DESIGN AND CHARACTERIZATION OF COLORIMETRIC PLASMONIC
NANOSTRUCTURES FOR IMAGING AND SENSING

BY
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DISSERTATION

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ABSTRACT

Increasing demand for early disease diagnostic techniques has attracted huge interest in plasmon-based optical sensors, which can detect small concentrations of chemical and bio-analytes that are not detectable by the conventional analytic optical tools. Advances in nanofabrication techniques have driven in-depth understanding of plasmons, which result from the interactions between nano-materials and the electromagnetic fields. Precisely designing and controlling unique optical properties of plasmons have shown better sensing limits than the conventional ones by amplifying optical signals as well as detecting sensitive plasmon resonance shift upon dielectric property change on the sensing surface.

In this dissertation, a series of experiments are undertaken using a colorimetric plasmonic nanocup array substrate with a single extraordinary optical transmission peak in the visible light range. Sensitive colorimetric sensing is demonstrated by detecting transmission peak shift upon the molecular adsorption or the dielectric property change on the surface. The surface modification of the plasmonic substrate using plasmonic metallic NPs is attempted in order to maximize the plasmonic sensitivity to the refractive index change through heterogeneous plasmon coupling. The plasmon coupling between the plasmons of NPs and nanostructure results in strong localized electric field and denser hot-spot formation; hence, the sensitivity is enhanced. Sensitive detections of specific bioanalytes that undergo antigen-antibody binding as well as bulk refractive index change
are detected through a plasmonic dark mode shift, resulted from the heterogeneous plasmon coupling.

Optical near-field interactions among plasmons, fluorophores, chromophores, and molecules are studied in order to amplify weak fluorescence, absorbance, and Raman signals from a small number of target molecules. Strong scattering field and large scattering cross-section at the plasmon resonance wavelength are the main factors for amplifying fluorescence, absorbance, and Raman scattering. Tuning plasmon resonance to target molecular optical characteristic wavelengths is critical in each application. The amplification of fluorescence is achieved by matching the plasmon resonance with the fluorescence emission band. The absorbance from chromophores, which are involved in conventional immunoassays, is enhanced by matching the chromophores’ absorbance peak with the plasmon resonance wavelength. The improved surface enhanced Raman scattering is accomplished by tuning the plasmon resonance to be close to the laser excitation wavelength. Understanding the signal amplification mechanisms from these results achieves two orders of magnitude lower limit-of-detection as well as improved sensitivity and signal-to-noise ratio. The colorimetric sensor, which is capable of enhancing fluorescence, absorbance and Raman signals from the nearby molecules, provides a versatile multifunctional sensing platform for chemical, biomedical, and environmental monitoring.
To my family
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>NP</td>
<td>nanoparticle</td>
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<tr>
<td>EOT</td>
<td>extraordinary optical transmission</td>
</tr>
<tr>
<td>MEF</td>
<td>metal-enhanced fluorescence</td>
</tr>
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<td>SERS</td>
<td>surface enhanced Raman scattering</td>
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<tr>
<td>FDTD</td>
<td>finite-difference time-domain</td>
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<tr>
<td>nanoLCA</td>
<td>nanoscale Lycurgus cup array</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>RIU</td>
<td>refractive index unit</td>
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<tr>
<td>LSPR</td>
<td>localized surface plasmon resonance</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscope</td>
</tr>
<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
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<tr>
<td>Q factor</td>
<td>quality factor</td>
</tr>
<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
</tr>
<tr>
<td>NP-nanoLCA</td>
<td>nanoparticle-assembled nanoscale Lycurgus cup array</td>
</tr>
<tr>
<td>SAM</td>
<td>self-assembled monolayer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>MCH</td>
<td>6-mercapto-1-hexanol</td>
</tr>
<tr>
<td>HSV</td>
<td>hue-saturation-value</td>
</tr>
<tr>
<td>LOD</td>
<td>limit-of-detection</td>
</tr>
<tr>
<td>HEX</td>
<td>hexachloro-fluorescein</td>
</tr>
<tr>
<td>TEX</td>
<td>sulforhodamine 101 acid chloride</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethylrhodamine</td>
</tr>
<tr>
<td>FLIM</td>
<td>fluorescence Lifetime Imaging</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>EF</td>
<td>enhancement factor</td>
</tr>
<tr>
<td>R6G</td>
<td>rhodamine 6G</td>
</tr>
<tr>
<td>BPE</td>
<td>trans-1,2-bis(4-bipyridyl)ethylene</td>
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CHAPTER 1
INTRODUCTION

