Development of nanoparticles-based biosensing assay for identification of enzyme activities

Liu, Rongrong

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**DEVELOPMENT OF NANOPARTICLES-BASED
BIOSENSING ASSAY FOR IDENTIFICATION OF ENZYME
ACTIVITIES**

**RONGRONG LIU
SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES
2011**

**Development of Nanoparticles-based Biosensing Assay
for Identification of Enzyme Activities**

RONGRONG LIU

School of Physical and Mathematical Sciences

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ABSTRACT

Nanoparticles provide a useful platform for wide range of applications in the fields of biochemistry and biomedicine. They have greatly attracted considerable attentions and have been widely used for biosensing, bioimaging and drug delivery systems. This research dissertation focuses on developing novel and convenient biosensing assays for detecting enzyme activities using metallic nanoparticles.

Metallic nanoparticles exhibit unique optical and physical features. Taking advantage of their excellent optical properties, we developed a gold nanoparticles and silver nanoparticles-based colorimetric assay for rapidly sensing β -lactamase activities and screening β -lactamase inhibitors. This simple and applicable method is efficient for screening class A and class C β -lactamases inhibitors *in vitro*. Moreover, this approach is also practicable for screening β -lactamases inhibitors in living bacterial strains. The detailed studies were presented in chapter two, three, and four. This easily operated method possesses the potential of wide applications for drug development and medicinal diagnosis.

Programming nanostructures of gold nanoparticles by enzyme activities were described in chapter five of this dissertation. Self-assembly and disassembly of gold nanoparticles were controlled by various enzyme activities. The designed peptide conjugate as enzyme substrate is responsive to esterase and protease. In the presence of esterase and substrate, gold nanoparticles were triggered to assembly by the peptide linker released from the hydrolyzed substrate. After further treatment with

protease, the peptide linker was specifically cleaved, which results in the disassembly of gold nanoparticles. Therefore, the nanostructures of gold nanoparticles were sequentially triggered to assembly and disassembly by enzymes. This method provides a new chemical tool for precisely controlling the nanostructures.

In addition, a controlled drug delivery system was also developed with mesoporous nanoparticles. The mesoporous silica nanoparticles with iron oxide core as drug carrier and capped by the oligonucleotides/oligonucleotides functionalized gold nanoparticles was studied in chapter six. Upon the treatment of enzyme with the oligonucleotides, the capped pores of mesoporous particles were opened to release the anticancer drugs. This system is multifunctional including controlled drug delivery and magnetic imaging. The multifunctional nanoparticles could serve as a new kind of stimuli-responsive drug delivery carrier and possess promising possibilities for biomedical applications.

Abbreviations

Å	Angstrom
AgNPs	silver nanoparticles
AuNPs	gold nanoparticles
Bla	β-lactamase
br	broad
BSA	bovine serum albumin
Bu	butyl
°C	degree centigrade
d	doublet
dd	doublet of a doublet
DIPEA	<i>N, N</i> -Diisopropylethylamine
DLS	dynamic light scattering
dsDNA	double-stranded DNA
ESI	electrospray ionization
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
m	multiplet
min	minutes
nm	nanometer

NMR	Nuclear Magnetic Resonance
NPs	nanoparticles
o	ortho
p	para
PBS	phosphate-buffered saline
PEG	polyethylene glycol
Ph	phenyl
ppm	parts per million
q	quartet
QDs	quantum dots
s	singlet
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERS	surface enhanced Raman scattering
SPR	surface plasmon resonance
ssDNA	single-stranded DNA
TEM	Transmission Electron Microscopy
TLC	thin layer chromatography
TMS	trimethylsilyl
UV-Vis	ultraviolet visible spectroscopy
δ	NMR chemical shift in ppm

Chapter 1

Introduction

1.1 Nanoparticles in biological sensing

In recent years, nanoscience and nanotechnology have attracted considerable attentions. Numerous areas have been studied in the fields of nanoscience, including development of novel synthetic methods for nanostructures, exploring new properties of nanomaterials, and expanding their wide applications.¹⁻⁵ As the developing platform for nanotechnology, nanomaterials have been viewed as one of the most significant fundamental and technological frontiers. They have been widely exploited in catalytic industry, public environment, medicinal chemistry and clinical diagnostics.⁶

Nanometer materials are in a unique length scale between the bulk solid and isolated atoms or molecules. This length scale is in the scale of electronic motion which determines the properties of materials. Thus, most of the physical and chemical properties of nanomaterials have changed tremendously compared with their bulk or molecular counterparts.⁷⁻¹⁰ For instance, nanocrystals show discrete energy level structures due to the strong quantum confining effect. In addition, nanomaterials possess similar size dimensions to important biomolecules such as proteins and oligonucleic acids. This property offers them great advantages for integration of nanotechnology to biotechnology. A large number of nanomaterials such as quantum dots, carbon nanotubes, silicon nanowires and various metallic and magnetic nanoparticles have been explored to be signaling probes, biosensors, and magnetic

energy storage. Some well-known nanomaterials and their corresponding ligands for surface functionalization and representative bioapplications are shown in Table 1.1. Nanomaterials and nanotechnology hold great promising development and applications.

Table 1.1. Nanomaterials and their representative applications.¹¹

Nanomaterials	Characteristics	Ligands	Representative applications
Au	Optical absorption, fluorescence and fluorescence quenching, stability	Thiol, disulfide, phosphine, amine	Biomolecular recognition, delivery, biosensing
Ag	Surface-enhanced fluorescence	Thiol	sensing
CdSe/ZnS	Luminescence, photo-stability	Thiol, phosphine, pyridine	Imaging, sensing
Fe ₂ O ₃ /Fe ₃ O ₄	Magnetic property	Diol, dopamine derivative, amine	MR imaging, biomolecular purification
SiO ₂	Biocompatibility	Alkoxysilane	Biocompatible by surface coating

Specifically, inorganic nanoparticles are particular attractive nanomaterials and have become the subject of extensive research for biodetection and biolabeling because of their highly interesting optical, electronic, and catalytic properties.^{6,12-13} Especially, silver and gold nanoparticles, noble metallic nanoparticles, have been widely employed as analytical tools in many fields of biology with their remarkable size-dependent optical, electrical and chemical properties.¹⁴⁻¹⁶ Hence, a variety of approaches using nanoparticles have been developed for sensitive detection of molecular recognitions including colorimetric detection,¹⁷⁻¹⁹ fluorescence resonance energy transfer (FRET)/quenching avenues,²⁰⁻²² surface plasmon resonance energy analysis,²³⁻²⁴ and light scattering based sensing.²⁵

1.1.1 Properties of Metallic nanoparticles

Metallic nanoparticles have received great interests in biochemical analysis. They could be easily prepared and also possess excellent optical and physical properties. Therefore, tremendous bioassays based on gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs) have been reported. These metallic nanoparticles based sensing assays could be readily monitored via surface plasmon resonance (SPR) spectroscopy, surface enhanced Raman spectroscopy (SERS), UV-Vis spectroscopy, fluorescence and electrochemical methods.

In 1951, Turkevitch *et al.* developed a method of reduction of Au(III) to Au(0) in water by citrate.²⁶ In this reaction, the citrate ion acts as both reductant and stabilizer. This method could produce ruby red citrate-stabilized gold nanoparticles in large quantities. Later this method was improved to prepare well controlled dimension of AuNPs with fairly narrow size distribution.

Following the Turkevitch method, a large variety of stabilizers were employed for fabricating the surface of AuNPs instead of citrate ligands. Different stabilizers such as surfactants, polymers, dendrimers and biomolecules were used based on appropriate biological interests.²⁷ Gierdrsig and Mulvaney firstly reported that AuNPs could be stabilized by alkanethiols through the strong Au-S bond between the soft acid Au and the soft thiolate base.²⁸ Subsequently, the Brust-Schiffrin method was developed based on Au-S coordination bond in biphasic solvents to prepare AuNPs with diameter in the range between 1.5 and 5.2 nm.²⁹⁻³⁰ From the equations 1.1 and 1.2 shown below, the two phase reaction was summarized. AuNPs could be easily

functionalized by introducing thiolates. For further modification of AuNPs with biological applications, AuNPs could be featured by thiolated oligonucleotides, polypeptides and PEGs through chemical substitution.

Figure 1.1. Equation of two-phase synthesis of gold nanoparticles by thiolates.²⁹⁻³⁰

1.1.1.1 Surface plasmon resonance (SPR) of metallic nanoparticles

According to the Mie theory, surface plasmon resonance (SPR) is a phenomenon that an electromagnetic frequency induces a resonant coherent oscillation of the free electrons at the surface of spherical nanoparticles if it is much smaller than the light wavelength.²⁹ Figure 1.2 shows the creation of surface plasmon in metallic nanoparticles.³⁰ The induced dipole oscillates in phase under the electric field of the incoming light. Surface plasmon absorption for gold and silver nanoparticles is in the visible spectrum region, despite the complexity of the physical principles of SPR. The localized surface plasmon resonance results in an enhanced electromagnetic field at the surface of metallic nanoparticles. When the two nanoparticles are in close proximity, the near-field coupling results in a red-shifted resonance wavelength peak.

Figure 1.2. Schematic illustration of the creation of surface plasmon in metallic nanoparticles due to interaction of electromagnetic radiation with the metal sphere.³⁰

The surface plasmon resonance of AuNPs could be observed when they are larger than 3 nm diameter. Based on the surface-enhanced optical properties, the extinction coefficients of AuNPs SPR bands are extremely higher several orders of magnitude than the organic dyes. AuNPs have absorption and scattering proportions which depend on their size. AuNPs mainly display absorption with the diameter below 20 nm, but when the size is larger than 80 nm, the ratio of scattering to absorption increases dramatically. Surface plasmon band of AuNPs is highly dependent on the composition, size, shape, interparticle distance and the surrounding environment (dielectric properties). Arising from SPR, AuNPs show an intense UV-Vis absorption peak from 500 to 550 nm varying from the size and shape.³¹ The SPR band is very sensitive to the changes in the solvent and the interparticle distance. A particular output signal is the red-shift of SPR band from that of a single particle and broadening of the plasmon band because of near-field plasmon coupling.³² This optical phenomenon provides AuNPs as a popular and applicable colorimetric biosensor in biological labeling, detection, and diagnosis.

1.1.2 Application of metallic nanoparticles in biological sensing

Over the centuries, gold colloids have been recommended for curing various diseases. The first scientific article was published in 1857 by Michael Faraday describing the red color of the colloidal nature of gold nanoparticles.³⁶ Gold nanoparticles possess high extinction coefficients which is 3 ~ 5 orders of magnitude higher than those of organic dye molecules.³⁷ In addition, the unique distance dependent optical property of gold nanoparticles makes them chemically programmed for specific target detection. As for silver nanoparticles, they have higher molar extinction coefficient than that of the same size of gold nanoparticle, which lead to improved detection sensitivity.

Nowadays, metallic nanoparticles have been employed in considerable applications in optics, catalysis, materials science and biomedical nanoscience. They are widely used for detection of various biomolecules including proteins,³⁸⁻⁴¹ enzymes,⁴²⁻⁴⁴ oligonucleotides,⁴⁵⁻⁴⁷ metal ions,⁴⁸⁻⁵² small molecules,⁵³⁻⁵⁶ and bacteria⁵⁷.

1.1.2.1 Colorimetric bioassays based on aggregation of nanoparticles

A lot of previous works have demonstrated that different agglomeration states of metallic nanoparticles can result in distinctive color changes.⁵⁸⁻⁵⁹ Colorimetric methods are extremely attractive because they can be easily monitored by naked eyes. For example, gold nanoparticles based colorimetric assay is well known to be a simple and sensitive method. It is suitable for high-throughput screening (HTS) system in a

multiple microwell plate format. The unique optical properties of AuNPs make them a good candidate for sensor applications. When the distance of inter-AuNPs is reduced to less than the AuNPs diameter, the surface plasmons on AuNPs couple with each other and the resonance shifts to longer wavelengths and the peak broadens.⁶⁰ Different inter-AuNPs distance displays different extent of red shift of resonance with various visible color (Figure 1.3).⁶¹ This phenomenon could be clearly visible by naked eye, which could be used for real-time and on-site detection.

Figure 1.3. (a) Visible color of 15 nm AuNPs for different inter-particles distance in nanometer.⁶¹ (b) Schematic diagram of distance dependent sandwich assay for polyvalent antigen (green circles) induced aggregation of AuNPs and a red shift in their extinction spectrum from red to blue.⁴⁵

Generally, the mechanisms for the colorimetric detection based on aggregation of AuNPs have two categories, crosslinking and noncrosslink aggregation of AuNPs (Figure 1.4). In most of assays, AuNPs were induced aggregation by the analytes as crosslinking molecules that have multiple binding sites for molecules immobilized on the surface of AuNPs. In this detection system, it requires the surface modification of AuNPs and the analytes recognition sites on the AuNPs surface. For noncrosslinking

method, there is no need to label the molecules or modify the surface of AuNPs. Neutralization of AuNPs surface charge by charged analytes induced the AuNPs aggregation. Besides simplicity, the noncrosslinking aggregation of AuNPs is much more rapid than the crosslinking aggregation.

Figure 1.4. Two mechanisms of colorimetric assays based on AuNPs aggregation.⁶²

Leuvinger pioneered the principle of connecting AuNPs with biological analyte for colorimetric detection.⁶³ Another remarkable example for AuNPs based colorimetric detection is the DNA-functionalized gold nanoparticles detection. Mirkin and co-workers first developed an entirely new colorimetric detection scheme for DNA in oligonucleotide functionalized gold nanoparticles based on the distance dependent optical properties of gold nanoparticles.¹⁸ Each AuNP was functionalized with several oligonucleotides. Polymeric network of nanoparticles formed when the target single stranded oligonucleotide was introduced. The hybridization between complementary oligonucleotides shortened the interparticle distance with concomitant color change from red to pinkish/purple. This methodology was also useful for colorimetric screening of target DNA and triplex DNA binders (Figure 1.5, Figure 1.6).⁵⁵ The well

known example of AuNPs based sensor is “*Northwestern spot test*” that is removing aliquots and spotting onto a C18 reverse-phase thin layer chromatography plate as the temperature is increased resulting in a visual record of the color change. This system reached a high sensitivity and could be detected visually. The method paved the way for studying the gold nanoparticles in the oligonucleotides detection and clinical diagnostics.

Figure 1.5. Illustration of AuNPs aggregation upon DNA hybridization.¹¹

Figure 1.6. Triplex DNA directed AuNPs assembly.⁵⁵

Several research groups subsequently studied the DNA-AuNPs interactions and developed quantitative detection of DNA sequence at very low concentration. Detection of genetic mutations is also achievable based on above principle. Franco group reported AuNPs based color change method to detect eukaryotic gene expression (RNA) without PCR amplification.⁶⁴ In this system, AuNPs were induced aggregation by salt concentration without hybridization-based crosslinking and the

mRNA from 0.3 μg of unamplified total RNA was detectable.

Lu and co-workers reported a highly sensitive and selective lead biosensor based on Pb^{2+} specific DNzyme-directed assembly of AuNPs.^{48,65} They used DNA functionalized AuNPs and substrate DNA strand to direct the assembly of AuNPs in head-to-tail or tail-to-tail manner (Figure 1.7). In the presence of Pb^{2+} ion, DNzyme activities were specifically catalyzed to cleave the substrate strand. This cleavage changed the aggregated AuNPs to disassembly with concomitant color change from blue to red. This approach is very selective to Pb^{2+} and could not be affected by other divalent metal ions.

Figure 1.7. Colorimetric detection of Pb^{2+} for DNzyme mediated assembly/disassembly of AuNPs. The nanoparticles are aligned in a head-to-tail or a tail-to-tail manner.⁴⁸

Jiang and co-workers also developed a method for visual detection of copper (II) by using AuNPs in terms of “click chemistry”.⁵¹ The detection of Cu^{2+} ion was mediated by the Cu (I)-catalyzed 1,3-dipolar cycloaddition of alkynes and azides on the surface of functionalized AuNPs, which resulted in the agglomeration of AuNPs associated with distinct color change. In this system, two sets of AuNPs were separately modified.

With the similar colorimetric approach, cocaine and some small molecules have been sensitively detected by using aptamer functionalized AuNPs. Figure 1.8 shows the schematic illustration of cocaine detection based on the disassembly of aptamer functionalized AuNPs.

Figure 1.8. Schematic depiction of cocaine triggered disassembly of AuNPs.⁵⁴

Moreover, gold nanoparticles-based colorimetric sensing methods have widely been employed in sensitively detecting enzymes and proteins. *Ricinus communis* agglutinin lectin, a bivalent galactose specific protein, could induce the aggregation of lactose-functionalized AuNPs and dissociate the aggregates when adding excess galactose. The sensitivity of this assay is high enough to reach 1 ppm, comparable to that of immunological assay methods such as ELISA. Subsequently, several glyconanoparticles were used for sensing concanavalin A and cholera toxin (Figure 1.9).⁶⁶⁻⁶⁹

Figure 1.9. Illustration of cholera toxin induced aggregation of lactose-gold nanoparticles.⁶⁹

Mirkin group extended the DNA-AuNPs method to a real-time screening assay for endonuclease activity.⁴² Stevens et al. developed thermolysin triggered disassembly of peptide stabilized AuNPs for sensitive detection of protease activities (Figure 1.10).⁷⁰ Others also developed this approach for sensing kinases⁷¹ and phosphatases⁷²⁻⁷³ (Figure 1.11). A number of noncrosslinking aggregation based AuNPs detection assays were also developed for sensing enzyme activities.^{62,74}

Figure 1.10. Disassembly of AuNPs-based assay for thermolysin activity.⁷⁰

Figure 1.11. Colorimetric assay for sensing phosphatase based on AuNPs aggregation.⁷²

Besides using AuNPs for simple colorimetric assay, they were also used for sensitive SERS detection. AuNPs could be modified with raman reporters and functional ligands which specifically bind the analyte. Upon binding to the gold particles, the Raman signal of this recognition from analyte and ligands is dramatically enhanced and allows for sensitive detection of analyte. Recent development of AuNPs modified with Raman active reporter molecules are used for detection of DNA and proteins not only in *vitro* but also in *vivo*.⁷⁵⁻⁷⁷

1.1.2.2 Metallic nanoparticles-based fluorescence biosensor

Metallic nanoparticles have excellent quenching ability. This property makes them potential materials for fluorescence resonance energy transfer (FRET)-based biosensors.^{22,78-79} In this approach, metallic nanoparticles function as efficient energy acceptors. It is found that all the gold nanoparticles not only increase the nonradiative rate of decay of the fluorescent dyes, but also decrease the radiative

rate.⁷⁸ Thus, gold nanoparticles have been successfully used in FRET applications.

Libchaber and co-workers reported a molecular beacon based on FRET for sensitively sensing target DNA.²² Gold nanoparticle is the core of this biosensor. Oligonucleotide molecules labeled with thiol group at one end and fluorophore at the other end are attached to the gold nanoparticle core. This hybrid structure constructs hairpin confirmation on the surface of gold nanoparticle with fluorescence quenching. Hybridization of target DNA with hairpin DNA opens up FRET restoring significant fluorescence (Figure 1.12). This molecular beacon is much more sensitive and has been used for monitoring single strand DNA and the cleavage process of DNA.⁸⁰⁻⁸¹ Siedel et al. demonstrated a gold nanoparticle-based FRET immunoassay for detection of atrazine on gold-coated well plates.⁸²

Figure 1.12. Schematic representation of gold nanoparticles-based molecular beacon for detection of target DNA.⁷⁸

Gold nanoparticles have also been employed as quencher for semiconductor Quantum Dots (QDs). QDs have several intrinsic photophysical properties including relatively high quantum yields, high molar extinction coefficients and high resistance

to photobleaching.⁸³⁻⁸⁶ All these make them ideal reagents for photoluminescence biolabels and bioimaging. Melvin and coworkers have reported quantum dots-gold nanoparticles conjugate as a FRET donor-accepter for DNA detection.⁸⁷ In the presence of complimentary DNA, the DNA labeled gold nanoparticles released from quantum dots, thus the quenched fluorescence restores from quantum dots. A similar approach was also used for sensing biomolecules such as avidin (Figure 1.13).⁸⁸ Kim and colleagues detected glycoprotein by using gold nanoparticle quenched dextran-conjugated quantum dots.⁸⁹

Figure 1.13. Schematic illustration of competitive inhibition assay for the detection of avidin by quantum dots and gold nanoparticles conjugation.⁸⁸

In comparison with organic dye quenchers, metallic nanoparticles have superior and excellent structural and optical properties for biosensing and bioimaging applications.

1.1.2.3 Electrochemical biosensors based on metallic nanoparticles

Metallic nanoparticles possess many important functions for electroanalysis. Their catalytic and conductivity properties have been widely applied in

electroanalytical and bioelectroanalytical applications.⁹⁰ The catalytic properties of nanoparticles permit their enlargement with metals and amplify electrochemical detection of the metal deposits. Moreover, the particles in nanoscale allow the electrical contact of redox centers in proteins with electrode surfaces. Therefore, the attachment of nanoparticles on the electrode could significantly enhance the conductivity and electron transfer from redox analytes.⁹¹

Mirkin and co-workers have reported an array based electrical approach with nanoparticle probe for sensitive detection of DNA.⁹² Oligonucleotide-functionalized gold nanoparticles were deposited between two electrodes associated with target DNA deposition by complementary hybridization (Figure 1.14). Silver was further deposited on the surface of gold nanoparticles by using silver salt and hydroquinone. Silver deposition enhanced conductivity change, providing the sensitivity of sensing target DNA as low as 500 femtomolar with a point mutation selectivity factor of $10^5:1$.⁹²

Figure 1.14. Schematic drawing of electrical detection of oligonucleotides.⁹²

Willner et al. have developed a bioelectrocatalytic system which connects the redox enzyme glucose oxidase onto gold nanoparticles functionalized with flavin adenine dinucleotide. The gold nanoparticle serves as an electron transfer media or “electrical nanoplug” for the arrangement of enzyme on the conductive support.⁹³

This efficient electrical sensing system offers an effective sensor for detecting glucose in the physiological concentration range.

1.1.2.4 Nanoparticles in drug delivery systems

Drug delivery systems could improve many properties of hydrophobic anticancer drugs such as solubility, stability, pharmacokinetics and enhancing their anticancer efficacy.⁹⁴ In this aspect, nanoparticles have been considered as effective carriers for hydrophobic drugs due to their optical properties, biocompatibility, and low toxicity. Recently, gold nanoparticles have been widely studied for targeted drug delivery and gene therapy.⁹⁵⁻⁹⁸ They could transport hydrophobic drugs as well as various biomolecules such as DNA, RNA and proteins to across the membrane barrier, which provide the access to targeted anti-cancer, gene therapy and protein-based treatment. Zubarev et al. have demonstrated that gold nanoparticles were covalently functionalized with chemotherapeutic drug, paclitaxel.⁹⁹ This approach offers a method for nanosized drug delivery system. He and co-workers reported transferrin-mediated gold nanoparticles for targeted tumor cell uptake.⁹⁵ In addition, Mirkin group developed a new approach for effective gene therapy using oligonucleotide-functionalized gold nanoparticles. This antisense nanoparticle could effectively suppress EGFP signal in C166 cells.¹⁰⁰

1.1.3 Programmed nanoparticles by biomolecular recognitions

The programmed self-assembly and disassembly of nanostructures with

controlled surface chemistry have attracted considerable attention for their potential applications in drug delivery, medical diagnosis and nanomechanical devices. However, among all the reported programmed nanostructures system, most of the sensing mechanisms were mainly based on one-directional assembly or disassembly and these two-stage methods irreversibly.

Reversible manipulating the structures of nanoparticles is promising in nanoscience for biological sensing and DNA nanodevices.¹⁰¹ Compared to the irreversible process, the reversible aggregation of nanoparticles is much more difficult because the nanoparticles assembly have the tendency to collapse to larger and insoluble materials. Recently, more and more research groups have reported reversible self-assembly and disassembly of nanoparticles systems in which the triggered changes in their assembled states were driven by some external factors such as pH, temperature, light, inorganic/organic molecules, metal ions or fueling oligonucleotide.¹⁰²⁻¹⁰⁸

Niemeyer and co-workers reported reversibly control DNA-AuNPs conjugate structure by using fueling oligonucleotides.¹⁰⁹ The assembled AuNPs structure was driven to disassembly by DNA strand displacement (Figure 1.15). A simpler approach was further developed by Choi group. They reported a proton-fueled switch to reversibly control the structure of “triplex-AuNP” conjugates.¹⁰⁸ The advantage of protons over oligonucleotides is averting the generation of waste duplex products.

