrating the percentage of live bacteria during the anti-AuSF nanoparticles concentration-dependent photothermal killing by using 1064 nm laser (1 W/cm^2). An amount of 1×10^7 CFU/mL of (a) *S. aureus* and (b) *E. coli* O157:H7 was used for each case, and images show the colonies of (c) *S. aureus* and (d) *E. coli* O157:H7 after photothermal killing by anti-AuSF nanoparticles.

4.3.7 SERS Detection of Bacteria

Antibody conjugated plasmonic AuSF nanoparticles offer the possibility of ultrasensitive Raman detection. As a proof of concept, we combined the AuSF nanoparticles with antibody-encoded magnetic nanochains (Figure 4.12a) for magnetic separation and quantitative immunoassay of *S. aureus* or *E. coli* O157:H7, respectively. As shown in the assay, both of the antibody-encoded AuSF nanoparticles and magnetic nanochains were mixed with bacteria in PBS buffer. After 1 h, TEM images show that the magnetic nanochains and AuSF

nanoparticles successfully targeted to bacteria to form a sandwich structure (Figure 4.12b and c).

Figure 4.12 (a) Schematic of the immune assay of using SERS-encoded AuSF nanoparticles and bioconjugated magnetic nanochains for bacterial detection. TEM images of (b) *S. aureus* or (c) *E. coli* O157:H7 and antibody-encoded AuSF nanoparticles captured by antibody-encoded magnetic nanochains.

Finally, the sandwich structure with targeted bacteria, *S. aureus* or *E. coli* O157:H7, were separated by a magnet and subjected to Raman detection. The results show that the SERS intensity gradually increases in a range from 10^2 to

10^8 CFU/mL as shown in Figure 4.13a and 4.13b. The specificity of detection was summarized in Figure 4.13c and 4.13d, PBS, LB buffer, BSA, *E. coli* O6, *E. faecalis* ATCC 29212 were chosen as control, and all the buffer, protein and bacteria in the same concentration show negligible signals. Therefore, the multi-functional AuSF nanoparticles make it possible for developing theranostic nanomedicine for bacteria detection.

Figure 4.13 SERS spectra of different concentrations of (a) *S. aureus* and (b) *E. coli* O157:H7. Inset: Raman intensity at 1341 cm^{-1} vs logarithm of the corresponding *S. aureus* and *E. coli* O157:H7. Specific detection of (c) *S. aureus* and (d) *E. coli* O157:H7 by Raman intensity at 1341 cm^{-1} of the magnetic separated sandwich structure from the assays of control buffers and various types of bacteria (10^7 CFU/mL).

4.4 Conclusion

In summary, we have developed a two-phase system technology that offers a convenient method in preparing the broad absorbance and multi-branches of plasmonic AuSF nanoparticles. We demonstrated that the slow reduction rate of Au precursors in the hexane-water interface allows easy designs of the NPs with different sizes. The Au (0) ions were deposited on Au seeds to form multiple sites to generate anisotropic branches. The strong coupling between adjacent branches leads to high enhancement Raman scattering efficiency, as well as red-shift of LSPR peak. The resulting AuSF nanoparticles represent excellent model systems that support NIR-II range optical properties. More importantly, optically active AuSF nanoparticles are of 52.7 and 53% of photothermal conversion value in 808 and 1064 nm laser, making it of great potential in PTT in NIR-II window. Combined with antibody-encoded magnetic nanochains, antibody-labeled AuSF nanoparticles can be used for selective SERS detection of bacteria. In addition, irradiation with NIR-II laser, it shows great potential in nanomedicine for pathogen photothermal killing.

Chapter 5 Broadband Magnetoplasmonic Nanocrystals

with Janus Core-Branch Structure: Biphasic Seeded

Growth for In Vivo Theranostic Applications

5.1 Introduction

The development of magnetoplasmonic nanomaterials is a topic of considerable interest because of the combination of plasmonic and magnetic properties.^{46, 52, 152} The plasmonic component allows optical detection,⁶³ PTT,^{101, 103} and PAI,¹⁵³ while the magnetic part provides magnetic targeting,¹⁶ MRI,^{146, 154} and magnetic hyperthermia.¹⁵⁵ However, each component has certain limitations, such as MRI suffers from limited spatial resolution, and optical imaging does not provide the penetration depth.¹⁵⁶ Multifunctional magnetoplasmonic nanoparticles, which can be utilized for synergistic optical/MRI imaging⁹⁵ and photothermal/hyperthermia therapy,¹⁵⁷ lead to the new approaches for overcoming the restrictions of current diagnostic tools.

AuNPs present good biocompatibility, low toxicity and easily tuned LSPR enabling them to widely use as nanomedicine.⁶ Consequently, intense research has focused on synthesis of hybrid magnetoplasmonic nanostructures,¹⁵⁸⁻¹⁶² and the existing hybrids in the field of biomedical applications are dimer,¹⁶³ Janus,¹⁶⁴ and core-shell¹⁰⁷ nanostructures. The spherical AuNPs of dimer or Janus magnetoplasmonic nanomaterials show weak LSPR band in NIR region,

thereby hindering in vivo applications because the visible light is almost fully absorbed or scattered by tissues.¹³⁰ Magnetoplasmonic NPs with core-shell nanostructure have been extensively investigated for their biomedical applications.^{43, 48, 165, 166} The Au shells, which have the excellent biofunctionalization and the capability to protect intracellular degradation of the magnetic core, are beneficial for long-term in vivo treatments.^{46, 157} Many groups, including ourselves, reported the encapsulation of magnetic core by an Au shell with the assistance of polymer or silica to prepare core-shell magnetoplasmonic nanomaterials.^{52, 167, 168} However, it involves multistep and is time-consuming to lower the overall yield. Furthermore, the sizes of assemblies are rather larger than 120 nm, which hinders the in vivo applications.^{45, 46} Another approach involves synthesis of magnetic core followed by epitaxial growth of Au shell.^{48, 49} However, the growth of magnetic core@Au nanospheres often needs multistep deposition of Au atoms to form a uniform Au shell, and they generally exhibit LSPR in the visible region. In order to enhance optical properties, anisotropic Au shells, such as Au nanostar,⁵⁰ popcorn,¹⁰⁷ nanorose⁴⁴, have been reported, exhibiting strong LSPR absorbance in NIR region. For example, Shi *et al.* reported the Au nanostars-coated magnetoplasmonic nanomaterials for in vivo dual-imaging and PTT of tumors by intratumoral injection of particles.⁴⁸ Yet, the growth of anisotropic Au shells always needs Ag⁺ ion or CTAB as ionic surfactants, which are toxic for biomedical applications.^{48, 50} In addition, these Au nanoshells lack precise

plasmonic control within a small size. Therefore, it is still challenging to develop NIR-active magnetoplasmonic nanomaterials with easy synthesis, suitable size, and low biological toxicity for biomedical applications.

