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Using functionalized nanoparticles as sensors for rapid monitoring of drinking water quality

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Using functionalized nanoparticles as sensors for rapid monitoring of drinking water quality

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CHAPTER 1

Introduction

1.1 Introduction

Drinking water can be defined as water delivered to the consumer that can be used as drinking, cooking, and washing, without risk of immediate or long term harm (Zuane 1996). To ensure the safety of the water, the water supplied to households, commercial and industrial facilities has to meet required drinking water standard posed by government agencies. Drinking water contaminated with disease vectors (i.e. pathogenic microorganisms or toxins) or unacceptable levels of dissolved poisonous chemicals may lead to acute and chronic illnesses (Sinclair, Jones et al. 2009). In many developing countries, contamination of the drinking water is a major cause of death. It has been estimated that 1.8 million deaths each year were caused by waterborne diseases around the world. (Prevention 2006)

The Environmental Protection Agency of the United States (USEPA) has set strict regulations on the allowable levels of certain contaminants in drinking water. EPA also requires standard procedures and methods to be followed for regular testing of water supplies to assure their safety. Based on the regulations, any water supply system must be adequately sampled to evaluate changes in water quality in time, in drought condition and in sudden deterioration of quality (USEPA 1995; Zuane 1996). Nowadays, large quantity of water samples are routinely collected from the field and then analyzed in laboratories

with highly sensitive water analysis methods. These methods normally require complicated and expensive instruments, which make them unsuitable for onsite implementation. Monitoring water quality in remote areas thus becomes a costly operation; water samples have to be collected and then transported to faraway sites for analysis (Nollet 2000). A field-deployable detection method could be of great value to meet the need of onsite water characterization. It can also be applied to monitor water quality of private wells, which are not regulated by USEPA.

Moreover, although the United States has one of the safest water supplies in the world, the national statistics doesn't reflect the specific quality of water for individual families.(USEPA 1999) The quality of water depends on the water treatment and delivery systems, which may vary from place to place. Quick onsite detection of dangerous levels of contamination to tap water could also be critical in a crisis management scenario when a terrorist's attack occurs to our public water supply system. A simple detection method that enables a quick, onsite evaluation of tap water quality is hence highly desirable.

One of the major inorganic contamination agents for water supplies is nitrite ion. Nitrite, as part of nitrogen cycle, widely exist in ground and surface water systems, due to contaminations of water sources by fertilizer, waste of livestock and other organic wastes.(USEPA 1995; Fanning 2000; Nollet 2000; Moorcroft, Davis et al. 2001) Even trace amount of nitrate and nitrite ions is unsafe to human health, and both are type A inorganic chemicals in water quality parameters which require close monitoring by

Health Authorities due to their potential toxicity.(USEPA 1995) Infants drinking water contaminated by nitrate or nitrite may suffer shortness of breath, blue baby syndrome, and even death if untreated.(Bruningfann and Kaneene 1993; Zuane 1996; Fanning 2000; Brender, Olive et al. 2004) The maximum containment levels (MCLs) of nitrate and nitrite, as recommended by the USEPA, are 10 ppm and 1 ppm (measured as Nitrogen), respectively.(USEPA 1995)

In the past two decades various methods have been developed to monitor the nitrite levels in fresh water, as reviewed in the next chapter. However, most of these methods are only suitable for water analysis in well-equipped laboratories. A fast and easy-to-use method for detection of trace amount of nitrite (around or below the EPA standard) in drinking water is still not widely available. With the advance of nanotechnology, crosslinking Au nanoparticles have been explored as nanosensors which can detect trace amount of nitrite in a colorimetric scheme (Daniel, Han et al. 2009). In this thesis, a new, non-crosslinking Au nanorod sensing scheme is developed that further improves the performance of the nanosensors and can easily be used in a portable sensing platform for in-field nitrite detection for drinking water.

Another major concern of water safety is contamination by pathogenic microorganisms. Approximately 76 million cases of foodborne/waterborne illness occur in the United States each year, causing enormous personal grievances and billions of dollars of loss to our economy (Mead, Slutsker et al. 1999). Our water supplies are also

susceptible to deliberate terrorist attacks by biological and chemical weapons (Prosnitz 2005). Therefore, foodborne/waterborne pathogens pose both a public health and a national security threat.

Efforts to deal with this significant threat are often times handicapped by the lack of effective surveillance systems to rapidly detect pathogenic contamination in the field where it occurs (Goyal 2006). Any method that can be applied to detect pathogens in the field needs to be rapid, cost efficient and easy-to-operate. Biosensor technology offers the best solution to meet this need because of its portability, sensitivity and potential for automation and online use. However, biosensor design, which is often dictated by specific applications, usually is not universally applicable for detection of a wide range of targets. By signaling mechanisms, biosensors can be grouped as electrochemical, optical and spectroscopic, thermometric, and mass-based. Among them optical biosensors are the most appealing due to their sensitivity, available instrumentation and relative ease of data interpretation (Geng and ABhunia 2007). Traditional optical biosensors rely on luminescent signals of chemical dyes attached to target-recognition agents. In the last decade, nanoparticles (e.g., Au/Ag plasmonic nanoparticles, semi-conductor quantum dots) have been utilized as optical reporters for novel biosensors that led to higher sensitivity and throughput (Stewart, Anderton et al. 2008; Wang and Irudayaraj 2008; Wang and Irudayaraj 2010). Due to the consistent surface chemistry of functionalizing these nanoparticles, a universal-applicable detection scheme using them as reporters can

be potentially developed for a wide range of foodborne/waterborne pathogenic targets.

Although applications of optical sensors for detection of a variety of pathogens have been developed over the years, many problems remain to be solved before optical biosensors can truly be applied to in-field pathogen detection as a universal platform. In most optical biosensors, specificity in target detection is achieved through molecular binding events between "captor" (i.e., antibodies and aptamers) and the targets. In order to eliminate false positive signals, separation of target-bound captors from unbound ones must be performed, usually through multiple, vigorous washing steps. This "washing" need adds cost and complexity to the biosensing operations, and diminishes the feasibility of quick in-field deployment. An alternative to this captor-based methodology is to utilize spectroscopic signatures of the target themselves for label-free detection in one single step. However, this approach requires high-precision spectral measurement which is not currently available in any portable devices; hence compromises any possibility for a field-deployable biosensing platform. In one of the first attempts to combine the advantages of molecular captors and spectroscopic fingerprinting, our group developed a mid-IR spectroscopic biosensor (Yu, Ganjoo et al. 2006), which was later advanced to incorporate a magnetic separation-based sampling step (Ravindranath, Mauer et al. 2009), that utilized fourier-transform infrared (FTIR) spectroscopic fingerprinting to achieve limit of detection (LOD) of 10^3 – 10^4 CFU of pathogenic bacteria in food matrices. Surface-enhanced Raman spectroscopy (SERS) biosensors will have the potential to

detect single bacterium because of the orders (10^8 – 10^{10}) of magnitude in signal enhancement possible by plasmonic SERS substrates (Doering and Nie 2002).

1.2 Hypotheses

Nano scaled molecular probes have been used to monitor drinking water quality, i.e., nitrite and nitrate concentrations at the lower extremes allowed by EPA standards. Nitrite sensor based on controlled crosslinking-induced aggregation of gold nanoparticles has been demonstrated to provide ppm level sensitivity for nitrite sensing (Daniel, Han et al. 2009). However, the formation of molecular crosslinks (i.e., chemical bonds) between nanoparticles is the rate determining step of the aggregation procedure, which limits how rapid the colorimetric response can be observed. Our working hypothesis is that a noncross-linking aggregation scheme based on disruption of the surface charge balance of gold nanoparticles can be designed to further decrease the detection time and yield a more rapid-response sensor for nitrite.

Our second working hypothesis is that by utilizing anisotropic gold nanorods to replace spherical nanoparticles, we can further improve the sensitivity of the nitrite sensor. Compared with spherical gold nanoparticles, which display only one single plasmonic vibrational mode, gold nanorods are more sensitive to changing of their dielectric environment to the existence of two plasmonic vibrational modes (i.e., transverse and longitudinal), which in theory will lead to higher sensitivity towards trace amount of nitrite. These two hypotheses were thoroughly tested in this study and a new

noncrosslinking nitrite sensor was developed.

The third hypothesis being tested in this study is the dual recognition mechanism for bacterial target identification that combines molecular and spectroscopic fingerprinting of bacterial targets. More details of this hypothesis are discussed in chapter 4. Briefly, Raman-labeled nano probes are functionalized with antibodies which could recognize and bind specifically to target bacterial cells. Upon binding to the targets, the nanoprobes serve as SERS enhancers. Raman signals of both the nano probes and the bacterial cells can hence be observed simultaneously and generate a superimposed spectroscopic signature indicative of the specific molecular binding events. Identifying these events will directly I.D. the bacteria without any further processing steps, and a single-step target detection will be realized.

1.3 Summary

In this thesis, filed-deployable nano sensors for onsite detection of nitrite and microorganisms were developed. The colorimetric nitrite ion sensor was designed utilizing 4-aminothiophenol (4-ATP) modified gold nanorods (GNR). In the presence of nitrite ions, the deamination reaction was induced by heating the 4-ATP modified GNR in ethanol solution, resulting in the reduction of the GNR surface charges, which led to aggregation of GNRs and a colorimetric response that was quantitatively correlated to the concentration of nitrite ions. This simple assay was rapid (≤ 10 minutes) and highly sensitive (< 1 ppm of nitrite), it can be used for rapid monitoring of drinking water

quality. Anisotropic nanoparticles (i.e., silver nanocubes, gold nanorods) based SERS molecular probe were also designed and fabricated for the rapid and specific detection of bacterial targets in a test-in-a-tube platform utilizing a novel dual-recognition mechanism. The probes were synthesized by covalently attaching Raman tags and bacteria-specific antibodies to the surface of silver nanocubes. Specific binding between the probes and bacterial targets ensured surface enhanced Raman spectroscopic (SERS) signatures of the targets to be observed alongside with the SERS signals of the Raman tags. The assessment through the dual signals (superimposed target and tag Raman signatures) established a specific recognition of the targets in a single step, no washing/separation steps were needed to separate target-bound probes from unbound ones, because unbound probes only yield tag signatures, and could easily be distinguished from the target-bound ones. The dual-recognition protocol implemented with a portable Raman spectrometer would become an easy-to-use, field-deployable spectroscopic sensor for onsite detection of pathogenic microorganisms.