Figure 1.15. Reversibly controlled nanostructure of DNA-AuNPs conjugate.¹⁰⁹

In addition, enzyme sensitive peptide decorated AuNPs was also used for reversibly programming nanostructures. Bhatia and co-workers reported that the reversible assembly and disassembly of nanoparticles were controlled by antagonistic kinase and phosphatase activities.¹¹⁰

A number of specific biomolecular recognition motifs such as biotin-avidin binding, antibody-antigen or enzymatic catalytic interactions have been extensively exploited for the control of AuNPs self-assembly. These methods provide a simple and specific sensing platform for systematic identification of a variety of molecular analytes including DNAs, bacterial toxins, proteins and enzymes.¹¹¹⁻¹¹²

Taking advantage of specific molecular recognitions, precisely control the network of nanoparticles will have a promising future in the advances of electronics, information technology, sensor development, and biomedical sciences.

1.2 Important roles of β -Lactamases

1.2.1 β -Lactam antibiotics and bacterial resistance

In the early 1940s, penicillin, a miracle drug, was first introduced in the clinical treatment to combat bacterial infections.¹¹³ Since then, β -lactams became the most widely used antibiotics in clinics. β -lactam antibiotics have structural similarity with the binding sites of bacterial substrates which render them inactivate the transpeptidases and hence block the bacterial cell wall synthesis.¹¹⁴ Originally, the β -lactams antibiotic family was limited to penicillin (sulfur-containing penams) and cephalosporin (sulfur-containing cephems), but now it includes natural and synthetic monocyclic β -lactams, carbapenems, oxapenams, carbacephems, and oxacephems (Figure 1.16 and Figure 1.17).¹¹⁵ They are all structurally related to the core structure, a four-membered β -lactam ring.

Figure 1.16. Structures of β -lactam antibiotics.

Figure 1.17. Structures of broad β -lactam antibiotics.¹¹⁵

β -Lactam antibiotics are preferred in hospitals and community settings for over the last 60 years after their introduction. The high clinical effectiveness, broad-spectrum activity and good safety profile afford their successful management of common bacterial infections. However, microorganisms are extremely adaptable to their surroundings and have survived over ages. One of the major phenomena of bacterial responses to the effects of antibacterial agents is the emergence of drug resistance. This greatly reduces the effectiveness of the antibiotics over a period of time and hence there is a need for better and more effective antibiotics to overcome the resistance. The threat of antibiotic resistance has been continuously evolving and the epideomicrological problem has now reached a point where some infectious

agents resist all the available antibiotics. Presently, the evolving resistance of bacterial infections to antibiotic treatment has become a major concern in the area of infectious disease treatment.¹¹⁶

1.2.2 Occurrence of β -lactamases

The prevalence of bacterial resistance has gained the attention of clinicians and researchers worldwide. It has been reported that there are four distinct mechanisms for bacterial resistance in response to the action of antibiotics.¹¹⁷

Firstly, the change in the characteristics and composition of the bacterial outer membrane prevents the entry of antibiotics into the cell. This mechanism is particularly prevalent in organisms such as *Pseudomonas aeruginosa*. The outer membrane of strains of *P.aeruginosa* contains similar number of outer membrane porins (channels - mainly water channels through which antibiotics travel to enter the cell). However, it has been shown that a particular antibiotic is not able to use these channels effectively in modified bacteria (exposed to antibiotics), and hence resistant to this antibiotic.

Secondly, as the bacterial resistance evolved with greater exposure to antibiotics, the target specificity of bacteria changes with the composition and nature of penicillin binding proteins (PBP's). Thus, bacteria resistance to particular antibiotics develops.

Thirdly, bacteria possess biological efflux pumps which involve an energy-dependent export of antibiotics which is an important detoxification process for the bacteria. The bacteria export the antibiotics among other substances, from

within either the periplasm or cytoplasm to the outside environment. These pumps were firstly identified in Gram negative bacteria where tetracycline was actively removed from resistant bacteria. Subsequently, numerous types of pumps were identified associated with various substrates including quinolones and β -lactam antibiotics.

Fourthly, the bacterial resistance arises from the secreted enzymes, which degrade the antibiotics before it can exert the desired effect. The bacterial β -lactamases are well known enzymes which could selectively cleave the amide bond in four-membered β -lactam ring of the β -lactam antibiotics.

Among the reported mechanisms, β -lactamases mediated resistance to β -lactam antibiotics is the most efficient and frequent mechanism of bacterial resistance. β -Lactamases destroy the β -lactam antibiotics before they reaches the penicillin binding protein target. Therefore, the β -lactam antibiotics are ineffective to bacterial infections due to the emergence of β -lactamases.

1.2.3 Classification of β -Lactamases

β -Lactamases (EC 3.5.2.6) (Blas) are plasmid or chromosome encoded bacterial enzymes that could efficiently hydrolyze the β -lactam ring. They are usually secreted into the periplasmic space of Gram negative bacteria and the outer medium of Gram positive bacteria. Although all the β -lactamases could hydrolyze the same β -lactam antibiotics, a large number of different β -lactamases have been isolated and characterized from different bacteria. They are highly diverse. Based on the structural comparisons of the amino acid sequence relationships among the diverse

series of β -lactamases, they are widely categorized into four classes (class A, B, C, D) following the initial work by Bush et al.¹¹⁸ The available representatives of each class display the crystal structures. Classes A, C, and D are serine enzymes and share a similar fold in which a serine residue initiates the catalytic function leading to the cleavage of β -lactam ring. These three classes constitute a majority of clinically important β -lactamases. Class B β -lactamases represent the zinc metalloenzymes in which zinc atom was involved in the catalytic mechanism instead of serine.¹¹⁵ They are completely different from the serine β -lactamases in terms of sequence, folding and catalytic mechanism.

Class A β -lactamases is a class of serine β -lactamases which was historically called “penicillinases” because of their higher hydrolytic ability to catalyze the penicillin than cephalosporins. Class A β -lactamases are closely related in sequence to low molecular weight class C PBPs such as PBP4 of *E. coli*, *H. influenza*, and *M. tuberculosis*.¹¹⁹ In terms of bacterial resistance, the predominant class A β -lactamases subclasses are the TEM/SHV (the historically Gram negative plasmid penicillinase), the *P. aeruginosa* PER/OXA/TOHO cephalosporinases and the CTX-M (NMC-A) carbapenemase subclasses.¹²⁰ The crystal structures of several Gram positive and Gram negative class A β -lactamases were solved in the late 1980s and early 1990s¹²¹⁻¹²⁴.

Figure 1.18(a) shows the TEM-1 β -lactamase ribbon structure which is formed of two domains.¹²⁴ One is α/β domain consisting of five stranded β -sheet and three α -helices, another is an α -domain consisting of eight α -helices.¹²¹⁻¹²² These two

domains sandwich the core of the active site of TEM-1 β -lactamase.

Figure 1.18. (a) The crystal structure of TEM-1 β -lactamase.¹²⁴ (b) The ribbon structure of class C β -lactamase of *Enterobacter cloacae* GC1.¹²⁵

Unlike class A β -lactamases, class C β -lactamases have a preference for cephalosporin substrate. They are mostly found in Gram negative bacteria and are chromosomally encoded in several organisms such as *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae*.¹²⁶ Figure 1.18(b) shows the three dimensional structure of β -lactamase from *Enterobacter cloacae* GC1. Class C β -lactamases have the similar mechanism with class A β -lactamases for β -lactam hydrolysis. However, there is a significant difference between them for deacylation at the catalytic level. The two classes use opposite faces of the acyl-enzyme species for the approach of the hydrolytic water.¹²⁷ In the class C enzymes, the water approaches from the β -direction.

Plasmid-encoded class C enzymes were found in *E.coli*, *K. pneumoniae*, *Salmonella spp.*, *C. freundii*, *E. aerogenes*, and *Proteus mirabilis*. The rate of incidences of these enzymes is the highest in *K. pneumoniae* and *E.coli* which are common to the hospital and community settings.¹²⁶

Class D β -lactamases were generally termed as oxacillinases because of their efficiency of hydrolyzing 5-methyl-3-phenylisoxazole-4-carboxy side chain penicillin class, exemplified by oxacillin and cloxacillin. A lot of variants of these enzymes are now known. Figure 1.19 displays the comparison of three similar serine-based β -lactamases (class A, C, D).

Figure 1.19. Three dimensional structure of (A) a class A β -lactamase (TEM-1; PDB code 1TEM), (C) a class C β -lactamase (AmpC; PDB code 1FCO), (E) and a class D β -lactamase (OXA-10; PDB code 1K57). Close-up stereo views of the active sites of the acyl-enzyme complex are shown as (B) TEM-1 with 6 α -hydroxymethylpenicillate, (D) AmpC with moxalactam, and (F) OXA-10 with 6 β -(1-hydroxy-1-methylethyl) penicillanic acid.¹¹⁵

Class B metallo- β -lactamases are zinc-dependent enzymes. They have broad β -lactam substrate tolerance. In general, the mechanism of catalysis by these

enzymes does not contain a covalent acyl-enzyme intermediate.

Although lots of subgroups of β -lactamases were known in the early 1970s, the number is still increasing over 470. These enzymes have been the major reason of the bacterial resistance to β -lactam antibiotics and have been the topics of extensive investigations in microbiology, biochemistry and genetic biology.

1.2.4 Mechanism of hydrolysis by β -lactamases

As we know, class A, C, and D are serine β -lactamases which could hydrolyze the β -lactam ring of antibacterial agents according to Figure 1.20a.¹¹⁶ However, class B metallo- β -lactamases have a different mechanism in which the zinc ion is involved in the major catalytic mechanism of hydrolysis as shown in Figure 1.20b.

Figure 1.20. Schematic drawing of the general mechanisms of hydrolysis of β -lactam by serine β -lactamases (a) and metallo β -lactamases (b).

1.2.5 β -Lactamases as biological tools in biotechnology applications

Although β -lactamases have mediated the bacterial resistance to antibiotics, they have been developed as powerful tool in biological studies. TEM-1 β -lactamase has several desirable features which meet the essential criteria for genetic reporter.¹²⁸ It is a relatively small (29 kDa) and monomeric enzyme which is well characterized in structure and function. Additionally, this enzyme is extremely versatile and can be fused to other proteins, retaining activity, and easily expressed. Furthermore, TEM-1 β -lactamase has no toxicity to prokaryotic and eukaryotic cells.

As a sensitive reporter enzyme, TEM-1 β -lactamase has been employed in monitoring and imaging several biological processes and interactions in single living mammalian cells. It is used for examining the activity of the promoter or regulatory elements.¹²⁹ Moreover, TEM-1 β -lactamase is also applied for imaging RNA splicing, monitoring viral infections and detecting protein-protein interactions.¹³⁰⁻¹³⁴

1.2.6. Biosensors for detection of β -lactamases

In recent years, clinicians and patients have been facing the challenges from antibiotic-resistant pathogenic bacteria for a long time. As β -lactamases are capable of hydrolyzing most potent β -lactam antibiotics and continue evolving, they presented a vexing clinical problem. In particular, a number of them are resistant to β -lactamase inhibitors. Due to the important roles of β -lactamases in clinical treatment and wide biological applications, it is essential to develop highly sensitive biosensors for detecting the presence of β -lactamases and screening their inhibitors.

Numerous methods have been devised for testing the β -lactamases. Rapid and

simple indicators usually use chromogenic cephalosporins, or link the hydrolysis of penicillin to a color change mediated by iodine or pH indicator. Chromogenic cephalosporins tests are much more specific to the action of β -lactamases. Although iodometric and acidimetric tests are easily performed in tubes and on paper strips, they do not specifically respond to β -lactamases. As a result, the need for running parallel control experiments is crucial to reduce the risk of false results.

Chromogenic probes such as cephacetrile, nitrocefin, pyridine-2-azo-*p*-dimethylaniline cephalosporin (PADAC) have been well developed for β -lactamases identification.¹³⁵⁻¹³⁶ Nitrocefin as a chromogenic cephalosporin changes color from yellow to red upon hydrolysis by β -lactamase. It was found to be a sufficiently sensitive substrate to indicate the presence of β -lactamases in small amounts of bacteria.¹³⁵ Therefore, it is commercially available and widely used in clinical laboratories. PADAC is another chromogenic cephalosporin substrate which changes color from purple to yellow after hydrolysis by β -lactamases. However, the color change occurs much more slowly than nitrocefin.

Fluorogenic substrates with high sensitivity are superior to chromogenic substrates as biosensors. The first fluorogenic biosensor for β -lactamases detection within living cells is CCF2/AM which is widely used (Figure 1.21).¹³⁷⁻¹³⁸ This membrane-permeable substrate connected coumarin (donor) and fluorescein (acceptor) based on fluorescence resonance energy transfer (FRET). Cleavage of β -lactam ring of the cephalosporin disrupted the FRET and reestablished fluorescence from coumarin.

Figure 1.21. Fluorogenic substrate CCF2/AM hydrolyzed by β -lactamase.

Bachmann and coworkers developed an oligonucleotide microarray for identification of the single nucleotide polymorphisms of TEM β -lactamases variants which are related to the extended-spectrum β -lactamase (ESBL) phenotype.¹³⁹ The target DNA was amplified and fluorescently labeled by polymerase chain reaction (PCR) technique. This microarray assay provides an attractive option for epidemiologic monitoring of TEM β -lactamases in routine clinical diagnostic laboratories. However, PCR technique is much more costly and time consuming.

Rao and co-workers later developed several novel fluorogenic substrates for imaging β -lactamases.¹⁴⁰⁻¹⁴¹ As shown in Figure 1.22, CC1 has no blue fluorescence because of electron transfer. In the presence of β -lactamase, the amide bond in four-membered ring of substrate was cleaved and the blue fluorescence of umbelliferone was detectable. Recently, Rao and co-workers reported that imaging β -lactamases activity *in vivo* by using the bioluminescent enzyme firefly luciferase

(fLuc).¹⁴²⁻¹⁴³ Due to the great sensitivity of luciferase-based bioluminescence imaging, they applied the assay in living animals for imaging β -lactamase activity (Figure 1.23).

Figure 1.22. Hydrolysis of fluorogenic substrate CC1 by β -lactamase.

Figure 1.23. Schematic illustration of the two-step reaction for detection of β -lactamase activity by using firefly luciferase.¹⁴³

Xu and co-workers also described a new approach to report the presence of β -lactamase by examining the formation of supramolecular hydrogels.¹⁴⁴ It is much

more simple and selectively respond to β -lactamase. This method is easy to be observed because of macroscopic hydrogelation.

1.3 Important roles of protease

Among enzyme family, proteases play a vital role in a multitude of physiological processes including simple digestion of food and highly-regulated cascades metabolic pathways. They are of great physiological importance by being an activation of a protein's function or a signal in a signaling pathway. Proteases can break specific peptide bonds to abolish a protein's function or digest it to its principal components. They are particularly relevant because proteolysis is the final step of expression of the activity of a variety of proteins.¹⁴⁵ Screening toxins and pathologies associated with the presence of specific protease are extremely desirable for the development of effective therapeutics.

1.4 Research topics and goals

In recent years, intense research has been focused on the nanomaterials. Developing various fabrication approaches to nanomaterials has been achieved. Besides that, nanomaterials have greatly attracted considerable attentions in the field of bioassays for sensing applications. Among all the nanomaterials, metallic nanoparticles have received much interest in colorimetric assays due to their advantages such as simplicity, high sensitivity and economy. In our study, we aim to employ the excellent properties of metallic nanoparticles for rapid sensing of

important enzyme activities and screening enzyme inhibitors. Based on our research, it is believed that an alternative and simple approach for highly sensitive and selective detection of enzyme activities was developed. The new method may have wide applications in drug developing process and medicinal or clinical diagnosis.

As a large family of bacterial enzyme, β -lactamases emerged and rapidly mutated along the occurrence of bacterial resistant to β -lactam antibiotics. With the increasing threat of bacterial resistance to antibiotics, developing rapid and sensitive detection method for β -lactamases activities is thus clinically important. Chapter 2 describes a novel gold nanoparticles-based colorimetric assay for detection of β -lactamase. Gold nanoparticles have unique surface plasmon resonance phenomenon which is distance-dependent that dispersed gold nanoparticles is red color whereas the aggregated gold nanoparticles is purple-blue. Taking advantage of the excellent optical properties of gold nanoparticles, β -lactamase substrate was designed to respond to the enzyme and subsequently change the interparticle distance followed by distinct color change. This rapid colorimetric assay is much simpler compared to other fluorometric methods and the novel approach has promising application for high throughput screening β -lactamase inhibitors *in vitro* and in living bacterial strains.

Among four traditional classes of β -lactamases family, class A and class C β -lactamases are the two major studied classes. Based on above developed simple gold nanoparticles based colorimetric assay, Chapter 3 presented the detailed study of sensing class A β -lactamase activities and screening their inhibitors *in vitro* and in

living bacterial strains.

Furthermore, class C β -lactamases are widely used in prodrug designs for treatment of various cancers. Thus, efficient biosensors for detection of their presence with high sensitivity are desirable. Chapter 4 describes the colorimetric approach for sensing class C P99 β -lactamase with gold nanoparticles and silver nanoparticles. Silver nanoparticles based colorimetric assay provides higher sensitivity due to their higher extinction coefficient compared to the same size of gold nanoparticles.

In chapter 5, programming the networks of gold nanoparticles by enzyme switch is described. Esterase and protease are chosen as model enzymes. The self-assembly and disassembly of gold nanoparticles were sequentially triggered by two enzymes actuations. It will provide a new chemical tool for manipulation of nanostructures.

Gold nanoparticles as drug carriers have been well developed. Chapter 6 shows a new drug delivery system using DNA functionalized gold nanoparticles as pore keeper for controlled drug release. Multifunctional nanoparticles were prepared as drug container. This system is applicable for simultaneous magnetic imaging and drug delivery.

1.5 References

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Chapter 2

Colorimetric Visualization of β -Lactamase Activity with Gold Nanoparticles

2.1 Introduction

Nanomaterials and nanotechnologies have received tremendous attention in recent years due to their potential biomedical applications in diagnosis and clinical therapy.¹ Intense research has been fueled by using nanomaterials to develop DNA and protein markers for sensing diseases. Nanomaterials based assays could offer significant advantages in biological sensing, imaging, and clinical diagnostics with regard to the sensitivity, selectivity, and practicality.²⁻³ Among the large number of nanomaterials explored in bioassay, gold nanoparticles (AuNPs) have gained much interest in versatile biological detection systems.⁴ They exhibit unique optical and electronic properties and have strong surface plasmon absorption band in visible spectrum range from 500 to 550 nm.⁵⁻⁶ Gold nanoparticles are important colorimetric indicators because of their distance-dependent optical properties. They appear red color in dispersed state, whereas the aggregated AuNPs result in a rapid color change from red to purple-blue with the interparticle distances substantially decreased to less than their average particle diameter. This distinctive color changes from red to purple-blue were also associated with the red shift and broadening of the surface plasmon absorption band. Moreover, gold nanoparticles possess high molar extinction coefficients which are several orders of magnitude more than those of

traditional organic chromophores.⁷ As a result, the colorimetric assay based on gold nanoparticles at nanomolar concentration allows sensitive detection of small amount of analytes. Additionally, AuNPs exhibit selectively strong binding to thiol-containing molecules by forming covalent Au-S bond which provides the principle for designing a linker between the dispersed AuNPs or modifying the surface of AuNPs. Therefore, AuNPs based colorimetric assay have been well applied for detecting various molecular recognitions such as antibody-antigen binding, protein-carbohydrate interactions, as well as measuring metal ions, and sensing enzyme activities.⁸⁻¹⁰

β -Lactamases (Blas) are important family of bacterial enzymes that catalyze the hydrolysis of β -lactam ring in penicillins and cephalosporins with high efficiency. With the occurrence of these enzymes, bacteria could be resistant to β -lactam antibiotics. The increased bacterial resistance has raised the attention of worldwide clinicians and currently has been a recognized problem in hospital therapy and community settings. Therefore, it is highly desirable to monitoring the β -lactamases activities before conducting effective clinical therapy toward bacterial infections. In the last few years, many groups have developed chromogenic and fluorogenic probes for successful sensing the β -lactamase activity.¹¹⁻¹⁵ However, all the existing methods are dependent on either sophisticated instruments or laborious sample preparation. The much more simple, rapid and economical assay is still highly needed to overcome the drawbacks of the current assays.

In this chapter, we reported a simple and convenient colorimetric assay for

Scheme 2.1. General design of the gold nanoparticles based colorimetric assay for sensing β -lactamase activity.

sensing β -lactamases activities by employing the excellent optical properties of AuNPs (Scheme 2.1). The nanoparticles changed color from red to purple-blue upon enzyme interaction with the designed cephalosporin substrates. This colorimetric assay facilitates the visualization of β -lactamase activity by naked eyes and simple colorimetric reader. This method could provide the possibility to rapidly identify the β -lactamase activity and has potential application for sensing enzyme activity in bacterial strains.

2.2 Results and Discussion

2.2.1 Cephalosporin biosensors for β -lactamase

It is known that cephalosporin substrates could be efficiently hydrolyzed by β -lactamase. As shown in Figure 2.1, upon the enzyme actuation, the β -lactam ring in cephalosporin was cleaved, generating an unstable intermediate. The intermediate spontaneously rearranges and releases the fragment on 3' position of cephalosporin. Based on this mechanism, we designed two cephalosporin substrates for sensing the β -lactamase.

Figure 2.1. Illustration of β -lactamase-catalyzed hydrolysis of cephalosporin.

In our design, we introduced the thiol group on the 3' position of cephalosporin substrates. Two cephem nuclei are connected through a dithiol modified 1,2-bis(2-aminoethoxy) ethane flexible linker. As an excellent leaving group, the thiol group could facilitate elimination of the fragment on the 3' position of cephalosporin upon enzyme treatment. Another advantage of introduction of thiol group is that it has strong interactions with gold surfaces. Taking advantage of the covalent Au-S bond, the dithiolated linker anchored on the surface of AuNPs resulting in shortening the interparticle distances, and leading to the aggregation of gold nanoparticles. Here, 1,2-bis(2-aminoethoxy) ethane was used to improve the solubility of substrate and minimize the steric interactions between the substrate and the enzyme. Moreover, in order to optimize the kinetic hydrolysis properties of the substrate, two different thiols, 2-mercaptoethylamine and 4-aminothiolphenol, conjugated to 1,2-bis(2-aminoethoxy) ethane linkers were connected to the 3' position of the cephem nucleus. Figure 2.2 illustrated the schematic β -lactamase hydrolysis of designed cephalosporin substrates. In the presence of β -lactamase, the two cephalosporins were efficiently hydrolyzed and eliminated dithiolated alkyl linker and aryl linker.

Figure 2.2. Illustration of hydrolysis of designed cephalosporin substrates for AuNPs-based colorimetric assay.

The cephalosporin substrates were synthesized from 7-amino-3-chloromethyl cephalosporanic acid benzylhydryl ester hydrochloride (ACLH). Following literature procedure, the dithiolated linkers were connected to the 3' position of cephalosporin in a week basic condition through nucleophilic substitution reaction.¹²⁻¹³ After

simple deprotection with trifluoroacetic acid to remove the diphenyl methyl ester, substrate (I) and (II) were obtained from HPLC purification in mild yields and determined by MS spectrum.

2.2.2 Colorimetric assays for β -lactamase activity using gold nanoparticles

To demonstrate our concept, above synthesized cephalosporin substrate (I) and (II) were exploited for the AuNPs based colorimetric assay for sensing the presence of β -lactamase.

The citrate-protected gold nanoparticles with 15 nm in diameter were prepared according to Turkevich method.¹⁶ Average size of prepared AuNPs was determined from transmission electron microscope images of around 200 particles. The UV-Vis absorption spectrum was measured to determine the concentration of gold nanoparticles. According to Lambert-Beer law, the concentration of nanoparticles is 2.6 nM with the molar extinction coefficient of $2.7 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}$ for 15 nm AuNPs at 520 nm.¹⁷

The aggregation of AuNPs induced by β -lactamase treated substrates was tested. Substrates (8 μM) were initially incubated with Bla (5 nM) in PBS buffer solution (pH 7.4) for 20 min. Then the resulting solutions were transferred into AuNPs suspensions. In order to stabilize the nanoparticles and to prevent the non-specific interaction of proteins with gold nanoparticles, 0.1% PEG 8000 was added into the reaction mixture. As shown in Figure 2.3, the color of the AuNPs suspension alone remained unchanged with time. When the intact substrates were added, no further

color changes in the AuNPs demonstrated that both of the two β -lactam substrates were stable under the experimental conditions. In the presence of Bla pretreated substrate (I), no detectable color change of nanoparticles was observed within 30 min. However, a distinct color change could be visualized after longer incubation time around 9 hr. In contrast, after adding the Bla pretreated substrate (II) into the AuNPs, the color dramatically changed from the pink-red into violet-blue within seconds. UV-Vis absorption of gold nanoparticles was also monitored within 30min after mixing the enzyme-treated substrate (II) with AuNPs (Figure 2.4). It was observed that both a decreased absorbance of plasmon band at 520 nm and an increased absorbance at 650 nm with increasing time. The shifted absorbance to a longer wavelength over time is correlated with the color change from pink-red to violet-blue by visual inspection.

