Surface plasmon resonance (SPR) enhanced biosensors

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**SURFACE PLASMON RESONANCE (SPR) ENHANCED
BIOSENSORS**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2016

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Abstract

A biosensor is a device that employs biomolecules or biomimetic components as capturing elements to detect analyte, record the information, which is translated mostly into electric signals. Different kinds of biosensors are based on different detection principles. Optical biosensors utilize the incident light as source to excite the molecules so that the signals, related to changes in refractive index, extinction/absorption, fluorescent emission and Raman scattering etc can be collected.

A plasmon is a collective oscillation of the free electrons in a noble metal. At the surface of a metal, plasmons take the form of surface plasmon polaritons (SPPs). When a surface plasmon is confined to a particle, free electrons participate in the collective oscillation and it is termed as localized surface plasmon (LSP), while plasmons propagating along the metal surface are defined as propagating surface plasmon (PSP). Surface plasmons are commonly excited by external electromagnetic radiation and a common feature of plasmons are that they concentrate the field to the near surface region, and can thus be used to enhance extinction, fluorescent, Raman and other signals. For example, binding of molecules in the vicinity of the surface induces the refractive index changes, which causes variations in the extinction spectra, eg. shift in peak position at a particular wavelength and/or line shape.

The sensitivity, specificity, selectivity, robustness, portability, speed and costs are the significant evaluation factors of a biosensor. The sensitivity and specificity are mainly related to the sensing mechanism and binding characteristics. The sensing principle in most of my works are SPR and SPR enhanced fluorescence. In the first part of my work, I designed two kinds of Au nanohole array, with (PAuNH array) and without (AuNH array) photoresist between the Au layer and the glass slides for the purpose of fluorescent enhancement using a model dye Alexa 647. This work is undertaken in close collaboration with researchers from IMRE, Singapore. The fabrication steps of the arrays were composed of electron-beam lithography and nano-imprint lithography. In comparison with the PAuNH array,

the AuNH array offered higher field enhancement for fluorescence when only LSP was excited. The detections operated on AuNH array using the collective effects of LSP and PSP turned out to be a promising combination in that it theoretically by simulation and experimentally revealed that the fluorescent enhancement was stronger in 2 orders of magnitude as compared with flat Au thin film substrate irradiated off-resonance.

In spite of intrinsic affinity of the receptor to the analyte, the external electric field provides extra force to increase the interaction frequency and probability, thus augment the signals of charged targets at increased speed. Non-specific binding is also reduced or even eliminated when the non-specific species possess different charge in relation to the analyte. I built an electro-focusing enhanced LSPR biosensor for the detection of human troponin I (cTnI). The limit of detection (LOD) in buffer can be improved by 2 orders of magnitude upon changing from no bias, 0 V, to -0.2 V bias applied on the sensor surface. The nonspecific adsorption of serum albumin (*i.e.* BSA) and rabbit serum is also significantly reduced at -0.2 V.

The point-of care device based on grating Au film for the detection of endotoxin (lipopolysaccharide, LPS) was demonstrated, which displayed excellent portability and good sensitivity. Relying only on the built-in LED light and CCD camera alleviated the size and the light 3D-printing sample holder furnished the device with control the light path and portability. Based on monitoring the resonant wavelength shift, the results showed the feasibility for the detection of LPS at detection limit of 32.5ng/ml.

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Abbreviations

2D	2 dimensional
3D	3 dimensional
A*STAR	Agency for Science, Technology, and Research
AFM	Atomic force microscopy
Ag/AgCl	Silver/silver chloride
AIT	Austrian Institute of Technology
ATR	Attenuated total reflection
Au	Gold
AuNH	Gold nanhole
BSA	Bovine serum albumin
BSE	Backscatter electron
c-Ab	Capturing PSA antibody
CBSS	Center for Biomimetic Sensor Science
CCD	Charge-coupled device
CD	Compact disk
Cl	Chlorine
Cr	Chromium
cTnI	Human troponin I
d-Ab	Detection antibody
d-Ab-AF647	Alexa 647 labeled PSA detection antibody
DNA	Deoxyribonucleic acid
DPN	Dip pen lithography
DUV	Deep Ultra- violet
EA	Ethanolamine
EBL	Electron beam lithography
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDX	Energy-dispersive X-ray
ELISA	Enzyme-linked immunosorbent assay
FDTD	Finite-difference time-domain
FEM	Finite element method

GC-SPR	Grating-coupled SPR
H ₂ O ₂	Hydrogen peroxide
HeNe	Helium-Neon
IMRE	Institute of Materials Research and Engineering
IPA	Isoproponal
LAL	Limulus amoebocyte lysate
LED	Light-emitting diode
LOD	The limit of detection
LPS	Lipopolysaccharide
LSP	Localized surface plasmon
LSPR	Localized Surface Plasmon Resonance
MEF	Metal enhanced fluorescence
MIBK	Methylisobutylketeone
MSE	School of Material Science and Engineering
N ₂	Nitrogen
NHS	N-Hydroxysuccinimide
NIL	Nano-imprint Lithography
O ₂	Oxygen
PAuNH array	AuNH Array with Photoresist
PBS	Phosphate-buffered saline
PBST	PBS tween buffer
PCR	Polymerase Chain Reaction
PMMA	Poly(methyl methacrylate)
PMT	Photomultiplier tube
PSA	Prostate specific antigen
PSP	Propagating surface plasmon
RIE	Reactive ion etching
RPT	Rabbit pyrogen test
RI	Refractive index
SA647	Alexa-labeled streptavidin
SAM	Self-assembled monolayer

SEI	Secondary Electron Images
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman scattering
SPF	Surface Plasmon enhanced fluorescence
SPFS	Surface plasmon enhanced fluorescence spectroscopy
SPM	Scanning probe microscopy
SPPs	Surface plasmon polaritons
SPR	Surface Plasmon Resonance
SPs	Surface plasmons
STM	Scanning tunneling microscopy
Thiol-PEG-COOH	Thiol capped Polyethylene glycol acid
TM	Transverse magnetically
UV	Ultra-violet
UV-vis	Ultra violet-visible
XRD	X-ray Diffraction

Chapter 1

Introduction

The first chapter gave a preview of this thesis, including the inspiration and motivation to do all the rest works, the aims and goals to be finished, an overview of the projects and the findings and outcomes of the next few chapters.

1.1 Hypotheses

Nanostructures can carry Surface Plasmon when excited by light and the SPR signal will depend on the local variations of the bounding medium. Such variations will translate into changes in the extinction/absorbance spectrum as well as in the fluorescence emission spectrum and can be used for biomolecular detection.

Hypothesis 1: Polarization/biasing of the AuNH array offers both improved sensitivity and selectivity.

The sensitivity, selectivity, robustness and portability are all essential design parameters for a biosensor¹. The sensitivity is the first factor considered when researchers develop a biosensor. Two general amplification strategies based on the target and the signal have been developed to improve the sensitivity of a biosensor, in which the target-based amplification (*i.e.* PCR, short for Polymerase Chain Reaction) is an effective straightforward way to boost the sensitivity simply by producing more targets available for binding. Along with the application and promotion of advanced spectroscopies, new materials (*i.e.* nanomaterials) and assays (*i.e.* based on SPR or SPR enhanced labelling methods), the signal-based amplification offers an alternative way to improve sensitivity. Carefully designed structures in the nanometer range with favorable optical properties, large surface to volume ratio and superb biocompatibility provide an attractive route to high sensitivity. The gold nanohole (AuNH) array is according to simulation sensitive enough for label-free detection, but it can also be adapted in fluorescence assays enhanced by Localized Surface Plasmon (LSP). Furthermore, the transfer of target molecules to the surface is also crucial to high performance biosensing. As a large fraction of biomolecules including peptides, proteins and DNA strands are not neutral in typical biofluids implies that they can be specifically attracted to differently polarized sensing surfaces. Thus I expect that an externally applied electric field is capable of improving the mass transfer kinetics of a charged target by tuning the electric field in a favorable direction. Simultaneously it can repel species of opposite charge. Setting the electric potential of the substrate (AuNH array in chapter 4) opposite to the target (cTnI) it can be selectively attracted to the surface whereas other interfering molecules of opposite charge to cTnI will be repelled. This application is valuable and of practical importance for detection of positively charged targets in humoral environment, *e.g.* in

plasma and serum, which both contain large amounts of negatively charged proteins (albumins).

Hypothesis 2: Properly designed synthetic peptides can replace antibodies in assays for sensitive and selective detection of biomedical targets in demanding environments.

Besides the experimental (biasing) and the materials properties (structure, size, morphology of the nanoparticles for LSP and film for PSP), the capturing element (the receptor) plays an equally important role in biosensing. The receptors in most of my works were synthetic peptides specifically synthesized for a given analyte. They offer tunable affinities in the sub-nM range; superior stability over antibodies under extreme condition (pH and temperature), facilitating a simple storing requirement and working condition; small size/short length leaving enough space for analyte to approach to the surface thereby inducing a more prominent signal change because the evanescent field of Surface Plasmon decays exponentially with the distance from the surface; easy and cheap to produce using peptide synthesizers; and high purity.

Hypothesis 3: The combination of LSP and PSP gives rise to an additional enhancement to the fluorescent signal resulting in improved sensitivity.

The confined electromagnetic field manipulated by LSP enables enhancement to the fluorescent signal under the condition when the resonance range of LSP matches the excitation/emission wavelengths of the fluorophore. Importantly, the size, period and thickness of the AuNH array can be tuned to fit the excitation range of the fluorophore. The continuous supporting film of the AuNH array is also capable to carry Propagating Surface Plasmon (PSP) when incident light illuminates from certain angle, and it can be excited simultaneously with LSP.

Hypothesis 4: The smartphone in conjunction with synthetic peptides provide an attractive avenue for detection of biomedically relevant targets on site.

Although sophisticated instruments and conventional approaches provide more accurate results and lower detection limits, they are not easily used in field applications. Moreover, they are also associated with high cost and require often well-trained operators. The smartphone is a ubiquitous platform that offers many features for the development of portable point-of-care devices for remote diagnosis on site and daily care. The built-in LED light source can be modulated to satisfy the excitation of PSP on well fabricated

grating Au film surface. The resolution of image detector of the phone is enough to record the spectra changes induced by the analyte binding when the resonant range of grating Au falls into the detecting range of the camera.

1.2 Objectives and Scope

In our center, CBSS, we work mainly on application of novel concepts, theory and methodology relevant to sensing. We follow the pioneering steps of our supervisor, Prof Bo Liedberg who opened the SPR biosensing field.

My ambitions are summarized in four aims:

- (1) Design PAuNH array and AuNH array substrates according to the simulation to fulfill biosensing application;
- (2) Establish a platform as LSPR label-free biosensor for human troponin I (cTnI). The sensitivity and selectivity (resistance to interfering molecules) can be improved by application of an electric field to focus the positively charged cTnI, thereby speeding up the binding rate of cTnI in Phosphate-buffered saline (PBS) solution and reduce the non-specific binding of the most abundant species as serum album with opposite negative charge;
- (3) Build a grating Au coupled label-free point-of-care device based on a smartphone for lipopolysaccharide (LPS, also named as endotoxin). The portability is accomplished by omitting external components. The sensitivity is improved by proper design and preparation of grating Au substrate and the selection of specific peptide for LPS;
- (4) Incorporate LSP and PSP into one sensor to furnish better directional fluorescence enhancement on AuNH array.

1.3 Dissertation Overview

The thesis addresses the design of LSP, combined of LSP and PSP and Surface Plasmon enhanced fluorescence (SPF) biosensors for application in various assay formats.

Chapter 1 provides a rationale for the research and outlines the goals and scope. It presents the problems and challenges in the fields I worked and the hypothesis of all the works afterwards. The objective of my research is stated in the second part of this chapter. A general overview of the whole thesis, which summarizes the contents of each chapter,

is elaborated.

Chapter 2 reviews the literature concerning the principle, theories and mechanism referred in my thesis. In the beginning, I briefly introduce the history of the biosensors and the works of the leading pioneers. The SPR-based biosensor, the sub area of optical biosensor is emphasized since it accounts for the main parts of my thesis. The basic knowledge is demonstrated in this chapter, for instance, enhancement and sensitivity improvement aspects.

Chapter 3 discusses the principles underlying the techniques employed and the unique instruments in our lab for the assays. This chapter is organized in the sorts of simulation, sample preparation and characterization. It also outlines the set-ups used in my works. In *simulation* part, I introduce the concept of finite element method (FEM). The electron beam lithography (EBL), the nano-imprint lithography (NIL), the spin coating method and thermal evaporation are illustrated after that. The characterization tools - ultra violet-visible spectroscopy (UV-Vis), scanning electron microscopy (SEM) and atomic force microscopy (AFM) are introduced and discussed. The main experimental instruments of my work are elaborated on in detail. Some of the process and operational instruction are explained for better understanding of the results of the assays. The data analysis methods (including the software) are discussed in this chapter as well as the fabrication and characterization of AuNH array.

The following chapters are arranged by content, starting from label-free detections followed by fluorophore-labelled assays.

Chapter 4 elaborates an electric field focusing and Localized Surface Plasmon Resonance enhanced label-free biosensors, which was based on the paper accepted in the RSC journal *Nanoscale*. The biosensor functionalized by electric field can give a faster and better assay for human troponin I (cTnI). The experimental section involves the materials, instruments and preparation of the experiments. The result and discussion reports the analytical data and conclusion.

Chapter 5 elaborates the work of grating-coupled Surface Plasmon Resonance enhanced label-free biosensors (manuscript in preparation). This chapter discusses lipopolysaccharide (LPS) detection using a smartphone and a grating Au substrate.

Chapter 6 describes a Localized and Propagating Surface Plasmon Resonance co-

enhanced directional fluorescent biosensor. Simultaneous excitation of LSP and PSP on metallic thin film indicated substantial enhancement of the fluorescence intensity. It was 100 times better as compared to a flat Au surface irradiated off-resonance and 3-fold higher than the substrate only supporting PSP resonantly excited.

Chapter 7 outlines some preliminary findings and highlights challenges for the future, for example, the selective modification of AuNH array to improve sensitivity.

Chapter 8 summarizes my thesis. The summary of all results are restated here. The chapter ends with a statement accounting for the original hypothesis. The opportunities and strategies for future work are listed.

1.4 Findings and Outcomes

This research led to several novel outcomes by:

1. Establishing a platform as LSPR label-free sensor for human troponin I, the limit of detection (LOD) of which was 2ng/mL using the X-nano instrument at an assay time of 30 min. For more than 15 years, cTnI has been known as a reliable marker of cardiac muscle tissue injury. It is considered to be more sensitive and significantly more specific in diagnosis of the myocardial infarction than the “golden marker” of last decades- Creatin Kinase. The clinical level of cTnI in blood is 1ng/mL. Thus, our platform has strong potential to meet it.

Figure 1. 1 Schematic illustration of (a) the setup made of PDMS and (b) the modified AuNH array chip applied with a negative bias for minimizing the nonspecific adsorption of BSA and simultaneously attracting the target cTnI to the sensor surface.

2. Building a smartphone platform for LPS with superb portability and detection limit of 32.5ng/mL in dry state. Excess LPS can create exaggerated inflammatory responses, which can then cause multiple organ failure, septic shock and even death. As a result, strict limits have been imposed on LPS level permitted in foodstuff and medical supplies, such as injectable drugs, dialysis baths and medical devices. In human blood, less than 10ng/mL of LPS can be neutralized by plasma. Our sensor possesses excellent portability as a point-of-care device. It also has relatively good performance for preliminary detection.

Figure 1. 2 (a) Schematic structure of the smartphone spectrometer relying on the in-built LED light, a CD diffraction substrate and CCD camera. Inset is a photo of 3d printing plastic sample chamber kit attached on HTC sensation XE smartphone. (b) Schematic illustration of the optical arrangement of the smartphone.

3. Incorporating LSP and PSP into one biosensor perform excellent fluorescence enhancement which accomplished the last aim stated in *Objective and scope*, about 2 times and 14 times better than that for the PSP enhanced fluorescence and LSP enhanced fluorescence, respectively.

Figure 1. 3 (a) Scheme of the geometry used for co-excitation of PSP and LSP modes on an AuNH array for fluorescence enhancement. ϑ_{max} is the maximal angle of fluorescence emission collected through the optics. (b) Images of a sensing spot acquired with a CCD camera upon reflection of the excitation laser beam from a flat Au film (left) and AuNH (right) at an angle of incidence of $\theta=62.3^\circ$ and 72.3° , respectively. Scale bar 2 mm.

References:

- [1] D. A. Giljohann and C. A. Mirkin. *Nature*. **2009**, 462, 461-464.

Chapter 2

Literature Review

In this part, I reviewed the history and pioneer works in biosensors. The fundamental principles and some of the remarkable works were introduced. The optical biosensor, which was a sub-category of biosensor, was then briefly presented, followed by the biosensor based on Surface Plasmon Resonance (SPR). Label-free SPR biosensor and SPR enhanced fluorescent biosensor were the two kinds applied in my works. After that, I deliberated the theories of interfacial molecular binding, diffusion limitation, limit of detection, the factors influencing the sensitivity of SPR biosensor and the methods to enhance or magnify the signal, for instance, employing electric field to reduce the nonspecific binding, utilizing enzyme which had stronger specificity and taking advantage of magnetic particles, etc..

2.1 Biosensor

Sensors are broadly classified into three categories according to the application areas: (1) Chemical sensor, detects chemical substances e.g. inorganic or organic volatile gases. (2) Physical sensor, measures the physical change, such as humidity, temperature, pressure, strain, distance, and volume, *etc.* and (3) Biosensor ¹, employs biomolecules or biomimetic components as capturing elements to detect analyte, record the information, which is translated mostly into electric signals.

This thesis focuses on biosensors. The first biosensor was developed to detect glucose using an enzymatic reaction and it dates back to 1960s ². A typical biosensor is composed of three parts (Figure 2.1): (1) a capturing/recognition element or layer, a sensitive bio-based/biomimetic unit that can recognize, bind to or interact with the target. It can be a cell surface receptor, antibody, enzyme, single stranded DNA, peptide, *etc.*; (2) a transducer, reports the binding or interaction events between the target and capturing molecules (can be based on gravimetric and optical phenomena *etc.*); (3) signal processor that amplifies and converts the transducer signal into a processable format and displays the signals in a user friendly way. ³

Figure 2. 1 Schematic illustration of a biosensor ¹

The function of the receptor is to recognize and capture the analyte. Carefully choosing the receptor ensures a good selectivity and specificity of a biosensor. The materials used as receptor can be biological molecules, such as protein, enzyme or nucleic acid, and biomimetic molecules, for instance synthesized peptide and molecularly imprinted polymers specific for the targets. Although proteins like antibodies have strong specificity and affinity to the targets, which are corresponding antigens, the difficulty of maintaining the biological activity reduced the practicability. Meanwhile, the large size of them needs more space to settle, which means in the same area of the sensor surface less targets can be captured. Especially for surface plasmon enhanced sensor, the electromagnetic field exponentially decreases with respect to the distance to the surface, The larger gap between the receptor and the surfaces weakens the signals as the refractive index change induced by the binding of the targets does not happen in the most sensitive place of the sensor. In my works, I used synthetic small peptides, usually composed of less than 20 amino acids and small biological molecules like biotin. The synthetic peptides overcome the mentioned drawbacks of large biological molecules like antibodies and ensure a good performance of the biosensors. Three ways are commonly used to immobilize the receptor onto the sensor surface: (1) direct immobilization. The receptors containing an end which has strong affinity or can react with the treated surface can directly settle themselves on the sensor surface, for example, a peptide with a cysteine unit at the terminal position to a gold surface; (2) interlayer immobilization. The receptors which have no affinity to the sensor surface can be attached to the sensor by an interlayer. This approach works for large molecules like antibody and the covalent attachment between the interlayer and the antibody requires the activation by the EDC/NHS coupling chemistry by coupling the -NH_2 groups of proteins with the -COOH groups on the surface of interlayer ⁴ (Figure 2. 2); (3) 3 dimensional (3D) matrix immobilization. In this approach, the receptors are embedded into the 3D matrix. ^{5,6} The first and second approaches usually form a 2 dimensional (2D) self-assembled monolayer (SAM) of the receptors or the combination of the interlayer and the receptors by incubating the sensor with solutions containing receptors or the mixture of receptors and interlayer molecules. In order for large targets binding, some short length spacers are mixed to separate the receptors. The first and second approaches were applied in my

works. In chapter 4, I made use of cysteine terminated synthetic peptides specific to cTnI, separated by five amino acid small peptide. For endotoxin detection, the SAM was only composed of the synthetic specific peptides.

