Development of Plasmonics-based Optical Nanoprobes for Medical Diagnosis

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2012
ABSTRACT

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Abstract

The development of practical and sensitive techniques for screening early biomarkers such as nucleic acid targets related to medical diseases and cancers is critical for early diagnosis, prevention and effective interventions. Recent advances in molecular profiling technology have made significant progress in the discovery of various biomarkers that could serve as important predictors of cancer risk and progression. Fast and precise measurement of biomarkers will help identify molecular signatures critical for the evaluation of cancer risk and early detection. Recently, there has been great interest in the design and fabrication of plasmonics-active biosensing platforms for a wide variety of applications ranging from biomedical diagnostics, food safety, environmental monitoring, to homeland defense. In particular, DNA-functionalized metal nanoparticles (e.g. gold and silver) have been utilized in the development of novel plasmonics-based analytical techniques for the detection of nucleic acid targets. In this study, two novel label-free approaches named “molecular sentinel (MS) nanoprobes”, and “plasmonic coupling interference (PCI) nanoprobes” have been developed for multiplex detection of disease biomarkers using surface-enhanced Raman scattering (SERS). The MS approach has been further extended into a unique “molecular sentinel-on-chip” (MSC) technology based on a SERS-active nanowire array substrate, leading to the development of a unique diagnostic tool having multiplexing and high-throughput
screening capabilities. Finally, a novel nanoparticle-based colorimetric assay has been developed and implemented for the detection of microRNAs (miRNAs). Direct detection of miRNAs in RNA samples from breast cancer cell lines has been demonstrated. Furthermore, the PCI technique has successfully detected miRNA biomarkers in biopsies of gastrointestinal cancer patients and the results are consistent with established techniques such qRT-PCR. The results of this study demonstrate that these plasmonics-based nanoprobes have great potential as useful point-of-care diagnostic tools for medical applications.
Dedication

I dedicate this dissertation to my lovely wife, Wei Xiong, and my parents, Chun-Chi Wang and Hsiu-Chun Chiang Wang, as well as the rest of the family. Without their presence, enduring love, support, and comprehension, I would have not achieved my goal.
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Chapter 1. Introduction

1.1 Hypothesis

1. Plasmonics-based nanoprobe technologies can be used for multiplex detection, and as a simple and rapid colorimetric biosensing technique.

2. Plasmonics-based nanoprobe technologies can be used for biomedical applications in cancer and disease diagnostics.

1.2 Specific Aims

This work involves two unique plasmonic nanoprobe technologies: (1) molecular sentinel (MS) nanoprobe and (2) plasmonic coupling interference (PCI) nanoprobe.

Specific Aim 1: Development and applications of the plasmonics-based molecular sentinel (MS) technique for biomarker detection

1.1. Develop plasmonic molecular sentinel (MS) nanoprobe technologies for multiplex detection of breast cancer biomarkers

1.2. Demonstrate plasmonic molecular sentinel (MS) nanoprobe technologies for viral infection diagnostics

1.3. Apply “Molecular Sentinel on Chip” (MSC) technique for SERS-based biosensing

Specific Aim 2: Development and applications of a novel plasmonic coupling interference (PCI) technique for nucleic acid detection
2.1. Develop SERS-based plasmonic coupling interference (PCI) nanoprobes for nucleic acid detection

2.2. Demonstrate the use of SERS-based PCI nanoprobes for label-free direct detection of microRNAs for cancer diagnostics

2.3. Develop and apply a simple nanoparticle-based colorimetric assay for direct detection of microRNAs for cancer diagnostics

1.3 Motivation

There is an increasing demand for the development of practical and sensitive techniques to screen and monitor various disease and cancer biomarkers for early diagnosis, prevention and effective interventions. Particularly, the early diagnosis of cancer is crucial for patient survival. Recent advances in molecular profiling technology such as DNA-microarray technology have made significant progress in the discovery of various biomarkers, which allow the characterization of diseases and tumors at the molecular level, and further guide the design of new drugs for targeted therapies. Appropriate biomarkers may also assist in early detection and diagnosis. According to the Surveillance Epidemiology and End Results (SEER) Program of the National Cancer Institute, the 5-year relative survival rates for localized and advanced breast cancer are 98.1% and 27.1%, respectively. This remarkable difference is assumed to be associated with improvements of early detection and/or treatment.[1, 2] Thus, fast and precise
detection of biomarkers is critical for the evaluation of cancer risk, early detection, and the assessment of treatment efficacy.

1.4 Background

Biosensing is generally defined by the measurement of the presence of biological molecules using an analytical device as the biosensor, which utilizes biomolecular probes to translate a biological response into a measurable physical signal (e.g. optical or electrical). A biosensor usually consists of two basic elements in spatial proximity: (1) a biorecognition element, such as nucleic acids, antibodies and enzymes that can interact specifically with target molecules, and (2) a sensor element (transducer) to convert a biochemical response into a measurable effect, such as an optical signal (e.g. a change in emission, scattering or absorption).[3, 4]

Figure 1: Biosensing principle

Among several optical assay readouts used in biosensing assays including fluorescence, absorbance, and luminescence etc., fluorescence-based techniques are the most commonly used detection approaches. Techniques that use fluorescence probes based on fluorescence resonance energy transfer (FRET) (e.g., molecular beacons) have been widely applied with great success in biosensing assays for nucleic acid or protein
detection. However, fluorescence-based techniques have intrinsic limitations for multiplex detection, such as requiring multiple light sources for excitation, and having broad and overlapped emission peaks making multiplex detection more difficult. Thus, the need for alternative, rapid, and selective multiplex assays has continued to encourage researchers to explore other technologies having comparable sensitivity as fluorescence but having additional unique and complementary advantages, and Raman spectroscopy is one of the most popular techniques. Raman spectroscopy is a spectroscopic technique used to measure the inelastic scattered light from a sample. Following laser irradiation of a sample, the inelastic light scattering process occurs in which the target sample absorbs one photon and emits another photon at the same time, the second photon being either at a lower frequency (i.e. Stokes scattering) or at a higher frequency (i.e. Anti-Stokes scattering) than the incident light frequency. The observed Raman shifts are equivalent to the energy changes involved in molecular vibrational transitions of the scattering species and are therefore exhibiting very narrow linewidths. For these reasons, Raman spectroscopy has now been considered as a powerful analytical technique for chemical and biological analysis due to the wealth of information on molecular structures, surface processes, and interface reactions that can be extracted from experimental data. The spectral selectivity associated with the narrow emission lines and the molecular specific vibrational bands of Raman labels make it an ideal tool for multiplex detection. Despite these strengths, use of Raman spectroscopy
for trace detection is generally limited by the small Raman scattering cross-sections - typically between $10^{-30}$ to $10^{-25}$ cm$^2$ per molecule - and the extremely low efficiency of the Raman scattering process (only one in every $10^6$-$10^8$ photons which scatter).

1.5 Surface-enhanced Raman scattering (SERS)-based biosensing

There has recently been great interest in the development and fabrication of plasmonics-active SERS biosensing platforms for a wide variety of applications. Plasmonics refers to the study of enhanced electromagnetic properties of metallic nanostructures. The term is derived from plasmons, the quanta associated with longitudinal waves propagating in matter through the collective motion of large numbers of electrons. According to classical electromagnetic theory, molecules on or near metal nanostructures experience enhanced fields relative to that of the incident radiation. When a metallic nanostructured surface is irradiated by an incident electromagnetic field (e.g., a laser beam), conduction electrons are displaced into frequency oscillations equal to those of the incident light. These oscillating electrons, called “surface plasmons,” produce a secondary electric field, which adds to the incident field. The origin of plasmon resonances of metallic nanoparticles are referred to as localized surface plasmons (LSPs). LSPs can be excited when light is incident on metallic nanoparticles whose size is much smaller than the wavelength of the incident light. At a suitable wavelength, resonant dipolar and multipolar modes can be excited in the nanoparticles, which lead to a significant enhancement in absorbed and scattered light
and enhancement of electromagnetic fields inside and near the particles. Hence, the LSPs can be detected as resonance peaks in the absorption or scattering spectra of the metallic nanoparticles. This condition yields intense localized fields which can interact with molecules in contact with or near the metal surface. In an effect analogous to a “lightning rod”, secondary fields can become concentrated at points of high curvature on the nanostructured metal surface. Nanoparticles of noble metals such as gold and silver resonantly scatter and absorb light in the visible and near-infrared spectral region upon excitation of their plasmon oscillations, and are therefore materials of choice for plasmon-related devices. Surface plasmons have been associated with important practical applications in surface plasmon resonance (SPR), surface-enhanced Raman scattering (SERS) and surface-enhanced luminescence (also referred to as metal-enhanced luminescence).

Discovery of the surface-enhanced Raman scattering (SERS) effect in the 1970s[5, 6] indicated that Raman scattering efficiency can be enhanced by factors of up to $10^6$ when the sample is located on or near nano-textured surfaces of plasmonics-active metals such as silver, gold, and transition metals. In general two basic mechanisms of SERS have been discussed – the electromagnetic enhancement (EME) where the SERS signals from molecules are related to the electromagnetic fields in the vicinity of the molecules[7-10] and chemical enhancement (CE), which is related to electronic coupling between the metallic substrate and the molecules thereby resulting in a change in the
Raman cross-section of the coupled molecule-substrate complex.[11, 12] There are two main sources of electromagnetic enhancement of SERS[9] – The first part of the enhancement is due to excitation of surface plasmons or electronic vibrations on the SERS substrate having a nanostructured metallic (e.g. Ag, Au, Cu) surface, which leads to significant enhancement in the localized electromagnetic fields in the vicinity of the substrate. Such an EM enhancement in the vicinity of the SERS substrates containing the Raman-active molecules leads to an increase in the Raman emission intensity, which is proportional to the square of the applied field at the molecule. Various models explaining important properties and characteristics of the surface plasmon effect have been reported.[7-10] Another part of the EM enhancement originates from the plasmonic enhancement of the Raman signal emanating from the Raman-active analyte molecules, thereby leading to an EM enhancement factor that is proportional to the fourth power of the electric field around the nanostructures.

SERS has recently attracted increasing interest in the development of novel analytical techniques for a variety of applications ranging from biomedical diagnostics, food safety, environmental monitoring and homeland defense. Particularly, SERS has been demonstrated that sensitive and specific detection of biomolecules such as DNA can be achieved by utilizing SERS-active metallic (e.g. silver and gold) nanoparticles (NPs) when molecules are adsorbed on or placed in close proximity to the metal surface.[13-15] In addition, it has been reported that nanoparticle-conjugated
oligonucleotides exhibit remarkably sharp melting profiles when compared to unmodified oligonucleotides.[16-18] By taking advantage of this unusual hybridization property, several nanoparticle-based DNA detection methods with improved sensitivity have been previously reported.[19-24]

1.6 Colorimetric-based Biosensing using Metal Nanoparticles

Colorimetric assays based on aggregation of metallic nanoparticles have attracted much attention for the detection of biomolecules as an alternative to fluorescence-based assays due to their simplicity, low cost and high sensitivity.[23, 25-27] Nanoparticles of noble metals such as silver and gold resonantly scatter and absorb light in the visible and near-infrared spectral region and exhibit interesting colors in solution. According to classical electromagnetic theory, when a metal nanoparticle surface is irradiated by an incident electromagnetic field, conduction electrons are displaced into frequency oscillations equal to those of the incident light. This phenomenon is called “surface plasmon resonance” (SPR) giving rise to unique optical properties of metal nanoparticles. It has been shown that the frequency of the surface plasmon band of metal nanoparticles as well as the color of nanoparticle solutions depend on various parameters including the size and shape of the particle, the type of metal, the dielectric properties of the medium, the distance between particles, and aggregate size. Typically, dispersed spherical silver nanoparticles have a yellow color in solution. If the particles aggregate, a color change from yellow to orange, to grey
depending on the aggregate size can be observed. For unaggregated gold nanospheres with the surface plasmon absorption band near 520 nm, the particle solution appears red, but changes to blue/purple upon aggregation.
Chapter 2. Development of plasmonic molecular sentinel (MS) nanoprobes for multiplex detection of breast cancer biomarkers¹ (Aim 1.1)

Recent interest in the functionalization of metallic (e.g. silver and gold) nanoparticles with DNA oligonucleotides [28-32] has led to the development of novel analytic tools based on surface plasmon resonance (SPR) [31, 33, 34], metal-quenched [35-37] or metal-enhanced [38] fluorescence, and surface-enhanced Raman scattering (SERS) [13, 15, 39-41] for the detection of nucleic acid targets of interest. These newly developed nanobiotechnologies utilize the unique optical and plasmonics-related properties of metal nanoparticles as signal transducers for reporting oligonucleotide hybridization events. Several nanoparticle-based DNA detection methods have been previously reported [15, 34, 39]. However, many techniques require labeling the targets and several washing steps following the reactions, which can complicate detection procedures. Herein, we have developed a novel label-free “molecular sentinel” (MS) nanoprobes that incorporates the SERS effect modulation scheme associated with metal nanoparticles and Raman-labeled DNA hairpin probes to detect nucleic acid sequences of interest.

2.1 Silver nanoparticle synthesis and functionalization with thiol-modified oligonucleotides

Nanoparticles of noble metals, such as silver and gold, resonantly scatter and absorb light in the visible and near-infrared spectral region and exhibit interesting colors in solution. Because of their unique optical properties, both silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) have been widely used in various SERS applications. However, it has been indicated that the enhancement factor for isolated AgNPs ($10^6 – 10^7$) is higher than that for isolated AuNPs ($10^3 – 10^4$) measured at their plasmon resonances of 407 and 514 nm, respectively.[42] Thus, in this dissertation, AgNPs were the main materials used as the signal-enhancing platform (nano-enhancer) for the development of novel plasmonics-based biosensing techniques discussed in the following chapters.