Figure 2.3. Colorimetric assay with Bla treated two substrates. Colors of the AuNP solution in the absence or presence of Bla-treated substrates; 1: AuNPs only; 2: AuNPs and substrate (II); 3: AuNPs and Bla-treated substrate (II); 4: AuNPs and substrate (I); 5: AuNPs and Bla-treated substrate (I).

Figure 2.4. UV-Vis spectra of AuNPs taken at 2 min intervals for 30 min after addition of Bla treated substrate (II).

2.2.3 Aggregation kinetics of gold nanoparticles

To assess the process of enzymatic hydrolysis reactions, the dynamic rates of β -lactamase induced AuNPs aggregates were monitored using UV-Vis spectrophotometric method. Figure 2.5 showed the time course for the absorbance of gold nanoparticles at 650 nm after addition of Bla pretreated substrates. For the substrate (I), no great absorbance change within 30 minutes demonstrated the slow process of the overall enzyme induced aggregation of nanoparticles. However, about two-third of change in the total absorbance occurred within the first five minutes suggested that this reaction process for substrate (II) was very fast. These results were in agreement with the color change observed by naked eyes.

Figure 2.5. Absorbance change at 650 nm of AuNPs in the presence of Bla-treated cephalosporin substrate (I) and (II) as a function of time.

Herein, we supposed that the colorimetric assay mainly consisted of a two-step interaction: one was the enzymatic hydrolysis with releasing the dithiol linkers and the other was the released dithiol linkers subsequently coagulate AuNPs to form aggregation. As shown in Figure 2.6, the absorbance change of aggregated AuNPs was recorded for the binding of free dithiol linkers to the AuNPs at different time points (alkyl linker: di-2-mercaptoethylamine conjugated 1,2-bis(2-aminoethoxy) ethane and aryl linker: di-4-aminothiolphenol conjugated 1,2-bis(2-aminoethoxy) ethane). Both of the linkers exhibited the similar coagulating abilities to AuNPs and their rapid binding process within seconds confirmed the kinetics of the system was limited to enzymatic hydrolysis reaction.

Figure 2.6. Absorbance change at 650 nm of AuNPs with free dithiol alkyl linker and aryl linker as a function of time.

Therefore, the colorimetric feature of AuNPs was applied to determine the hydrolysis kinetic parameters of Bla. The schematic enzyme reaction process was shown in Figure 2.7, where the E is the enzyme representing β -lactamase, S is the β -lactam substrate, ES is the noncovalent enzyme-substrate complex, ES* is acyl-enzyme adduct, and P is the hydrolyzed product.

Figure 2.7. Illustration of Mechanism of β -lactam hydrolysis by β -lactamase.

Spectrophotometric method was applied to monitor the hydrolysis of the cephalosporin substrates by β -lactamase with the aid of the AuNPs. The initial rate of hydrolysis occurring in the first 10 min was determined in duplicate at each of five different substrate concentrations (pH 7.4) at 25°C. Further analysis from

Michaelis-Menten equation (equation 2.1) and Lineweaver-Burk plot (Figure 2.8) of the enzymatic hydrolysis for the two cephalosporin substrates revealed $k_{cat} = (0.33 \pm 0.1) \text{ s}^{-1}$, $K_m = (140 \pm 11) \mu\text{M}$ for substrate (I) and $k_{cat} = (8.69 \pm 1.3) \text{ s}^{-1}$, $K_m = (113 \pm 8.0) \mu\text{M}$ for substrate (II) (where $k_{cat} = k_2k_3 / (k_2 + k_3)$ is turnover number and K_m is Michaelis-Menten constant).

$$\frac{1}{V_{\max}} = \frac{K_m + [S]}{V_{\max}[S]} = \frac{K_m}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}} \dots\dots\dots(2.1)$$

Figure 2.8. Double reciprocal plots of substrate (I) (a) and substrate (II) (b) hydrolyzed by enzyme per second (v) versus substrate concentrations.

The K_m values of these two substrates are comparable which suggest that substrate (I) and substrate (II) have the similar enzyme affinities towards β -lactamase. However, the small value of k_{cat} for substrate (I) demonstrated that the leaving group based on alkyl thiol linker was less efficient than that of thiolphenol used in substrate (II). Although the ES and ES* complexes were accumulated, subsequently released hydrolyzed product for substrate (I) were deficient. Thus, the alkyl thiol based

substrate (I) prefers longer incubation time for enzyme hydrolysis and concomitantly exhibited a slow response to induce AuNPs aggregation. It is clear that substrate (II) appeared to be a good substrate for this colorimetric assay.

In this colorimetric assay, enzyme hydrolyzed substrate (II) with dithiolated aryl linker faster responded to induce the aggregation of nanoparticles compared with substrate (I). Higher concentrations of substrate (II) after enzyme hydrolysis could induce much greater extent of crosslinking of AuNPs which were accompanied with different colors of aggregated nanoparticles. Figure 2.9 shows the colorimetric images of AuNPs after adding Bla treated various concentrations of substrate (II) ranging from 0 to 12 μM . The final concentration of Bla was maintained at 5.0 nM. From the color change, 8 μM of substrate (II) with Bla could induce the modest extent of aggregation of AuNPs with distinct color change and no precipitated agglomeration.

Figure 2.9. Aggregation of AuNPs after mixing with different concentrations of substrate (II). 1:AuNPs suspension without substrate; 2:AuNPs mixed with Bla treated 4 μM of substrate (II); 3:Bla treated 6 μM of substrate (II); 4:Bla treated 8 μM of substrate (II); 5:Bla treated 10 μM of substrate (II); 6:Bla treated 12 μM of substrate (II).

By employing the distinct color change of gold nanoparticles, β -lactamase activity could be indirectly monitored. To examine the sensitivity of this assay for sensing β -lactamase, we investigated the enzymatic reaction by incubating the different Bla concentrations with substrate (II) at room temperature. Then the mixtures were applied for AuNPs-based colorimetric assay. The final concentration of substrate (II) was maintained at 8 μ M. Photographs in Figure 2.10 show the color change of AuNPs and it is detectable for 60 pM of Bla by using nanoparticles color change.

Figure 2.10. Aggregation kinetic of AuNPs after mixing with different Bla concentrations within 30 min. 1: AuNPs suspension without substrate; 2: AuNPs mixed with 0 μ M Bla treated substrate (II); 3: AuNPs and 60 pM Bla treated substrate (II); 4: AuNPs and 1.0 nM Bla treated substrate (II); 5: AuNPs and 5.0 nM Bla treated substrate (II).

2.2.4 Characterization of gold nanoparticles in the colorimetric assay

Transmission electron microscopy was performed to characterize the morphology of AuNPs in the colorimetric assay. As substrate (II) is kinetically robust

to enzyme hydrolysis, the different aggregation behaviors of substrate (II) with Bla in AuNPs suspension were monitored. As shown in Figure 2.11a, intact substrate (II) ($8\ \mu\text{M}$) was stable and unable to induce the aggregation of AuNPs suspensions. Upon treatment of the same concentration of substrate (II) with Bla ($5\ \text{nM}$), the enzyme interaction triggered the release of the dithiolated linker, thus inducing the crosslinking of AuNPs to increase the significant aggregation (Figure 2.11b). Although a little bit of agglomerations could be detected in Figure 2.11a, which was possibly caused by self-assembly during the drying process in the sample preparation, most of the AuNPs were dispersed randomly in the solution with $15\ \text{nm}$ in diameter.

Figure 2.11. TEM images of a) substrate (II) ($8\ \mu\text{M}$) in AuNPs and b) incubation of substrate (II) ($8\ \mu\text{M}$) with Bla ($5\ \text{nM}$) in AuNP solutions. Scale bars: $200\ \text{nm}$.

Dynamic light scattering (DLS) measurements further confirmed a well-dispersed population of AuNPs in the solution with substrate (II) only, and

highly aggregated AuNPs in the solution with Bla pretreated substrate (II) (Figure 2.12). The light scattering measurements clearly indicated the monodispersity of AuNPs in average hydrodynamic diameters of 17.2 ± 1.3 nm in the solution only treated with substrate (II) and highly aggregated AuNPs in average diameters of 182.9 ± 22.5 nm in the solution treated with Bla and substrate (II).

Figure 2.12. Hydrodynamic size distribution of AuNPs with substrate (II) only (a) and with Bla treated substrate (II) (b).

Both TEM images and DLS measurements proved that cephalosporin substrates

were stable in the AuNPs suspension and the aggregation of AuNPs was indeed specifically triggered by the interactions between the substrates and Bla. The dithiolated linker of the cephalosporin substrate serves as coagulant and interacts with nanoparticles.

2.2.5 Inhibition assay of β -lactamase

To evaluate the potential of current colorimetric assay in enzyme inhibitors screening, we determined the β -lactamase inhibition by using this simple and sensitive AuNPs assay. One commonly used Bla inhibitor, sulbactam was chosen and the effect of the enzyme inhibition was evaluated using our system. After incubating different concentrations of inhibitor pretreated with Bla and substrate (II), the mixture was transferred into AuNPs solution and a different color would be observed which indicated the different abilities for the enzyme inhibition. The IC_{50} value (concentration of inhibitor that reduces enzyme activity to 50% of the activity of the native enzyme) was found to be about 4.4 μ M, which is similar to the previously reported value (Figure 2.13).¹⁸ This result supports that this colorimetric assay has potential for a quantitative analysis of Bla activity and for screening the Bla inhibitors.

Figure 2.13. AuNPs-based inhibition assay by sulbactam and Bla treated substrate (II).

2.2.6 Colorimetric assay in antimicrobial bacteria strains

To further evaluate substrate (II) would respond to β -lactamase in the living bacteria, different strains of bacteria ($\sim 10^8$ cfu/mL) such as wild type *E. coli* B121, antibiotic-resistant plasmid encoded *E. coli* B121 and one clinically isolated β -lactam resistant *K. pneumoniae* (ATCC 700603) were incubated with substrate (II). As shown in Figure 2.14a, wild type *E. coli* B121 treated substrate (II) will not lead to the AuNPs color change because it is unable to express Bla. However, significant color change was observed within 30 min after adding plasmid encoded *E. coli* B121 or *K. pneumoniae* treated substrate (II) into AuNPs suspensions. The different color changes (blue in Bla encoded *E. coli* B121 and reddish purple in *K. pneumoniae*) were attributed to the different subclass of Bla expressions. These two β -lactam resistant bacterial strains contain TEM-1 β -lactamase in plasmid encoded *E. coli* B121 and SHV-18 β -lactamase in *K. pneumoniae*, respectively. The different types of β -lactamase exhibited different enzymatic conversion capabilities for the same

substrate, which is consistent with the results reported recently.¹⁵

Figure 2.14. (a) Colorimetric assay of AuNPs incubated with bacterial strains after 30 min. 1: AuNPs; 2: wild type BI21 with substrate (II) (8 μ M); 3: plasmid-encoded BI21 with substrate (II) (8 μ M); 4: *K. pneumoniae* with substrate (II) (8 μ M). (b) Fluorescence spectra of CC1 hydrolyzed by three kinds of bacterial strains. Excitation wavelength is 360 nm.

The enzyme activities in different bacterial strains were also monitored by fluorescence assay with CC1. CC1 is a sensitive fluorescent sensor for determining the Bla activity (Figure 2.15).¹⁹ As shown in Figure 2.14b, no significant fluorescent signal was detected in wild type *E. coli* BI21 which has no β -lactamase expression.

Figure 2.15. Hydrolysis of CC1 by Bla releases the blue fluorescence.

The fluorescence emission from CC1 in *E.coli* Bl21 (with Bla expression) was about 4 times higher than that in *K. pneumoniae*, which confirmed the highest enzyme activity in the Bla (TEM-1) encoded *E.coli* Bl21 strains. As a commonly used fluorogenic probe, CC1 was more sensitive to detect β -lactamases compared to the colorimetric assay. However, CC1 itself was not so stable and easily occurred spontaneous hydrolysis. In addition, the whole fluorescent assay had to be performed with specific instrumentations.

Figure 2.16. Colorimetric assay by nitrocefin with different bacterial strains. **1:** Nitrocefin solution (8 μ M) only; **2:** Nitrocefin (8 μ M) mixed with wild type *E. coli* Bl21; **3:** Nitrocefin (8 μ M) mixed with β -lactam antibiotics resistant *E.coli* Bl21; **4:** Nitrocefin (8 μ M) mixed with clinical isolate *K. pneumoniae* (ATCC 700603) strains.

The colorimetric assay in living bacterial strains was also performed by using nitrocefin, a standard β -lactamase indicator (Figure 2.16). The result indicated that nitrocefin could induce the similar color change in both of the β -lactam resistant bacteria strains from yellow to pink. There was no color difference between the Bla

encoded *E.coli* Bl21 and *K. pneumoniae* strains. Moreover, the pink color was also detected in the wild type *E. coli* Bl21 bacteria where no Bla is present, possibly due to the nonspecific hydrolysis of nitrocefin. Therefore, compared to the nitrocefin based colorimetric assay, the significantly different color change observed in different β -lactam resistant bacteria, and no background in wild type bacterial strains in the AuNPs based colorimetric assay indicate that the latter has a higher reporting threshold than that of nitrocefin assay.

The AuNPs based enzymatic assay provides a particularly useful sensing approach for systems that have significant background, which would cause a false positive signal in the nitrocefin assay. This new developed AuNPs based colorimetric assay induced by enzymatic reaction provides an alternative approach for simple and specific indication of living drug resistant bacteria in real time.

2.3 Conclusions

In summary, we described a simple, economical assay to rapidly visualize β -lactamase activities by using gold nanoparticles. This assay arises from the use of Bla to cleave the dithiol modified linker from the cephalosporin substrate and subsequently induce the crosslinking of AuNPs. This process does not require specific instrumentation or complicated experiment steps. Thus the merit of simplicity for this colorimetric assay should be appreciated. It can offer an alternative platform to evaluate the enzyme kinetic reactions and to screen the β -lactamases inhibitors in real time. We believe that it may provide useful practical applications for the rapid and specific detection of antibiotic resistant bacteria in clinical settings.

2.4 Experimental Section

Materials and Chemicals

7-Amino-3-chloromethyl 3-cephem-4-carboxylic acid diphenylmethyl ester hydrochloride (ACLH) was provided from Otsuka chemical Co. Ltd. Nitrocefin was purchased from Merck. β -lactam resistant *K. pneumoniae* bacterial strains (ATCC 700603) were purchased from ATCC. β -lactamases were obtained from Biologics Process Development, Inc, CA, USA and Sigma-Aldrich (6–18 units/mg corresponds to the amount of enzyme which hydrolyzes 1 μ mol of benzylpenicillin per minute at pH 7.0 and 25°C or 50–150 units/mg protein by using cephaloridine). All the other starting materials were obtained from Sigma or Aldrich. Commercially available reagents were used without further purification, unless noted otherwise. The solvents were dried according to regular protocols. Silica gel (40 μ m average particle size) was used for flash column chromatography. All other chemicals were analytical grade or better. Copper specimen grids (200 mesh) with formvar/carbon support film were used for transmission electron microscopy measurements. Deionized water (18 $\text{m}\Omega\text{ cm}^{-1}$) was used in all the colorimetric experiments. All glassware used in the nanoparticles based experiments were soaked in aqua regia (HNO_3 : HCl 1:3) and rinsed thoroughly with deionized water.

Instrumentation and Characterization

The synthesized compounds were characterized using ^1H NMR (Bruker Advance 400 MHz) using CDCl_3 as the solvent. Chemical shifts (δ) are given in ppm relative to

tetramethylsilane. The coupling constants (J) are in hertz (Hz). Abbreviations used are s = singlet, bs = broad singlet, d = doublet, t = triplet, and m = multiplet. ESI-MS spectrometric analyses were performed on the Thermo Finnigan LCQ Deca XP Max and transmission electron micrograph were taken on JEOL 2000 EX TEM. Absorbance spectra were measured on Beckman Coulter DU 800 UV-Vis spectrophotometer. Dynamic light scattering measurement was conducted at 90 Plus particale size analyzer to study the particle size distribution in solution. Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer.

Analytical reverse-phase high performance liquid chromatography (HPLC) was performed on Alltima C-18 column (250×3.0 mm) at a flow rate of 1.0 mL/min and semi-preparative HPLC was performed on the similar C-18 column (250×10 mm) at a flow rate of 3 mL/min. An eluting system consisting of A (water with 0.1% TFA) and B (acetonitrile with 0.1% TFA) was used under a linear gradient to elute the products, which was monitored by UV-Vis absorbance at 280 nm. The linear gradient started from 80% solution A and 20% solution B, changed to 20% solution A and 80 % solution B in 30 minute and to 0% solution A and 100% solution B in the following 5 minutes, and then back to 80% solution A and 20% solution B in the next 5 minutes.

Synthesis of cephalosporin substrates

Cephalosporin substrate (I) and substrate (II) were prepared according to synthetic routes as shown in Figure 2.17 and Figure 2.18, respectively.

Figure 2.17. The synthetic route for cephalosporin substrate (I).

Figure 2.18. The synthetic route for cephalosporin substrate (II).

Preparation of 1: 7-Amino-3-chloromethyl cephalosporanic acid benzylhydryl ester hydrochloride (ACLH) (451 mg, 1 mmol) was suspended in dichloromethane. Then acetyl chloride (78.5 mg, 1 mmol) was added drop wise into the suspension. Finally 2,6-lutidine (214 mg, 2 mmol) was added into the reaction mixture and the solution was stirred for 2 hrs under nitrogen. After the removal of the solvent on the rotary evaporator, the residue was purified by flash chromatography on silica gel (eluent: ethyl acetate / hexane =1/1) to afford 223.5 mg (yield: 85.4%) of title compound. ^1H NMR (400 MHz, CDCl_3) δ 7.47-7.45 (m, 2H), 7.41-7.31 (m, 7H), 7.28-7.26 (m, 1H), 6.98 (s, 1H), 5.90 (dd, J = 8.9 and 4.92 Hz, 1H), J = 4.96 (d, J =4.95 Hz, 1H), 4.38 (s,

2H), 3.55 (d, $J = 18.36$ Hz, 1H), 3.45 (d, $J = 18.36$ Hz, 1H), 2.02 (s, 3H). ESI-MS observed $[M+H]^+$: 457.7, calculated $[M+H]^+$: 457.5.

Preparation of 2: A mixture of **1** (155 mg, 0.34 mmol) and sodium iodide (253 mg, 1.7 mmol) in 5mL of acetone was stirred for 1 hr at room temperature. The reaction mixture was concentrated on the rotary evaporator and diluted with 5mL water. The suspension was extracted with 25 mL of ethyl acetate, and the organic phase was washed with 10% sodium thiosulfate (5 mL $\times$ 2), brine (5 mL $\times$ 3) and dried over anhydrous magnesium sulfate. The slightly orange powder **2** (152 mg, 0.45 mmol) was used without further purification.

Preparation of 3: 2-Mercaptoethylamine hydrochloride (261.4 mg, 2.3 mmol) was added to the solution of chloro triphenylmethane (557.6 mg, 2 mmol) in 1.0mL of anhydrous dichloromethane. Then trifluoroacetic acid (TFA, 0.4 mL) was added to afford dark brown solution. The solution was stirred for 2 hrs under nitrogen. The reaction was quenched by 1N NaOH (3 mL). The suspension was extracted with 10mL of ethyl acetate, and the organic phase was washed with brine (5 mL $\times$ 3) and dried over anhydrous magnesium sulfate. The solvent was removed and residue was purified by flash chromatography on silica gel (eluent: ethyl acetate/hexane = 1/3) to afford 543 mg of desired product (85%). ^1H NMR (400 MHz, CDCl_3) δ 7.50-7.48 (m, 6H), 7.34-7.26 (m, 6H), 7.25-7.23 (m, 3H), 2.63 (t, $J = 6.5$ Hz, 2H), 2.36 (t, $J = 6.5$ Hz, 2H), ESI-MS: observed $[M+\text{Na}]^+$: 342.5, calculated $[M+\text{Na}]^+$: 342.3.

Preparation of 4: To a clear solution of **3** (150mg, 0.47mmol) in anhydrous dichloromethane (1.5mL) was added iodoacetic anhydride (200mg, 0.56mmol) at room temperature, followed by *N, N*-diisopropylethylamine (DIPEA, 106 μ L, 0.61mmol). The reaction mixture was stirred under room temperature for 12 hours. After the removal of the solvent in vacuum, the residue was applied to flash chromatography on silica gel (eluent: ethyl acetate/hexane =1/8) to afford 80 mg of title compound (70%). ^1H NMR (400 MHz, CDCl_3) δ 7.46-7.45 (m, 15H), 3.63 (s, 2H), 3.10 (dd, J = 6.08 Hz and J = 12.36 Hz, 2H), 2.45 (t, J = 6.2 Hz, 2H). ESI-MS observed $[\text{M}+\text{Na}]^+$: 510.5, calculated $[\text{M}+\text{Na}]^+$: 510.4.

Preparation of 5: To a cooled (ice bath) and stirred solution of compound **4** (75mg, 0.154mmol) in 3.0mL anhydrous dichloromethane was added *N, N*-diisopropylethylamine (DIPEA, 25 μ L, 0.33mmol). Then 2, 2'-(ethylenedioxy) bis-ethylamine (10 μ L, 0.069mmol) was added drop-wise into this solution. The solution was stirred overnight. The reaction mixture was concentrated on the rotary evaporator. Purification of the crude product by flash chromatography on silica gel (eluent: methanol/ dichloromethane =1/20) afforded the desired product 50 mg (41%). ^1H NMR (400 MHz, CDCl_3) δ 7.38-7.37 (m, 12H), 7.28-7.25 (m, 10H), 7.21-7.20 (m, 8H), 3.81 (t, J = 5.48 Hz, 4H), 3.62 (m, 4H), 3.59 (s, 4H), 3.47 (s, 2H), 3.22(dd, J = 7.36 Hz and J = 7.32 Hz, 4H), 3.09 (m, 2H), 2.88 (m, 2H), 2.40 (m, 2H) ESI-MS: observed $[\text{M}+\text{Na}]^+$: 891.1; calculated $[\text{M}+\text{Na}]^+$: 891.1.

Preparation of 6: To a cooled solution of **5** (20mg, 0.024mmol) in 350 μ L of anhydrous dichloromethane was added trifluoroacetic acid (1mL) and triisopropylsilane (50 μ L) with cooling (ice bath). The mixture was stirred for 1 hr at the same temperature, then the solvent was removed under reduced pressure. The residue was washed with cold hexane (1 mL $\times$ 3) to afford 15.5 mg of the light yellow crude product. The crude product was used for next step reaction without further purification. The product was added drop-wise to a solution of compound **2** (23.3 mg, 0.051 mmol) in 0.1mL anhydrous *N, N*-dimethylformamide (DMF), followed by addition of *N, N*-diisopropylethylamine (DIPEA, 8.8 μ L, 0.05 mmol) and 2, 6-lutidine (28 μ L, 0.24 mmol). The mixture was stirred at room temperature for 5 hrs. Then, the reaction mixture was diluted with water (5 mL) and extracted by ethyl acetate (10 mL). The organic phase was washed by brine (5 mL) and dried over anhydrous magnesium sulfate. The solvent was removed and the crude product was further purified by RP-HPLC to collect 3.5 mg of compound **6**. ESI- MS observed $[M+Na]^+$: 1246.6, calculated $[M+Na]^+$: 1246.1.

Preparation of cephalosporin substrate (I): A solution of **6** (3.0mg, 0.00245mmol) was dissolved in 150 μ L of anhydrous dichloromethane. Then trifluoroacetic acid (100 μ L) and anisole (4.5 μ L) were added. The mixture was stirred for 1 hr at the cooled temperature (ice bath). The solvent was removed under reduced pressure. The precipitate was collected and washed with hexane (1mL $\times$ 3) and then purified by RP-HPLC to afford 1.3 mg (60%) of the title product. ESI- MS observed $[M+H]^+$:

891.6, calculated $[M+H]^+$: 891.2.

Figure 2.19. Chromatography profile of purified cephalosporin substrate (I).