As early diagnoses of diseases critically influence the survival chance or the cure rate, lowering the detection limit and improving the sensitivity are the primary demands for the next-generation sensors. In addition, a development of cost-effective and user-friendly diagnostic tools has become more critical with growing recognition of the resource-constrained underdeveloped environment in the world. In light of this interest, colorimetric sensors with high sensitivity became popular for eliminating bulky and expensive equipment and for allowing straightforward detections of target analytes through color changes or emergence. Nanoscale materials have drawn huge interest by exhibiting colorimetric properties as well as their unique optical properties. Strong light absorption and scattering at the surface plasmon resonance (SPR) wavelength allow the metallic NP (NP) solutions or metallic nanostructure to show visible color to the naked eyes. The light scattering, extinction, and absorption properties are dependent on the morphology of the NPs or the nanostructures; thus, engineering efforts have been placed on precise fabrications of different sizes and shapes of NPs or geometries of nanostructures in order to maximize the dynamic range of color change.

Even though these plasmonic substrates were used for label-free detections based on colorimetric properties, the use of fluorophores and chromophores in biomedical and chemical fields still plays a key role in identifying the target analytes in complex media. The local electric field confinement and strong scattering properties of nanoscale metals are capable of amplifying optical signals from chemicals (e.g., fluorescence, absorbance
from chromophores, and Raman scattering. Quantum efficiency enhancement from increased radiative decay rate from an outcoupling of surface plasmons into photons enables fluorescence enhancement. Strong scattering field near the surface plasmon provides more photons that are absorbable by the nearby chromophores and molecules; thus, absorbance and Raman scattering enhancement is achieved. The primary design rule of the plasmonic structure for fluorescence, absorbance and Raman scattering enhancement is to control the degree of scattering field intensity, the scattering peak wavelength, and the density of “hot spots” on the metallic surface.

This dissertation consists of five main topics: (1) fabrication of a colorimetric plasmonic nanostructures, (2) a modification of colorimetric substrate for the refractometric sensitivity improvement, (3) the plasmonic effect on fluorescence enhancement, (4) the plasmonic effect on absorbance enhancement, and (5) the plasmonic effect on Raman scattering enhancement. Before moving on to each topic, the general properties of the surface plasmons, the fluorescence enhancement mechanisms and the Raman signal enhancement by surface plasmons are covered in this chapter.

1.1 Overview of surface plasmons for colorimetric sensing

1.1.1 Colloidal NPs for colorimetric sensing

Colorimetric properties using plasmonic metallic particles have been known for centuries. One of famous plasmon-based products is the Lycurgus Cup using gold NPs in the fourth century A.D. Since then, controlled and precise fabrications of colloidal NPs have been developed, while theoretical studies started to establish from the early twentieth century. Solving Maxwell’s equation on a nanosphere, so-called Mie theory, revealed that
the scattering of light is mostly determined by the radius of a sphere. Many colloidal NPs show absorbance peaks as a result of the plasmons developed on the surface of NPs.\textsuperscript{2–4} Multiple plasmon resonance modes appear depending on the size and shape of the NP and their orientation to the incident light. For example, nanorods show two distinctive plasmon resonances, which correspond to a transverse and a longitudinal oscillation of free electrons.\textsuperscript{5} Each plasmonic mode sometimes shows different sensitivity to the surrounding dielectric property changes.\textsuperscript{6}

A precise study of plasmonic properties by changing the morphology and the orientation of the NPs has been conducted. For the morphology control, the techniques for synthesizing uniform NP shapes became critical in NP-based plasmon studies. Mock \textit{et al.}\textsuperscript{7} demonstrated red, green and blue Ag NPs with different particle shapes by nucleating silver salts on gold NPs (Figure 1.1). Typically the triangular NPs show redder color than the spherical NPs of the same size. Regardless of shape, larger NP sizes resulted in larger plasmon resonance wavelengths.\textsuperscript{8,9}