Figure 2. 2 Schematic illustration of the EDC/NHS coupling chemistry

The principles behind the detecting methods that biosensors are based on usually refer to thermal, electrical, optical, magnetic, piezo-electric⁷⁻¹⁰ and the combination of these properties. The biosensor also can be categorized in terms of the interaction mechanisms by which the sensor signal is generated. For example, when a capturing molecule (e.g. an antibody) binds to the target molecules (e.g. an antigen) forming an antibody/antigen complex then one refers to an affinity biosensor. In other cases the capturing molecule (immobilized or free in solution) become catalytically converted or decomposed into smaller products that can be detected by the transducer. The product can be molecular fragments, ions and electrons. This is how many enzymes work and one often refers to catalytic or enzymatic biosensors (see example below for glucose sensing). My thesis focuses on optical/affinity biosensors.

The working process for an optical/affinity biosensor can be described as follows. The target molecule is recognized and binds to the capturing molecule. This binding creates a change in the local environment at the transducer surface and if we are talking about non-labeled target molecules then the complex formed will change the local refractive index and/or thickness of the layer formed on the surface. In case of a labeled, e.g. with a fluorophore, then the fluorescence emission will increase upon binding. The transducer will capture the signal and translate it into measurable electronic signal. The most well-known optical biosensor that relies on affinity binding is the SPR-based BIAcore system¹¹.

The third element of a biosensor is the signal producer which can amplify and convert the transducer signal into a processable format and display the signals in a user friendly way. The most well-known catalytic/enzymatic biosensor is the glucose sensor that can measure the concentration of the glucose in blood. Theoretically, in the sensor, glucose is first digested by glucose oxidase enzyme, producing gluconic acid and glucose oxidase enzyme is reduced at the same time. Glucose oxidase enzyme is recycled by another reaction consuming oxygen (O_2) to form the hydrogen peroxide (H_2O_2). Extra electron is released during the process and is measured to determine the glucose concentration. A diagram of the working process of the glucose sensor is depicted in Figure 2.3.¹

Figure 2. 3 Diagram of glucose biosensor reaction¹

Common problems in label free affinity biosensing detection are selectivity and non-specific binding, meaning that unexpected molecules in the analyte solution also bind to the capturing molecule or the transducer surface and generate a signal. This creates false readings and constitutes huge problem in affinity biosensing of complex analytes. Numerous strategies have been employed to tackle non-specific binding. Researchers have developed coating which are resistant to non-specific binding. This includes, for example, co-immobilization of capturing molecules with oligoethylene glycol tails¹²⁻¹⁴ or

immobilization of the capturing molecule in a 3D sensing matrix¹⁵. The capturing molecule also can be derivatized in order to resist binding to non-target molecules.

Compared to other approaches, such as chromatography and spectrometry often used in environmental industry and immune-assaying for medical and biological purposes, biosensors are becoming more and more popular. Some offer very high sensitivity and selectivity. Many researchers are finding ways to push the limit of detection (LOD). Others are developing sensors for field applications. The development of innovative fabrication processes of sensing surfaces (biochips) and advanced and efficient production of capturing molecules has dramatically reduced the cost and need of trained personnel in modern biosensors.

2.1.1 Optical Biosensor

Optical biosensors utilize the incident light as source to excite the platform so that the interaction of the target can be detected in the form of changes of optical properties, for instance, reflection, absorption, extinction, refraction, interference, fluorescence and Raman signals, *etc*¹⁶⁻²¹, can be detected. Advances have been made in various aspects, such as the detection mechanism(s), biochip and transducer design, sampling techniques *etc.* Among all the kinds of biosensors, Surface Plasmon Resonance based sensors have attracted more and more interests since the prototype of Surface Plasmon Resonance sensor was first demonstrated by Bo Liedberg *et. al.* in the early 1980s.^{22, 23}

2.1.2 Surface Plasmon Resonance Biosensor

Plasmons are collective oscillations of free electrons in metals. Upon exposure to external electromagnetic field, the whole electron gas can be excited to oscillate with respect to the positive charged cores.^{24, 25} It is called Surface Plasmon Resonance (SPR) when it happens on the surface of the metal. The phenomenon of Surface Plasmons propagating on metallic thin film was observed by Wood^{26, 27} and Fano²⁸ upon excitation of electromagnetic waves. The theory was then established and developed through a series of studies by several scientists.²⁹⁻³³ Noble metals are particularly useful and applied in SPR because of their dielectric (free electron or gas-like) properties. The excitation is largely dependent on the optical properties of the metal, the dielectric function. The dielectric functions of metals have two parts, a negative real one and a positive imaginary

one. There are two kinds of Surface Plasmon Resonance: one occurs when light is incident on a macroscopic planar surface and they are referred to as Propagating Surface Plasmons (PSPs) (Figure 2.4a)^{34, 35}; Surface Plasmon also can be localized (Figure 2.4b)^{34, 35} when the light illuminates particles smaller than the wavelength of the light and they are called Localized Surface Plasmons (LSP).

Figure 2. 4 Scheme of (a) Propagating Surface Plasmon Resonance and (b) Localized Surface Plasmon Resonance ³⁴

When SPR is excited by incident light, the light is partially scattered by the metal surface. The remaining part is absorbed by the metal surface. In order to quantify the distribution of light scattering and absorption, Gustav Mie developed a method to solve Maxwell's

equations and applied it into spherical particles' LSPR in the year of 1908. Assuming that light incidents to a metal spherical particle, the extinction, scattering, and absorption cross-sections are given below ³⁶:

$$\sigma_{ext} = \frac{2\pi}{|k|^2} \sum_{L=1}^{\infty} (2L+1) [Re(a_L + b_L)] \quad (2.1)$$

$$\sigma_{sca} = \frac{2\pi}{|k|^2} \sum_{L=1}^{\infty} (2L+1) (|a_L|^2 + |b_L|^2) \quad (2.2)$$

$$\sigma_{abs} = \sigma_{ext} - \sigma_{sca} \quad (2.3)$$

$$a_L = \frac{m\psi_L(mx)\psi'_L(x) - \psi'_L(mx)\psi_L(x)}{m\psi_L(mx)\chi'_L(x) - \psi'_L(mx)\chi_L(x)} \quad (2.4)$$

$$b_L = \frac{\psi_L(mx)\psi'_L(x) - m\psi'_L(mx)\psi_L(x)}{\psi_L(mx)\chi'_L(x) - m\psi'_L(mx)\chi_L(x)} \quad (2.5)$$

Here k stands for incident light wave vector, while L is symbol of scattering mode, which must be an integer. x in the Riccati-Bessel functions ψ_L , χ_L is equal to the product of particle radius r and wave number in the medium k_m ($k_m = \frac{2\pi}{\lambda_m}$). The other independent variable of ψ_L , χ_L , $m = \frac{\tilde{n}}{n_m}$ (Note that $\tilde{n} = n_R + (n_I)i$, where $\tilde{n}$, n_R and n_I stand for the real and imaginary part of the metal refractive index, respectively, and n_m is the refractive index of the surrounding medium).

In the case that the particle size is much smaller than the wavelength, $x \ll 1$, b_L will be 0 and a_L can be simplified as a_s :

$$a_s \approx -\frac{(2x^3)i}{3} \cdot \frac{m^2 - 1}{m^2 + 2} \quad (2.6)$$

Since $m = \frac{\tilde{n}}{n_m}$ and $\tilde{n} = n_R + in_I$, a_s can be rewritten as:

$$a_s \approx -\frac{(2x^3)i}{3} \cdot \left(1 - \frac{3n_m^2}{n_R^2 + (2n_R n_I)i - n_I^2 + 2n_m^2}\right) \quad (2.7)$$

The dielectric function of the metal and the surrounding medium are substituted into a_s :

$$\tilde{\epsilon} = \epsilon_1 + \epsilon_2 i \quad (2.8)$$

$$\epsilon_1 = n_R^2 - n_I^2 \quad (2.9)$$

$$\epsilon_2 = 2n_R n_I \quad (2.10)$$

$$\epsilon_m = n_m^2 \quad (2.11)$$

$$a_s \approx \frac{2x^3}{3} \cdot \left[i - \frac{3\varepsilon_m}{(\varepsilon_1 + 2\varepsilon_m)i - \varepsilon_2} \right] \quad (2.12)$$

Assume the volume of the particle is V . This simplifies the equations for σ_{ext} and σ_{sca} which can be rewritten as

$$\sigma_{ext} = \frac{18\pi\varepsilon^{1.5}V}{\lambda} \cdot \frac{\varepsilon_2(\lambda)}{[\varepsilon_1(\lambda) + 2\varepsilon_m]^2 + [\varepsilon_2(\lambda)]^2} \quad (2.13)$$

$$\sigma_{sca} = \frac{32\pi^4V^2\varepsilon_m^2}{\lambda^4} \cdot \frac{(\varepsilon_1 - \varepsilon_m)^2 + (\varepsilon_2)^2}{(\varepsilon_1 + 2\varepsilon_m)^2 + (\varepsilon_2)^2} \quad (2.14)$$

The scattering and extinction equations show that LSPR is influenced by the particle size, wavelength of the incident light, the dielectric function of the metal and the surrounding medium, especially under a specific condition that $\varepsilon_1 = -2\varepsilon_m$. It demonstrates the way that LSPR peak position is affected by surrounding environment.

Although the previous equations are not accurate for calculate σ_{ext} and σ_{sca} of bigger particles, whose size is bigger than 10nm, nor of particles of complex shape, they can be used to predict optical properties of spherical particles.³⁷

Recently, Localized Surface Plasmons have attracted much attention. Numerous researchers have become aware of the potential of applying LSP in other fields including biology, microelectronics, physics, materials science *etc.* Thus, LSPs show great promise in many multidisciplinary areas of fundamental and applied nature. The direct shift of the LSPR peak, for example, is a very useful sensing method to detect bulk refractive index changes in the local environment of the particle. Because of the localization of the surface plasmon to the metal particle it can also be used to enhance various optical signals as fluorescence emission and Raman scattering. Thus, LSPR in conjunction with these methods have proven to be useful for label as well as label-free detection of targets at the molecular level.³⁷

Propagating Surface Plasmon biosensors commonly measure the refractive index changes near the surface of metallic thin films in the form of reflectivity changes. In spite of the fact that PSP can be excited by bombardment of electron with intense energy, the optical methods are more commonly employed. Two approaches are used practically in experiments – the prism- and grating-coupler. For prism-coupler excitation, two different configurations have been proposed and theoretically and experimentally elucidated by

Kretschmann (Figure 2. 5a) and Otto (Figure 2. 5b) ³³, respectively. Among the two, the Kretschmann configuration is widely adopted in the sensing and thin film communities.

(a)

(b)

Figure 2. 5 Attenuated total reflection method with the (a) Kretschmann and (b) Otto configuration for the excitation of surface plasmons ^{32, 33}

They are both based on the attenuated total reflection (ATR) method. For the Kretschmann configuration, the metallic thin film is deposited directly on the base of the prism facing the surrounding dielectric medium. The refractive index of the prism (ξ_p) is

chosen to be larger than that of the dielectric medium (ξ_d). Under such circumstance and when the propagation vector of the SP matches that of the incident wave, the Surface Plasmon can be excited at the interface between the dielectric medium and the metallic thin film, resulting in a concentrated electromagnetic field. The electric field from the SP is attenuated perpendicular to the surface and it is normally referred to as the evanescent field. The extension of this into the medium defines the penetration or sensing depth of the PSP. The surface plasmon is not confined in a small area but propagates along the interface along with a propagation or decay length of about 10 μm at visible wavelengths. In the Otto configuration (Fig. 2.5b) the metal is deposited on a dielectric medium $\xi_d < \xi_p$ or it can be separated from the prism via a small air gap, typically 500-1000nm wide. The strength of Surface Plasmon lies in its thickness and refractive index sensitivity of the surrounding medium. The resonance position which can be seen as a dip in the angular reflectivity curve at a specific angle θ (Figure 2. 6) is determined by the integrated effect of the refractive index of the prism, metallic thin film and the dielectric medium according to the following equation:

$$\theta = \sin^{-1} \text{Re} \left(\sqrt{\frac{\epsilon_m \epsilon_d}{(\epsilon_m + \epsilon_d) \epsilon_p}} \right) \quad (2.15)$$

According to the Fresnel equations, the angular reflectivity curve can be predicted by calculating the transfer matrices.³⁸⁻⁴³

For a grating-coupler approach (Figure 2. 7), a simpler implementation is utilized since the excitation of Surface Plasmon depends on the topography (periodicity) of the surface of the metallic thin film and not on the properties of an external prism. In this structure, the light is directly shed on the periodically modulated surface at an incident angle of θ so as to the diffraction matches the propagation vector of both the incident wave and Surface Plasmon. The reflectivity is related to the incident angle and is sensitive to refractive index changes of surroundings.

Figure 2. 6 An illustration of angular reflectivity spectra for Au ($\zeta_{Au} = -12.24+0.7i$) thin film

Figure 2. 7 Schematic illustration of periodic grating-coupler excitation structure.

2.2 Field Enhancement

Field enhancement is a consequence of the localization of the Surface Plasmon and the dielectric properties of the bounding media, resulting in the augment of electromagnetic field. The enhancement of Surface Plasmon excited on certain boundary of metallic thin film and external dielectric and located at the dielectric medium is characterized by

enhancement factor K . The intensity of the incident light and the light at the boundary (accumulated electromagnetic field intensity) denoted as I_s and I_0 , respectively. Then the K is calculated in the formula:

$$K = \frac{I_s}{I_0} \quad (2.16)$$

The K is a function of incident angle θ for many interface configurations, not limited to glass-metal-dielectric. It gradually augments along with the increase of θ and hits the ceiling at certain angle value. The K decreases afterwards. For a normal glass-water interface configuration, the maximum K_{g-w} is about 4 at critical angle (which is determined by the dielectric constant of the two materials creating the boundary), while the maximum K_{m-w} reaches almost 16 for a 50nm Au film caused by the excitation of Surface Plasmon.

2.2.1 SPR Enhanced Label Free Biosensors

More and more attentions have been brought to biosensors based on SPR, which are applied for detection of biological and chemical molecules⁴⁴, ions and other spices. SPR enhanced label free biosensors measure the variation of resonance in the form of resonant peak wavelength, angle, intensity *etc.* directly resulting from targets binding and inducing refractive index change of the surrounding medium⁴⁵. Localized surface plasmon enhanced label free biosensors involving noble metal particles in solution and nanostructures randomly and periodically distributed on chips⁴⁶⁻⁴⁸ utilizes signals coming from the transmittance or absorbance spectra. On the contrary, measurement of reflectivity related changes is the main tool in propagating surface plasmon resonance enhanced label free biosensors. The combination of nano particles and nano-scale thin films brings out new promise for SPR enhanced label free biosensors⁴⁹. In the assay, the targets are captured by the recognition elements immobilized on the metallic surface. For biological targets, the assay is usually employed in liquid environment while it is more convenient for gas detection to occur in air or another carrier gas. Theoretically, the sensitivity to the bulk refractive index change of the LSPR enhanced label free biosensors is more than 10 times smaller than that of the PSPR biosensors⁵⁰. This weak point is balanced by the advantage of a smaller sensing volume near the surface, which in some cases can be used for trace detection close to the metal particle surface. LSPR enhanced

label free biosensors recently extend into new avenues for single molecule detection^{51,52}. The limit of detection has been demonstrated in the femtomolar regime⁵³, even down to attomolar⁵⁴ with the assistances of carefully prepared recognition elements such as RNA and proper design of the structures.

2.2.2 SPR Enhanced Fluorescent Biosensors

Figure 2. 8 The Jablonski diagram illustration of the electronic and vibrational states of a fluorophore⁵⁵

Fluorescence is one category of luminescence phenomenon that involves photons absorption and re-emission and consists of three stages (Figure 2. 8): excitation stage (S_0 to S_1'), excited stage (S_1' to S_1) and fluorescence emission stage (S_1 to S_0). The molecules with the ability of emit fluorescence are called fluorophores, which usually has strongly delocalized electrons. At first stage, excitation occurs by absorption of the energy $h\nu_{ex}$ from external source (*i.e.* laser light) resulting in excitation to and higher excited stage, S_1' . The excited stage usually lasts several nanoseconds depending on the on the fluorophore. The conformational adjustment of the excited fluorophore happens, as well as quantities of various interactions and reactions with the environment. After dissipation of the energy (via molecular vibrations), the fluorophore ends up in S_1 from which relaxation to the ground state S_0 occurs. This results in emission with energy of $h\nu_{em}$, S_1 to S_0 . Some other processes also accompany with fluorescence process, such as quenching, k_q and fluorescence energy transfer, k_{et} .

The fluorophore often suffers from photo bleaching caused by too large or extended exposure to the external light source leading to photochemical destruction owing to photoreaction involving reactive oxygen molecules.

The enhancing capability of Surface Plasmon because of the localization of the electromagnetic field is the basic principle for many spectroscopic techniques. The cooperation of surface plasmon resonance spectroscopy and fluorescence spectroscopy is referred to as surface plasmon enhanced fluorescence spectroscopy (SPFS). When a fluorophore locates in the vicinity of the surface of a noble metal particle or thin film, it can be effectively excited by the evanescent field of surface plasmon and the signals created can be magnified up to several orders of magnitude with respect to the free fluorophore (without metal).^{56, 57} The enhancement of surface plasmon to a fluorophore is decided by not only the parameters of the metallic nanostructures (size, shape, periodicity etc.), but also the distance between the nanostructures and the fluorophore: the non-radiative decay (quenching) of the fluorophore within the distance of approximately 10nm is the dominating process. For distances between 10nm to 20nm, the quenching decreases because some of the fluorophores re-coupled to surface plasmon along with the increasing distance. Afterwards, only the fluorescence happens with the consideration of disturbance from light source and the reflective light of the metal⁵⁸. A lot of works have been done utilizing simple nanoparticle⁵⁹, core shell structure⁶⁰, novel metallic thin film^{61, 62} and other structures^{63, 64} to detect chemical and biological molecules based on surface plasmon enhanced fluorescence spectroscopy (Figure 2.9). The detection limit of this technique was upgraded to attomolar⁶⁵.

Figure 2. 9 Schematic illustration of (a) nanoparticle enhanced fluorescent detection,⁵⁹ (b) the preparation of fluorescent core-shell Ag@SiO₂ (MEF) nanoballs and fluorescent nanobubbles⁶⁰, (c) Biotin/avidin fluorescent detection enhanced by Au film⁶² and (d) surface functionalization, gold NP attachment, and gold nanoshell growth on the upconversion NCs for fluorescence⁶³.

2.3 Binding Kinetics

In a biosensor, the target recognition and binding are the initial step when it works, including target molecules mass transfer in the bulk solution to the surface, interaction between the receptor and the target molecules and the target “settling” on the surface. In liquid environment, the last settling stage is a kinetic condition other than static one, which involves association and dissociation. As a result, the amount of target bond on the surface (by receptors) and the binding rate is influenced by the mass transfer limit (target molecules diffusion in the solution) and affinity of analyte to receptor (the association and dissociation constants).

A and R represent the analyte and the receptor, respectively. The binding kinetics is a process shown below:

Where A_b stands for the analyte molecules in bulk solution, A_{s0} for those approaching to the surface, A_{s1} for the ones associated with receptors. k_m , k_a , and k_d are the mass transfer coefficient, association constant and dissociation constant, respectively.

