Study of biomolecules with gold nanoparticles

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Study of Biomolecules with Gold Nanoparticles

LO Kin Man

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

Principal Supervisor: Dr. LI Hung Wing

Hong Kong Baptist University

August 2014
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration of the degree of MPhil at Hong Kong Baptist University, and has not been previously included in a thesis, dissertation submitted to this or other institution for a degree, or other qualification.

Signature: ____________

Date: ____________
ABSTRACT

Gold nanoparticle (AuNP) is used for the detection of biomolecules and study of the interaction between bio-molecules with the aid of dark field microscopy (DFM). AuNP exhibits unique optical properties and ability to conjugate with different biomolecules either by covalent binding or physical absorption, which allow the AuNP possessing a variety of biological application.

We reported a sensitive detection system for measuring DNA–protein interaction at single plasmonic metal nanoparticles level by Localized Scattering Plasmon Resonance (LSPR) spectroscopy. As a proof of concept, DNA molecules were conjugated to gold nanoparticles (AuNPs) through gold–thiol chemistry and the resulted complex was served as single-particle probes of human topoisomerase I (TOPO). By recording the changes in Rayleigh light scattering signal of the individual nanoparticles upon protein binding, DNA–protein interaction was monitored and measured. The $\lambda_{\text{max}}$ shifts in LSPR spectrum of individual AuNP was found to be highly correlated with the amount of TOPO that bound onto.

We presented an immunosensing platform to detect cancer biomarkers by collecting the LSPR signal of immune-target conjugated gold nanoparticle (AuNP). Prostate specific antigen (PSA), which is a FDA-approved biomarker for prostate cancer, was chosen as an example. Herein, the immunoreaction of PSA, capturing PSA antibody (CHYH1) (Ab1), and detecting PSA antibody (CHYH2) (Ab2) was studied with a spectrometer coupled-dark field microscope. LSPR of immunotarget conjugated AuNP was directly measured. In brief, Ab1 and Ab2 were covalently conjugated with AuNPs separately, followed by addition of PSA for the formation of sandwiched immuno-complex in PBS solution. Then, the complex was immobilized on surface of glass slide for capturing dark-field images and LSPR spectra.
Besides, to study the ligand-receptor interaction, we prospect a detection system at single plasmonic metal nanoparticle level by LSPR spectroscopy. Glucocorticoid receptor protein (GR) was chosen as example with two ligands ginsenoside-Rg1 (Rg1) and dexamethasone (DEX). Herein, dsDNA molecules were covalently conjugated with AuNPs and the resulted complex was used as single particle probes of GR. The binding of GR to the dsDNA could be promoted by the agonistic ligands. DNA-GR interaction in the presence of ligands was monitored and measured by recording the changes of LSPR upon protein binding.

This technique provides a sensitive and high-throughput platform to screen and monitor accurately the specific biomolecular interactions. It is capable of revealing information such as particle–particle variations that might be buried in conventional bulk measurement.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. Hung-Wing Li for her guidance, encouragement and grateful help throughout my research progress. I would also like to thank colleagues in H.-W. Li group, Dr. Ho Man Chan for his grateful help and advice, Dr. Zhong-ping Li, Ms. Yi Wong, Ms. See-Lok Ho, Ms Chung-Yan Poon, and Mr. Chung-Yin Lai for their continuous support.

I devote my gratitude to all collaborators. I thank Dr. Dik-Lung Ma from the Department of Chemistry, HKBU, for providing ssDNA and topoisomerase for DNA-protein assay; Prof. Ricky Ngok-Shun Wong from the Department of Biology, HKBU, for providing dsDNA, receptor and ligands for receptor-ligand assay.

I also thank for the technical supports from all the technicians of the department and the faculty, especially Mr. Benson Siu-Cheong Leung from the Department of Physics, HKBU, for capturing TEM images of gold nanoparticles.

I appreciate all my friends and family, who gave their infinite supports and encouragement to me.

Finally, the General Research Fund (HKBU201309) from the Research Grant Councils of the Hong Kong Special Administrative Region is gratefully acknowledged.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>( \lambda_0 )</td>
<td>Original peak position</td>
</tr>
<tr>
<td>( \lambda_{spr} )</td>
<td>Peak position</td>
</tr>
<tr>
<td>( \lambda_{\text{max}} )</td>
<td>Maximum absorption wavelength</td>
</tr>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>D</td>
<td>diameter</td>
</tr>
<tr>
<td>N</td>
<td>number density of particles</td>
</tr>
<tr>
<td>( \rho )</td>
<td>density</td>
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<tr>
<td>R</td>
<td>radius</td>
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<td>V</td>
<td>volume</td>
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**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AFP</td>
<td>alpha-fetoprotein</td>
</tr>
<tr>
<td>AgNPs</td>
<td>Silver nanoparticles</td>
</tr>
<tr>
<td>APTES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>AuNPs</td>
<td>Gold Nanoparticles</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Csa</td>
<td>Magnitude of visible light scattering</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-couple device</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
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<tr>
<td>CEIA</td>
<td>Capillary electrophoretic immunoassays</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
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<tr>
<td>DFM</td>
<td>Dark field microscopy</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immuno-absorbant assay</td>
</tr>
<tr>
<td>EM</td>
<td>Electromagnetic</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor Protein</td>
</tr>
<tr>
<td>LSPR</td>
<td>Localized surface plasmon resonance</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>mM</td>
<td>millimolarity</td>
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<tr>
<td>N.A.</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>ng</td>
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<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<td>nm</td>
<td>Nanometer</td>
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<td>PBS</td>
<td>phosphate buffer saline</td>
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<td>Picomolarity</td>
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<td>Prostate-specific antigen</td>
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<td>Rg1</td>
<td>Ginsenoside-Rg1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SERS</td>
<td>Surface-enhanced Raman scattering</td>
</tr>
<tr>
<td>SMD</td>
<td>Single-molecule detection</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TBS</td>
<td>Tric-HCL Buffer Saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron electron microscopy</td>
</tr>
<tr>
<td>TIRFM</td>
<td>Total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>TOPO</td>
<td>DNA topoisomerase</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultra violent-visible</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>MicroLiter</td>
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<tr>
<td>µm</td>
<td>Micrometer</td>
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Chapter 1   Introduction

1.1 Nanoparticle

Particles in nanoscale have a high surface area to volume ratio that provides unique physical and chemical properties. Development and application of functionalized nanoparticles have emerged as a broad discipline in research areas of material science, biotechnology and environmental fields. Noble metallic nanoparticles such as gold and silver nanoparticles (AuNPs and AgNPs) exhibit their own characteristic optical and chemical properties depending on their sizes and shapes. Nanoparticles prepared by various methods were demonstrated, characterized and applied in many different aspects, such as biological and chemical sensing, imaging, catalysis, nonlinear optics\textsuperscript{1-3}.