Preparation of 7: To a solution of chloro triphenylmethane (557.6mg, 2mmol) in 1.5mL of anhydrous dichloromethane was added 4-aminothiophenol (275.4mg, 2.2mmol). Then trifluoroacetic acid (TFA, 0.35mL) was added to afford dark brown solution. The solution was stirred for 2 hrs at ambient temperature under nitrogen. After the removal of the solvent on the rotary evaporator, the residue was quenched by 1N NaOH (3 mL). The suspension was extracted with 10mL of ethyl acetate, and the organic phase was washed with brine (5 mL×3) and dried over anhydrous magnesium sulfate. The solvent was removed and residue was purified by flash chromatography on silica gel (eluent: ethyl acetate/hexane =1/3) to afford 625 mg of desired product (85%). The crystal structure of this compound was also obtained. ^1H NMR (400 MHz, CDCl_3) δ 7.43 (d, J = 7.24 Hz, 6H), 7.28-7.19 (m, 9H), 6.78 (d, J = 8.4 Hz, 2H), 6.35 (d, J = 8.4 Hz, 2H), 3.66 (s, 2H). ESI-MS: observed $[M+H]^+$: 368.4, calculated $[M+H]^+$: 368.3.

Preparation of 8: To a clear solution of **7** (150mg, 0.41mmol) in anhydrous dichloromethane (1.5mL) was added iodoacetic anhydride(173mg, 0.49mmol) at room temperature, followed by *N, N*-diisopropylethylamine (DIPEA, 107μL, 0.61mmol). The precipitate was formed within 3 minutes. The reaction mixture was stirred under room temperature for 12 hours. After the removal of the solvent in vacuum, the residue was applied to flash chromatography on silica gel (eluent: ethyl acetate/hexane =1/8) to afford 120 mg of title compound (79%). ¹H NMR (400 MHz, CDCl₃) δ 7.44-7.39 (m, 6 H), 7.29-7.18 (m, 12 H), 6.96 (d, *J* = 8.64 Hz, 2 H), 3.82 (s, 2 H). ESI-MS: observed [M+Na]⁺: 558.3, calculated [M+Na]⁺: 558.4.

Preparation of 9: To a cooled (ice bath) and stirred solution of compound **8** (75mg, 0.135mmol) in 3.0mL anhydrous dichloromethane was added *N, N*-diisopropylethylamine (DIPEA, 21.7μL, 0.29mmol). Then 2,2'-(ethylenedioxy) bis-ethylamine (8.0μL, 0.055mmol) was added drop-wise into this solution. The solution became clear after three hours and the reaction mixture was stirred overnight. The solvent was removed in vacuum. Purification of the crude product by flash chromatography on silica gel (eluent: methanol/ dichloromethane =1/20) afforded title compound 38.6 mg (73%). ¹H NMR (400 MHz, CDCl₃) δ 7.42-7.40 (m, 12 H), 7.30-7.20 (m, 22H), 6.93 (d, *J* = 8.0 Hz, 4 H), 3.59-3.40 (m, 8H), 3.40 (s, 4H), 2.84 t, *J* = 4.7 Hz, 4H). ESI-MS: observed [M+H]⁺: 965.4; calculated [M+H]⁺: 965.3.

Preparation of 10: To a cooled solution of **9** (15mg, 0.018mmol) in 350 μ L of anhydrous dichloromethane was added trifluoroacetic acid (1 mL) and triisopropylsilane (45 μ L) with cooling (ice bath). The mixture was stirred for 1 hr at the same temperature, and then the solvent was removed in vacuum. The residue was washed with cold hexane (1mL $\times$ 3) to afford 13 mg of the light yellow crude product. The crude product was used for next step reaction without further purification. The product was added drop-wise to a solution of compound **2** (23mg, 0.045mmol) in 0.1 mL anhydrous *N,N*-dimethylformamide (DMF), followed by addition of *N,N*-diisopropylethylamine (DIPEA, 7 μ L, 0.04mmol) and 2, 6-lutidine (28 μ L, 0.24mmol). The mixture was stirred at room temperature for 5 hrs. Then, the reaction mixture was diluted with water (5 mL) and extracted by ethyl acetate (10mL). The organic phase was washed by brine (5mL) and dried over anhydrous magnesium sulfate. The solvent was removed and the crude product was further purified by RP-HPLC to collect 3.0 mg of compound **10**. ESI-MS observed $[M+Na]^+$: 1342.5; calculated $[M+Na]^+$: 1342.4.

Preparation of cephalosporin substrate (II): A solution of **10** (3.0mg, 0.0023mmol) was dissolved in 150 μ L of anhydrous dichloromethane. Then trifluoroacetic acid (150 μ L) and anisole (50 μ L) was added under the cooling condition (ice bath). The mixture was stirred for 1 hr at the same temperature. The solvent was removed in vacuum. The precipitate was collected, washed with hexane (1mL $\times$ 3) and then further purified by RP-HPLC to afford 1.5 mg (66.7%) of the title

product. ESI- MS: observed $[M+H]^+$: 988.7: calculated $[M+H]^+$: 988.2.

Figure 2.20. Chromatograph profile of purified cephalosporin substrate (II).

Preparation of citrate-stabilized gold nanoparticles: Gold nanoparticles (15 nm) were prepared by citrate reduction of hydrogentetrachloroaurate (HAuCl_4).¹⁶ The aqueous solution of HAuCl_4 (100 ml, 0.25 mM) was refluxed for 5-10 min, and 5 ml of 0.5% trisodium citrate solution was added quickly and reflux was continued for another 30 min until the color of the solution would change gradually from faint yellowish to wine-red. After filtration through 0.45 μM Millipore syringe to remove the precipitate, the filtrate was stored at room temperature for a period of time.

Preparation of TEM samples: TEM grids were treated by oxygen plasma in a Harrick plasma cleaner/sterilizer for 1 min to improve the surface hydrophilicity. TEM samples were prepared by directly dropping 20 μl sample solution on the formvar/carbon TEM grid, dried in air for 15 min. Transmission electron microscope images were captured on JEOL 2000 EX TEM at 200kV.

Dynamic light scattering (DLS) analysis for size distribution: The size and size population distributions of gold nanoparticles in substrate (II) (8 μM) treated AuNPs suspensions and Bla pretreated substrate (II) (8 μM) AuNPs suspensions were determined on a Brookhaven Instruments spectrophotometer. The instrument was equipped with a compass 315M-150 laser that was used at a wavelength of 660 nm. Dust-free solution vials were used for the aqueous solutions, and measurements were performed at an angle of 90° under room temperature. The CONTIN algorithm was used for analyze the DLS data.

Enzyme hydrolysis of substrate (II) by β -lactamase (Bla): Substrates solutions were prepared in deionized water and Bla was dissolved in PBS buffer (pH 7.4) to make different Bla concentrations. Then Bla solution (10 μL) was mixed with 190 μL of substrates for the enzyme interactions. The final substrate concentrations were maintained at 8 μM . The enzymatic reaction was performed by incubating the different Bla concentration with substrates for 20min at room temperature. All the tests were performed in triplicates.

Bla solution (10 μL) was mixed with 190 μL of different concentrations of substrates for the enzyme interactions. The final concentration of Bla was maintained at 5.0 nM. The substrates concentrations were ranged from 4 to 12 μM . The enzymatic reaction was performed by incubating the different concentration of substrate with Bla for 20 min at room temperature.

Colorimetric assays and plasmon absorption shifts upon AuNPs aggregation:

After 20min enzyme treatment, the resulting substrate solution was mixed with AuNPs suspension (15 nm, 800 μ L). The pictures were captured at each 2-minute intervals to observe color change of AuNPs. UV-Vis spectrum were also collected at different time intervals after mixing the enzyme-treated substrates with AuNPs. As a control, AuNPs suspension (800 μ L) was mixed with 200 μ L DI water, the color and the UV-Vis spectrum of the suspension were analyzed following the same procedure.

Kinetics of AuNPs based colorimetric enzymatic assays: The kinetic experiments were carried out at 25°C in PBS buffer with pH 7.4. The absorbance change at 650nm was measured by UV spectrophotometer. To a series of different concentration of substrates (range from 160 to 20 μ M) were added a solution of β -lactamase. The reaction mixture was then added into AuNPs suspension (2.6 nM). The rate of enhancement in absorbance at 650nm was applied to determine the kinetic properties of enzyme hydrolysis. The values of the kinetic parameters (K_m and k_{cat}) were determined from the double-reciprocal plot of the hydrolysis rate versus substrate concentrations (Lineweaver-Burk plot).

Inhibition assay for Bla activity by using gold nanoparticles: For the inhibition assay of Bla activity, the procedure is the same as that in the enzyme reaction for aggregation of AuNPs. The final concentrations of substrate and Bla solutions were maintained at 8.0 μ M and 5.0 nM, respectively. One commonly used β -lactamase

inhibitor; sulbactam (10 μ L in PBS buffer, pH 7.4) was mixed with Bla solution (10 μ L in PBS, pH 7.4) first. Then the mixture was incubated at room temperature for 20 minutes to inhibit Bla activity. The inhibitor pre-treated Bla solution was added into 180 μ L solution of substrate for the further enzyme interactions. Finally, after 20 min incubation, 200 μ L of the substrate solution (with inhibitor pre-treated Bla) was added into 800 μ L of AuNPs suspension to induce the aggregation of AuNPs. The absorbance change at 650 nm was analyzed every 3 min for 30 min at room temperature by Beckman Coulter DU 800 UV-Vis spectrophotometer.

Preparation of bacterial cultures: The Gram negative *E.coli* strain BL21 (DE3) was used as the host to express the TEM-1 β -lactamase. The bacterial strain was grown on a nutrient agar plate containing 100 μ g/mL carbenicillin, and the plate was incubated at 37°C overnight. Taking the single bacterial colony and inoculate it into 50 mL of sterile Luria-Bertani (LB) broth. The inoculated broth was incubated at 37°C with orbital shaking at 280 rpm overnight. The clinical isolate *K. pneumoniae* (ATCC 700603) bacterial strain was cultured according to the same methods.

β -lactamase activity in β -lactam antibiotic resistant bacterial strains: When the optical density (OD) at 600 nm of bacterial strains reached 0.8, the suspension was chilled on ice for 5 min, 1ml aliquots were taken into 1.5mL vial, and bacteria were harvested by centrifugation at 10,000 rpm for 5 min. After centrifugation, supernatant was removed and cells were washed three times with 1mL of PBS buffer

(approximately 1×10^8 cfu/mL). The bacterial cells were then re-suspended in 2.5 mL deionized water for CC1 assay. Fluorogenic substrate CC1 was prepared according the literature.¹⁹ 5 μ L of CC1 (1 mM in PBS, pH 7.4) was added into 2.5 mL of bacteria suspensions, Fluorescence spectra were recorded on Varian Cary Eclipse fluorescence spectrophotometer. The excitation wavelength was 360 nm and 10 nm of slit was used for detection. The enhancement of fluorescent signal at 450 nm was detected every ten minutes until no any further fluorescence increase. In contrast, wild type *E.coli* B121 strains without β -lactam resistant gene were also used as negative control. The different fluorescent signal in wild type *E. coli* B121, β -lactam antibiotics resistant *E.coli* B121 and clinical isolate *K. pneumoniae* (ATCC 700603) strains were recorded at the same conditions.

Colorimetric assays in β -lactamase bacterial resistance strains: Bacterial strains ($\sim 10^8$ cfu/mL) were suspended in 200 μ L of deionized water which contained substrates under the room temperature. The suspension was incubated for 20 minutes for further enzyme interactions. After centrifugation, supernatant was applied for colorimetric image. The 200 μ L of the bacterial solution was added into 800 μ L of AuNPs suspension to induce the aggregation of AuNPs. The color change of the AuNPs was recorded at different time intervals.

Colorimetric assay by using standard β -lactamase indicator, nitrocefin in living

bacteria: Bacterial cells ($\sim 10^8$ cfu/mL) were suspended in 1ml of Tris buffer (pH 7.4).

Nitrocefin solution (from Merck) was incubated with bacteria for enzyme interactions.

The final concentration of nitrocefin was maintained at 8 μ M, which was the same as the AuNPs based enzymatic assay. After 20 min interaction and centrifugation, supernatant was applied for colorimetric image.

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Chapter 3

Colorimetric Screening of Class A β -Lactamase Activity and Inhibition with Gold Nanoparticles

3.1 Introduction

β -Lactam antibiotics as chemotherapy reagent have been widely used in the treatment of bacterial infections in clinics over the past several decades. They could prevent growing bacteria from building the cell wall. However, the evolution of bacterial resistance to β -lactam antibiotics has been an ever-present threat to human health since the β -lactams were introduced in clinical therapy. Among a number of sources of resistance, the prevalent reason for bacteria resistance is the production of β -lactamases (Blas).

The production of β -lactamases has been widespread among Gram-positive and Gram-negative bacteria. A large number of β -lactamases variants have been identified and have been categorized into four classes (class A, B, C, and D).¹ Among the different classes of β -lactamases, class A β -lactamases constitute the largest family with diverse catalytic properties. There are several subclasses in class A β -lactamases including TEM/SHV β -lactamases, the *P. aeruginosa* OXA cephalosporinases and CTX-M carbapenemase.² They could efficiently hydrolyze the β -lactam ring in penicillin and cephalosporin resulting in ineffectiveness of β -lactam antibiotics. Therefore, it is valuable to identify the β -lactamases activity before conducting efficient treatment for the bacterial infections. Moreover, to respond to this bacterial

resistance, the family of β -lactamase inhibitors has raised considerable attention and largely expanded. This in turn brings about a challenge in screening potent candidates for β -lactamase inhibitors. Thus, it is highly desirable to develop a simple, quantitative and sensitive method to detect β -lactamase in *vitro* and in living bacterial strains.

In recent years, nanotechnology has been extensively developed in biological detections. Numerous studies have been focused on nanomaterials-based bioassays due to their scientific and potential economic importance. Gold nanoparticles (AuNPs) as one of the metallic nanomaterials have received much attention because of their excellent intrinsic characteristics such as ease of preparation, biocompatibility, stability, and unique physical and optical properties. They not only possess high extinction coefficients, but also exhibit distance-dependent optical properties which make them a good candidate for colorimetric tools.³⁻⁵ Dispersed gold nanoparticles solution displays red color, whereas the aggregated AuNPs changed to purple-blue. This significant color change associated with different status of AuNPs is detectable by naked eyes. Therefore, gold nanoparticles are widely used in varieties of colorimetric bioassays for sensitively detecting different analytes such as DNA, metal ions, proteins, enzymes, and small molecules.^{4,6-9}

In this chapter, we developed a simple and specific colorimetric assay based on aggregation of gold nanoparticles for sensing the class A β -lactamase activity in *vitro* and in the β -lactamase-secreting bacteria strains. This method was extended from our previously reported colorimetric assay in chapter 1. It could provide an alternative platform for sensitively detecting class A β -lactamase activity. Additionally, a

high-throughput screening of β -lactamase inhibitor candidates could be achieved by performing this simple method in a 96-well microplate.

3.2 Results and Discussion

3.2.1 Colorimetric assay for sensing class A β -lactamase

In our design, we synthesized a cephalosporin substrate which could be hydrolyzed by class A β -lactamase. A flexible 2-(4-mercaptophenyl) acetic acid coupled 1,2-bis(2-aminoethoxy) ethane linker is connected to the 3'-position of cephalosporin through iodo-thiol substitution. Thiol group was introduced in this sensing system owing to its several advantageous properties. Firstly, in the presence of β -lactamase, the β -lactam ring in cephalosporin was hydrolyzed and opened the four-membered ring, resulting in fast electron rearrangement. As a good leaving group, the thiol group could facilitate the release of the flexible linker after the enzyme hydrolysis. As shown in Figure 3.1, the thiolated linker was eliminated after the hydrolysis reaction between the substrate and class A β -lactamase.

Figure 3.1. Schematic illustration of the hydrolysis of cephalosporin substrate in the presence of class A Bla.

Moreover, the strong interactions between gold nanoparticles and thiol group which have been widely used in large number of colorimetric bio-detections were also an advantage properties of thiol group in this system. After the release of thiol-modified fragment, the free thiol terminal and positively charged amino groups anchored gold nanoparticles by Au-S bond and electrostatic interaction between the charged amino group and the citrate ions on the surface of gold nanoparticles. These interactions induced the aggregations of AuNPs which were accompanied by significant color change from red to blue due to the red-shifted plasmon band of the AuNPs. In contrast, when in the presence of potent Bla inhibitors, the activities of Bla were significantly suppressed and the substrate could not be efficient hydrolyzed without the release of coagulant linker to induce the aggregation of AuNPs. The distinct color change could not be detectable in AuNPs solution. Thus, this

red-shifting aggregation can be used as a colorimetric sensor to identify Bla activity in the absence and presence of Bla inhibitors. The efficiency of inhibitors of the enzyme can be screened by the specific color changes of gold nanoparticles. Figure 3.2 depicted the general concept for the colorimetric assay based on our design.

Figure 3.2. Schematic illustration of the colorimetric assay based on the aggregation of gold nanoparticles.

In a typical assay, the substrate (8.0 μM) was initially incubated with transformed TEM-1 Bla (2.0 nM) in a PBS buffer (pH 7.4) for 20 min in the absence of inhibitor. The resulting solution was subsequently transferred into AuNPs suspension. As shown in inset of Figure 3.3a, a significant color change from red to blue occurred within seconds. Both a decreased absorbance at 520 nm and an increased absorbance at 650 nm were observed in the UV-Vis spectrum along the time increased (Figure 3.3a). The

blue color of AuNPs and the red shift of spectrum indicate the highly aggregated state of gold nanoparticles.

Figure 3.3. UV-Vis spectra of AuNPs based colorimetric assay. (a). UV-Vis spectra of AuNPs before (black) and after (red) incubation with Bla-treated substrate in the absence of inhibitor. (b). Similar tests to a), but in the presence of an inhibitor (2.0 μM). The inset shows the color change of AuNPs corresponding to the UV-Vis spectra. 1: AuNPs only; 2: AuNPs and Bla treated substrate; 3: AuNPs only; 4: AuNPs and inhibited-Bla with substrate.

Furthermore, in order to demonstrate the inhibition assay, the Bla was pretreated

with potent class A Bla inhibitor (tazobactam) and then was used for the colorimetric assay for sensing the Bla activity. Tazobactam is a triazolyl-substituted penicillanic acid sulfone which was studied to be a strong Bla inhibitor. Typically, the transformed TEM-1 Bla which pre-incubated with tazobactam was mixed with substrate for 20 min in PBS buffer. After that, above mixture was transferred into AuNPs suspension. As shown in Figure 3.3b, there is no distinct spectrum shift and color change. This proves the Bla activity has been significantly inhibited and thiol modified fragments were not enough to trigger the detectable aggregation of AuNPs.

Control experiments were performed to eliminate the influence of nonspecific interactions between gold nanoparticles and environment. In this colorimetric assay, the effects of concentrations of PBS buffer and pH values on AuNPs were conducted for comparison.

Figure 3.4. Absorbance of gold nanoparticles at 520 nm in different concentration of PBS buffer solution.

AuNPs suspension showed no distinct aggregation in the PBS buffer with the concentration below 3 mM. (Figure 3.4) However, when the concentration of PBS buffer is higher than 3 mM, the obvious decrease of absorbance at 520 nm were observed which indicates the AuNPs were not stable and result in the aggregation.

pH effect on the aggregation of AuNPs was evaluated by adjusting various pH values of AuNPs suspension by adding HCl or NaOH. As shown in Figure 3.5, AuNPs were much stable with pH values larger than 5.5.

Figure 3.5. Absorbance of gold nanoparticles at 520 nm in different pH solution.

In our colorimetric assays, AuNPs aggregations were performed with 2 mM PBS buffer under pH 7.4. This condition has largely minimized the nonspecific interactions in AuNPs suspension and the aggregation of AuNPs was mainly induced by the enzymatic hydrolysis of substrate.

3.2.2 Characterization of morphology of AuNPs by transmission electron microscopy (TEM) and dynamic light scattering (DLS)

The different aggregation states of AuNPs in the presence or absence of enzyme inhibitor were monitored by TEM and DLS. AuNPs suspension with substrate was monodispersed in the solution with the hydrodynamic diameter of (17.3 ± 0.7) nm (Figure 3.6a, 3.7a). Substrate itself could not induce the aggregation of AuNPs. However, in the presence of Bla, substrate was hydrolyzed resulting in eliminating the thiol and amino group terminated linker which induced a dramatic aggregation of AuNPs with increased diameter of (117.7 ± 20.1) nm (Figure 3.6b, 3.7b). For the enzyme inhibition assay, the inhibitors suppressed the enzyme activity and inhibited the enzyme hydrolysis. As a potent inhibitor of class A Bla, tazobactam was pre-incubated with Bla and then employed in the colorimetric assay. No significant crosslinking of AuNPs was observed from TEM images and the hydrodynamic diameter of AuNPs is (23.4 ± 1.8) nm (Figure 3.6c, 3.7c). These results are consistent with that of the colorimetric assays and corresponding red shifts of UV-Vis spectra.

Figure 3.6. TEM images of (a) substrate ($8.0 \mu\text{M}$) in AuNPs; (b) incubation of substrate ($8.0 \mu\text{M}$) with Bla in the absence and (c) presence of inhibitor tazobactam ($2.0 \mu\text{M}$) in AuNPs solutions. Scale bar: 50 nm.

Figure 3.7. Hydrodynamic diameter of AuNPs. (a) Substrate (8.0 μM) in AuNPs. (b) AuNPs and substrate (8.0 μM) with Bla in the absence of inhibitor. (c) Incubation of substrate (8.0 μM) with Bla in the presence of inhibitor tazobactam (2.0 μM) in AuNPs solutions.

3.2.3 Sensitivity of the colorimetric assay

In order to evaluate the sensitivity of this method, various concentrations of Bla solutions were mixed with substrate for the enzyme interactions. The final concentration of substrate was maintained at 8.0 μM . After the enzymatic reaction performed for 20 min by incubating Bla solution with substrate at room temperature, the mixture was transferred into AuNPs suspension to induce the aggregation of AuNPs. The crosslinking of AuNPs increases with increasing enzyme concentration. As shown in Figure 3.8, the absorbance change at 650 nm indicates the aggregation of AuNPs, thus as low as 1 pM transformed TEM-1Bla could be detectable by using this AuNPs-based colorimetric assay. This sensitivity is much higher than previously reported one.¹⁰

Figure 3.8. Absorbance change at 650 nm of gold nanoparticles with substrate and various concentrations of transformed TEM-1Bla.

3.2.4 Screening inhibitors of class A β -lactamase *in vitro*

In a typical screening experiment, we chose four class A Bla inhibitors: aztreonam

(ATM), clavulanate acid (CA), tazobactam (TZB) and sulbactam (SUL) (Figure 3.9).

These four β -lactams are well recognized inhibitors to suppress class A Bla activities in clinics. To prove the potential application of the colorimetric assay for high throughput screening, all the inhibition reactions were performed in a 96-well microplate.

Figure 3.9. Chemical structures of four inhibitors.

These four inhibitors have different capabilities to suppress the enzyme activity. Figure 3.10a shows the colorimetric images for inhibition screening. We could easily visualize the different inhibited-Bla activities from the different extent of aggregated AuNPs. The red solution reveals the potent enzyme inhibition, while a blue solution indicates the least inhibition and aggregation of AuNPs induced by the enzymatic reaction. From the colorimetric visualization by naked eyes, we obtained the inhibition trend is TZB > CA > SUL > ATM. This result is in agreement with the control experiment performed by using standard indicator nitrocefin. When increasing the

concentration of inhibitors to 3 μM , the similar inhibition trend was obtained in nitrocefin assay (Figure 3.11). However, no difference between the color changes was observed by using nitrocefin under the same condition as that of AuNPs (Figure 3.10a).

Figure 3.10. (a) Colorimetric images of inhibition assay by using AuNPs (2.5nM) and nitrocefin (20 μM) with the TEM-1 Bla (2.0 nM) inhibited with inhibitors (0.1 μM). (b) Absorbance change ratio of Bla inhibition assay in the absence and presence of different inhibitors (inhibitor concentration: 0.1 μM).

Figure 3.11. Colorimetric images of the inhibition assay using nitrocefin (20 μM) with TEM-1 Bla (2 nM) and inhibitors (3 μM).

The observed inhibition order is consistent with the results of reported binding affinity of the inhibitors to the different types of class A β -lactamase.¹¹ Comparing with the nitrocefin assay, AuNPs-based colorimetric assay suggests that it is much more efficient approach for screening β -lactamase inhibitors.

3.2.5 Quantitative identification of class A β -lactamase inhibitors

Based on above colorimetric assay for screening inhibitors, we quantitatively assessed the efficiency of inhibitors towards β -lactamase. The IC_{50} values (concentration of inhibitor that reduces enzyme activity to 50% of the activity of the native enzyme) of inhibitors were detected by addition of various β -lactamase inhibitors (TZB, CA, SUL and ATM) with different concentrations into Bla solution (2.0 nM). Above mixture was incubated for 20 min at room temperature to inhibit Bla activity. Then, the pre-treated Bla solution was mixed with substrate for 20 minutes allowing enzyme hydrolysis and added into AuNPs suspension to induce the aggregation of AuNPs. By measuring the absorbance at 650 nm of gold nanoparticle, we evaluated the inhibited enzyme activity accordingly.