In current study, NIR-II laser shows advantages for imaging and therapy because of the deeper penetration and high signal-to-noise ratio due to low scattering and absorption of tissues.^{135, 169, 170} Many Au nanomaterials, such as Au/Ag nanocages,¹³⁷ Au rod-in-shell,¹⁰⁵ and Au nanoechinus,¹¹¹ have shown excellent optical and photothermal properties irradiated by NIR-II laser. However, magnetoplasmonic nanomaterials with LSPR in NIR-II region for biomedical applications have rarely been studied. Here, we report that a magnetoplasmonic nanomaterial with C-B nanostructure synthesized by growth of Au branches on the Janus Au-MFO NPs in hexane-water phases at room temperature (Figure 5.1). Firstly, the C-B NPs with small size (40 nm) show broad LSPR peak from visible to NIR-II region (600 to 1350 nm) which can be easily controlled by adjusting the molar ratio of Au precursors to Janus seeds. Therefore, the relatively small size of the C-B NPs may permeate barriers more rapidly including membranes and leaky vasculature in cancers based on the EPR effect.⁴⁴ Secondly, these multifunctional C-B NPs provide a theranostic platform to combine magnetic and plasmonic properties. Magnetic measurements show C-B NPs are superparamagnetic with a high T_2 relaxivity. Simultaneously, C-B NPs show high SERS intensity and photothermal conversion efficiency. Thirdly, the Au branches which grow around the

magnetic core to protect it from separation were formed in absence of Ag^+ ion and CTAB to lower the cytotoxicity. We have demonstrated that the C-B NPs show low cytotoxicity, guidance of MRI and Raman monitoring, highly efficient tumor eradication by NIR-II laser for in vivo mice model.

Figure 5.1 Scheme of in vivo imaging and therapy.

5.2 Experimental Section

5.2.1 Materials and Characterization

Materials. $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was purchased from Alfa Aesar. Iron(III) acetylacetonate ($\text{Fe}(\text{acac})_3$), oleic acid, benzyl ether, propidium iodide (PI), Manganese(II) acetate ($\text{Mn}(\text{OAc})_2$), oleylamine 70% and 90%, dimethylformamide (DMF), chloroform (CF), ethanol, hexane, hydroxylammonium chloride ($\text{HONH}_2 \cdot \text{HCl}$), N,N'-dicyclohexylcarbodiimide (DCC), 4-nitrothiophenol were purchased from Sigma-Aldrich. Carboxyl-poly(ethylene glycol)-thiol (MC-PEG-SH, $M_n = 3400$), methoxypoly(ethylene glycol)-thiol (PEG-SH, $M_n = 5000$) were received from Laysan Bio, Inc. Janus nanoparticles seed (Janus NP), and mPEG-phosphonate was synthesized according to our previous report.¹⁷¹ 10×TAE buffer pH 8.0 and agarose were purchased from 1st base (Singapore). 10×TAE buffer pH 8.3 and agarose were purchased from Promega (Madison, USA). Sodium chloride was purchased from Merck (Darmstadt, Germany).

Characterization. TEM images and UV-vis-NIR absorbance spectra were obtained according to chapter 3. Dark field images were collected by an Olympus IX71 inverted microscope with the aid of oil-immersion dark field condenser, and a photometrics coolSNAP-cf cooled CCD camera. The single-particle Raman spectra were obtained by using a system which consists of an inverted microscope equipped with a dark-field condenser, camera

(Princeton Instrument PIXIS back illuminated CCD with eXcelon technology, 1340x100 pixels, 20x20um pixel size), a spectrometer (Princeton Instrument Acton series SP-2150), a grating (600 G/mm with 750 nm blaze wavelength), a 785 nm laser (TOPTICA iBeam smart Wavelength-Stabilized diode laser), a filter for 785nm laser (Semrock 785 nm MaxLine® laser clean-up filter), a beamsplitter (Semrock 785nm RazorEdge Dichroic™ laser-flat), and an edge filter (Semrock 785 nm EdgeBasic™ best-value long-pass). The X-ray diffractometer (XRD) were tested by a Bruker AXS D2 Advanced X-ray diffractometer with Cu K α radiation ($\lambda=0.15406$ nm). The magnetization curves were obtained by using a MPMS-VSM. Inductively coupled plasma mass spectroscopy (ICP-MS) was obtained on a Prodigy High Dispersion ICP-MS.

5.2.2 Synthesis of Au-MFO Seed

The hydrophobic AuNPs were prepared as shown in last chapter. A solution of 0.2 mmol of Fe(acac)₃, 0.1 mmol of Mn(OAc)₂, 4 ml of oleylamine 70%, and 2 mL of oleic acid were mixed and then increased temperature to 80 °C. 5 mL of above Au NPs solution was added, and then water and hexane were evaporated in the solution at 150 °C. Then the solution was slowly increased to 260 °C under a nitrogen flow, and reacted at this temperature for 20 min. After the mixture cooled to 25 °C, the products were precipitated by adding ethanol and separated with a magnet. The Au-MFO was dispersed in 10 mL of hexane.

5.2.3 Synthesis of C-B NPs

A novel water-oil phase mixture system was employed to prepare the C-B NPs. First, a precursor solution was prepared using 0.48 mL of 50 mg/mL $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ in acetonitrile, 0.6 mL of oleylamine and 12 mL hexane. The solution was sonicated, and then the liquid phase was removed. The as-fabricated HAuCl_4 and oleylamine complex in 15 mL of hexane was reserved for further usage. Second, 0.2 mL of 400 nM Au-MFO Janus NPs in hexane and 0.2 mmol of $\text{HONH}_2 \cdot \text{HCl}$ in 3 mL DI water were added into 15 mL of hexane under vigorous stirring. Third, the precursor solution (1, 5, or 12 mL) was added into the mixture of Janus NPs and $\text{HONH}_2 \cdot \text{HCl}$. Afterward, 1 mL of ethanol was added into the mixture and the as-fabricated core-branch nanoparticles were aggregated. The C-B NPs were collected with a magnet and redispersed in CF, and the C-B NPs with 1, 5, or 12 mL of precursor solutions were named as C-B1, C-B2, and C-B3, respectively. 0.05 mL of 4-nitrothiophenol solution (1 mg/mL) was added, and the mixture was sonicated for 15 min for sufficient loading of Raman dye on C-B3 NPs surface. Afterward, 5 mg of HS-PEG (MW = 5000) and 4 mg of mPEG-phosphonate (MW=5000) were added for modification of Au and IO parts, respectively, and then stirred for another 12 h at room temperature. The mixture was precipitated in hexane and separated by a magnet to obtain C-B3 NPs modified by Raman dye in aqueous solution.