1.1.1 Chemical Synthesis of Nanoparticles of Different Shapes and Sizes

To prepare metal nanoparticles (AuNPs and AgNPs) in aqueous solution, there are several synthetic methods which are well established to produce nanoparticles of uniform and controllable sizes in a simple manner. Among these methods, tetrachloroauric acid (HAuCl\textsubscript{4}) is commonly used as a core material of AuNPs, while sodium borohydride, thiocyanate, white phosphorus and sodium citrate are introduced as general reducing agents. Sodium citrate however is conventionally used due to its simplicity and high stability in the aqueous synthesis of AuNPs.\textsuperscript{4}

Representative synthesis of gold nanoparticles by reduction of gold chloride and sodium citrate in boiling water was pioneered by Turkevich et al. (1951)\textsuperscript{5} and refined by Frens (1973)\textsuperscript{6}. AuNPs of diameter in a range of 15-150 nm was controlled by the regulating the molar ratios of gold chloride and sodium citrate. To prepare AuNPs of smaller sizes (<10 nm), a stronger reducing agents such as sodium borohydride, has to be used as described by Tschopp et al\textsuperscript{7}. The Brust method\textsuperscript{8} transferred gold ions
from the aqueous medium to organic medium followed by sodium borohydride reduction, resulted in small gold nanoparticles (~2 nm) synthesized in organic phase. Microemulsion method yielded nanoparticles of various shapes (rods, stars, cubes, etc.) by reducing the gold chloride in the micelles templates in aqueous solution\textsuperscript{9-11}. Obtaining AuNPs with a wide range of diameter was also allowed by seed method. During the seed-mediated growth process, the ellipticity, size, and shape of the nanoparticles can be obtained with careful controlling of the relative amount of seed, gold chloride and reducing reagent.\textsuperscript{10, 11}

1.1.2 Nanoparticle and biomolecule Interaction

Nanoparticles could be functionalized to recognize biomacromolecules specifically and thus be utilized for a wide variety of biological applications such as biosensing, drug and gene delivery, and bioimaging.\textsuperscript{12} Nanoparticles composed by various metal and semiconductor materials exhibited different characteristics and applications. For example, platinum is used as bio-catalyst; iron (III) oxide is used for MR imaging and biomolecular purification with its magnetic property; biocompatible silica core with surface coating treatment potentially for various bio-applications; functionalized carbon nanotube is developed for biosensor that measures electric conductivity; quantum dots (CdSe, ZnS, CdTe), which are of high quantum yield and photo-stability, are widely used in biosensing and bioimaging. On the other hand, noble metals especially gold and silver offer unique optical properties and chemical stability, they are introduced for bio-molecular recognition, drug delivery, bioimaging and sensing. Among all the nanoparticles, synthetic methods of AuNPs are well established for the biological optical imaging and sensing applications for their biocompatibility and high affinity to various proteins, which allows monitoring of protein-protein interaction and cellular imaging.
1.1.3 Preparation of AuNP probe

Biomolecules such as DNA, plasma protein and antibodies can be conjugated with AuNP through either non-covalent binding or covalent bond. The non-covalent binding of proteins to nanoparticles is based on three separate but dependent phenomena: (i) ionic interaction, (ii) dative binding to nitrogen or sulfur atom in the protein, and (iii) hydrophobic attraction. When AuNPs are prepared from the reduction of sodium citrate, the negative citrate ions act as stabilizers surrounding the nanoparticles surface. The particles carrying negative charges have a binding affinity towards the positive surface residues of proteins in neutral or physiological pH by electrostatic interaction.\textsuperscript{13, 14} For the dative binding, the metallic surface of the nanoparticles has a strong interaction towards the electronegative nitrogen and sulfur atoms. Displacement of citrate is possible on the surface of nanoparticle by protein upon adsorption with several amino acids, including lysine (amine), histidine (imidazole), and cysteine (thiol).\textsuperscript{14} Lastly, hydrophobic interaction between proteins and AuNPs surface forming a self-assembled monolayer is favored.\textsuperscript{15, 16} In addition, hydrophobic groups of the proteins interact together to form a protein-multilayers,\textsuperscript{14} which enhances the surface coverage of proteins on the nanoparticles.

Besides the non-covalent adsorption, proteins can attach to nanoparticle surface through covalent bonds with linker via amine or thiol groups.\textsuperscript{8} Alkanethiol is a commonly used linker. It contains thiol groups that can covalently bind to the surface of the AuNPs, and consists of functional groups such as carboxylic acid or amine available to bind with proteins in the presence of carbodiimide crosslinker. The crosslinking agent activates carboxylic groups for rapid reaction with primary amines forming amide bonds.

To achieve stable and functionally active nanoparticle that conjugated with protein, several parameters should be considered: (i) isoelectric point of the protein,
(ii) pH of the reaction buffer solution, (iii) stability of the nanoparticles and (iv) the amount of the protein conjugated to the nanoparticles.\textsuperscript{17} As compared with smaller nanoparticles, larger nanoparticles with larger surface area allow a greater number of proteins binding onto their surface, and hence the average area covered by the proteins is enhanced.\textsuperscript{18} However, proteins loaded on the surface of nanoparticle generally exhibited electrostatic repulsion and steric hindrance, which protect the modified nanoparticle from self-aggregation. This is very important for developing AuNP probes for biological sample studies where the citrate-coated AuNPs are likely to self-aggregate while the protein-coated nanoparticles are much stable in high salt content solution.\textsuperscript{17}

The conjugation and immuno-activity of protein-nanoparticle can be characterized by UV-visible absorption, \textsuperscript{19, 20} fluorescence spectroscopy,\textsuperscript{21} localized surface plasmon resonance (LSPR) spectroscopy,\textsuperscript{21, 22} surface-enhanced Raman scattering (SERS),\textsuperscript{20, 23} light scattering (DLS),\textsuperscript{17, 19} transmission electron microscopy (TEM), enzyme-linked immuno-absorbent assay (ELISA)\textsuperscript{23} and electrochemistry measurements.\textsuperscript{19}

1.2 Importance of Bioassays for biomarkers detection

According to the American Cancer Society, the 5-year relative survival rate for all cancers diagnosed between 2001 and 2007 was 67\%, increased from 49\% in 1975-1977. The improvement in survival reflects progress in diagnosing certain cancers at an earlier stage and improvements in treatment. Genetic analysis provides the opportunity to detect disease-associated genes or even to predict diseases before the onset of physical changes in the cells. Significant progress in genomics research has been made during the last decade. Many disease-related mutations or genetic markers have been identified, and numerous genetic analysis methods have been
established. In clinical diagnosis, detection of biomarkers provides clinical information for all of the various cancer-related diseases crucial to the stage of tumorigenesis, monitoring of treatment, and the status of the patient.\textsuperscript{24}

1.2.1 Cancer biomarker

Cancer biomarkers lead to promising and informative diagnosis of cancer because of their unique association of genomic changes in cancer cells as the disease proceeds. The evaluative cancer biomarkers are divided into several classes: (i) mutations in certain DNA fragments; (ii) over/under expression of gene activity monitored by messenger RNAs; (iii) amount of proteins present in serum or circulating tumor cells.\textsuperscript{24} Biomarker-base diagnostics include risk assessment, noninvasive screening for early stage disease, detection and localization, disease stratification and prognosis, response to therapy and for screening of disease recurrence. Some important cancer biomarkers, alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and prostate-specific antigen (PSA), have been detected by immunoassay methodology for the diagnosis of liver, colon, and prostate cancers, respectively\textsuperscript{25}

1.2.2 Prostate Specific Antigen (PSA)

Prostate cancer is the most common cancer other than skin cancer found in American men. Over 240,000 new cases of prostate cancer were diagnosed and about 33,720 people died of prostate cancer in the United States in 2012, predicted by American Cancer Society. From the statistics, about 1 in 6 men will develop prostate cancer during his life time and about 1 in 36 men will die of prostate cancer. The risk of having prostate cancer is highly age correlated. Men before the age of 40 seldom have the prostate cancer, but the risk of having the cancer rise rapidly after the age of
Prostate cancer is still one of the most common cancers all over the world. Even in Hong Kong, the number of new cases registered of the prostate cancer raised from 597 in 1999 to 1484 in 2009 reported by Hong Kong Cancer Registry. In order to diagnose and monitor treatment response of these cancers, prostate specific antigen (PSA) is introduced and approved of U.S. Food and Drug Administration (FDA). PSA blood test beginning at the age of 50 for early prostate cancer detection is recommended by American Cancer Society. On the other hand, PSA level is a standard indicator for monitoring the response along cancer treatment as well as recurrence after therapy.

1.2.3 DNA Topoisomerase (TOPO) and Glucocorticoid Receptor Protein (GR)

Protein and DNA interaction is a key step for regulation of development and metabolism of animal bodies. DNA topoisomerase (TOPO) is a type of enzyme that has ability to bind with a set of single – and double-stranded oligonucleotide, relax negatively supercoiled DNA and convert it into a less-supercoiled conformation. The topoisomerase is involved in various biological processes such as DNA transcription, recombination, and replication. Almost every type of cell contains topoisomerase and essential for cell growth.