It is noteworthy that at low inhibitor concentrations the red shift of the spectrum of gold nanoparticles is linearly dependent on the logarithm of the inhibitor concentration. Based on this dependency, IC_{50} values of the inhibitors can be estimated directly from the graph. As shown in Figure 3.12, the IC_{50} values of TZB, CA, SUL, ATM obtained by AuNPs-based assay are 2.0, 4.7, 6.9, 60.3 μ M, respectively.

Figure 3.12. IC_{50} values of TZB, CA, SUL and ATM for transformed TEM-1 β -lactamase by using substrate (8 μ M) and gold nanoparticles.

As control, IC_{50} values of the inhibitors were also determined by standard indicator: nitrocefin (Figure 3.13). From the nitrocefin assay, the IC_{50} values of TZB, CA, SUL, ATM are 1.6, 3.5, 5.8, 49 μ M which are similar to that of AuNPs-based assay. Therefore, the AuNPs-based colorimetric assay provides an efficient manner to quantitatively estimate the inhibitors efficiency *in vitro*.

Figure 3.13. IC_{50} values of TZB, CA, SUL and ATM for transformed TEM-1 β -lactamase by using nitrocefin (20 μ M).

3.2.6 Screening inhibitors of class A β -lactamase in living bacteria strains

In order to evaluate the applicability of this efficient colorimetric assay in living bacteria strains, we chose four β -lactam resistant bacterial strains. They are TEM-1 transformed *E. coli* Bl21, TEM-1 *E. coli* (ATCC 35218), *Bacillus cereus* (ATCC 13061), and *K. pneumoniae* (ATCC 700603), which contained different kinds of class A Bla such as transformed TEM-1, TEM-1, *B.cereus* PenPC, and SHV-18, respectively. The enzyme activities were identified in the absence and presence of Bla inhibitors. Wild type *E. coli* Bl21 was used as a negative control because it could not express Bla. As shown in Figure 3.14, the clear color changes in plasmid-transformed

E. coli B121 exhibited the inhibition trends similar to those observed *in vitro* measurement. A red color in TZB indicated the relatively potent Bla inhibition, a much reddish color in CA exhibited a weaker inhibition activity than that in TZB but stronger than that in SUL. Based on the color change, CA and TZB don't have distinct differences for inhibition activity in this bacterial strain. A blue color in ATM, which color was close to the solution without inhibitor treatment, demonstrated the least activity for the enzyme inhibition. The absorbance ratios at 650 nm and 520 nm also confirmed the same inhibition order (TZB > CA > SUL > ATM) as observed in color change (Figure 3.16). As control experiment, the enzyme inhibitor screening was also performed in nitrocefin assay. However, the nitrocefin based colorimetric assay could not differentiate the inhibition activity under the same conditions as that of AuNPs assay. There is no difference between the absorbance ratios at 486 nm and 390 nm for the four inhibitors (Figure 3.16). The identical inhibition trends were achieved with increasing the concentration of inhibitors up to 3.0 μ M in nitrocefin assay (Figure 3.15).

Figure 3.14. AuNPs-based colorimetric inhibition assays for Bla activity in a 96-well microplate with four inhibitors and four class A Bla contained living bacteria (A: transformed TEM-1 *E.coli* Bl21, $\sim 10^8$ cfu/ml, B: TEM-1 *E.coli*, $\sim 10^9$ cfu/ml, C: *Bacillus cereus*, $\sim 8 \times 10^9$ cfu/ml, and D: *K. pneumoniae*, $\sim 3 \times 10^8$ cfu/ml). Bacteria without inhibitor as positive control. Wild type *E.coli* Bl21 (no Bla) and AuNPs solutions as negative controls (inhibitors concentration: 0.1 μ M).

Figure 3.15. Nitrocefin based colorimetric assay for inhibitors screening. The concentration of inhibitors was 3.0 μ M.

Figure 3.16. Absorbance change ratio of class A Bla inhibition assay with AuNPs or nitrocefin in the absence and presence of different inhibitors (inhibitors concentration: 0.1 μ M) in transformed TEM-1 *E. coli* (A), TEM-1 *E. coli* (B), *B. cereus* (C) and *K. pneumoniae* (D).

Similar screening results were obtained in TEM-1 *E. coli*, and *Bacillus cereus* strains, although a large amount of bacterial strains had to be used due to the lower enzyme activities in these two bacteria strains. *K. pneumoniae* bacteria strains were also employed for testing the enzyme inhibition screening. *K. pneumoniae* is one clinically isolated β -lactam resistant bacterial strain, which contained the extended-spectrum β -lactamase (ESBL): SHV-18. In the AuNPs-based assay, a red color in CA demonstrated the efficient enzyme inhibition, which was more effective than TZB. It is in accordance with the known activities of these inhibitors in *K. pneumoniae*.¹² The blue color in ATM suggested weak enzyme inhibition. This result

demonstrated that different inhibitors would exhibit various inhibition activities toward the same bacterial enzyme. Moreover, the different Bla inhibitions observed in *E. coli*, *Bacillus cereus*, and *K. pneumoniae* strains were attributed to the various inhibition activities of the same inhibitor to the different subclass of enzymes in bacteria. As control experiment, the inhibitors alone were incubated with bacterial strains and no detectable aggregation of gold nanoparticles was observed under the same condition as the colorimetric assay. It has eliminated the nonspecific interaction between the inhibitors and the living bacteria.

Therefore, gold nanoparticles-based colorimetric assay could efficiently screen the Bla inhibitors in the living bacteria strains with small amount of inhibitors. It offers an alternative approach to screen class A β -lactamase inhibitors.

3.3 Conclusions

In conclusion, we have developed a simple and effective AuNPs based colorimetric method for efficient screening of class A β -lactamase activity and inhibitors both *in vitro* and in bacterial strains. The colorimetric method based on the aggregation of AuNPs can be used not only to sensitively identify the enzyme activity, but also to provide valuable information on the efficiencies of simultaneous screening of different enzyme inhibitors *in vitro* and in the variedly enzyme expressed bacteria. It is easy to monitor and indicate the relative inhibition capabilities. This screening method without the aid of sophisticated instruments may provide an alternative platform to study the inactivation of β -lactam antibiotics for the treatment of antibacterial drug resistance. This colorimetric method may find its application in pharmaceutical industry for the discovery of new antimicrobial enzyme inhibitors.

3.4 Experimental Section

Materials and General methods

Chemicals: 7-Amino-3-chloromethyl 3-cephem-4-carboxylic acid diphenylmethyl ester hydrochloride (ACLH) was provided from Otsuka chemical Co. Ltd. Nitrocefin was purchased from Merck. β -lactam resistant *K. pneumoniae* bacterial strain (ATCC 700603), TEM-1 *E. coli* strain (ATCC 35218) and *Bacillus cereus* strain (ATCC 13061) were purchased from ATCC. The purified transformed TEM-1 β -lactamase was obtained from Biologics Process Development, CA, USA. All the other starting materials were obtained from Sigma or Aldrich. Commercially available reagents were used without further purification, unless noted otherwise. The solvents were dried according to regular protocols. All other chemicals were analytical grade or better.

Instrumentation: The synthesized compounds were characterized by using ^1H NMR (Bruker Advance 400MHz) using CDCl_3 as the solvent. ESI-MS spectrometric analyses were performed at the Thermo Finnigan LCQ Deca XP Max and transmission electron micrograph on a JEOL 2000 EX TEM. Absorbance spectra were measured on Beckman Coulter DU 800 UV-Vis spectrophotometer. HPLC experiments were conducted on Shimadzu LC-20A.

Analytical reverse-phase high performance liquid chromatography (HPLC) was performed on Alltima C-18 column (250 $\times$ 3.0 mm) at a flow rate of 1.0 mL/min and semi-preparative HPLC was performed on the similar C-18 column (250 $\times$ 10 mm) at a flow rate of 3 mL/min. An eluting system consisting of A (water with 0.1% TFA) and B (acetonitrile with 0.1% TFA) was used under a linear gradient to elute the products,

which was monitored by UV-Vis absorbance at 280 nm. The linear gradient started from 80% solution A and 20% solution B, changed to 20% solution A and 80% solution B in 30 minute and to 0% solution A and 100% solution B in the following 5 minutes, and then back to 80% solution A and 20% solution B in the next 5 minutes.

Synthesis and characterization of cephalosporin substrate.

Enzyme substrate was prepared according to Figure 3.17.

Figure 3.17. Synthetic route for the cephalosporin substrate.

Preparation of 1: 7-Amino-3-chloromethyl cephalosporanic acid benzylhydryl ester hydrochloride (ACLH) (451 mg, 1 mmol) was suspended in dichloromethane. Then acetyl chloride (78.5 mg, 1 mmol) was added drop wise into the suspension. Finally 2, 6-lutidine (214 mg, 2 mmol) was added into the reaction mixture and the solution was stirred for 2 hrs under nitrogen. After the removal of the solvent on the rotary evaporator, the residue was purified by flash chromatography on silica gel (eluent: ethyl acetate / hexane =1/1) to afford 223.5 mg (yield: 85.4%) of title compound. ¹H NMR (400 MHz, CDCl₃) 7.47-7.45 (m, 2H), 7.41-7.31 (m, 7H), 7.28-7.26 (m, 1H), 6.98 (s, 1H), 5.90 (dd, J = 8.9 and 4.92 Hz, 1H), 5.01 (d, J=4.95 Hz, 1H), 4.40 (s, 2H), 3.65 (d, J = 18.3 Hz, 1H), 3.51 (d, J = 18.3 Hz, 1H), 2.02 (s, 3H). ESI-MS observed [M+H]⁺: 457.7, calculated [M+H]⁺: 457.9.

Preparation of 2: A mixture of **1** (155 mg, 0.34 mmol) and sodium iodide (253 mg, 1.7 mmol) in 5mL of acetone was stirred for 1 hr at room temperature. The reaction mixture was concentrated on the rotary evaporator and diluted with 5 mL water. The suspension was extracted with 25 mL of ethyl acetate, and the organic phase was washed with 10% sodium thiosulfate (5 mL×2), brine (5 mL×3) and dried over anhydrous magnesium sulfate. The slightly orange powder **2** (152 mg, 0.45 mmol) was used without further purification.

Preparation of 3: 3,6-Dioxaoctyl-1,8-diamine (244 mg, 1.65 mmol) was stirred in dry dichloromethane (1mL), then di-*tert*-butyl carbonate (120 mg, 0.55 mmol) in dry

dichloromethane (1 mL) was added slowly over 2 hours. The reaction was stirred overnight at room temperature, the solvent evaporated, and the residue purified by column chromatography (silica gel, ethanol:ethyl acetate:triethylamine eluent = 5:4:1). The product was obtained as a pale yellow oil (96 mg, 71%). $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 3.67 (s, 4H), 3.55-3.62 (m, 4H), 3.34-3.40 (m, 2H), 2.94 (t, J = 5.2 Hz, 2H), 1.49 (s, 9H). ESI-MS observed $[\text{M}+\text{Na}]^+$: 271.2, calculated $[\text{M}+\text{Na}]^+$: 271.3.

Preparation of 4: 4-mercaptophenylacetic acid (369.6 mg, 2.2 mmol) was added to the solution of chlorotriphenylmethane (557.6 mg, 2 mmol) in 2.0 mL dichloromethane. The solution was stirred for 2 hrs under nitrogen. The reaction was quenched by 1N NaOH (3 mL). The suspension was extracted with 10 mL of ethyl acetate, and the organic phase was washed with brine (5 mL $\times$ 3) and dried over anhydrous magnesium sulfate. The solvent was removed and residue was purified by flash chromatography on silica gel (eluent: ethyl acetate/hexane = 1/3) to afford 713.4 mg of desired product (87%). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.43-7.40 (m, 6H), 7.26-7.17 (m, 9H), 6.93 (m, 4H), 3.52 (s, 2H) ESI-MS: observed $[\text{M}+\text{Na}]^+$: 433.6, calculated $[\text{M}+\text{Na}]^+$: 433.5.

Preparation of 5: To a cooled (ice bath) and stirred solution of compound **3** (74.4 mg, 0.30 mmol) in 1.0 mL anhydrous dichloromethane was added compound **4** (123.2 mg, 0.30 mmol). Then *N,N'*-dicyclohexylcarbodiimide (123.6, 0.30 mmol) in dry dichloromethane (1 mL) was added slowly over 2 hours. The solution was stirred overnight. The reaction mixture was concentrated on the rotary evaporator.

Purification of the crude product by flash chromatography on silica gel (eluent: methanol / dichloromethane = 5/95) afforded the desired product 65.8 mg (60%). ^1H NMR (400 MHz, CDCl_3) δ 7.41-7.38 (m, 6H), 7.27-7.15 (m, 9H), 6.91 (m, 4H), 3.52-3.49 (m, 8H), 3.42-3.27 (m, 4H), 3.27 (m, 2H), 1.43 (m, 9H) ESI-MS: observed $[\text{M}+\text{Na}]^+$: 663.7; calculated $[\text{M}+\text{Na}]^+$: 663.8.

Preparation of 6: To a cooled solution of **5** (43.8 mg, 0.12 mmol) in 0.5 mL of anhydrous dichloromethane was added trifluoroacetic acid (1 mL) and triisopropylsilane (80 μL) with cooling (ice bath). The mixture was stirred for 1 hr at the same temperature, then the solvent was removed under reduced pressure. The residue was washed with cold hexane (1 mL $\times$ 3) to afford 25.6 mg of the light yellow crude product. The crude product was used for next step reaction without further purification. The product was added drop-wise to a solution of compound **2** (82.3 mg, 0.051, 0.15 mmol) in 0.5 mL anhydrous *N,N*-dimethylformamide (DMF), followed by addition of *N,N*-diisopropylethylamine (DIPEA, 26.4 μL , 0.15 mmol) and 2, 6-lutidine (84 μL , 0.72 mmol). The mixture was stirred at room temperature for 5 hrs. Then, the reaction mixture was diluted with water (5 mL) and extracted by ethyl acetate (10 mL). The organic phase was washed by brine (5 mL) and dried over anhydrous magnesium sulfate. The solvent was removed and the crude product was further purified by RP-HPLC to collect 8.9 mg of compound **6**. ^1H NMR (400 MHz, CDCl_3) δ 7.41-7.28 (m, 10H), 7.13 (d, J = 8.0 Hz, 2H), 7.06 (d, J = 8.0 Hz, 2H), 6.80 (s, 1H), 5.74 (dd, J = 8.9 and 4.92 Hz, 1H), 4.93 (m, 3H), 4.07 (d, J = 13.2 Hz, 1H), 3.75 (d, J = 13.2 Hz,

1H), 3.63-3.52 (m, 12H), 3.46 (m, 2H), 3.05 (m, 2H), 2.06 (s, 3H). ESI- MS observed $[M+Na]^+$: 741.6, calculated $[M+Na]^+$: 741.8.

Preparation of substrate: A solution of **6** (3.5 mg, 0.0049 mmol) was dissolved in 150 μ L of anhydrous dichloromethane. Then trifluoroacetic acid (200 μ L) and anisole (9.0 μ L) were added. The mixture was stirred for 1 hr at the cooled temperature (ice bath). The solvent was removed under reduced pressure. The precipitate was collected and washed with hexane (1 mL $\times$ 3) and then purified by RP-HPLC to afford 1.8 mg (65%) of the title product. ESI-MS observed $[M+H]^+$: 553.4, calculated $[M+H]^+$: 553.6.

Preparation of Citrate-coated Gold Nanoparticles.

Gold nanoparticles (15 nm) were prepared by citrate reduction of $HAuCl_4$. $HAuCl_4$ (100ml, 0.25mM, 2.5×10^{-5} mol) was dissolve in 95 ml of deionized water. The aqueous solution was refluxed for 10 min and followed by addition of 5 ml of 0.5% sodium citrate solution. The mixture was refluxed for another 30min until the color of the solution would change gradually from faint yellowish to wine-red. The pH value was adjusted to 7.4 by using 0.1M NaOH. After filtration through 0.45 μ M Millipore syringe to remove the precipitate, the filtrate was stored at room temperature for a period of time.

Transmission Electron Microscopy (TEM) Measurements

TEM samples were prepared by pipetting a 20 μL portion of gold nanoparticles solution on a carbon coated copper grids. The grids were then dried with a tissue paper and air dried. This grid preparation method minimized the unwanted aggregation due to evaporation. TEM measurements were performed on a JEOL 2000 EX TEM instrument at 200 kV.

Enzyme hydrolysis of substrate by class A β -lactamase

Substrate solutions were prepared in deionized water and Bla was dissolved in PBS buffer (pH 7.4). Then Bla solution was mixed with substrates for the enzyme interactions. The final substrate and Bla concentrations were maintained at 8.0 μM and 2.0 nM, respectively. The enzymatic reaction was performed by incubating Bla solution with substrates for 20 min at room temperature. Finally, the substrate solution was added into AuNPs suspension to induce the aggregation of AuNPs. The absorbance change at 650 nm was analyzed every 2 min for 30 min at room temperature by Beckman Coulter DU 800 UV-Vis spectrophotometer.

Inhibition assay for class A β -lactamase activity by using gold nanoparticles

For the inhibition assay of Bla activity, the procedure is similar with that in the enzyme reaction for aggregation of AuNPs. The final concentrations of substrate and Bla solutions were maintained at 8.0 μM and 2.0 nM, respectively. Various β -lactamase inhibitors were mixed with Bla solution first. Then the mixture was

incubated at room temperature for 20 minutes to inhibit Bla activity. The pre-treated Bla solution was mixed with substrate for additional 20 minutes. Finally, the substrate solution with inhibitor pre-treated Bla was added into AuNPs suspension to induce the aggregation of AuNPs. The absorbance change at 650 nm was analyzed every 2 min for 1 hour at room temperature by Beckman Coulter DU 800 UV-Vis spectrophotometer.

Sensitivity Detection of the AuNPs-based colorimetric assay

Substrate solutions were prepared in deionized water and a range of concentrations of Bla were prepared in PBS buffer (pH 7.4). Then Bla solution was mixed with substrates for the enzyme interactions. The final concentration of substrate was maintained at 8.0 μ M. The enzymatic reaction was performed by incubating Bla solution with substrate for 20 min at room temperature. Finally, the mixture was transferred into AuNPs suspension to induce the aggregation of AuNPs.

Colorimetric inhibition assay by using nitrocefin

Various β -lactamase inhibitors (3.0 μ M) were mixed with Bla (2.0 nM) solution first. Then the mixture was incubated at room temperature for 20 minutes to inhibit Bla activity. After that, the inhibitor pre-treated Bla was added into nitrocefin solution. The final concentration of nitrocefin was maintained at 20 μ M. After 10 min interaction, the solution was applied for colorimetric image.

Culture of bacterial strains

The bacterial strains were cultured on nutrient agar at 37°C overnight. Then pick a single colony with a sterile disposable pipet tip into 5 mL of LB broth (Fischer) and grow at 37°C in orbital shaker. When the optical density (OD) of bacteria strains at 600 nm reached 0.8, the suspension was chilled on ice for 5 min, 1ml aliquots were taken out and put into 1.5mL vial, then bacteria were harvested by centrifugation at 3,000 rpm for 10 min. After centrifugation, supernatant was removed and bacteria were washed three times with 1mL of PBS buffer.

Aggregation tests with gold nanoparticles in bacterial strains

The harvest bacterial cells were suspended in deionized water which contained substrate under room temperature. The mixture was incubated for 20 minutes of enzyme interactions. Then, the bacterial solution was added into AuNPs suspension to induce the aggregation of AuNPs. After 40 min interactions, AuNPs solution was separated from bacteria by centrifugation at 3,000 rpm for 3 min. The supernatant solution was applied for colorimetric image.

Colorimetric assay by using β -lactamase inhibitors and gold nanoparticles in bacteria strains

Bacterial cells (β -lactam antibiotics resistant gene transformed *E. coli* BL21 $\sim 10^8$ cfu/mL, TEM-1 *E. coli* (ATCC 35218) $\sim 10^9$ cfu/mL, *Bacillus cereus* (ATCC 13061) $\sim 8 \times 10^9$ cfu/mL, and clinical isolate *K. pneumoniae* (ATCC 700603) $\sim 3 \times 10^8$ cfu/mL)

were suspended in PBS buffer (pH 7.4). Inhibitor solutions were incubated with bacteria for further enzyme interactions. Then the pretreated bacterial suspension and substrate solution were added into AuNPs suspension to induce the aggregation of AuNPs. The final concentration of substrate and inhibitors were maintained at 8 μM and 0.1 μM , respectively. After 40 min interaction, AuNPs solution was separated from bacteria by centrifugation at 3,000 rpm for 3 min. And then the supernatant was applied for colorimetric image.

Colorimetric assay by using β -lactamase inhibitors and nitrocefin in bacteria.

Bacterial cells (β -lactam antibiotics resistant gene transformed *E. coli* BL21 $\sim 10^8$ cfu/mL, TEM-1 *E. coli* (ATCC 35218) $\sim 10^9$ cfu/mL, *Bacillus cereus* (ATCC 13061) $\sim 8 \times 10^9$ cfu/mL, and clinical isolate *K. pneumoniae* (ATCC 700603) $\sim 3 \times 10^8$ cfu/mL) were suspended in PBS buffer (pH 7.4). Inhibitor solutions were incubated with bacteria for further enzyme interactions. The pretreated bacterial solution were added into nitrocefin solution. The final concentration of nitrocefin and inhibitors were maintained at 20 μM and 3.0 μM , respectively. After 20 min interaction, the solution was separated from bacteria by centrifugation at 3,000 rpm for 3 min. And then the supernatant was applied for colorimetric image.

Absorbance change ratio for β -lactamase inhibitors and gold nanoparticles or nitrocefin in bacteria strains.

β -lactam antibiotics resistant bacterial strains were suspended in PBS buffer (pH

7.4). Inhibitor solutions were incubated with bacteria for further enzyme interactions. The bacterial solution and substrate solution were added into AuNPs or nitrocefin solution. The final concentration of substrate, nitrocefin and inhibitors were maintained at 8.0 μM , 20 μM and 0.1 μM . After interaction, AuNPs and nitrocefin solution were separated from bacteria by centrifugation at 3,000 rpm for 3 min. And then the supernatants were used to measure the absorbance ratio at 650 nm/520 nm and 486 nm/390 nm, respectively.

3.5 References

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Chapter 4

Gold and Silver Nanoparticles-based Bioassay for Screening Class C

P99 β -Lactamase Activity and Inhibition

4.1 Introduction

Nanosized metallic particles have attracted a great deal of interest in nanobiotechnology during the last decades. The noble metals with novel optical and electronic properties exhibit strong surface plasmon resonance (SPR) which allows them present the intense color in the colloidal solution.¹ The exact surface plasmon absorption is dependent on several parameters such as shape, size, medium, the interparticle distance, and the type of metals.² For example, the dispersed gold nanoparticles (AuNPs) around 16 nm of diameter have red color with a surface plasmon absorption band centered at 520 nm. When the interparticle distance decreases to less than the diameter of the particle, the coupling interactions result in a red-shift of the resonance wavelength and lead to significant aggregation of AuNPs with the distinctive color change from red to purple-blue.³⁻⁴

As for silver nanoparticles (AgNPs), they possess the similar optical properties as AuNPs. The monodispersed silver nanoparticles in solution are vivid yellow color with the size dependent surface plasmon resonance between 390 and 420 nm. Upon aggregation, the silver nanoparticles appear orange-red color with the resonance band shift to longer wavelength.⁵ The color change associated with nanoparticles aggregation possesses the high extinction coefficients which are usually over thousand

times larger than those of traditional organic chromophores.⁶ Therefore, the distance-dependent optical properties of gold and silver nanoparticles have been exploited in wide biological sensing. They have been extensively exploited in the development of colorimetric assay for the detection of nucleic acids, proteins, metal ions, and small molecules etc.^{5,7-13} Similar analytical methods have also been described as efficient tools for the control of the formation of nanoparticles assemblies, identification of enzyme activities or screening of their inhibitors.