The effect of the NPs’ orientation to the incident light was studied mostly by patterning the NPs on a flat surface using electron-beam lithography.\textsuperscript{10} The orientation of the NP dimers to the polarization direction of the incident light determines the direction of plasmon resonance peak shift from an isolated NP’s resonance wavelength. When the dimers are oriented parallel to the light polarization direction, the resonance wavelength red-shifts from that of a single particle; however, the dimers, arranged perpendicular to the polarization direction, result in the blue-shift of the resonance wavelength.\textsuperscript{11,12} This is mainly because the restoring force of the electronic oscillation in each NP changes with the
light polarization directions.\textsuperscript{10} When more than two NPs exist in a system by forming an array, the number of NPs in these 1D or 2D arrays affects the resonance wavelength. More NPs in an array typically show a red-shift of the plasmon resonance.\textsuperscript{13}

The morphologies and the orientation of NPs determine the surface plasmon’s optical properties, but the distance between two or more NPs significantly changes the resonance condition. When the distance between two identical nanospheres decreases, the plasmon resonance wavelength red-shifts or a new plasmonic mode emerges.\textsuperscript{14} During the past decades, this idea was adopted to many chemical and biological detection mechanisms. Detections of polynucleotides,\textsuperscript{15} proteins,\textsuperscript{16} antigen-antibody conjugations\textsuperscript{3} and ion concentrations of an ionic solution\textsuperscript{17} have been demonstrated. The term, ‘molecular ruler’, was also developed by utilizing the plasmon resonance condition change upon the distance variation.\textsuperscript{18} Recently, pressure and stress were also measured by this distance-dependent plasmon resonance change, which eventually changed the scattering color.\textsuperscript{19} These results show the versatility and the effectiveness of the use of nanoscale materials to the colorimetric sensing with high sensitivities.

One of the greatest advantages of using bottom-up synthesized NPs is the low-cost in synthesis. However, the use of colloidal NPs suspended in solution has not been attractive for many applications due to some disadvantages of their properties. One of the drawbacks with the colloidal NPs in colorimetric sensing is the irreversible aggregation of NPs.\textsuperscript{16} Larger particles are less stable in solutions and therefore do not preserve the original scattering color after the aggregation. This limits the selection in the sizes of NPs, which will eventually restrict the dynamic color range. Another critical limitation with
colloidal NPs is the necessity of solvent or buffering media that can make the stable separation among the NPs. Thus, controlling the separation of the particles without the presence of the liquid or solvent is critical to broaden applications of these sensing platforms in ambient air. In addition, sizes and shapes of NPs are not usually perfectly uniform during the bottom-up synthesis. This could discourage the use of colloidal plasmonic NPs when the precise control of the colorimetric properties is required.

1.1.2 Nanostructures for colorimetric sensing

In order to excite surface plasmons on a planar film, the energy momentum should be provided by prism coupling or the grating structure. The nanostructure that will be introduced in this dissertation is based on the grating structures, which do not require a high refractive indexed prism or bulky angle-variable laser equipment (i.e., SPR equipment). Various geometries of the grating on the surface have been previously studied; surface plasmons can be focused using circular gratings\textsuperscript{20,21} or surface plasmons can be guided to a specific direction.\textsuperscript{22,23} Among grating structures, nanohole array structure shows extraordinary optical transmission (EOT) phenomena from an optically thick metallic layer. When the transmission peak is in the visible light range, one can observe the transmission light at a specific wavelength, showing as a color of a substrate. The first observation of the EOT by Ebbesen et al.\textsuperscript{24} was in the near infra-red region. The subsequent studies on EOT substrates reveal that the controls over materials and geometries enable one to make the resonance wavelengths in the visible light range.

The periodicity of nanohole arrays and the size and depth of the hole were varied to identify the effect of these controllable geometrical parameters on the SPR. Among these
geometrical parameters, the periodicity plays the critical role in determining the plasmon resonance wavelength.\textsuperscript{25}

\[
\lambda_{sp}(i,j) = \frac{p}{\sqrt{\varepsilon_d \varepsilon_m + \varepsilon_i + \varepsilon_j}}
\]

\(p\) is the periodicity, \(i\) and \(j\) represent the scattering order of the array, \(\varepsilon_d\) and \(\varepsilon_m\) stand for the dielectric functions of the dielectric and the metal.\textsuperscript{25} When comparing with a single hole in a film, the transmission intensity from the nanohole arrays is larger.\textsuperscript{26} The hole diameter in an array determines the wavelength and the intensity of the transmission. When the hole diameter decreases, both transmission intensity and the transmission wavelength become smaller.\textsuperscript{27}

The materials selected for the EOT structure have been metals such as gold,\textsuperscript{28,29} silver,\textsuperscript{30} copper\textsuperscript{31} and aluminum.\textsuperscript{32} For the colorimetric sensing, gold and silver have been the most preferred materials due to their bulk plasmon resonance wavelengths residing in the visible light range. Recently, researchers showed that aluminum nanohole arrays, having EOT in the near ultra-violet light region, shows larger dynamic range in the visible light range,\textsuperscript{33} as the SPR wavelength red-shifts with increasing dielectric constants. Ag-Au bimetallic or alloy nanohole arrays have been also studied.\textsuperscript{34} Continuous efforts on finding optimal plasmon resonance condition will be placed on engineering a variety of materials properties as well as the structure geometries.