Glucocorticoid receptor protein (GR) is a type of transcriptional regulatory protein that interacts with cortisol and other glucocorticoids and bind to specific nucleotide sequence. The receptor-glucocorticoid complex regulates the inflammatory system, further controls the metabolism and immune response of human body. Ginsenoside-Rg1 (Rg1) and dexamethasone (DEX) are agonistic ligands for GR which activate the GR function to bind with specific sequence of dsDNA. Rg1 is a compound extracted from *panax ginseng*, a medicinal plant cultivated in north Asian countries such as China and Korea. DEX is a synthetic glucocorticoid compound
which has a high binding ability with GR. Study of the protein-DNA interaction is important for understanding of gene expression, immune response and further drug development.

1.3 Bioassay and detection systems for biomarker proteins

Immunoassay is the most commonly utilized approach for specific protein-protein interaction. There has been many different detection systems developed for enhanced sensitivity and selectivity. For examples, microfluidic-based immunosensing microchips for these cancer related biomarkers have been developed with various detection methods, including optical (fluorescence\textsuperscript{29,30}, luminescence, or enzyme conjugates\textsuperscript{31}), electrochemical,\textsuperscript{32} and capillary electrophoretic immunoassays (CEIA).\textsuperscript{33}

1.3.1 Fluorescence spectroscopy

Among all transduction modalities in biosensing platform, fluorescence spectroscopy is still the most common method. Conventional fluorescent labels include organic dyes, fluorescent proteins and lanthanide chelates. Such fluorophores have several advantages in biosensing including (i) availability of different chemical structure to cover a large spectral range ; (ii) well- developed standard bioconjugation protocols are available; (iii) their small molecular size provides ability towards single molecule detection sensitivity; (iv) and high solubility in water or psychological condition.

Fluorescence Resonance Energy Transfer (FRET) is a useful tool in observing conformational changes, intramolecular and intermolecular interactions of single biomolecules. During FRET, the donor fluorophores on the biomolecules transfer energy to the acceptor fluorophores within a close proximity (typically less than 10
nm) and results in a change of emission intensity. However, there is always a trade-off between photostability and fluorescence intensity in FRET. This limits the observation time and hence fluorescence signal detected. Quantum dots and fluorophore-tagged latex/silica nanobeads are viable alternative to the conventional labeling fluorophores. They have better photo-stability and enable for longer observation times of analytes without noticeable bleaching. But, they have several existent challenges needed to overcome. Although quantum dots have a better photo stability, they are unstable in aqueous solution and possibly toxic. The core-shell nanobeads are too big (over 100 nm) for labeling and tracking of molecules. The beads also have a risk of fluorophore leakage. Therefore, gold and silver nanoparticles are introduced, with non-bleached properties, good biocompatibility and tunable size, charge and hydrophobicity.

1.3.2 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is another technique for studying molecular interactions. SPR is an oscillation of surface conduction electrons excited by light (or EM radiation). It requires no labeling or chemical amplification on analyte. For the detection of biomolecules by SPR, the biomolecules would be immobilized on a flat-film of metal layer, such as gold film, for the detection of surface plasmon. Refractive index change resulted from the binding of biomolecules to the surface of metal spots facilitates a real-time analysis. However, complex optical instrumentation and precise temperature control is necessary for SPR. SPR is still not widely used in clinical immunoassays or other non-research-related application yet.
1.4 Localized Surface Plasmon Resonance (LSPR)

Localized Surface Plasmon Resonance (LSPR) is an alternative method to determine the molecular interaction. LSPR can be excited by light and oscillate locally around nanometer-sized metallic structures, alloy or semiconductor, and it is sensitive to the nanoparticle’s size, shape, composition, orientation and local dielectric environment (Figure 1.1). The LSPR signal of the nanoparticles can hence be manipulated by controlling these parameters during the fabrication process with chemical synthesis and lithographic techniques. Sherry et al. have demonstrated the LSPR of different morphologies of silver nanostructures including nanoprism and nanocube. As the nanoparticles absorb and scatter light, the particles can be directly visualized with dark-field microscopy. Upon binding analytes to nanoparticles surface, a shift in spectral extinction peak would be resulted. Large molecules such as antibodies and proteins result in an even greater change in the local dielectric environment per adsorbed molecules. Attaching noble-metal nanoparticles such as gold and silver to biomolecules can facilitate intermolecular interaction analysis. As an advantage over FRET analysis, unlike fluorophores and quantum dots, plasmonic metal nanoparticles do not blink or photobleach even lengthy light exposure is applied. Long intervals real-time observation of biomolecules interaction and thermodynamic and kinetic data acquisition is feasible. Compared with SPR, LSPR is non-temperature sensitive and it requires simpler optical extinction measurement. Immobilization of analyte molecules can be avoided. Sonnichsen et al. have developed a molecular ruler to monitor the separation between single pairs of nanoparticles (Gold and Silver) in real-time for the study of kinetics of single DNA hybridization events. McFarland et al. demonstrated the use of Ag nanoparticles as real-time optical sensors in flow cell. Other application of LSPR including biochemical sensors, labels for immunoassays and surface-enhanced
Nanoparticles compared with fluorophore as a labeling tool

Based on the LSPR of gold and silver nanoparticles, the resonance induces an intense scattered light which is much stronger than the fluorescence emission of the common fluorophores. A typical gold nanosphere has an optical cross-section which is 4-5 orders of magnitude higher than the conventional fluorophores for imaging. For a 80 nm gold nanosphere, the magnitude of visible light scattering (Csa) is \(1.23 \times 10^{-14}\) m\(^2\) at 560 nm, corresponding to a molar scattering coefficient of \(3.22 \times 10^{10}\) M\(^{-1}\)cm\(^{-1}\).

The light emission from fluorescein, which is a high quantum yield fluorophore commonly used for imaging, has an emission coefficient of \(9.23 \times 10^{4}\) M\(^{-1}\)cm\(^{-1}\) at 483 nm. The light scattering from 80 nm AuNP is 5 orders of magnitude higher than that of fluorescein. Consider a 300 nm polystyrene nanospheres with a light scattering \(1.77 \times 10^{-14}\) at 560 nm, the 80 nm AuNP with smaller size exhibits a comparable scattering intensity. The remarkable scattering from the nanoparticles is non-bleaching compared with a fluorescence dye which blinks under exposure of strong excitation light. Long exposure time is allowed to collect and accumulate scattering signal. This favors the usage of AuNPs in optical imaging and sensing of biological samples.
1.4.2 Principle of Dark Field Microscopy

Dark field microscopy (DFM) is one of the most widely used microscopic techniques in biological and materials science\textsuperscript{40,46}. Dark field is a technique used to illuminate the sample and induce scattering. The directly transmitted light will not be collected by the objective lens, while only the scattering light from the samples reaches the objective lens. Figure 1.2a shows the principle of DFM. From the diagram, an emitting light source (halogen lamp) producing a continuous wavelength spectrum illuminates the condenser. An opaque disc (or patch stop) is placed underneath the condenser lens, blocking the light from directly focus on the sample and leaving a ring of illumination at the edge of condenser. Instead of coming up through the sample, the light is scattered by the sample (cells or particles) within the cover slides. The directly transmitted light is omitted and only light that is scattered by sample can reach the objective lens. The low N.A. objective equipped in the DFM system is used to avoid the reflection of transmitted light in order to maintain a dark background. The collected scattering light is analyzed by the spectrometer. The DFM image and the diffracted scattering light are captured by CCD camera. The scattering spectrum (LSPR spectrum for AuNP) from single particle is obtained by conversion with imaging programs.
1.4.3 DFM Studies of Biomolecules with Metallic Nanoparticle

Gold and silver nanoparticles produce an intensive scattering light which is dominated by LSPR. The resonance intensity is proportion to the particles size. Particle size larger than 20 nm can be observed by the DFM. AuNPs and AgNPs with 40 to 80 nm are often used as they have large absorption and scattering cross-sections in DFM imaging. By coupling the DFM to spectrometer and CCD camera, The LSPR spectra from single nanoparticles can be readily measured.