2.1.1 Silver Nanoparticle Synthesis and Characterization

In this dissertation, a novel type of SERS-active AgNPs using hydroxylamine hydrochloride as the reduction agent was prepared according to a method reported by Leopold and Lendl.[43] Briefly, a hydroxylamine hydrochloride solution (1 mL of a 1.5 x $10^2$ M solution containing 3 x $10^2$ M NaOH) was rapidly added to a silver nitrate solution (9 mL of a 1.11 x $10^3$ M solution) under vigorous stirring for 30 min at room temperature.

There are several advantages of using this type of AgNPs. First of all, they can be easily and rapidly prepared. Second, it has been shown that hydroxylamine-reduced
AgNPs exhibited comparable SERS enhancement to other types of AgNPs such as citrate-reduced AgNPs. Third, it has been indicated that they can provide a large surface available for the adsorption of analytes comparable to that of citrate-reduced AgNPs since chloride ions acting as stabilization agents adsorbed on their surfaces are smaller than citrate. The size of the AgNPs was determined to have an average diameter of 43.7 ± 9.3 nm using transmission emission microscopy (Figure 2a). The quantity of the nanoparticles was estimated to be ~ 4.8x10^{11} particles/mL measured by using NanoSight NS500 (NanoSight Ldt. Amesbury, UK). Figure 2b shows that the surface plasmon absorption band of the AgNPs was observed at 410 – 412 nm.

Figure 2: (a) TEM image of hydroxylamine-reduced silver nanoparticles. (b) UV-vis spectrum of silver nanoparticles.
2.1.2 Silver Nanoparticle Functionalization with Thiol-modified Oligonucleotides

In this dissertation, thiol-modified oligonucleotides were used as the biorecognition elements for the development of plasmonics-based biosensing techniques to detect specific nucleic acid sequences. Thus, preparation of oligonucleotide-AgNP conjugates is the critical first step. Various methods to modify metal nanoparticles with oligonucleotides have been reported. However, most of the approaches utilized AuNPs in a number of different applications and required time-consuming salt aging steps.\[17, 25, 26, 44\] Typically, thiol-modified oligonucleotides were first incubated with nanoparticles for about 18 hours. To prevent nanoparticle aggregation, a NaCl solution was then gradually added over 3 to 5 days to give a final NaCl concentration of at least 0.3 M. Although the preparation time can be reduced to within a day using a modified method,\[45\] it was still necessary to add NaCl gradually in increments of 50 - 100 mM to reach a final concentration of 1 M.

Although this salt-aging method has been adapted for citrate-reduced AgNPs,\[23, 46\] it has not been used for hydroxylamine-reduced AgNPs. It was found that hydroxylamine-reduced AgNPs were unstable and still tended to aggregate during the slow salt-aging process. To overcome this difficulty, we have successfully used MgCl\(_2\) instead of using NaCl to functionalize hydroxylamine-reduced AgNPs with thiol-modified oligonucleotides. To determine the optimal concentration of MgCl\(_2\), the unmodified AgNPs (0.9 mL) were first incubated with or without 0.5 \(\mu\)M thiolated
oligonucleotides for 1 hour followed by addition of different concentrations (from 0 to 0.5 mM) of MgCl₂ (0.1 mL). The UV-Vis spectrum of each sample was then measured at different time points. Figure 3 shows that oligonucleotides can prevent AgNP aggregation in MgCl₂ solutions at concentrations up to 0.25 mM for 22-mer thiolated oligonucleotides with sequence 5’-TCAACATCAGTCTGATAAGCT-3’, and up to 0.5 mM for 40-mer thiolated oligonucleotides with sequence 5’-AAAAAGGCTATACACAACA-GGTACCACACACATAATAGCC-3’. A ~ 4 nm red-shift of the surface plasmon band was observed indicating the adsorption of oligonucleotides onto the nanoparticle surface (Figure 4).
Figure 3: MgCl₂-mediated adsorption of oligonucleotides onto AgNPs. UV-Vis spectra of AgNPs incubated with different concentrations (from 0 to 0.5 mM) of MgCl₂ for 2 and 20 hrs in the absence (a, b) or presence of 22 (c, d) or 40-mer (e, f) thiolated-oligonucleotides. The presence of thiolated-oligonucleotides prevented the aggregation of AgNPs (indicated by the changes in the absorbance at ~412 nm) in MgCl₂ solutions at concentrations up to 0.25 mM and 0.5 mM for 22-mer and 40-mer oligonucleotides, respectively.
Figure 4: UV-Vis spectra (400 – 450 nm) showing the red-shift of the surface plasmon band of oligonucleotide-AgNP conjugates in the presence of MgCl₂: (a) in the absence of thiolated-oligonucleotides, (b) in the presence of 0.5 µM 22-mer thiolated-oligonucleotides, (c) in the presence of 0.5 µM 40-mer thiolated-oligonucleotides. The observed ~ 4 nm red-shift of the surface plasmon band indicates the adsorption of oligonucleotides onto the nanoparticle surface.

After the incubation with MgCl₂, the conjugates were back-filled with mercaptohexanol (MCH) to displace non-specifically adsorbed DNA and to passivate the silver surface,[47] or other desired polymers/stabilizers such as Polyethylene glycol (PEG) described in Chapters 5 and 6. The conjugates were then purified by repeated centrifugation to remove unbound oligonucleotides. To determine the number of oligonucleotides bound to a silver nanoparticle, a ligand exchange process described previously[48] was performed using Cy3-labeled oligonucleotides. Briefly, the dye-labeled oligonucleotide-AgNP conjugates were incubated with Mercaptoethanol (0.5 M) for at least 16 hours at room temperature to release dye-labeled oligonucleotides from the nanoparticle surface. The solutions were centrifuged at 12,000 rpm for 10 min to isolate oligonucleotides from AgNPs. The fluorescence emission of the collected
supernatants was then measured using the FLUOstar Omega microplate reader (BMG Labtech, Inc.). For Cy3-labeled oligonucleotides, the supernatants were excited at 550 nm and the emission was collected at 580 nm. The concentrations of the released reporter oligonucleotides were determined according to a standard curve. The number of oligonucleotides per particle was then determined by dividing the total number of bound oligonucleotides by the number of nanoparticles. Using this process, the number of Cy3-labeled 40-mer oligonucleotides immobilized on a AgNP was estimated to be ~70, and ~80 strands/particle after incubation with 0.25 mM MgCl₂ for 2 and 20 hrs, respectively, and ~80 and 90 strands/particle after incubation with 0.5 mM MgCl₂ for 2 and 20 hrs, respectively.

2.2 Molecular sentinel nanoprobe design and operating principle

Figure 5 schematically illustrates the operating principle of the MS nanoprobe. The MS nanoprobe is composed of a DNA hairpin probe (~30 - 45 nucleotides in length) and a silver nanoparticle. One end of the hairpin probe is tagged with a SERS-active label as a signal reporter. At the other end, the probe is modified with a thiol group, which is designed to conjugate covalently to the silver nanoparticle via thiol-metal interaction. The sequence within the loop region of the hairpin probe is complementary to a specific target gene sequence of interest.
Figure 5: The operating principle of the SERS-based molecular sentinel (MS) nanoprobe. The MS nanoprobe is composed of a Raman-labeled DNA hairpin probe and a silver nanoparticle. In the absence of the complementary target DNA, a strong SERS signal is observed due to the hairpin conformation adopted by the MS nanoprobe (left: closed state). In the presence of the complementary target DNA, the hairpin conformation of the MS nanoprobe is disrupted and the SERS signal is quenched due to the physical separation of the Raman label from the surface of the silver nanoparticle (right: open state).

In the absence of the target, the Raman label is in close proximity to the metal surface due to the stem-loop configuration ("closed" state), and a strong SERS signal is produced upon laser excitation. The metal nanoparticle is used as a signal enhancing platform for the SERS signal associated with the label. The enhancement is due to a nanostructured metal surface scattering process (nano-enhancers) which increases the
intrinsically weak normal Raman scattering. Theoretical studies of electromagnetic effects have shown that the SERS enhancement \( G \) falls off as \( G = \frac{r}{(r+d)^{12}} \) for a single analyte molecule located a distance \( d \) from the surface of a metal nanoparticle of radius \( r \). The electromagnetic SERS enhancement strongly decreases with increased distance, due to the decay of a dipole over the distance \( (1/d)^3 \) to the fourth power, thus resulting in a total intensity decay of \( (1/d)^{12} \). Therefore in designing the SERS MS nanoprobe, the hairpin configuration has the Raman label in contact or close proximity (< 1 nm) to the nanoparticles thus inducing a strong a SERS signal (Figure 5, left). However, in the presence of the specific DNA target, hybridization between the target and DNA probe disrupts the stem-loop configuration and spatially separates the Raman label from the metal surface (“open” state) as depicted in Figure 5 (right). Since the SERS enhancement \( G \) depends strongly on the distance \( d \) between the Raman label and the surface of the metal nanoparticle \( (G \) is proportional to \( d^{-12} \)), the SERS signal is significantly reduced (quenched) in the open state of the MS nanoprobes.

### 2.3 Development of MS nanoprobes for the detection of breast cancer biomarkers

The ability to simultaneously detect multiple oligonucleotide sequences is critical for many medical applications such as early diagnosis, high-throughput screening and systems biology research. In particular, the development of practical and sensitive detection techniques with multiplexing capability can lead to improved accuracy for cancer diagnosis, since a variety of molecular alterations in multiple genes are usually
involved in tumorigenesis and progression of various cancers. With this important medical application, there has been an increased number of research activities aimed at developing multiplex detection technologies [49-51].

In this study, we show for the first time the feasibility of multiplex detection using the MS technology in a homogenous solution without washing or separation steps. We have designed two MS nanoprobes, ERBB2-MS and KI-67-MS, with sequences as follows to target the erbB-2 and ki-67 genes, respectively. (ERBB2-MS: 5’-SH-
CGCCATCCACCCCAAGACCACGACCAGCAGAATGGCG-Cy3-3’; KI-67-MS: 5’-SH-GCGTATTCTGCACACCTCTTGGACACTCCGATACGC-TAMRA-3’). These two genes, which are well known as critical biomarkers for breast cancer, have been adopted for diagnostic use in clinical practice. The erbB-2 gene (also known as ERBB2 or HER2/neu) encodes a transmembrane glycoprotein, which belongs to the epidermal growth factor receptor (EGFR) family and possesses intrinsic tyrosine kinase activity. Overexpression of ERBB2 is observed in 20% to 30% of invasive breast cancers [52, 53]. Ki-67 is known as a cell proliferation marker and can potentially be used as an early predictor for treatment efficacy [54]. The expression of Ki-67 is well regulated throughout the cell cycle. In quiescent cells, Ki-67 expression is low. In proliferating tissues and tumors, Ki-67 is overexpressed [55]. Several studies have shown that the overexpression of ERBB2 is usually associated with the increased expression of Ki-67 in breast cancer [56, 57]. However, the functional relationship between these two genes is
not well understood. Simultaneous detection of these two genes can further provide valuable information for insight into the functional relationship between these two genes and may lead to an improved early detection and diagnosis of breast cancer.

The Raman spectra from the ERBB2-MS and the KI-67-MS nanoprobes are shown in Figure 6(a) and 6(b), respectively. The high intensity of the major SERS bands in each case indicates that with the stem-loop configuration intact, the Raman labels at the 3’-end of the two molecular sentinels (Cy3 and TAMRA) were near the surface of silver nanoparticles (closed state) and the Raman signals were plasmonically enhanced. The major Raman peaks of the two labels are marked in Figure 6 and used throughout this report to illustrate the changes in SERS spectra upon hybridization to specific DNA sequences. It is noteworthy that even though the fluorescence spectra of these two dyes Cy3 and TAMRA strongly overlap, their Raman spectra are clearly distinguishable from one another due to the narrow SERS peaks. This feature underlines the advantage of the SERS-based MS nanoprobes over fluorescence-based assays for multiplex detection.
Figure 6: SERS spectra of the ERBB2-MS (a) and KI-67-MS (b) nanoprobes in 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM MgCl2. The major Raman bands are labeled as follows: ERBB2-MS: (1) 1197 cm\(^{-1}\), (2) 1393 cm\(^{-1}\), (3) 1468 cm\(^{-1}\), (4) 1590 cm\(^{-1}\). KI-67-MS: (1*) 1218 cm\(^{-1}\), (2*) 1354 cm\(^{-1}\), (3*) 1508 cm\(^{-1}\), (4*) 1535 cm\(^{-1}\), (5*) 1650 cm\(^{-1}\).

We have investigated the effect of MgCl\(_2\) concentration on the stem-loop configuration of the MS nanoprobes. It has been indicated that DNA hairpins can be effectively formed in the presence of MgCl\(_2\), since magnesium ions can stabilize the double-stranded stem regions [58, 59]. Figures 7(a) and 7(b) respectively show that the
SERS intensity of the ERBB2-MS nanoprobes at 1393 cm\(^{-1}\) (peak #2) and the KI-67-MS nanoprobes at 1354 cm\(^{-1}\) (peak #2*) increases with increasing MgCl\(_2\) concentration up to 5 mM in 20 mM Tris-HCl buffer (pH 8.0). When the concentration of MgCl\(_2\) was over 5 mM, precipitation of MS nanoprobes was observed, which may be due to the fact that the maximum ionic strength has been reached.