The hole shape also influences the plasmon resonance conditions for the EOT. When the hole is a simple cylindrical shape in a sub-wavelength size, multiple transmission peaks develop in the visible to near infra-red regions. Each peak represents
the surface plasmons excited at different materials interfaces (e.g., metal-dielectric substrate, metal-dielectric superstrate) and plasmonic modes of different array orders. Controlling the geometries of holes or the materials can excite additional plasmonic modes or suppress some or them. The sensitivity of the plasmon resonance response to the dielectric change is also affected by the geometries of holes. When the shape of the hole is asymmetric, for example, different plasmonic modes are excited in different directions; thus, the polarization dependent optical properties are found. In addition, the sensitivities to a refractive index change from these asymmetric hole shapes were reported to be better than the circular hole arrays.

### 1.1.3 Fabrication of nanostructures

Several fabrication methods have been attempted to produce nanostructures including nanohole arrays. Electron beam lithography and focused ion beam are the commonly adopted processes for nanostructure fabrications, but high costs and low productivity of these technologies prevent scaling up the substrate. Nanosphere lithography is often adopted as an alternative, but many have experienced non-uniformity in a large wafer-scale and most resulting geometries are restricted to hexagonal arrays. One of the most cost-effective methods is a laser interference lithography with a combination of the replica molding technique. The patterns produced by the laser interference lithography are easily tuned by the laser beam’s angle adjustment and the use of filters. The patterns are generated without masks; so, better uniformity and precision of patterns are achieved when compared to the conventional photolithography techniques. The replica molding process utilizes a mold, which is typically fabricated by the lithography procedure. Infinitely many substrates can be replicated as long as the mold is not damaged and thus
the fabrication cost is tremendously reduced. In this dissertation, a colorimetric substrate is fabricated by replica molding process with a mold that was produced by the laser interference lithography. The fabrication process will be covered in detail in Chapter 2.

1.2 Fluorescence enhancement by plasmons

The use of fluorophores in bio- and chemical fields still plays a key role in identifying the target molecules or organisms by exceptionally easy identification with conventional microscopes. Despite their usefulness, the organic fluorophores have critical disadvantages of photobleaching and its high volume requirement to be detected under the conventional microscope.\textsuperscript{45–47} As a result, the enhancement of the fluorescence intensity with a prolonged lifetime has become critical for the optical detection of bio-molecules as well as targeted chemical activities. When the metal-enhanced fluorescence (MEF) phenomena was revealed, the dependence of the fluorophore’s distance to the metallic surface and the influence of geometrical factors of the metallic NPs or nanostructures on fluorescence have been extensively investigated.\textsuperscript{48–50}

1.2.1 Parameters affecting fluorescence

There are many parameters that affect the MEF. The typical parameters are the radiative and non-radiative decay rate, quantum yield and lifetime. The relationships among these parameters are shown below:\textsuperscript{51}

\[
\eta = \frac{k_r}{k_r + k_{nr}} \quad (2)
\]

\[
\tau = \frac{1}{k_r + k_{nr}} \quad (3)
\]
\[ k_r = \frac{\eta}{\tau} \]  

\( \eta \) is the quantum yield, \( k_r \) and \( k_{nr} \) are the radiative and the non-radiative decay rate, and \( \tau \) is the lifetime. The quantum yield increases when the radiative decay rate increases and the non-radiative decay rate decreases. The increase in the radiative decay rate usually involves the decrease in the lifetime.