AuNP and AgNP have a good affinity to biomolecules such as DNA, plasma protein and antibodies, and scattering signal of the nanoparticles could be controlled by changing particle size, shape and composition, this allows a variety application of those nanoparticles in biological aspect. Schultz S. et al. have demonstrated a typical biological assay using colloidal silver as optical reporters; Huang X.H. et al. used the gold nanorods as contrast agents for cancer cell imaging, with addition of
photothermal cancer therapy; \(^{21}\) and other literatures reported.\(^{25, 36, 42, 48, 49}\)

### 1.5 Summary of following chapters

In this study, TOPO, PSA and GR detection will be focused in the following chapters. For TOPO study, we describe a LSPR technique at single-nanoparticle level to directly monitor DNA-protein interaction. Human topoisomerase I (TOPO) (100 kDa) and a DNA sequence (25-base) were chosen as a demonstration model of protein-DNA interaction. TOPO is known to be able to relax the supercoiled DNA and convert it into a less-supercoiled conformation. DNA topoisomerases are essential for cell growth and it is one of the target enzymes of anti-cancer drugs. By monitoring the changes in LSPR spectrum of each individual nanoparticle upon binding to target molecules, this nanoscale bionsensing platform could be extended to screening a wide variety of biomarkers of interested.

For PSA study, we extend the LSPR technique to provide a high sensitive and high throughput multiplexed assay for biomolecules, while retaining all the capabilities of existing nanoparticle arrays and SPR. To illustrate the proposed system, cancer biomarker, PSA, is chosen and detected using an immunoassay method within the fabricated microchip using LSPR as the detection method.

For GR study, we extend the LSPR technique at single-nanoparticle level to directly monitor DNA-protein interaction and further for ligand-receptor interaction. Glucocorticoid receptor protein (GR) (85.6 kDa) and a dsDNA sequence (23-base) were selected to demonstrate the ligand-receptor interaction in the presence of ligands, ginsenoside-Rg1 (Rg1) and dexamethasone (DEX). The role of GR is to interact with the ligands and bind with specific DNA sequence, in order to control immune response of human bodies. The agonistic binding ability of the ligands is determined by the changes in LSPR response from AuNPs.
Chapter 2. Materials and methods

2.1 Preparation of Au nanoparticles (AuNP)

Gold nanoparticles (AuNPs) were prepared by citrate stabilized seeded growth method\textsuperscript{50}, NH\textsubscript{2}OH stabilized seeded growth method\textsuperscript{10} and citrate-stabilized method\textsuperscript{6,51} as reported. The distilled water (Watsons Water, HK) used in the experiment was filtered through 0.22 μm nylon membrane prior to use. All glassware used in the synthesis were cleaned with aqua regia solution (HCl/HNO\textsubscript{3}, v/v 3:1) and rinsed thoroughly with water. Gold (III) chloride trihydrate, trisodium citrate, 3-aminopropyltriethoxysilane (APTES) were purchased from Sigma Aldrich.

2.1.1 Citrate stabilized seeded growth method

For synthesis of AuNP seeds, 30 mL of 2.2 mM trisodium citrate was refluxed with vigorous stirring in a 100 mL three-necked round bottomed flask. 0.2 mL of HAuCl\textsubscript{4} (25 mM) was added into the vortex of the boiling solution. Under continuous stirring and boiling for 15 min, the solution color gradually changed from colorless, grey to pale wine red, which indicated the formation of AuNP seeds. The solution was cooled down to 90 °C. 0.2 mL of HAuCl\textsubscript{4} (25 mM) was injected into the vortex of the solution and allowed to react for 30 min. This process was repeated twice. Then, 11 mL of the solution was extracted and 10.6 mL of filtered distilled water and 0.4 mL of 60 mM trisodium citrate was sequentially injected. The diluted solution was used as seed solution and the process was repeated again for the growth of large AuNP. The citrate-stabilized seeded growth AuNP was used throughout the detection of TOPO, PSA and GR.

2.1.2 NH\textsubscript{2}OH stabilized seeded growth method

For synthesis of AuNP seeds, 25 mL of HAuCl\textsubscript{4} (1 mM) was refluxed with
vigorous stirring in a 50 mL round bottomed flask. 2.5 mL of 38.8 mM trisodium citrate was added into the vortex of the boiling solution quickly. After continuous stirring and boiling for 15 min, the solution color gradually changed from yellow, grey to wine red, which indicated the formation of AuNP seeds (12 nm, 17 nM). The solution was cooled to room temperature for the growth step.

The growth solution of AuNP was prepared by addition of 50 μL of 1% HAuCl₄ and 21 μL of 0.4 M hydroxylamine solution into 9.5 mL of water in a 20 mL glass vial. 120 μL of AuNP seed solution was added immediately into the vortex of the growth solution. Under continuous stirring 1 min, the solution color gradually pale wine red to pink, which indicated the formation of large AuNP.

2.1.3 Citrate stabilized method

For synthesis of citrate stabilized AuNP, 50 mL of HAuCl₄ (0.25 mM) was refluxed with vigorous stirring in a 100 mL round bottomed flask. 0.625 mL of 1% sodium tricitrate was added into the vortex of the boiling solution. Under continuous stirring and boiling for 10 min, the solution color gradually changed from colorless, grey to reddish purple, which indicated the formation of AuNP. The solution was cooled down and stored at room temperature.

2.2 Preparation of buffer solution

Phosphate buffer was prepared by mixing sodium monophosphate, sodium diphosphate and 0.1 M sodium chloride and diluted with autoclaved distilled water to 50 mL. The pH of phosphate buffer was adjusted to 7.0. PBS solution was then filtered through a 0.22 μm nylon membrane filter, autoclaved prior to use. Tris buffer containing 20 mM pH 8.0 Tris-HCl was prepared with autoclaved distilled water as the hybridization buffer. The pH of the Tris buffer was adjusted to 7.4 by adding of 1
M HCl dropwise was then filtered through a 0.22 μm nylon membrane filter, autoclaved prior to use.

2.3 Preparation of flow cell

All cover slides were pre-washed prior to experiments. Briefly, 22 x 22 mm and 22 x 32 cover glass (Thermo-Menzel) were used as upper and bottom glass slide respectively to prepare the flow cell. The slides were sonicated sequentially in water for 15 min twice, in ethanol for 15 min and washed with water. Then, they were immersed in piranha solution (H$_2$SO$_4$/H$_2$O$_2$, v/v 3:1) twice for 30 min with extensive rinsing by water after each piranha immersion. After that, the slides were sonicated in water for 30 min twice respectively. The pre-washed bottom glass slides were dried with nitrogen before immersing in 2% (v/v) (3-Aminopropyl)triethoxysilane (APTES) in ethanol solution for 1 hr. After modification, slides were rinsed several times with ethanol and water, followed by dried under a stream of nitrogen. The slide was placed in oven at 110 °C for 1 h. The upper glass slides were dried with nitrogen and into 0.5% Bovine serine albumin (BSA) in PBS for 15 min. Then, the slides were rinsed with water and dried with nitrogen. A home-made flow cell was made with glass slides with double side tapes. Following procedure of TOPO, PSA and GR detection were referred to Chapter 2.6, 2.7 and 2.8 respectively.

2.4 Dark Field Microscopy Imaging System and Scattering Spectroscopy

An inverted model Eclipse Ti-U microscope (Nikon, Japan) was equipped with a non-polarized 100-W halogen lamp to illuminate sample at large angles from above through a dark-field condenser (Nikon, dry 0.95–0.80). The numerical aperture (NA) of the Plan Fluor 60x oil-immersion objective (Nikon, oil 0.5–1.25) was tuned to ~ 0.7. The scattering signal of the gold nanoparticles was acquired with a PIXIS 100F
front-illuminated charge-coupled device (CCD) camera (Princeton Instruments, Princeton, NJ, USA), which mounted on an Acton SP2300 grating spectrometer (Princeton Instruments, Princeton, NJ, USA) equipped with a grating (grating density, 300 lines/nm) and connected to the trinocular port of the microscope. Typical acquisition time was 10s. Scattering spectra were obtained with the WinSpec/32 software (Version 2.5.23.0, Downingtown, PA) provided by Princeton Instruments. The wavelength range of the spectra was 450-700 with a resolution of 0.5 nm.