The situation is simplified when ignoring the dissociation of R on the surface. The process reveals three key factors influencing the binding kinetics. Take $[X]$ as the concentration of X . When considering all the factors, the conditions are described by the following equations:

$$\frac{d[A_{s0}]}{dt} = \frac{k_m([A_b] - [A_{s0}]) - k_a[A_b][R] + k_d[A_{s1}R]}{\frac{Dh^2wl}{v_{max}}} \quad (2.17)$$

$$\frac{d[A_{s1}R]}{dt} = k_a[A_{s0}][R] - k_d[A_{s1}R] \quad (2.18)$$

$$D = \frac{k_B T}{6\pi r \eta} \quad (2.19)$$

where $D^{66, 67}$ denotes the diffusion coefficient, v_{max} stands for the max velocity in the flow-cell, k_B for Boltzman constant⁶⁸, T for the absolute temperature, η for the viscosity of the fluid. The parameters of the flow-cell, height, width and length are h , w , and l , respectively.

When the affinity of the analyte molecules to the receptors is relatively strong, which means the binding kinetics is limited by the first part:

The binding rate equation is simplified:

$$\frac{d[A_{s1}R]}{dt} = k_m[A_b] \quad (2.20)$$

This situation is called the mass transfer controlled kinetics.

On the contrary, when the target molecules diffuse quickly to the surface, k_m is large enough, then the interaction affinity becomes critical to the process. It's the interaction controlled kinetics.

When the process reaches equilibrium, the saturation concentration of A - R complex is $[(A_sR)_{max}]$ and the association and dissociation of the analyte molecules to the receptor is stable and equal:

$$\frac{d[A_{s1}R]}{dt} = k_a[A_{s0}][R] - k_d[A_{s1}R] = 0 \quad (2.21)$$

Thus, $[A_{s1}R]$ can be calculated:

$$[A_{s1}R] = \frac{k_a[A_b][(A_sR)_{max}]}{k_a[A_b] + k_d} \quad (2.22)$$

The signal, S can be in various forms. In our experiments, it is mostly SPR signal. It is commonly linearly related to the complex concentration $[A_{s1}R]$. Then the relationship can be expressed as $S = F_{conv}[A_{s1}R]$, where F_{conv} is a factor converting the concentration to S value (including the factor of the molecular mass). For mass transfer controlled kinetics, according to equation 2.22, the binding signal S solely increases linearly with time with k_m . In the case of the interaction controlled kinetics, the surface concentration of free R (not occupied by analyte) is:

$$[R] = [(A_sR)_{max}] - [A_{s1}R] \quad (2.23)$$

based on equation 2.23, equation 2.18 can be transformed into the following one:

$$\frac{dS}{dt} = k_a[A_{s0}](S_{max} - S) - k_dS \quad (2.24)$$

where S_{max} is the theoretical maximum value of the signal in saturation ($[(A_sR)_{max}] = [R_0]$, $[R_0]$ is the surface concentration before binding). By integration, the equation 2.24 can be solved:

$$S_a(t) = (S_{max} - S_0)(1 - e^{-(k_a[A_{s0}] + k_d)(t - t_0)}) + S_0 \quad (2.25)$$

where S_0 is the signal at t_0 .

For dissociation phase, the concentration of the analyte is 0,

$$\frac{dS}{dt} = -k_dS \quad (2.26)$$

Then the dissociation should be:

$$S_d(t) = (S_n - S_0)e^{-k_d(t - t_n)} + S_0 \quad (2.27)$$

where S_n is the signal at t_n (the dissociation starts).

When reaching equilibrium, the signal S_{eq} can be expressed,

$$S_{eq} = \frac{k_a[A_{s0}]S_{max}}{k_a[A_{s0}] + k_d} = \frac{[A_{s0}]S_{max}}{[A_{s0}] + \frac{k_d}{k_a}} \quad (2.28)$$

The time to reach the equilibrium can be calculated as following:

$$t = \frac{-\ln\left(1 - \frac{S}{S_{eq}}\right)}{k_a[A_{s0}] + k_d} \quad (2.29)$$

For example, it costs about $\frac{1}{k_d}$ period of time to reach 90% association equilibrium for

$$[A_{s0}] = \frac{k_d}{k_a}.$$

(Equations 2.17-2.29 were referred to ⁶⁹)

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Chapter 3

Experimental Methodology

This chapter fundamentally discussed the underlying principle of the commercial experiment equipment, the set-up we built, the theories involved in simulation and the fabrication of the substrates and the molecules, as well as the characterization methods. Since the Au nanohole (AuNH) array substrates were used in the following three projects, the simulation to design the parameters, the fabrication process and the characterization of the substrates are introduced in this chapter.

3.1 Simulation

3.1.1 Finite Element Method

Finite element method (FEM) ^{1,2} is invented to systematically and numerically solve the boundary problems for partial differential equations. It dated back to the 1950s for the computations of dam construction in China. Referring to the idea of approaching a circle by connecting tiny straight lines, FEM was applied to solve many engineering problems, including electromagnetic field distribution as the basis of computational modeling softwares, *i.e.* COMSOL.

3.1.2 Simulation Process

The Au nanohole array has been used as a label free transducer substrate in chapter 4 and to enhance fluorescence excitations for biosensors on highly ordered nanohole array³⁻¹⁰ for the work in chapter 6 and 7.

In order to design the nanohole array as the platform to perform fluorescent experiment (the absorption range of the nanohole array has to partially overlap that of the dye, Alexa647 for the works in chapter 6 and 7), the period, the diameter and the height of the nanohole wall were simulated in the commercial software COMSOL based on time harmonic analysis of finite element method (FEM). The 3-D Maxwell's equations were solved to draw the spectra and predict the optical field distribution of the gold nanohole array. ^{11,12} In the simulations, the time-harmonic analysis hypothesizes that all variables are sinusoidal, and the solution is solved as a stationary problem with a complex value to represent the magnitude and the phase. To obtain the spectrum of a gold nanohole array, the wavelength is varied in the spectral range by assuming a perfect white light illumination. The dielectric constant of gold is wavelength dependent and is taken from the Palik's handbook ¹³, and the refractive indexes for air, glass, and water used in our simulations are 1, 1.52, and 1.33, respectively.

In the initial gold nanohole array design, the light is assumed to be incident perpendicularly to the glass chip with the gold nanoarray immersed in water. As presented in Figure 3. 1a, two plasmonic modes, denoted as α and β , were generated on the gold nanoarray, where α and β are related to the electromagnetic fields distributed at

the top and bottom rims of the gold nanoholes, respectively. Since the field distribution at the top rims dominates the fluorescence excitations in future sensing applications, the design is to optimize so that the plasmonic peak of the α mode matches the Alexa 647 excitation wavelength of 647 nm. Accordingly, the various parameters of the gold nanohole structure including the nanohole radius R , pitch p and gold thickness T were adjusted in the simulation to obtain the desired plasmon wavelength.

Figure 3. 1 The simulated absorption spectra of gold thin film (thickness of 100nm) and gold nanohole array ($p = 400\text{nm}$, $R = 75\text{nm}$, $T = 100\text{nm}$) in water, with the light incidence normal from the water side. Insets are the field distribution of gold nanohole at the α and β plasmonic modes as related to the two absorption peak in the spectra. (b) The simulated field distribution of α mode at the x -direction on the surface of gold nanohole, with the hole array pitch $p=400\text{nm}$, while the radius R and gold thickness T vary as indicated in the spectra. Inset is an example of the field distribution on the gold nanohole surface when $R = 75\text{nm}$ and $T = 100\text{nm}$

The general method of identifying the suitable parameters for fluorescence excitation at a specific dye wavelength has been reported in earlier work.¹¹ With the reported method, the simulations indicate that the pitch influences the plasmonic wavelength, and 400nm is considered to be the most suitable pitch to match the excitation wavelength of Alexa 647. Figure 3. 1b demonstrates that maximum field intensity on the nanohole surface varies with the thickness and radius of the gold nanohole array. The maximum intensity of the plasmonic signal reaches the highest value of about $1 \times 10^6 \text{ W/m}^2$ (in the simulations, the

incident power was 1 nW within a quarter of a single nanohole area, which was a $200\text{nm} \times 200\text{nm}$ area for a 400nm pitch nanohole array) when the gold nanohole array has the dimensions of pitch $p = 400\text{nm}$, radius $R = 75\text{nm}$, and thickness $T = 100\text{nm}$. At these dimensions, the maximum absorption peak for α and β plasmonic modes are located almost at the wavelengths of $\lambda_\alpha = 647$ and $\lambda_\beta = 730\text{nm}$, respectively. Furthermore, it has been shown that the maximum fluorescence intensity of a dye absorbed on a nanostructure is strongly dependent on the overlap between the LSPR spectrum of the nanostructure and the excitation and emission spectra of the dyes.¹⁴ The shape of excitation spectrum of Alexa 647¹³ matches very well with that of the α mode's spectrum in Figure 3. 1a. Figure 3. 1b also illustrates that of the α mode, the strongest plasmonic signal intensity is always at the top rim of the gold nanoholes. Moreover, intensity drops a half when the distance is 6nm axially off the rim.

For dye excitations, Chen et. al reported that a LSPR scattering peak about $40\text{-}120\text{meV}$ higher in energy than the emission peak of the fluorophores will generate the brightest fluorescence signal.¹⁴ According to the following equation (where the units of energy (E) and wavelength are eV and nm , respectively, and equation 3.2 is the differential result of equation 3.1):

$$E = \frac{1240}{\lambda} \quad (3.1)$$

$$\frac{dE}{d\lambda} = -\frac{1240}{\lambda^2} \text{ or } d\lambda = -\frac{\lambda^2 \times dE}{1240} \quad (3.2)$$

for the emission wavelength λ of 665nm , the optimal excitation wavelength should be blue shifted $\Delta \lambda = 14.3 \sim 43\text{nm}$, which is $620 \sim 650\text{nm}$ according to the Equation 3.2. Figure 3. 2 reveals the relationship of the plasmonic absorption wavelength with the pitch, diameter and thickness of a circular gold nanohole array.

Figure 3. 2 The resonant wavelength for the principle mode α as a function of (a) the pitch (p), (b) the diameter (D) and (c) the thickness (T) of a gold nanohole array, when the light is normally incident from the top of the chip in water. In (a), $D = 150\text{nm}$, $T = 100\text{nm}$; in (b), $p = 400\text{nm}$, $T = 100\text{nm}$; in (c), $p = 400\text{nm}$, $D = 150\text{nm}$

By reviewing Figure 3.2, it is obvious that 200nm of pitch variation (from 300 to 500nm) causes a linear peak wavelength shift of 180nm (from 555 to 735nm), and 50nm of diameter variation ΔD (from 125 to 175nm) induces a wavelength shift of $\Delta\lambda=10\text{nm}$ (645 to 655nm), while 50nm of thickness variation ΔT (from 50 to 100nm) shifts the peak wavelength of $\Delta\lambda=27\text{nm}$ (from 620 to 647nm). It is also well known that engineering errors are unavoidable in nanofabrication. Thus, based on Figure 3. 2, we fixed the pitch to 400nm to be very strict, while the diameter and the thickness of the gold nanoholes were adjusted to the size region where a small variation of the parameters induces minimal variation of the plasmonic peak in relationship with the fluorescent excitation of the dye Alexa647. The $\Delta\lambda$ was calculated by equation 18:

$$\Delta\lambda = \frac{\partial\lambda}{\partial D} \times \Delta D + \frac{\partial\lambda}{\partial T} \times \Delta T \quad (3.3)$$

Accordingly, a diameter of $D=140\text{nm}$ and thickness of $T=80\text{nm}$ were selected, the variation of the plasmonic peak is calculated to be 12.8nm for the 80nm thick gold nanohole array, and $\Delta\lambda = 29\text{nm}$ for a 50nm thick gold nanohole array. These variations still allow the plasmonic peak ($636 \sim 652\text{nm}$) located close to the optimal wavelength range which might offer the brightest fluorescence excitation.¹⁴ All in all, the parameter of the gold nanohole array was decided: $D=140\text{nm}$, $p=400\text{nm}$ and $T=50\text{nm}$. (Note: T stands for the whole thickness of the gold (45nm) and the adhesion layer chromium (5nm)). The LSPR-enhanced electric field distribution was simulated by FEM in Figure 3. 3a and b.

In chapter 4, the design of AuNH array for the works in both chapter 6 and 7 was employed since it can also satisfy the requirement in the troponin I assay.

Figure 3. 3 (a) Simulation by FEM of LSPR-enhanced electric field distribution illuminated by 620nm light, normally shedding from the water side of gold nanohole array without photo resist. (b) is the corresponding illustration of (a).

3.2 Synthesis and Fabrication

3.2.1 Au Nanohole Array Substrate

Two kinds of AuNH array were used in different projects, among which the AuNH array directly on glass slides was used in the projects presented in chapter 4, 6 and 7. The design, fabrication and characterization of this array are elaborated on in this chapter, while the one with a photoresist layer between the Au layer and glass slides was only utilized in chapter 7 and is described in that chapter.

3.2.1.1 Electron Beam Lithography

It is important for nanotechnology to integrate large amounts of identical units in a small area. Modern lithography technique makes it possible to fabricate such kind of device to fulfill a variety of functionalities. Figure 3. 4 illustrates the general lithography process.

Figure 3. 4 Illustration of general process to fabricate a pattern including lithography step

Although the shape and size of each unit depends on quality of the mask, the resolution is determined by the frequency (or wavelength) of the energy source in the exposing step because of diffraction. So the resolution of a lithography system is limited by Rayleigh equation⁴⁵:

$$R = \frac{\kappa \cdot \lambda}{NA} \quad (3.4)$$

Here, NA is the numerical aperture of exposing system. The value of a process dependent factor, κ ranges from 0.25 to 1. λ is the wavelength of the illumination system. This demonstrates that the resolution will be improved when the wavelength is shortened.

The light source with wavelength longer than 10^{-7} m is unusual for lithography, especially for commercial products. The wavelength is in the ultraviolet range for commercial products, such as 157nm for 45nm line with components.^{15, 16}

In the electromagnetic spectrum (Figure 3. 5), X-rays and γ -rays are much shorter than ultraviolet. But since X-rays and γ -rays cannot be properly focused, they are not a feasible source for lithography systems. Compared with X-rays and γ -rays, electron beams are more practical because of its shorter wavelength and capability to be focused.

Figure 3. 5 Category and spectrum of the electromagnetic field ⁴⁵

The ability to control electron beams and the discovery of Poly(methyl methacrylate) (PMMA) as the resist, the first electron beam lithography machine was based on scanning electron microscopy. In general, the process is to create the designed pattern on the substrate by scanning electron beam causing reactions to occur at certain parts of the resist according to the setting parameter. The reaction mechanisms of resist when exposing to electron beam, are different for positive and negative resist. The energy from

electron beam breaks the chains in the positive resist, while new bonds are formed when negative resist is exposed to the electron beam. The mechanism of PMMA degradation is shown in Figure 3. 6 ¹⁷. A number of resists are listed in Table 3.1 along with their properties.

Figure 3. 6 There are two types of electron emitting sources (guns): (1) thermal emission gun, electron emitted from a heated metal filament and (2) field emission gun, excited filament emanating electron by an electric field. Recently, field emission gun took the place of first one because of the strong emitting capability and stability of the electron beam. At the same time, the voltage of the electric field can be tuned from tens to several hundred of keV ¹⁷

After exposure, the resist must be developed in order to remove the unexpected part. The developing process will be stopped by other solvent rinsing. At present, the developer and stopper are commercially designed and supplied along with the resist. Usually, the PMMA developer is a mixture of Methylisobutylketone (MIBK) and isopropanol (IPA) with a ratio of 3 to 1. The corresponding stopper which can stop the photo crosslinking process is pure IPA. The pattern on resist is supposed to appear at this step, after which deposition can take place.

Table 3. 1 Properties of some resist in electron beam lithography ¹⁸⁻²⁰

Resist	Film thickness (nm)	Accelerating voltage (keV)	Sensitivity ($\mu\text{C}/\text{cm}^2$)	Resolution (nm)
ARCH	300	50	10	100
Poly(p- vinylphenol)	500	50	2	200
CAMP	300	50	60	250
SAL 605	450	20	1.6	50
AZPN 114	1000	10	1	750
AZDX 561	1000	30	12	200

E-beam lithography is popular not only because of higher resolution, but also because of several other advantages. The pattern can be precisely designed by software and the dose of energy (by voltage) and spot size can be controlled by computer and machine. It improves the traditional lithography process by not using the mask. So e-beam lithography is often used to prepare the mask for other processes.

On the other hand, e-beam lithography has some disadvantages. As described before, it needs a very complicated controlling system for pattern designing and operation is needed. The procedure is typically performed in a Scanning Electron Microscope (SEM). The process is time consuming. For a sample of $2\mu\text{m} \times 2\mu\text{m}$, it takes almost one day to finish the process. The errors caused by this technique can be classified into two kinds. As a result of the improper deflection, the shape will be falsely transferred to the pattern on the resist. The second one is caused by a variety of reasons; including in backscattering, long range reflection of backscattered electrons, sample or filament contamination, sample charging, mechanical error and so forth. These errors are classified as physical errors.

3.2.1.2 Nano-imprint Lithography

Nano-imprint lithography is a reliable and repeatable technique for nano or micro meter scale pattern fabrication. It is massive production technique to replicate the mold or the complimentary part of the mask made by electron beam lithography. It is a relatively simple method.

Generally, the procedure involves three steps: coating resist on substrate, imprinting using a mold, removal of the mold and post-treatment. Two types of nano-imprint lithography differ from each other due to the resists. For one type using thermal resist, the coating materials are thermal resists. The thermal resist is heated to its glass transition temperature, while the mold is pressed into the soft resist to create the complimentary part of the mold on thermal resist. After cooling down, the mold is separated from the thermal resist, which still need post-treatment such as reactive ion etching to transfer the pattern to the substrate. For photo imprinting, ultra-violet (UV) light illuminates the mold and substrate during imprinting to cure the resist. So the substrate and mold must be transparent to UV light. The next step is similar to thermal imprinting. Figure 3.7 illustrates the two types of nano-imprint lithography.²¹

The trend of small size nanostructure overcome the constraint of limited access to sample, which is crucial to certain areas of biomolecule detection and sensing. A motor now is used to precisely control the shape in three dimension. Other improvement such as nano-tips array was proposed to be equipped to fabricate polymer.

Figure 3. 7 Illustration of Nano-imprint lithography process with thermal or photo resist²¹

3.2.1.3 Spin Coating

Spin coating is a technique widely used to apply polymer thin film on a solid flat substrate for several decades.²²⁻³² The typical process is starting from loading the substrate on the stage. Then certain volume of solution is dropped on the center of the substrate. The rotation step aims to spread the fluid to the surface according to the setting speed procedure. The excessive solution is spun off the substrate. The thickness depends on the polymer properties such as viscosity, drying rate, percent solids, surface tension, and spin process.

Figure 3. 8 (a) Scheme of spin coater stage; (b) Typical spin coating process²²⁻³²

The whole process comprises three steps (Figure 3. 8b). The second step can be fulfilled by two methods: static dispensing, depositing solution before stage rotation and dynamic dispensing, dropping solution on substrate under a lower speed of stage rotation. In order to get a homogeneously flat film, a step-acceleration is necessary, a lower spin speed to unify the distribution of content on the surface and a higher spin speed to spread off the solvent and excess solution to form a film. For some purpose, the substrate needs to be pre-treated (cleaning, heating, *etc.*). The technique is now able to fabricate film with thickness about several nanometers.³³

3.2.1.4 Thermal Evaporation

Figure 3. 9 (a) The software page of the source controlling part. (b) A picture of one source boat in the chamber.

The deposition process of a thermal evaporator is analogous to boiling water in a pot, consisting of two continuous steps: melting the source materials and vapor condensing on the substrate. Extremely high vacuum is critical to the evaporating condition since the chamber is in high temperature which could cause interaction between the vapor and ambient contents and increase the impurity of the film of substrate. The machine in our

lab (Sean Campbell Angstrom Engineering, Inc., Canada) evaporates source in the boat by high electric voltage. Two boats are used in the chamber (Figure 3. 9). The substrate is attached on top of the chamber. The whole process of vacuum and deposition is automatically controlled by software installed together with the machine.