### 1.2.2 Metal-enhanced fluorescence

When a fluorophore is near a metallic surface, additional radiative decay rate term by metal is added to \( k_r \) term. The radiative decay rate typically increases by the presence of metal; therefore, the lifetime decreases and the photostability of a fluorophore is improved.\(^{52} \) The metallic surface can be considered as a mirror, reflecting the incoming light. The local photonic mode density is changed due to this reflected electromagnetic field.\(^{53,54} \) The reflected light excites the fluorophores and thus higher chance of fluorescence emission is resulted. The reflection from the metallic surface, however, is not the only factor for a fluorescence enhancement. Larger scattering cross section of metals than that of fluorophores is considered a main factor for the fluorescence enhancement. In addition, strong scattering field by a surface plasmon excitation on the metallic surface affects the excitation and the emission efficiency of a fluorophore. A gold bowtie antenna, for example, achieves an enhancement factor of 1,340 by improving light absorption and the radiative decay rate.\(^{55} \) The EOT structure, which can transmit electromagnetic field through the holes, also shows a fluorescence enhancement by effectively exciting the fluorophore with a localized electric field on the surface.\(^{56} \) Various hole diameters and
periodicities of the array have been tested in order to find an optimum design for larger fluorescence enhancement.\textsuperscript{50}

The surface plasmons excited on a metallic NP or a nanostructure affect the shape of the fluorescence emission spectra as well. Ringler \textit{et al.}\textsuperscript{57} showed dramatic fluorescence spectral shape change when using dimers with different spacing between them. A numerical calculation verified that the scattering light from the metallic surface influences the emission of the fluorescence. Chen \textit{et al.}\textsuperscript{58} reported that the SPR peak, which is responsible for the strong light scattering, should be $\sim$40 meV higher than the fluorescence emission peak in order to have the maximum fluorescence enhancement. As the plasmon resonance wavelength should overlap with the fluorescence absorption or emission spectra for a fluorescence enhancement, a precise tuning of the plasmon resonance by varying the size, shape, and geometry of NPs or nanostructures is required to maximize the fluorescence enhancement factor.

1.2.3 \textbf{Fluorescence quenching by plasmons}

On the other hand, the fluorescence is sometimes quenched when a fluorophore is too close to a metallic NP or nanostructure. When fluorophores and plasmons are in close proximity and when the fluorescence emission wavelength is matched with the plasmonic absorption band, fluorescence quenching occurs. This process is typically called a surface-energy-transfer.\textsuperscript{59} Dulkeith \textit{et al.}\textsuperscript{60} showed that the non-radiative decay rate near the metal increases by $\sim 10^9$/sec within a 4 nm region from the NP surface, while the radiative decay rate decreases to $10^7$/sec. Various shapes and sizes of the NP have different fluorescence quenching distances between the fluorophore and the metallic surface.\textsuperscript{61} Depending on the
distance, the radiative and non-radiative decay rates compete and determine whether the fluorescence will be quenched or enhanced.

1.3 **Surface enhanced Raman scattering (SERS)**

Highly localized electric or magnetic field near the surface of metals has driven the development of spectroscopic tools for dark field imaging or enhanced Raman scattering. Raman scattering, which traces unique molecular vibrational modes for different molecules, helps identify and differentiate molecules of interest in a complex medium. Since the cross-sections of Raman scattering from molecules are much smaller than those of fluorophores, the use of metals is critical for the Raman scattering enhancement by increasing the scattering cross-section. The cross sections of the normal Raman scattering are $10^{-14}$-fold smaller than those of fluorophores, which cross-sections are typically around $10^{-16}$ cm$^2$ per molecules. The enhancement of these optical signals near the plasmonic surface allows detection of a small number of target analytes and overcomes the detection limit with less laser power. As the surface plasmonic properties determine the enhancement factor, designing and fabricating such plasmonic substrate structures have become extremely important in SERS technology. A careful selection of substrate materials as well as a geometrical design of such substrates is required. Furthermore, a cost-effective fabrication method for a mass-production of wafer-scale SERS substrate is in demand.

1.3.1 **Physical explanations for SERS**

Two physical explanations for silver or gold NP based SERS have been proposed: the plasma resonance model based on the electromagnetic theory and the charge transfer
processes at a so-called active site on a metallic surface. The first theory explains that the Raman enhancement is attributed to the electromagnetic interaction between molecules and a metal, which is considered as a polarizable body. This supports different SERS enhancement factors for various shapes of a metal. The latter theory explains that the metal-molecule active site interaction enhances the chemical’s optical signal through the molecule-metal charge transfer processes. The active site theory, however, only considers a monolayer of molecules adsorbed on the surface of the metal. This is contrary to the electromagnetic theory, which can support the enhancement of the Raman signals from molecules present in more than 10 Å from the metallic surface. As many SERS studies have reported ultrasensitive detections of molecules with enhancement factors of up to $10^{14}$, the contribution from the enhanced electromagnetic field is much more significant for SERS detection than the charge-transfer mechanisms.