5.2.4 Characterization of C-B NPs

The size and nanostructure of C-B NPs were observed by a transmission electron microscope (TEM). The UV-vis-NIR absorbance spectra of PEGylated C-B NPs before and after transfer from CF to DI water were recorded on a UV-vis-NIR spectrophotometer. The stability of PEGylated C-B3 NPs in different solution, containing DI water, 50 mM MES (pH = 5.0) buffer, 1×PBS buffer, 10 mM tris-HCl (pH = 8.5) buffer, 50 mM borax / sodium hydroxide (pH=10) buffer, CF, DMF, and ethanol, were tested by a UV-vis-NIR spectrophotometer. The electrophoresis of C-B3 NPs modified by HS-PEG:HS-PEG-CM (1:0, 3:1, 1:1, 1:3, 0:1) were loaded in 1 % agarose gels using 0.5×TBE buffer as running buffer. Gel wells were sealed with 2.5 % agarose gels. The images were taken after that gels ran at 100 V, 8 V/cm for 10 min.

5.2.5 MRI Characterization

C-B3 NPs were prepared in water with different iron concentrations from 0 to 0.16 mM. The samples were placed in an MR scanner and tested by using a number of MR sequences. The T_2 relaxation time of the suspension was computed using MATLAB V7.1. The T_2 relaxation rates were plotted versus iron concentrations. The relaxivity was computed using linear regression analysis.

5.2.6 NIR Laser-Induced Heat Conversion

The aqueous solution of C-B1, C-B2, and C-B3 NPs with 30 ppm Au element concentration were irradiated by 1064 and 808 nm laser (1 W/cm², 8 min). Pure water and AuNRs was used as a negative control. Real-time temperature of the samples was recorded using a FLIR thermal camera. The η value of C-B NPs was calculated according to the method in last chapter.

5.2.7 In Vitro Cytotoxicity

MCF7 cells were seeded in 96-well plates at a density of 1.0×10^4 cells/well and incubated for 24 h. The cells were exposed to PEGylated C-B3 NPs with or without magnetic attraction for 20 min on the bottom of 96-well plates, and then incubated for 2 h at 37 °C. The concentrations of C-B3 NPs were 0, 0.03, 0.05, 0.1, and 0.15 mg/mL. After that, the medium was changed with fresh medium, and cultured in the incubator for 24 h. Cell survival efficiency was measured using the standard CCK-8 assay. The data represent the average results of triplicate measurements.

5.2.8 PPT of MCF7 Cells Loaded with C-B3 NPs

MCF7 cells in 96-well plates were exposed to 0.2 mL of 0.025 or 0.05 mg/mL PEGylated C-B3 NPs solution. After with or without magnetic targeting for 20 min on the bottom of 96-well plates, the cells were then irradiated with 1064 nm laser (1 W/cm², 6 min). After PTT, MCF7 cells were washed with

fresh medium, and incubated in fresh medium for another 24 h before the cell viability was calculated using the CCK-8 assay. The data reported represent the means of triplicate measurements. In the live/dead assay, MCF7 cells were stained with calcein AM and propidium iodide.

5.2.9 Live Cell Raman Imaging

The glass slides were covered with MCF-7 cells, and treated with PEGylated C-B3 NPs modified by 4-nitrothiophenol with or without 20 min magnetic targeting. And the Raman mapping was recorded by a 785 nm laser. The laser focal spot was 1 μm and mapping measurements at 1341 cm^{-1} were carried out as raster scans in 1 μm steps with 1 s as the integration time per step.

5.2.10 Animal Preparation

All animal experiments were carried out in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes approved by the Ethics Committee of Union Hospital, Huazhong University of Science and Technology. Female BALB/c nu/nu mice (provided by Beijing HFK Bioscience Co. Ltd.), aged 4-6 weeks, were supplemented with 17β -estradiol (3 mg/kg per week) to support the growth of the estrogen-dependent MCF-7 tumors. Breast tumors were established by orthotopic injection of 5×10^6 MCF-7 cells suspended in 100 μl of serum-free medium: Matrigel mixture (1:1 ratio) to mammary fat pad of each mouse.

5.2.11 In Vivo MR/Infrared Thermal Imaging

For MR imaging, the mice bearing tumors were anesthetized with pentobarbital sodium (40 mg/kg body weight) by intravenous injection. Before and after administration of C-B3 NPs (4 mg/mL, 0.1 mL) through the tail vein, transversal cross-sectional scan images were acquired with a 3.0 T MRI system using spin echo sequence. The T₂-weighted sequence parameters were as follows: FOV=80 mm, base resolution=192×192, slice thickness=1.2 mm, TE=62 ms, TR=3000 ms, and scanning time=3 min.

For infrared thermal imaging, the mice were injected intravenously with C-B3 NP solution (1 mg/mL, 100 µL) or PBS. 24 h after injection, the tumors were irradiated with a 1064 nm NIR laser (1.5 W/cm², 5 min). Thermographic pictures were taken using a FTIR camera.

5.2.12 Photothermal Treatment with NIR Light and Pathologic Analyses

When the tumor size reached ~75 mm³, the MCF-7 tumor mice were randomly divided into 4 groups (5-7 mice/group). For the treatment group (n=6), mice were injected intravenously with C-B3 NP (1 mg/mL, 100 µL) and then irradiated by the 808 nm laser (1.5 W/cm²) for 5 min. The control groups of mice included mice with PBS administration (control, n=5), mice with C-B3 NP administration only (C-B3, n=6), and mice with PBS administration subjected to laser irradiation (PBS+1.5 W/cm², n=6). The tumor sizes were measured and

calculated as the volume (V) = $ab^2/2$, where a is the length and b is the width of the tumor, respectively.

For pathologic analyses, the tumor-bearing mice were sacrificed twenty-four hours after the laser treatment. The tumors were fixed in 4% paraformaldehyde and processed for H&E staining.