3.2.1.5 Fabrication Procedure of AuNH array directly on glass slides

Figure 3. 10 Schematic structure of the platforms, parameter: 140nm diameter circle at a pitch of 400nm, the height of the gold nanohole wall is 50nm (yellow squares stands for gold, 45nm and brown squares is chromium, 5nm)

The AuNH array was prepared through a serial of steps involving nickel mold fabrication by electron beam lithography (EBL) and AuNH array production by Nano-imprint Lithography (NIL).^{34, 35} An area of 2mm $\times$ 2mm nanohole array (140nm diameter circle at a pitch of 400nm) was first written on a silicon substrate by e-beam lithography, and then the silicon substrate was electroplated to form a nickel mold (with nanoholes). The structure was then transferred to the photo resist coated glass substrate to form a nano-pillar array structure. Then, the photo resist was treated with RIE for 2s, to etch the indented photo resist down to the glass substrate. Afterwards, 5nm of chromium (Cr) as an adhesive layer and 45nm thick of gold were sequentially deposited on the polymer nano-pillar array through thermal evaporation. Finally, the photo resist was removed by plasma etching (5sccm O₂, power of 18W) for 5min and rinsed with acetone and isopropyl.

3.2.2 Grating Au Substrates

The design and characterization of the grating Au substrates were described elsewhere (chapter 5) since they were only applied in one work.

3.3 Characterization

3.3.1 Ultra violet-visible Spectroscopy

Before the experiments, the spectra of the nanohole array were measured with an Ultra violet-visible (UV-vis) spectrometer (Perkin Elmer, Lambda 35) by controlling the incident light normally passing through the nanohole array. The area of nanohole array was so small that the rest part of chip was covered in case of the absorption of gold film (Figure 3. 11). For comparison, the spectrum of AuNH in air and PBS buffer were measured for chapter 4, 6 and 7.

Figure 3. 11 Schematic illustration of the set-up inside the chamber of UV-vis spectrometer

The extinction spectra of AuNH array substrate were measure (Figure 3. 12) using the set-up in Figure 3. 11.

Figure 3. 12 The extinction spectra of AuNH array (1) in air (black solid), (2) water (red dashed) and (3) in air after the surface was coated with 5 μL of 6.3 μM PSA antibody-Alexa647Ab-AF647

The UV-vis spectra of AuNH array substrate (Figure 3. 12) shows α and β mode plasmon peak of $\lambda_\alpha = 586\text{nm}$ and $\lambda_\beta = 696\text{nm}$ in air, which shift to $\lambda_\alpha = 621\text{nm}$ and $\lambda_\beta = 721\text{nm}$ in PBS, respectively. The α mode of plasmon peak is comparable with the simulated results as indicated in Figure 3. 1. After the surface was covered with 5 μL of 6.3 μM PSA antibody-Alexa647 Ab-AF647 and dried in air, α and β mode peak are shown at $\lambda_\alpha = 602\text{nm}$ and $\lambda_\beta = 721\text{nm}$, respectively. Both the red-shift of α and β mode peaks are ascribed to the high refractive index of PSA antibodies and dye which are around $n_{\text{ab}}=1.40$ ³⁶ and $n_{\text{dye}}=1.56$ at the wavelength of 600–650 nm³⁷, respectively. Additionally, the more red-shift of β mode peaks indicate that antibodies were more accumulated in the nanoholes rather than on the surface.

3.3.2 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is a microscopic technology utilizing focused electron beam to reach down to nanometer/micro world. The prototype of scanning electron microscope machine was invented in 1937 in the hand of Manfred von Ardenne

in order to resolve the optical diffraction limitation of visible light in microscope, which can be reached by accelerated electrons to achieve better resolution, *i.e.* smaller than 700nm. He not only built the set-up³⁸ but also the base of the technology, explaining the principle and further pointing out the mode of detection and developing prospects.³⁹ The Cambridge group promoted and commercialized the scanning electron microscope.⁴⁰

Figure 3. 13 Schematic illustration of a Scanning Electron Microscope⁴⁰

Theoretically, a SEM (Figure 3. 13) can measure the topography of the surface by secondary electron imaging (SEI) mode and detect the condition of thin layers under the surface in the backscattering electron (BSE) mode. The composition of the surface is able to be characterized by energy-dispersive X-ray (EDX) mode, similar but less sensitive to

X-ray Diffraction (XRD). Other information of the sample characterized by signals such as specimen current and transmitted electrons.

A SEM is applied more than a normal characterization tool. It is also widely used as a lithography operation platform, which has been introduced in detail in this chapter.

The images of AuNH array (Figure 3. 14) substrates were captured by SEM machine (JSM-7600F, JEOL, USA).

Figure 3. 14 An SEM image of Au nanohole array substrates. The scale bar was 200nm. The inset was an image of one chip, nanhole array located in the center.

3.3.2 Atomic Force Microscopy

Atomic Force Microscopy (AFM) is another ubiquitous scanning probe microscopy (SPM) technique to characterize the topography of the sample surface, the mechanical parameters *i.e.* hardness, as well as the properties like electric potentials, which was an extension from scanning tunneling microscopy (STM).

An atomic force microscope machine (Figure 3. 15) is composed of optical signal part (a laser, a photo diode), piezo stage and a probe part, which is a cantilever with a sharp tip on the top. The mechanical components are controlled by central controlling computer by software. The feedback from the optical part as well adjusts the movement and relative displacement of the tips to the sample surface. In principle, the AFM brings the tip with radius curvature in close proximity to the surface. By interacting with the surface atoms the tip is adjusted to keep at a distance and the force serves as the feedback, according to Hooke's Law ⁴¹. The tip moved back and forward to record the average height of one scanning line in all three imaging mode: contact mode, tapping mode and non-contact mode. The resolutions of AFM in vertical and lateral direction are limited in various ways.

The vertical resolution Δz is mainly hindered by the thermal noise of the deflection detecting system following the equation:

$$\Delta z \sim \sqrt{\frac{4k_B T}{3k}} \quad (3.5)$$

Where k_B stands for Boltzmann constant; T is the absolute temperature and k is the spring constant. The most advanced commercial AFMs can achieve 0.01nm resolution in the vertical direction, while the lateral resolution or the minimum detectable distance d is influenced by both tip curvature R and vertical resolution Δz :

$$d = \sqrt{2R} \times (\sqrt{\Delta z} + \sqrt{\Delta z + \Delta h}) \quad (3.6)$$

Recently, the AFM was developed to be multi-function equipment. For instance, the dip pen lithography (DPN)^{42, 43} make use of the AFM system to directly deliver molecules on polymer to a substrate.

The topographic images of the AuNH array substrates were captured by Atomic Force Microscope DI-3000 (Digital Instruments, Italy) in tapping mode. From the characterization results, the nanoholes were homogenously patterned. AFM measured the real two times pitch is 826.17nm (Figure 3. 16a) and four times pitch is 1664 μ m (not shown). So the average pitch is 414.5nm. The average diameter and thickness are 136 nm and 49nm, respectively.

(a)

(b)

Figure 3. 15 (a) Schematic illustration of Atomic Force Microscope. (b) Van der Waals force curve⁴⁴

Figure 3. 16 AFM image of gold nanohole array (a) two times of pitch $2p=826.17\text{nm}$, (b) diameter $D=136.72\text{nm}$ and (c) thickness $T=49.614\text{nm}$. (d) 3D AFM image of a 3×4 hole array area.

3.4 Other Experimental Equipment

3.4.1 X-nano Localized Surface Plasmon Spectroscopy

The X-nano (Insplorion AB, Sweden) system (Figure 3.17) is an analytical instrument to detect processes and interactions at nanomaterial interfaces. It is a derivative and evolution product of UV-vis spectroscopy, and equipped with a much faster detector making it possible to scan a spectrum within one second. As a result, this instrument is convenient for real time monitoring of binding events on a surface.

The X-nano machine is composed of four main components: the sample box, a light, an integrated optical and thermal detectors box and a controlling computer with operational software. A white light beam is emitted and rasterized by a raster and certain wavelength of light incidents through a fiber to the sample inside the sample chamber. The rest of the unabsorbed light passes through the fiber and is detected by the optical detector. The temperature is controlled and detected by the thermal detector and feedback system. The micro-pump provided by the company Insplorion recycles or injects liquid into the chamber.

The installed Insplorer® Software records the signal and analyzes it to output three types of data: peak position (the wavelength of peak within a range), extinction difference (the extinction value difference of two fixed wavelengths picked by the user, *Ext_d*) and centroid (a mathematical calculation central wavelength of a peak).

Figure 3. 17 (a) Schematic illustration of an X-nano Localized Surface Plasmon Spectroscope. (b) The extinction spectra of AuNH array typically shift to longer wavelength upon molecular binding, leading to decrease of the extinction difference $Ext d$ derived from two preselected wavelengths. The extinction difference is defined as $\Delta Ext d = Ext d_2 - Ext d_1$.

3.4.2 Prism-coupled Surface Plasmon Resonance Spectroscopy

The experimental instrument in chapter 6 was a home-built prism-coupled Surface Plasmon Resonance spectroscope (Figure 3. 18) operating in the Kretschmann configuration. It is composed of a light source, a chopper, two pieces of lenses, one sample holder attached with a right triangle prism, one detector (D1) monitoring the reflectivity, one fluorescent detector (D2) behind and a controlling computer. The light source is a Helium-Neon (HeNe) laser beam, 5 mW, $\lambda = 632.8$ nm manufactured by Uniphase. The frequency of the chopper is 1331Hz with a built-in amplifier. In between of the chopper and the sample holder is two polarizers (Glan-Thompson), modulating the plane of polarization of the laser beam, as well as the intensity. The prism ($n=1.85$ at 633 nm, Schott, LASFN9), which is the key component to excite surface plasmon on Au and reflect the laser beam to the detector is attached to the sample. Special oil with the same refractive index is needed to extrude air between sample and the prism. The chopper and two polarizers are in line with the laser source and the sample holder. The degree movement of D1 is two times along with sample holder precisely implemented by two 2-circle 414 co-axial goniometers with the precision 10^{-3} degree (Huber AG, Germany) respectively. Loading a flow cell, the system can be utilized to detect signal change in solution and real-time assay. Liquid sample is pumped into the cell and circulated by a peristaltic pump.

The fluorescent detector is set to the back of the prism to eliminate laser beam influence. The fluorescent signal intensity is recorded by a H6240-01 photomultiplier tube (PMT, Hamamatsu, Japan). In case of photo bleaching, an automatically controlled shutter blocks the laser in a pulse program, 5s on and 60s off.

Figure 3. 18 Schematic illustration of Prism-coupled Surface Plasmon Resonance Spectroscopy

Not only the static reflectivity angular spectra (Figure 3. 19a) but the kinetic measurement (Figure 3. 19b) can be operated by the instrument. For the reflectivity angular spectra, the angles of two scanning ends, the steps and intervals are fully customized on the computer. The reflectivity of the chosen angle range of the substrate can be measured and recorded. The shape of the spectrum of typical Au thin film is illustrated in Figure 3. 19a. The black curve is the response for a bare surface, the red-shift of the dip, related to the Surface Plasmon Resonant angle happened (from the black to the red one) when the analyte approaching to the Au surface, due to an increasing refractive index near the surface. So did the red-shift from the red curve to the blue curve. Fitting the spectra by the software Winspall (Max Planck Institute for Polymer Research in Mainz, Germany) using the transfer matrix based model, the refractive index and thickness of the layer of each condition on the substrate surface can be determined. For kinetic measurement, the reflectivity at a preset angle can be monitored in a set period of time. Compared to the angular scanning mode, the whole process of the analytes binding to the surface is able to be monitored. The selected angle is recommended to be on the steep slope and is most sensitive to the shift of the curve.

3.4.3 Limit of Detection

The limit of Detection (LOD) or detection limit is a criterion of a detection method and a data analysis approach. It is the lowest measurable quantity of analyte by a platform. It is determined by the calibration curve of the read-out and three times of standard deviation of a blank sample in my works.

Figure 3. 19 Illustration of (a) reflectivity angular spectra of three different conditions and (b) kinetic measurement by Prism coupled SPR spectroscopy

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Chapter 4

Electric field focusing and Localized Surface Plasmon Resonance Enhanced Label Free Biosensors

An Au nanohole array (AuNH array) was fabricated by nano-imprint lithography. Modified by specific peptide for human troponin I (cTnI) and spacer, the platform can detect concentration of 110ng/mL by extinction difference change (ΔExt_d) which was calculated from real-time localized surface plasmon resonance (LSPR) signal. Application of negative bias on AuNH array improved the limit of detection (LOD) to 2ng/mL because of attraction positively charged cTnI in buffer (pH=7.4), and repulsion to other negatively charged contents like bovine serum albumin (BSA), which reduced the possibility of nonspecific binding. The external bias facilitated an extra electrostatic force to adsorb complementary charged target molecule and resulted in faster diffusion and binding rate, as well as slower rate for nonspecific molecules with the same kind of charge of the bias.

4.1 Introduction

Surface plasmon resonance (SPR) originates from collective oscillations of the free electron cloud in metals.¹ Due to the high field enhancement near the metal surface and high sensitivity of SPR to refractive index changes, it has been extensively applied on photo-catalysis,²⁻⁴ opto-electronic devices⁵ and biosensors⁶⁻⁸ based on label-free,⁹⁻¹² fluorescence enhancement¹³⁻¹⁷ and surface enhanced Raman scattering¹⁸⁻²¹. Label-free SPR biosensors typically provide a LOD in the 0.1-10 ng/cm² regime.⁶ There are several key factors limiting the sensitivity of SPR sensors, including 1) the bulk refractive index sensitivity of SPR, 2) the diffusion limited mass transport of the target molecules to the sensor surface which slows down the molecular binding and limits the sensor response, and 3) the high nonspecific adsorption of undesirable molecules on the sensor surface such as albumin in serum samples which might lead to erroneous responses *e.g.* due to blocking of specific binding sites. Several methods have been employed to boost the sensitivity including well-designed nanostructures with improved refractive-index sensitivity,¹¹ amplification of the SPR signal with nanoparticles²²⁻²⁵ and enzyme labelling,^{26, 27} tuning the pH of samples to minimize the nonspecific adsorption,²⁸ and applying magnetic field^{29, 30} and electric field³¹⁻³⁴ to promote the molecular diffusion. For instance, magnetic fields enabled rapid capture of magnetic nanoparticle-targeted hCG and E.coli bacteria to a grating-coupled SPR sensor surface.^{29, 30} In addition, the electric field applied to a LSPR sensor chip offered a route to control the adsorption of molecules to the sensor surface.^{33, 34} Furthermore, the influences of the applied electric field on the LSPR resonance have been discussed,³⁵⁻⁴⁰ and several groups integrated electric field biasing or electrochemistry with LSPR for biosensing.^{33, 41, 42} However, limited research has been devoted to studies aiming at reducing the nonspecific adsorption of abundant molecules such as serum albumin, while enhancing the binding of target molecules to the sensor surface.

Figure 4. 1 (a) Troponin complex in which the green chain represents troponin I, blue for troponin C and magenta for troponin T⁴³. (b) and (c) are illustration of troponin complex function in inhibited state and in activated state in muscle, respectively.

The target in this work was human troponin I (cTnI), a biomarker of myocardial infarction. TnI is one of the three components of troponin complex (Figure 4. 1a),

troponin T, troponin C and troponin I, which plays a critical role in muscle contraction. Troponin complex stays in the gap between tropomyosin and actin filaments. Troponin I occupies the attachment site of actin, while troponin T stabilize the whole complex to tropomyosin when the muscle is in relaxed state (Figure 4. 1b). The stimulus causes an action potential releasing calcium ion to bind to troponin C so that troponin changes its shape, leaving a vacant site on actin for myosin, resulting in muscle contracting (Figure 4. 1c). From the last two decades, cTnI has been known as a reliable biomarker for cardiac muscle damage relevant to not only myocardial infarction, the most severe disease, but also to disorders including renal failure ⁴⁴.

In this chapter, I present a combined LSPR electrofocusing platform for the detection of cTnI. An AuNH array is employed both as the LSPR sensor chip and as the working electrode. The LSPR sensor responses upon binding of cTnI and the nonspecific adsorption of bovine serum albumin (BSA) were investigated on the AuNH substrate under different biases. As the isoelectric point of cTnI ($pI = 9.87$) is higher than for BSA ($pI = 4.7$), a negative bias applied on the sensor surface is expected to favour the specific capture of cTnI while repelling BSA molecules from the sensor surface (Figure 4.2b). In addition, the bias applied on the substrate creates a high electric field gradient at the edge of the nanohole, thereby offering an efficient mean of capturing molecules at the edge of the nanoholes where the plasmon field provides the highest sensitivity to refractive index changes.⁴⁵ Therefore, the sensitivity for selective detection of cTnI is expected to increase significantly under negative biases applied on the sensor chip.

Figure 4. 2 Schematic illustration of (a) the setup made of PDMS and (b) the modified AuNH array chip applied with a negative bias for minimizing the nonspecific adsorption of BSA and simultaneously attracting the target cTnI to the sensor surface. (c) The extinction spectra of AuNH array typically shift to longer wavelength upon molecular binding, leading to decrease of the extinction difference Ext_d derived from two preselected wavelengths λ_1 and λ_2 . The change of Ext_d is defined as $\Delta Ext_d = Ext_{d2} - Ext_{d1}$.

4.2 Experimental Section

4.2.1 Materials

cTnI full length protein (MW ca. 24kDa, 0.48mg/mL) was purchased from Abcam. Phosphate buffered saline (PBS), bovine serum albumin (BSA, ca. ~66kDa, $\geq 96\%$) and rabbit serum (R9133) were purchased from Sigma-Aldrich. CALNN-(PEG)₄-FYSHSFHENWPS and CALNN were purchased from GL Biochem(Shanghai). Absolute ethanol (99.9%) was purchased from Merck.

4.2.2 AuNH Array Fabrication and Modification

The AuNH array was prepared through a serial of steps involving nickel mold fabrication by EBL and AuNH array production by nano-imprinting.^{46, 47} An area of $2 \times 2 \text{ mm}^2$ consisting of a layer of 50 nm Au coated on glass with periodic (pitch of 400 nm) holes 140 nm in diameter is located at the center of a chip. The AuNH array was characterized by SEM, AFM and UV-vis spectroscopy UV-vis (mentioned in chapter 3, 3.3), all indicating a periodic and uniform hole array.

In order to modify the AuNH array, the AuNH array chip was treated with oxygen plasma for 30 s under 10 sccm oxygen at pressure of about 500 mtorr and power of 18 W (plasma cleaner PDC-32G). Then, it was incubated in an ethanol solution containing a mixture of CALNN and CALNN-PEG-FYSHSFHENWPS⁴⁸ at molar ratio of 1:1 (total concentration 50 μM). After one days' incubation, the chip was rinsed by ethanol and dried by nitrogen gas. The chip (working electrode) was fixed into a reaction chamber made off PDMS in which a Pt wire served as a counter electrode and Ag/AgCl as the reference electrode. The sensor chip was covered with a thin layer of PDMS with a hole (about 2 mm diameter) to allow the AuNH array to be contacted with the solution in the chamber. The incident light was normally illuminated through the backside of the AuNH chip. The transmittance spectra were acquired every second by a spectrometer (Insplorion Xnano instrument, Sweden). The electrodes were connected with the Multi Autolab (Metrohm Autolab B.V., Netherlands) for electric bias application.

4.2.3 Detection Process

The plasmon resonance peak typically shifts to higher wavelength upon molecular binding to the surface of the nanohole, leading to a decrease of the extinction difference *Ext_d*. The *Ext_d* was obtained from the extinction intensity difference at two wavelength, e.g. λ_1 and λ_2 (Figure 4.2 c). Comparing the two versions of data, peak shift and *Ext_d*, upon applying several rounds of +0.5V and -0.5V bias, the *Ext_d* was chosen as the signal to noise ratio was higher than that of the peak shift. (Figure 4.3 b and c)

In the detection of cTnI, about 4 μL of cTnI solutions were sequentially injected into the chamber to achieve cTnI concentrations of 1ng/mL, 10ng/mL, 100ng/mL and 1 $\mu\text{g/mL}$ in the buffer solution. The *Ext_d* values (i.e. the extinction difference between the wavelength at about 700nm and 730nm) were monitored in real time (i.e. $Ext_d(t) = Ext_{730}(t) - Ext_{700}(t)$). The ΔExt_d of each concentration was estimated as the *Ext_d* after 30 min incubation of cTnI subtracted to the background value. The electric bias was applied on the sensor chip using the multi Autolab. Voltage values are reported with reference to an Ag/AgCl electrode.