![Graph](image)

Figure 7: The effect of MgCl\(_2\) concentration on the SERS intensity of one of the major Raman bands (peak #2: 1393 cm\(^{-1}\)) from the ERBB2-MS nanoprobes (a) and (peak #2*: 1354 cm\(^{-1}\)) from the KI-67-MS nanoprobes (b). (•): 5 mM, (△): 4 mM, (+): 3 mM, (-): 0 mM.
2.4 Detection Specificity of the MS nanoprobes

To test the effectiveness of the designed MS nanoprobes, the ERBB2-MS and KI-67-MS nanoprobes were separately incubated with their complementary target DNA in hybridization buffer (20 mM Tris-HCl buffer, pH 8.0, 5 mM MgCl₂) and allowed to react at 37 °C for 1 hour. Figures 8 and 9 respectively show the SERS spectra of the ERBB2-MS and KI-67-MS nanoprobes in the presence or absence of 0.5 μM target DNA. In the case of presence of target DNA (lower spectra), the SERS intensity of the major Raman bands was significantly decreased (marked by arrows), indicating that the Raman labels were physically separated from the silver nanoparticles upon hybridization to their targets when the stem-loop configuration of the hairpin probes was disrupted (open state). On the other hand, in the absence of DNA (blank: upper spectra) or the presence of non-complementary DNA (negative control: middle spectra), the SERS intensity of the major Raman bands remains high, indicating that the MS nanoprobes remain in the stem-loop configuration (closed state).
Figure 8: SERS spectra of the ERBB2-MS nanoprobes in the presence or absence of complementary DNA targets. Upper spectrum: blank (no target DNA present). Middle spectrum: in the presence of 0.5 µM non-complementary DNA (negative control). Lower spectrum: in the presence of 0.5 µM complementary target DNA (positive diagnostic).
Figure 9: SERS spectra of the KI-67-MS nanoprobes in the presence or absence of complementary DNA targets. Upper spectrum: blank (no target DNA present). Middle spectrum: in the presence of 0.5 µM non-complementary DNA (negative control). Lower spectrum: in the presence of 0.5 µM complementary target DNA (positive diagnostic).
2.5 Multiplex detection of breast cancer biomarkers using the MS nanoprobes

We demonstrated the multiplexing capability of the MS technique by mixing two different and separately prepared ERBB2-MS and KI-67-MS nanoprobes. According to the SERS signal of the individual ERBB2-MS and KI-67-MS nanoprobes shown in Figure 6, the SERS intensity from the ERBB2-MS nanoprobes is relatively higher than that from the KI-67-MS nanoprobes. To obtain comparable SERS signals from both MS nanoprobes in the mixture, the ERBB2-MS and KI-67-MS nanoprobes were mixed with volume ratio of ERBB2-MS to KI-67-MS of 1:2 in 20 mM Tris-HCl buffer. Five mM MgCl₂ were then added to the mixture sample and allowed to react at room temperature for at least 30 min. The upper spectrum in Figure 10 represents the SERS spectrum of the mixture of the two MS nanoprobes. Note that when compared to the SERS spectra of the individual MS nanoprobes (shown in figure 6), all major Raman peaks of the Raman labels used for the ERBB2-MS and KI-67-MS nanoprobes can be easily identified in the new SERS spectrum obtained by mixing the two MS nanoprobes.
Figure 10: SERS spectra of the composite MS nanoprobes (ERBB2-MS + KI-67-MS) in the presence or absence of target DNA. The major Raman bands from ERBB2-MS are marked with black number, and the major Raman bands from KI-67-MS are marked with red number with (*) sign. Upper spectrum: blank (in the absence of any target DNA). Lower spectrum: in the presence of two target DNA complementary to both MS nanoprobes.

Demonstration of multiplex detection was performed in the presence of both target DNA sequences (0.5 μM for each target) in hybridization buffer, and allowed to react at 37 °C for 1 hour. Figure 10 shows that the SERS signal from the composite MS nanoprobes was significantly quenched in the presence of both targets. The SERS intensity of all major Raman peaks (in the lower spectrum) was greatly decreased when both MS nanoprobes hybridized with their complementary DNA targets.

To further demonstrate the specificity and selectivity of the composite MS nanoprobes, the hybridization assays were then performed in the presence of individual
complementary DNA target (i.e. only one of the two complementary targets). The middle and lower spectra in Figure 11 show the resulted SERS signal from the composite MS nanoprobes targeted to 0.5 µM target DNA complementary to ERBB2-MS and KI-67-MS nanoprobes, respectively. The result indicates that only the SERS peaks associated with the complementary MS nanoprobes was significantly quenched (indicated by arrows) when in the presence of its target DNA. For example, in the middle spectrum only the SERS peaks 1-4, associated with the ERBB2-MS nanoprobes were quenched when the erbB-2 DNA target is present. In this case, only the ERBB2-MS nanoprobes were in an open state. In contrast, the SERS signal of the Raman peaks associated with the second MS nanoprobes (KI-67-MS) remained high, indicating that the second nanoprobes were in a closed state due to the absence of its DNA target. Note that the second MS nanoprobe can be designed to serve as an internal control in multiplex detection due to the specificity and selectivity of the MS technique.
Figure 11: SERS spectra of the composite MS nanoprobes (ERBB2-MS + KI-67-MS) in the presence or absence of single target DNA. The major Raman bands from ERBB2-MS are marked with black number, and the major Raman bands from KI-67-MS are marked with red number with (*) sign. Upper spectrum: blank (in the absence of any target DNA). Middle spectrum: in the presence of single target DNA complementary to the ERBB2-MS nanoprobes. Lower spectrum: in the presence of single target DNA complementary to the KI-67-MS nanoprobes. The arrow signs illustrate the decreased SERS intensity of the major Raman bands in the presence of corresponding target DNA.
2.6 Summary

In summary, we have developed a facile one-step method to functionalize hydroxylamine-reduced AgNPs with thiolated oligonucleotides in the presence of low concentration of MgCl₂. In general, oligonucleotide loading can be slightly increased by increasing the incubation time from 2 to 20 hours. After conjugation of oligonucleotides to AgNPs, the silver surfaces are still available for further functionalization with desired small thiol molecules or polymers. This method provides a new route to prepare oligonucleotide-AgNP conjugates within a few hours to a day, and may facilitate the development of novel AgNP-based biosensing platforms.

We have demonstrated for the first time the feasibility of using the MS technique for qualitative multiplex DNA detection in a homogeneous solution. Two separately prepared MS nanoprobes were mixed and used to detect specific DNA sequences associated with erbB-2 and ki-67 breast cancer biomarkers present in the sample solution. The results of this study demonstrate the specificity and selectivity of the MS nanoprobes, as well as the ability to use multiple MS nanoprobes for multiplexed DNA detection. Furthermore, the SERS measurements were performed immediately following the hybridization reactions using a homogeneous assay without washing steps, which greatly simplifies the assay procedures. The results of this study demonstrate that the MS nanoprobe technique can provide a useful tool for multiplexed DNA detection in a homogeneous solution for medical diagnostics and high throughput bioassays.
Chapter 3. Demonstration of plasmonic molecular sentinel (MS) nanoprobes for viral infection diagnostics (Aim 1.2)

3.1 Host Biomarker for Infectious Diseases

There is a strong need to develop diagnostic technologies that can be used at the point-of-care to detect infectious diseases. A promising approach involves detection of the host response to various pathogens by evaluating changes in gene expression in peripheral blood samples, induced in response to infection.[60-63] These studies utilized laboratory-based cDNA microarray systems to characterize changes in RNA transcript abundance as a response to infectious challenges. Based upon research results, the development of host gene expression-based classification systems for discriminating types of infection is quite promising; thus, there is a strong need to develop practical diagnostic systems for use at point-of-care settings.

As a proof of concept, we sought to develop a point-of-care RNA detection and quantification approach using the molecular sentinel (MS) technique to ultimately increase applicability of this strategy. Thus, we chose a single transcript, the human radical S-adenosyl methionine domain containing 2 (RSAD2) gene as the test system for use in development of this novel RNA detection method. The RSAD2 gene is involved in antiviral defense and is directly induced by human cytomegalovirus (HCMV). The RSAD2 gene is a component of the group of genes found to accurately classify acute respiratory viral infection in comparison to no infection or bacterial infection,[63] and is
up-regulated in the nasal epithelium of individuals infected with rhinovirus.[64] The human RSAD2 gene encodes a protein known as Viperin, first identified in HCMV infected fibroblasts.[65] Upon viral infection, the type I interferons (IFNs) are produced and secreted by infected cells to initiate a complex signaling cascade, leading to the induction of hundreds of genes that limit viral infection. RSAD2 has been recognized as one of the most highly induced genes upon interferon stimulation or infection with various viruses, including HCMV, influenza virus, hepatitis C virus (HCV), dengue virus, alphaviruses, and retroviruses such as human immunodeficiency virus (HIV).[66] The RSAD2 protein (Viperin) was shown to localize to the endoplasmic reticulum (ER) and lipid droplets, where it exerts its antiviral function. It has been postulated that RSAD2 may alter lipid droplet formation or the ability of the viral proteins to localize to this organelle, leading to inhibit the replication of various DNA and RNA viruses.[66]

By evaluating changes in host gene expression profiles in response to viral infection, Zaas et al. have developed a robust blood mRNA expression signature that distinguishes individuals with symptomatic acute respiratory infections (ARIs) from uninfected individuals with over 95% accuracy.[63] This “acute respiratory viral” bio-signature encompasses 30 transcripts of genes known to be related to the host immune response to viral infection. In particular, RSAD2 was the most highly expressed gene in symptomatic individuals from all three human viral challenge studies with live rhinovirus, respiratory syncytial virus, and influenza A.
3.2 RSAD2-MS nanoprobe design

To demonstrate the potential of the MS technique for detection of respiratory diseases, a MS nanoprobe (RSAD2-MS) with sequence 5’-thiol-AAAAAGGCTATACACAC-AGGTACCACACATAATAGCC-Cy3-3’ for the RSAD2 gene was designed. The RSAD2-MS nanoprobe consisted of a 40-base DNA hairpin probe modified with the Cy3 Raman dye on the 3’ end and a thiol substituent at the 5’ end. The underlined sequences represent the complementary arm sequences which form a stem-loop structure. The 23-base loop region between the two complementary “arms” was designed to be complementary to a portion of the RSAD2 gene sequence. For the formation of hairpin structures, the MS nanoprobes were incubated in a 20 mM Tris-HCl buffer solution containing 2.5 mM MgCl₂. Figure 12 shows the specificity of the RSAD2-MS nanoprobes using 100 nM of synthetic DNA as the target molecules. The hybridization was carried out in a glass tube containing 0.1 mL of 20 mM Tris-HCl buffer solution (pH 8.0) and 2.5 mM MgCl₂. The result indicates that the SERS intensity is significantly reduced in the presence of its complementary DNA targets (lower spectrum). On the other hand, the SERS intensity remains high in the absence of DNA (upper spectrum) or the presence of non-complementary DNA (middle spectra), indicating that the MS nanoprobes are in the closed (stem-loop) state. The decreased SERS signal indicates that hybridization with the complementary target DNA molecules opens the stem-loop structure of the probe DNA molecules, thereby separating the SERS dye Cy3 from the silver surface.
Figure 12: SERS spectra of the RSAD2-MS nanoprobes in the presence or absence of complementary DNA targets. Upper spectrum: blank (no target DNA present). Middle spectrum: in the presence of 100 nM non-complementary DNA (negative control). Lower spectrum: in the presence of 100 nM complementary target DNA. Five SERS measurements were performed per sample and averaged into a single spectrum.

To demonstrate that the observed reduction in SERS intensity is due to the plasmonic modulation effect (upon stem-loop opening) and not due to dissociation of the hairpin probes from Ag nanoparticles, we also measured the fluorescence signal of the labels on the MS probes. The fluorescence intensities of the target-incubated MS
samples were compared before and after centrifugation. As shown in Figure 13, the fluorescence intensity of the supernatant was significantly less than that of the target-incubated MS nanoprobe solutions before and after centrifugation, thus indicating that most of the hairpin probes were still attached to the nanoparticles after hybridization to the complementary targets.

Figure 13: Fluorescence intensities of the target-incubated MS samples before and after centrifugation. The target-incubated MS samples were centrifuged at 12,000 rpm for 10 min. The supernatants were collected and the precipitates were redispersed in the same volume of hybridization buffer. The MS samples and supernatants were excited at 550 nm and the emissions were collected at 580 nm using the FLUOstar Omega microplate reader (BMG Labtech, Inc.). The experiments were repeated three times.
3.3 Quantitative analysis

We performed further studies to demonstrate the possibility for semi-quantitative DNA detection for the RSAD2 gene. In a series of measurements, we incubated the RSAD2-MS nanoprobes with various concentrations of RSAD2 target DNA between 0 and 100 nM. Five SERS measurements were performed per sample and averaged into a single spectrum. Figure 14 illustrates that the reduction of SERS intensity at 1196 cm\(^{-1}\) was greater with increasing RSAD2 DNA target concentration. The result confirms that more Raman labels on the RSAD2-MS nanoprobes were being separated from the silver surface during the hybridization process with increasing concentration of target molecules. By monitoring the SERS intensity of specific peaks, the concentration of DNA target can be estimated indirectly. As shown in the inset of Figure 14, a power fit with a correlation coefficient \(r\) of 0.9904 over the DNA target concentration from 1 to 100 nM was obtained. The results indicate that the MS technique can detect 1 nM and lower target DNA. At higher target concentrations, SERS signals from samples having over 50 nM target DNA decreased and reached a plateau, which indicated that the probe was fully hybridized and the observed signal was from the background. The general trend in this study indicates the potential of using the MS nanoprobes for semi-quantitative analysis. Together with the multiplex capability of the MS technique shown in our previous study,[67] the MS nanoprobes could be a useful
3.4 Point-of-care potential of using the MS nanoprobes

To demonstrate the potential of using the MS nanoprobes to detect RSAD2 RNA targets for point-of-care applications, we used a small, portable Raman spectrometer (Advantage 633, DeltaNu) for SERS measurements, and purified RNA samples as the specimen. For the initial “proof-of-concept” test, the total RNA from human lymph node
tissue was used for the detection of RSAD2 background expression levels. The RSAD2-MS nanoprobes (0.05 mL) were incubated with human lymph node total RNA (purchased from Applied Biosystems) followed by addition of a 0.05 mL of Tris/MgCl₂ buffer solution (MgCl₂ final concentration: 2.5 mM). The sample solution was allowed to react at room temperature for at least 20 min prior to SERS measurements.