### 1.3.2 Materials for SERS substrates

In the Drude model, the dielectric function of metal is given in Equation (5),

$$\varepsilon = \varepsilon_b + 1 - \frac{\omega_p^2}{\omega^2 + i\omega\gamma}$$  \hspace{1cm} (5)

$\varepsilon_b$ is interband transition contribution to the dielectric function, $\omega_p$ is the plasma resonance frequency and $\gamma$ is the electronic scattering rate, which is inversely proportional to the conductivity of the metal. Considering the polarizability of a metallic sphere with dielectric constant $\varepsilon(\lambda)$ given in Equation (6), the modified polarizability combined with Equation (5) is given in Equation (7).
\[
\alpha = R^3 \frac{\varepsilon - 1}{\varepsilon + 2}
\]  

(6)

\[
\alpha = \frac{R^3(\varepsilon_b \omega^2 - \omega_p^2) + i \omega \gamma \varepsilon_b}{(\varepsilon_b + 3)\omega^2 - \omega_p^2 + i \omega \gamma (\varepsilon_b + 3)}
\]  

(7)

The width of the resonance is given as \(\gamma(\varepsilon_b + 3)\), which is responsible for the strong interband optical absorption and damping of the plasmonic oscillation in the metal. Metals with a large \(\varepsilon_b\) in its dielectric function exhibit low conductivity as well as larger width of the plasmon resonance peak, resulting in a reduced SERS enhancement. Materials such as transition metals with the inter-band transitions in the visible light range, thus, become the worst choices for the SERS materials. In addition, this explains why silver shows better SERS performance than gold and copper.  

1.3.3 SERS substrate design

The key component for SERS is the metallic NPs or nanostructures with surface plasmonic effects. The size and shape of the metallic NPs and geometries of nanostructures determine the SERS performance. As SERS is mainly dependent on the fourth power of the local electric field intensity, designing metallic substrates to maximize the local electric field is most critical in order to lower the detectable analyte concentration; down to multiple or single-molecular levels. In addition to the electric field intensity, the number density of NPs or plasmons in a sensing platform also determines the degree of Raman scattering enhancement. Lastly, an ideal SERS substrate should allow molecules of interest to be placed right at the “hot spots” where the electric field is maximized. Lee et al. found that a factor of more than 100 has achieved by making the analytes accessible to the gap between nanowires.
1.4 Overview of thesis

This dissertation will focus on fabricating a colorimetric plasmonic substrate with the EOT properties. The unique optical properties of the substrate will be studied using a finite-difference time-domain (FDTD) simulation, which will also be used for improving sensitivity. A new substrate design for a better colorimetric sensing is proposed in Chapter 3 based on numerical calculations by using FDTD simulations. The application of the new substrate design to the existing colorimetric sensing platform is performed and detailed optical property changes are discussed in Chapter 4. The fluorescence and absorbance enhancements on the plasmonic substrates are discussed in Chapter 5 and Chapter 6. Making full use of the advantages of the colorimetric substrate is demonstrated for maximizing fluorescence and absorbance enhancements; the plasmon resonance wavelength is easily tuned to match the molecular optical characteristic wavelengths by controlling the surrounding refractive index. The absorbance enhancement on the colorimetric substrate demonstrates improved limit-of-detection and sensitivity, particularly in a low target analyte concentration regime that is not detectable by conventional immunoassays. Finally, in Chapter 7, a surface modification of the colorimetric substrate will provide improved SERS activity with a high hot-spot density and a strong electric field confinement on the sensing surface.
1.5 Figures

Figure 1.1 Optical properties with different shapes and sizes. Adapted from [Mock et al., 2002].
CHAPTER 2
COLORIMETRIC SENSING THROUGH NANOPLASMONIC SUBSTRATE

This chapter introduces a colorimetric substrate, which is used as a refractive index sensor, fluorescence and absorbance amplifier, and a surface enhancement Raman scattering (SERS) substrate. This chapter primarily focuses on the fabrication and the optical characterization of the substrate. The content is reproduced with permission. Copyright 2013, Wiley-VCH.