Two papers have been published in peer-reviewed journals partially based on the work reported in this thesis:

Xiao, N., and Yu, C. (2010). “Rapid-Response and Highly Sensitive Noncross-Linking Colorimetric Nitrite Sensor Using 4-Aminothiophenol Modified Gold Nanorods.” Analytical Chemistry **82** (9): 3659-3663

Wang, Q., Xiao, N., and Yu, C. (2010). “Detection and Identification of Microorganisms

in Mixed Cultures by Nanoparticle-Induced NanoSPR Enhanced FTIR Spectroscopy and Chemometrics.” Transactions of the ASABE **53**(3): 999-1006

One more manuscript is under preparation.

Xiao, N. and Yu, C. “A Single-Step Dual Recognition Detection of Microorganisms Using Surface Enhanced Raman Scattering Nanoprobes in a Test-in-a-tube Platform”.

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CHAPTER 2

Background and Literature Review

2.1 Detection of trace amount of nitrite in drinking water

Various methods have been developed for the detection of trace amount of nitrite in water over the last two decades. Some of the typical methods are summarized in Table 1. (Nollet 2000) Nitrate and nitrite ions could be directly analyzed by UV spectroscopy and electrochemical methods (Stanley, Maxwell et al. 1994; Thomas, Theraulaz et al. 1997), but these methods are susceptible to interference from other compounds and particles in water. Ion chromatography (Pobozy, Swerydakrawiec et al. 1993; Rokushika, Yamamoto et al. 1993) or other pretreatment methods (i.e. a sulphonated DuPont Nafion fiber, precolumns and an additional valve) (Jackson and Jones 1991; Dahllof, Svensson et al. 1997) were used to separate nitrite and nitrate from other inorganic anions, and led to highly sensitive detection of low levels of nitrate and nitrite ions, but the high instrumentation cost excluded them from being viable in-field detection choices. Capillary electrophoresis is another direct detection method for nitrite and/or nitrate(Guan, Wu et al. 1996) that is highly sensitive; recently a portable capillary electrophoresis system was developed by Hauser and coworkers(Kuban, Nguyen et al. 2007) that could potentially support in-field monitoring of nitrite/nitrate, but it was still quite sophisticated and costly.

Nitrite can be indirectly detected in colorimetric assays by utilizing the highly

selective diazotation reaction between nitrite and sulphanilamide (Ahmed, Stalikas et al. 1996), and the subsequent coupling of the product with N-(1-naphthyl)-ethylenediammonium dichloride to form azo dye that can be detected with high sensitivity using spectroscopic methods, such as photometry and fluorescence spectroscopy. Since nitrate can be easily reduced to nitrite using enzyme or metallic catalysts, colorimetric assays for nitrite eventually can be used for nitrate detection as well (Ahmed, Stalikas et al. 1996; Daniel, Han et al. 2009).

Table 1 Typical Standard Methods for Detection of Nitrite

Technique	Water Type	Detection Range	Ref.
Online Direct UV Measurement	Natural Water, Wastewater	10.9 – 543.5 μM/L NO_2^-	(Thomas, Theraulaz et al. 1997)
Direct Amperometric Detection Reduce Nitrate to Nitrite	River and Well Water	0.007 – 13.6 mM/L NO_3^- - N	(Stanley, Maxwell et al. 1994)
Photometry Reduce Nitrate to Nitrite	Environmental Waters	0.22 – 47.8 μM/L NO_2^-	(Ahmed, Stalikas et al. 1996)
Fluorescence Detection	Natural Water	19.6 – 300 μM/L NO_2^-	(Perezruiz, Martinezlozano et al. 1992)
Chromatographic Method Conductivity / UV as Detector	Seawater	4.3 μM/L NO_2^-	(Rokushika, Yamamoto et al. 1993)
Ion-interaction Chromatography	Natural water	0.043 μM/L NO_2^-	(Pobozy, Swerydakrawiec et al. 1993)
Capillary Electrophoresis	River water	0.017 – 1.70 mM/L NO_2^-	(Guan, Wu et al. 1996)

Colorimetric assay is highly desirable when in-field monitoring of nitrite and/or nitrate is needed, as in many environmental, agricultural and food control applications,

due to its simplicity and portability. However, most colorimetric assays based on chemical dyes are not sensitive enough for detecting nitrite and/or nitrate at low levels. Recently, Mirkin and co-workers developed a crosslinking colorimetric method based on gold nanoparticles to detect low level of nitrite ions. (Zhao, Chiuman et al. 2007; Daniel, Han et al. 2009) However, in their approach two types of gold nanoparticles functionalized differently were needed to react with nitrite ions to trigger the colorimetric response, and each type of nanoparticles needed specific chemical functionalization. The complex chemical procedures involved limit the applicability of the approach. Also it took 25 minutes for the assay to register a colorimetric response for nitrite levels at the upper limit of the EPA standard (1 ppm). A more rapid assay will certainly be welcome for onsite water quality applications.

Crosslinking colorimetric nano sensors that utilize the distance-dependent optical properties of gold nano particle clusters require the formation of molecular crosslinks that connect individual nanoparticles. The formation of the molecular crosslinks can be the rate-determining step that limits how rapidly a colorimetric response may develop. Also the need to chemically form the molecular crosslinks adds to the complexity of the chemical functionalization of the nanoparticles. Cross-linking is not always required for a colorimetric assay. Aggregation of nanoparticles due to electrostatic manipulation of the surface charges has also been utilized to detect variety of molecular targets such as, ATP (Zhao, Chiuman et al. 2007), peptides (Oishi, Asami et al. 2008) and DNA (Li and

Rothberg 2004). Electrostatic aggregation can occur more rapidly than cross-linking, (Li and Rothberg 2004) leading to faster assays. Utilizing electrostatic aggregation of gold nanorods (GNR), a more rapid colorimetric nitrite sensing system was developed in this thesis. Taking advantage of the higher plasmonic sensitivity of large aspect ratio GNR than spherical gold nanoparticles, a truly colorimetric detection of nitrite at as low as 0.07 ppm (significantly below the EPA recommended MCL) was achieved with simple visual inspection in less than 10 minutes. Since the plasmonic properties of GNRs can be tuned by controlling their aspect ratios, the sensitivity of the GNR-nitrite sensor can be further improved.

2.2 Detection of pathogenic microorganisms in water/food systems

2.2.1 Biosensors being the fastest growing technology for pathogen detection

Foodborne/waterborne pathogens comprise bacteria, viruses, protozoa, molds, nematodes and infective proteins. For a detection platform to be universally applicable to these various culprits, it has to be built on a simple mechanism that can be adapted to fit individual needs. Although biosensor technology currently only ranks fourth in the area of pathogen detection (Fig. 1A) (Lazcka, Del Campo et al. 2007), it is the fastest growing due to its promises of rapid and accurate detection, portability and automation (Fig. 1B). Biosensors have recently been defined as analytical devices incorporating a biological material (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products, etc.), a biologically derived material (e.g., recombinant

antibodies, engineered proteins, aptamers, etc.) or a biomimic (e.g., synthetic catalysts, combinatorial ligands and imprinted polymers) intimately associated with or integrated within a physicochemical transducer or transducing microsystem. Grouped by the ways of signal transduction, they can be categorized into electrochemical, optical (including spectroscopic), thermometric, and mass-based (piezoelectric and surface acoustic).

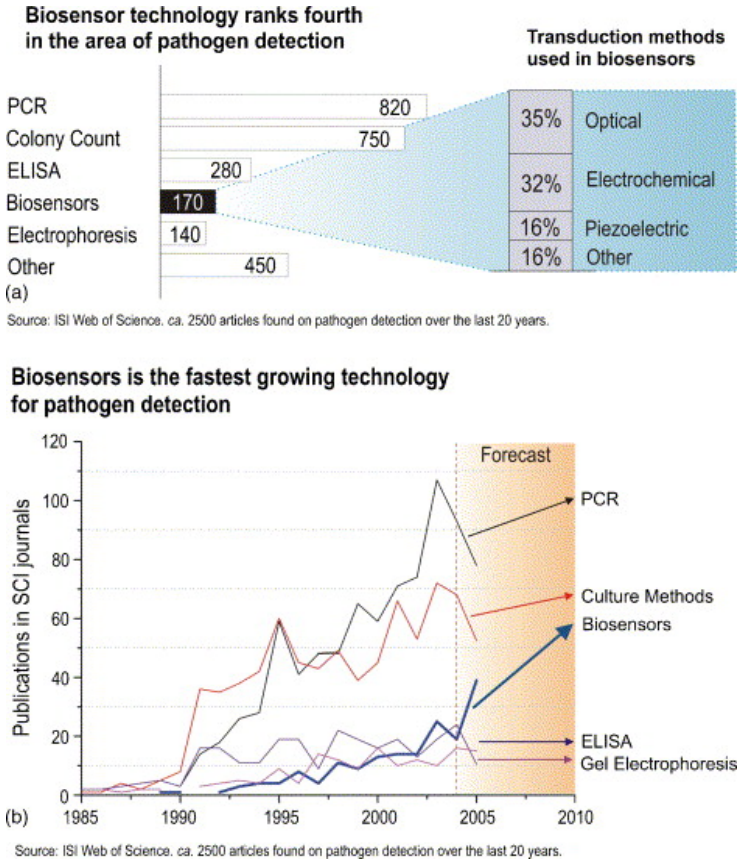

Fig. 1 (A) Number of articles using different detection techniques for pathogens over the last 20 years; (B) Trends showing biosensors being the fastest growing technology (Lazcka, Del Campo et al. 2007)

Among these options, optical sensors appear to be most appealing because of their sensitivity, available instrumentation, and relative ease of data interpretation (Geng and Bhunia 2007). However, much research and development work is still needed before

biosensors become a real and trustworthy alternative.

2.2.2 Antibody-based biosensing for microorganism detection

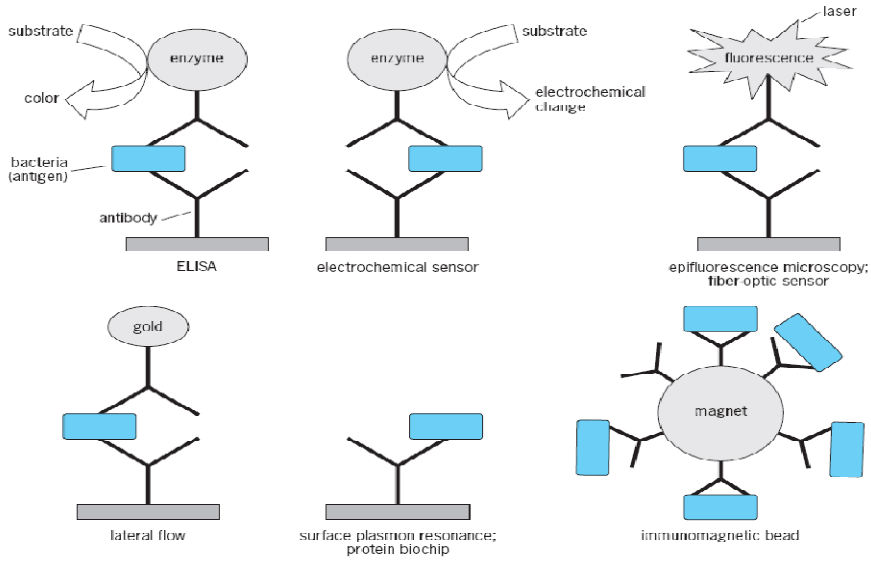

Fig. 2 Various antibody-based detection schemes for pathogens (Bhunia and Lathrop 2003)

The most widely used molecular recognition method is the antibody method. The basic principle of the antibody-based detection (immunoassay) is the highly specific binding of antibodies to a target antigen, followed by the detection of the antibody-antigen complex (Fig. 2). Highly specific antibodies are produced in laboratory animals against a specific invading antigenic component of the pathogen or toxin, or via genetically-engineered microorganisms. Nowadays monoclonal antibodies can be produced in large quantities that only recognize the target antigen even in the presence of other organisms and interfering food components (Bhunia and Lathrop 2003).