As discussed in chapter 1, 2, and 3, β -lactamases (Blas) are an important family of bacterial enzymes which could efficiently and irreversibly cleave the amide bond of β -lactam ring. As a result, β -lactam antibiotics were rendered ineffective toward the bacterial infection by the hydrolysis of β -lactamases. Intense studies have been focused on elaborating β -lactamases. Based on molecular structures and preferred substrates, β -lactamases have been classified into four classes A, B, C and D.¹⁴ Among the different members in β -lactamase family, classes A and C β -lactamases are the most clinically important enzymes that are responsible for the antibiotics resistance in bacteria. Class A Blas have been the most thoroughly studied one and have the high hydrolysis capabilities for penem and penam antibiotics. They have been widely studied for combating the increased antibiotic resistance in clinical therapy and for imaging the gene expressions *in vitro*, in living cells and animals.¹⁵⁻¹⁷ In comparison with the well-exploited class A Blas counterparts, class C β -lactamases are relatively less characterized. They are a significant factor in the resistance of Gram negative bacteria to β -lactam antibiotics.¹⁸ Class C β -lactamases were termed as

cephalosporinases with larger molecular weight than class A Blas.¹⁹ They are usually chromosomally encoded in Gram negative bacteria such as *Escherichia coli*, *Citrobacter freundii* and *Enterobacter cloacae*.¹⁶ Although the geometries of the active sites in class C β -lactamases are similar to those in class A enzymes, there are significant differences in the arrangements of secondary structure elements in these two different classes of bacterial enzymes. Moreover, along with their important roles in antimicrobial drug resistance, class C Blas have also been reported as an efficient antibody directed enzyme prodrug therapy (ADEPT) platform in cancer research to maximize the concentration of the cytotoxic agent at the tumor.²⁰⁻²¹ For example, *Enterobacter cloacae* P99 β -lactamases has been used in the cephalosporin-doxorubicin conjugate for cancer therapy.²² Hydrolysis of cephalosporin β -lactam ring by P99 β -lactamases leads to a secondary elimination reaction, resulting in the expulsion of doxorubicin which is a cytotoxic compound used for cancer chemotherapy. This strategy is also used in the design of one class C P99 β -lactamases and tumor antibody conjugates reacting with cephalosporin prodrugs which localized on the targeted tumor cell surface. Cleavage of cephalosporin β -lactam ring could trigger the controlled release of antitumor agents previously attached to the 3' position of cephalosporin, resulting in tumor selective drug delivery.²³ In terms of these important bifunctional properties, the development of a simple and reliable bioassay to efficiently identify class C β -lactamases activity and to screen their inhibitors will be clinically significant to combat bacterial resistance and improve the efficacy for the prodrug release in cancer therapy. Therefore, based on our

previously developed colorimetric assay, we aim to extend it for sensing class C P99 β -lactamases activity.

In this chapter, we presented a practical and easily operated colorimetric aggregation method by taking advantages of the significant color change and red-shift of surface plasmon resonance bands of gold and silver nanoparticles for systematic determination of class C *E. cloacae* P99 β -lactamase activity and screening its inhibitors.

Figure 4.1. Illustration of class C *E. cloacae* P99 β -lactamase induced aggregation of AgNPs and AuNPs.

Figure 4.1 depicts the general principle and molecular design of this simple assay. The short polyethylene glycol (PEG) modified 2-(4-mercaptophenyl) acetic acid was

attached to the 3' position of cephalosporin, which is well known as good substrate for class C *E. cloacae* P99 β -lactamase. Under the enzyme hydrolysis, the β -lactam ring in cephalosporin derivative is opened and leads to releasing the fragment containing the free thiol and positively charged amino group, which substitutes the citrate ions on the surface of silver and gold nanoparticles and thereby results in the aggregation of these metallic nanoparticles due to the electrostatic interactions and silver or gold-thiols interactions. The aggregated silver and gold nanoparticles demonstrate the obvious color change and red-shift of their plasmon absorption bands. Exploiting the significant color change from silver or gold nanoparticles, it is possible to construct a simple and effective colorimetric assay for efficient detection of class C *E. cloacae* P99 β -lactamase activity and screening of its inhibitors by either naked eyes or simple UV-Vis absorbance measurement.

4.2 Results and Discussion

4.2.1 Colorimetric assays using AgNPs and AuNPs

This nanoparticles-based colorimetric assay for sensing β -lactamases was first developed as described in chapter 2. Taking advantage of the excellent optical properties of silver nanoparticles, we extended the colorimetric sensing assay by using AgNPs. As we know, hydrolysis of the β -lactam ring in cephalosporin derivative could induce spontaneous elimination of leaving groups attached to the 3'-position. In a typical experiment, the designed β -lactam substrate was initially incubated with class C *E. cloacae* P99 Bla in PBS buffer (pH 7.4). The aliquot of solution was then transferred

into the dispersed AgNPs solution or AuNPs solution. As shown in Figure 4.2, the remarkable color change from vivid yellow to orange-red was found within seconds. The color change of AgNPs was also accompanied by the red-shift of surface plasmon resonance (SPR) peak from 400 nm to 550 nm upon the addition of the enzyme treated substrate into AgNPs solutions. As a control, the initial AgNPs solutions containing intact cephalosporin substrate were vivid yellow in color, demonstrating that the AgNPs solution were stable and the observed color change and spectrum shift were from the enzymatic interaction induced silver nanoparticles aggregation.

Figure 4.2. UV-Vis absorption spectra of AgNPs before (black line) and after addition (red line) of *E. cloacae* P99 Bla (3.0 nM) treated substrate (5.0 μ M). The inset shows the color change of AgNPs. (1) AgNPs with substrate only and (2) AgNPs with Bla treated substrate.

The aggregation of silver nanoparticles was a dynamic process as a function of time. As shown in Figure 4.3, both the decreased absorbance at around 400 nm and increased

absorbance at around 550 nm of silver nanoparticles were observed with time increasing. Highly aggregated state of AgNPs was achieved after 15 mins and the UV-Vis spectra of AgNPs displayed the maximum red shift.

Figure 4.3. Time course of AgNPs aggregation in the presence of Bla treated substrate.

Gold nanoparticles with the diameter of 16 nm were also used in this colorimetric assay. The AuNPs solution showed a red color with a typical plasmon absorption band around 520 nm, and addition of intact β -lactam substrate to the AuNPs solution did not induce color change and absorption spectral shifts. However, the obvious color change from red to blue was observed upon addition of *E. cloacae* P99 Bla treated substrate into AuNPs solution shown in Figure 4.4. A decreased absorption at 520 nm and an increased absorption at 620 nm were observed in the UV-Vis spectrum with a prolonging the reaction time. These results demonstrated the hydrolysis of cephalosporin by *E. cloacae* P99 Bla is the trigger of the aggregation of nanoparticles.

Figure 4.4. UV-Vis absorption spectra of AuNPs before (black line) and after addition (red line) of *E. cloacae* P99 Bla (3.0 nM) treated substrate (5.0 μ M). The inset shows the color change of AuNPs. (1) AuNPs with substrate only and (2) AuNPs with Bla treated substrate.

Through the distinct color change, the activity of β -lactamases could be monitored by naked eyes. The apparent color change and spectral shifts indicated that both silver and gold nanoparticles could be used for visualization of enzymatic activity.

4.2.2 Sensitivity of the colorimetric assay

The metallic nanoparticles based colorimetric assay provided a platform for quantitatively sensing the process of class C *E. cloacae* P99 Bla enzymatic hydrolysis. Figure 4.5 showed the quantitative relationship between the absorbance change and the concentration of *E. cloacae* P99 Bla for both silver and gold nanoparticles.

Silver nanoparticles possess the higher extinction coefficient than that of the same size of gold nanoparticles. The significant absorbance intensity enhancement from

dispersion to full aggregation was observed in 2.5 nM of silver nanoparticles solutions. From Figure 4.5A, higher absorbance of aggregated AgNPs was induced by higher concentration of P99 Bla, indicating more cephalosporin substrate were cleaved by enzyme. However, the absorbance of aggregated AgNPs reached a plateau when the concentration of P99 Bla was higher than 600 pM. In addition, a near-linear correlation between the absorbance and the enzyme concentration is in the range of 0 ~ 0.1 nM Bla. (Figure 4.6A) This condition provided an effective range for colorimetric assay used for *E. cloacae* P99 Bla detection with a sensitivity as low as 5.0 pM. Therefore, the dynamic range for AgNPs based assay is from 5.0 pM to 600 pM.

The similar results were obtained for gold nanoparticles based assay. The overall color change and absorbance intensity enhancement in the linear range is 0.015 to 0.08 nM class C *E. cloacae* P99 Bla. It enabled the effective enzyme detection with the lowest concentration down to 16 pM. The dynamic range of AuNPs assay was determined from 16 pM to 90 pM. Compared to the colorimetric assay based on AuNPs, AgNPs were more sensitive due to their higher extinction coefficients relative to AuNPs of the same size.⁶

Figure 4.5. (A) *E.cloacae* P99 Bla concentration versus absorbance at 550 nm of AgNPs solution with 5.0 μ M substrate. (B) *E.cloacae* P99 Bla concentration versus absorbance at 620 nm of AuNPs solution with 5.0 μ M substrate.

Figure 4.6. (A) The near-linear relationship between *E.cloacae* P99 Bla concentration and the absorbance at 550 nm of AgNPs solutions. (B) The near-linear relationship between *E.cloacae* P99 Bla concentration and the absorbance at 620 nm of AuNPs solutions.

4.2.3 Transmission Electron Microscopy (TEM) and Dynamic light scattering (DLS) characterisation

The observed color change and absorption spectral shifts caused by the aggregation of silver or gold nanoparticles were confirmed by transmission electron microscope (TEM) and dynamic light scattering (DLS) measurements. As shown in Figure 4.7, in the absence of *E. cloacae* P99 Bla, both the silver and gold

Figure 4.7. TEM images of AgNPs and AuNPs. (A) substrate (5.0 μM) in AgNPs; (B) substrate (5.0 μM) with *E. cloacae* P99 Bla in the absence and (C) presence of inhibitor aztreonam (1.0 μM) in AgNPs solutions. (D) substrate (5.0 μM) in AuNPs; (E) substrate (5.0 μM) with *E. cloacae* P99 Bla in the absence and (F) presence of inhibitor aztreonam (1.0 μM) in AuNPs solutions. Scale bar is 50 nm.

nanoparticles were well dispersed and the cephalosporin derivative itself or enzyme inhibitors were unable to induce the aggregation of silver or gold nanoparticles (Figure 4.7A, D). The hydrodynamic diameters of AgNPs and AuNPs are (20.1 ± 3.2) nm and (16.7 ± 0.4) nm, respectively (Figure 4.8 A, D). However, upon treatment with *E. cloacae* P99 Bla, cleavage of the β -lactam ring in cephalosporin induced the release of the free thiol and positively charged amino group contained flexible linker which induced the significant aggregation of silver and gold nanoparticles (Figure 4.7 B, E) with the corresponding hydrodynamic diameters are (903.7 ± 60.1) nm and (151.6 ± 14.2) nm (Figure 4.8 B, E). As expected, in the presence of sufficient amount of efficient *E. cloacae* P99 Bla inhibitor such as aztreonam (ATM), the enzymatic activity would be dramatically suppressed. Therefore, there was no significant nanoparticles aggregation observed from TEM results which were very similar to those of AgNPs and AuNPs solutions without enzyme treatment (Figure 4.7 C, F). The dynamic diameters are (32.8 ± 10.1) nm and (25.0 ± 1.8) nm for AgNPs and AuNPs, respectively (Figure 4.8 C, F). These results clearly demonstrated that the enzyme interactions between the cephalosporin derivative and class C *E. cloacae* P99 Bla played important role in the aggregation of silver and gold nanoparticles. Upon treatment with effective enzyme inhibitors, both the AgNPs and AuNPs aggregation would be significantly decreased.

Figure 4.8. Hydrodynamic diameters of AgNPs and AuNPs. (A) substrate (5.0 μM) in AgNPs; (B) substrate (5.0 μM) with *E. cloacae* P99 Bla in the absence and (C) presence of inhibitor aztreonam (1.0 μM) in AgNPs solutions. (D) substrate (5.0 μM) in AuNPs; (E) substrate (5.0 μM) with *E. cloacae* P99 Bla in the absence and (F) presence of inhibitor aztreonam (1.0 μM) in AuNPs solutions.

4.2.4 Stability of silver nanoparticles and gold nanoparticles with substrate

The cephalosporin substrate has amino terminal which is sensitive to the electrostatic environment and could induce the unexpected aggregation of citrate-coated AgNPs or AuNPs. To investigate the stability of nanoparticles with substrate, Figure 4.9

shows the effect of the substrate concentrations on the aggregation of AgNPs and AuNPs. It was evaluated by monitoring the absorbance change at 550 nm and 620 nm for AgNPs and AuNPs, respectively. No distinct aggregation of nanoparticles were observed at low concentration of substrate. The critical coagulation concentration of substrate is around 20 μM for AgNPs and 8 μM for AuNPs. Thus, it was concluded that the substrate could not cause AgNPs and AuNPs aggregation in the experimental concentration (5 μM).

Figure 4.9. Absorbance change of AgNPs at 550 nm (A) and AuNPs at 620 nm (B) versus the concentration of substrate.

4.2.5 Screening Inhibitors by colorimetric assays

One important feature of this bioassay is that the color change is rapid and can be used for evaluating the efficiency of *E. cloacae* P99 Bla inhibitors, and therefore could potentially be utilized in drug screening. In the typical screening experiment, β -lactam substrate (5.0 μM) was first incubated with *E. cloacae* P99 Bla (3.0 nM) in PBS buffer (pH 7.4) in the presence of one of the following β -lactamase inhibitors (0.3 μM):

aztreonam (ATM), clavulanate acid (CA), tazobactam (TZB), and sulbactam (SUL) (Figure 4.10). Most of them are well known inhibitors that efficiently suppress class C Bla activities in clinics. The resulting solutions were then transferred into silver or gold nanoparticles suspensions. The absorbance spectral variation of silver or gold nanoparticles at 550 nm or 620 nm was monitored as a function of time, and the color change of the nanoparticles solutions were determined with naked eyes and simple UV-Vis absorbance measurement.

Figure 4.10. Chemical structures of four *E. cloacae* P99 β -lactamase inhibitors.

In silver nanoparticles based enzyme inhibition assay, yellow solution revealed the potent enzyme inhibition and an orange-red color demonstrated the weak inhibition and aggregation of AgNPs occurred at this stage. As shown in Figure 4.11A, the observed yellow colors in ATM indicated the significant Bla inhibition. The slight orange color in TZB revealed the weaker inhibition than ATM and the orange color in SUL exhibited a

weaker enzyme inhibition than those in ATM and TZB but stronger than that in CA. Similarly, in gold nanoparticles based enzyme inhibition assay, a red color implied significant effectiveness in the enzyme inhibition against aggregation whereas a blue color indicated the least inhibition and allowed the enzymatic reaction to proceed, resulting in the aggregation of AuNPs (Figure 4.11B). The different colors associated with the different extents of aggregation provided the following enzyme inhibition trend: ATM > TZB > SUL > CA in AgNPs assay. The observed inhibition trend for class C *E. cloacae* P99 Bla in AuNPs based assay was identical with that using AgNPs. Both of these data were in agreement with the result as determined by the standard indicator nitrocefin when the higher inhibitor concentration was used in the enzyme inhibition assay (Figure 4.12). This result was also similar to the reported inhibitors binding affinities toward class C *E. cloacae* P99 Bla.^{14,24} No significant color change among the different inhibitors could be observed in the nitrocefin assay under comparable conditions (Figure 4.11C), demonstrating that both AgNPs and AuNPs based colorimetric inhibition assay exhibited more efficient properties to effectively screen the class C *E. cloacae* P99 Bla inhibitors.

Figure 4.11. Colorimetric assay for *E. cloacae* P99 Bla inhibition in 96-well microplate with four different inhibitors. (A) AgNPs based assay with 0.3 μ M inhibitors; (B) AuNPs based assay with 0.3 μ M inhibitors; (C) Nitrocefin assay with 0.3 μ M inhibitors.

Figure 4.12. Colorimetric inhibition assay for *E. cloacae* P99 Bla in 96-well microplate with four kinds of inhibitors (5 μ M) by using nitrocefin.

The nanoparticles-based inhibition assays were also monitored by using UV-Vis spectrophotometer. As shown in Figure 4.13, the absorbance of AgNPs at 550 nm was monitored as a function of time. ATM as the potent inhibitor of P99 Bla could significantly inhibited the enzyme activity and the lowest absorbance for aggregated AgNPs was observed comparing with other three inhibitors. The AuNPs-based assay got the similar inhibition trend as AgNPs-based assay (Figure 4.14).

Figure 4.13. Time-course measurement of *E. cloacae* P99 Bla inhibition assay with AgNPs. Substrate (5.0 μ M) and *E. cloacae* P99 Bla treated inhibitors (0.3 μ M) with AgNPs.

Figure 4.14. Time-course measurement of *E. cloacae* P99 Bla inhibition assay with AuNPs. Substrate (5.0 μ M) and *E. cloacae* P99 Bla treated inhibitors (0.3 μ M) with AuNPs.

4.2.6 Quantitative analysis of β -lactamase inhibitors

Based on the significant color change and absorption intensity enhancement in AgNPs and AuNPs, the effect of enzyme inhibition was also quantitatively estimated by the colorimetric assay on the basis of AgNPs and AuNPs aggregation.

Figure 4.15. Inhibition assay of *E. cloacae* P99 Bla activity using ATM (A), TZB (B), SUL (C), and CA (D). The IC₅₀ values were calculated from the absorbance change of AgNPs at 550 nm.

As shown in Figure 4.15, the corresponding IC₅₀ values (concentration of inhibitor that reduces enzyme activity to 50% of the activity of the native enzyme) of the four inhibitors ATM, TZB, SUL and CA for *E. cloacae* P99 Bla were identified by AgNPs to be 0.0027, 0.157, 5.1 and 753 μ M, respectively. Similarly, the IC₅₀ values of these

four inhibitors were also respectively evaluated to be 0.004, 0.144, 4.8 and 900 μM based on the AuNPs-based assay (Figure 4.16). When using nitrocefin which is a standard indicator for Bla, IC_{50} values of ATM, TZB, SUL and CA were 3.0 nM, 165.9 nM, 5.5 μM and 1.12 mM, respectively (Figure 4.17). Thus, the IC_{50} values from nanoparticles-based assay were consistent with values obtained from nitrocefin assay and were also comparable with values previously reported (Figure 4.17).

Figure 4.16 Inhibition assay of *E. cloacae* P99 Bla activity using ATM (A), TZB (B), SUL (C), and CA (D). The IC_{50} values were calculated from the absorbance change of AuNPs at 620 nm.

Figure 4.17. Inhibition assay of *E. cloacae* P99 Bla activity using ATM (A), TZB (B), SUL (C), and CA (D). The IC_{50} values were calculated from the absorbance change of nitrocefin at 486 nm.

All these results suggest that metallic nanoparticles (such as silver or gold nanoparticles) based colorimetric bioassay could be used for the efficient identification of class C *E. cloacae* P99 Bla activity and high throughput screening of class C *E. cloacae* P99 Bla inhibitors.

4.3 Conclusions

In conclusion, a simple and practical colorimetric assay for class C *E. cloacae* P99 β -lactamase activity and inhibitors screening has been successfully established with silver, gold nanoparticles and β -lactam cephalosporin substrate. This method is easily monitored by visual inspection or simple spectrophotometer. Based on the hydrolysis of enzyme, the β -lactam ring in cephalosporin is cleaved and results in the release of the free thiol and positively charged amino containing linker which further induces the aggregation of silver or gold nanoparticles through the cross-linking interactions between the flexible linkers and the citrate ions on the surface of these metallic nanoparticles. The silver nanoparticles proved to provide enzyme assay with higher sensitive than that based on gold nanoparticles. Both metallic nanoparticles exhibit the unique feature for efficient screening of various enzyme inhibitors by naked eye and simple UV-Vis absorbance measurement. It clearly indicates that the metallic nanoparticles based colorimetric assay may offer a new way to study the efficacy for the effect on the inhibition of bacterial drug resistance. The quantitative measurements presented in this work may also have other relevant applications in prodrug development for cancer therapy.

4.4 Experimental Section

Materials and Chemicals

7-Amino-3-chloromethyl 3-cephem-4-carboxylic acid diphenylmethyl ester hydrochloride (ACLH) was provided from Otsuka chemical Co. Ltd. Nitrocefin was purchased from Merck. The purified Class C *Enterobacter cloacae* P99 β -lactamase was obtained from Sigma-Aldrich. The purity and isoform components of enzyme were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All the other starting materials were obtained from Sigma or Aldrich. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were analytical grade or better. The cephalosporin derivative was prepared as reported previously.

Instrumentation

The synthesized product was purified by reverse-phase HPLC (Shimadzu LC-20A) and characterized by using ^1H NMR (Bruker Advance 400MHz). ESI-MS spectrometric analysis was performed on the Thermo Finnigan LCQ Deca XP Max. Transmission Electron Microscope was operated on JEOL 2000 EX, 120 kV. Absorbance spectra were measured on Beckman Coulter DU 800 UV-Vis spectrophotometer.

Preparation of Silver and Gold Nanoparticles

Silver nanoparticles (AgNPs) around 16 nm were prepared by chemical reduction of silver nitrate in sodium borohydride according to the reported method. Briefly,

silver nitrate (250 μ l of 100 mM) and trisodium citrate (250 μ l of 100 mM) were added into 100 mL water followed by the addition of fresh NaBH₄ solution (5 mM, 6 mL) under vigorously stirring. The mixed solution was stirred for additional 30 min and was left overnight before using. The pH of the vivid yellow colloid solution was adjusted to 7.4.

The 16 nm citrate-capped gold nanoparticles (AuNPs) were prepared by reduction of hydrogen tetrachloroaurate (HAuCl₄). The aqueous solution of HAuCl₄ (1 mM in 95 mL of deionized water) was refluxed for 20 min and followed by addition of 3 mL of 1% trisodium citrate solution. The mixture was heated under reflux for another 30 min until the color of the solution change to wine-red. After cooling down to room temperature, the pH was adjusted to 7.4 and filtered through 0.45 μ M Millipore syringe to remove the precipitate; the filtrate was stored at room temperature.

Transmission Electron Microscope (TEM, JEOL 2000 EX, 120 kV) was used to provide the images of the as-synthesized silver and gold nanoparticles. The concentrations of AgNPs and AuNPs were determined by surface plasmon resonance absorbance at 400 nm and 520 nm, respectively.

Identification of Class C *E. cloacae* P99 β -lactamase

The purity and isoform components of Class C *E. cloacae* P99 β -lactamase was analyzed by SDS-PAGE. As shown in Figure 4.18, the purified *E. cloacae* P99 β -lactamase exhibited one band with molecular weight of 39 KDa which indicated

the higher molecular weight than that of transformed TEM-1 β -lactamase (29 KDa).

The result is consistent with the reported values.

Figure 4.18. SDS-PAGE analysis of *E. cloacae* P99 class C Bla (Lane 1, 12 μ M) with molecular weight of 39 KDa and transformed TEM-1 class A Bla (Lane 2, 12 μ M) with molecular weight of 29 KDa.

Colorimetric assay for class C *E. cloacae* P99 Bla activity with silver and gold nanoparticles

In a typical experiment, β -lactam substrate was initially incubated with class C *E. cloacae* P99 Bla in phosphate buffered saline (PBS buffer, 10 mM, pH 7.4) at room temperature for 20 min. Then the mixed solution was transferred into the dispersed Ag or Au nanoparticles solution to afford 5.0 μ M of substrate and 3.0 nM of *E. cloacae* P99 Bla. The color change and UV-Vis absorbance were monitored as a function of time. The control experiments were performed by Ag or Au nanoparticles solution containing intact substrate without enzyme treatment.

Colorimetric assay for class C *E. cloacae* P99 Bla Inhibition with silver and gold nanoparticles

In the inhibition assays, class C *E. cloacae* P99 Bla was initially incubated with different inhibitors in phosphate buffered saline (PBS buffer, pH 7.4) for 10 min at room temperature to inhibit the enzyme activity. The cephalosporin based β -lactam substrate was subsequently added into the inhibitor-treated enzyme solutions for additional 20 min incubation. Then, the aliquot of above mixed solution was immediately transferred into the dispersed nanoparticles solution affording 5.0 μ M of substrate and 3.0 nM of class C *E. cloacae* P99 Bla. The color change and UV-Vis absorption spectra of AgNPs or AuNPs suspension were collected every two minutes for 30 min at 25°C by Beckman Coulter DU 800 UV-Vis spectrophotometer. The quantitative IC₅₀ measurements were conducted on the basis of absorbance change of nanoparticles at 5 min time point upon the addition of reaction mixtures into nanoparticles solutions. The control experiments indicated that substrate, class C *E. cloacae* P99 β -lactamase and inhibitors would not induce the non specific aggregation of AgNPs and AuNPs.