As shown in Figure 15, the SERS signal of the RSAD2-MS nanoprobes (solid lines) was relatively reduced in the presence of 0.5 µg (spectrum b) and 1 µg total RNA (spectrum c) compared to the blank sample (spectrum a), and the SERS signal was further reduced in the presence of more RNA sample. To confirm the detection specificity, we have utilized a Cy5.5-labeled MS nanoprobe targeted to the gene sequences of the Influenza A virus (H1N1) nucleocapsid protein (NP) as the control nanoprobe, which is not expected to detect complementary targets in the normal human RNA samples. The results from the control experiments (dotted lines shown in Figure 15) show that the intensity of the major Cy5.5 SERS peaks at 1339 cm⁻¹, 1461 cm⁻¹ and 1625 cm⁻¹ remain high in the presence of normal human total RNAs, confirming the detection specificity using the RSAD2-MS nanoprobes.
Figure 15: Demonstration of the potential of using the MS nanoprobes to detect RSAD2 RNA targets for point-of-care applications using a small, portable Raman spectrometer (Advantage 633, DeltaNu) for SERS measurements. (a) blank (no RNA present). (b) in the presence of 0.5 and (c) 1 µg total RNA from human lymph node. Solid lines: using RSAD2-MS nanoprobes. Dotted lines: using control-MS nanoprobes.

The current DeltaNu Advantage 633 Raman spectrometer only provides a spectral resolution of 10 cm\(^{-1}\) due to the use of a low dispersion grating. As a result, the SERS spectra measured here were relatively broader than those using the confocal Raman microscope with a spectral resolution of 1 cm\(^{-1}\) (as shown in Figure 12). However, the result shown in Figure 15 demonstrates that the spectral resolution
provided by the portable spectrometer is adequate for diagnostics. It is noteworthy that we used a portable Raman spectrometer (small suitcase size) as a reader of the SERS signals. The portable Raman spectrometer has several advantages, such as low cost (~$10,000) and high portability (10-times smaller than the Renishaw confocal Raman microscope, which costs ~$300,000), which make it practical for point-of-care applications. This work also demonstrates that the MS technique can be easily integrated with a small portable Raman spectrometer for point-of-care applications, and the MS technique has the potential for RNA biotarget detection.

3.5 Summary

In summary, we have demonstrated the feasibility and specificity of using the molecular sentinel technique to detect human DNA and RNA targets, which can be used as a novel host marker in response to viral respiratory infection. We have also shown that the reduction of SERS intensity was dependent on the concentration of target molecules, illustrating the capability to use MS nanoprobes for semi-quantitative analysis. Due to narrow absorption bands and large spectral range, Raman provides great possibilities for multiplexing detection. The multiplex capability, which allows the monitoring of a large number of molecular processes simultaneously, is an important feature in medical diagnostics of a large number of biotargets.

We have also demonstrated for the first time the potential for point-of-care implementation of the MS diagnostics technology by using a portable Raman
spectrometer for detection. The results of this study demonstrate that the MS technique can provide a novel diagnostic approach for detecting RNA. The capability to detect and quantify nucleic acid molecules (DNA/RNA) could allow the MS diagnostics technology to play an important role in the diagnosis of infection using host-based transcriptional changes as medical diagnostics biomarkers.
Chapter 4. Application of the “Molecular Sentinel-on-Chip” technique for SERS-based biosensing (Aim 1.3)

4.1 Design and operating principle of the MSC technique

In this chapter, the MS approach was further extended into a unique quantitative “molecular sentinel-on-chip” (MSC) technology. The application of the MSC system based on a SERS-active nanowire chip is investigated and compared to previous studies on solution-based MS nanoprobes for the detection of DNA sequences. This approach involves using triangular-shaped nanowire (TSNW) arrays to enhance the SERS signals from the MSC hairpin probes located inside or near nanoscale gaps between plasmonically active metallic nanostructures.[10, 68-71] Figure 16 schematically illustrates the operating principle of the MSC using a TSNW substrate. The sequence within the loop region of the hairpin probe is complementary to a specific target gene sequence of interest. The hairpin probe having a Raman label at one end is then immobilized onto a metallic TSNW substrate via a thiol group attached on the other end. The MSC detection strategy is based on the dependence of SERS enhancement on the distance between the metallic surface and the Raman label. In the absence of target DNA molecules, the hairpin configuration has the Raman label in close proximity to the metallic surface (closed state) and exhibits a high SERS signal as depicted in Figure 16 (left). However, when complementary DNA targets are recognized by the MSC nanoprobe, hybridization occurs and the Raman label is separated away from the surface (open state) as depicted in Figure 16 (right). As a result, the SERS signal of the
Raman label is significantly reduced, indicating target recognition and capture. Moreover, we also employ the fact that a decrease of the overall SERS signal can also occur due to the moving away of some of the SERS-active dye molecules (attached to the probe molecules) from the inverted triangular SERS hotspots - due to opening of stem-loop structure of some of the probe DNA molecules upon hybridization with a complementary target molecule - formed at the bottom of the gap between the gold-coated triangular nanowires. Along with demonstrating the MSC technology, this paper also presents a relatively simple process of developing SERS substrates on a large area (6-inch wafers) in a reliable and controllable manner, such that these substrates contain plasmonic nanostructures with sub-10 nm gaps (that are potential 'SERS hotspots') between the nanostructures.
Figure 16: The operating principle of the MS-based DNA detection on a TSNW substrate. The hairpin probe modified with a Raman label at one end is immobilized onto a metallic TSNW substrate via a thiol group attached on the other end. In the absence of the complementary targets, a strong SERS signal is observed due to the hairpin conformation adopted by the hairpin probe (left: closed state). In the presence of the complementary targets, the hairpin conformation of the DNA probe is disrupted and the SERS signal is reduced due to the physical separation of the Raman label from the surface of the metallic surface (right: open state).

4.2 Development of nanowire arrays

It has been previously demonstrated that single-molecule SERS can be achieved using metal nanoparticle arrays or aggregates when analytes are located in the “hot spot” (i.e. unusually intense electromagnetic field produced inside nanoscale gaps or nanoparticle junctions).[72-75] However, the main challenge lies in developing SERS-active substrates having controlled sizes, shapes and sub-10 nm gaps that can be fabricated in a reliable and reproducible manner.
In this study, we employed a combination of deep UV lithography, atomic layer deposition, and metal deposition to fabricate the TSNW substrates having controlled sub-10 nm gap nanostructures over an entire 6-inch wafer (Figure 17B). As shown in Figure 17A, the fabrication of the TSNW array substrate involves the following steps: (1) development of silicon TSNWs on a 6-inch wafer, (2) coating with a spacer layer, and (3) deposition of gold film using E-Beam deposition. The TEM cross-sections of the TSNWs, with the individual nanowires having a subwavelength length, are illustrated in Figure 17C–E. Theoretical studies of rectangular grooves in zero-order silver gratings indicated substantial increase in the electric field intensity inside the grooves as the spacing between the metallic gratings decreased.[76] The coupling of p-polarized incident radiation to surface plasmon polariton (SPP) modes in short-pitch metallic gratings have also been investigated.[7, 77, 78] The narrow width of the grooves leads to strong coupling between surface charges on opposing walls of an individual grating groove, thereby leading to formation of standing SPP modes localized inside the groove. The coupling between the SPP modes inside the individual grooves increases with decreasing groove width. Figure 17C-E show TEM cross-sections of triangular shaped silicon nanowires. Sub-10 nm gaps between the gold film deposited on top of the triangular-shaped silicon nanowires can be clearly observed, especially at the bottom region of the triangular-shaped nanowires – labeled as inverted triangular nanowire regions ‘G’ in Figure 17A).
Figure 17: (A) Schematic showing the development steps of triangle-shaped nanowires (TSNW). (B) Picture showing half of a 6-inch SERS substrate wafer with nanowire structures. (C), (D) TEM cross-section of triangular silicon nanowires. (E) Enlarged view of the TEM cross-section region highlighted in (C) showing triangular silicon nanowires (in off-white color) over-coated with a silicon germanium layer (in light gray color) and a hafnium oxide layer (in dark gray color) and finally over-coated with a thin gold film layer (in black color).
As the Deep UV Lithography process used to develop the silicon nanowires can only allow a minimum gap of 100 nm between the nanowires to be achieved, we employ the growth or deposition of conformal thin film layers on top of the silicon nanowires to decrease the effective gaps between the nanowires. Subsequently, a plasmonics-active metal layer (e.g., Au, Ag) is deposited onto the nanowires. The TEM cross-sections (in Figure 17D-E) also show the hafnium oxide spacer layer that was developed by employing atomic layer deposition (ALD) to conformally reduce the gap between the neighboring silicon nanowires before deposition of the gold film layer using E-Beam deposition. The silicon nanowires were coated with a layer of silicon germanium employing ultra high vacuum rapid thermal chemical vapor deposition as an optional step to reduce the gap between the nanowires before the metal deposition. The SERS substrates were evaluated for their uniformity for the different regions in which nanowires of different dimensions and spacings were developed (each region having nanowires of a certain dimension and periodicity). Upon taking five SERS measurements using a laser spot of ~ 5 µm, which ensures that several nanowires - on each 1 mm × 300 µm region in which nanowires of the same dimension and periodicity were developed - it was observed that there was less than 5% variation of the SERS signals over each region.
4.3 Demonstration of DNA detection using the MSC technique

To demonstrate the applicability of the MS-based detection on the TSNW arrays, we chose a single transcript, the Ki-67 gene as the test system for use in the development of a nucleic acid detection platform.[54, 79] As mentioned in previous chapter, the Ki-67 gene can potentially be used as an early predictor for cancer treatment efficacy and has been recognized as a critical breast cancer biomarker.

In this study, the MSC hairpin and target oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The oligonucleotide sequences used in this study are as follows: KI67-MSC: 5’-SH-GCGTATTTCTGCACACCTCTTTGACAC-TCCGATACGC-TAMRA-3’. Complementary target DNA: GCACTTTGGAGAGCAAGTCTGTCAGAGAGTAACGCGGAGTGTCAAGAGGTGTGCAGAAAATCCAAAGAGGCTGAGGACAATG-3’. Non-complementary DNA: 5’-GCCAGCGTCGAGTTGGTTTGCGAGCTCCTGA-3’.

The MSC hairpin probe (KI67-MSC) for the Ki-67 gene was carefully designed with a 6 base-pair stem sequence (underlined sequences) with the melting temperature (Tm) ≈ 46 ºC allowing the formation of stable hairpin structure at room temperature in the absence of a complementary DNA target. The loop region of the hairpin probe (bold sequences) was designed to incorporate a partial sequence with 23-mer complementary to the Ki-67 gene sequences. The 3’-end of the KI67-MSC nanoprobe was labeled with a SERS-active dye 5-carboxytetramethylrhodamine (TAMRA) as a signal reporter, and the
5'-end of the nanoprobe was modified with a thiol group to covalently attach the KI67-MSC nanoprobe to the gold-coated nanowire substrate. In the presence of a complementary DNA target, the Tm of the probe-target hybrid is higher than 65 °C, which would allow a more stable open-state configuration of the hairpin probe to form, resulting in the separation of the Raman label from the surface of the metal substrate and a decreased SERS signal. With the hairpin probe with 35-mer, the separation distance between the Raman label and the metal surface in the open state of the KI67-MSC nanoprobe was estimated to be over 10 nm. As the plasmonic enhancement of the incident EM fields is the highest at the surface of the gold-coated nanowires (TSNWs) and decreases as the distance away from the metal surface, this distance is large enough to significantly reduce the SERS signal.

The MKI67-MSC nanoprobes were immobilized on a TSNW substrate by first incubating the substrate in a 1 µM probe solution containing 1M NaCl and 10 mM sodium phosphate buffer (pH 7.0) for two hours at room temperature and then rinsing unattached DNA probes thoroughly with deionized water. The functionalized substrate was next exposed to 1-mM 6-Mercapto-1-hexanol (MCH) for 5 min followed by rinsing thoroughly with deionized water to displace non-specifically adsorbed DNA probes and to passivate the gold surface.[47] The functionalized TSNW substrate was stored in 20 mM Tris-HCl buffer (pH 8.0) at 4 °C. To form the hairpin structure effectively, the MSC nanoprobe-coated TSNW substrate was incubated with 20 mM Tris-HCl buffer (pH 8.0)
containing 10 mM MgCl2 for at least 30 min at room temperature before SERS measurements.