Antibody-based target recognition has been widely used in biosensor development and applied to foodborne pathogen detection, with variety ways of signal transduction. In

Surface plasmon resonance (SPR) biosensor, antigen binding to the SPR chip leads to refractive index change occurring at the surface of the SPR chip, which is directly measured either through change of angle of incidence or change in the intensity of light at fixed incident angle (Cooper 2003). SPR biosensors have been successfully applied to pathogen detection (Oh, Lee et al. 2003; Taylor, Yu et al. 2005). Their main strength lay in their label-free nature; however, their operation is complex and their equipment cost is high.

Another development in optical biosensor technologies is the evanescent field-based platform, including resonant mirror (RM) sensing scheme and fiber-optic sensors, in which light propagates inside a dielectric waveguide and the evanescent field excites fluorescence-labeled antibody-antigen complexes that are situated on top of the waveguide (Rasooly and Rasooly 1999; Hayman 2008). The evanescent field platform supports single-step sensing in principle, because the evanescent field only excites surface-bound targets that are within 100 nm to the surface, hence unbound targets will not lead to any detectable signal. However, these assays cannot work for whole cells and/or viral particles, precisely because their sizes are out of the range for evanescent field. For all the other schemes shown in Fig. 2, multiple washing steps are still necessary to eliminate unbound detecting antibodies, and in general further improvement over the sensitivity is needed for the sensing scheme to be practically applicable, where 100-1000 cells/ml level sensitivity is needed.

For multiplex detection, array biosensors are often used. Taitt et al used a patterned array of antibodies against multiple antigens immobilized on the surface of a planar waveguide (microscope slides) to capture antigen. Fluorescent-labeled tracer antibodies were then applied to bind to the antigen and subsequent excitation with diode laser launched at the edge of the glass slide emitted a fluorescent signal, which was captured by a CCD camera. Multiple pathogens, including *B. anthracis, S. aureus* toxin B, cholera toxin, ricin, *Franciscella tublarensis and Brucella abortus* were detected using this scheme (Taitt, Anderson et al. 2005), bacterial cells were detected at the range of 10^3 to 10^6 cfu/ml, and toxins were in the ng/ml range. It was also used to detect *S. Typhimurium* with a detection limit of 8×10^4 cfu/ml from different food matrices such as cantaloupe, chicken washings, sprouts and liquid eggs (Taitt, Shubin et al. 2004). A multiple analyte array biosensor (MAAB) was also developed by Shriver-Lake and coworkers that could detect multiple bacterial cells at 10^3-10^6 cfu/ml (Sapsford, Shubin et al. 2004).

All fluorescent-based optical detection schemes suffer from photo bleaching and limited availability of high-performance fluorescent dyes. A detection scheme that utilizes non-fluorescent labeling techniques would be of great interests for development of new biosensor technology.

2.2.3 Spectroscopic biosensing for pathogen detection

Pioneered by Naumann and coworkers in 1990s, FT-IR and later Raman spectroscopy has been successfully applied to identify differentiate and classify

pathogenic microorganisms based on their unique spectroscopic signatures (Naumann, Helm et al. 1991; Timmins, Howell et al. 1998; Naumann 2000). More recently, Grow et al. described a μSERS system in which individual bacteria cells were captured selectively on a biochip by antibodies (Grow, Wood et al. 2003). Antibodies specifically targeting a bacterium were immobilized onto a SERS active biochip, and antigens were captured. Surface-enhanced Raman spectral fingerprints were then collected and compared with a database for identification. The system was able to differentiate viable from nonviable cells and could detect *Listeria* species, *Legionella* and *Cryptosporidium* oocysts at subspecies and strain levels. In this approach, high-quality Raman spectra acquired through a Raman microscope are essential for the identification.

The ability to distinguish viable from non viable cells is of great importance in evaluating water quality, especially when the water is contaminated with the viable but non culturable (VBNC) microorganisms where the conventional microbiological analysis will yield faulty results. Quick spectroscopic screening of drinking water to identify presence of pathogenic microorganisms has the potential to become a powerful tool for water quality and safety control applications.

2.2.4 SERS molecular probing

Raman scattering is a rare event compared to elastic Rayleigh scattering, only 10^{-6} to 10^{-8} of scattered photons are Raman photons, and this limits the sensitivity of Raman detection. Surface enhanced Raman scattering overcomes this problem. When the

incident light is applied to a roughened metal surface or metallic nanoparticles, localized surface plasmon are excited, especially when the plasmon frequency is in resonance with the radiation. This results in an exceptionally large electromagnetic field being created at the close proximity of the surface or nanoparticles. If a molecule is absorbed onto the surface, or stay within a close distance to the nanoparticles, its Raman cross section will be greatly increased. A chemical resonance enhancement can also be at play in which a charge transfer occurs between the metallic nanostructure and the molecule. The two enhancement mechanisms combined can yield a 10^{13}-10^{14} increase in Raman intensity (Doering and Nie 2002).

Another way to utilize SERS is to create molecular probes by attaching capturing antibodies and SERS-active labels to metallic nanoparticles. These nanoparticles will then bind specifically to the antigens, whereas the antigen-bound complexes can be visualized by their SERS signatures. The concept has been demonstrated by our previous work with SERS-labeled DNA probes for detection of alternative splicing isoforms of BRCA1 gene (Sun, Yu et al. 2007; Sun, Yu et al. 2008). Since all molecules have their unique Raman spectroscopic signatures, the reservoir for SERS labels is much bigger than that of fluorescent labels, and SERS labels do not photo bleach.

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Chapter 3

Rapid-response and Highly Sensitive Non-crosslinking Colorimetric Nitrite Sensor Using 4-aminothiophenol Modified Gold Nanorods

Abstract

A novel colorimetric nitrite ion sensor was developed utilizing 4-aminothiophenol (4-ATP) modified Gold Nanorods (GNR). In the presence of nitrite ions, deamination reaction was induced by heating the 4-ATP modified GNR in ethanol solution, resulted in the reduction of the GNR surface charges, which led to aggregation of GNRs and a colorimetric response that was quantitatively correlated to the concentration of nitrite ions. This simple assay was rapid (≤ 10 minutes) and highly sensitive (< 1 ppm of nitrite), it can be used for rapid monitoring of drinking water quality.

3.1 Introduction

Nitrate and nitrite, as part of nitrogen cycle, widely exist in ground and surface water systems, due to contaminations of water sources by fertilizer, waste of livestock and organic wastes (Agency 1995; Fanning 2000; Maria Teresa Oms 2000; Moorcroft, Davis et al. 2001). Even trace amount of nitrate and nitrite ions is unsafe to human health, and both are type A inorganic chemicals in water quality parameters which require close monitoring by Health Authorities due to their potential toxicity (Zuane 1996). Infants drinking nitrate or nitrite contaminated water may suffer shortness of breath and blue baby syndrome and, if untreated, may die (Bruningfann and Kaneene 1993; Agency 1995;

Zuane 1996; Fanning 2000; Brender, Olive et al. 2004). The maximum containment levels (MCLs) of nitrate and nitrite, as recommended by the U.S. Environmental Protection Agency (EPA), are 10 ppm and 1 ppm (measured as Nitrogen), respectively (Agency 1995).

In the past two decades various methods have been developed to monitor the nitrate and nitrite levels in fresh water. Some of the typical methods are summarized in Table 1.(Maria Teresa Oms 2000) Nitrate and nitrite ions could be directly analyzed by UV spectroscopy and electrochemical methods, (Stanley, Maxwell et al. 1994; Thomas, Theraulaz et al. 1997) but these methods are susceptible to interference from other compounds and particles in water. Ion chromatography (Pobozy, Swerydakrawiec et al. 1993; Rokushika, Yamamoto et al. 1993) or other pretreatment methods (i.e. using a sulphonated Dupont Nafion fiber, precolumns and an additional valve)(Jackson and Jones 1991; Dahllof, Svensson et al. 1997) were used to separate nitrite and nitrate from other inorganic anions, and led to highly sensitive detection of low levels of nitrate and nitrite ions, but the high instrumentation cost excluded them from being viable in-field detection choices. Capillary electrophoresis is another direct detection method for nitrite and/or nitrate(Guan, Wu et al. 1996) that is highly sensitive; recently a portable capillary electrophoresis system was developed by Hauser and coworkers(Kuban, Nguyen et al. 2007) that could potentially support in-field monitoring of nitrite/nitrate, but it was still quite sophisticated and costly.

Nitrite can be indirectly detected in colorimetric assays by utilizing the highly selective diazotation reaction between nitrite and sulphanilamide,(Perezruiz, Martinezlozano et al. 1992; Ahmed, Stalikas et al. 1996) and the subsequent coupling of the product with N-(1-naphthyl)-ethylenediammonium dichloride to form azo dye that can be detected with high sensitivity using spectroscopic methods, such as photometry and fluorescence spectroscopy. In most colorimetric assays, nitrate is reduced to nitrite using enzyme or metallic catalysts and detected indirectly (Ahmed, Stalikas et al. 1996; Daniel, Han et al. 2009).

Colorimetric assay is highly desirable when in-field monitoring of nitrite and/or nitrate is needed, as in many environmental, agricultural and food control applications, due to its simplicity and portability. However, most colorimetric assays based on chemical dyes are not sensitive enough for detecting nitrite and/or nitrate at low levels. Recently, Mirkin and co-workers developed a crosslinking colorimetric method based on gold nanoparticles to detect low level of nitrite ions.(Daniel, Han et al. 2009) However, in their approach two types of gold nanoparticles functionalized differently were needed to react with nitrite ions to trigger the colorimetric response, and each type of nanoparticles needed specific chemical functionalization. The complex chemical procedures involved limit the applicability of the approach. Also it still took 25 minutes for the assay to register a colorimetric response for nitrite levels at the upper limit of the EPA standard (1 ppm).