5.2.13 Biodistribution

The mice were sacrificed at 3 h and 24 h intravenously postinjection. Heart, lung, liver, spleen, kidney, brain and tumor were collected for gold content analysis. The organs were weighed and digested in 2 mL of boiling aqua regia for ICP-MS analysis.

5.3 Results and Discussion

5.3.1 Synthesis of C-B NPs

Preparation of magnetoplasmonic heterostructures, which provides a synergic efficiency, such as in vivo MRI, magnetic targeting, and photothermal cancer therapy, has attracted significant research attention in the past decades. Taking advantage of slow nucleation reactions in a water-hexane two-phase system, we successfully get 35 nm magnetoplasmonic C-B NPs with Au branches surrounding the surface of MFO core. In this two-phase synthesis system, hydrophobic Janus Au-MFO NPs (Janus seed NPs) composing an 8 nm AuNP and a 10 nm MFO (Figure 5.2a) were dispersed in 6 mL of hexane, and 0.2 mM $\text{NH}_2\text{OH}\cdot\text{HCl}$ in 1 mL of water was mixed with hexane phase by stirring vigorously. Then, the Au precursor prepared by dissolving AuCl_4^- and oleylamine in hexane (see Experiments) was slowly dropped into above mixture with a speed of 0.1 mL/min at room temperature. In order to tune the length of Au branches, different amount of Au precursor was added, and then precipitated by adding ethanol and purified by magnetic separation. Importantly, supernatant is colorless, suggestive of no AuNPs self-nucleated in the reaction. The C-B NPs were characterized by TEM as shown in Figure 5.2. By increasing the molar ratio of Au precursors to Janus seeds (5:1, 12:1, 35:1), the lengths of Au branches can be modulated to 5, 15 and 30 nm (C-B1, C-B2, and C-B3 NPs, respectively) with a narrow size distribution.

Figure 5.2 TEM images of (a) Janus seed NPs, (b) C-B1 NPs, (c) C-B2 NPs, and (d) C-B3 NPs.

The XRD patterns of MFO, Janus seed, C-B1, C-B2, and C-B3 NPs (Figure 5.3) provide additional evidence for the forming of Au branches. Interestingly, the Au branches originated from the AuNP of Janus seeds and grow toward the MFO part, different from heteroepitaxial nucleation for core-shell structures or one nucleated site on subnanometer particles. For example, Liz-Marzan reported that the Au branches protruded from Au-silica Janus seeds opposite the silica part only in the presence of silver ions, otherwise

Au just overgrew rather than forming Au branches.¹⁷² Sun reported the growth of Au on Au-IO NPs in octadecene at 80 °C, and found that the epitaxial growth of a new AuNP on Au-IO NP. At longer growth time, the new AuNP can pull the Au part away from the Fe₃O₄ NPs because of increasing surface tension.¹⁷³ By contrast, the magnetoplasmonic C-B NPs in this work show an interesting structure as close-up of a hand holding a ball. The Au branches around the MFO core can serve as a cage avoiding the MFO parts detaching from magnetoplasmonic NPs. Compared to the common seed-mediated growth of Au branches, no silver ions or CTAB are needed in this protocol, thus providing a magnetoplasmonic C-B NPs with low cytotoxicity for biomedical applications.

Figure 5.3 XRD patterns of MFO, Janus seed, C-B1, C-B2, C-B3 NPs.

To understand the growth of C-B NPs, the morphologies of the Janus seed and C-B1 NPs were examined by HRTEM. From Figure 5.4a, Janus seed NPs show that the lattice fringes are about 0.24 nm and 0.25 nm related to (111) planes of Au and (311) plate of MFO, respectively.^{173, 174} The HRTEM image of C-B1 shows the (111) lattice fringes of Au branch aligned parallel to (311) lattice fringes of MFO NPs as shown in Figure 5.4b, which means that the MFO also directed epitaxial growth of Au branches. As shown in Figure 2b, all the Au branches can grow around surface of MFO and generate a bend rod at early stage. After the branches extending the MFO parts, the newly formed Au branches became straight rods. For comparison, when we change the Janus seed NPs with AuNPs, the Au branches became straighter than that on Janus seed NPs (Figure 5.5). Therefore, the epitaxial relationship between MFO and Au branches leads to the unique core-branch growth of Au branches in this work.

Figure 5.4 HRTEM images of (a) Janus seed and (b) C-B1 NPs.

Figure 5.5 (a) UV-vis-NIR absorbance spectra of Au seed and C-B NPs synthesis based on Au seed. (b) TEM images of branches nanoparticles synthesis based on Au seed.

5.3.2 LSPR of C-B NPs

Figure 5.6a shows the LSPR of the magnetoplasmonic Janus NP seeds, C-B1, C-B2, and C-B3 NPs in UV-vis-NIR region. Initially, Janus NP seeds display a narrow LSPR at 541 nm. When the Au branches start to grow, a new peak in NIR region emerges with increasing the length of Au branches. The C-B3 NPs with the longest Au branches showed a broad absorbance covering the whole NIR window (650-1350 nm), due to the strong coupling of closely-packed Au branches. Therefore, the C-B3 NPs which cover both NIR-I and NIR-II windows were chosen for biomedical applications. In order to examine the uniformity of C-B3 NPs, the optical behavior of C-B3 NPs was further examined by dark field microscope. The scattering spectra (Figure 5.6b) of randomly selected single C-B3 NP showed a similar signal from 400 to 1000

nm, and the scattering light showed homogenous signals in the view. The scattering color of the C-B3 NPs shows almost identical yellow-white color due to the broad LSPR and small size.

Figure 5.6 (a) UV-vis-NIR absorbance spectra of Janus seed, C-B1, C-B2, and C-B3 NPs. (b) Scattering spectra of C-B3 NPs. The inset: the dark field image of single C-B3 NPs.

Figure 5.7 Photographs of PEGylated C-B1, C-B2 and C-B3 NPs before and after transferring from CF to water, and photographs of PEGylated C-B3 NPs in (1) water, (2) MES (30 mM, pH=5.0) buffer, (3) 1×PBS buffer, (4) tris-HCl (30 mM, pH=8.5) buffer, (5) borax / sodium hydroxide (pH=10) buffer, (6) CF, (7) DMF, and (8) ethanol.