The upper spectra (blank sample) in Figure 18 shows the intense SERS signal from the KI67-MSC nanoprobes immobilized on a gold nanowire sample region. The strong SERS intensity of the major Raman peaks indicates that the MSC nanoprobes remain in the stem-loop configuration (closed state) on the TSNW substrate. Detection of unlabeled Ki-67 DNA target sequence was performed by incubating the functionalized TSNW substrate in a sample solution containing 1 µM complementary DNA targets for one and a half hours to allow hybridization between the complementary ‘probe’ and KI-67 ‘target’ DNA. The SERS measurements were carried (using a laser beam spot of ~ 5 µm) out in the same region (each region having nanowires of a certain dimension and periodicity) and in the same section of the region before and after the incubation. As the variation of the SERS signals in each region (1 mm by 300 µm) was observed to be less than 5%, carrying out measurements around the same section in each region before and after the incubation gives reliable SERS measurements values for the cases when the target DNA molecules are detected or not. The lower spectra (positive diagnostics) in Figure 18 shows a decrease in SERS signal of the KI67-MSC nanoprobes after addition of the complementary KI-67 target DNA (indicated by arrow signs). Although preliminary studies reported here for the demonstration of the molecular sentinel on chip concept
involved 1µM detection of KI-67 DNA target, thorough measurements to determine the limit of detection of the MSCs will be carried out in future studies.

Figure 18: SERS spectra of the immobilized KI67-MSC nanoprobes in the presence or absence of complementary DNA targets. Upper spectrum: blank (no target DNA present). Middle spectrum: in the presence of 1 µM non-complementary DNA (negative control). Lower spectrum: in the presence of 1 µM complementary target DNA (positive diagnostic). The arrow signs illustrate the decreased SERS intensity of the major Raman bands in the presence of complementary target DNA.
The decreased SERS signal indicates that hybridization with the complementary target DNA molecules causes opening of the stem-loop structure of the probe DNA molecules (open state), thereby separating the SERS dye, TAMRA, away from the plasmonics-active gold-coated nanowire surface, where the EM enhancement of the incident optical fields is the highest. Moreover, following the opening of stem-loop structure of some of the probe DNA molecules upon hybridization with a complementary target molecule, the decrease of the overall SERS signal can also be attributed to the moving away of SERS dye molecules attached to some MSC probes from the SERS “hot spots” formed at the top and bottom regions in the gap between the gold-coated triangular nanowires. On the other hand, in the presence of non-complementary DNA (negative control: middle spectra in Figure 18), the SERS intensity of the major Raman bands remains high, indicating that the MSC nanoprobes remain in the stem-loop configuration (closed state). According to the SERS intensity shown in Figure 18, we estimated the SERS quenching efficiency to be 40–50 % of the original value upon hybridization of the KI67-MSC nanoprobe to the complementary target DNA. The remaining SERS intensity in the presence of the complementary target DNA may be contributed from those un-reacted nanoprobes located inside or near the high E-field regions on the TSNW substrate.
4.4 Summary

We have demonstrated the feasibility of SERS-based DNA detection using the molecular sentinel-on-chip nanoprobes on a well-controlled and reproducible TSNW array with sub-10 nm metallic nanostructures over an entire 6-inch wafer, thus enabling tuning the localized surface plasmon resonance wavelength of the nanostructures with the incident light. This new approach utilizing a novel nano-platform could lead to the development of a reliable and useful tool for molecular diagnostics, potentially having multiplexing and high-throughput screening capabilities - application of the MSC technology for these capabilities will be evaluated in future studies. With the reproducible and controllable substrate fabrication process on a very large area (6-inch to 12-inch wafers), the array-based SERS molecular sentinel-on-chip technology could contribute to the development of novel DNA diagnostic tools. The label-free MSC approach of combining plasmonic modulation with DNA hairpin probes allows the SERS measurements to be performed immediately following the hybridization reaction without washing steps, which greatly simplifies the assay procedures.
Chapter 5. Development of SERS-based plasmonic coupling interference (PCI) nanoprobes for nucleic acid detection\(^1\) (Aim 2.1)

5.1 Rationale

It has been observed that the EM field is particularly strong in the interstitial space between the particles in addition to the EM enhancement contributed from individual particles.\(^80\) It is believed that the anomalously strong Raman signal originates from “hot spots”, i.e., regions where clusters of several closely-spaced nanoparticles are concentrated in a small volume. The high-intensity SERS then originates from the mutual enhancement of surface plasmon local electric fields of several nanoparticles that determine the dipole moment of a molecule trapped in a gap between metal surfaces. This effect, also referred to as interparticle coupling or plasmonic coupling in a network of NPs, can provide a further enhancement effect besides the EM enhancement from individual particles. It has been reported that SERS enhancement from the “hot spots” between two or more nanoparticles (i.e. nanoparticle dimers, aggregates or nano-networks) can reach \(10^{11} - 10^{15}\) for resonant Raman dye molecules (such as rohdamine 6G), allowing single-molecule detection and making SERS highly competitive with fluorescence-based assays.\(^{73, 81, 82}\)

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“hot spots” is of the order $10^{11}$. Other enhancement processes such as the contribution of chemical effects are likely to induce further enhancement effects.[80] Depending on the SERS measurement conditions, the average SERS enhancement factor as high as $\sim 10^8$ have been obtained.[83, 84]

The calculation of the electromagnetic field in the gaps between metal nanoparticles under optical illumination has attracted interest in recent years because of the very large field enhancements induced in the particle gaps arising from surface plasmon resonances. The dimer is the prototypical example of a "hot spot" since it exhibits the key properties of EM enhancement in the gap between the particles and its dependence on the gap size relative to the particle size. For this reason the enhancement characteristics of nanoparticle dimers and the influence of plasmon resonances on this enhancement have been the subject of many studies.[85-91] Using a semi-analytical method to compute the electric field in the gaps between two spheres and between two spheroids over a range of frequencies indicates the occurrence of very large field enhancements in the gaps between nanoparticles.[92] For a pair of 20 nm silver nanospheres with a 2.5 nm gap, a total SERS enhancement of over $10^7$ was estimated at the peak of the plasmon resonance.[86] A spatially averaged enhancement will, of course, be much less than this peak value. Two different numerical methods have been used to compare the calculations of the electric field at a point in the gap midway between the two silver nanoparticles.[70] The first calculation was performed using the
FEM-based commercial software package Comsol Multiphysics and the second was a semi-analytical solution based on a multipole expansion (ME) of the fields. The results, which demonstrate that the agreement between the Comsol and ME calculations is within 5% across all geometries with respect to amplitude, wavelength offset and plasmon resonance bandwidth, showed very strong plasmonic coupling enhancement in the gap regions between the nanoparticles.

It has been observed that metal nanoparticles usually aggregate in a high ionic strength salt solution. However, this aggregation process is generally uncontrollable, thus seriously affecting the reproducibility of SERS measurements. To practically control the separation distance between particles, DNA oligonucleotides have been utilized as spacers and linkers to assemble nanoparticles into a network of NPs in a controllable manner.[25, 26, 93] Moreover, DNA sequences of interest have been designed as the interparticle linkers to induce the plasmonic coupling effect leading to an increased SERS signal of the Raman reporters absorbed on the Au nanoparticle surface.[94, 95]

Herein, we have developed a new approach referred to as plasmonic coupling interference (PCI) for DNA/RNA detection using SERS. The PCI method described here combines the plasmonic coupling phenomenon with the nucleic acid hybridization process, leading to the development of a label-free (i.e., the targets do not need to be labeled) detection approach for DNA/RNA target. The PCI method utilizes specific DNA sequences of interest to induce interference in the plasmonic coupling effect. As a result,
the reduction in SERS intensity of Raman-labeled DNA probes can be used as a parameter for a new biosensing modality.

5.2 Operating Principle of the PCI Approach for Nucleic Acid Detection

The operating principle of the PCI detection strategy using functionalized Ag NPs is illustrated in Figure 19. In this approach, we first aim to couple nanoparticles using the shortest separation distance in order to induce a strongest plasmonic coupling and a maximum SERS enhancement of a Raman label located between two adjacent silver nanoparticles, Previous studies have shown that nanoparticles can be coupled using DNA oligonucleotides with over 8 bases.[26] However, due to the thermal instability of short DNA-DNA duplexes, it is difficult to use DNA oligonucleotides shorter than 8 bases for assembling nanoparticles into a nano-network. To overcome this problem, we utilized short locked nucleic acids (LNAs) with 7 bases in order to couple nanoparticles in a separation distance between 2 to 3 nm. It has been previously reported that LNAs can offer a high salt and thermal stability for coupling nanoparticles.[96, 97] Nonetheless, longer LNA or DNA oligonucleotides were also tested in this study, e.g. 17-base probes for SNP detection in Section 5.5, and 22-base probes for miRNA detection in Section 6.2.

As shown in Figure 19a, Ag NPs are first functionalized with 0.5-µM thiolated LNAs with the sequence of 5’-dithiol-\textbf{GGGCGG}-3’ (referred to as capture-NPs with LNA bases underlined) or the complementary dye-labeled probes with the sequence of
3'-CCCG(Cy3)CCC-dithiol-5’ (referred to as reporter-NPs). A Raman dye, Cy3, is used as the signal reporter, which is internally attached to the guanine (G-base) in the middle of the reporter oligonucleotide. However, the labeling site is not restricted to the middle of the oligonucleotides. The Raman dye can also be labeled at the 3’-end. These functionalized NPs (both capture-NPs and reporter-NPs) are then further conjugated with low molecular weight thiolated poly(ethylene glycol)s (HS-PEGs). It has been indicated that short PEGs can provide the Ag NPs stability in sodium phosphate buffer solution containing 100 mM NaCl. [95] The number of labeled-oligonucleotides immobilized on a Ag NP was estimated to be ~220 oligonucleotides per particle for the 7-base dithiol probes, ~130 oligonucleotides per particle for the 17-base dithiol probes described in Section 5.5, and ~300 oligonucleotides per particle for the 13-base alkanethiol probes described in Section 6.2.
Figure 19: Scheme showing the preparation of functionalized Ag nanoparticles (NPs) and the operating principle of the detection strategy. (a) Ag NPs are functionalized with thiolated LNAs (capture-NPs) or complementary DNA probes labeled with Cy3 Raman dye (reporter-NPs). (b) Plasmonic coupling between adjacent NPs is induced by the formation of LNA/DNA-probe duplexes which couple NPs in a short separation distance. (c) Plasmonic coupling is interfered by the formation of LNA-target DNA duplexes. Due to the same sequences of the reporter-probes and the target DNA, target DNA strands compete with reporter-NPs for binding to capture-NPs.

The detection strategy of the PCI method is schematically shown in Figures 19b and 19c. To induce plasmonic coupling effect, capture-NPs and reporter-NPs are mixed in a volume ratio of 1:1 in order to form LNA-DNA duplexes. The mixture is allowed to react at room temperature for 20 min in a 10-mM Tris-HCl buffer solution (pH 8.0) containing 50 mM NaCl and 2.5 mM MgCl₂. The duplex formation assembles
nanoparticles into a three-dimensional nano-network of NPs having the Cy3 label located between adjacent NPs (Figure 19b). In this situation the Cy3 label molecules experience a strong plasmonic coupling effect, leading to an increased SERS signal of the Raman labels upon laser excitation. Figure 19c depicts the mechanism for the detection of particular nucleic acid sequences (target DNA). In this approach, the sequences of DNA probes (reporter-NPs) are designed to have the same sequence as the DNA targets. Therefore, the target DNA strands are then used as competitors of the reporter-NPs in a competitive binding process. As a result, the SERS signal is not enhanced as in Figure 19b since the plasmonic coupling is interfered by the target strands.

5.3 Demonstration of the PCI-based Nucleic Acid Detection

Figure 20 shows the increased SERS intensity of the Cy3 Raman peaks in the presence of both capture-NPs and reporter-NPs (upper spectrum: blank) as compared to the SERS intensity in the presence of target DNA strands (lower spectrum). The enhanced SERS signal indicates that the plasmonic coupling was induced by the hybridization reaction between the LNA and the labeled-DNA strands. To demonstrate the detection of DNA by using the concept of plasmonic coupling interference, capture-NPs and target DNA strands (1 μM) were mixed 1 hour prior to addition of a solution of reporter-NPs in order to ensure that the target DNA can effectively react with LNA strands. After adding reporter-NPs, the mixture was allowed to react for 20 min at room temperature and immediately followed by SERS measurements without washing steps.
Figure 20: SERS spectra of the Cy3 Raman peaks in the presence or absence of target DNA strands. Upper spectrum: in the presence of both capture-NPs and reporter-NPs (blank: without target DNA present). Lower spectrum: in the presence of 1 µm target DNA strands in the mixture of capture-NPs and reporter-NPs. Inset: photographs of sample solutions showing the dramatic color change in the absence of target DNA.

The lower spectrum in Figure 20 shows the quenched SERS signal in the presence of target DNA strands in the mixture of capture-NPs and reporter-NPs, thus indicating that the plasmonic coupling effect was interfered in the presence of target DNA strands. Noteworthy is a dramatic color change from greenish yellow to clear-grey observed over the course of 20 min indicating that Ag NPs were aggregated in the blank sample, but not in the presence of target DNA strands (as shown in the inset of Figure 20). Time-dependent monitoring of the nucleic acid-cross-linked Ag-NP aggregates was
performed using the absorption spectroscopy by mixing capture-NPs and reporter-NPs (Figure 21). After a 10 min reaction time, it was found that the surface plasmon band was red-shifted from ~410 to ~700 nm, indicating that Ag NPs were aggregated via nucleic acid hybridization. This result indicates the potential use of the PCI approach as a simple and rapid screening tool based on simple visual examination of color changes of the sample.