Crosslinking colorimetric nano sensors, based on distance-dependent optical properties of gold nano particle clusters, require the formation of molecular crosslinks that connect individual nanoparticles. The formation of the molecular crosslinks can be the rate-determining step that limits how rapidly a colorimetric response may develop. Also the need to chemically form the molecular crosslinks adds to the complexity of the chemical functionalization of the nanoparticles. Cross-linking is not always required for a colorimetric assay. Aggregation of nanoparticles due to electrostatic manipulation of the surface charges has also been utilized to detect variety of molecular targets such as, ATP(Zhao, Chiuman et al. 2007), peptides(Oishi, Asami et al. 2008) and DNA(Li and Rothberg 2004). Electrostatic aggregation can occur more rapidly than cross-linking (Oishi, Asami et al. 2008) leading to faster assays. Utilizing electrostatic aggregation of gold nanorods (GNR), a more rapid colorimetric nitrite sensing system was developed in this report. Taking advantage of the higher plasmonic sensitivity of large aspect ratio GNR than spherical gold nanoparticles, a truly colorimetric detection of nitrite at as low as 0.07 ppm (significantly below the EPA recommended MCL) was achieved with simple visual inspection in less than 10 minutes. Since the plasmonic properties of GNRs can be tuned by controlling their aspect ratios, the sensitivity of the GNR-nitrite sensor can be further improved.

3.2 Experimental section

3.2.1 Fabrication and functionalization of Gold Nanorods

Gold nanorods with different aspect ratios, as indicated by different longitudinal surface plasmon resonance peaks (SPR_{long}), were synthesized via seed-mediated growth method.(Nikoobakht and El-Sayed 2003; Desai, Villalba et al. 2009) Details of the procedure were also reported elsewhere (Yu and Irudayaraj 2007). Hexadecyltrimethylammoniumbromide ($C_{16}TAB$, 99%) and benzyldimethylammoniumchloride hydrate (BDAC, 99%), Sodium borohydride (99%), L-ascorbic acid, Gold (III) chloride hydrate (>99%) and Silver nitrate (>99%) were all purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Nanopure deionized and distilled water (18.2 MΩ) was used for all experiments. Gold nanorods were made through the seed-mediated growth. Gold nanoparticles with diameter around 4 nm were made as follows as seeds: 0.6mL, 0.01M freshly prepared, ice-cold NaBH4 solution was added to a mixture solution composed of 5 mL, 0.2 M CTAB, 0.25 mL, 0.01M HAuCl4 and 4.75 mL water. The solution was vigorously mixed for 2 min. The seeds were then kept at 27 °C for 3 hours to allow complete degradation of the remaining NaBH4, and were then used in subsequent nanorods growth: 5mL of 0.2 M CTAB solution and 5mL of 0.1 M BDAC solution were mixed to get bisurfactant solution. 80 μL of 0.01 M $AgNO_3$ and 0.5 mL of 0.01 M $HAuCl_4$ were added to the bisurfactant solution. After gentle mixing, 65 μL of 0.1 M ascorbic acid was added. The mixture solution was mixed until color of the solution changed from yellow to colorless. After addition of 12 μL of seed solution, the growth solution was incubated at 27 °C overnight.

The GNRs were further functionalized by 4-aminothiophenol (4-ATP). Briefly, 4 mL of 3 nM gold nanorods were reacted with 0.5 mL of 10 mM 4-ATP dissolved in acidic water (pH = 2) under vigorously stirring at 60 □ for 3 h. The solution was then centrifuged and washed twice with 3 mM CTAB acidic aqueous solution (pH = 4). Finally, 4-ATP modified gold nanorods were resuspended in 2.5 mL of acidic water (pH = 4).

3.2.2 Nitrite sensing using 4-ATP functionalized GNR

In a typical experiment for detecting nitrite, 0.8 mL of sample was placed in 1.5 mL eppendorf tube. 8 µL of 1 M phosphoric acid was added to keep the sample under acidic condition. Then 4-ATP modified GNR was added to make the final extinction of SPR_{long} equal to 0.9. After a quick vortex mixing, 0.2 mL of ethanol was added to the mixture. The solution was incubated at 95 □ in water bath incubator for certain amount of time (the heating time is quantitatively recorded and correlated to the nitrite concentration in the sample) and then put on ice briefly to cool down the solution. All environmental water samples were filtered with 50 µm paper filter.

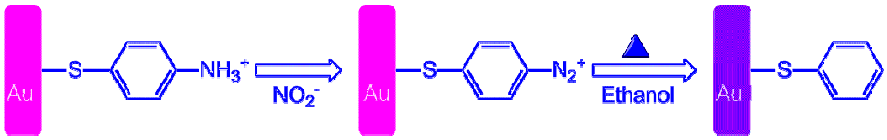

Fig. 3 Non-crosslinking colorimetric detection of nitrite with 4ATP modified GNR

The whole detection strategy is illustrated in Fig. 3. Under acidic condition, amines attached to the benzene ring are ionized yielding ammonium cations. GNR colloids are

stable under such condition due to the electrostatic repulsion among positively charged ammonium cations. In the presence of nitrite ions, the primary aromatic amines react with nitrite ions to form diazonium salt in presence of mineral acid. In aqueous solution, diazonium salts are unstable and tend to lose the diazonium groups. In the presence of ethanol, amine groups in aromatic amines are subsequently dissociated from benzene rings under heating in the deamination reaction, replaced by hydrogen in the primary products, leading to the reduction of surface charges of the GNRs. The disruption of the surface charge balance leads to aggregation of gold nanorods, yielding a dramatic color change, which can be observed via naked eye.

3.3 Results and discussion

3.3.1 SERS Characterization of surface chemistry of the gold nanorods

Surface enhanced Raman spectroscopy (SERS) was used to confirm diazo bond formation and deamination reaction. As shown in Fig. 4, SERS spectrums of GNR in 20% ethanol were recorded after modified with 4-ATP, reacted with nitrite ions and heated to 95 °C. The strongest band at 1074 cm^{-1} is identified as the stretching vibration of C-S, And the C-C stretching vibration of benzene rings is observed at 1577 cm^{-1}(Zheng, Zhou et al. 2003). Importantly, N=N stretching vibration of diazonium salt is shown in 1394 cm^{-1},(Jiao, Niu et al. 2005) which only appears in nitrite treated GNR_4-ATP. The 1142 and 1439 cm^{-1} bands are assigned as b2 modes of benzene ring vibrations (Osawa, Matsuda et al. 1994). The intensities of these two bands are directly correlated to the

charge transfer from the gold nanorods to the diazonium ions attached to their surfaces, which is influenced by the conjugation between benzene ring and diazo bond (Jiao, Niu et al. 2005). The absence of these two bands in 4-ATP modified GNR and GNR_4-ATP after deamination reaction confirmed the formation of diazo bonds between 4-ATPs and nitrite ions, as illustrated in step 2 of the Fig. 3.

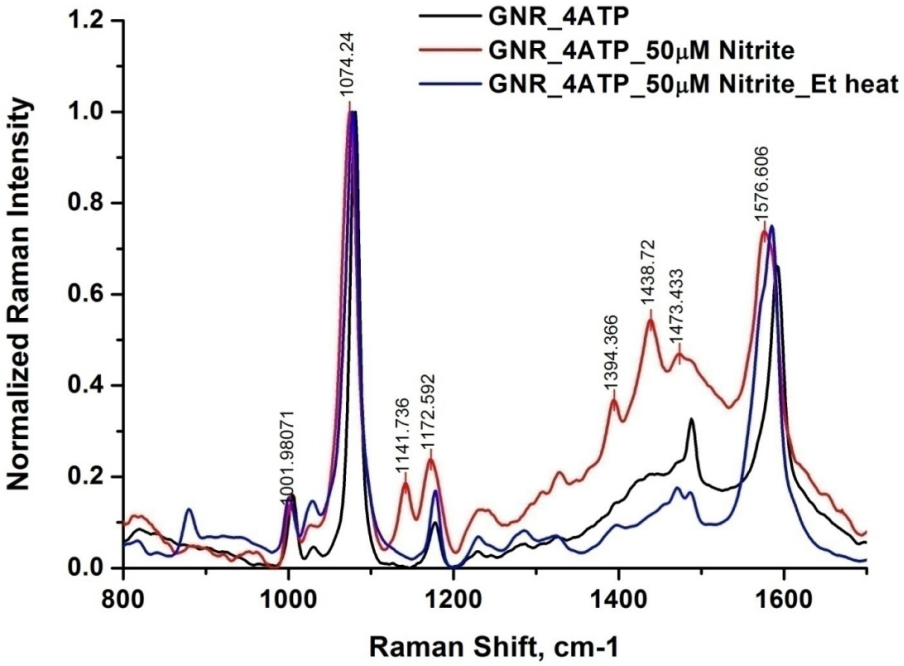

Fig. 4 Raman spectra of 4ATP modified GNR (black), GNR-4ATP reacted with nitrite (red) and heated at 95□(blue) in 20% ethanol.

3.3.2 Aggregation induced colorimetric nitrite sensing using the 4-ATP GNR sensors

In Fig. 5A, differences between the UV-Vis spectra of GNR sensors treated with different samples clearly showed the aggregation of the GNR sensors when nitrite ions were present in the samples. Surface functionalization of the GNRs with 4-ATP under acidic condition did not introduce significant change to the surface charges of the GNRs,

the slightly-blue shifted longitudinal plasmonic band indicated stable GNR colloids in aqueous solution. Without the presence of nitrite ions, the GNR_4-ATP sensors remained stable for weeks. However, after reacting with nitrite ions and the subsequent deamination reaction, neutralized surface molecules (benzene) significantly reduced the overall surface charges on the GNR surfaces, and led to aggregation of the GNRs, as evidenced by the significant red shift and broadening of the longitudinal band (SPR_{long}). The peak at 1150 nm is identified as ethanol peak. A colorimetric response was recorded (red to purple), as shown in Fig. 5B.