Colorimetric inhibition assay for class C *E. cloacae* P99 Bla activity by using Nitrocefin

Class C *E. cloacae* P99 Bla was initially incubated with different inhibitors in phosphate buffer saline (PBS buffer, pH 7.4) for 10 min and subsequently β -lactam substrate was added for additional 20 min incubation. Then, the aliquot of above

mixture solution was transferred into nitrocefin solution affording 10 μM of nitrocefin, 5 μM of inhibitor and 3 nM of *E. cloacae* P99 Bla.

4.5 References

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Chapter 5

Programmed Self-Assembly and Disassembly of Gold Nanoparticles by Enzyme Switch

5.1 Introduction

The controllable assembly and manipulation of nanostructures is of great interest in recent years since the first report on gold nanoparticles (AuNPs) functionalized with oligonucleotides.¹⁻² Programmed network of AuNPs have been exploited in wide range of biomolecular interactions based on specific recognition motifs such as antibody-antigen recognition, biotin-avidin binding and lectin-sugar association.³⁻⁷ In addition, complementary oligonucleotide hybridization and enzymatic catalytic reactions have also been extensively exploited for the control of AuNPs assembly, which provide a simple and specific sensing platform for the systematic identification of a variety of molecular analytes including DNAs, bacterial toxins, proteins and enzymes by performing colorimetric or surface enhanced Raman scattering (SERS) measurements.⁸⁻⁹ However, most of the reported molecular recognitions were mainly based on one directional AuNPs aggregation or dispersion and these assembly or disassembly processes were usually achieved in a separate population of gold nanoparticles.¹⁰⁻¹² A few recognition processes with various degrees of continuous two-step self-assembly of AuNPs have been reported, in which the triggered changes in their assembled states were driven by a physical or chemical perturbation such as pH, temperature, light, concentration of inorganic/organic molecules, or fueling

oligonucleotides.¹³⁻¹⁹ Especially, the development of AuNPs network where the self-assembly and disassembly are associated with multiple enzyme stimuli within one population of nanoparticles has not been well exploited yet.

Inspired by the specificity of enzymatic cleavage, we introduced a special level of controllably bidirectional self-assembly and disassembly of AuNPs system by enzyme switch. In this study, we presented a novel “trimethyl lock” based dual enzyme-responsive conjugate which can be utilized to control the self-assembly and disassembly of AuNPs in one population of nanoparticles. Figure 5.1 depicted the general design of this programmed AuNPs network by enzyme switch.

Figure 5.1. Schematic illustration of programming self-assembly and disassembly of AuNPs by enzyme switch.

The trimethyl lock is an *o*-hydroxyphenylpropionic acid derivative in which the

strong steric interactions between the three methyl groups could facilitate rapid intramolecular lactonization to form a hydrocoumarin.²⁰⁻²¹ Thus, upon unmasking of the hydroxyl group in trimethyl lock, a facile and spontaneous intramolecular cyclization leads to the formation of lactone and liberate the moieties attached to the carboxyl group (Figure 5.2).²² This unique “trimethyl lock” lactonization reaction was widely used in biochemical and biological research such as designing the latent fluorophores for sensing enzyme activity, biomolecular imaging, protein labeling, and designing esterase-sensitive prodrugs.²³⁻²⁸

Figure 5.2. Intramolecular lactonization of substituted “trimethyl lock”.

Herein, we employed the “trimethyl lock” effect in our system to trigger the self-assembly of AuNPs for colorimetric assay. In this design, the enzyme-responsive AuNPs conjugate consists of two sections: 1). A unique “trimethyl lock” lactonization section to release the peptide linker upon the esterase treatment. This peptide linker was modified with amino and thiol group at each end which could lead to the self-assembly of AuNPs through electrostatic interactions and strong Au-S bond. 2). A protease active section to cleave the peptide linker and further induce AuNPs disassembly (Figure 5.3).

It is known that both esterase and protease are abundant in nature and essential for

Figure 5.3. Mechanism of “trimethyl lock” peptide conjugate regulating the AuNPs self-assembly and disassembly based on the esterase and protease.

many important biological processes. They are also involved in various disease states such as HIV, cancer, Alzheimer’s and heart diseases.²⁹ Based on above specific enzymatic hydrolysis, both the self-assembly and disassembly of AuNPs can be achieved in the same nanoparticles system and this sequential two-step process will be easily monitored by naked eye, simple spectrophotometer and surface enhanced Raman scattering (SERS) measurements. Moreover, this approach may have potential applications for sensing enzyme activity in biological diagnostics.

5.2 Results and Discussion

5.2.1 Preparation of “trimethyl lock” peptide conjugate

To demonstrate our proof of concept, we synthesized a “trimethyl lock” peptide

conjugate which consists two fragments and responds to the dual enzymes. One fragment is the acetyl “trimethyl lock” which was firstly synthesized by the modification of hydroxyl group of “trimethyl lock” with acetyl group. Then, the other fragment of a flexible 4-mercaptophenylacetic acid modified peptide (SH-Ph-CH₂-Gly-Gly-Gly↓Phe-Gly-Gly-Lys(NH₂)-CONH₂ or NH₂-K(CONH₂)GGF-GGG-CH₂-Ph-SH) was synthesized according to the solid-phase Fmoc peptide synthesis protocol. This peptide sequence could be specifically cleaved by thermolysin between Gly and Phe as arrow indicated. Then, above two fragments were connected to afford the thiol ester and amide bond at each end of the peptide sequence through coupling carboxyl group on the acetylated “trimethyl lock” (Figure 5.4). The advantage of introducing “trimethyl lock” in this investigation is to achieve the effective esterase hydrolysis for the further release of peptide linker based on lactonization reaction, and also to significantly minimize the self-aggregation of AuNPs caused by enzyme substrate itself as reported in previous study.³⁰

Figure 5.4. Synthetic route of the acetyl “trimethyl lock” peptide conjugate.

In order to confirm the specific cleavage site of conjugate by enzymes, we

performed the RP-HPLC analysis. The hydrolysis of “trimethyl lock” peptide conjugate catalyzed by esterase and thermolysin was monitored respectively. As shown in Figure 5.5b, upon the esterase treatment, a peak with an elution time of 10.7 min was identified which corresponded to the polypeptide fragment $\text{NH}_2\text{-K}(\text{CONH}_2)\text{GGF-GGG-CH}_2\text{-Ph-SH}$ as determined by ESI-MS: Found $[\text{M}+\text{H}]^+$: 727.6, calculated $[\text{M}+\text{H}]^+$: 727.3. Similarly, in the thermolysin proteolysis product, a peak at $t = 14.6$ min was observed which were corresponding to the fragment acetyl trimethyl lock-SH-Ph-CH₂-GGG as determined by ESI-MS: Found $[\text{M}+\text{H}]^+$: 585.9, calculated $[\text{M}+\text{H}]^+$: 585.6 (Figure 5.5c).

Figure 5.5. Cleavage experiments by RP-HPLC. Chromatogram profiles of original substrate (trace a), after treatment with esterase (trace b) and after exposition to thermolysin (trace c). The peaks at 10.7 min (trace b) and 14.6 min (trace c) correspond to the fragment KGGFGGG and acetyl trimethyl lock-SH-Ph-CH₂-GGG, respectively.

These results proved our design and confirmed that “trimethyl lock” peptide conjugate could be specifically cleaved when exposed to esterase and thermolysin.

5.2.2 Colorimetric assay in gold nanoparticles

Typically, the “trimethyl lock” peptide substrate (8.5 μM) was firstly incubated with esterase (1.25 $\mu\text{g/mL}$) in monodispersed bis (*p*-sulfonatophenyl) phenylphosphane stabilized AuNPs suspension (pH 7.4) for 2 hrs. The enzyme hydrolysis catalyzed by esterase resulted in the cleavage of the thiolester bond and removal of acetyl group from the “trimethyl lock” peptide substrate. Unmasking of phenolic oxygen would facilitate the lactone formation with concomitant release of peptide moiety previously attached to the carboxyl groups. The free thiol and positively charged amino group at each end of released peptide initiated the formation of aggregated AuNPs clusters through Au-S bond and electrostatic interactions with the functional groups on the surface of bis (*p*-sulfonatophenyl) phenylphosphane stabilized AuNPs, thus resulting in the significant color change from red to purple-blue. Meanwhile, both an increased absorption band at 600 nm and a decreased absorption at 520 nm were observed in the UV-Vis spectrum (Figure 5.6, state 2). The dynamic UV-Vis spectra were also recorded at 0.5 hr time interval (Figure 5. 7). As a control, the incubation of AuNPs with only intact “trimethyl lock” peptide substrate could not induce any further color change and spectral shifts (Figure 5.6, state 1), indicating the substrate was stable and the self-assembly of AuNPs was mainly from the esterase reaction.

Figure 5.6. Color image (a) and UV/Vis spectrum (b) for the self-assembly and disassembly of AuNPs induced by the enzyme reactions. State 1: AuNPs with “trimethyl lock” peptide substrate (8.5 μM) only; State 2: AuNPs with esterase (1.25 $\mu\text{g/mL}$) treated substrate (8.5 μM); State 3: Aggregated AuNPs in state 2 treated with thermolysin (60 $\mu\text{g/mL}$).

Furthermore, in order to examine whether the second enzyme could disassemble the aggregated AuNPs, the aggregated AuNPs were exposed to protease thermolysin (60 $\mu\text{g/mL}$) at 37°C for 4 hrs. The amide bond between Gly and Phe in the peptide sequence which was connecting to the AuNPs would be selectively recognized and cleaved. The AuNPs aggregation was interrupted and the disassembly of AuNPs resulted in the color change from purple-blue to red. In addition, the maximum plasmon resonance peak shifted back from 600 nm to 520 nm (Figure 5.6, state 3). These results clearly demonstrated that addition of thermolysin could induce the redispersion of aggregated AuNPs in the solution. This colorimetric assay based on self assembly and disassembly of gold nanoparticles could be easily observed by naked eyes.

Figure 5.7. UV-Vis spectra of gold nanoparticles with “trimethyl lock” peptide conjugate (8.5 μM) treated with esterase (12.5 $\mu\text{g/mL}$) as a function of time. The time interval is 0.5 hr from state 1 to state 5.

In this colorimetric assay, the different concentrations of substrate affected the dynamic rate of self-assembly and disassembly of gold nanoparticles. Higher concentration of substrate induced the aggregation of gold nanoparticles in shorter time (Figure 5.8a). Then, the aggregated gold nanoparticles were further incubated with thermolysin (60 $\mu\text{g/mL}$), the aggregated clusters were driven to disassembly with different dynamic rates, which higher concentration of substrate displays longer dispersion time (Figure 5.8b). This self-assembly and disassembly process were also monitored by naked eyes and UV-Vis spectrophotometer.

Figure 5.8. Absorbance change of gold nanoparticles at 600nm as a function of time with “trimethyl lock” peptide substrate 4.3 μM (square), 8.5 μM (circle), 12.9 μM (triangle), respectively. (a) “Trimethyl lock” peptide substrate treated with esterase and gold nanoparticles. (b) Self-assembled gold nanoparticles treated with thermolysin for disassembly.

5.2.3 Sensitivity of the colorimetric assay

This bidirectional self-assembly and disassembly of AuNPs system is dependent on the catalytic efficiency of dual enzymes. The different amount of enzyme could induce different extent of assembly or disassembly of AuNPs. The state of AuNPs was observed to be highly aggregated with higher concentration of esterase and the absorbance reached a plateau when the enzyme concentration was higher than 1 $\mu\text{g/mL}$ (Figure 5.9(a)). The near linear relationship between the esterase concentration and the corresponding absorbance at 600 nm was shown in Figure 5.10(a). The minimum concentration of esterase that could induce the AuNPs self-assembly was found to be 18.5 ng/mL. This disassembled process could also be easily achieved with the thermolysin enzyme concentration as low as 34.1 ng/mL (Figure 5.9(b)). The near

linear correlation of thermolysin and absorbance at 520 nm was shown in Figure 5.10(b).

Figure 5.9. (a) Esterase concentration versus absorbance at 600 nm of AuNPs aggregation after addition of esterase treated “trimethyl lock” conjugate. (b) Thermolysin concentration versus absorbance at 520 nm of AuNPs redispersion upon hydrolysis of peptide linker.

Figure 5.10. (a) The near linear relationship of esterase concentration versus absorbance at 600 nm of AuNPs aggregation after addition of esterase treated “trimethyl lock” peptide conjugate. (b) The near linear relationship of thermolysin concentration versus absorbance at 520nm of AuNPs redispersion upon hydrolysis of peptide linker.

The enzyme action of thermolysin is limited and much less efficient maybe due to the steric inaccessibility of the enzyme active sites in a dense crosslinking networks of AuNPs.

5.2.4 Transmission electron microscopic and dynamic light scattering measurements

Further evidence of the AuNPs self-assembly and disassembly based on the dual enzyme reactions was obtained from transmission electron microscopy (TEM) and dynamic light scattering (DLS).

Figure 5.11. Transmission electron microscopy images of AuNPs. a), AuNPs with “trimethyl lock” peptide conjugate (8.5 μM); b), AuNPs with esterase (1.25 $\mu\text{g/mL}$) treated “trimethyl lock” peptide conjugate (8.5 μM); c), Aggregated AuNPs in (b) further treated with protease thermolysin (60 $\mu\text{g/mL}$). Scale bar: 50nm.

Transmission electron microscopic measurements were performed to determine the different self-assembly and disassembly states of AuNPs (Figure 5.11). The data revealed that intact “trimethyl lock” peptide conjugate could not induce the

aggregation of AuNPs. Once upon the treatment of esterase, the monodispersed AuNPs were induced highly aggregated by the released peptide linker. The aggregated AuNPs dissociated along time after further reacting with thermolysin. Therefore, the dual enzymatic reactions were crucial for the continuous assembly and disassembly of AuNPs, which is consistent with the results in previous spectrophotometric measurements.

The similar results were also obtained from dynamic light scattering measurements. As shown in Figure 5.12, the “trimethyl lock” peptide substrate itself was unable to induce the assembly of the AuNPs and the hydrodynamic size of the monodispersed AuNPs was determined to be 9.6 nm. The narrow width of the size distribution suggests that AuNPs with the “trimethyl lock” peptide substrate was stable in the AuNPs solution without observed aggregation. Upon the esterase treatment, the specific lactonization of the “trimethyl lock” triggered the release of thiol and amino groups at each end of the peptide, which initiated the distinctive self-assembly of AuNPs. The nanoparticles aggregated into larger clusters with the hydrodynamic diameter increasing to 153.2 nm. Furthermore, following the addition of thermolysin into the assembled AuNPs for 4 hrs, DLS showed a population of well-dispersed AuNPs with the average size reducing to 14.6 nm, indicating the disassembly of AuNPs based on the protease cleavage. Compared to the monodispersed AuNPs, the slight size increasing in the disassembled particles revealed the presence of cleaved peptide fragment on the surface of redispersed AuNPs.

Figure 5.12. The hydrodynamic size and size distribution of AuNPs. (a), AuNPs with “trimethyl lock” peptide conjugate ($8.5 \mu\text{M}$); (b), AuNPs with esterase ($1.25 \mu\text{g/mL}$) treated “trimethyl lock” peptide conjugate ($8.5 \mu\text{M}$); (c), Aggregated AuNPs in (b) further treated with thermolysin ($60 \mu\text{g/mL}$).

5.2.5 Gel electrophoresis for gold nanopartilces

Gold nanoparticles were analyzed by gel electrophoretic mobility shift assay. In this assay, concentrated stabilized gold nanoparticles migrate in 1% agarose gels at

100V, 30min. The $1\times$ TBE buffer (pH 8.0) was used as running buffer. As shown in Figure 5.13, the assembled gold clusters have the lowest gel mobility and stay in the sample well. Whereas, the gel bands for monodispersed and redispersed gold nanoparticles have similarly higher mobility shifts. This result displays the formation of large gold clusters were induced by the esterase treated “trimethyl lock” peptide conjugate and gold nanoparticles. The dissociation of aggregated AuNPs was triggered by the thermolysin hydrolysis.

Figure 5.13. Electrophoretic mobility assay. 1: gold nanoparticles with substrate only; 2: gold nanoparticles with esterase treated substrate; 3: aggregated gold nanoparticles in (2) further treated with thermolysin.

5.2.6 Zeta Potential analysis of gold nanoparticles

Zeta potential measurements were performed to analyze the surface charge of gold nanoparticles (Figure 5.14). The zeta potential of stabilized AuNPs incubated with “trimethyl lock” peptide conjugate is -23.56 mV at pH 8. This clearly indicated that

the surface of AuNPs maintained negative charge and the AuNPs were monodispersed in the solution because of the electrostatic repulsion. After adding esterase treated substrate into AuNPs, the zeta potential of the particles increased to -8.42 mV. The huge increase in the zeta potential is ascribed to the attachment of peptide fragment to the surface of AuNPs, which induced the aggregation of particles. Upon further treatment with thermolysin, the zeta potential of aggregated AuNPs decreased to -18.58 mV. This indicated the electrostatic repulsion between the AuNPs increased and AuNPs were driven to dispersion after the treatment by thermolysin. Above results are in agreement with the distinct color change which could be visible by naked eyes.

Figure 5.14. Zeta potential measurements for dispersed AuNPs (1), self-assembled AuNPs (2), and redispersed AuNPs (3).

5.2.7 Surface enhanced Raman Scattering Measurements

It has been well known that resonant surface plasmon excitation of the free electrons in metal nanostructures can enhance localized electromagnetic fields around

the surface. This enhancement effect becomes particularly strong in the interstitial spaces of aggregated nanoparticles, which is very suitable as platform for surface enhanced Raman scattering (SERS) studies. In order to monitor the self-assembly and disassembly processes of AuNPs induced by the multiple enzyme interactions, the SERS enhancement measurements were performed directly by using stabilizer, dipotassium bis (*p*-sulfonatophenyl) phenylphosphine dihydrate as molecular reporter.

Figure 5.15. Raman spectra of (a) 10^{-1} M dipotassium bis (*p*-sulfonatophenyl) phenylphosphine dihydrate solution; (b) AuNPs stabilized with dipotassium bis (*p*-sulfonatophenyl) phenylphosphane; (c) Stabilized AuNPs with esterase treated substrate and (d) Aggregated AuNPs treated with thermolysin, Laser wavelength: 633 nm.

As depicted in Figure 5.15(b), the stabilized AuNPs without esterase treatment did not have plasmonic coupling and SERS signals from these dispersed particles were very weak. However, intense signals were detected from AuNPs aggregation upon the addition of “trimethyl lock” peptide conjugate treated with esterase (Figure 5.15(c)). The observed SERS signals included the ν (CH) at 765 cm^{-1} , δ (CH) at 1098 cm^{-1} , and ν (CC) at 1603 cm^{-1} which were in accordance with characteristic bands of stabilizer in aqueous solution (Figure 5.15(a)).³¹ After further treatment with thermolysin, the peptide sequence in the aggregate was cleaved and the aggregated AuNPs went back to the dispersed state and no SERS signals could be observed as shown in Figure 5.15(d).

5.3 Conclusions

In conclusion, we have demonstrated a unique design for the control of self-assembly and disassembly of AuNPs on the basis of dual enzyme reactions in one population of nanoparticles. This process is illustrated by a series of enzymatic reactions in which randomly dispersed AuNPs are first converted into an aggregated structure, and subsequently to a redispersed state upon different enzyme treatments. Based on the consecutive color change from red to blue and finally to red again, visualization of the self-assembly and disassembly of AuNPs in the same nanoparticles system can be observed by the naked eye, simple spectrophotometer and surface enhanced Raman scattering (SERS) measurements. With more sophisticated design of the substrate or peptide sequence, this strategy could also be readily extended to other enzymatic systems. This sequential two-step assembly and disassembly process initiated by specific enzyme reactions can help us to understand the mechanisms of biomolecular recognitions. It may also have the potential to serve as a valuable platform for multiple enzyme detection and drug screening in biological studies or clinical settings.

5.4 Experimental Section

5.4.1 Materials and Chemicals

The purified esterase from hog liver (131 units/mg protein) was purchased from Fluka. Thermolysin from *Bacillus thermoproteolyticus* rokko (lyophilized powder, 68 units/mg protein) was purchased from Sigma. Fmoc-amino acids and rink amide polystyrene resins were obtained from Sigma-Aldrich. 10 nm gold nanoparticles were purchased from Sigma. Their average diameter measured by TEM (100 particles) was found to be 8.8 ± 1.0 nm. Dipotassium bis (*p*-sulfonatophenyl) phenylphosphine dihydrate was purchased from Aldrich. All the other commercially available reagents and chemicals were obtained from Sigma or Aldrich and used without further purification unless noted. Milli-Q water (18.2 M Ω) was used, obtained from an ultrapure water system (Millipore) with 0.22 μ m filter. The solvents were analytical grade or better and dried over according to regular protocols. HPLC grade acetonitrile and methanol were used for peptide purification.

Instruments for Characterization and Purification

^1H NMR was taken on Bruker Advance 300 MHz. When deuterated chloroform with TMS was used as a locking agent, TMS ^1H (0 ppm) peaks were used as a reference. ESI-MS spectrometric analyses were performed at the Thermo Finnigan LCQ Deca XP Max. Analytical reverse-phase high performance liquid chromatography (HPLC) was performed on Alltima C-18 column (250 $\times$ 3.0 mm) at a flow rate of 1.0 mL/min and semi-preparative HPLC was performed on the similar C-18 column (250 $\times$ 10 mm) at a flow rate of 3 mL/min. UV-Vis absorption spectra

were recorded on Beckman Coulter DU 800 UV-Vis spectrophotometer using quartz cuvettes. Background adjustments were made using deionized water. Solid phase peptide was synthesized on WS180° Shake synthesizer.

5.4.2 Preparation and characterization of “trimethyl lock” peptide conjugate

Synthetic route of “trimethyl lock” as shown in Figure 5.16.

Figure 5.16. Synthetic route of “trimethyl lock” substrate.

Preparation of compound 1: 3, 5-dimethylphenol (2.1 g, 17.3 mmol) and 3, 3-dimethylacrylate (2.15 mL) were dissolved in 15 mL benzene solution. Then 1.2 mL concentrated sulfuric acid was added forming a dark yellow solution. The mixture was stirred for 4 hours under reflux. After removal of solvent from the rotatory evaporator, the reaction mixture was washed with ethyl acetate (50 mL), water (20 mL), 1.0 M sodium hydroxide (10 mL), brine (20 mL x 3) and finally dried over anhydrous

magnesium sulfate. The solvent was removed under vacuum affording 2.512 g (71.6 %) yellow oil compound. ^1H NMR (300 MHz, CDCl_3): δ 6.77 (s, 1H), δ 6.57 (s, 1H), δ 2.61 (s, 2H), δ 2.50 (s, 3H), δ 2.28 (s, 3H), δ 1.46 (s, 6H). ESI-MS: found $[\text{M}+\text{H}]^+$: 205.44, calculated $[\text{M}+\text{H}]^+$: 205.3.

Preparation of compound 2: Compound **1** (2.512 g, 12.3 mmol) was suspended in 30 mL anhydrous THF under ice bath with subsequent addition of lithium aluminum hydride (467.8 mg, 12.3 mmol). The mixture was stirred for 1 hr under nitrogen. Then the reaction was quenched with 1.0 M HCl (10 mL) and filtrated. After the solution was concentrated under reduced pressure, it was washed with ethyl acetate (30 mL), water (15 mL), brine (15 mL x 3) and dried over anhydrous magnesium sulfate. The residue was purified with column chromatography (silica gel) with eluent hexane/ethyl acetate (3:2) to afford 2.203 g white powder product (87.7 %). ^1H NMR (300 MHz, CDCl_3): δ 6.51 (s, 1H), δ 6.36 (s, 1H), δ 3.64 (t, $J = 7.26$, 2H), δ 2.50 (s, 3H), δ 2.28 (t, $J = 7.26$, 2H), δ 2.19 (s, 3H), δ 1.58 (s, 6H). ESI-MS: found $[\text{M}+\text{H}]^+$: 209.18, calculated $[\text{M}+\text{H}]^+$: 209.3.

Preparation of compound 3: To a solution of compound **2** (2.203 g, 10.6 mmol) and 4-dimethylaminopyridine (2.025 g, 16.6 mmol) in anhydrous THF (20 mL) was added *tert*-butyldimethylsilyl chloride (1.8 g, 12.0 mmol). The reaction was stirred under an ice bath for 14 hours. Then the mixture was extracted with ethyl acetate (20 mL), water (10 mL), brine (10 mL x 3) and dried over anhydrous magnesium sulfate. The

residue was then concentrated under reduced pressure and purified with flash chromatography with eluent hexane/ethyl acetate (3:2) to afford 3.2g (93.9%) white solid. ^1H NMR (400 MHz, CDCl_3) δ 6.54 (s, 1H), δ 6.44 (s, 1H), δ 3.69 (t, $J=7.35$, 2H), δ 2.54 (s, 3H), δ 2.28 (t, $J=7.35$, 2H), δ 2.24 (s, 3H), δ 1.63 (s, 6H), δ 0.97 (s, 9H), δ 0.13 (s, 6H). ESI-MS: found $[\text{M}+\text{Na}]^+$: 345.06, calculated $[\text{M}+\text{Na}]^+$: 345.5.