Meanwhile, the color of colloid solution changed is controlled by their structural parameters, such as size, shape, local environment, and chemical composition. The magnetoplasmonic C-B NPs show a color change accompanied by increasing the length of Au branches as shown in Figure 5.7. The Increase in the length of Au branches can result in a red-shift in surface plasmon resonance band, leading to red to black solution. The mechanism of red-shift is that no uniform electric field of the nanomaterials forms. In order to make water-soluble C-B NPs, the NPs were modified by PEG, and the PEGylated C-B NPs were well-stabilized into water (Figure 5.7). Although the surface ligands of C-B NPs were changed from oleylamine to HS-PEG, the color of C-B NPs solution didn't changed as well as their UV-vis-NIR absorbance spectra (Figure 5.8a). Importantly, the PEGylated C-B3 NPs were readily dispersed in different solutions (Figure 5.8b) to show a high stability for broad biomedical applications.

Figure 5.8 (a) C-B NPs before and after transferring from CF to water. (b) UV-vis-NIR absorbance spectra of PEGylated C-B3 NPs in different solutions.

5.3.3 Electrophoresis of C-B NPs

Electrophoresis was used for test the HS-PEG-Carboxymethyl-modified C-B3 NPs. The Figure 5.9 shows that C-B3 NPs moved faster after coating with high ratio of HS-PEG:HS-PEG-Carboxymethyl (1:0, 3:1, 1:1, 1:3, 0:1). The C-B3 NPs with carboxymethyl groups on the surface of NPs have great potential for further bioconjugated applications.

Figure 5.9 Electrophoretic analysis of C-B3 NPs modified by HS-PEG:HS-PEG-CM = 1:0, 3:1, 1:1, 1:3, and 0:1 from left to right.

5.3.4 SERS of C-B NPs

The strong plasmon band in NIR region and easy modification through Au-S bonds allow us to investigate the SERS property of C-B3 NPs. Same Au molar concentration of Janus seeds and C-B NPs modified by 4-nitrothiophenol were dispersed in water and irradiated by 785 nm laser. As shown in Figure 5.10a, Raman tag-labeled C-B3 NPs show the highest SERS intensity compared to the Janus seeds, C-B1, and C-B2 NPs. The calculated Raman enhancement factor (EF) of the C-B1, C-B2, and C-B3 NPs are about 3×10^5 , 1.2×10^6 and 8

$\times 10^6$, respectively. The C-B3 NPs with the longer and sharp Au branches contain higher number of electrons excited than C-B1 and C-B2 NPs,⁶⁸ and the coupling of adjacent Au branches lead to stronger electromagnetic fields, results in the highest signal intensity of the C-B3 NPs⁶³ The Raman tag-labeled C-B3 NPs also shown strong Raman signal when excited by 633 nm laser (Figure 5.10a), indicating an dual-wavelength SERS by C-B3 NPs. Figure 5.10b shows the averaged single-particle SERS spectra (n=6) of Raman tag-labeled C-B1, C-B2, and C-B3 NP, respectively. The SERS spectra show the characteristic peaks of 4-nitrothiophenol at 1078, 1341, and 1575 cm^{-1} , same with previous normal Raman spectra. The single-particle SERS spectra of C-B3 NPs with the strongest Raman intensity allow the single-particle SERS detection.

Figure 5.10 (a) SERS spectra of Janus seed, C-B1, C-B2, and C-B3 NPs labeled by 4-nitrothiophenol. (b) The averaged SERS spectra (n = 6) obtained from the single C-B1, C-B2, and C-B3 NPs, respectively. The inset: SERS intensity of C-B NPs at 1341 cm^{-1} .

5.3.5 Magnetic Properties of C-B NPs

The PEGylated C-B3 NPs containing a MFO core exhibited excellent magnetic properties. Magnetic measurement (Figure 5.11a) shows that the MFO, Janus seed NPs, and C-B NPs are superparamagnetic with a saturation magnetization. The saturation magnetization of MFO and Janus seed NPs are 51 and 30 emu/g at 300 K, respectively. The saturation magnetization of C-B3 NPs is about 4 emu/g because of much Au element on it. The spin-spin relaxation time (T_2)-weighted MR images (Figure 5.11b) show that the reduced intensity is significant at high iron concentration, leading to an appreciable negative contrast enhancement (dark signal) in MRI. The relaxation rate of the C-B3 NPs increases with higher iron concentrations. As reported by Cheon, the M_s value of the Mn doped C-B NPs is higher than that of IONPs.¹⁷⁵ Consistent with the magnetization results, C-B3 NPs showed a high T_2 relaxivity, $r_2=138 \text{ mM}^{-1}\text{s}^{-1}$.

Figure 5.11 (a) Room-temperature magnetization measurements of the MFO, Janus seed, and C-B3 NPs. (b) T_2 -weighted MR images of C-B3 at different concentrations, and corresponding T_2 relaxation rate of C-B3 NPs as a function of the iron concentration (mM).

5.3.6 Photothermal Conversion of C-B NPs

Another important attribute of magnetoplasmonic C-B NPs is the photothermal effect. The temperature changes of C-B NPs aqueous solutions (30 ppm of Au concentration in 0.5 mL) under 808 nm (1 W/cm^2) were recorded every 20 s by a FLIR camera (Figure 5.12a). The C-B3 NPs aqueous solution showed a rapid temperature increase and reached 73.1°C in 8 min. In contrast, water shows minor temperature increase, indicating the high photothermal effect of C-B NPs. For C-B2 and C-B1 NPs aqueous solutions, the temperatures only reached 61.6 and 49.4°C , respectively. Then the η value of C-B3, C-B2, and C-B1 NPs are calculated to be 53.5%, 45.3%, and 42.8%, respectively (Figure 5.12c). The C-B3 NPs show higher η value than C-B2 and C-B1 NPs, and it should be attributed to the high optical absorption cross section. The control experiment was carried out by AuNRs with LSPR at NIR region (Figure 5.13). The AuNR solution with same OD_{808} as C-B3 solution, the temperature of solution can reach 64.5°C after irradiation by 808 nm laser for 8 min. The η value of this AuNR solution was calculated to be 45.2%. According to the Mie theory, both the absorption and scattering cross sections increase with larger particles sizes. However, the scattering cross sections increases at a faster rate that is proportional to V^2 (V : volume of the particles), and the absorption cross sections increases at a rate proportional to V .⁵³ The higher scattering cross section decreases the photothermal efficiency, while the absorption of light promotes the converting photons or vibrations of crystal

lattice into heat.¹⁰¹ Thus, the C-B3 NPs show a higher η value may be due to that the C-B3 NPs have smaller size than AuNRs.