All images and spectra analysis were performed with Image J (version 1.43u, NIH, USA), a public domain and Java-based image processing program, and OriginPro 8 (version 80725 B725, OriginLab Corporation. USA ), a data analysis and graphing software. Each individual particle followed by the subtraction of background within the same area in the same image was selected as a region of interest for the LSPR spectrum. With the use of the functions in OriginPro 8, the LSPR spectra were fitted with the Lorentzian peak function computationally. Also, the distribution of peak position of 100 AuNPs in the LSPR spectra were obtained and fitted with Gaussian area function.

2.5 Transmission electron microscopic (TEM) and UV-vis measurements

TEM images were recorded with a Tecnai G2 20 S-TWIN microscope (FEI, USA) equipped with a bottom-mounted FEI Eagle high-resolution CCD camera. Samples of 5 μL of AuNP or surface modified AuNP was placed onto a carbon-coated TEM copper grid (Electron Microscopy Sciences, Hatfield, PA) and the film was kept under vacuum overnight. More than 100 particles from the enlarged TEM images were analyzed for size distribution of the particles. UV-vis measurements were performed by Cary 300 UV-Vis Spectrophotometer (Agilent Technologies, CA, USA) using a standard 1 mm path length quartz cuvette with a reference beam of sample
using blank solution.

2.6 Detection of DNA Topoisomerase

2.6.1 Preparation of ssDNA-modified Au nanoparticles (AuNP-ssDNA)

For the detection of TOPO, thiolated single stand DNA oligonucleotides of sequence, 3’-TAAAAATTTTTCCAGTCTTTTTTC-5’ was purchased from Invitrogen. ssDNA-modified Au nanoparticles probes were synthesized by mixing 100 μL of an aqueous 64 nm diameter Au nanoparticles solution (0.95 nM) with 10 μL of thiolated ssDNA (15.3 μM). After standing for 16 hours, the solution was brought to 0.1 M NaCl, 20 mM phosphate buffer (pH 7) and allowed to stand for 40 hours, followed by centrifugation for 5 min at 6000 rpm to remove excessive reagents. After removing the supernatant, AuNPs was resuspended in 100 μL of 0.1 M NaCl, 20 mM phosphate buffer (pH 7).

2.6.2 Preparation of TOPO conjugated AuNP-ss-DNA complex in PBS solution

Human Topoisomerase I (TOPO) was obtained from Topogen, Inc. TOPO was incubated with ssDNA-AuNP by mixing 5 μL TOPO I (4 unit/μL) with 5 μL AuNP-ssDNA in 37 °C water bath for 1 hour. Different amounts of TOPO were added in the concentration dependent study.

2.6.3 Preparation of TOPO conjugated AuNP-ss-DNA complex for DFM measurement

To immobilize ssDNA-AuNP on the ATPES glass slide surface, the ssDNA-AuNP solution was diluted 10 times with buffer solution prior to the experiment. Solution was flowed into the APTES treated channel and incubated for 10
min. After immobilization of ssDNA-AuNP, the channel was rinsed with PBS. 20 μL of 0.5% BSA in PBS was added and incubated for 30 min in room temperature in order to prevent the non-specific interaction of ATPES glass slide surface. The excess BSA was removed by rinsing the channel with PBS, followed by addition of 10 μL of TOPO and incubated in 37 °C water bath for 1 hour. Different amounts of TOPO were added in the concentration dependent study. Then, the prepared flow cell was placed on the microscopy stage for the dark field measurement.

2.7 Detection of Prostate Specific Antigen

2.7.1 Preparation of antibodies AuNP conjugated probe

To conjugate antibodies with AuNP, a cross-linker MUA was first prepared in the ethanol. 4 mL of 0.08 nM pH 9 (tuned by 0.1 M NaOH) citrate stabilized seeded growth AuNP was mixed with 14.8 μL of 0.5 mM MUA solution and stood overnight. Then, the solution was centrifuged and resuspended into water. 1 μL of 0.1 mM EDC and 1 μL of 0.1 mM NHS were freshly prepared in water and added into 500 μL of the MUA-AuNP solution. After reacted for 30 min at room temperature, 1 μL of 100 g/L antibody (CHYH1 or CHYH2) was added to the AuNP mixture and incubated for another 1 hour at room temperature. The result antibody-AuNP conjugated solution was centrifuge and resuspended in PBS for PSA detection.

2.7.2 Immunoassay

The 3 μL of CHYH1-AuNP and 3 μL of CHYH2-AuNP were mixed with 6 μL 2xPBS with 0.2 % BSA. 1.3 μL of PSA solution was added into the mixture and reacted for 2 hours at 37. Different amounts of PSA were added in the concentration dependent study. The resulted solution was filled into the ATPES-flow cell and
incubated for 10 min for the AuNP probes immobilization. Finally, the channels were washed with PBS before imaging.

2.8 Detection of Glucocorticoid Receptor Protein

2.8.1 Preparation of ssDNA-modified Au nanoparticles (AuNP-dsDNA)

For the detection of GR, thiolated single stand DNA oligonucleotides of sequence, 3’-TATCAGAACAGAGTGTCTGGCG-5’-C6-SH and its complementary single stand DNA 3’-CGCCAGAACAAGCTGTCTGATA-5’ were purchased from Tech Dragon Limited. The thiolated ssDNA (15 μM) and its complementary ssDNA (15 μM) were first hybridized in Tris buffer (pH 7.4, 0M of NaCl), and allowed to stand for 1 hour in room temperature after annealing at 90 °C for 2 min. dsDNA-modified AuNP probes were synthesized by mixing 10 μL of thiolated dsDNA (15.3 μM) with 100 μL of an aqueous 64 nm diameter Au nanoparticles solution (0.95 nM). After standing for 16 hours at room temperature, the solution was centrifuged for 5 min at 6000 rpm to remove excess reagents. dsDNA-AuNP was resuspended to the final volume of 100 μL of Tris buffer.

2.8.2 Preparation of GR conjugated AuNP-ds-DNA complex for DFM measurement

To immobilize dsDNA-AuNP on the ATPES glass slide surface, the dsDNA-AuNP solution was diluted 20 times with Tris buffer solution prior to the experiment. Solution was flowed into the APTES treated channel and incubated for 10 min. After immobilization of dsDNA-AuNP, the channel was rinsed with PBS. 20 μL of 0.5% BSA in PBS was added and incubated for 30 min in room temperature in order to prevent the non-specific interaction of ATPES glass slide surface. The excess
BSA was removed by rinsing the channel with PBS, followed by addition of 10 μL of GR-Rg1-DEX mixture and incubated in a 37 °C water bath for 2 hour. Different amounts of GR, Rg1 and DEX were added in the concentration dependent study. Then, the prepared flow cell was placed on the microscopy stage for the dark field measurement.
Chapter 3       Result and Discussion

3.1 Characterization of AuNPs

The unique absorption and LSPR properties of nanoparticles are highly dependent on their sizes and morphologies. In this work, AuNPs prepared by three different methods were employed for comparison and to illustrate the morphology-spectral signal relationship of these nanoparticles by the developed LSPR technique. The size and shape of the prepared AuNPs were characterized by TEM and UV-Vis spectroscopy (Figure 3.1). As shown in Figure 3.1a-f, with respect to the size and shape uniformity, the AuNPs generated by the citrate-stabilized seeded growth method was better than those of direct citrate-stabilized and \( \text{NH}_2\text{OH} \) seeded growth. Using trisodium citrate as the reducer and stabilizer produced large AuNPs of elongated shape and of broad size distribution while \( \text{NH}_2\text{OH} \) seeded-growth gold nanoparticles resulted in a mixture of rod and triangular shape AuNPs. The AuNPs obtained from citrate-stabilized seeded-growth were spherical and of good monodispersity. Nonetheless, as depicted in Figure 3.1(g-i), there were no significant differences in the absorption peaks in the UV-vis spectra of these three types AuNPs. Throughout the following experiments, AuNPs of 52 nm synthesized by citrate seeded-growth was adapted for their uniform size and shape.
Figure 3.1. Transmission Electron Microscopic images, size distribution and UV-Vis spectra of citrate-stabilized seeded growth AuNPs (a,d,g), citrate-stabilized AuNPs (b,e,h) and hydroxyamine stabilized seeded growth AuNPs (c,f,i). The average sizes are 52.1±5.4, 54.5±8.8 and 47.8±8.1 nm respectively.