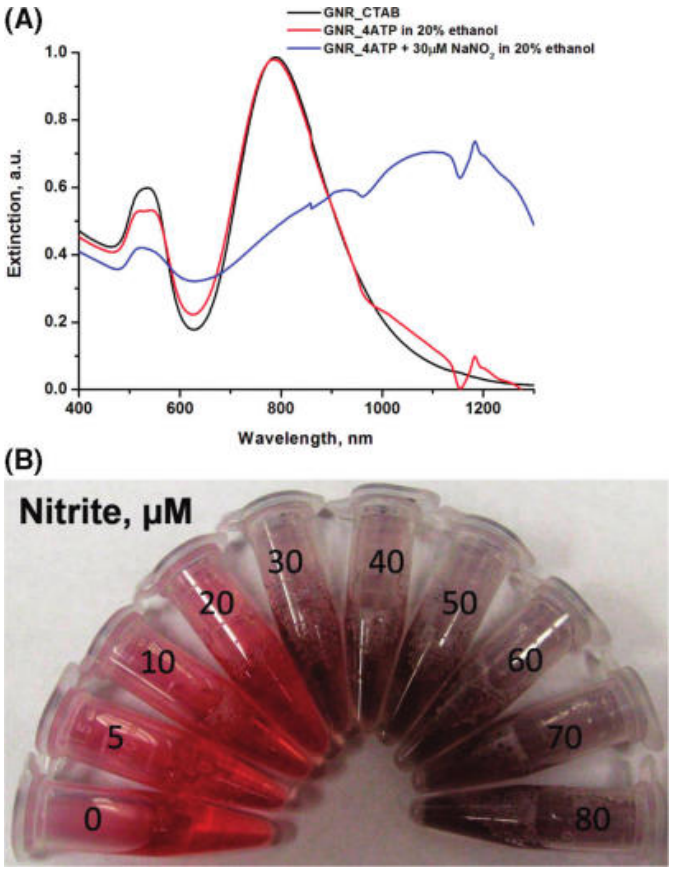

Fig. 5 (A) Absorption spectra of CTAB covered GNR (black), GNR-4ATP in 20% ethanol solution (red), and aggregated GNR after heating in 20% ethanol (blue). (B) Photograph of GNR-4ATP reacted with various concentrations of nitrite after incubation in 20% ethanol at 95 ℃

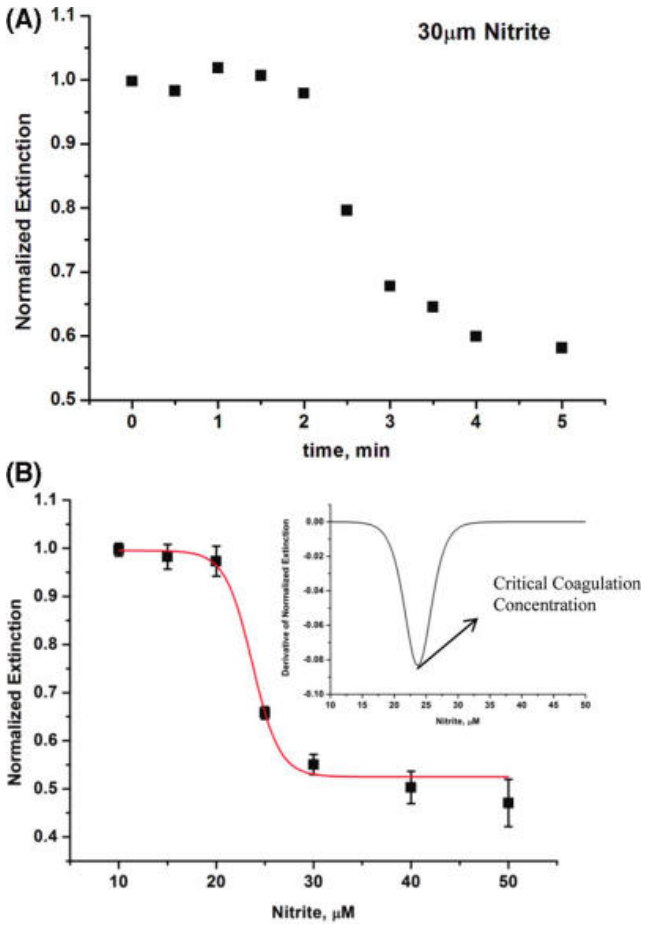

Fig. 6 (A) Extinction of GNR-4ATP at wavelength of its SPR_{long} band, 785 nm, reacted with 30 μM nitrite, as a function of incubation time at 95 °C. (B) Extinction of GNR-4ATP at wavelength of its SPR_{long} band, 680 nm, after 5 min incubation at 95 □ as a function of nitrite concentration.

The amount of diazonium ions undergoing deamination reaction was dependent on the heating time of the mixture. As the heating time increased, more diazonium ions were decomposed with the elimination of the -N=N groups, which led to the reduction of the positive surface charges of the GNRs. When the surface charges were lowered down to a critical level, aggregation of the GNRs was induced. The extinction intensity of the SPR_{long} band of the GNR colloids was continuously monitored throughout the heating process. As shown in Fig. 6A, extinction of SPR_{long} band started to decrease significantly

after 2 minutes of heating, indicating the onset of the aggregation of GNRs. The onset and progression of the aggregation was also dependent on the nitrite concentration in the sample. More nitrite led to more diazonium ions being formed, which subsequently resulted in a higher rate of deamination reaction and the earlier onset of the aggregation. As shown in Fig. 6B, at a fixed heating time, a critical coagulation concentration (CCC) of nitrite could be identified that represents the minimum concentration of nitrite that induces the aggregation of the GNRs.

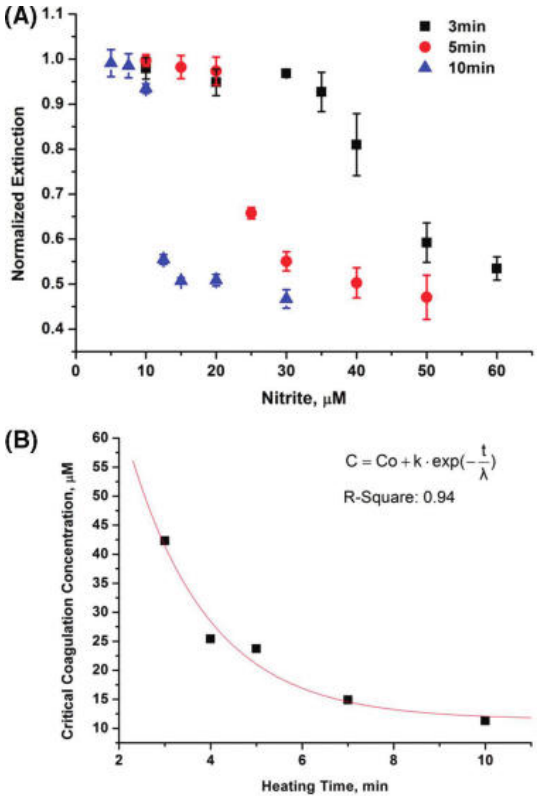

Fig. 7 (A) GNR, with SPR_{long} at 680nm, particle solution extinction at 680nm after heating at different nitrite concentration and different heating time (B) CCC of GNR-4ATP with SPR_{long} at 680 nm as a function of heating time

The CCC represents the concentration of nitrite that would trigger the colorimetric response from the GNR sensors. As shown in Fig. 7A, the CCC is dependent on the

heating time. By adjusting the heating time, the GNR sensors can be used to detect nitrite at different concentrations, with a sensitivity of ~ 10 μM reached at 10 minutes of heating. A response curve was created for GNR sensors made from GNRs with aspect ratio of 1.5 (λ_{log} = 680 nm), as shown in Fig. 7B. The CCC was correlated with the heating time of the sample-sensor mixture. By recording the time when the aggregation occurs, which can be easily determined through simple visual inspection, the concentration of the nitrite in the sample can be quickly determined. It should be noticed that the CCC appeared to be exponentially correlated to the heating time, hence to increase the sensitivity of the detection beyond 10 μM, the heating time will need to be significantly increased.

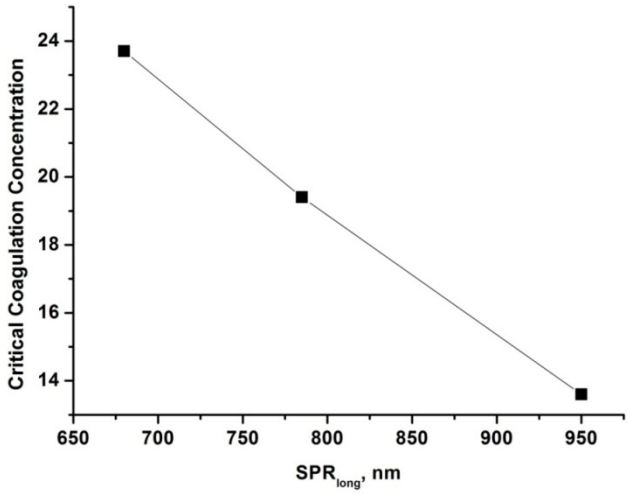

Fig. 8 CCC of GNR-4ATP with different aspect ratios as a function of SPR_{long} band

To further improve the sensitivity of the GNR sensors, we took advantage of the tunable plasmonic properties of the GNRs by controlling their aspect ratios. It is well known that larger aspect ratio GNRs is more sensitive towards changes in their dielectric environment (Yu and Irudayaraj 2007). As the aspect ratio increases, lower concentration

of nitrite is needed to trigger the aggregation of the GNRs. At fixed heating time (5 minutes), the CCC (representing the sensitivity of the assay) decreases as the aspect ratios of the GNRs increase (as demonstrated by the red-shift of the longitudinal plasmonic band), as shown in Fig. 8. The lowest CCC observed in this study was 5.2 μM (~0.07 ppm) using GNR sensors with λ_{log} = 806 nm (data not shown), which is significantly lower than the recommended EPA standard (1 ppm or 71 μM). It is reasonable to believe the sensitivity can be further improved if GNRs with larger aspect ratios are used.

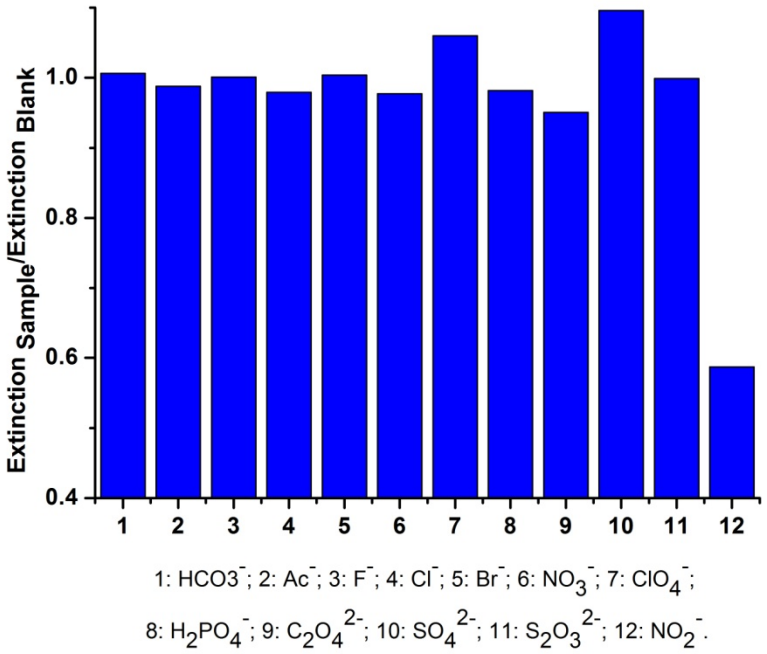

Fig. 9 Selectivity of GNR nitrite sensor towards nitrite and other anions (The concentration of SO_4^{2-} and $S_2O_3^{2-}$ were 0.1 mM, the concentration of NO_2^- was 30 μM and the other anions concentration were 1 mM.)