Figure 21: Time-dependent monitoring of the nucleic acid-cross-linked Ag-NP aggregates. The decrease in the absorbance of the surface plasmon band at ~ 410 nm indicates that the aggregation of AgNPs occurs by mixing capture-NPs and reporter-NPs in a hybridization buffer solution.
5.4 Possibility for Quantitative Analysis

The capability for quantitative analysis is an important feature of any bioanalytical technique. In this work we further investigated the quantitative aspects of this approach. In a series of measurements, we kept the concentrations of the capture-NPs and reporter-NPs constant and varied the concentration of target DNA (competitors) from 0 to 1 µM. Figure 22 shows that the SERS intensity of reporter-NPs at 1195 cm\(^{-1}\) decreased with increasing target DNA concentrations indicating that plasmonic coupling effect between capture-NPs and reporter-NPs was interfered to different degrees by changes in the concentration of target DNA competitors. The minimum target DNA concentration that can cause detectable interference in the current unoptimized system is about 200 pM with the dynamic range from 200 pM to 100 nM. Although the application of plasmonic coupling interference for quantitative DNA analysis requires further investigation, the results of this study demonstrate the potential of this PCI technique for quantitative nucleic acid diagnostics.
Figure 22: SERS spectra showing the major Raman peak of Cy3 label at 1195 cm\(^{-1}\) with varying concentrations of target DNA strands from 0 to 1 µm. The concentrations of capture-NPs and reporter-NPs were kept constant in a series of measurements.

5.5 Application of the PCI Technique for Single-Nucleotide Polymorphism (SNP) Detection

Single-nucleotide polymorphisms (SNPs) are the most common genetic variations which could contribute to disease risk by creating genetic instability. To demonstrate the biomedical application of the PCI technique for SNP detection, a pair of capture-NPs and reporter-NPs with the sequence of 5’-dithiol-A\(_{10}\)-GACGGAC-3’ (Val654-capture with LNA bases underlined) and 3’-Cy3-CTGCCTG-A\(_{10}\)-dithiol-5’
(Val654-reporter with Cy3 labeling at 3’-end), respectively, were designed to detect a rare single-nucleotide polymorphism (SNP) (Ile654Val) of the ERBB2 gene. The ERBB2 gene (also known as HER2/neu) belonging to the epidermal growth factor receptor (EGFR) family is well known as a critical biomarker for breast cancer. The Ile654Val SNP linked to another more frequent Ile655Val SNP resides within the transmembrane domain. [98] It has been suggested that the rare ERBB2 variant Ile654Val is associated with an increased familial breast cancer risk. [99] However, due to the close vicinity of the Ile654Val and Ile655Val SNPs, it is difficult to detect the Ile654Val SNP by using DNA probes such as TaqMan probes. To overcome this difficulty, the 7-base LNA (Val654-capture) complementary to the Ile654Val SNP sequence was used to increase single nucleotide discrimination. As shown in the lower spectrum in Figure 23, the reduction in SERS intensity indicates that the plasmonic coupling effect was significantly interfered in the presence of 10 nM SNP sequences (lower spectrum: 5′-GTCCGTC-3’). However, in the blank sample (upper spectrum) or in the presence of 10 nM wild-type sequences (middle spectrum: 5′-GTCCATC-3’), the SERS signals were enhanced in both cases indicating that the plasmonic coupling effect was induced through the formation of nano-networks composed of the Val654-capture-NPs and Val654-reporter-NPs. The result of this study demonstrates that the PCI technique can be used as a novel tool to identify significant SNPs for medical diagnostics.
Figure 23: ERBB2 Ile654Val SNP detection using PCI nanoprobes. Upper spectrum: blank sample containing a mixture of Val654-capture-NPs and Val654-reportor-NPs. Middle spectrum: in the presence of 10 nm wild-type sequences. Lower spectrum: in the presence of 10 nM SNP sequences.

5.6 Summary

In summary, we have reported a label-free approach for SERS-based nucleic acid detection with a novel PCI detection mechanism that utilizes target oligonucleotides as competitive binding products to interfere with plasmonic coupling effect in nanonetworks. In this study, 7-base LNAs were developed and used as a proof of concept to
demonstrate the capability for coupling nanoparticles in a very short separation distance with high thermal stability. In this paper, we have demonstrated that this PCI approach can be used as a simple and rapid screening tool for cancer diagnosis to identify and discriminate DNA sequences with SNPs. Furthermore, the highly specific and narrow SERS spectral peaks could allow multiple assays to be performed simultaneously in a single sample solution when using multiple Raman labels. There is a strong need to develop nucleic acid bioassays that are label-free and can be used in a variety of applications ranging from biomedical diagnostics, food safety, environmental monitoring and homeland defense. The initial results of this study lay the foundation for the development of novel nucleic acid diagnostic tools that could have potential for a wide variety of applications based on nucleic acid detection to address the above sensing needs.
Chapter 6. Demonstration of the use of SERS-based PCI nanoprobes for label-free direct detection of microRNAs for cancer diagnostics (Aim 2.2)

6.1 Rationale

MicroRNA (miRNA) is a class of small noncoding endogenous RNA molecules (18-24 nucleotides) found in almost all species. Currently, thousands of miRNAs have been identified in plants, animals and viruses.[100, 101] It is estimated that up to 1000 genes in the human genome encode miRNAs. Moreover, it has been indicated that many miRNAs are highly conserved in closely related species and some of them are conserved in distantly related species, e.g. conserved in Caenorhabditis elegans and humans.[101, 102] This observation implies their functional importance. Recent studies have shown that miRNAs play an important role in the regulation of gene expression either, directly through cleavage of specific messenger RNA (mRNA) molecules (post-transcriptional regulation), or indirectly, through translational repression by binding to the 3’ untranslated region (3’UTR) of their target mRNAs.

It has been shown that miRNAs are involved in a variety of critical biological processes including embryonic development, cell differentiation and proliferation, apoptosis, metabolism and tumorigenesis.[103-105] Moreover, alterations in the expression levels of a single or multiple miRNAs have been shown to be linked with cancer types, disease stages and response to treatment.[106]
In recent years, there has been great interest in the development of new methods for the detection of miRNAs including northern blotting, microarray analysis and qRT-PCR. However, the short length of mature miRNAs makes detection more difficult than the detection of genomic DNA or messenger RNA (mRNA). To date, the most standardized and widely used method for the direct detection of miRNAs is northern blotting, which is laborious and time-consuming. Thus, there is a strong need to develop a simple, rapid, selective and sensitive method to detect miRNA molecules.

6.1.1 MicroRNAs as novel biomarkers for breast cancer

Breast cancer is a complex, phenotypically diverse genetic disease, involving a great deal of changes in gene expression and structure. Recent advances in molecular profiling technologies have made significant progress in resolving the molecular taxonomy of breast cancer, which has shed new light on the etiology of the disease and also holds great potential for the development of novel diagnostic biomarkers and therapeutic targets. Because of this, miRNAs have generated great interest in the clinical and scientific communities. The recent discovery that miRNA expression is frequently dysregulated in cancer has revealed an entirely new repertoire of molecular factors upstream of gene expression, which warrants extensive investigation to further elucidate their precise role in malignancy [107, 108]. Recent discovery of quantifiable circulating cancer-associated miRNAs indicates great potential for their use as novel, minimally invasive biomarkers for breast cancer diagnostics [109-112]. Fast and precise
measurement of miRNAs will help address miRNA function, their putative role as oncogenes or tumor suppressors, and their potential role in breast cancer management, particularly in improving current prognostic tools.

There is an increasing need in development of nanoprobes for detection of multiple nucleic acid biomarkers associated with breast cancer risk in patient cells. For instance, aggressive triple-negative breast cancers have been shown to have loss of expression of miR-200, 203, 149 family members and gain of miR-21;[113, 114] these changes are associated with epithelial mesenchymal transition (EMT) and cancer stem cell (CSC) regeneration. The miRNA expression profiles may serve as useful tests for cancer and disease diagnostics.

6.1.2 MicroRNAs as novel biomarkers for gastrointestinal cancer

An increasing number of studies have investigated the diagnostic and prognostic values of miRNAs in gastrointestinal cancer. For instance, Ueda et al. identified 22 upregulated and 13 downregulated miRNAs in gastric cancer versus non-tumor mucosa after analyzing the expression of 237 miRNAs in 353 gastric samples by microarray.[115] Eighty-three percent of the samples could be distinguished correctly by this signature. A recent study analyzed the miRNA expression profile in 100 gastric cancer patients and identified a seven-miRNA signature (miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p and miR-126) that could be used as an independent predictor for overall survival and relapse-free survival shown by multivariate analysis.[116]
Development of sensitive and specific biomarkers for gastrointestinal cancer will improve current management of the malignant disease including cancer early detection, differentiation, progression and recurrence monitoring, and treatment response evaluation. The advantages of using miRNAs as biomarkers are as follows: (a) miRNAs are involved in tumorigenesis; (b) miRNAs are tissue-, tumor- or even pathology-specific; and (c) some miRNAs are related to treatment response or patients’ survival.

MiRNAs could also serve as minimally invasive biomarkers. Recent studies have shown that tumor-derived miRNAs are present in circulation in stable form and the levels of circulating miRNAs are detectable and quantifiable with current available methods. A recent study examined the concentration of tumor-derived miRNAs in patients with gastric cancer to assess their clinical application for diagnosing and monitoring diseases. They showed that levels of four miRNAs (miR-17-5p, miR-21, miR-106a, miR-106b) were significantly elevated and let-7a was lowered in gastric cancer patients, with miRNAs being significantly reduced in postoperative samples.[117]

6.2 PCI nanoprobe design and demonstration for miRNA detection

To demonstrate the detection of miRNAs using the PCI technique, a pair of unlabeled complementary probes (capture-(miR21)-NPs) and Cy3-labeled probes (reporter-(miR21)-NPs) with the sequences 5’-SH-TCAACATCAGTCTGATAAGCTA-3’ and 5’-SH-TAGCTTATCAGACy3-3’, respectively, were designed to detect the mature human miRNA-21 (miR-21) molecules with sequences of 5’-UAGCUUAUCAG-
ACUGAUGUUGA-3’. It has been shown that miR-21 functions as an oncogene and is overexpressed in a variety of different tumors including breast cancer and gastrointestinal cancer.[118-120] The unlabeled capture-(miR21)-probes are fully complementary to the mature miR-21 sequences, and the Cy3-labeled reporter-(miR21)-probes are complementary to a partial sequence of the unlabeled capture-(miR21)-probes. The melting temperature for the duplex of the capture-(miR21)-probes and Cy3-labeled reporter-(miR21)-probes is estimated at 33.4 °C in a 50-mM NaCl solution. Thus, the hybridization-mediated nanoparticle aggregation could be expected to take place at room temperature. As a proof of concept, we tested the PCI technique for the miR-21 detection using synthesized miRNA with sequence of 5’-UAGCUUAUCAGACUGAUG-UUGA-3’ as the target molecules. As shown in the lower spectrum in Figure 24, the reduction in SERS intensity indicates that the plasmonic coupling effect was significantly interfered in the presence of 100 nM complementary miR-21 targets. However, in the absence of target miRNA sample (upper spectrum: blank sample) or in the presence of 100 nM non-complementary DNA samples with sequence of 5’-TCATCCATGACAACT-TTGGTATCGTGGAAAGGACTCATGAC-3’ (middle spectrum: negative control), the SERS signals were enhanced in both cases indicating that the plasmonic coupling effect was induced through the aggregated nanoparticles. The result of this study demonstrates that the PCI technique can be used as a novel tool to detect miRNA molecules for medical applications.
Figure 24: Demonstration of the miRNA detection using PCI nanoprobes and synthetic miRNA targets. Upper spectrum: blank sample containing a mixture of capture-(miR21)-NPs and reporter-(miR21)-NPs. Middle spectrum: in the presence of 100 nm non-complementary DNA sample as the negative control test. Lower spectrum: in the presence of 100 nm complementary miRNA sample.

6.3 Evaluation of the Stability of PCI Nanoprobes

To improve the stability of the nanoprobes in high ionic-strength salt solutions, different polyethylene glycol (PEG) polymers were used to functionalize AgNP surfaces. Figure 25 shows the UV-Vis spectra of three different PEGylated nanoprobes incubated in six Tris buffer solutions containing different concentrations of NaCl (50, 150 and 300 mM) and MgCl₂ (2.5 and 5 mM) for 2 hours at room temperature. We found that the
PEGylated nanoprobes with a mixture of 100 µM thiolated PEG-COOH (low-molecular-weight PEG thiol acid, MW 458.56) and 2 µM thiolated long-chain PEG polymer (mPEG-5000) appeared to be highly stable in Tris buffer containing 0.15 M NaCl and 2.5 mM MgCl₂. Increasing the concentration of mPEG-5000 to 5 µM with the same concentration of PEG-COOH can further increase the stability. However, the hybridization efficiency was significantly decreased due to the long chain of mPEG-5000 (Figure 26). Thus, a mixture of 100 µM PEG-COOH and 2 µM mPEG-5000 was used to prepare nanoprobes throughout this study.
Figure 25: Evaluation of the stability of three different PEGylated nanoprobes by measuring the UV-Vis spectra in six different salt buffer solutions (2-hr incubation at room temperature) at different ionic strength. Nanoprobes were functionalized with (a) 100 µM PEG-COOH (low-molecular-weight PEG thiol acid, MW 458.56), (b) 100 µM PEG-COOH and 2 M mPEG-5000, (c) 100 µM PEG-COOH and 5 µM mPEG-5000. The nanoprobes functionalized with a mixture of PEG-COOH and mPEG-5000 appeared to be highly stable in Tris buffer containing up to 0.15 M NaCl and 2.5 mM MgCl₂ for (b) nanoprobes, and up to 0.3M NaCl and 5 mM MgCl₂ for (c) nanoprobes. Buffer 1: 50 mM NaCl and 2.5 mM MgCl₂, Buffer 2: 50 mM NaCl and 5 mM MgCl₂, Buffer 3: 150 mM NaCl and 2.5 mM MgCl₂, Buffer 4: 150 mM NaCl and 5 mM MgCl₂, Buffer 5: 300 mM NaCl and 2.5 mM MgCl₂, Buffer 6: 300 mM NaCl and 5 mM MgCl₂.
Figure 26: Time-dependent monitoring of the aggregation (hybridization) efficiency of two different PEGylated nanoprobes functionalized with (a) 100 µM PEG-COOH and 2 M mPEG-5000, (b) 100 µM PEG-COOH and 5 µM mPEG-5000 in a Tris-Salt buffer solution containing 150 mM NaCl and 2.5 mM MgCl₂. When using the (b) nanoprobes, the hybridization efficiency was significantly affected by the long chain of mPEG-5000 on the particle surface.
6.4 Effect of Reaction time and aggregate size on SERS intensity

To investigate the effect of reaction time on SERS intensity, a time-dependent monitoring (0 – 60 min) of the SERS signal was carried out using the nanoprobes prepared with a mixture of 100 µM PEG-COOH (low molecular weight PEG) and 2 µM mPEG-5000 described in the previous section. As shown in Figure 27, over the 60-min measurement period, it was found that the SERS signal increased over time indicating that the aggregation is a continuous process.