Figure 4. 3 (a) Extinction spectra of the AuNH array applied with -0.5 V and +0.5 V biases. The time dependent (b) peak position changes and (c) extinction difference (*Ext_d*) upon applying different biases on the sensor surface.

4.2.4 Data Analysis

The calibration curve of the signals was fitted in the sigmoidal model:

$$y = START + (END - START) * \frac{x^n}{k^n + x^n} \quad (4.1)$$

Where, *END* stands for the y value of the end; *START* stands for the y value of the start; *k* and *n* are two parameters.

The boundary conditions of this equation are: $k > 0$, $x \geq 0$ and $n > 0$.

4.3 Results and Discussion

Figure 4. 4 LSPR responses of modified AuNH array under voltage sweeping measured in PBS buffer before (solid line) and after incubation of 3 μ g/ml cTnI for 30 min (dashed line). The voltage was swept (three cycles) from 0V, 0.6V, -0.6V and back to 0V at scanning rate of 100 mV/s.

In order to investigate the effect of applied electric bias on LSPR response, three cycles of voltage scans (from 0V, 0.6V, -0.6V and back to 0V at a scanning rate of 100 mV/s vs Ag/AgCl electrode) were applied on the peptide-modified AuNH array before and after incubation with cTnI in the PBS buffer (Figure. 4. 4). The *Ext_d* value shifted synchronously with the applied bias. Positive bias resulted in an increasing *Ext_d* value (less negative), indicating red-shift of the LSPR peak, while negative bias induced a blue-

shift (Figure 4. 3a). The relative magnitude of the observed shifts is attributed to the higher polarizability of the chlorine (Cl^-) ion ($4.17 \text{ \AA}^3$) with respect to water molecules ($1.43 \text{ \AA}^3$) and Na^+ ions ($0.148 \text{ \AA}^3$).⁴⁹ According to the DLVO theory, the applied electric field on the surface influences the electric double layer in solution. Different strength of the applied potential results in a redistribution of ions within the double layer, thus varying the refractive index near the surface.⁵⁰ Interestingly, the chip after binding with cTnI showed higher *Extd* changes (ΔExtd) under voltage sweeping from 0.6 V to -0.6 V (Figure 4. 4). This is most likely due to an increase in the effective surface area of the AuNH after the binding of positively charged molecules cTnI (Figure 4. 5 indicates the increase of surface roughness due to the binding of cTnI on the AuNH sensor chip), resulting in an increasing concentration of highly polarizable Cl^- ions near the surface.

Figure 4. 5 AFM imaging of the AuNH array a) before and b) after 30-min incubation of 3 $\mu\text{g/ml}$ cTnI in PBS buffer under -0.2 V bias applied on the sensor chip.

Real-time measurements of the LSPR response upon injection of cTnI (in PBS) into the reaction chamber were carried out under different biases applied to the sensor chip (vs silver/silver chloride (Ag/AgCl) electrode), as shown in Figure 4. 6a. The ΔExtd (with the background *Extd* offset to zero) slowly increased after the injection of 100 ng/mL cTnI under no bias and the response increased with the concentration of cTnI beyond 100ng/mL. Control experiment carried out on the sensor chip without the specific peptide modification (only CALNN) indicated negligible nonspecific adsorption (Figure 4. 7). The ΔExtd value of each concentration was calculated by subtracting the background value ($\Delta\text{Extd}(t < 60\text{min})$) from that after 30min incubation. The calibration curve under no

bias (triangles in Figure 4. 6b) was fitted by a sigmoidal model (Equation 4. 1), and the LOD was estimated to ~ 110 ng/mL for our platform under no bias. The noise level was calculated as 3 times the standard deviation of the Ext_d before injection of cTnI in the solution.

Figure 4. 6 (a) Real-time ΔExt_d measured on the AuNH array with a series of injections of cTnI in PBS. Sensor response for no bias (black curve), -0.2 V (blue curve) and 0.2 (red curve). (b) Ext_d changes (ΔExt_d) as function of the cTnI concentration measured under no bias (triangles), -0.2V bias (squares) and 0.2V bias (circles). Dotted line is 3 times of standard deviation.

Assuming the binding events are limited by the mass transport diffusion, the surface coverage of molecules on the sensor surface during the incubation time of t can be simplified as $\Gamma = 2C_{bulk}\sqrt{Dt/\pi}$,^{34, 51, 52} where C_{bulk} is the bulk concentration of analyte in the solution, D is the diffusion constant of the molecules. Based on an LOD of 110 ng/ml achieved for 30-min incubation (no bias), we are able to estimate the smallest detectable surface mass of $\Gamma_{LOD} = 2.9 \text{ ng/cm}^2$. Thus our results indicate that it is necessary to allow for 252 days of incubation to achieve LOD of 1 ng/ml.

Figure 4. 7 LSPR response (ΔExt_d) of the AuNH array modified by CALNN under no bias, after the injection of cTnI at the concentration of 1 ng/mL, 10 ng/mL 100 ng/mL and 1 μ g/mL.

Table 4. 1 Result of AuNH array as sensor under different bias.

Medium	Bias (V)	LOD (ng/mL)
PBS	No bias	110
	0.2	170
	-0.2	1.8
BSA	-0.2	8*
Serum	-0.2	25**

* with 3 mg/ml BSA in PBS

** in 10 $\times$ diluted rabbit serum

However, the binding rate and the sensor response were significantly improved under -0.2 V bias applied to the sensor chip. For instance, the initial binding rate for 10 ng/ml cTnI is comparable to 1 μ g/ml cTnI under no bias. The LOD is improved by two orders of magnitude to 1.8 ng/ml under -0.2 V bias (Figure 4. 6b, Table 4. 1), for a reaction time of 30 min, which is theoretically more than 3000 times faster than the diffusion limited assay $t \sim 252$ days at no bias. Positive bias applied on the sensor surface hinders the binding of cTnI to the sensor surface, thereby worsens the LOD to 170 ng/ml (Figure. 4. 6, Table 4. 1).

Figure 4. 8 Real-time monitored LSPR response ($\Delta Extd$) to the incubation of 3 mg/ml BSA on the peptide-modified AuNH array under no bias and -0.2V.

Figure 4. 9 (a) Real-time LSPR signals of AuNH array under -0.2V bias. Inset is the calibration curve for the detection of cTnI spiked in PBS with 3 mg/ml BSA under -0.2V bias. (b) The real-time Ext_d changes measured on AuNH array under -0.2 V bias for the detection of cTnI in 10 $\times$ diluted rabbit serum.

While, the negatively charged molecules such as BSA (pI=4.7) are repelled from the sensor surface the nonspecific adsorption is expected to decrease. For instance, after injection of 3 mg/ml BSA in PBS, the LSPR sensor response due to BSA adsorption decreased by a factor of 2 at -0.2V bias (Figure 4. 8). To investigate the possibility of detecting cTnI in complex samples at -0.2 V bias, we started by adding various concentrations of cTnI (1 ng/ml to 3 μ g/mL) in PBS containing 3 mg/ml BSA. The extinction changes were monitored in real time before and after the injection of the cTnI

in the BSA containing buffer solutions (Figure 4. 9a). Sequential injection at BSA solution spiked with cTnI revealed a slow increase of the ΔExt_d was seen upon the injection of 3 mg/ml BSA. A significant response at 100 ng/ml cTnI, and a LOD of about 8 ng/ml was estimated for the detection of cTnI in 3 mg/ml BSA, see insert Figure 4. 9a and Table 4. 1. This result indicated the feasibility for the detection of cTnI in series of high BSA concentration, and potentially also for more complex biofluids like serum or plasma.

Figure 4. 10 (a) Real-time monitored ΔExt_d upon the injection of 10× diluted rabbit serum on the peptide-modified AuNH array under bias of no bias and -0.2V. (b)

Calibration curve for the detection of cTnI in 10× diluted rabbit serum. Dash line shows the noise level 3σ .

The nonspecific adsorption of a 10-time diluted rabbit serum was subsequently investigated (Figure 4. 9b). The nonspecific adsorption was reduced by a factor of 2 by monitoring the ΔExt_d after applying -0.2 V bias (Figure 4. 10a). However, the response induced by the nonspecific adsorption ($\Delta Ext_d = 0.001$) was still higher than the specific sensor response to 3 $\mu\text{g/ml}$ cTnI (Figure 4. 9b), suggesting that other molecules/proteins than serum albumin (*e.g.* globulins and antibodies) may adsorb to the sensor surface. Nevertheless, the sensor response to cTnI at 100 ng/ml was comparable to that measured in PBS buffer. A detection limit of 25 ng/ml was achieved in serum samples (Figure 4. 10b). Control experiments carried out under bias of 0.4 V and -0.4 V indicated a poor long-term stability of the sensor response, which might be ascribed to the dissociation of the thiol molecules from the sensor surface and the minor oxidation/chlorination of the gold film. Taken together, electro-focusing at -0.2 V offers a route to reduce non-specific adsorption of negatively charge proteins to the peptide modified AuNH array thereby allowing for detection of cTnI in biofluids of increasing complexity.

4.4 Conclusion

I have successfully demonstrated an electro-focusing-enhanced plasmonic sensor based on a gold nanohole array for the detection of cTnI in buffer, BSA solution and rabbit serum. The LOD in buffer can be improved by 2 orders of magnitude upon changing from no bias to -0.2 V bias applied on the sensor surface. The nonspecific adsorption of serum albumin (*i.e.* BSA) and rabbit serum is also reduced at -0.2 V. This method provides an avenue for highly sensitive detection of biomolecules in complex fluids like serum without tedious sample purification and pretreatment.

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Chapter 5

Grating-coupled Surface Plasmon Resonance Enhanced Label Free Biosensors

In this work, I have developed a smartphone detection platform by employing grating-coupled surface plasmon resonance (GC-SPR) on an Au substrate for detection of lipopolysaccharide. The sensor system relies on the smartphone's built-in flash light source and camera. The detection limit for the lipopolysaccharide assay was approximately 32 ng/mL. Due to the portability and simplicity, the smartphone device shows great promise for point-of-care and remote detection of biomedical and environmental targets.

5.1 Introduction

Portable and compact point of care (POC) systems for biomolecular detection have attracted huge attention because of increasing interest in performing early diagnosis in the patient's home or at the doctor's office. Environmental monitoring at remote locations where conventional instruments and advanced laboratory infrastructure are not available^{1,2} is another key application area for such systems. Standard diagnosis in a clinical setting usually involve well-trained personnel, sophisticated set-ups and complicated assay procedures, which may include examinations (X-ray, tomographic and ultrasonic imaging, *etc.*) as well as lab-tests (mass spectroscopy, electrochemical assay and enzyme-linked immunosorbent assay (ELISA) *etc.*).² These methods have definitely advanced modern diagnostics in the developed part of the world. In spite of the high sensitivity and accuracy, they are less useful for near patient application because of their bulkiness. The running costs are also high making them less available in resource-limited areas of the world.

Multiple strategies have been proposed to address the technical challenges in the areas of POC and field diagnosis.³⁻⁷ For example, paper-based devices for HIV⁸ and colorimetric immunoassays⁹ have been proposed. Although these simple colorimetric assays can be read by the naked eye, more sensitive and precise analyses require amplification, image capturing and data processing. Among the external auxiliary tools, the smartphones are gradually standing out because of the global ubiquity, steady upgrading functions and proven usefulness for bioanalysis on the basis of fluorescent imaging^{5, 10}, photonic crystal resonance¹¹ and SPR¹². The smartphone also can be used for tests of contaminants in drinking water and food. It can also serve as a first hand tool for the characterization of injectable biofluids prior to use. Lipopolysaccharide (LPS) or endotoxin¹³ is one of such targets present in the outer membrane of Gram-negative bacteria. It consist of three covalently bond parts, an inner and outer core, and O-antigen. LPS is an inflammatory stimulator that may result in septic shock.¹⁴ Septic shock is a severe pathophysiological syndrome in response to an infection that in many cases is fatal as it can lead to organ failure.¹⁵ Human beings are exposed to LPS trough drinking

water and injectable biofluids (including various supplements and infusion liquids). The first detection method for detection of LPS, rabbit pyrogen test (RPT), dates back to 1940s.¹⁶ Other approaches have been established since then including the widely used limulus amoebocyte lysate (LAL) test.¹⁷ Apart from these complicated methods, some of which involves animals, methods that are fully synthetic and are easy to operate are in great demand.

Figure 5. 1 (a) Schematic structure of the smartphone spectrometer relying on the in-built LED light, a CD diffraction substrate and CCD camera. Inset is a photo of 3d printing plastic sample chamber kit attached on HTC sensation XE smartphone. (b) Schematic illustration of the optical arrangement of the smartphone.

There are several technical limitations in the application of smartphones for biodetection. First of all, the limited resolution of the built-in camera influences both accuracy and sensitivity. Secondly, external components, especially the light source for optical assay is a great burden for the subscribers. Last but not the least, the influence of the ambient is hard to be controlled or compensate for, resulting in a noisy signal or unstable read-out.

In this work I demonstrate a portable LPS detection platform based on a smartphone and a grating-coupled SPR (GC-SPR) add on. The lack of external light source and detector reduces the size of the whole platform. In order to ensure a stable sample position and a fixed incident angle of the light, a plastic sample chamber kit, designed especially for the HTC sensation XE model was produced by 3D printing (Figure 5. 1a). SPR was excited by the LED flash light (white light), which was then passed through a polarizer and directed to the grating gold substrate at an incident angle of about 5° , and reflected back to the camera through a compact disk (CD) with 640 lines/mm as a diffraction substrate to form a “rainbow” band on the charge-coupled device (CCD) camera. The rainbow band with approximately $900 \text{ pixels} \times 50 \text{ pixels}$ was selected to yield to single intensity value for every wavelength of the spectrum. Five spectra gathered from independent consecutive images are finally averaged to obtain a single spectrum (i.e. $I(\lambda)$).

5.2 Experimental Section

5.2.1 Materials

LPS was purchased from Abcam. Peptide KC-13, specific for LPS used were customized and purchased from GL Biochem(Shanghai), whose sequence is KKNYSSSISSIHIC. UV curable polymer, NOA72 was obtained from Norland (Cranbury, NJ). The plastic sample chamber kit, including a chamber and several sample holders were 3d printed with acrylonitrile butadiene styrene (ABS). Other chemicals, including bovine serum albumin (BSA, ca. $\sim 66\text{kDa}$, $\geq 96\%$), were purchased from Sigma-Aldrich. The UV lamp with the two wavelengths of 254nm and 365nm and power of 6W was purchased from UVP, LLC (CA, USA).

5.2.2 Grating Au substrate Fabrication and Modification

The grating Au substrates were prepared by a series of steps starting from a sinusoidal silicon master ($\Lambda=500\text{nm}$ and modulation depth of $d=60\text{nm}$), from which the substrates were replicated (Figure 5. 2a). The master was made by reactive ion beam lithography reported elsewhere^{18, 19}. A complementary secondary master was prepared by pouring PDMS solution onto the silicon master and thermally curing it at 65°C . The sinusoidal topography was transferred to glass slides and the sinusoidal surface was first made of UV-curable polymer NOA72. The surface solidified under UV treatment for 3h by UV illumination at a wavelength of $\lambda = 365\text{ nm}$ operating at 6W. It was then aged for several hours. Afterwards, a layer of Cr (2nm) followed Au (70nm) film was thermally deposited on top of the UV-cured polymer.

The substrates were cleansed by O_2 plasma for 3min followed by incubation in an aqueous solution containing $50\mu\text{M}$ KC-13. The sample was then rinsed by distilled water and dried in N_2 .

Figure 5. 2 (a) Photo of grating Au substrate. (b) AFM image of grating Au substrate. (c) The profile of the section denoted by straight black line.

5.2.3 Characterization of the grating Au substrate

Figure 5. 3 (a) Reflectivity of grating Au substrates under a series of incident angles from 0° to 30° with the augment step of 0.1° and wavelength range between 400nm to 1000nm. (b) Reflectivity of grating Au substrates under 10 different degrees (0° , 3° , 4° , 5° , 6° , 7° , 10° , 15° , 20° , 25°).

The grating Au surface was characterized by AFM (Figure 5. 2b and c). The results indicated homogeneously distributed sinusoidal strips. The average depth was found to be about 60nm and the period 500nm, Figure 5. 2c.

The reflectivity spectra of the Au grating substrates measured under a series of incident angles in air were plotted in Figure 5. 3. The incident angle is defined as the angle between the surface normal and the light beam (Figure 5. 4). The SPR resonant wavelength increases linearly from about 570 nm to 730 nm with the incident angle increase from 4 ° to 25 °, as shown in Fig. 5. 3a. The SPR coupling efficiency is high and the resonant dip is narrow (width 20-50 nm) for incident angles from 5 ° to 20 °. The reflectivity intensity is also low (~ 0) at wavelength $\lambda < 450$ nm and $\lambda > 950$ nm.

Figure 5. 4 The optical system for the measurement of angular-reflectance spectra under angles from 0 to 30 ° with wavelength range from 400 to 1000 nm.

5.2.4 LPS measured by prism coupled surface plasmon resonance spectroscopy on KC-13 modified Au film

The affinity of peptide KC-13 to LPS was detected by a home-built SPR system, as described elsewhere²⁰. Briefly, a light beam emitted from a HeNe laser (2 mW, $\lambda = 632.8$ nm) passed through a polarizer to become transverse magnetically polarized beam and was coupled to a LASFN 9 glass prism (90 °, $n_p = 1.845$) with optically matched sensor chip to its base by using immersion oil. A flow-cell with the volume of approximately 20 μ L was pressed against the sensor surface to enable sample exposure via a peristaltic pump operating at the flow rate of 0.25 ml/min. The excitation of surface

plasmons (SPs) was observed from the angular reflectivity spectra measured using a photodiode detector and a rotation stage. The resonant coupling of the incident light beam and the SPs is manifested as resonant dip in the reflectivity spectrum.

The Au chip (consist of 2 nm Cr and 47 nm Au) was modified with KC-13 and exposed to 1, 10 and 100 $\mu\text{g/ml}$ LPS in water. The time-dependent reflectivity at an incident angle of 56 degrees was measured as shown in Figure 5. 8. The signal-to-noise ratio for 1 $\mu\text{g/ml}$ LPS is about 8.8, where the noise level is 0.135% calculated as 3 times standard deviation of baseline signal.

5.3 Results and Discussion

In the smartphone spectrometer, a mirror was placed on the sample holder and several filters were employed to calibrate the smartphone spectrometer. These filters include a 550nm long pass filter and five band pass filters: 470nm $\pm$ 20nm, 482nm $\pm$ 17.5nm, 525nm $\pm$ 20nm, 536nm $\pm$ 20nm 670nm $\pm$ 20nm, whose spectra were measured by UV-vis spectrometer (Figure 5. 5 solid curves). For the grating-coupled SPR measurement, the reference spectrum, $I_0(\lambda)$, was measured based on the reflection of the light on a mirror which was placed on the sample holder. The reflectivity of Au grating substrate was then measured to obtain the sample reflectivity spectra $I_s(\lambda)$. The grating-coupled SPR reflectivity spectra was then calculated based on the following equation, $R(\lambda) = -\log(I_s(\lambda)/I_0(\lambda))$. A mirror was inserted into the sample holder and the images of this mirror without any filter in the light path was served as the reference when analyzing the images on the software to draw the spectra curves of the filters (Figure 5. 5 dotted curves). The smartphone spectrometer with detectable spectra range from 430 nm to 650 nm was accordingly determined based on the calibration. Considering the SPR coupling efficiency and the detectable range of the smartphone spectrometer, the angle of sample holder to the incident light was accordingly determined to be approximately 5°, which was designed in Figure 5. 1 for 3D printing.