Preparation of compound 4: The compound from **3** (3.2 g, 9.94 mmol) was further reacted with anhydrous acetic anhydride (1.74 mL, 18.5 mmol) with the addition of triethylamine (2.79 mL, 20 mmol) and 4-dimethylaminopyridine (313.7 mg, 2.57 mmol). The mixture was stirred for 2 hours under nitrogen at room temperature. Then the solution was quenched and purified by column chromatography (silica gel) with eluent hexane/ethyl acetate (3:2) which later gave 3.402g (94%) yellow oil title product. ^1H NMR (300 MHz, CDCl_3): δ 6.88 (s, 1H), δ 6.66 (s, 1H), δ 3.61 (t, $J=7.35$, 2H), δ 2.61 (s, 3H), δ 2.31 (s, 3H), δ 2.30 (s, 3H), δ 2.15 (t, $J=7.35$, 2H), δ 1.59 (s, 6H), δ 0.97 (s, 9H), δ 0.09 (s, 6H). ESI-MS: found $[\text{M}+\text{H}]^+$: 365.21, calculated $[\text{M}+\text{H}]^+$: 365.6.

Preparation of compound 5: To 3.402 g (9.34 mmol) of compound **4** was added 20 mL of anhydrous THF and 10 mL of deionized water. Glacial acetic acid (20 mL) was added into above solution and stirred overnight under nitrogen in an ice bath. The mixture was concentrated under reduced pressure and extracted with ethyl acetate (30 mL), water (10 mL), 10 % sodium bicarbonate (50 mL) and brine (10 mL x 3). Then

the residue was dried over anhydrous magnesium sulfate and purified with silica gel with eluent hexane/ethyl acetate (1:1) to afford title compound (2.013 g, 85.8 %). ^1H NMR (300 MHz, CDCl_3) δ 6.80 (s, 1H), δ 6.56 (s, 1H), δ 3.40 (t, $J = 7.65$, 2H), δ 3.06 (br, 1H), δ 2.49 (s, 3H), δ 2.22 (s, 3H), δ 2.20 (s, 3H), δ 2.00 (m, 2H), δ 1.46 (s, 6H). ESI-MS: found $[\text{M}+\text{H}]^+$: 251.11, calculated $[\text{M}+\text{H}]^+$: 251.2.

Preparation of compound 6: The 2.013 g (8.05 mmol) of compound **5** was dissolved in 10 mL of dichloromethane. Then pyridine chlorochromate (3.45 g, 16 mmol) was added into above solution forming a black suspension. The mixture was stirred for 1 hour under room temperature and was filtered to collect the eluent. After evaporation of solvent, the residue was purified with column chromatography with eluent hexane/ethyl acetate (2:3) to obtain 1.5 g (75.0 %) of the title product. ^1H NMR (300 MHz, CDCl_3) δ 9.53 (t, $J = 2.58$, 1H), δ 6.84 (s, 1H), δ 6.62 (s, 1H), δ 2.81 (d, $J = 2.55$, 2H), δ 2.53 (s, 3H), δ 2.26 (s, 3H), δ 2.22 (s, 3H), δ 1.56 (s, 6H). ESI-MS: found $[\text{M}+\text{H}]^+$: 248.31, calculated $[\text{M}+\text{H}]^+$: 248.3.

Preparation of compound 7: Compound **6** (1.5 g, 6.04 mmol) was dissolved in 10 mL of acetone and 10 mL of deionized water. Then potassium permanganate (VII) (955 mg, 6.04 mmol) dissolved in 5 mL of deionized water and 5 mL of acetone was added dropwise to the above solution. The reaction mixture was stirred for 17 hours under ambient temperature. After evaporation of solvent, the product was extracted with dichloromethane (30 mL), washed by water (5 mL), brine (5 mL x 3) and finally

dried over anhydrous magnesium sulfate. The mixture was then concentrated under reduced pressure and purified with flash chromatography with eluent hexane/ethyl acetate (1:4) to obtain solid powder of “trimethyl lock” product (942 mg, 66.2 %). ^1H NMR (300 MHz, CDCl_3) δ 6.88 (s, 1H), δ 6.66 (s, 1H), δ 2.90 (s, 2H), δ 2.60 (s, 3H), δ 2.34 (s, 3H), δ 2.29 (s, 3H), δ 1.64 (s, 6H). ESI-MS: found $[\text{M}+\text{H}]^+$: 265.33, calculated $[\text{M}+\text{H}]^+$: 265.3.

Synthesis and purification of polypeptide: The polypeptide sequence, SH-Ph-CH₂-Gly-Gly-Gly-Phe-Gly-Gly-Lys (NH₂)-CONH₂, which could be cleaved by thermolysin, was synthesized on Fmoc-Rink amide polystyrene resin by standard Fmoc-Solid Phase Peptide Synthesis strategies.¹ Typically, each amino acid was coupled in sequence for two hours with TBTU/HOBT as coupling reagent in anhydrous DMF. Finally, 4-thiol phenyl acetic acid was coupled to the N-terminus of above polypeptide, which contains amide at the C-terminus, in anhydrous DMF following the solid phase coupling method. After cleavage and deprotection from resin in trifluoroacetic acid solution, the above crude polypeptide was obtained and dried in vacuum. Then, the polypeptide was purified by reverse-phase semi-preparative HPLC using 20% - 80% water/acetonitrile gradient eluting system containing 0.1% TFA, which was monitored by UV-Vis absorbance at 280 nm. After frozen and lyophilized, 8.6 mg white powder of pure peptide was obtained. Figure 5.17 shows the analytical HPLC chromatogram profile for purified polypeptide. ESI-MS Found $[\text{M}+\text{H}]^+$: 728.3; calculated $[\text{M}+\text{H}]^+$: 728.3.

Figure 5.17

Synthesis of “trimethyl lock” conjugated polypeptide substrate. To a stirred suspension of “trimethyl lock” compound **7** (15.6 mg, 0.06 mmol) in 200 μL anhydrous dichloromethane, thionyl chloride (43 μL , 0.59 mmol) was added dropwise at cooled temperature (ice bath). The reaction mixture was warmed slowly to room temperature and stirred 18 h. The solvent was evaporated in vacuum to give the oil product without purification. Then, the oil product was reacted with above purified polypeptide (8.6 mg, 0.012 mmol) in 150 μL anhydrous DMF followed by addition of triethylamine (13 μL , 0.093 mmol) under nitrogen atmosphere for 12 hours. After removing DMF, the crude product was purified by reversed-phase semi-preparative HPLC with 20% - 80% water/acetonitrile gradient system containing 0.1% TFA to give the final “trimethyl lock” peptide substrate product 7.4 mg (53.1%). As shown in Figure 5.18, the purified substrate was analyzed by analytical HPLC. ESI-MS Found $[\text{M}+\text{H}]^+$: 1220.63, $[\text{M}+\text{Na}]^+$: 1242.85; calculated $[\text{M}+\text{H}]^+$: 1220.6.

Figure 5.18

5.4.3 Cleavage of “trimethyl lock” conjugated peptide substrate with esterase/thermolysin

The “trimethyl lock” conjugated peptide substrate (85 μM) was incubated with esterase (125 $\mu\text{g/mL}$) in PBS buffer (pH 7.4) for 2 hrs. Separately, another part of “trimethyl lock” peptide substrate (85 μM) and thermolysin (600 $\mu\text{g/mL}$) were mixed in PBS buffer (pH 7.4) for 4 hrs. Both of the reactions were monitored by RP-HPLC (20% - 80% water/acetonitrile containing 0.1% TFA linear gradient system).

5.4.4 Stabilization of gold nanoparticles

Purchased gold nanoparticles were stabilized by co-dissolving with freshly prepared dipotassium bis (*p*-sulfonatophenyl) phenylphosphine dihydrate solution (0.5 mM) for more than 10 h.³² After centrifuging and harvesting the gold nanoparticles,

PBS buffer solution was used to redissolve gold nanoparticle with the concentration of 8.2 nM. The stabilized gold nanoparticles were stable in PBS buffer at room temperature over a period of days.

5.4.5 Colorimetric assay for enzyme hydrolysis of “trimethyl lock” substrate

To the solution of 0.4 mL of monodispersed dipotassium bis (*p*-sulfonatophenyl) phenylphosphane stabilized AuNPs (10 nm), the peptide substrate and esterase in 0.05 mL of PBS buffer (pH 7.4) were added to afford their final concentrations of 8.5 μ M and 1.25 μ g/mL, respectively. The mixture was incubated at 37°C for enzyme hydrolysis 2 hrs.

5.4.6 Transmission electron microscopy (TEM) for particle size analysis

All the TEM photographs of gold nanoparticles were taken on JEOL 2000 EX transmission electron microscope at 200 kV. The gold nanoparticles solution was dropped onto the carbon-coated copper grids (200 mesh) which had been pre-treated by UV-light to reduce static electricity. Then the nanoparticles solution was allowed to settle on grids for 5 mins before the excess solution was wicked away with filter paper.

5.4.7 Dynamic Light Scattering (DLS) Measurements

Dynamic light scattering (DLS) measurements were performed using a 90 Plus particle size analyzer (Brookhaven Instruments Corporation). The DLS instrument

was operated at 25°C, 90 degree detector angle with an incident laser wavelength of 660 nm. Size and size distribution of gold nanoparticles were determined in solution. All the samples were measured for 3 min, and the reported values are the average of five repeated consecutively measurements.

5.4.8 Surface Enhanced Raman Scattering (SERS) Measurements

Gold nanoparticles solution dropped on glass slides (approximately 10 μ L) was used for SERS measurement. The spectra were excited using 3.5mW of power at He-Ne 632.8 nm laser. The laser beam was then focused onto the sample via a dichroic mirror and through an Olympus 40 $\times$, 0.90 NA microscope objective. Raman signals were collected and focused into a 400 μ m optical fibre (Ocean Optics, Inc.) which delivered the signals to a single-stage monochromator (DoongWo, Inc.). Spectrum acquisition was started and an integration time is 40 sec for all SERS measurements.

5.5 References

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Chapter 6

Multifunctional Nanocontainers Capped with Oligonucleotides for Controlled Drug Delivery and Magnetic Imaging

6.1 Introduction

Controlled drug delivery systems have promising applications for treatment of various human diseases in clinic and represent a developing field for biomedical material science.¹⁻² The drug delivery system usually possesses unique features and could control the period of drug delivery and minimize the nonspecific drug release. It can target the specific areas *in vivo*. Controlled drug delivery systems could maintain the therapeutic levels of active drugs during the treatment period, which are superior to traditional therapies with a saw-tooth curve of drug concentration in plasma.²

Recently, the rapid development of nanotechnology has motivated the researchers to exploit the nanomaterials for biomedical applications. It is found that the nanosized particles are particularly efficient in evading the reticuloendothelial system and could be used for encapsulating hydrophobic drugs.³ Mesoporous silica nanoparticles (MSNs) are the widely used nanomaterial in drug delivery systems due to their biocompatibility and high quantities of drug encapsulation.⁴ However, the mesoporous silica nanoparticles based drug delivery system has one problem that the efficacy of drugs was decreased before reaching the target tissues. Subsequently, several MSN-based controlled-drug release systems with the “zero-premature release” property have been developed and synthesized by using different kinds of pore caps,

such as nanoparticles, organic molecules, and supramolecular assemblies.⁵⁻⁷ Various stimuli-responsive strategies have been applied as gate triggers for uncapping the pores and releasing the drug molecules from MSNs. These triggers include chemicals, pH, electrostatic interaction, enzyme, redox, and photoirradiation.⁶⁻¹¹ Nanocontainers with stimuli-responsive gatekeepers could provide unique advantages for precise and controlled release of drugs. Despite these developments of drug delivery systems, many of the MSN-based controlled drug release systems are unable to function under physiological conditions. In order to efficiently control release of toxic drugs *in vitro* and *in vivo*, it would be desirable to design a capped MSN nanomaterial that would respond to a noninvasive and internally controllable trigger, such as cancer cell over-expressed enzyme activation under physiological conditions.

Superparamagnetic iron oxide nanoparticles are of great interest in recent years. They have demonstrated various biological applications such as drug delivery, gene delivery, magnetic resonance imaging, cell or protein separation, and thermal tumor therapy.¹²⁻¹⁸ A few examples of controlled drug delivery based on magnetic nanoparticles have been reported.¹⁸ In these systems, the magnetic nanoparticle serves as core with the shells in which pharmaceutical drugs were encapsulated. These core-shell materials could simultaneously image tumor sites by magnetic resonance imaging (MRI) and effectively treat cancer cells with the anticancer drug.

In this study, we integrated the mesoporous silica nanoparticles with superparamagnetic iron oxide nanoparticles to form core-shell nanocontainer (designated as Fe₃O₄@mSiO₂). This core-shell nanocontainer has great potential for

multifunctional applications including magnetic imaging and drug delivery. The mesoporous structure was further capped with double strand oligonucleotides or oligonucleotide-functionalized gold nanoparticles, which could form DNA/gold capped Fe_3O_4 -MSN nanohybrid (Figure 6.1). The oligonucleotides respond to enzyme to loosen the double helix, which gives the intracellular stimuli-responsive controlled drug release. We demonstrated that this multifunctional nanoparticle could potentially be used for simultaneous magnetic imaging and therapeutic treatment.

Figure 6.1. Schematic illustration of multifunctional nanocontainer system.

6.2 Results and Discussion

6.2.1 The preparation of core-shell nanoparticles

A typical procedure for the synthesis of multifunctional nanospheres is shown in Figure 6.2. The oleic acid-capped magnetic iron oxide nanoparticles (Fe_3O_4) were

synthesized by thermal decomposition method according to the previously reported one.¹⁹ Typically, iron (III) chloride hexahydrate and sodium oleate were dissolved in a mixture of absolute ethanol, water and hexane. The solution was refluxed for 4 h. Above mixture was then washed with water and evaporated the hexane by rotary evaporator. Iron-oleate complex was obtained. Then, iron-oleate complex was dissolved in a solution of oleic acid and octadecene. Fe_3O_4 nanocrystals were prepared by gradually elevating the temperature to 320°C . The as-prepared 15 nm sized Fe_3O_4 nanocrystals are stabilized with hydrophobic oleic acid and are dispersed in hexane.

Figure 6.2. Schematic illustration of the preparation of core-shell nanoparticles.

To conduct sol-gel reaction for forming mesoporous silica shell, hydrophobic ligand-capped Fe_3O_4 were transferred from organic phase to aqueous solution with cetyltrimethyl ammonium bromide (CTAB).²⁰⁻²¹ CTAB is a kind of surfactant and serve as phase transfer agent (Figure 6.3).

Figure 6.3. Chemical structure of CTAB.

The oleate-capped Fe_3O_4 was transferred to water solution by dissolving in chloroform and CTAB aqueous solution and evaporating the organic solvent (Figure 6.4). The hydrophobic tail of CTAB surfactant interacts with the oleate ligands on the surface of the Fe_3O_4 , and the hydrophilic headgroup of CTAB make the Fe_3O_4 soluble in water. This phase transfer process was effective.

Figure 6.4. Phase transfer process for Fe_3O_4 nanocrystals by CTAB.

Subsequently, the tetraethyl orthosilicate (TEOS) and (3-mercaptopropyl) methyldimethoxysilane in an aqueous solution containing CTAB stabilized Fe_3O_4 and small amount of ethyl acetate was initiated the sol-gel reaction by NaOH in alkaline condition affording Fe_3O_4 embedded mesoporous silica nanoparticles. Here, CTAB-stabilized Fe_3O_4 acted as seeds for the formation of mesoporous silica particles. During the formation process of mesoporous structure, CTAB also served as the organic template for the formation of the mesoporous nanospheres. The mesoporous $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles have the mercapto-functional group on the surface which could facilitate labeling the oligonucleotides as capping agent.

Furthermore, in order to load cargo molecules such as hydrophobic drugs into the

pores of the mesostructure, the CTAB surfactants were removed from the mesopores by using an ion exchange method. Ammonium nitrate solution was used for removing CTAB from the materials.

6.2.2 Characterization of the core-shell nanoparticles

After obtaining the above mesoporous nanostructure, the transmission electron microscope (TEM) was performed to characterize the morphology of nanoparticles. As shown in Figure 6.5, the diameter of Fe_3O_4 is around 15 nm upon calculated over 100 particles. The Fe_3O_4 embedded mesoporous silica nanoparticles are with the diameter of 120 ~ 130 nm.

To observe the magnetic properties of $\text{Fe}_3\text{O}_4@\text{mSiO}_2$, the magnet was placed on the side wall of the vial containing magnetic nanoparticles. Figure 6.6 showed the suspended $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ was dispersed in aqueous solution, whereas the distinct attraction force between the particles and magnet accumulate the particles in the vicinity of magnet.

Figure 6.5. TEM images of the $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles

Figure 6.6. (a) The $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles were suspended in aqueous solution and placed next to the magnet. (b) The $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles were collected in the presence of magnetic field.

6.2.3 Functionalizing nanoparticles with oligonucleotides

In order to demonstrate our design, we functionalized the $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles with oligonucleotide. The modification procedures were illustrated in Figure 6.7. Typically, the as-synthesized mercapto- $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ particles were linked to the bifunctional NHester reagent through thiol group. The bifunctional reagent was further reacted with amino-group modified oligonucleotide A by forming amide bond.

Figure 6.7. Schematic illustration of $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticle functionalized by oligonucleotide.

Here, the (3-mercaptopropyl) methyltrimethoxysilane was used to introduce the mercapto group on the surface of $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ particles and MAL-(PEG)₂-NHester as the bifunctional linker (Figure 6.8). After modification of particles surface, we performed the Fourier Transform Infrared spectroscopy (FTIR).

Figure 6.8. Chemical structure of (3-mercaptopropyl)methyldimethoxysilane (a) and MAL-(PEG)₂-NHester linker (b).

FTIR spectra showed that the synthesized mercapto Fe₃O₄@mSiO₂ particles with distinct absorbing peaks at 2843 cm⁻¹ and 1083 cm⁻¹ corresponded to the S-CH₂ stretching and the Si-O stretching respectively, which were from the pure (3-mercaptopropyl)methyldimethoxysilane (Figure 6.9 & Figure 6.10). Subsequently, MAL-(PEG)₂-NHester linker was attached to the surface of mesoporous nanoparticles. Compared with the pure linker and the mercapto particles, the noticeable C=O stretching peak at 1707 cm⁻¹ was from PEG linker (Figure 6.11 & Figure 6.12). Above nanoparticles were further coupled with oligonucleotide A, the C=O stretching peak from succinimide group in linker clearly decreased (Figure 6.13). It indicates that the succinimide group has reacted with the amino group functionalized oligonucleotide. These result supported that the oligonucleotide A has been anchored on the surface of mesoporous nanoparticles.

Figure 6.9. FTIR spectrum of the (3-mercaptopropyl)methyldimethoxysilane.

Figure 6.10. FTIR spectrum of $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles with mercapto-silane.

Figure 6.11. FTIR spectrum of MAL-(PEG)₂-NHester linker.

Figure 6.12. FTIR spectrum of the Fe₃O₄@mSiO₂ nanoparticles functionalized with the MAL-(PEG)₂-NHester linker.

Figure 6.13. FTIR spectrum of $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles functionalized with oligonucleotide A.

6.2.4 Drug loading assay

To prove our concept, we subsequently chose doxorubicin to investigate the drug release assay using prepared mesoporous nanoparticles. Doxorubicin (Dox) is one of the most potent and well-known anticancer drug which has shown great efficiency against wide range of neoplasms. It is a member of the anthracycline ring antibiotics and widely used in various cancer therapies (Figure 6.14).²² Despite this oncology drug being widely used, its clinical application is limited by several undesirable side-effects such as dose-dependent cardiotoxicity and myelosuppression. Moreover, the hydrophobic drug is restricted to pass through the cellular membrane resulting in minimal drug internalization and its ability to overcome biological barriers such as

blood-brain barrier. Thus, various approaches have been developed to improve the drug efficacy and safety. Many groups have reported the Dox conjugates as prodrugs for efficient drug delivery and cancer treatment.²³⁻²⁴ In addition, nanoparticles as drug carriers were also well developed.²⁵⁻²⁶

In our study, we loaded the Dox into the mesoporous nanostructure by soaking them in concentrated drug-DMSO solution as reported method. The drug loaded nanoparticles were collected by centrifugation, washed three times, and then resuspended in PBS buffer. The drug loaded nanoparticles which were previously modified with oligonucleotide A, were reacted with its complementary oligonucleotide A' in PBS buffer to cap the Dox in the mesopores of particles.

Figure 6.14. Chemical structure of doxorubicin.

The oligonucleotide A' responsive to the enzyme thrombin which could lead to the change of its confirmation to G-quadruplex, thus the double strand complementary structure were loosen and destroyed.²⁷ Based on this design, upon the thrombin treatment, the capped nanoparticles were switched on, leaking out the loaded drug gradually from the mesopores.

6.2.5 Drug release assay in *vitro*

After obtaining the capped drug-loaded mesoporous nanoparticles, we evaluated the efficiency of drug release in the presence of thrombin in PBS buffer. As shown in Figure 6.15, the thrombin triggered capped nanoparticles could release Dox gradually, whereas no capped particles liberated Dox like a blast after 40 hr. As control, fully capped nanoparticles did not release Dox under the same condition. Based on this preliminary result, the capped $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles with oligonucleotide can potentially be served as drug carrier to store and deliver anticancer drug with controlled release.

Figure 6.15. Dox release profile of capped $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ nanoparticles in the absence (circle) and presence of thrombin (triangle) and no capped nanoparticles (square).

6.3 Further work and Perspective

Although the preliminary study of this enzyme responsive controlled drug delivery system proved our hypothesis, more experiments are still needed for further investigation. The multifunctional nanocarrier capped with oligonucleotide-functionalized gold nanoparticles should be evaluated, which may have higher capping capacities compared to the current results. Moreover, magnetic imaging assay could be examined *in vitro* and in cancer cells. Cytotoxicity assay should be done for assessing the efficacy of this drug delivery system.

6.4 Experimental Section

Materials

Iron(III) chloride hexahydrate was purchased from Sigma. Oleic acid and octadecene were purchased from Merck. Cetyltrimethylammonium bromide, (3-mercaptopropyl) methyldimethoxysilane (95%), and tetraethylorthosilicate were obtained from Sigma-Aldrich. Doxorubicin hydrochloride was purchased from Sigma-Aldrich. Single strand Oligonucleotide A, B were purchase from 1st Base Pte Ltd (Singapore). The sequences of oligonucleotides are 3'-CAACACCGACCTTT-(CH₂)₆-NH₂ (oligonucleotide A) and 3'-GGTTGGTGTGGTTGGTTTTTT-(CH₂)₆-SH (oligonucleotide A'). All the other reagents were purchased from Sigma-Aldrich.

Transmission electron microscopy (TEM) measurements

The samples for TEM measurement were prepared by directly dropping 20 μ l

nanoparticles solution on the formvar/carbon coated copper grid, dried in air for 15 min. Transmission electron microscope images were then captured on JEOL 2000 EX TEM at 200kV. The particle size analysis was taken around 100 particles.

Fourier transform infrared spectroscopy (FTIR) measurements

FTIR spectra of mesoporous nanoparticles were recorded on SHIMADZU IR Prestige 21fourier transform infrared spectrophotometer in the diffuse reflectance mode at a resolution of 4 cm^{-1} in the range of $400 - 4000\text{ cm}^{-1}$ in KBr pellets. For comparison, FTIR spectrum of pure KBr was also recorded as background.

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List of Publications

Peer-reviewed Papers:

1. **Rongrong Liu**, Roushen Liew, Jie Zhou, Bengang Xing*. A Simple and Specific Assay for Real-Time Colorimetric Visualization of β -Lactamase Activity by Using Gold Nanoparticles. *Angewandte Chemie International Edition*, **2007**, 46, 8799-8803.
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4. **Rongrong Liu**, Weiling Teo, Siyu Tan, Huajun Feng, P. Padmanabhan, Bengang Xing*. Metallic nanoparticles bioassay for *Enterobacter cloacae* P99 β -lactamase activity and inhibitor screening. *Analyst*, **2010**, 135, 1031-1036. (Selected as Back cover)

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1. **Rongrong Liu**, Roushen Liew, Zhou Jie, Bengang Xing*. Novel colorimetric assay for Real-time Visualization of Bacterial Enzymes by Using Gold Nanoparticles. *International Symposium on Catalysis and Fine Chemicals*, **2007** Nanyang Technological University, Singapore.

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