2.1 Introduction

A nanoscale Lycurgus cup array (nanoLCA) substrate was developed as a colorimetric bio- and chemical sensor, which is based on detections of dielectric property change near the metallic surface. It has similar characteristics of the Lycurgus cup created by ancient Romans, which transmits green color but reflects orange-red color in air (Figure 2.1a). The EOT is observed from the nanoLCA by exhibiting strong scattering electric fields at the edge of the cup. The strong field comes from the surface plasmon excited by the periodic structure of each nanocup; therefore, the surface plasmon resonance property change due to the surrounding dielectric change results in the changes in the EOT wavelength. This enables one to achieve colorimetric detections of target analytes that present in a bulk medium or are adsorbed on the surface of the nanoLCA.

Many EOT structures show extraordinary transmission at specific wavelengths and most structures show the multiple peaks in the visible light range and in the near infra-red region. Unlike many grating-based EOT structures (e.g., periodic nanohole arrays), the
nanoLCA shows a single prominent transmission peak, which is the SPR wavelength. The precise control over the geometrical parameters of the periodic nanocup structure is the main factor that determines the position and the number of the EOT peak (or SPR wavelength). We employed the FDTD method in order to theoretically calculate and estimate the desired geometry for achieving a single EOT wavelength. When a single transmission peak is observed rather than multiple peaks, the changes in the SPR properties can be directly correlated to the transmission color or the spectra. Furthermore, in comparison to the case of colorimetric structure with multiple EOT peaks, the same SPR shift shows higher contrast in transmitted color change when a single EOT peak is present.

2.2 Materials and Methods

2.2.1 Fabrication of the nanoLCA

A replica molding technique was adopted to fabricate the nanoLCA by using a mold, composed of 500 nm-tall and 180 nm-diameter nanopillar arrays with a periodicity of 350 nm. The pattern was replicated by using an ultraviolet (UV)-curable polymer (NOA 61). After curing the polymer by exposing UV light for 1 min, the polymeric nanocup arrays were produced. After depositing 9 nm Ti and 90 nm Au or Ag on this pattern, top and bottom metallic films and sidewall NPs are formed on the nanocup array structure (Figure 2.1b).

2.2.2 FDTD simulations

The three-dimensional (3D) FDTD analysis was performed using a commercial software package (FDTD solutions, Lumerical Solutions, Inc. Vancouver, Canada). A plane wave with normal incidence angle, polarized in x-direction and propagating in +z
direction was used for exciting the surface plasmon on the nanoLCA. A uniform mesh is overrideed on the structure with the mesh size of 1 nm (x, y, z-directions). The dielectric properties of Ag in the spectral range from 300 nm to 1100 nm were taken from Palik. In order to avoid wrong calculations affected by any light reflection at the z-boundaries, perfectly matched layers were imposed at +z and −z boundaries normal to the light propagation directions. The periodic boundary conditions were used at the x and y-boundaries.

2.3 Results and Discussion

2.3.1 Optical properties of the nanoLCA substrate

The Ag nanoLCA substrate achieves a single prominent resonance in the refractive index range from 1 refractive index unit (RIU) to 1.5 RIU. The resonance wavelength red-shifts with increasing surrounding refractive index, resulting in transmitting redder color as shown in Figure 2.1c. When using Au nanoLCA, the transmission peak wavelength also shifted with gradually increasing concentration of glycerol solutions shows a linear relationship between the resonance wavelength and the refractive index (Figure 2.1e). The sensitivity, defined as the amount of peak shift per unit refractive index change, is 202 nm/RIU for bulk glycerol concentration detection.

2.3.2 Theoretical studies on the nanoLCA substrate

In order to understand the extraordinary transmission phenomena of the nanoLCA structure with a single EOT peak, we performed 3D FDTD simulations by constructing five different (labeled 1-5 in Figure 2.2a) nanohole shapes. Nanoholes without sidewalls (model 1 and 2) showed multiple transmission peaks, similar to what have observed from
many nanohole arrays. However, when there were discrete metallic layers on the sidewalls (model 4 and 5), similar spectral shapes and transmission peak position to the experimental result were observed. Although the actual nanoLCA had NPs randomly attached on the sidewall unlike the simulation models, the overall spectral results from model 4 and 5 agreed well with the experimental results. This implies that the presence of the metallic layer on the sidewall of the nanocup helps coupling the plasmonic modes excited on the top metallic film (outside of the cup) and the bottom metallic layer of the cup. If the EOT peak were responsible solely for the sidewall metallic structure, the EOT spectra would have been different with different geometries of the metallic structure and the gap between the NPs on the sidewall.