Figure 5. 5 Spectra of filters measured by UV-vis spectrometer (solid curves) and drawn by software from the images captured by the CCD camera.

Figure 5. 6 a) Images of grating Au before (upper one) and after 1mg/mL BSA binding, measured under the incident angle of about 6° . The white line and red arrow indicate the

valley on the color band. (b) Spectra of the images measured by smartphone in the air under the circumstances of bare grating Au (black curve) and that of non-specific bond by 1mg/mL BSA (red curve).

As a proof-of-concept experiment, a high concentration of BSA (1 mg/mL) was incubated with the grating Au chip and measured before and after the adsorption of BSA under the incident angle of about 6° , as shown in Figure 5. 6. The rainbow band shows a dark band corresponding to the SPR resonant band that shifts to higher wavelength (Figure 5. 6a). The SPR spectra also indicated the resonant wavelength shifts from 587 nm to about 595 nm after the adsorption of BSA on the sensor chip. Therefore, due to the high sensitivity of SPR to refractive index changes, such a smartphone SPR spectrometer shows the feasibility for biomolecule detection. We accordingly modified the grating Au substrate with a specific peptide for the detection of LPS (from *Klebsiella pneumonia*). Peptide-functionalized metallic surface (nanostructures) has been widely applied in our group for sensing applications including the detection of toxins,^{21, 22} ions and protein biomarkers.^{23, 24} In this smartphone SPR spectrometer, we modified the grating Au substrate with KC-13 peptide (KKNYSSSIHHC). The peptide shows high affinity to the binding of LPS as reported previously²⁵. After incubation of the KC-13 modified sensor chip with various concentrations of LPS in PBS buffer for 20 min, the SPR spectra were measured in the smartphone spectrometer. As shown in the rainbow band changes upon exposure to different concentration of LPS (Figure 5. 7a); small changes on the dark band can be observed (give wavelength range). Accordingly, the SPR reflectivity spectra show the resonant wavelength shifts to higher wavelength upon increasing the concentration of LPS applied on the sensor chip. A resonant wavelength shift of 1.46 nm was observed upon the incubation of 10 ng/mL LPS on the sensor surface, and wavelength shifts up to 5.71 nm at presence of 10 μ g/mL LPS. The signal-to-noise ratio for 1 μ g/ml LPS is about 2.4 (Figure 5. 7c) which is about 3.6-fold lower than that measured with conventional prism-coupled SPR setup (Figure 5. 8). This is due to the higher noise of smartphone SPR spectrometer and the chip-to-chip variations. The calibration curve based on monitoring the resonant wavelength shift indicated that the LOD for the detection of LPS down to 32.5ng/ml can be achieved with the smartphone SPR spectrometer. The LOD was determined as the concentration of LPS at which the

response is 3 times of the standard deviation of blank sample. Although the LOD is still need to be improved to achieve the clinically relevant level of 10 ng/ml, our method offers a LOD that is at least an order of magnitude lower than those reported for fluorescence based LPS detection ($\sim 1.5 \mu\text{g/ml}$)²⁶ and fluorescence turn-on detection (280 ng/ml),²⁷ and other methods.²⁸ We expect that this compact, low-cost smartphone SPR spectrometer can be generally applied for point-of-care diagnosis, food safety control and environment monitoring in near future.

Figure 5. 7 (a) Color bands of mirror and different subjects added on grating Au substrate, added subject denoted on the left of each band. The vertical blue line illustrates the

resonance position change. (b) The spectra of grating Au substrate with different. (c) The sigmoidal fitted calibration curve (red) and the dashed black curve is 3 times of standard deviation.

Figure 5. 8 The time-dependent reflectivity changes of KC-13 modified Au chip after incubation with 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ LPS in water, as measured at incident angle of 56° .

5.4 Conclusion

I have built a grating-coupled SPR smartphone spectrometer and applied it for detection of a model target – lipopolysaccharide. This spectrometer doesn't require any external light source but relies on the built-in LED flash light, the CCD camera of the smartphone and a CD diffraction substrate. The spectrometer is capable of measuring spectra in the range of 430-650nm, which matches the grating-coupled SPR spectra acquired using the grating Au substrate. A peptide (KC-13) modified sensor chip was applied for the detection of LPS based on the smartphone SPR system. Based on monitoring the resonant wavelength shift, I demonstrate the feasibility for the detection of LPS at a limit of detection of 32.5ng/ml and expect that the smartphone SPR spectrometer with small, low-cost add on will be widely applied in field and POC testing in near future.

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Chapter 6

Localized and Propagating Surface Plasmon Resonance Co-enhanced Directional Fluorescent Biosensor

Localized surface plasmons (LSPs) and propagating surface plasmons (PSPs) are able to be excited simultaneously on metallic thin film with distributed nanostructures. An AuNH array with such kind of features was utilized to enhance fluorescence intensity for biosensing application. The experimental results verified the simulations that the collective effect of LSPs and PSPs in a Kretschmann configuration facilitated a two orders of magnitude stronger enhancement as compared to a planer Au film excited off-resonance. Furthermore, the combination of LSPs and PSPs elevated the fluorescence enhancement 3-fold as compared to the individual contributions. The fluorescence collection efficiency on the nanohole array substrate was also using the nanohole array.

*This is a collaboration work with others which I mainly doing the experiments other than the simulations.

6.1 Introduction

Surface plasmons (SPs) are optical resonances originating from excitation of free electron oscillations at the surface of metals. They allow for strong electromagnetic field confinement in vicinity to such surfaces which have found diverse applications in analytical technologies,^{1, 2} photo-catalysis,³⁻⁷ and opto-electronic devices.⁸⁻¹² Besides enabling direct detection of molecular binding events due to local refractive index changes, plasmonic nanostructures featuring strong enhancement of the electric field offer powerful means for amplifying spectroscopic signals in surface-enhanced Raman scattering (SERS)^{13, 14} and metal enhanced fluorescence (MEF).¹⁵⁻¹⁷ The fluorescence emission from organic dyes or quantum dots can be enhanced by coupling of their excitation (at wavelength λ_{ex} close to the absorption band) or emission (at emission wavelength λ_{em}) transitions with SPs. The SP-driven excitation at λ_{ex} allows for local enhancement of the excitation rate without increasing the background while the SP-mediated emission can be used to control the angular distribution of emitted light. For instance, reverse Kretschmann configurations utilized for out-coupling of fluorescence light emitted via PSP^{18, 19} as well as LSPs supported by plasmonic nanoantennas²⁰⁻²³ have proven effective for obtaining highly directional fluorescence emission.²⁴ Up to now, various metallic (nano)structures have been employed for the enhancement of fluorescence emission by combined coupling of emitter absorption and emission with SPs including continuous thin metallic films supporting PSPs,¹⁵ and metallic nanostructures such as nanocubes,²⁵ nanoholes,²⁶⁻²⁹ nanorods,^{30, 31} nanodisks,³² core-shell nanoparticles,^{33, 34} DNA-assembled nanoparticles,³⁵ antennas-in-box³⁶ and bowtie nanoantennas³⁷ that support LSPs.³⁸ The amplified fluorescence signals have been implemented in various bioassays for highly sensitive detection of proteins and nucleic acid analytes^{39, 40} with LOD reaching femtomolar concentration levels. In general, plasmon-enhanced fluorescence biosensors typically provide sensitivities that are 4 to 5 orders of magnitude better than classical label-free SPR biosensors and 1 to 2 orders of magnitude better than ELISA.⁴¹

Optical excitation of PSPs in the red and infrared part of spectrum at the surface of noble metals such as gold and silver provides electric field intensity enhancement $|E/E_0|^2$ of $\sim 10^2$ due to the field confinement in the direction perpendicular to the surface. This

confinement can be quantified by the penetration depth L_p (defined as a distance from the metal surface where the surface plasmon electric field amplitude drops by a factor of $1/e$) which for PSP equals $\sim 10^2$ nm. The field intensity can be further enhanced by coupling PSP with modes possessing low Ohmic losses such as long range surface plasmons,⁴¹⁻⁴⁴ which typically translates into an enhancement of fluorescence light intensity by a factor up to $EF \sim 10^2$. Another efficient means to confine the light intensity and amplify fluorescence signal is offered by LSPs that exhibit stronger confinement of the electromagnetic field at distances L_p smaller than a few tens of nanometers. The tight field confinement of LSPs have been utilized for the amplification of fluorescence intensity which can reach an enhancement factor of $EF > 10^2$ for an surface-averaged and even $> 10^3$ for individual emitters placed directly at a plasmonic “hot spot”.³⁷ Note that plasmonic hot spot refers to a small volume at which metallic nanostructures confine the electromagnetic field by resonant excitation of LSPs.

In heterogeneous plasmon-enhanced fluorescence bioassays, the surface of the metallic (nano)structures is functionalized with biomolecular recognition element (e.g., a capturing antibody) that specifically bind target analyte of interest. Typically, the captured analyte is immersed in a solution containing a secondary antibody (the detection antibody) that is labeled with a fluorescence emitter (e.g. organic dye, quantum dot). The analyte binding events are detected by monitoring the fluorescence intensity emitted upon probing with confined SP field. Probing by LSPs can lead a substantial enhancement of the fluorescence signal. The enhancement is typically limited by the distribution of the plasmonic hot spots that occupy just a small fraction of sensing spot area. Therefore, the probability of capturing the analyte at the hot spots is low which severely impedes the overall assay sensitivity.

In this work, the simultaneous excitation of LSP and PSP modes on Au film with an array of nanoholes was investigated for the amplification of fluorescence emission by combining the advantages of LSPs (increased field confinement at hot spots) and PSPs (large surface area that is probed by SP field). Similar structures were studied for other modalities of plasmonic biosensors. For instance, nanoporous metallic films supporting LSPs and PSPs had been used to study molecular binding events.⁴⁵ Similarly, silver nanowell substrates that were prepared from an anodized aluminum oxide template

enabled simultaneous excitation of LSP and PSP, in which the PSP was expected to collect the energy of the incident light and re-excite LSP for SERS enhancement.⁴⁶ In addition, the coupling of LSP on Au nanoparticles with PSP on Au film was explored for increasing the field intensity in the gaps between a nanoparticle and a metal surface. This approach was reported to provide SERS enhancement up to 10^7 ,⁴⁷ and fluorescence enhancement yielding up to $EF \sim 10^3$.⁴⁸ Furthermore, dye molecules placed in a small gap between a silver nanocube and Au film provides highly directional fluorescence emission.²⁵ However, the use of these approaches for practical applications is limited by the size of the gap. It has to be rather narrow (typically 3~5 nm) which does not allow for accommodating large molecules such as antibodies. We herein investigated the field distribution and fluorescence emission on LSP and PSP co-excited mode on Au nanohole arrays and compared the fluorescence enhancement with PSP mode on flat Au film. Such a LSP and PSP co-enhanced fluorescence spectroscopy was achieved for biomolecules detection with the fluorescence enhancement comparable to the simulation results.

6.2 Experimental Section

6.2.1 Materials

The triethylene glycol mono-11-mercaptopundecylether (Thiol-PEG, #673110), PBS buffer tablets, and Tween-20 were purchased from Sigma-Aldrich (Singapore). Biotinylated PEG alkane thiol (Thiol-biotin, #CMT015) was obtained from Nanoscience Instrument, Inc. (USA). Alexa 647 labeled streptavidin (SA647, #S21374) was purchased from Invitrogen (Singapore).

6.2.2 Optical Setup, Design, Fabrication, Characterization and Modification of the AuNH array substrate

An attenuated total reflection (ATR) method was used for the excitation of localized and propagating surface plasmon on the sensor surface (Figure 6. 1a). A transverse magnetically (TM, p-polarization) polarized beam from a HeNe laser ($\lambda=632.8$ nm) was coupled to a LASFN9 glass prism for the excitation of LSP and PSP. Onto the prism base, a sensor chip with a structure supporting surface plasmon was optically matched with

matching oil ($n = 1.700$, Cargille lab. NJ, USA). Aqueous samples (with a refractive index close to $n_b = 1.333$) were pumped at the flow rate of 0.4 mL min^{-1} through the flow-cell using a peristaltic pump. The analyzed samples circulated in the fluidic system with a total volume of $800 \text{ }\mu\text{L}$. The fluorescence light emitted from the sensor surface was collected through the flow-cell by a lens (numerical aperture $\text{NA} = 0.3$), passed through two band-pass filters (transmission wavelength of $\lambda = 670 \text{ nm}$) and its intensity was detected by a photomultiplier tube (PMT) from Hamamatsu (H6240-01, Japan). The LASFN9 glass prism was mounted on a motorized rotation stage and angular reflectivity spectra $R(\theta)$ were measured by using a photodiode detector and lock-in amplifier.

We have designed an Au structure with an array of nanoholes (AuNH) for the excitation of a routinely used chromophore in fluorescence assays - Alexa Fluor 647 - which is excited at a wavelength of $\lambda_{\text{ex}}=647 \text{ nm}$ and emits fluorescence light at a wavelength of $\lambda_{\text{em}}=670 \text{ nm}$. By rational design of the pitch and diameter of nanohole array arranged in a square lattice, the LSP resonance was tuned to overlap with λ_{ex} of the emitter, which were elaborated in chapter 3.

For the investigation of Alexa-labeled streptavidin (SA647) binding, the flat Au film and AuNH substrates were first immersed into a mixed thiol ethanol solution with 0.01 mM Thiol-biotin and 0.09 mM Thiol-PEG overnight. The substrates were dried with nitrogen (N_2) stream before use.

Figure 6. 1 (a) Scheme of the geometry used for co-excitation of PSP and LSP modes on an AuNH array for fluorescence enhancement. θ_{max} is the maximal angle of fluorescence emission collected through the optics. (b) Images of a sensing spot acquired with a CCD camera upon reflection of the excitation laser beam from a flat Au film (left) and AuNH (right) at an angle of incidence of $\theta=62.3^\circ$ and 72.3° , respectively. Scale bar 2 mm.

6.2.3 Detection Process

The SA647 at concentrations from 0 to 10 nM in PBST buffer (PBS with 0.05% tween-20) were pumped into the flow-cell contact with biotinylated Au substrates for 20 min, followed by 5 min rinsing with PBST for each concentration of SA647.

6.2.4 Simulation

The simulations were performed by the collaborators. We used a classical fluorescence model in which a fluorophore was approximated with oscillating adsorption μ_{ab} and emission μ_e dipoles. The excitation rate γ_e of a fluorophore obtained upon irradiation by an incident wave at the absorption wavelength λ_{ab} can be expressed as:

$$\gamma_e \propto |\vec{E}(\lambda_{ab}) \cdot \mu_{ab}|^2 \quad (6.1)$$

Note that this Equation 6. 1 holds for small amplitude of electric intensity $|\vec{E}|$ when the excitation rate is far from saturation. Electric field $\vec{E}(\lambda_{ab})$ given in Equation 6.1 was calculated with a single unit cell and a mesh size of 1 nm. After its excitation, the fluorophore can return to its ground state by emitting a photon at a higher wavelength λ_{em} (radiative decay rate γ_r) or without emitting a photon (non-radiative decay rate γ_{nr}). Further, we assumed an intrinsic radiative decay rate γ_r^0 and non-radiative decay rate γ_{nr}^0 for an emitter in homogenous aqueous environment with the quantum yield of $\eta_0 = \gamma_r^0 / (\gamma_r^0 + \gamma_{nr}^0)$. When the emitter is brought in vicinity to a metallic structure, decay rates are altered leading to a change in the quantum efficiency η :

$$\eta = \frac{\frac{\gamma_r}{\gamma_r^0}}{\frac{\gamma_r}{\gamma_r^0} + \frac{\gamma_{abs}}{\gamma_r^0} + \frac{(1 - \eta^0)}{\eta^0}} \quad (6.2)$$

where, γ_r/γ_r^0 refers for the normalized radiative decay rate and γ_{abs}/γ_r^0 for an additional non-radiative decay rate associated with the absorption by the metal. These ratios can be obtained from finite-difference time-domain (FDTD) simulations as $\gamma_r/\gamma_r^0 = P_r/P_r^0$ and $\gamma_{abs}/\gamma_r^0 = (P_{em} - P_r)/P_r^0$, where P_r^0 is the power radiated to far field by identical dipole in homogenous dielectric medium.

The directionality of surface plasmon-coupled emission was taken into account by using a parameter named collection efficiency CE . We assumed that only light emitted at λ_{em} into a range of polar angles $\vartheta = 0$ - ϑ_{max} can contribute to a measurable signal in a realistic biosensor system (e.g., fluorescence light was collected by a lens with a numerical aperture $NA = n \sin[\vartheta_{max}]$). As following Equation 6.3 shows, the CE was defined as the emitted power that can be collected within assumed range of polar angles which was normalized to the total power emitted to the far field:

$$CE = \frac{\int_0^{2\pi} \int_0^{\vartheta_{max}} P_r(\vartheta, \varphi) \sin \vartheta d\varphi d\vartheta}{\int_0^{2\pi} \int_0^{\pi} P_r(\vartheta, \varphi) \sin \vartheta d\varphi d\vartheta} \quad (6.3)$$

Figure 6. 2 The scheme of the nanohole structure and the location of the fluorophore which was 8 nm away from the metal surface.

The simulations of the fluorescence emission were performed by using the FDTD method implemented in a commercially available package FDTD Solutions (Lumerical Solutions Inc., Canada). Considering the length of the self-assembled monolayer and the diameter of the streptavidin, a fluorophore was placed at 8 nm away from the AuNH and flat Au film surface (Figure 6. 2). The fluorophore was represented as an oscillating dipole. In order to calculate the angular distribution of field intensity emitted from an individual dipole, a super-cell comprising an array 49×49 periods (400 nm) was used similar to other works. The central part including the oscillating dipole was simulated with a mesh size of 1 nm, while the rest of the supercell was simulated with a maximum mesh size of 5 nm. Total emitted power from a dipole P_{em} was calculated by the integration the energy flux through walls of a cube closely surrounding the dipole (cube edge length of 10 nm). Quantum yield of an emitter η that was altered due to the coupling with metallic nanostructures was obtained as a ratio of the energy emitted to the far field P_r and the total emitted energy P_{em} . The energy emitted to far-field was simulated by using a two dimensional detector placed in the plane above and below the nanohole arrays. Near-field components of the electric and magnetic field intensity were recorded and transformed into the far-field dependence of P_r on the polar ϑ and azimuthal φ angles. Considering the

surface area of the nanohole wall (i.e. position 1) was about 8-fold smaller than the gold surface at position 2, the *EF* was estimated only for the fluorophores located at position 2, Figure 6. 2.

6.3 Results and Discussion

Figure 6. 3 (a) Experimental (black) and simulated (red) UV-vis transmission spectra of the AuNH array on a glass substrate in contact with an aqueous environment. (b) Experimental and simulated angular SPR reflectivity from flat Au and AuNH substrates at a wavelength of $\lambda=632.8$ nm.

When brought in contact with aqueous samples, the AuNH sample shows two LSP transmission peaks at 645 and 795 nm, respectively (Figure 6. 3a). The resonance measured at normal incidence ($\theta=0$) qualitatively agreed with the simulations. Moreover, the simulations revealed that the resonance at 645 nm was associated with the excitation of LSPs at the top rim of the nanoholes that was in direct contact with aqueous phase.

The resonance at 795 nm was due to the coupling to LSP at the bottom rim of the nanoholes in contact with the glass substrate. The peak associated with LSPs at the top rim was further used as it allows probing an area that was better accessible to molecules diffusing from the aqueous phase above the structure. Note that the distinct peak at $\lambda=500$ nm was due to the interband transition of Au.^{49, 50}

By using the Kretschmann configuration, the excitation of LSP and PSP modes was observed by measuring reflectivity at wavelength of $\lambda=632.8$ nm upon tuning the incident angle θ (Figure 6. 2a). As shown in Figure 6. 3b, the excitation of PSP modes on flat Au and AuNH substrates manifested themselves as dips in reflectivity spectra at similar angles of incidence $\theta \sim 72^\circ$, which qualitatively agreed with simulations. The overall reflectivity change of the AuNH array was lower than for flat Au due to the excitation of LSPs at the metallic nanoholes that were not sensitive to variation in the angle of incidence θ . Moreover, our data revealed that the resonance on the AuNH array was significantly broader than for the flat Au film. This was most likely due to radiation losses associated with the diffraction of the periodic array of nanoholes and to a change in the dispersion relation of PSP modes due to the coupling to LSPs. The difference in the coupling angle between the experiment and simulations of the AuNH sample was likely due to the roughness of prepared structures⁴⁵ which was not taken into account in the simulations. As shown in Figure 6. 2b, the scattering intensity on the AuNH substrate at the angle of incidence of $\theta=62.3^\circ$ (off resonance) and 72.3° (at resonance) were about 10 and 3-fold higher than on flat Au film at the corresponding angles, respectively.