The C-B3 NPs with broad absorbance were performed as a photothermal agent by laser in the NIR-II window. The temperature change of C-B NPs solution under 1064 nm at a density of 1 W/cm² was also recorded (Figure 5.12b). Similar to 808 nm laser, the solution of C-B3, C-B2 and C-B1 NPs showed a rapid temperature increase from 22 °C to 64.8, 56.3, and 37.9 °C in 8 min, respectively. The η value of C-B3 is estimated to be 36.1%, which is higher than C-B2 (35.2%) and C-B1 NPs (27.9%). The AuNRs solution containing same OD₁₀₆₄ was taken as control, and both the elevated temperature (62 °C) and η value (33.7%) are lower than C-B3 NPs. Furthermore, the photothermal stability of C-B3 NPs was tested by absorbance spectra and TEM (Figure 5.14). After the C-B3 solution was exposed to 1064 nm laser, the structure and UV-vis-NIR spectra of C-B3 NPs remain same before and after laser excitation.

Figure 5.12 Plots of temperatures as a function of irradiation time for C-B3, C-B2, C-B1, and AuNR NPs aqueous solution by (a) 808 nm and (b) 1064 nm lasers. The Au concentration of C-B3, C-B2, and C-B1 are 30 ppm. AuNR as a control was adjusted to give same OD with C-B3 NP at 808 nm and 1064 nm, respectively. Plot of cooling time versus negative natural logarithm of the temperature driving force obtained from the cooling stage at (c) 808 nm and (d) 1064 nm.

Figure 5.13 UV-vis-NIR absorbance spectra and TEM image of AuNR NPs.

Figure 5.14 Photothermal stability comparison before and after irradiation with a 808 nm laser ($1\text{W}/\text{cm}^2$), (a) UV-vis-NIR absorbance spectra, (b) infrared thermal image of C-B3 NPs after laser irradiation, TEM images of C-B3 NPs (c) before and (d) after laser irradiation.

5.3.7 In Vitro PTT

Encouraged by high η value of C-B3 NPs, we next investigated PTT for MCF-7 cancer cells by 1064 nm laser. A live/dead assay with calcein AM staining live cells and PI highlighting dead/late apoptotic cells reveals the photothermal effect of C-B3 NPs (Figure 5.15). In the case of incubation with C-B3 NPs and then exposure to $1.5\text{ W}/\text{cm}^2$ of 1064 nm laser for 6 min, most MCF-7 cells showed red color after calcein AM and PI staining, indicating MCF-7 cells had been killed by the PTT of C-B3 NPs. In the control groups, almost all the cells displayed a green fluorescence indicating low therapeutic

efficiency by only C-B3 NPs or laser.

The viability of corresponding MCF-7 cells was further measured quantitatively using CCK-8. As shown in Figure 5.16, no obvious cytotoxicity was shown for the MCF-7 cells exposed to 1064 nm laser (up to 1.5 W/cm^2) or treated with C-B3 NPs (up to 0.15 mg/mL) alone. The MCF-7 cells were incubated with C-B3 NPs (0.05 mg/mL) for 20 min without magnetic enrichment of C-B3 NPs to the surface of cells. Afterward, the supernatant was replaced by PBS and exposed to 1064 nm laser, 91% of cells remained alive. However, after the magnetic enrichment of C-B3 NPs to the cells for 20 min and then removal of free samples, the Raman mapping (Figure 5.17) with signal at 1337 cm^{-1} shows a significant accumulation of C-B3 NPs on the surface of cells by an external magnet. The control experiments without adding C-B3 NPs or only C-B3 NPs without magnetic enrichment show weak Raman signal. The cell incubation with C-B3 NPs in the presence of a magnetic field, and then exposed to 1064 nm laser after removing free samples, a considerably better therapeutic effect than that without magnetic enrichment. This is because the heat diffuses into medium from the C-B3 NPs and only leads to an elevated temperature of the surrounding medium.¹⁷⁶ When the laser power was 1 W/cm^2 , 82% and 90% of MCF-7 cells died for the 0.025 and 0.05 mg/mL of C-B3 NPs, respectively. Therefore, this makes magnetoplasmonic C-B3 NPs an attractive choice as magnetically-targeted PTT agents for effective treatment of cancer cells.

Figure 5.15 After MCF-7 cancer cells incubation with 0.05 mg/mL PEGylated C-B3 NPs with magnetic enrichment, confocal images of MCF-7 cancer cells after PTT irradiated by an 1064 nm laser (1.5 W/cm^2 , 6 min). Cells were stained with calcein AM and PI after 24 h post incubation. (a) No laser and C-B3 NPs. (b) Laser only. (c) C-B3 NPs. (d) Laser and C-B3 NPs. Scale bar = 100 μm .

Figure 5.16 (a) Viability of MCF-7 incubated without (black bar) and with (blue bar) PEGylated C-B3 NPs exposed to 1064 nm laser for 6 min, and incubated with 0.025 mg/mL (red bar) or 0.05 mg/mL (dark yellow bar) PEGylated C-B3 NPs with magnetic enrichment and then exposed to 1064 nm laser for 6 min. (b) Viability of MCF-7 cancer cells incubated different concentration of C-B3 NPs with or without 20 min magnetic attraction.

Figure 5.17 Bright-field (left) and Raman mapping (right) of (a) the MCF-7 cells, (b) labeled with PEGylated C-B3 NPs without magnetic attraction, and (c) labeled with PEGylated C-B3 NPs with 20 min magnetic attraction.

5.3.8 Biodistribution

We next sought to investigate the biodistribution of C-B3 NPs in mouse model. The PEGylated C-B3 NPs (200 μ L, 2 mg/mL) were intravenously injected through the tail vein, and the individual tissues of mice at 3 and 24 h postinjection were analyzed by ICP-MS (Figure 5.18). The tumor uptake of the C-B3 NPs reached $\sim 3.5\%$ ID/g at 3 h and $\sim 8\%$ ID/g at 24 h, suggesting the significant accumulation of C-B3 NPs in tumors by the EPR effect. The lung and heart which are bloodstream organs show correspondingly high Au content at 3 h and progressive clearance to 24 h. It can suggest that the PEGylated C-B3 NPs actively circulated in the bloodstream at early stage.¹⁰¹ The C-B3 NPs

show a high uptake in liver ($\sim 28\%$ ID/g at 3 h and $\sim 35\%$ ID/g at 24 h) due to its abundance of Kupffer cell for eliminating C-B3 NPs through phagocytosis.¹⁷⁷ The spleen uptake of C-B3 NPs reached $\sim 15\%$ ID/g at 3 h and $\sim 25\%$ ID/g at 24 h may attribute to the accumulation of injected NPs from body into spleen.¹⁷⁸ The Au concentration in kidney and lung decreased over time indicating possible clearance of C-B3 NPs through the renal system.¹⁷⁹ In addition, the brain with low uptake of C-B3 NPs reached about only 1% at both 3 and 24 h, and it may be attribute to the blood-brain barrier (BBB).