Figure 27: Investigation of the effect of reaction time on SERS intensity by time-dependent monitoring (0 – 60 min) of the SERS signal of PCI nanoprobes incubated in a Tris-Salt buffer solution containing 150 mM NaCl and 2.5 mM MgCl₂ at room temperature. The result showed that the SERS signal increased over time indicating that the aggregation is a continuous process within 1 hr.
To investigate the corresponding aggregate size responsible for the increased SERS signal, the size of the nanoprobes (a mix of capture and reporter probes) in a hybridization buffer solution was then monitored over time (0 – 60 min) at room temperature using NanoSight NS500 (NanoSight Ldt. Amesbury, UK). As shown in Figure 28, we found that large aggregates (100 – 200 nm) significantly increased over time consistent with the finding in Figure 27.

Figure 28: Investigation of the effect of aggregate size on SERS intensity by time-dependent monitoring (0 – 60 min) of the size of PCI nanoprobes incubated in a Tris-Salt buffer solution containing 150 mM NaCl and 2.5 mM MgCl₂ at room temperature. Large aggregates (100 – 200 nm) were found and significantly increased during the measurement period.
6.5 Multiplex capability of PCI nanoprobes

6.5.1 Two-mix PCI nanoprobes

The multiplexing capability of the PCI technique was first demonstrated by mixing two different sets of PCI nanoprobes to detect miR-21 and let-7a sequences. It has been shown that the let-7a microRNA regulates estrogen receptor alpha signaling in estrogen receptor positive breast cancer. [121] The reporter-NP for miR-21 was labeled with Cy3 dye, and the reporter-NP for let-7a was labeled with 5-carboxy-tetramethyl-rhodamine (TAMRA) dye. The (a) spectrum in Figure 29 represents the SERS spectrum of the mixture of the two sets of the PCI nanoprobes. To demonstrate the specificity and selectivity of the composite PCI nanoprobes, the hybridization assays were performed in the presence of individual complementary target sequences (i.e. only one of the two complementary targets). The (b) and (c) spectra show the resulting SERS spectra from the composite PCI nanoprobes targeted to 100 nM targets complementary to miR-21 and let-7a PCI nanoprobes, respectively. The result indicates that only the SERS peaks associated with the complementary PCI nanoprobes was significantly reduced when in the presence of its target sequences. The (d) spectrum in the figure shows that the SERS intensities of all major Raman peaks were greatly decreased in the presence of both targets.
Figure 29: (a) SERS spectrum of the mixture of the two sets of the PCI nanoprobes. (b) and (c) SERS spectra show the resulted SERS signal from the composite PCI nanoprobes targeted to 100 nM targets complementary to miR-21 and let-7a PCI nanoprobes, respectively. (d) SERS spectrum shows that the SERS intensities of all major Raman peaks were greatly decreased in the presence of both targets. The major Raman bands from miR-21-PCI nanoprobes are marked with black number, and the major Raman bands from let-7a-PCI nanoprobes are marked with red number with (*) sign. The arrow signs illustrate the decreased SERS intensities of the major Raman bands in the presence of corresponding target miRNAs.
6.5.2 Four-mix PCI nanoprobes

To further demonstrate the multiplexing capability of the PCI technique for more than two targets, four sets of nanoprobes were prepared for miR-149, miR-125b, let-7a and miR-21. The reporter probes were labeled differently with TYE563, Rhodamine Red, TAMRA and Cy3, respectively. Figure 30 shows the corresponding SERS spectra of the four individual nanoprobes (absence of target sequences). The major SERS peaks for each set were marked with the corresponding wavenumbers. As can been seen, the unique SERS peaks (marked with asterisks) for each nanoprobe can be identified at 1383 cm\(^{-1}\) for miR-149-nanoprobe, 731 cm\(^{-1}\), 1283 cm\(^{-1}\) and 1360 cm\(^{-1}\) for miR-125b-nanoprobe, 502 cm\(^{-1}\), 628 cm\(^{-1}\), 1217 cm\(^{-1}\) and 1354 cm\(^{-1}\) for let-7a-nanoprobe, and 1196 cm\(^{-1}\) for miR-21-nanoprobe.

Figure 30: SERS spectra of the four different nanoprobes labeled differently with TYE563, Rhodamine Red, TAMRA and Cy3 for the detection of miR-149, miR-125b, let-7a and miR-21, respectively. The major SERS peaks for each label were marked with the corresponding wavenumbers, and the unique SERS peaks for each nanoprobe were marked with asterisks.
These four sets of nanoprobes, miR-149, miR-125b, let-7a, and miR-21, were then mixed in a volume ratio of 2:1:1:1, respectively, for multiplex detection. Figure 31a shows the resulting composite SERS spectrum obtained from the mixture. The unique SERS peaks for each nanoprobe were easily identified in the composite spectrum, and marked with numbers 1 to 4 for miR-149, miR-125b, let-7a and miR-21 nanoprobes, respectively. To demonstrate multiplexed detection of miRNAs, the hybridization assays were performed in the presence of individual complementary target sequences. Figures 31b-e show the resulting SERS spectra in the presence of 100 nM miR-149, miR-125b, let-7a and miR-21, respectively. The result indicates that only the SERS peaks associated with the corresponding PCI nanoprobes were significantly reduced when in the presence of the target sequences. The reduction can be easily observed at the unique SERS peaks for each nanoprobe. The results of this study demonstrated that the PCI technique has great potential for the detection of multiple miRNAs simultaneously without washing or separation steps, and could be a useful diagnostic tool for multiplexed miRNA detection.
Figure 31: Multiplexed detection of 4 miRNAs using 4-mix PCI nanoprobes (a) in the absence of targets (blank), or in the presence of 100 nM of (b) miR-149 targets, (c) miR-125b targets, (d) let-7a targets, and (e) miR-21 targets. The result showed that the unique SERS peaks associated with the corresponding PCI nanoprobes were significantly reduced (arrow signs) when in the presence of the target sequences.
6.6 Discrimination between mature miRNAs and pre-miRNAs

In chapter 6.2, we have used a synthetic RNA corresponding to the mature form of hsa-miR-21 as test target and a synthetic non-complementary DNA to assess the specificity of the miR21-nanoprobes. Here, we further investigated the capability of the PCI method to differentiate between mature miR-21 and its non-active stem-loop precursor (pre-miR-21). It has been a challenge for the direct detection of mature miRNAs in clinical samples using hybridization methods, because when a sample containing both pre-miRNAs and mature miRNAs is studied, short oligonucleotide probes can also non-specifically bind to the complementary region of the pre-miRNAs resulting in false positive results.[104] As shown in Figure 32, the SERS signal remained high in the presence of 100 nM synthetic 60-mer DNA (spectrum a) corresponding to a portion of pre-miR-21 sequences. However, in the presence of 100 nM mature miR-21 sequences (spectrum b), the SERS signal was significantly reduced comparing to the pre-miR-21 sample (spectrum a) and blank sample (spectrum c). This result indicates that the PCI method can accurately discriminate mature miRNAs from their pre-miRNAs. This ability could be attributed to the absence of spacers between oligonucleotide probes and nanoparticle surfaces, so that pre-miRNAs with longer sequences cannot be hybridized to the capture probes.
Figure 32: The PCI technique can distinguish pre-miRNAs from mature miRNAs. SERS spectra of the miR-21 PCI nanoprobes in the presence of (a) 100 nM pre-miR-21 sequences, (b) 100 nM mature miR-21 sequences, and (c) in the absence of complementary sequences (blank).

6.7 Control of aggregation using stoppers

To control the aggregation of nanoprobes, a stopper complementary to a partial sequence of the capture probes was designed. As shown in Figure 33, the stopper solution is added at a time after initiation of nanoprobe aggregation. The stoppers work as competitors to the reporter probes. Thus, the aggregation stops when all or most of the capture probes are bound to the stoppers. Figure 34 shows the reduction of the absorbance at 416 nm was stopped at 30 min after addition of a 1 µM stopper solution (comparing to Figure 26a). This strategy can minimize the effect of reaction time on the
signal changes during multiple SERS measurements (i.e. the SERS signal increased over time as discussed in Section 6.4).

Figure 33: Schematic showing the control of nanoprobe aggregation using stoppers. The stoppers work as competitors to the reporter probes, and stop the aggregation when all or most of the capture probes are bound to the stoppers.

Figure 34: Effectiveness of use of the stopper solution in controlling aggregation. Time-dependent monitoring of the nanoprobe aggregation by measuring the UV-Vis spectra before and after the addition of a stopper solution shows that the aggregation was stopped at 30 min after the addition of the stoppers.
6.8 Direct miRNA detection for breast cancer diagnostics

To demonstrate the potential of using the PCI technique to detect miR-21 for breast cancer diagnostics, a blind study was performed with two enriched small RNA samples extracted from two different breast cancer cell lines. To improve the detection sensitivity for small amount of enriched small RNA (50~100 ng), 5 µL sample volume was used with ~ 5 pM nanoprobes for the blind trial. The reaction was carried out at 37 °C for 30 min followed by addition of a PCI-stopper solution (10 µM) to control the aggregation as described in the previous section. The sample was then loaded into a glass capillary tube to prevent or slow down the evaporation of the sample solution during the SERS measurement. Figures 35 and 36 show that the characteristic Raman peaks of the nanoprobes obtained in the presence of sample #1 (green columns) were found to be less intense than those obtained in the presence of sample #2 (blue columns) in both 100 ng (A) and 200 ng (B) tests. The result indicates that the miR-21 expression in sample #1 was relatively higher than that in sample #2. This result was consistent with the result of qRT-PCR showing that the miR-21 expression in sample #1 obtained from cell line SUM149PT was approximately 3-fold higher than that in sample #2 obtained from cell line AU565.
Figure 35: A blind study showing the direct detection of miR-21 using PCI nanoprobes with (A) 100 and (B) 200 ng enriched small RNA extracted from two breast cancer cell lines: blank (red columns); sample #1 (green columns) obtained from cell line SUM149PT; sample #2 (blue columns) obtained from cell line AU565. The characteristic Raman peaks of the nanoprobes obtained in the presence of sample #1 were found to be less intense than those obtained in the presence of sample #2 in both 100 ng (A) and 200 ng (B) tests, indicating that the expression level of miR-21 was relatively higher in sample #1 than in sample #2. Insets: corresponding SERS spectra measured in the presence or absence of RNA samples.
Figure 36: Relative SERS Decrease Index showing that the SERS intensities of the characteristic Raman peaks of the nanoprobes obtained in the presence of RNA sample #1 from breast cancer cell line SUM149PT (having higher expression of miR-21) were decreased more than those obtained in the presence of RNA sample #2 from breast cancer cell line AU565 in both 100 ng (A) and 200 ng (B) tests. The decrease index was calculated as the ratio of the decreased SERS intensity in the presence of the test samples to the SERS intensity of the blank.
6.9 Direct miRNA detection for gastrointestinal cancer diagnostics in patient biopsies

To demonstrate the potential of using the PCI technique to detect miR-21 for gastrointestinal cancer diagnostics, a blind study was performed with 500 ng of enriched small RNA extracted from two endoscopic biopsies (samples #4929 and #170) from patients evaluated in Duke gastroenterology clinic who agreed to donate tissue for research purposes. The assay was carried out at room temperature for 1 hr using ~ 0.1 nM PCI nanoprobes (100 µL sample volume) and 500 ng of RNA samples. As shown in Figures 37 and 38, the SERS intensity obtained from sample #170 was less than that obtained from sample #4929 in the presence of 500 ng of RNA from sample #170 (esophageal adenocarcinoma) than that from sample #4929 (control healthy squamous esophageal tissue). In other words, the expression level of miR-21 was relatively higher in sample #170 than in sample #4929. This result was consistent with the result of qRT-PCR (Figure 39) showing that the miR-21 expression in sample #170 obtained from esophageal adenocarcinoma was approximately 4.24-fold higher than that in sample #4929 obtained from the control squamous esophageal tissue.
Figure 37: A blind study showing the direct detection of miR-21 using PCI nanoprobes with 500 ng enriched small RNA extracted from two endoscopic biopsies from patients: blank (red columns); sample #170 – RNA extracted from esophageal adenocarcinoma (green columns); sample #4929 – RNA extracted from a control healthy squamous esophageal tissue (blue columns). The characteristic Raman peaks of the nanoprobes obtained in the presence of sample #170 were found to be less intense than those obtained in the presence of sample #4929, indicating that the expression level of miR-21 was relatively higher in sample #170 than in sample #4929. Insets: corresponding SERS spectra measured in the presence or absence of RNA samples.
Figure 38: Relative SERS Decrease Index showing that the SERS intensities of the characteristic Raman peaks of the nanoprobes obtained in the presence of 500 ng RNA sample #170 from esophageal cancer tissue (having higher expression of miR-21) were decreased more than those obtained in the presence of 500 ng RNA sample #4929 from control normal tissue. The decrease index was calculated as the ratio of the decreased SERS intensity in the presence of the test samples to the SERS intensity of the blank.
Figure 39: qRT-PCR analysis of the relative expression levels of miR-21 in the patient samples #4929 and #170, showing that the miR-21 expression in sample #170 was ~4.24-fold higher than that in sample #4929.