The simulations were carried out to study the near field enhancement of the electric field due to the excitation of PSP and LSP modes. The excitation of PSP waves at $\theta=72^\circ$ on a flat Au film confines the incident field perpendicular to the surface (Figure 6. 4a). For the AuNH array under coupling of the incident wave at $\theta=62.3^\circ$, excitation occurs for LSPs located at the upper and lower rim of the Au nanoholes (Figure 6. 4b). When the angle of incidence increased to $\theta=73^\circ$, the PSP and LSP were co-excited which led to an increase of the field amplitude of LSP (Figure 6. 4c). A maximum field intensity enhancement of $|E/E_0|^2=1.6 \times 10^3$ was predicted at the rim of the metallic nanoholes upon co-excitation of LSP and PSP modes. This value was about an order of magnitude higher than that of the

PSP mode on flat Au film ($|E/E_0|^2=130$) and 8 times higher ($|E/E_0|^2\approx 200$) than that observed for excitation of LSPs at the angle $\theta=62.3^\circ$.

Figure 6. 4 Spatial distribution of electric field amplitude $|E/E_0|$ for (a) resonant excitation of PSP mode on flat Au film, (b) LSP mode on an AuNH substrate and (c) co-excitation of LSP and PSP modes on an AuNH substrate at $\lambda=632.8$ nm. The amplitude of the p-polarized plane wave incident at the indicated angles of incidence θ was set to 1. Scale bars were 100 nm.

In order to explore the details of the field enhancement upon coupling to PSP and LSP modes, cross-sections of the electric field amplitude $|E/E_0|$ in the vicinity to nanoholes were plotted in Figure 6. 5. These plots showed that field amplitude decays exponentially away from the surface for PSP excitation on the flat film (Figure 6. 5a). The co-excitation of PSP and LSP modes at the nanohole rim led to about 4-times stronger field amplitude at the surface, but the field decays faster away from the surface. For instance, one can see that at distances z larger than ~ 40 nm from the surface, the field for co-excited LSP and PSP on the AuNH substrate was lower than for the PSP on the flat Au film. Furthermore, Figure 6. 5b displayed the lateral field distribution along the y -axis for the excitation of PSPs on a flat Au film and co-excited PSP and LSP at AuNH substrate. It indicated that the field intensity due to co-excited LSP and PSP was stronger than that occurring for the coupling to PSP only in vicinity to the Au nanohole at a perimeter of ~ 100 nm away from

the edge of the nanohole. At distances further away from the hole the flat surface provided stronger field enhancement.

Figure 6. 5 (a) Comparison of cross-sections of the electric field amplitude $|E/E_0|$ as a function of distance z from the surface for the resonant excitation of PSP on flat Au film (dashed) and co-excited LSP and PSP modes on AuNH substrate (solid) at $\lambda=632.8$ nm. The field distribution is plotted for $x=0$ and $y=70$ nm, green dot (inset). (b) The cross-section of electric field amplitude $|E/E_0|$ as a function lateral distance along the y-axis (inset) for different heights above the surface $z=1, 5, 10$ nm for PSP mode on flat Au film (dashed) and the co-excited LSP and PSP modes (solid) as indicated in the inset figure $x=0$.

Figure 6. 6 Comparison of the averaged angular distribution of emitted fluorescence intensity $P_r(\vartheta, \varphi = 0)$ for emission at the wavelength of $\lambda_{em}=670$ nm on (a) flat Au and (b) AuNH substrate. The maximum acceptance polar angles are shown as red dashed lines.

The near field coupling of surface plasmon-enhanced field with emitters that serve as labels in fluorescence assays was studied using FDTD model as described in previous work.³² In these simulations the emitters were represented by their absorption and emission dipoles. The angular distribution of fluorescence intensity $P_r(\vartheta, \varphi)$ emitted into the substrate (BK7 glass) and surroundings (aqueous phase) was simulated for the randomly oriented emitters located on the flat Au and AuNH substrate. The distance between emitters and Au surface was set to 8 nm which approximately agreed with to the distance between the dye and the surface (Figure 6. 2). In these simulations, we assumed that only fluorescence light emitted into aqueous medium within a cone defined by the

maximum polar angle $\vartheta_{max}=13^\circ$ contributed to the signal (corresponding to numerical aperture $NA=0.3$ of the optics for collecting the emitted light).

The results (Figure 6. 6a) showed that for a flat Au film the majority of fluorescence light intensity was emitted into the substrate. This was mainly due to the fact that the far field emission strongly couples by near field to PSPs that are subsequently out-coupled via reverse Kretschmann configuration. This led to the occurrence of a highly directional lobe emitted into the substrate at polar angle $\vartheta = \pm 110^\circ$. When introducing periodic perforation of the metallic film, diffraction provided competing means for extracting of the emitted light intensity from the surface to the far field. The data (Figure 6. 6b) revealed that the lobes associated with reverse Kretschmann out-coupling were suppressed and the emission was dominantly channeled to the far field via diffraction at polar angles of $\vartheta = \pm 13^\circ$ into water and $\vartheta = \pm 169^\circ$ into the glass substrate. The diffraction-coupled emission in water carried the emitted energy predominantly at angles below the acceptance angle ϑ_{max} . From these data, we calculated that a fraction of photons emitted towards aqueous medium at $\vartheta < \vartheta_{max}$ (Equation 6. 1) was ~ 2.2 times higher for the AuNH with respect to the flat Au film.

The model describing the interaction of emitter with the metallic surface was used to predict the overall fluorescence intensity enhancement (EF):

$$EF \sim \langle \gamma_e \times \eta \times CE \rangle \quad (6.4)$$

where γ_e , η and CE were the enhanced excitation rate, changed quantum yield and collection efficiency for random orientation of dyes.

The excitation rate γ_e was proportional to the field intensity enhancement $|E/E_0|^2$ at λ_{ex} and the collection efficiency CE quantifies the fraction of photons emitted at λ_{em} that were delivered within the cone defined by the NA . The intrinsic quantum yield of $\eta^0=0.3$ was assumed according to the producer (Life Technologies) for Alexa Fluor 647 dye in water. The simulations revealed that the excitation and emission via co-excited PSP and LSP modes increased the fluorescence intensity F emitted within the NA by a factor of 1.5 with respect to the probing with PSP modes only. Comparing the probing by PSP and LSP (co-excited at $\theta=72.3^\circ$ and $\lambda=632.8$ nm on AuNH) and by PSP (excited at $\theta=72.3^\circ$ and $\lambda=632.8$ nm on flat Au film) with that for the probing at off-resonance regime

(excited at $\theta=62.3^\circ$ and $\lambda=632.8$ nm on flat Au film), the fluorescence enhancement by a factor of 72 and 47, respectively, was predicted by the simulations.

Figure 6. 7 The angular SPR reflectivity (solid lines) and fluorescence spectra (line with symbols) measured on (a) flat Au and (b) AuNH substrates upon the affinity binding of SA647 at concentrations of (black) 0, (red) 10 pM, (green) 100 pM, (blue) 1 nM and (orange) 10 nM. (c) The kinetics of fluorescence signal on (1) AuNH and (2) flat Au film upon the sequential binding of SA647 from solutions with a concentration of (a) 1 pM, (b) 10 pM, (c) 100 pM, (d) 1 nM and (e) 10 nM. (d) The fluorescence intensity changes as a function of SA647 concentration on (blue circle) (red dot) an AuNH array and (green square) (black diamond) flat Au film at incident angles of 72.3° and 62.3° , respectively.

In order to experimentally evaluate the potential of co-excited LSPs and PSPs for the amplification of a model fluorescence assay, we prepared substrates with flat Au and AuNH and modified them with a thiol self-assembled monolayer (SAM) containing terminal biotin groups (Figure 6. 1). These substrates were used for the excitation of

either PSP or co-excited LSP and PSP modes for probing of affinity binding of streptavidin that was labeled by Alexa Fluor 647 dye (SA647). The angular SPR reflectivity and fluorescence spectra measured for the flat Au film after binding of SA647 at the concentrations from 0 to 10 nM show that the fluorescence intensity F increases at the resonant angle $\theta=72.3^\circ$ from 4×10^3 to 3.5×10^5 cps (Figure 6. 7a). While on the AuNH array the affinity binding of SA647 results in a 2-3 times stronger fluorescence signal (from 8×10^3 to 1.2×10^6 cps) (Figure 6. 7b).

The fluorescence kinetic measurement at the resonant angle also indicated 2-3 times higher fluorescence intensity on AuNH substrates upon the binding of SA647 with respect to the flat Au film (Figure 6. 7c). The fluorescence intensity saturated after incubating SA647 at a concentration higher than 10 nM, which was not the case for the reflectivity measurement. This was due to the high fluorescence intensity that ran out of the photomultiplier linear range and approached to the maximal detectable intensity (3.6×10^6 cps) of the photomultiplier. The calibration curves for the affinity binding of SA647 on flat Au and AuNH substrates at on-resonant and off-resonant angles were shown in Figure 6. 7d. The LOD for the detection of SA647 was determined as the concentration of SA647 at which the response was 3 times of the standard deviation of fluorescence fluctuation. The highest sensitivity was achieved on AuNH at on-resonant angle (i.e. upon co-excitation of LSP and PSP) with LOD of 0.7 pM, which was about 2 times and 14 times better than that for the PSP enhanced fluorescence and LSP enhanced fluorescence, respectively. Even though the LSP excited at 62.3° shows the maximum intensity enhancement up to $|E_{sp}/E_0|^2=200$ fold (Figure 6. 4b), which is higher than that of PSP, the LSP enhanced fluorescence shows about 7-fold lower sensitivity (Figure 6. 7d). This is because of the small sensing volume on the AuNH located at the rim of the nanohole.

The experimental and simulated EF on flat Au film and AuNH substrate were normalized to the flat Au film at off-resonance angle (62.3°). The experimental results on AuNH substrate showed higher EF than expected from the simulations. Note that in the simulation, the molecules located on the wall of the nanoholes were not considered because the wall area was very small in comparison to the overall surface area. In addition, the molecules were assumed homogenous distribution on the metallic surface.

Figure 6. 8 (a) The fluorescence kinetics and fittings for the binding of 1 nM and 10 nM SA647 on flat Au film. (b) The fluorescence intensity changes as a function of the number of molecules on 400 nm $\times$ 400 nm surface area of the flat Au film and AuNH substrates.

One may argue that the larger surface area of the AuNH with respect to the flat Au film contributes the fluorescence enhancement. Essentially, the surface area of AuNH substrate can be estimated as $S_{hole} = n(p^2 - \pi R^2 + 2\pi R H_0)$, where R and n were the radius and the number the nanoholes, respectively. p was the pitch of the nanohole array, and H_0 was the thickness of the Au film (Figure 6. 1). For the same size of flat Au film, the surface area is $S_{flat} = np^2$. Accordingly, the surface area of AuNH was about 1.04 times higher than the Au film. This 4% larger surface area was negligible for the maximum 3 times higher fluorescence enhancement on AuNH as compared with flat Au film. The

number of molecules bound on the nanohole substrate was estimated to equal 7 to 696 molecules per $400 \times 400 \text{ nm}^2$, after 20 min incubation of 1 pM to 100 pM, respectively, based on fitting the kinetic curves (Figure 6. 8). The fluorescence enhanced sensitivity on co-excited LSP and PSP mode is about $G=4.88 \times 10^{-6}$ cps/molecular, which is about 3.2 times higher than the PSP enhanced fluorescence.

6.4 Conclusion

In summary, the co-excitation of LSP and PSP can significantly enhance the field intensity around the nanohole that allowed for improved fluorescence enhancement as compared with geometries where only individual PSP or LSP modes interacted with an emitter. The simulation indicated that maximal field enhancement up to $|E/E_0|^2 = 1600$ occurred at the edge of the nanoholes and that highly directional fluorescence emission can be observed on AuNH substrate as compared with flat Au film. Furthermore, the investigation on the fluorescence measurement upon the binding of Alexa647-labeled streptavidin (SA647) on AuNH substrate revealed a fluorescence enhancement of about 10^2 as compared to a flat Au surface irradiated off-resonance. The fluorescence enhancement can be further improved by the selective modification of nanohole array to allow the molecular binding only on the “hotspot” such as the edge of nanohole.^{51, 52} This method benefited both the advantages of the stronger electromagnetic field of LSP and longer penetration depth (higher probing volume) of PSP, and the high direction fluorescence emission for ultrasensitive sensing applications.

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Chapter 7

Preliminary observation and new directions

This chapter outlines some preliminary findings and highlights challenges for the future: 7.1 Localized Surface Plasmon Resonance Enhanced Fluorescent Biosensor and 7.2 the selective modification of AuNH array to improve sensitivity.

7.1 Localized Surface Plasmon Resonance Enhanced Fluorescent Biosensor

Metal nanostructures attract more and more attentions since they are able to resonate at certain wavelength of external incident light and redistribute the electromagnetic field of the light source at the nanostructured surface. The power of the light source is concentrated in the very narrow areas, for instance, at sharp corners and edges of the nanostructures. This capability of focusing the electromagnetic field extends the application of such nanostructures since the concentrated field can strongly enhance fluorescence and other phenomena. Diverse shapes of nanostructures including nanoparticles and nanofilms have been studied and utilized for label-free¹⁻⁴ and fluorescence biosensing⁵⁻¹¹. The noble metal materials, Au¹²⁻¹⁴ and Ag¹⁵ are the ones that have been most extensively studied¹⁶⁻¹⁹. For Au nanostructures, although surface plasmon can be generated on Au nanofilms, certain sample arrangements are necessary to fulfill the momentum-matching requirement. Nanostructures can support localized surface plasmon with neither many sample arrangements nor complicated apparatus. They also offer better confinement of the electromagnetic field which largely augments the concentration of external light, thus paving the way for improved enhancement. Recently, we have gained better understanding of the Au nanohole array structure and the application of such structures for fluorescence based biosensing^{20, 21}. Four factors should be considered to achieve favorable localized surface plasmon resonance enhanced fluorescence: the distance between the fluorescent dye and the nanostructure; the physical and chemical properties of the nanostructure; the overlap extent of the two spectra (the excitation spectrum of the dye and the absorption spectrum of the nanostructures); and the regularity of the distribution of the nanoobjects. First of all, the distance between the fluorescent dye and the surface is crucial since it controls the quenching/enhancement capacity of the metal nanostructure. Secondly, the materials the nanostructures are made of, as well as physical parameters, the size, the shape, and *etc.* determine their optical properties. The third factor, the extent of overlap of the extinction (nanostructure) and emission (dye) spectra influences the enhancement. It has been demonstrated that the brightest fluorescence intensity requires a situation where the the localized surface plasmon scattering peak appears at higher energy (40–120 meV) compared to dye's emission peak²². Fourthly, regular distribution of the nanostructures contributes to a

shaper resonance spectrum, which further improves the fluorescence enhancement. The advancement in fabrication methods, including electron beam lithography, focused ion beam lithography, *etc.*²³, together with high throughput methods such as nano-imprinting lithography²⁴, lithography using atomic force microscope²⁵ and other microscopic tools facilitate a precise production of sophisticated designs on the nanometer length scale. In spite of the use of colloidal lithography for fabrication of AuNH arrays, flawless nanohole arrays were hard to achieve even by finest control in this method²⁶. Other methods such as stencil lithography²⁷ suffer from different problems, including blurring of edges and sharp corners of the nanostructures. I demonstrated an easy way to fabricate AuNH arrays (with or without photoresist) with high efficiency, precision and throughput by the combination of electron beam lithography and nano-imprinting lithography, with the aid of UV curable photoresist. Both the PAuNH array and the AuNH array were utilized for fluorescence enhancement and the PAuNH array platform was applied for PSA fluorescent detection through accurate design and fabrication. The developed methodology offers great potential for other targets and point-of-care applications.

7.1.1 Experimental Section

The UV curable photoresist mr-UVCur21-300nm was purchased from Micro Resist Technology, GmbH. Au pellet purity 99.99% was bought from A.CHEM.A PTE LTD. PSA, PSA capturing antibody and PSA detection antibody (d-Ab) were purchased from SunnyLab of SCIPAC Ltd. Alexa Fluor® 647 Protein Labeling Kit was purchased from Molecular Probes, Invitrogen. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Tokyo Chemical industry Co. LTD.. Cr (Sigma-Aldrich) and other normal chemicals and solvents were purchased from Sigma-Aldrich.

The PAuNH array was also designed to enhance the fluorescent intensity of Alexa 647 fluorophore as discussed in chapter 3. The simulation result and corresponding scheme are shown in Figure 7. 1.

Figure 7. 1 (a) Simulation by FEM of LSPR-enhanced electric field distribution obtained upon illuminated by 620nm light incident perpendicularly to the sample from the water side of PAuNH array (cross-section view). Image (b) is the corresponding illustration of (a).

The fabrication process of the PAuNH array was similar to that of AuNH array on glass. A $2\text{mm} \times 2\text{mm}$ nanohole array (140nm diameter holes at a pitch of 400nm) was first written on a silicon substrate by e-beam lithography, and then the silicon substrate was electroplated to form a nickel mold (with nano-pillars, which are the reversed patterns of nanohole). The mold was used to transfer the nanostructure to a 300nm thick layer of mr-UVCur21-300nm by nano-imprint lithography. The photo resist substrates were then etched with RIE in O_2 flow of 10sccm and at power of 50W for 40s. Then a 50nm thick gold film was deposited on the PAuNH array (Figure 7.2).

Figure 7. 2 Schematic structure of PAuNH array, parameter: 140nm diameter circle at a pitch of 400nm, the height of the gold nanohole wall is 50nm (yellow areas refer to gold, 45nm thick, and the brown areas in between of the gold and polymer/glass are chromium, 5nm)

The PAuNH array substrates were characterized by UV-vis spectroscopy (schematic setup in Figure 3. 11), SEM and AFM (Figure 7. 3), which consisted of 50nm of gold film (including adhesion layer) coated on top a 50nm thick polymer square nanohole array ($p=400\text{nm}$, $D=140\text{nm}$). The nanopatterns were in the central $2\text{mm} \times 2\text{mm}$ area of a $1\text{cm} \times 1\text{cm}$ glass substrate, and the yield of the sample fabrication was almost 100% when observing the sample at different locations. The gold nanohole with 50 nm thick photo resist showed α and β mode peak of about $\lambda_{\alpha} = 589\text{nm}$ and $\lambda_{\beta} = 728\text{nm}$ in air, and at $\lambda_{\alpha} = 609\text{nm}$ and $\lambda_{\beta} = 745\text{nm}$ in water, which are further shifted to $\lambda_{\alpha} = 627\text{nm}$ and $\lambda_{\beta} = 762\text{nm}$ upon modification with dye PSA antibody (Figure 7. 3a). The shape of each hole was not a perfect circle due to the mold. The experimental results were consistent with the simulated spectra, in which the absorption intensity of the α mode was lower than that of the β mode.

Figure 7. 3 (a) The UV-vis absorption spectra of the PAuNH array which was measured in (1) air, (2) water and (3) in air after the surface was coated with 10 μL of 63nM PSA antibody-Alexa647Ab-AF647. (b) SEM and (c) AFM images of the PAuNH array.

The PSA detection antibody (d-Ab) was labeled with Alexa647 dyes and subsequently used as a model recognition-reporter system in the biomarker detection experiment. The procedure of labeling the PSA d-Ab was based on the manual of the kit. UV-vis measurement indicated that each detection antibody was labeled on average with seven Alexa 647 dye molecules.