Figure 5.18 Biodistribution of C-B3 NPs in tumor, heart, liver, spleen, lung, kidney and brain at 3 and 24 h postinjection (n=3).

5.3.9 Optical Imaging

The magnetoplasmonic C-B3 NPs can provide multi-imaging by the advantages of magnetic and plasmonic properties for tumor resection surgery. The MFO core of C-B3 NP provides a MRI detection agent for delineating the

margins of tumors in mouse model. The T_2 -weighted spin echo images were recorded at 0, 1, 3, and 24 h after intravenous injection of C-B3 NPs (Figure 5.19). The results showed that the T_2 signal intensity of tumor was darkened significantly after 1 h of C-B3 NPs injection and marked negative enhancement at 24 h. The MRI analysis provides an efficient approach to determine the macroscopic outline of the tumor. The mice were sacrificed and tumor was analyzed by Raman detection after removing the epidermis. Figure 5.20 shows the Raman spectrum of tumor at 0 and 24 h after intravenous injection of C-B3 NPs. The Raman signal of tumor at 24 h intravenous postinjection is much stronger than tumor at 0 h. Because of the sensitive and specific signature of the SERS spectrum, it is benefit for accurate resection of tumor for intraoperative monitoring.

Figure 5.19 In vivo MR images obtained at (a) 0, (b) 1, (c) 3, and (d) 24 h after intravenous injection of C-B3 NPs into BALB/c nu/nu mice bearing subcutaneous MCF-7 breast tumors.

Figure 5.20 Raman spectrum of tumor before and after intravenous injection of C-B3 NPs.

5.3.10 In Vivo PTT

We firstly studied the photothermal conversion of the C-B3 NPs using a mice tumor model. At 24 h postinjection with PBS or C-B3 NPs solution, the mice were irradiated upon 1064 nm laser at a power of 1.5 W/cm². For the mouse injected with C-B NPs, the infrared thermal imaging shows that the local tumor temperature increased by ~20 °C within 5 min (Figure 5.21). The thermal images recorded at 5 min indicated that the temperature in tumor region was higher than the other parts of body. The temperature change in vivo is sufficient to provide the irreversible tumor damage for a postoperative oncotherapy. In contrast, the mice treated with PBS only showed 3.6 °C higher than the initial tumor temperature after 5 min exposure to the laser. Therefore, the tumor cells can be destructed only the mice treatment with both C-B3 NPs and laser.

The in vivo therapeutic efficacy of C-B3 NPs was evaluated as shown in Figure 5.22a. Tumor-bearing mice (5 mice/group) were intravenous injected with 200 μL of the PEGylated C-B3 NPs (2 mg/mL) or 200 μL of the saline, respectively. At 24 h postinjection, the tumor sides were exposed to 1064 nm laser ($1.5 \text{ W}/\text{cm}^2$, 5 min), and all the tumors were completely ablated leaving black scars at their original sites without reoccurrence over a course of 12 days (Figure 5.22b). The tumor-bearing mice in all three control groups showed similar growth rate, suggesting that laser irradiation or C-B3 NPs alone did not affect the tumor growth (C-B3 NP vs control, $P < 0.0001$). Furthermore, no significant body weight variation and side effects was noticed after the PTT treatment, which indicated that the PEGylated C-B3 NPs exhibited biocompatibility.

Figure 5.21 Infrared thermal image of the tumor-bearing mouse treated (a, b) without and (c, d) with C-B3 NPs before and after 1064 nm laser irradiation.

Figure 5.22 (a) Relative tumor volume of the tumor-bearing mice after intravenous injection of the samples and exposed to the 1064 nm laser (1.5 W/cm², 5 min), and corresponding photographs of tumor from mice (Inset). (b) Tumor-bearing mice treated with C-B3 NPs (above), and 12 days after tumor-bearing mice treated with C-B3 NPs and 1064 nm laser.

5.3.11 Histopathological Analysis

The histopathological analysis of the tumors specimens of the control groups (Figure 5.23) shows that there are no differences regarding the cells' size, shape, nuclear modifications, or necrosis after irradiation, indicating limited therapy from only laser or C-B3 NPs treatment. In contrast, the common features of thermo-necrosis present on tumor section in C-B3 NPs plus laser treated group, such as intensive necrosis area stained by eosin, malignant cells shrunken, loss of contact, eosinophilic cytoplasm, and nuclear damage (pyknotic and fragmentized nuclei). Few viable tumor cells with highly pleomorphic nuclei and many mitoses were found within the tumor tissue. It indicated that the tumor under treatment with both magnetoplasmonic NPs and

lasers showed obvious cancer necrosis. However, the mice irradiated by laser or treated with PBS and C-B3 NPs did not show any tumor necrosis. Therefore, the results suggest that C-B3 NPs have a great superiority as a photothermal agent for the PTT of tumor by NIR-II laser.

Figure 5.23 Hematoxylin and eosin (HE) staining of tumor sections collected from treatment group (a) control, (b) only C-B3 NPs, (c) only laser, and (d) C-B3 NPs + laser of mice at day 1.

5.4 Conclusion

In summary, a new type of magnetoplasmonic C-B NPs was synthesized in water-hexane phase system for combination of dual-imaging and PTT for cancer therapy. One key finding is that the hexane-water system and magnetic core could be benefit for growth of Au branches from Au part to cove MFO part. The C-B NPs show a unique structure similar to Au branches holding a magnetic core to provide the magnetoplasmonic NPs with a high stability. The LSPR peak of C-B NPs can be easily tuned to NIR-II region by increasing the length of Au branches. More importantly, the C-B3 NPs with high absorbance in NIR-II region show a small size (40 nm), which promotes a high accumulation in tumor by intravenous injection. The multifunctional C-B3 NPs provide a synergetic imaging and therapy for tumor treatment. The magnetic properties of C-B3 NPs allow for MRI imaging at preoperative, and the plasmonic properties of that can be used for Raman monitoring at intraoperative, and PTT at postoperative. In addition, the mice which were treated with both NIR-II laser and C-B3 NPs showed excellent cancer necrosis with no side effects. Therefore, this unique imaging at different stages of operative combination with effective PTT of C-B3 NPs open new possibilities to enable more accurate tumor diagnosis and resection in future clinical applications.