Concentration of the AuNPs can be calculated by the gold salt content and size of the AuNP determined by TEM image. Herein, the citrated-stabilized AuNP was used as an example. The average weight of each AuNP was estimated from the following equation 1 reported by Liu X. et al. \(^{13}\)

\[
m = \rho \times V = \rho \times \left(\frac{4}{3} \pi R^3\right) = \rho \times \left(\frac{4}{3} \pi \left(\frac{D}{2}\right)^3\right) = \frac{\pi}{6} \rho D^3
\]

(1)

The gold nanoparticles were assumed in spherical shape and fcc crystal structure, where \(m\) is the average weight of the AuNP, \(\rho\) was the density (19.3 g/cm\(^3\) for fcc crystal structure), \(V\) was the average volume, \(R\) was the average radius, and \(D\) was the
average diameter of the AuNP. D was determined from the TEM image result, and the average weight of the AuNP was found to be $1.2 \times 10^{-6}$ ng for AuNPs with average diameter of 54.5 nm.

Assumed that all the gold salt was consumed to produce AuNP, the concentration of AuNP in solution was then calculated by equation 2,

$$c = \frac{m'}{m \times N_A} \quad (2)$$

where $c$ was the concentration of AuNPs in molm$^{-1}$, $m'$ was the gold content concentration for the formation of AuNPs in ngmL$^{-1}$, $N_A$ was $6.023 \times 10^{23}$, $m$ was weight of AuNP determined by previous equation. The concentration of AuNP was found to be 0.046 nM.

Figure 3.1 h shows the UV-vis spectrum of citrate-stabilized AuNP solution. the size and concentration of citrate coated AuNPs could be approximated by the spectrum reported by Haiss W. et al. $^{52}$

$$d = \frac{\ln\left(\frac{\lambda_{spr} - \lambda_0}{L_1}\right)}{L_2} \quad (3)$$

The equation was limited to calculate the particle diameters ($d$) ranging from 35 to 100 nm, where $\lambda_{spr}$ is the peak position, $\lambda_0 = 512$, $L_1 = 6.53$, $L_2 = 0.0216$. Consequently, the concentration of AuNPs could be determined by following equation

$$N = \frac{A_{450} \times 10^{14}}{d^2 \left[ -0.295 + 1.36 \exp\left( -\left( \frac{d - 96.8}{78.2} \right)^2 \right) \right]}$$

where $A_{450}$ was the absorbance at $\lambda = 450$ nm, $d$ is the particle diameter, and $N$ was the number density of particles in solution. From the figure 3.1 h, the AuNP spectrum shows a peak located at 531 nm and absorbance at 450 nm was 0.309. The approximation of size and concentration of AuNPs were found to be 49.4 nm and
0.041 nM, respectively. The results were comparable with that of calculation from TEM images.

In a typical dark-field microscopic (DFM) image, each bright spot represents a single plasmonic nanoparticle which gives Rayleigh light scattering signal. Each nanoparticle can be served as an individual biosensor. Figure 3.2a (left) shows a representative image for nanoparticles immobilized on a glass slide. A narrow slit was placed between the objective lens and the spectrograph so that only a narrow vertical region of interest was imaged. The Rayleigh light scattering signal from all AuNPs located in the slit region was then dispersed simultaneously into the first order image (spectroscopy) as shown in Figure 3.2a (right) by the spectrograph that coupled to the DFM. A high-throughput measurement is hence allowed. The LSPR spectrum of each individual particle was then reconstructed as demonstrated in Figure 3.2b. Taking the great advantages that AuNPs are photostable and the detection approach is free of photobleaching, long exposure time is allowed to enhance the observed signal-to-noise ratio of single particle spectrum with good spectral resolution. Besides analyzing spectrum of each single particle, the spectral properties for examples distribution of peak position of large number of individual AuNPs could also be determined for the purpose of achieving statistical data as illustrated in Figure 3.3a-c. LSPR spectra of individual particles shows intrinsic particle-particle variations in their optical properties while collecting data from large enough number of particles gives the bulk averaged measurement. Figure 3.3a-c shows the \( \lambda_{\text{max}} \) distribution of 100 particles each for those AuNPs prepared by the three methods as mentioned in Figure 3.1a-c. It was obvious that the LSPR \( \lambda_{\text{max}} \) and peak profile of these AuNPs were notably different and highly correlated to the size dispersity and shape of the as-prepared AuNPs. Generally, AuNPs of larger size gave longer \( \lambda_{\text{max}} \). AuNPs of broader size distribution produced broaden LSPR \( \lambda_{\text{max}} \) distribution. LSPR
measurement of single particles was capable of providing richer information as compared to conventional absorption spectroscopy. One can obtain the optical properties particle by particle to elucidate any chemical environment changes at the sensitivity of a single particle.

Figure 3.2(a). Dark field image of AuNPs refined with a narrow slit of 3 µm (left). The first order image of the AuNPs generated by a spectrograph that coupled to the dark-field microscope (right).

Figure 3.2 (b). Typical LSPR spectrum of AuNP with Lorentzian fitting.
Fig. 3.3 (a-c). LSPR $\lambda_{\text{max}}$ distribution of citrate stabilized seeded-growth AuNP, citrate-stabilized AuNP and NH$_2$OH stabilized seeded-growth AuNP with Gaussian Fitting. Peak maximum were at 561.1, 609.5 and 561.5 nm respectively.
3.2 DNA-Protein interaction by UV-vis measurement

To study DNA-protein interaction, TOPO and a DNA sequence were chosen as a DNA-protein model for proof-of-concept; AuNP was chosen as a model plasmonic metal nanoparticle for its promising surface modification and its strong LSPR signaling for detection purpose. The oligonucleotide of interest was conjugated to AuNPs via gold-thiol covalent bonding. It is commonly known that single-stranded DNA (ssDNA) protects AuNPs from aggregation by the highly negatively charged oligonucleotide backbone. Many research work developed methods based on DNA-AuNP aggregation formation for the sensing of DNA, protein and metal ions. Color change could be visualized in the presence of the target analytes of high enough concentration (in nM). Figure 3.4 shows the UV-vis spectra of AuNPs before and attachment of ssDNA. There were no significant changes in \( \lambda_{\text{max}} \) and absorbance showing that the ssDNA-AuNPs were still well dispersed in solution. Upon incubation, the target TOPO molecules (pI = 8.1-8.3)\(^{19} \) favorably bound to ssDNA sequence attached AuNPs. Once TOPO bound onto the ssDNA-conjugated AuNP in solution, the negative charges of the DNA backbone were shielded because of the fact that TOPO protein is rather neutrally charged at pH 7.4. The electrostatic repulsion among the nanoparticles was thus minimized and cluster formation among the AuNPs was favored. The interaction between ssDNA-conjugated AuNPs and TOPO of various concentration (0.05 – 1.2 unit/µL) was confirmed by UV-vis absorption spectroscopy as shown in Figure 3.5a. It was showed that the absorbance significantly decreased as TOPO concentration increased although no obvious peak shift (\( \lambda_{\text{max}} \)) was observed. The relationship between the peak absorbance and the concentration of TOPO added was depicted in Figure 3.5b. On the other hand, the resulted reaction mixtures were visualized under DFM. The images consistently showed that the initial individual AuNPs formed larger but less populated bright spots upon the addition of
TOPO. The size of the clusters formed increased correspondingly with the TOPO concentration (Figure 3.6a-g). This enlarged spot size was attributed to the favorable formation of AuNPs aggregation/clusters due to the minimized electrostatic repulsion among the nanoparticles. The cluster formation thus hindered measurement of the optical properties changes of individual nanoparticles in solution-base for biomolecules detection.