Figure 7. 4 Illustration of fluorescence enhancing capability experiments, (a) comparison of PAuNH array and gold film with bare glass from top to the bottom, respectively, using 10 μL of 63nM d-Ab-AF647; (b) comparison of AuNH array and PAuNH array with bare glass from top to the bottom, respectively, using 5 μL of 6.3 μM d-Ab-AF647.

The fluorescence spectra of PAuNH, gold film and bare glass were measured with a fluorescence spectrophotometer (Figure 7. 4a), in which the light was illuminated from the top of the chip, and the fluorescence emission was detected at an off-specular angle of 56 degree with respect to the surface normal. The fluorescence enhancement experiment was carried out by depositing a drop (10 μL) of 63nM Alexa 647 labeled PSA antibody (d-Ab-AF647) on the above mentioned PAuNH array sample (drop size of around 10mm²). The fluorescence spectrum was recorded after drying at an excitation wavelength of 620nm (2nm slit widths) and an incident angle (θ) of 70°.

The fluorescence enhancement of AuNH array was compared with the glass and the PAuNH platform (Figure 7. 4b). The experimental procedure was similar to that used for PAuNH array (Figure. 7.4 a). Only the amount of d-Ab-AF647 was changed to a 5 μ L (concentration condensed to 6.3 μ M).

The PAuNH substrate was modified with thiol capped polyethylene glycol acid (Thiol-PEG-COOH) by incubating the substrate in a 1mM Thiol-PEG-COOH ethanol solution overnight. Afterwards, the substrate was rinsed with ethanol and dried with nitrogen. The substrate was fixed in a flow-cell with a volume of about 100 μ L. The cell was circulated with buffer solution using a peristaltic pump. The carboxylic acid groups on the substrate were activated with fresh prepared EDC (37.5 mg/ml) and NHC (11.5 mg/ml) water solution. The capturing PSA antibody (c-Ab), 50 μ g/ml, was then immobilized on the surface (2 hour incubation). Circulation in ethanolamine (EA) for 15min blocked the unreacted carboxyl groups to prevent unspecific binding.

The PSA detection was carried out in the fluorescence spectrometer, in which the sample substrate was fixed at an incident angle of 70 degrees with respect to the normal axis of the substrate (in the normal plain to the substrate surface), and the fluorescence intensity was measured. A series of different concentration of PSA (10ng/ml, 100ng/ml, 1 μ g/ml and 10 μ g/ml) were analyzed. PBS tween buffer (PBS with 0.05% tween-20) spiked with PSA was incubated on the substrate with flow-cell for 30 min, after 2-min rinsing with PBST buffer, a 10nM d-Ab-AF647 was injected into the flow-cell and incubated for 20 min. The fluorescence spectra were measured before and after the incubation of d-Ab-AF647. The four concentrations used the same chip and the previous binding of PSA and d-Ab-AF647 were dissociated by several seconds washing in 0.1M hydrochloride. (Figure 7. 5)

Figure 7. 5 Illustration of PSA detection by fluorescence spectrometer on PAuNH array, where the incident angle was 70° and the detector was perpendicular to the incident light.

7.1.2 Result and Discussion

The fluorescent enhancement of both PAuNH array and AuNH array substrates were examined. Because of the high density of dye on the surface, the emission wavelength at about 680 nm obtained in contact with air was about 10 nm red-shifted as compared with the free Alexa 647 in water solution (Figure 7. 6a). The fluorescence peak intensity at the wavelength of 680 nm showed a 9 fold higher of PAuNH array as compared to glass substrate, which almost matched the simulation result of the field enhancement factor (which is 10) of the PAuNH array with only α mode. The fluorescence peak intensity at the wavelength of 680 nm of PAuNH array was about 2-fold higher than that of the Au film. The higher fluorescence intensity on Au film can be ascribed to the localized surface plasmon of Au film which was raised from the rough of Au film.

Figure 7. 6 Comparison of fluorescence enhancement measured by fluorescent spectroscopy (a) between PAuNH array, Au film and bare glass, 10 μ L of 63nM d-Ab-AF647. (b) between AuNH, PAuNH array, and bare glass, incident wavelength was 620nm at angle of 70 °; 5 μ L of 6.3 μ M d-Ab-AF647.

Compared with PAuNH array, the fluorescence enhancement capability of AuNH array was two-fold higher (Figure 7. 6b). The fluorescence peak intensity at the wavelength of 680 nm showed a 7.5 fold higher of PAuNH array with respect to glass substrate in Figure 7. 6b, which was not coincident with Figure 7. 6a. The inconsistent may partly be attributed to the difference in the concentration of d-Ab-AF647, which means that rarer the dye was, more sensitive the platform was.

Figure 7. 7 (a) Fluorescence spectra by fluorescence spectrometer on the PAuNH array for the sandwich immunoassay of PSA, the concentrations from 0 to 10 μ g/ml. (b) The linearly fitted calibration curve for the detection of PSA by fluorescence spectrometer for the PAuNH array.

In non-dynamic PSA detection, the PAuNH were modified thiol-COOH and activated with EDC/NHS for the immobilization of capture antibody against PSA. The fluorescence intensity spectra were measured according to the fluorescence intensity changes after the binding of 10nM d-Ab-AF647 on the PAuNH array, which were pre-incubated with PSA at concentrations various from 0ng/ml to 10 μ g/ml. Fluorescence spectra for the detection of PSA based on sandwich immunoassay on PAuNH array showed the intensity increased as the increase of PSA concentration. The gauss

distributed curves (the smoother and thicker curves in Figure 7.7a) were fitted according to the fluorescence spectra from which the maximum intensity was determinate. The fluorescence intensity at the maximum intensity increased an order of magnitude when the concentration of PSA increased from 10ng/ml to 10 μ g/ml. The calibration curve for the detection of PSA on PAuNH array is shown in Figure 7. 7b, in which the maximum fluorescence intensity was fitted as the function of the PSA concentration. The LOD of 10ng/ml (300pM) was determined as the concentration of PSA at which the signal response was three times of standard deviation of fluorescence response for blank samples ($3\sigma=3\times 10^4$). The calibration curve showed a linear response at the PSA concentration from 10ng/ml to 10 μ g/ml.

7.1.3 Conclusion

In summary, I have in this work designed nanohole array platform by simulation which can carry LSPR for fluorescence enhancement and label-free detection. The resonance wavelength of the nanohole array was designed at around 650nm for excitation of dyes such as Alexa 647 by adjusting the parameters of the holes and periodicity of the array. The AuNH array showed a maximum fluorescence enhancement factor around 15 fold as compared with glass substrate, while the PAuNH array displayed 9 fold higher fluorescence enhancement factor with respect to glass substrate experimentally. The LOD for the detection of PSA was about 10ng/ml measured by fluorescence spectrometer on PAuNH array substrate. In the next step, AuNH array can be used as the platform for PSA sandwich detection since it has higher enhancement capability than PAuNH array.

7.2 Selective modification of Au nanohole array

On the nanohole array, simulations suggest that the edge surface of each unit is the hot spot where electromagnetic field is concentrated. Therefore, the most edge surface of each unit can give the largest enhancement for fluorescence and Raman signals. The concentrated electromagnetic field could offer the possibility for multi-photon polymerization or scission of polymers coated on top, while the rest part could not supply enough intensity for those processes. As a result, I postulate that the hot spot could be isolated by covering the rest part by positive photo resist or highlighted by negative photo

resist through the enhanced scission or polymerization process.

7.2.1 Experimental Section

In this experiment, the platforms used were 140nm diameter gold nanohole array with wall height of 50nm without photo resist on the bottom, the period of which was 400nm. (Figure 7. 8)

Selective modification of the gold nanohole array started from spin coating a layer of photo resist. PMMA and SU-8 were used as the modification materials. Preheating was undertaken to make a smooth surface. After certain period of illumination, with certain kind of light, the chips were post-heated, and then immersed into the developer for 2min and rinsed by stopper for 1min. The chips before spin coating, after spin coating and after illumination were characterized by SEM and the spectra were taken by UV-vis.

For SU8 modification, the thickness of SU8 was about 120nm. The temperature preheating and post-heating was 95 °C. Three chips were illuminated under the light ruling out the part whose wavelength was shorter than 550nm by a filter for 20s, 40s, and 60s, respectively.

For PMMA modification, the thickness of the polymer layer was about 130nm. The temperature preheating and post-heating were 180 °C and 100 °C, respectively. In the exposure step, two conditions were used, one light longer than 550nm for 1min and the other by 603 ± 3nm light. The power was about 20W. I also compared the spectrum before, after preheating to see the heating influence. One chip went through the whole process except exposure and the spectra of this chip were served to eliminate the others steps' effects.

Figure 7. 8 Scheme of SU8 and PMMA modified nanohole array. (a) top-down view and (b) cross-section. Yellow part stands for Au surface, grey one is SU-8 layer. Purple areas are PMMA.

7.2.2 Result and Discussion

Figure 7. 9 The comparison spectra of three exposures, (a) 20s, (b) 40s and (c) 60s. In (a) (b) (c), black curves correspond to the spectra of nanohole array, red curves after SU8 coverage, blue curves after exposure and developing

For SU8 modified gold nanohole array, Figure 7. 9 was the comparison of the three chips of different exposure time, 20s (Figure 7. 9a), 40s (Figure 7. 9b), 60s (Figure 7. 9c). Black curves were the spectra of the nanohole array; Red curves were measured after spin coating 120nm SU8 and the blue ones were gotten after exposure and developing. All the peaks of α mode in black curves were not sharp of three chips. For the chip exposed 20s, the bare nanohole array absorption peak of α mode was located at around 573nm. It red-shifted to 601nm because the environment just beside the nanohole array changed from air to SU8, whose refractive index (RI) is larger than air. After exposure and developing, α peak shifted back to the previous wavelength. It meant that the environment beside the nanohole array after exposure was the same as bare nanohole array. So the exposure time was not long enough for photo-polymerization. The changes of 40s chip was similar to 20s one, peak from 569nm to 610nm back to 570nm. When the chip was illuminated for 60s, α peak changed from 569nm to 615nm then to 581nm, 12nm deviation from the black curve and 34nm red-shift from the red curve. α peak shifts in the blue curve from black and red ones demonstrated that the environment around the chip after 60s exposure was different from the bare nanohole array or wholly covered by SU8. Since nothing except SU8 was involved, the change of the peak only can be explained that the nanohole array was partly covered by SU8, only the edge part of each unit.

Normally, the SU8 was polymerized under deep UV (DUV) light. A broad band less than 350nm of DUV can induce its photo-polymerization. One photon in this range of light can lead to one reaction to form single crosslinker. But its chain almost didn't crosslink under visible and longer wavelength light. The developer cannot dissolve the crosslinked SU8 part but the uncrosslinked chain. The result that some part was still covered by SU8 after developing demonstrated that enhanced multi-photon polymerization must happen under longer wavelength light in my experiment.

For PMMA modification, the Figure 7. 10 compared three conditions. α peak after exposure and developing red-shifted 36nm to that of the bare nanohole array and blue-shifted 17nm to that of the array wholly covered with PMMA. After spin coating PMMA, the red-shift was similarly due to the RI change. But in the Figure 7. 10b, peak shifts differed from those in the Figure 7. 10a for the different light used, 50nm to the black curve's and 7nm to the red one's. The deviations of the blue curve's peak from both

black and red curves' peaks also can be attributed to the partial coverage. And according to the relationship between the peak wavelength and shift and the RI, the nearer α peak of the blue curve to that of one's curve, the more similar the coverage situation to that. It demonstrated that more part of the chip in the Figure 7. 10b was covered by PMMA than that in the Figure 7. 10a.

Figure 7. 10 The comparison spectra of three condition, (a) illuminated by light longer than 550nm for 1min, (b) illuminated by single wavelength (603nm) light for 10min and (c) not exposed under light but going through the rest steps. In (a) (b) (c), black curves correspond to the spectra of nanohole array, red curves after PMMA coverage. Blue curves in (a) and (b) were measured after exposure developing, and in (c) was measured after developing

The absorption light of PMMA fell in the range of less than 300nm around, centered at around 200nm. The reaction in PMMA after exposure was opposite to that in SU8, the chains of PMMA breaking other than crosslinking. And only the breaking chain can be dissolved by PMMA developer. In the experiments, the chain broke when irradiated by longer wavelength light. In the second experiments, the wavelength 603nm was the middle of the peak position before and after spin coating one layer of PMMA. This wavelength was around three times of PMMA absorption light. Thus, the scission of

PMMA chain was proved to be induced by three photons. While in the first experiment, two or more photons must involve in one point scission reaction of the PPMA chain.

The third chip which comparison spectra was plotted in Figure 7. 10c went through all the other steps except exposure. The blue curve resembled the red one in peak position and curve shape. The result ruled out other steps influence, especially developing.

The result in Figure 7. 10c and Figure 7. 10a and b prove matched the hypothetical schemes of SU8 and PMMA coverage on the nanohole array. In Figure 7. 10c, a peak red-shift to that of the black one was only 12nm, accounting for 26.08% of the total shift when it was wholly covered by SU8 (46nm red curve to black curve). While the percentage were 67.92% (36nm to 53nm) and 87.72% (52nm to 57nm) in Figure 7. 10a and b respectively. It matched with the scheme in principle that SU8 can only cover the very narrow edge part of the nanohole while PMMA covered rest part of the nanohole array. Although the relationship between peak position change and the energy and materials was not very clear and the percentage was not definitely liner to the coverage, the result was side evidence, to some extent.

7.2.3 Conclusion

In addition, to further improve the sensitivity of LSPR, we selectively modified the nanohole edge through plasmon induced photo-polymerization or degradation of SU8 or PMMA. This method allows us selectively employ the hot spot (i.e. the strong electromagnetic field) for molecule binding and fluorescence excitation. The extinction spectra demonstrated that the surfaces of nanohole array were successfully modified by SU8 or PMMA. Next step of this work, we will focus on characterize the surface of the modified nanohole array. If SEM and AFM cannot clearly display the surface profile, we may try to analyte the composition of surface. Indirect measurement with UV-vis could indicated the α -mode and β -mode changes upon the selective modification of SU8 or PMMA, which gives the information where the polymer was functionalized, as well for monitoring of molecular interactions. The selectively modified gold nanohole surface can be further functionalized with thiol terminated molecules and APTES for bare gold and SU-8, respectively. The PSA antibody will be immobilized for further PSA detection either with label-free or fluorescence measurement. The displacement of extinction peak

position means that the surface environment change. Unmodified nanohole array will serve as control for the indirect comparing experiments. After the surface is successful modified, it will be carried out for PSA detection.

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Chapter 8

Research Summary, Statements of Hypotheses and Outlooks

The summaries of all results are restated in this chapter. The statements accounting for the original hypothesis are listed here .The chapter ends with the outlooks.

8.1 Research summary

In this thesis, I first explained how we designed two kinds of PAuNH array and AuNH array to cover the excitation range of the fluorescent dye by α plasmonic mode (two modes are able to be excited on the Au nanohole array surface, α mode and β mode) according to the simulations. Both of PAuNH array and AuNH array were characterized to display homogeneously and periodically distributed nanoholes. The AuNH array performed two times better fluorescent enhancement than the PAuNH array. The collective effects of LSP and PSP were simulated to significantly enhance the field intensity around the nanohole which allowed for improved fluorescence enhancement as compared with geometries where only individual PSP or LSP modes interacted with an emitter. The augment in enhancement was experimentally verified that the investigation on the fluorescence measurement upon the binding of SA647 on AuNH substrate revealed a fluorescence enhancement of about 10^2 as compared to a flat Au surface irradiated off-resonance.

The sensitivity and efficiency of a biosensor are not only hindered by individual factors of signal mechanism. The binding kinetics is critical to a biosensor in another way that the affinity and association and dissociation equilibrium decide the amount of analyte creating the signal and the consumed time. The applied external electric field (set according to the electric polarization of the analyte) is capable to increase the interaction frequency and probability in order to reduce the saturation time and improve the signal, as well as lessen nonspecific binding of the molecules with the same electric polarization. By connecting the AuNH array substrate to direct current electric source, an electro-focusing-enhanced plasmonic sensor was employed for the detection of cTnI in buffer, BSA solution and rabbit serum. The LOD in buffer can be improved by 2 orders of magnitude upon changing from no bias to -0.2 V bias applied on the sensor surface. The nonspecific adsorption of serum albumin (*i.e.* BSA) and rabbit serum was also reduced at -0.2 V. Lack of tedious sample purification and pretreatment facilitates this method to be an effective platform for highly sensitive detection of biomolecules in complex fluids.

In spite of the high sensitivity and strong selectivity, portability and robustness of a biosensor are attracting more attentions recently since daily health care and field and onsite detection tools brought more interests as supplements and preliminary detecting

methods. On the basis of PSPR excited on grating Au thin film, we built a point-of-care device for LPS, also known as endotoxin which was measured by smartphone and taken no use of external accessories but only a 3D-printed plastic holder. The measurable range of the smartphone spectrometer we designed was around 430-650nm which included the key range of reflectivity spectra of the grating Au substrate. Based on monitoring the resonant wavelength shift, the results showed the feasibility for the detection of LPS at detection limit of 32 ng/ml. The system showed promising for the detection of other biomolecules as the conventional SPR system did. Therefore, the smartphone SPR spectrometer with small, low-cost merits is expected to be widely promoted in near future.

8.2 Statements of Hypotheses

Statement 1: Polarization/biasing of the AuNH array offers both improved sensitivity and selectivity.

From the work in chapter 4, the LOD in buffer can be improved by 2 orders of magnitude upon changing from no bias to -0.2 V bias applied on the sensor surface. Meanwhile, the nonspecific adsorption of serum albumin (*i.e.* BSA) and rabbit serum was also reduced at -0.2 V. Thus, an externally applied electric field improved the mass transfer kinetics of a charged target by tuning the electric field in a favorable direction. Simultaneously it can repel species of opposite charge. Setting the electric potential of the substrate opposite to the target (cTnI) it can be selectively attracted to the surface whereas other interfering molecules of opposite charge to cTnI will be repelled. The first hypothesis was supported. The sensitivity and selectivity can be further improved by further tuning down the polarization/biasing of the AuNH array.

Statement 2: Properly designed synthetic peptides can replace antibodies in assays for sensitive and selective detection of biomedical targets in demanding environments.

In chapter 4 and 5, the properly designed synthetic peptides played significant role. The specific peptide for cTnI in chapter 4 survived from the external bias and stayed stable on the substrate surface and performed good affinity to the target. The specific peptide for LPS also displayed strong stability over antibodies and selectivity to the target, which contributed to the sensor to work in demanding environments. They are cheaper, experimentally easy to be immobilized on the substrates and stored. These results are in

favor of Hypothesis 2.

Statement 3: The combination of LSP and PSP gives rise to an additional enhancement to the fluorescent signal resulting in improved sensitivity.

Both the simulation and the experimental results in chapter 6 support the hypothesis 3 that the collective effects of LSP and PSP significantly enhanced the field intensity around the nanohole which allowed for improved fluorescence enhancement as compared with geometries where only individual PSP or LSP modes interacted with an emitter.

Statement 4: The smartphone in conjunction with synthetic peptides provide an attractive avenue for detection of biomedically relevant targets on site.

The work in chapter 5 illustrated a smartphone based point-of-care assay for LPS, which gave a LOD of 32ng/mL. The usage of smartphone with a built-in LED light source together with the detector of the phone exempted the external components but only a light plastic holder. The synthetic peptides as mentioned in statement 2 performed good affinity to the target. The combination of the smartphone and synthetic peptides indeed provided an attractive avenue for biomedical assay on site, which greatly supported the fourth hypothesis. However, some works can be done to improve the selectivity and the development of a software to analyze data directly by smartphone is in great need.

8.3 Outlooks

Some of the future directions related to my works are listed below:

1. Although the fabrication procedure of Au NH array has a lot of advantages, it can still be improved to increase the quality of the AuNH array for larger bias to be applied.
2. The corresponding software for data analyzing directly on the smartphone can make it more convenient for assay on site.
3. The simultaneous excitation of LSP and PSP on metallic thin film with other kinds of distributed nanostructures can be studied in the same way in chapter 6, which may give better enhancement than AuNH array.

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