Any detection schemes based on surface charge balance would inevitably be interfered by other anions in the sample if they were present at high enough concentrations. To evaluate the selectivity of the GNR sensing scheme, its responses

towards other anions, including HCO_3^-, CH_3COO^-, F^-, Cl^-, Br^-, NO_3^-, ClO_4^-, $H_2PO_4^-$, $C_2O_4^{2-}$, SO_4^{2-} and $S_2O_3^{2-}$, were investigated (Fig. 9). No colorimetric responses were observed for anions other than SO_4^{2-} and $S_2O_3^{2-}$ up to the concentration of 1 mM. For SO_4^{2-} and $S_2O_3^{2-}$, 0.1 mM appeared to be the limit without triggering a colorimetric response. Therefore, for anionic concentration below 100 μM, the GNR sensors are specific towards nitrite. Combining it with an enzymatic or metallic-catalytic reduction of nitrate, the GNR sensing scheme can be applied to highly sensitive nitrate detection as well.

3.3.3 Analysis of environmental water samples

Environmental water samples collected from four locations were tested with this method (Fig. 8A). No aggregation is observed for all samples after 8 minutes heating, which means nitrite concentration in these water samples are below 15 μM. Then concentrated nitrite solutions were added to these samples to make the final nitrite concentrations 1 ppm (71 μM, the MCL of nitrite) and 0.9 ppm (64 μM). Since the accuracy of this method is at its best with nitrite concentration between 15 and 40 μM, all samples were tested after diluted four times. A heating time of 5.5 minutes was used to discriminate 1 ppm and 0.9 ppm nitrite concentration after four times dilution of water samples. With 0.9 ppm nitrite ions, no significant decrease of the extinction was observed. However, with 1 ppm nitrite in the water, normalized extinction of the solutions dropped from around 1 to 0.5, in conjunction with visible color changes, as shown in Fig.10 A.

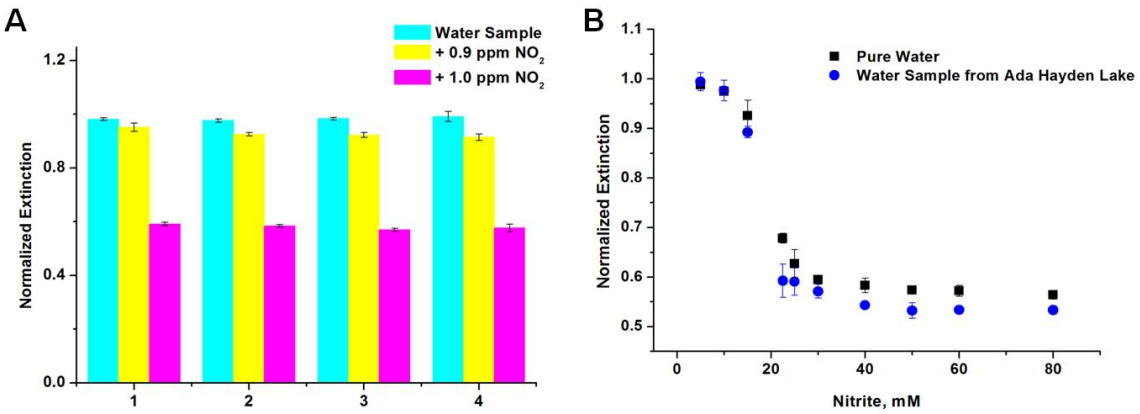

Fig. 10 (A) Analysis of filtered water samples, 1. tap water of Ames 2. tap water of Iowa City 3. Nashua water monitoring well 4. Ada Hayden Lake; (B) Compare the test results between pure water and filtered water from Ada Hayden Lake.

Also, we challenged this method with complex environmental samples. Previous result has shown that the concentration of nitrite of water sample from Ada Hayden Lake is below the detection limit of this test, so we considered it as 0 μM. To mimic nature water samples with nitrite, different amounts of concentrated nitrite solution were added to filtered lake water samples. As illustrated in Fig. 10B, there are no significant differences between distilled water and filtered environmental water samples. It is concluded that the nanosensors can be used to analyze water samples collected from natural sources with minimal pre-processing.

3.4 Conclusion

In conclusion, a novel GNR nitrite sensor, taking advantage of tunable optical property of GNR, was developed. This noncrosslinking aggregation assay was rapid and highly sensitive with a detection limit as low as 5.2 μM (0.07 ppm). With the colorimetric response, no sophisticated instrumentation is required. It can serve as an easily applicable,

user-friendly, portable assay to monitor low-level nitrite contaminations in drinking water or relatively high purity water.

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CHAPTER 4

Dual Recognition Mechanism for Bacteria Detection Using Surface Enhanced Raman Spectroscopic Nanoprobes

Abstract

Anisotropic nanoparticles (i.e., silver nanocubes, gold nanorods) based SERS molecular probe were designed and fabricated for the rapid and specific detection of bacterial targets in a test-in-a-tube platform utilizing a novel dual-recognition mechanism. The probes were synthesized by covalently attaching Raman tags and bacteria-specific antibodies to the surface of gold nanorods and silver nanocubes. Specific binding between the probes and bacterial targets ensured surface enhanced Raman spectroscopic (SERS) signatures of the targets to be observed alongside with the SERS signals of the Raman tags. The assessment through the dual signals (superimposed target and tag Raman signatures) established a specific recognition of the targets in a single step at a sensitivity of 10^2 cfu/mL with no washing/separation steps. The dual-recognition protocol implemented with a portable Raman spectrometer would become an easy-to-use, field-deployable spectroscopic sensor for onsite detection of pathogenic microorganisms.

4.1 Introduction

The presence of pathogenic bacteria in food and drinking water poses a threat to both public health and security. Approximately foodborne diseases cause 76 million illnesses and 5,000 deaths in the United States each year (Mead et al., 1999). To deal with

this threat, the first step is to detect them at the earliest possible moment, preferably in-field. However, the conventional methods require series of enrichment and sample preparing steps which limit their in-field deplorability. API, as one of the most popular biochemical tests for bacterial identifications, requires a series of biochemical tests for organism culture. It takes several days to culture the cell, run the test and analyze the results (Jarvis and Goodacre 2008) in this process. Sandwiched ELISA (Enzyme-linked immunosorbent assay), another widely used bacterial identification technique, also needs multiple washing steps to separate bound antibodies from unbound ones, which is also unfavorable for in field deployment. (Lam and Kostov 2009).

Raman spectroscopy has long been explored as a tool for biological targets detection and identification (Kneipp, Kneipp et al. 1999), especially with the development of surface enhanced Raman spectroscopy (SERS) that increases the sensitivity of Raman spectroscopy up to $10^{11\text{-}13}$ times (Doering and Nie 2002; Le Ru, Blackie et al. 2007). Noble metal nanoparticles (such as silver, gold etc.) were utilized as uniform, highly sensitive, and reproducible SERS substrates for pathogen biosensor applications (Tripp, Dluhy et al. 2008). Green *et al.* (Green, Chan et al. 2009) demonstrated that different species of *Listeria* could be differentiated based on their SERS spectral signatures analyzed with statistical multivariate discriminant method. With SERS and a novel barcode data processing procedure, Ziegler and coworkers (Patel et al., 2008) reported more than 50 bacteria could be differentiated. In general, direct

differentiation/identification of bacterial targets through their unique Raman spectroscopic signatures require high-quality spectral data in conjunction with statistical analysis built upon known spectral fingerprints of bacterial species, which limit its field-deployability: high quality spectral data are difficult to acquire by a portable instrument, even with SERS; and the statistical recognition procedure won't work if the target to be detected is not in the existing spectral database.

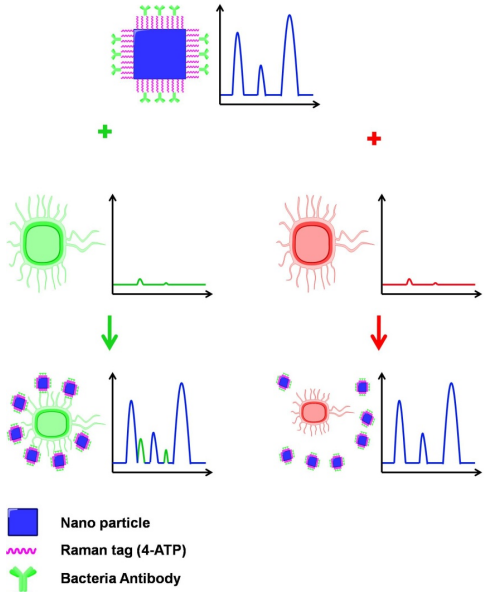

Fig. 11 Scheme of dual recognition bacterial detection SERS nano probe

An alternative strategy to utilize SERS for detection of biological targets was also explored by several groups-- the approach of SERS-based molecular nanoprobing. (Porter, Lipert et al. 2008; Huang, Tay et al. 2009) In this approach nanoparticles functionalized with specific antibodies and Raman reporter molecules are deployed as molecular probes. These probes could specifically recognize bio-targets by antibody-antigen binding, and

report these binding events through the Raman reporters. Their application bears great similarity to fluorescence-labeled antibodies, with the advantages of broader reporter library (>1000 Raman-active molecules) and no photobleaching. However, in this approach, separation of target bound probes from unbound ones through multiple washing steps is still needed, which reduces its field deployability.

In this study we developed a dual-recognition mechanism utilizing SERS molecular probes to achieve target bacteria detection in one single step, which is more suitable for in-field applications. As shown in Fig. 11, in the dual-recognition mechanism, Raman-labeled and functionalized anisotropic nanostructures (e.g., gold nanorods, silver nanocubes) are constructed as SERS nanoprobes that display specific label signatures (*probe signal*), and through covalently-bound antibodies they could bind to their target bacteria specifically. The antibody-antigen binding ensures that the target cells would attract enough nanoprobes to bind to them, and measureable SERS signals from the bacteria would be generated (non-target would NOT have enough nanoprobes bound to them, and their SERS signal would be non-measurable). Observation of superimposed SERS signals of the probe and the target indicates the binding events, and subsequently definitely identifies the target in one single step; no washing or separation is needed. Furthermore, since the specificity of the target detection is provided by the antibodies, it is no longer critical to have high-quality spectra: as long as a few key signatures from the bacterial cells are recognized, a positive identification of the target can be reached. A

portable Raman sensor thus becomes feasible under the dual-recognition scheme.