Figure 3.4 UV-Vis spectrum of citrate stabilized AuNPs and ssDNA-AuNPs.
Figure 3.5 (a) UV-Vis spectra of ssDNA-conjugated AuNPs with TOPO of concentration 0, 0.05, 0.1, 0.2, 0.3, 0.4, 1.2 unit/µL respectively; (b) relationship between the peak absorbance and TOPO concentration.
Figure 3.6 (a-g). dark-field images of ssDNA-conjugated AuNPs with TOPO of concentration 0, 0.05, 0.1, 0.2, 0.3, 0.4, 1.2 unit/µL respectively.
3.3 DNA-Protein interaction by LSPR measurement

To overcome the obstacle introduced by the solution-base cluster formation of nanoparticles, we adopted a LSPR biosensing assay at single particles level on surface-base. The ssDNA-AuNPs were firstly immobilized electrostatically on a APTES-treated glass slide. We took the great advantage of a flow cell that allows multi-consequent reactions to occur on surface; while rinsing out excessive reagent is also possible to remove any possible matrix interferences. Here, excessive AuNPs in free solution was rinsed off with buffer solution. The self-aggregation of the AuNPs in later reaction was thus prevented. To the same TOPO-DNA reaction, before the introduction of TOPO that binds to ssDNA-AuNP, loss of TOPO due to non-specific interaction/adsorption onto positively charged the APTES should be eliminated. In this work, the vacant APTES sites on the slide were blocked by 0.5 % BSA in PBS in order to minimize the loss of target TOPO via non-specific adsorption. After that, TOPO was then flowed in and the reactants were allowed to incubate at physiological conditions for most favorable interaction between TOPO and ssDNA sequence. Once TOPO protein molecules bound to the ssDNA-AuNP, a change in the local dielectric environment is resulted and hence the LSPR response varies. The Rayleigh light scattering signal before and after the target binding onto individual particles could be directly measured and compared to verify the protein-DNA interaction.

To demonstrate the capability of the LSPR system for detecting DNA-protein interaction, we studied the LSPR response of ssDNA-AuNPs in the presence of different TOPO concentration (0-5 unit/µL). After the on-chip addition and incubation of TOPO with ssDNA-AuNP, DFM images and LSPR spectroscopy of individual nanoparticles were collected. A representative LSPR spectrum of a nanoparticle before and after the binding of TOPO was depicted in Figure 3.7. A $\lambda_{\text{max}}$ shift (from
570.3 to 575.0 nm) was observed for this particular particle. It was noted that the LSPR response varied of individual particles as illustrated in Table 3.1 where the $\lambda_{\text{max}}$ shift of six particles before and after TOPO binding were listed. This variation was attributed to the subtle differences in the size, morphology and in chemical and biological environment among particles. By collecting and analyzing the LSPR spectrum of groups of 100 or more nanoparticles in each experimental condition, the $\lambda_{\text{max}}$ distribution was established as shown in Figure 3.8a for TOPO concentration range of 0-5 unit/µL. A response curve ($\lambda_{\text{max}}$ shift of LSPR) as a function of TOPO concentration in the range of 0-5 unit/µL was established as illustrated in Figure 3.8b. It was observed that the higher the applied TOPO concentration, the longer the $\lambda_{\text{max}}$ red-shift of the resulted LSPR signals. The magnitude in red-shifting was level-off when TOPO concentration was higher than 2 unit/µL and this implied that the ssDNA-AuNPs were sufficiently saturated with TOPO molecules. To verify the specificity of the assay, 0.5 % BSA in PBS was used as a non-specific protein for a control experiment. The resulted $\lambda_{\text{max}}$ distribution in the presence of BSA was of no significant difference as compared to that of ssDNA-AuNPs (572.6 vs 572.1 nm, see Figure 3.9a). This result elucidated that the BSA molecules did not interact to some extent with the ssDNA AuNPs. This was also confirmed by UV-vis spectroscopy as shown in Figure 3.9b that there was no obvious $\lambda_{\text{max}}$ shift and change in absorbance being observed. It demonstrated that this established assay is promising for measuring DNA-protein interaction at single nanoparticles level of high specificity.
Figure 3.7. A representative LSPR spectrum of a ssDNA-AuNP before and after addition of 2 unit/µL TOPO. $\lambda_{\text{max}}$ shift from 570.4 to 575.0 nm was observed.

<table>
<thead>
<tr>
<th>ssDNA-AuNP</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\lambda_{\text{max}}$ after TOPO addition (nm)</th>
<th>$\lambda_{\text{max}}$ shift (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>570.4</td>
<td>575.0</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td>568.7</td>
<td>571.3</td>
<td>2.7</td>
</tr>
<tr>
<td>3</td>
<td>576.1</td>
<td>581.3</td>
<td>5.2</td>
</tr>
<tr>
<td>4</td>
<td>565.5</td>
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<td>6.1</td>
</tr>
<tr>
<td>5</td>
<td>560.6</td>
<td>564.3</td>
<td>3.7</td>
</tr>
<tr>
<td>6</td>
<td>560.8</td>
<td>566.0</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Table 3.1. LSPR $\lambda_{\text{max}}$ of six ssDNA-AuNPs before and after addition of 2 unit/µL TOPO.
Figure 3.8 (a). The $\lambda_{\text{max}}$ Distribution of 100 ssDNA-AuNPs in the presence of 0, 0.05, 0.1, 0.5, 1, 2, and 5 unit/$\mu$L of TOPO. The $\lambda_{\text{max}}$ were 568.2, 569.2, 570.2, 571.8, 573.3, 576.9, and 576.3 nm respectively. (b) The LSPR $\lambda_{\text{max}}$ shift as a function of TOPO concentration.
Figure 3.9. (a) ssDNA-AuNP with 0 and 0.5% BSA, LSPR distribution value are 572.6 and 572.1 nm respectively. (b) UV-Vis spectrum of ssDNA-AuNP with 0 and 0.5% BSA.
It is noteworthy that the DNA-protein interaction at single-nanoparticle level cannot be achieved from the conventional UV-vis bulk measurement due to the cluster formation of the NPs in solution-based as mentioned above. However, one could also observe the changes in LSPR signal from AuNP seeded glass slide provided that (i) the fabrication offers reasonably densely packing of the AuNP seeding; (ii) particle-particle interaction is eliminated and; (iii) sufficient amount of the analyte is applied such that every nanoparticles has even distribution of target analyte in order to produce reasonable statistical averaged signal changes. As a consequence, the sample preparation would be more demanding and complicated. Unlike the single particle nanosensors, the methodology and sensitivity of this approach would be similar to traditional SPR (typically in nM concentration).

It is demonstrated that LSPR assay developed here is a selective and sensitive tool that theoretically only a single AuNP was involved and the change in LSPR signal could be resulted from attachment of low number of target molecules. The advantages of single LSPR nanosensor over ensemble measurement are (i) information that could be buried in ensemble measurement, for instances; the particle-particle variation would be revealed. It is anticipated that when the number of sampling getting large enough and the data could then be averaged to provide the ensemble properties; (ii) small sample consumption and high sensitivity, theoretically down to single molecules level and; (iii) direct measurement and simple sample preparation.
3.4 Protein-protein interaction by dark field imaging

To study protein-protein interaction, immunoreaction between PSA and two pairs of monoclonal antibodies, CHYH1 (Ab1) and CHYH2 (Ab2) were chosen as an example. AuNP was selected as a probe for its ability to conjugate protein on surface and its strong LSPR scattering for detection purpose. Figure 3.10 shows the schematic diagram of immunoassay of PSA with AuNPs probes. The two pairs of antibodies were conjugated to AuNPs through gold-thiol covalent bonding in the presence of linkers MUA, EDC and NHS. Protein as a large molecule, could cover the AuNPs surface and protect AuNPs from aggregation. The antibodies could be directly conjugated onto the AuNPs through electrostatic interaction between protein and AuNPs.