Chapter 6 Conclusions and Future Work

6.1 Conclusions

In this thesis, we have successfully developed a series of magnetoplasmonic nanomaterials to apply for biomedical detection and therapy. Multifunctional Au nanomaterials here exhibit many important properties, such as catalysis, optical sensing, SERS, photothermal conversion, and other optical properties. The magnetic chains or NPs here show self-mixing, magnetic separation and targeting, and MRI properties. The combination of magnetic and plasmonic nanomaterials then has been used for several biomedical applications.

Firstly, we fabricated a novel type of magnetic nanochains-supported AuNPs for GOx-mimicking catalysis, self-mixing in ultrasmall volume, and colorimetric detection of DNA. The PDA coated magnetic nanochains can reduce Au precursors to produce small size of AuNPs *in situ*. The magnetic nanochains@AuNPs possesses a combination of magnetic and catalytic properties, showing better catalytic activity when in the presence of an external magnetic field. However, the catalytic activity of AuNPs can be blocked by ssDNA. Therefore, this magnetic nanochains@AuNPs has been successfully used for DNA detection.

Secondly, we put forward a strategy to design anisotropic Au

nanomaterials with multi-branches to exhibit enhanced plasmonic coupling for optical sensing applications. Here, the hexane-water phases can help to slowly reduce the Au precursors, which results in anisotropic growth of Au branches from seeds. This multi-branches Au nanomaterials show a broad LSPR peak, high SERS activity, and high photothermal conversion efficiency. Combination with the bioconjugated magnetic nanochains, this assay can be used for specific SERS detection and photothermal killing of bacteria.

Finally, we prepared a magnetoplasmonic nanomaterial to combine multiple sensing and therapy properties for in vivo tumor imaging and therapy. By introducing hydrophobic Janus seeds in hexane-water phases, a novel C-B nanostructure with small size can be achieved, and it shows excellent imaging and therapy efficiency for cancer cells. More importantly, it achieved NIR-II laser-active PTT for in vivo mice model.

6.2 Future Research

Our designs here have been proved to be versatile and facile methods to fabricate a diversity of multifunctional magnetic and plasmonic nanomaterials for biomedical applications. It will provide synergistic properties for us to enhance the existed technologies.

The future work will be proposed in the following aspects.

1) Currently, we have incorporated MRI, SERS, PTT into the

magnetoplasmonic nanostructures. The further work needs to explore the properties of PAI, PDT, and radiotherapy for combination therapy of tumor, which significantly improves the sensitivity and spatial resolution of imaging, as well as synergistic therapy. For example, the C-B NPs can be firstly coated by a layer of mesoporous silica to form C-B@Si NPs. Secondly, the C-B@Si NPs can be dispersed into Tris buffer to induce the self-polymerization of dopamine to obtain the C-B@Si@PDA NPs. In this step, the drugs or photosensitizers can be loaded with mesoporous silica. Thirdly, the C-B@hollow PDA NPs can be prepared via etching process to remove the mesoporous silica from C-B@Si@PDA NPs. This magnetoplasmonic Au nanomaterial with hollow structure is a multifunctional platform that allows PTT, MRI, PAI, and drug delivery. In addition, the PDA layer enables easy loading of antibody or aptamer for biomedical applications.

- 2) As we discussed above, we investigated the PEGylated magnetoplasmonic nanomaterials for in vivo cancer imaging and therapy by ERP effect. Furthermore, C-B NPs can be modified by antibody or aptamer for targeted detection. For example, the angiopep can target the lipoprotein receptor-related protein which is over-expressed on the glioma cells. The CM-PEGylated C-B3 NPs can conjugate with angiopep-2 peptides *via* EDC and NHS reactions. In addition, PEGylated C-B3 NPs can also be coated by a layer of PDA, and then conjugated with angiopep-2 peptides. Therefore,

angiopep-2 peptides labeled C-B NPs are prospective in penetrating blood brain barrier to target the brain tumor. MRI and PAI imaging, magnetic hyperthermia and photothermal therapy can be applied to promote the theranostic efficiency for brain tumor model.

- 3) In the previous work, the NIR-II laser-active Au nanomaterials have been used for PTT of cancer cells with laser power of 1.5 W/cm^2 . Appropriate design of nanomaterials with higher NIR-II laser photothermal conversion efficiency is needed to prevent the potential skin damage for clinical applications. In the further, we will develop Au nanomaterials-based theranostic platform at a low laser power in NIR-II region for in vivo biomedical applications. For example, QDs have been developed as imaging agents within the NIR-II window. In future, we can prepare Au-QDs Janus NPs, and synthesize QDs@Au branches NPs for enhancing the optical density in NIR-II region. In addition, we can prepare now C-B NPs with thinner and more closely-packed Au branches, which may also contribute to the plasmonic coupling of the Au branches.

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List of Publications

1. **Peng Wang**, Jiajing Zhou, Zheng Fang, Jinghua Ren, Hongwei Duan, Broadband Magnetoplasmonic Nanocrystals with Janus Core-Branch Structure: Biphasic Seeded-Growth for in vivo Theranostic Applications. In preparation.
2. **Peng Wang**, Paramita Das, Chenxu Wang, Qirong Xiong, Hongwei Duan, Glucose Oxidase-mimicking, Magnetic Nanochain-Supported Gold Nanoparticles for Colorimetric Biosensing. In preparation.
3. **Peng Wang**, Qirong Xiong, Jiajing Zhou, Jieli Ma, Hongwei Duan, Biphasic Synthesis of Gold Snowflake Nanoparticles as NIR-II-Window Light Mediated Theranostics. In preparation.
4. Chenxu Wang, Jiajing Zhou, **Peng Wang**, Wenshan He, and Hongwei Duan. Robust Nanoparticle–DNA Conjugates Based on Mussel-Inspired Polydopamine Coating for Cell Imaging and Tailored Self-Assembly. *Bioconjugate Chem.*, 2016, 27, 815–823.
5. Jiajing Zhou, **Peng Wang**, Chenxu Wang, Yi Ting Goh, Zheng Fang, Phillip B. Messersmith, and Hongwei Duan. Versatile Core-Shell Nanoparticle@MetalOrganic Framework Nanohybrids: Exploiting Mussel-Inspired Polydopamine for Tailored Structural Integration *ACS Nano*, **2015**, 9, 6951–6960.
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