4.2 Experimental section

4.2.1 Anisotropic nanoparticle fabrication and functionalization

The detail experimental procedure of fabricating and modifying GNR was described in experimental section of Chapter 3.

Silver nanocubes were synthesized using the method suggested by Skrabalak and Xia (Skrabalak, Au et al. 2007). In four 20 mL glass reaction vials, 6 mL of ethylene glycol were added to each vial. Then these vials were incubated at 150 ^{o}C for 1 hour to evaporate the water in the system. After heating, 70, 80, 90 and 100 μL of 3 mM Na_2S in ethylene glycol were pipette to each vial. The mixture solution were heated for 8 to 10 minutes. Then 1.5 mL of 0.02 g/mL PVP (Polyvinylpyrrolidone) in ethylene glycol was added to each vial. Immediately, added 0.5 mL of 0.048 g/mL $AgNO_3$ in ethylene glycol. Finally, the whole mixture solutions were heating for up to 20 minutes until the solution becoming ochre-colored.

The reaction was quenched by putting the vials in water bath at room temperature. To rinse the nanocubes, twice of the volume of acetone were added to each vial spin down for 30 minutes at 2000*g*.Then particles were washed with deionized water for three times and finally resuspended in 4 mL of deionized water.

For 0.9 mL of as synthesized silver nanocubes, 0.1 mL of 10 mM 11-mercaptoundecaonic acid (MUDA) was added to the solution. Then the mixture

solution was stirred at room temperature for 12 hours. To wash out the extra PVP and unbounded MUDA, the solution was spin down and washed with ethanol once and 10 mM phosphate buffer (pH = 7.4) for twice (Rycenga, Kim et al. 2009).

Well established EDC-NHS coupling protocol was used to conjugate anti-bacteria antibody to MUDA modified silver nanocubes.(Yu, Nakshatri et al. 2007) Briefly, 0.2 mL of as made MUDA modified silver nanocubes was treated with 50 μL a mixture of EDC (0.4 M) and NHS (0.1 M). 25 μL of 1 mg/mL anti-bacteria antibody was dded to the solution immediately. The mixture solution was then sonicated in ice water for 30 minutes. The unbounded antibody and coupling reagents were removed by centrifuge and resuspend in 10 mM phosphate buffer (pH = 7.4).

4.2.2 Bacteria cell culture and bacteria nanoprobe interaction

Two bacterial strains (*E. Coli* and *L. monocytogenes*) were grown in LB medium at 37^{o}C for 18 hours. The bacterial cells were then collected by centrifugation and washed with PBS buffer for two times and finally redispersed in PBS buffer. The final bacterial cell concentration was determined by optical density (OD) measurement at 600nm. The concentration of the bacterial cells was 10^{9}cfu/mL when the OD of the solution equaled to 1.

Certain concentration of bacteria was added to anti-bacterial antibody-conjugated nanoprobe solution. The mixture was incubated at room temperature for around 30 minutes. After incubation one drop of the mixture solution was placed on a mesh for

TEM imaging using a 2007 JEOL 2100 200 kV STEM.

4.2.3 Raman Spectroscopic Measurement

Raman spectra were measured using a DXR Raman microscope (Thermo Scientific, Inc., Madison, WI) with 780 nm excitation and 14 mW laser power. Around 5 μL of the solution was spotted on gold coated glass slide and spectra of the droplet were measured before it was dried.

4.3 Results and discussion

4.3.1Raman characterization of gold nano probe modification

With the help of thiol groups, a layer of 4-ATP molecules were anchored on the surface of gold nanorods after incubation with GNRs solution (Frey, Stadler et al. 2001). SERS was used to verify the modification procedure. As illustrated in Fig. 12, band at 1074 cm^{-1} is the stretching vibration of C-S bond and band at 1578 cm^{-1} is the C-C stretching vibration of benzene ring in 4-ATP (Zheng, Zhou et al. 2003). The appearance of these bands, instead of original CTAB bands, in SERS spectrum indicates successfully substitution of CTAB with 4-ATP on the gold surface.

After react with nitrite ions in acid condition, diazonium salt was produced, which subsequently reacted with histidine residues in proteins. Peak at 1391 cm^{-1} is assigned as N=N stretching vibration of diazonium salt. This new peak in SERS spectrum after protein conjugation proves the formation of diazonium bond (Jiao, Niu et al. 2005) (Fig. 12). However it is difficult to identify peaks related to protein after antibody conjugation.

One possible reason is that the concentration of antibodies on the surfaces of the nanoparticles is still too low to be detected. To detect protein by SERS, either high concentration of protein (Drachev, Thoreson et al. 2004) or extrinsic Raman label (Porter, Lipert et al. 2008) were needed.

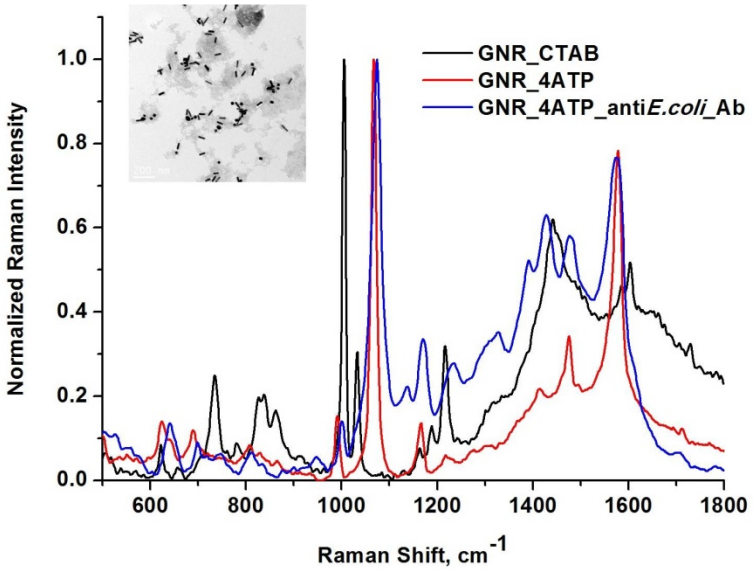

Fig. 12 SERS spectra of gold nanorods covered with CTAB (black), 4-ATP (red) and 4-ATP and anti-*E. coli* antibody (blue). TEM image is GNRs modified by 4ATP and anti-*E. coli* antibody.

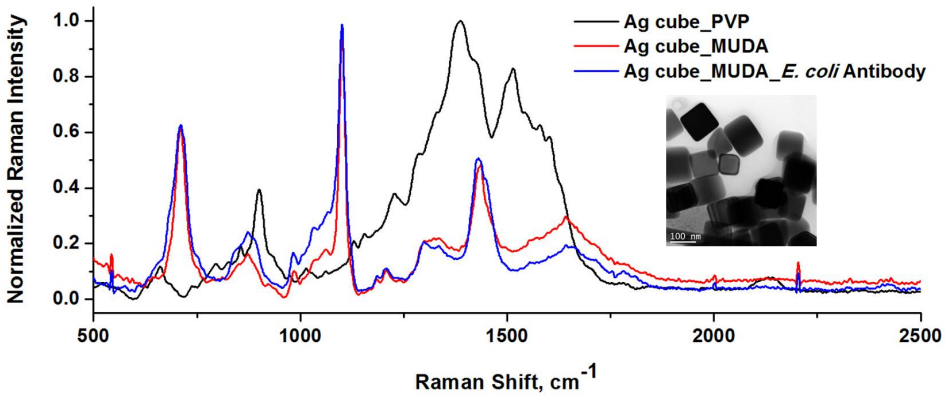

Fig. 13 SERS spectra of silver nanocubes covered with PVP (black), MUDA (red) and MUDA and anti-*E. coli* antibody (blue). TEM image is silver nanocubes modified by MUDA and anti-*E. coli* antibody.

SERS spectra of silver nanocubes covered with PVP, MUDA and MUDA linked

with *E. coli* antibody were shown in Fig. 13. Replacement of PVP from surface of silver nanocubes with MUDA significantly altered the SERS signature of the nanocubes. However, similar as observed for GNRs, the attachment of antibodies to the MUDA-modified silver nanocubes did not introduce identifiable signatures in the Raman spectra of the silver nanocubes, due to weak signals resulted from the low concentration of antibodies on the nanocube surfaces.

4.3.2 Dual recognition to detect target bacteria

Fig. 14 shows the TEM images of anti-*E. coli* antibody coated GNRs binding with *E. coli* (Fig. 14A) and *L. monocytogenes* (Fig. 14B). Binding between *E. coli* cells and the nanoprobes could be identified in TEM image, although the bacteria were not fully covered by nanoprobes. Further optimization of the binding conditions is needed in future work. For the *Listeria* which is a non-target for the nanoprobes, there appeared to be no binding of the nanoprobes to the *Listeria monocytogenes* cells.

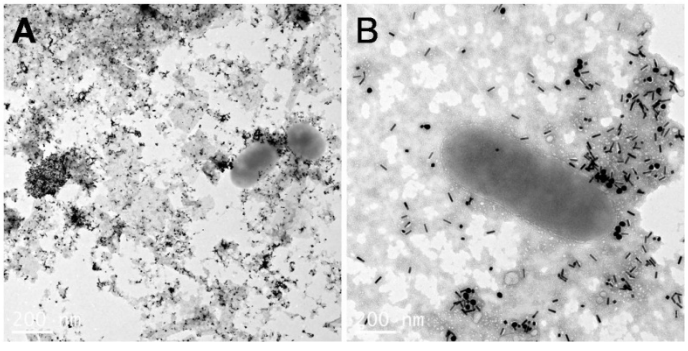

Fig. 14 TEM image of gold nano probe with anti-*E. coli* antibody incubated with (A) *E. coli* and (B) *L. monocytogenes*.

Although colloidal nanoparticles can be absorbed onto microbial cell wall from all

directions due to electrostatic interaction, simply mixing bacterial cells with nanoparticles usually do not bring enough particles to the surface of bacterial cells to generate measurable SERS signals (Kahraman, Zamaleeva et al. 2009). With our specifically functionalized nanoprobes, however, as shown in the TEM image in Fig. 14, more nano probes were attached to the surface of *E. coli* due to the anti-*E. coli* antibodies conjugated on the surface of GNRs.