Figure 3.10. Schematic illustration of immunoassay of PSA with AuNPs probes.

The sandwich type immunoassay was performed with the aid of antibodies conjugated AuNPs. The two pairs of AuNP probes could be aggregated in the presence of the target analytes PSA at high concentration. Then, the complex was immobilized electrostatically on a APTES-treated glass slide for capturing dark field images and LSPR spectra. During the incubation in buffer solution, the two monoclonal antibodies Ab1 and Ab2 conjugated with AuNPs were bound on specific part of the PSA molecules. The sandwich type immunoreaction resulted in cluster formation among AuNPs. The interaction between PSA and AuNP probes was
confirmed by dark field images as shown in Figure 3.11 (a-d). Upon the increase of PSA concentration from 0 – 500 pM, the AuNP probes became larger and brighter. The number and size of the cluster formed increased correspondingly with the PSA concentration. Different from many research works based on AuNP aggregation formation for sensing the DNA, protein and metal ions, the cluster formation by PSA immunoreaction did not show an obvious color change for visual detection. In order to suppress the non-specific interaction of the detection, BSA was introduced to block the nonspecific adsorption between the AuNP probes. The BSA protein did not interact with the two pairs of antibodies AuNP probes, but over suppression was occurred at a high BSA amount (Figure 3.12, 3.13). Excessive BSA protein may overlap with each other and formed a massive BSA layer on the AuNPs surface. The BSA layer shielded the PSA binding towards the antibodies on AuNPs surface and inhibited the formation of cluster.
Figure 3.11 (a-d) Dark-field images of antibodies conjugated AuNP with 0, 50, 100 and 500 pM PSA in the present of 0.1 % BSA in PBS solution respectively.
Figure 3.12 (a-d) Dark-field images of antibodies conjugated AuNP in the present of 0.1, 0.5, 1 and 2 % BSA in PBS solution respectively.
Figure 3.13 (a-d) Dark-field images of antibodies conjugated AuNP with 500 pM PSA in the presence of 0.1, 0.5, 1 and 2 % BSA in PBS solution respectively.

3.5 Protein-protein interaction by LSPR measurement

To illustrate the capability of the LSPR system for detecting protein-protein interaction, we studies the LSPR response of AuNPs cluster in the presence of different PSA concentration (0 – 500 pM). After immunoreactions of PSA with antibodies-AuNPs probes in solution, DFM images and LSPR spectroscopy of individual nanoparticles were obtained. The $\lambda_{\text{max}}$ distribution of 100 AuNPs-antibodies in the presence of 0 -500 pM of PSA was established as shown in
Fig 3.14. It was observed that the higher the PSA concentration, the longer the $\lambda_{\text{max}}$ red shift of the resulted LSPR signals. Besides, the distribution curve was flattened when the PSA amount increase. Upon the solution-base cluster formation of AuNP, the AuNP probes bound together to form cluster in the present of PSA with vary of size. It was noted that the size of the clusters affected the LSPR peak wavelength and result in boarder LSPR $\lambda_{\text{max}}$ distribution as shown in Figure 3.15a-f. The magnitude of $\lambda_{\text{max}}$ red shift was induced by the boarding of LSPR $\lambda_{\text{max}}$ distribution according to the cluster formation. The cluster formation had a high variation and hindered measurement of the optical properties changes of individual nanoparticles in solution-base for quantitative biomolecules detection.

![Graph showing the distribution of LSPR wavelengths for different PSA concentrations](image)

Figure 3.14 The $\lambda_{\text{max}}$ Distribution of 100 AuNPs-antibodies in the presence of 0, 10, 50, 100, 200 and 500 pM of PSA. The $\lambda_{\text{max}}$ were 559.1, 560.0, 558.4, 563.1, 562.9 and 572.3 nm respectively.
Figure 3.15 (a-f) LSPR peak wavelength distribution of AuNPs-antibodies in the presence of 0, 10, 50, 100, 200 and 500 pM of PSA with gauss fit peak maximum at 559.1, 560.0, 558.4, 563.1, 562.9 and 572.3 nm respectively.
3.6 Ligand-receptor interaction by LSPR measurement

To study ligand-receptor interaction, GR and two ligands, Rg1 and DEX, were chosen as a ligand-receptor model for proof-of-concept. Based on the sensitive detection system used in the DNA-TOPO interaction, we adopted a LSPR biosensing assay at single particles level on surface-base for the ligand-GR interaction. Double stranded DNA-AuNPs were firstly immobilized electrostatically on a ATPES-treated glass slide, and 0.5 % of BSA was used to block the vacant on ATPES site in order to minimize the non-specific interaction and optimize the GR-DNA interaction. After on-chip addition and incubation of GR with dsDNA-AuNP in the presence of ligands, DFM images and LSPR spectroscopy of individual nanoparticles were collected. LSPR spectrum of groups of 100 nanoparticles in each experimental condition were analyzed and the $\lambda_{\text{max}}$ distribution was established as shown in Figure 3.16 a-e for 50 nM of GR in the presence of 500 nM of Rg1 or DEX. In the presence of GR, the receptor could nonspecifically bind with the dsDNA, and about 3 nm of $\lambda_{\text{max}}$ red shift was obtained from the dsDNA-AuNP. Also, it was observed that the GR-DEX mixture had a $\lambda_{\text{max}}$ red shift up to 4 nm. It was because the activation of DEX to the GR, promoted the binding ability of GR to specific dsDNA sequence. For the GR-Rg1 mixture, the activation ability of Rg1 was much less than DEX, so that the no $\lambda_{\text{max}}$ red shift was observed. To study the effect of DEX toward the GR-DNA ability, different amount of DEX was used for the GR-DNA-AuNP experiment. The resulted $\lambda_{\text{max}}$ distribution was established as shown in Figure 3.17a for DEX: GR ratio from 0:0 to 20:1. A response curve as a function of DEX: GR ratio from 0:0 to 20:1 was established as illustrated in Figure 3.17b. It was observed that the higher the DEX:GR ratio, the longer the $\lambda_{\text{max}}$ red shift of the resulted LSPR signals. This result indicated that the DEX ligand interact to GR and increased GR-dsDNA binding ability.
Figure 3.16 (a-d). LSPR peak wavelength distribution of dsDNA-AuNP-GR, dsDNA-AuNP-GR-DEX, dsDNA-AuNP-GR-Rg1, and dsDNA-AuNP (e) the $\lambda_{\text{max}}$ distribution of 100 dsDNA-AuNP in the presence of PBS, GR-Rg1, GR-DE and GR only. The $\lambda_{\text{max}}$ were 564.4, 562.7, 568.4 and 567.2 nm respectively.
Figure 3.17. (a) The \( \lambda_{\text{max}} \) Distribution of 100 dsDNA-AuNPs in the presence of 75 nM of GR and 1500-75 nM of DEX. The \( \lambda_{\text{max}} \) of DEX:GR from 0:0, 0:1, 1:1, 2:1, 10:1 and 20:1 were 567.2, 571.5, 571.4, 571.6, 572.5 and 573.9 nm respectively. (b) The LSPR shift as a function of DEX:GR ratio.
4. **Conclusion**

We reported a sensitive detection system for DNA-protein interaction, protein-protein interaction and ligand-receptor interaction at single particles level. This will be a valuable biosensing device to screen and monitor accurately the specific biomolecular interactions with a massively parallel detection capability in a high-throughput, simple and low-cost system. It is a direct, label-free and amplification-free method. Those nanoscale detection assays are of particular importance when the amount of sample is very limited as in conventional bulk measurement.
5. **List of Reference**


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Aug 2014