To further improve the detection sensitivity for smaller amounts of enriched small RNA (50~100 ng), the final sample volume was reduced from 100 µL to 5 µL with ~5 pM nanoprobes for second trial. The same assay procedure described in the previous section using 5 µL of sample volume and capillary tubes for SERS measurements was followed in this study. Figures 40 and 41 show that the SERS intensity obtained from sample #202 (cancer tissue) was less than that obtained from sample #5176 (non-cancer tissue) in both 50 ng (a) and 100 ng (b) tests. The result indicates that the miR-21 expression in sample #202 was relatively higher than that in sample #5176. This result was consistent with the result of qRT-PCR (Figure 42) showing that the miR-21 expression in sample #202 was approximately 12-fold higher than that in sample #5176.
Figure 40: A blind study showing the direct detection of miR-21 using PCI nanoprobes with (A) 50 and (B) 100 ng enriched small RNA extracted from two endoscopic biopsies from patients: blank (red columns); sample #202 (green columns); sample #5176 (blue columns). The characteristic Raman peaks of the nanoprobes obtained in the presence of sample #202 were found to be less intense than those obtained in the presence of sample #5176, indicating that the expression level of miR-21 was relatively higher in sample #202 than in sample #5176. Insets: corresponding SERS spectra measured in the presence or absence of RNA samples.
Figure 41: Relative SERS Decrease Index showing that the SERS intensities of the characteristic Raman peaks of the nanoprobes obtained in the presence of RNA sample #202 from esophageal cancer tissue (having higher expression of miR-21) were decreased more than those obtained in the presence of RNA sample #5176 in both 50 ng (A) and 100 ng (B) tests. Sample #5176 was from squamous tissue from a patient with chronic gastroesophageal reflux disease but did not have Barrett's esophagus or esophageal cancer. The decrease index was calculated as the ratio of the decreased SERS intensity in the presence of the test samples to the SERS intensity of the blank.
6.9 Summary

In summary, we have demonstrated the direct detection of miRNAs using PCI nanoprobes, as well as the capability to discriminate mature miRNAs from their non-active precursors, pre-miRNAs, and the multiplexing capability using two-mix or four-mix PCI nanoprobes. To improve the nanoprobe stability in high ionic-strength salt solutions, the functionalization of nanoprobes with different polyethylene glycol (PEG) polymers was investigated. Moreover, the results of the investigations of the effect of reaction time and aggregate size on SERS intensity show that the SERS signal and aggregate size increased over time, indicating that the aggregation is a continuous process. To control aggregation, a PCI-stopper was designed to stop the reaction at the desired time.
To demonstrate the direct miRNA detection using the PCI nanoprobes for cancer diagnostics, three blind studies were performed with enriched small RNA samples extracted from breast cancer cell lines, and endoscopic biopsies from patients evaluated in Duke gastroenterology clinic who agreed to donate tissue for research purposes. The minimum sample amount of 50 ng can be achieved by reducing the final sample volume from 100 µL to 5 µL and using capillary tubes for SERS measurements. The results of this study show that the PCI technique has great potential as a novel label-free tool to detect miRNAs for medical applications.
Chapter 7. Development and applications of a simple nanoparticle-based colorimetric assay for direct detection of microRNAs for cancer diagnostics (Aim 2.3)

7.1 Rationale

Colorimetric assays based on aggregation of metallic nanoparticles have received increasing interest due to their simplicity, low cost and high sensitivity. Unmodified metallic nanoparticles usually aggregate in a high ionic strength salt solution. However, this salt-induced aggregation process is generally uncontrollable, thus seriously affecting the reproducibility of the detection. To practically control the aggregation process, DNA has been used to assemble nanoparticles into aggregates through DNA hybridization in a sandwich hybridization scheme.[26] Although it has been indicated that silver nanoparticles (AgNPs) with a higher extinction coefficient can offer a higher sensitivity than gold nanoparticles (AuNPs), most of the approaches utilized AuNPs, and few published studies have used oligonucleotide-functionalized AgNPs in colorimetric assays due to their limited stability and laborious preparation.[23, 122]

Herein, we report for the first time a new colorimetric non-sandwich assay based on the aggregation of AgNPs for the direct detection of miRNAs in total small-RNA from clinical samples of gastrointestinal cancer. The operating principle of the colorimetric detection strategy using oligonucleotide-functionalized AgNPs (nanoprobes) is illustrated in Figure 43. In this approach, two types of nanoprobes, probe-A and probe-B, are prepared for one specific miRNA sequence. Probe-A is designed to be complementary to a specific miRNA sequence. To induce aggregation, probe-B is designed to have
sequences complementary to the probe-A. Our colorimetric approach then utilizes the target sequences as competitors of the probe-B in a competitive binding process. As a result, the aggregate formation is disrupted by the target molecules, resulting in different colors depending on the aggregate size.

Figure 43: Operating principle of the colorimetric detection strategy. Probe-A: complementary to a specific miRNA target sequence. Probe-B: complementary to the probe-A sequence. In the absence of targets, probe-A and probe-B were assembled into aggregates. In the presence of targets, the aggregate formation is disrupted by the target molecules, resulting in different colors.

### 7.2 Specificity and sensitivity analysis

In this study, a mature miRNA, miR-21, was used as the test target because it has been identified as part of miRNA signatures for gastrointestinal cancer. To detect miR-21, the miR21-probe-A and the miR21-probe-B with sequences 5’-SH-TCAACATCAGT-CTGATAAGCTA-3’ and 5’-SH-TAGCTTATCAGAC-3’, respectively, were designed and
prepared following the same procedure described in Chapters 5 and 6. The specificity (Figure 44) and sensitivity (Figure 45) of this approach have been assessed with a non-complementary sequence 5’-TAGCAGCACGTAATATTGCG-3’ as a negative control, and different concentrations of complementary targets. The colorimetric assays were carried out by mixing miR21-probe-A and miR21-probe-B in a volume ratio of 1:1 with or without target molecules. The mixture was allowed to react at room temperature for 1 hr in a 10-mM Tris-HCl buffer solution (pH 8.0) containing 150 mM NaCl, 2.5 mM MgCl₂ and 0.01% Tween-20 (final volume: 0.1 mL). The UV-Vis absorption spectrum of the mixture was then measured using the FLUOstar Omega microplate reader (BMG Labtech, Inc.). The total absorbance difference (ΔA) of a sample was calculated by subtracting the absorbance of the blank at 414 and 550 nm, which are the characteristic absorption bands for isolated AgNPs and AgNP aggregates, respectively (Figure 44). The results show that our approach can detect as low as 10 fmol (0.1 nM) target molecules (ΔA = 0.0086+/−0.0009 OD) (see inset in Figure 45) with a high specificity (ΔA = 0.0059+/−0.0011 OD for 10 pmol (100 nM) non-complementary negative control samples) (see inset in Figure 44). In the presence of sufficient targets, the solution color remained yellow or pale yellow indicating that particles were well-dispersed or partially aggregated depending on the target concentration. As shown in Figure 45, a noticeable color difference can be observed in the corresponding photographs at target concentrations ranging from 1 nM to 100 nM.
Figure 44: Assessment of colorimetric assay specificity. Photographs and UV-Vis absorption spectra (with blank subtraction) of the miR21-nanoprobes – (a) in the presence of 100 nM complementary target sequences, and (b) in the presence of 100 nM non-complementary sequences. Inset: total absorbance differences (ΔA) calculated by subtracting the absorbance of the blank at 414 and 550 nm, which are the characteristic absorption bands for isolated AgNPs and AgNP aggregates, respectively.
7.3 Direct miRNA detection in clinical samples

To demonstrate the potential of our approach for clinical applications, a blind study was performed with 500 ng of enriched small RNA extracted from two endoscopic biopsies (samples #4929 and #170) from patients evaluated in Duke gastroenterology clinic who agreed to donate tissue for research purposes. As shown in Figure 46, the absorbance of sample-170 at 414 nm was higher than that of sample-4929, and the absorbance of sample-170 at 550 nm was lower than that of sample-4929, indicating that the expression level of miR-21 in sample-170 was relatively higher than that in sample-
4929. The total absorbance difference (ΔA) at 414 and 550 nm was calculated to be 0.0078+/−0.0002 OD and 0.0128+/−0.0008 OD for samples 4929 and 170, respectively (see inset in Figure 46). By fitting the data to the calibration curve (0.1 to 1 nM range) shown in Figure 45, the miR-21 expression levels in samples-4929 and-170 were estimated to be 0.078+/−0.006 and 0.308+/−0.053 nM, respectively (Figure 47). Thus, the relative expression level of miR-21 in sample-170 (obtained from esophageal adenocarcinoma) was found to be 3.96+/−0.78-fold higher than that in sample-4929 (obtained from control healthy squamous esophageal tissue). This colorimetric result was consistent with the estimated relative expression level (~4.24-fold) using qRT-PCR shown in Figure 39.

![Direct Detection of miR-21 miRNA in Clinical Samples](image)

**Figure 46:** UV-Vis spectra (with blank subtraction) of a blind study using 500 ng of enriched small RNA from two endoscopic biopsies: (a) patient sample-170 (esophageal adenocarcinoma), (b) patient sample-4929 (control healthy squamous esophageal tissue). Inset: total absorbance differences (ΔA) calculated by subtracting the absorbance of the blank at 414 and 550 nm.
Figure 47: The expression levels of miR-21 in patient samples 4929 and 170 estimated by fitting their total absorbance difference (ΔA) values to the calibration curve (0.1 to 1 nM range) shown in Figure 42.

7.5 Summary

The results from this study demonstrate that the nanoparticle-based colorimetric approach can be used as a novel label-free diagnostic tool for the direct detection of miRNAs for medical applications. It is noteworthy that this approach does not require washing steps, which greatly simplifies the assay procedures, thereby providing great potential as a simple and rapid diagnostic test for point-of-care.
Chapter 8. Conclusions and future perspectives

The development of simple and sensitive techniques for the detection of specific nucleic acid sequences is critical for medical applications. Recently, there has been increasing interest in using oligonucleotide-metal nanoparticle conjugates as the detection platform due to their unique optical and plasmonic properties, such as SERS, high extinction coefficient in the visible wavelength region, SPR, fluorescence quenching, and surface-enhanced fluorescence (SEF). However, most techniques require labeling of the target molecules and washing steps, which can make the assays time-consuming and laborious. In this work, we have developed two novel plasmonics-based nanoprobes, MS and PCI, for label-free detection of disease biomarkers using SERS spectroscopy and colorimetric assays. For the first time, we have demonstrated (1) the feasibility of using the MS technique for multiplex DNA detection in a homogeneous solution without washing steps, (2) the application of the MS approach in the detection of a host marker for viral infection diagnostics, (3) the feasibility of SERS-based DNA detection using the MS nanoprobes on a nanowire array, (4) the feasibility and multiplex capability of a novel approach for the detection of specific DNA or RNA sequences using the PCI nanoprobes, (5) direct detection of miRNAs in as little as 100 ng and 50 ng enriched small RNA from breast cancer cell lines and endoscopic biopsies from esophageal adenocarcinoma patients using the SERS-based and colorimetric-based PCI nanoprobes.
These novel approaches developed in this dissertation could lead to the development of a simple, rapid and point-of care diagnostic tool for medical applications.

There is an increasing need for practical and sensitive techniques for biomarker detection and quantification analysis. Many existing detection techniques are not ideal yet for clinical use. For practical clinical applications, several challenges need to be overcome, such as ultrasensitive detection, quantitative multiplex detection, and in-situ/in-vivo detection. For example, the detection capability of metal nanoparticle-based probes may be affected by binding to blood proteins when detecting circulating miRNAs directly in serum or plasma. To address these issues, several parameters such as the types of nanoparticles (AgNPs, AuNPs and nanoshells), effects of particle sizes on cellular uptake, and surface coating with protein resistant polymers for biomarker detection in complex media, need to be further investigated and optimized to meet the needs of clinical practice. Ultimately, we hope that our work will provide useful guidelines for the development of future plasmonics-based detection platforms for medical diagnostics.
References


Biography

Hsin-Neng Wang received his M.S. degree in bioinformatics at the University of Tennessee, Knoxville, in 2003. His Master’s research focused on the development of Laboratory Information Management System (LIMS). Meanwhile, the progress in biomedical research promoted by advanced techniques greatly inspired him to continue his study in biotechnology and nanotechnology. With a strong interest to explore advanced biomedical technologies, he came to Duke to pursue his Ph.D. degree in Biomedical Engineering. Wang’s Ph.D. research focuses mainly on applying biophotonics, molecular spectroscopy and nanotechnology to biosensor development for disease diagnosis and therapeutics.

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**Publications prior to joining DUKE**


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