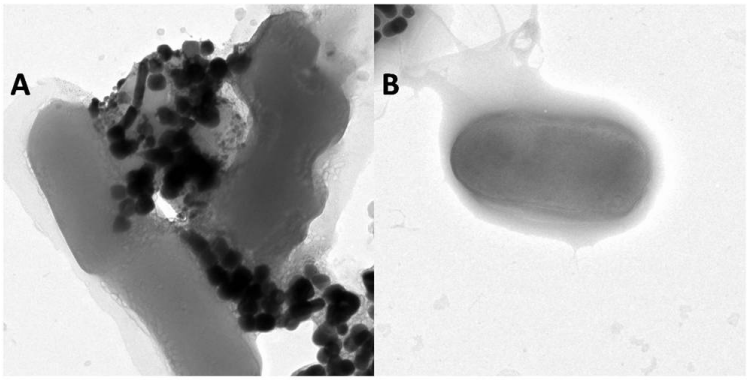

Fig. 15 TEM image of silver nano probe with anti-*Listeria* antibody incubated with (A) *L. monocytogenes* and (B) *E. coli*.

We also use anti-*Listeria* antibody modified silver nano probe to test the binding effect between *L. monocytogenes* and anti-*Listeria* antibody. As demonstrated in Fig. 15, *Listeria* antibody could recognize and bind with *L. monocytogenes* instead of *E. coli*. This result is consistent with what we observed with gold nano probes: nanoprobes modified with anti-bacteria antibody could bind to the surface of target bacteria, due to the specific recognition between antibody and antigen.

Fig. 16 shows the SERS spectra of gold nanoprobes (specific towards *E. coli*) incubated with *E. coli* and *Listeria monocytogenes*, respectively. The illumination volume

of the Raman microscope was not sufficient to cover a whole bacterial cell and adjacent nanoprobes, average spectra over 10 measurement was used to illustrate the SERS signatures (Jarvis and Goodacre 2008). In Fig. 16, peak at 723 cm^{-1} only appeared upon the binding of probes to their specific bacterial targets (*E. coli*). This peak represents adenine from flavin, NAG and NAM (Kahraman, Zamaleeva et al. 2009).

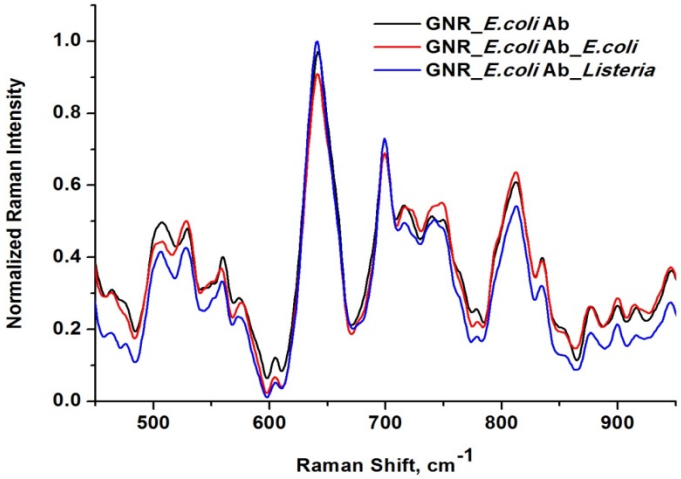

Fig. 16 SERS spectra of gold nano probe covered with anti-*E. coli* antibody (black), incubated with *E. coli* (red) and *Listeria* (blue).

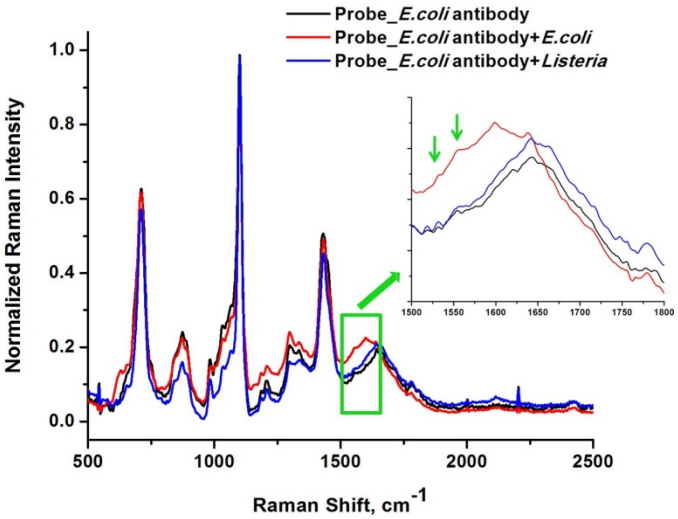

Fig. 17 SERS spectra of silver nano probe covered with anti-*E. coli* antibody (black), incubated with *E. coli* (red) and *Listeria* (blue).

Fig. 17 shows the SERS spectra of silver nanoprobes (specific towards *E. coli*) incubated with *E. coli* and *Listeria monocytogenes*, respectively. The 1599 and 1554 cm^{-1} peaks only appeared upon the binding of probes to their specific bacterial targets (*E. coli*) (Kahraman, Zamaleeva et al. 2009). The appearance of these peaks indicated the existence of *E. coli* in the sample solution. Moreover, these peaks were easier to be identified that the peaks introduced by gold nano probe. The main reason is that silver nanocubes are superior SERS enhancer than gold nanorods (Rycenga, Kim et al. 2009), they would be favored as choice of SERS nanoprobes.

4.3.3 Sensitivity and Selectivity of the Dual-recognition probing scheme

The sensitivity and selectivity of the superior silver nanocube probes were investigated. As shown in Fig. 18A, the two bacterial peaks were identified at 10^2 cfu/mL *E. coli* concentration, comparable with that of high-end ELISA assay, without going through any washing steps.

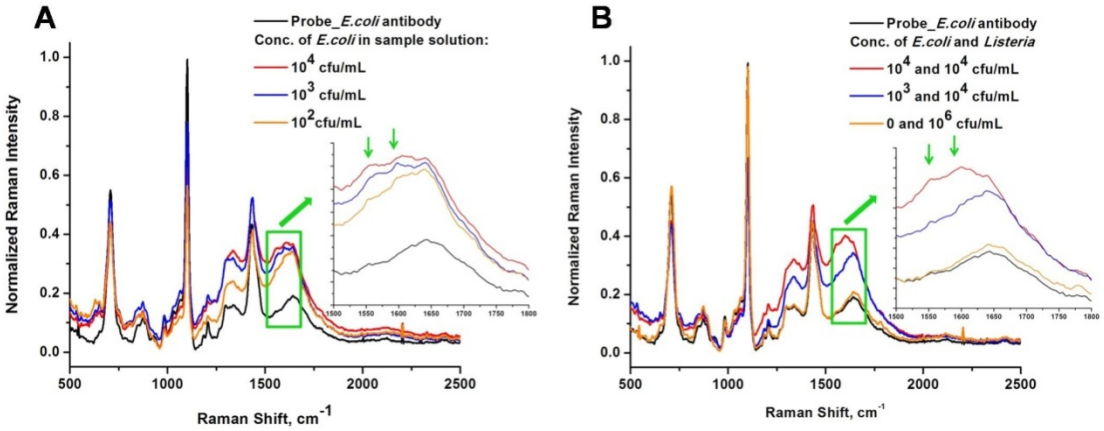

Fig. 18 SERS spectra of silver nano probe covered with anti-*E. coli* antibody interacted with: (A) *E. coli* sample solution with different *E. coli* concentration; (B) *E. coli* and *Listeria* mixture sample solution.

Also, for a sample solution with concentration of 10-fold higher *Listeria monocytogenes* than target *E. coli*, the specific *E. coli* peaks could still be identified, as shown in Fig. 18B. Relatively high level of interferences from other bacteria (*Listeria monocytogenes*) did not diminish the sensitivity and accuracy of the dual-recognition probing scheme, indicating that this scheme would be extremely attractive to in-field pathogen detection applications, where interference from other co-existed microorganism species will be omnipresent.

4.4 Conclusion

A dual recognition mechanism was successfully established for single-step detection of bacterial target in a lab-in-a-tube setting using SERS spectroscopic sensing. To prove the possibility of this mechanism *E. coli* and *Listeria*, gram negative and positive bacteria, were used as sample bacteria. The detection time for this method is relatively short, taking about 30 minutes incubation of nanoprobes with a sample to achieve a definite spectral signal to determine whether or not the targets are present in the sample. The sensitivity of the dual-recognition probing scheme is high (100 cfu/ml), and it was demonstrated that interference from other sources was minimal even at high concentrations (10 times higher than the targets).

The binding efficiency between nanoprobes and target bacteria needs to be further improved to obtain stronger SERS enhancement of the finger printing peaks of the bacterial targets. Also, multiplexing detection scheme will be developed with a

multi-channel sensor design in the near future.

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CHAPTER 5

Future Prospective

In conclusion, to meet the needs of quick and portable assay for drinking water, two kinds of nano sensor was developed to detect danger level of nitrite and microorganisms. The colorimetric nitrite sensor utilizes electrostatic aggregation of GNR to analyze trace amount of nitrite with simple visual inspection. Dual recognition scheme makes it possible to detect low level of microorganisms by portable Raman sensor. These two sensors demonstrated the potential of employing nano particles to analyze low level of contaminants in drinking water.

The GNR nitrite sensor could also be used for nitrate detection by reducing nitrate to nitrite. If the reduction-oxidation (Redox) reaction doesn't change the surface stability of GNR, nitrite reduced from nitrate could still trigger the colorimetric change of the sensor. I tried to use nitrate reductase to reduce nitrate to nitrite. However, the products of the reaction were not able to induce aggregation of GNR. It is speculated that the main reason of this failure is that the protein (nitrite reductase), as a macromolecule, protected nanoparticles from aggregation. The colorimetric nano sensor may be able to detect nitrate if nitrate reductase is removed from mixer solution after redox reaction. For example, protein could be separated from solution by filtration or aggregation. Also, other catalysts (i.e., metallic catalysts) that catalyze the redox reaction from nitrate to nitrite that do not influence the stability of nanoparticles could be utilized.

Moreover, 4-ATP modified GNR may be used as Raman sensor to detect nitrate by using portable Raman spectrometer. As illustrated in Chapter 3, before and after diazotation reaction, Raman signals of 4-ATP modified GNR are significantly different. So it is possible to use this nano sensor as Raman sensor to detect nitrate after reduced to nitrite by nitrate reductase. Strong Raman signals from 4-ATP enables the usage of low sensitive portable Raman spectrometer for the analysis of trace amount of nitrate, which means this sensor is still applicable as an in-field detection method.

For microorganism Raman sensor, in this thesis, the feasibility of dual recognition mechanism is proved. However, the SERS signal from bacterial cells is still not strong enough for them to be effectively detected by portable Raman system. The next step is to further optimize the design of the nanoprobes and the functionalization chemistry to yield SERS probes that can bind to bacterial targets more efficiently, and yield more intensive bacterial spectroscopic signatures that can are measurable with portable Raman systems. Also microfluidic devices can be introduced to pre-concentrate the target cells in a sample and further improve the detection limits of the Raman-based methods.

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