The peak at \( \lambda = 450 \) nm represents the localized surface plasmon resonance (LSPR) mode at the gap between the NPs on the sidewall and at the rim of the nanoLCA. The corresponding variation in the electric field intensity (\(|E|^2\)) is shown in Figure 2.2b. The top (x-y) and the cross-sectional (x-z) views of the electric field distribution show the LSPR mode at the interface between NPs and the sidewall.

2.4 Conclusions

The nanoLCA substrate demonstrates colorimetric properties by showing redder color with increasing surrounding refractive index. The SPR shift proportional to the target analyte’s concentration allows to sense refractive index change colorimetrically. This eventually enables one to find the concentration of an unknown refractive index by calibrating the SPR peak position and the surrounding refractive index. The FDTD simulation shows the electric field confined at the edge and the sidewall of the nanocup.
The sidewall metallic structure plays a key role in suppressing multiple transmission peaks, achieving a single SPR wavelength in the visible light range.
2.5 Figures

Figure 2.1 (a) Schematic of colorimetric properties of the nanocup array structure. (b) Scanning electron microscope (SEM) of the Ag nanoLCA. (c) Color transmitted from Ag nanocup array surface with a variety of solvents. (d) Transmission spectra of Au nanoLCA and (e) their peak shifts with increasing concentration of glycerol solutions (from 0 % to 60 %).
Figure 2.2 (a) FDTD simulated transmission spectra of five different Ag nanocup array structure. All models have 500 nm depth and Ag thickness of 90 nm. (b) Electric field distribution at the resonance wavelength.
CHAPTER 3

VERTICALLY STACKED PLASMONIC NANOPARTICLES IN A CIRCULAR ARRANGEMENT: A KEY TO COLORIMETRIC REFRACTIVE INDEX SENSING

Linear spectral response of a single peak in the visible light range to the surrounding media refractive index variation achieves true colorimetric sensing. FDTD simulations were performed to find an optimal substrate design in order to accomplish a single plasmon resonance wavelength in the visible light range. Design parameters that influence the plasmonic properties were introduced and analyzed in order to achieve true colorimetric sensing. This chapter is reproduced by permission of The Royal Society of Chemistry.  

3.1 Introduction

In this chapter, a new substrate design for a colorimetric environmental sensing is proposed: a tunable plasmonic circular array of NPs on the solid template fabricated by top-down process. A linear relationship between the plasmon resonance peak shift and the refractive index variation of the surrounding media were observed. Even though the particles were not closely spaced in a several nm scale, the circular array of NPs showed a single prominent resonance peak in the visible wavelength and the resonance peak position was easily tuned by varying the substrate refractive index and the morphology of the particle.

In addition, a simple and easy way to achieve a higher signal-to-noise ratio (SNR) is demonstrated by introducing additional vertical plasmon coupling of the circular array.
Achieving a high SNR is critical for improving the detection limit as well as accurately measuring the plasmonic peak shift upon the refractive index change. The use of bimetallic layers was reported as a method of improving SNR.\textsuperscript{73} Many methods for enhancing the SNR attempt to reduce the electronic noise by improving the instruments and the measurement systems,\textsuperscript{74,75} rather than demonstrating the direct way to amplify the optical signals from the surface plasmons. Here, introducing the out-of-plane coupling by simply adding more layers is suggested as an effective way to improve SNR; increasing signal intensity linearly with the number of layers was demonstrated. The enhancement of the smoothness of spectra and the quality factor (Q factor) was also observed with reduced full width at half maximum (FWHM).

3.2 Materials and Methods

Eight hemispheric Ag NPs with 30 nm in diameter attached on the sidewall were modeled using the 3D FDTD simulation (Lumerical Solutions Inc.). Figure 3.1a and Figure 3.1b show a schematic of eight NPs in a circular array. A circular array of eight hemispheres and a toroid structure on the flat polymer substrate with a refractive index of 1.56 were also modeled to see the effect of the discreteness of the metallic circular array on the plasmon resonance, since the toroid structure is considered as an infinite number of hemispheres arranged in circle. The silver dielectric properties were taken from Palik\textsuperscript{70} and the uniform 0.8 nm mesh was overridden on the structure. Periodic boundary conditions with a periodicity of 320 nm in the x- and y- directions were imposed.
3.3 Results and Discussion

3.3.1 Tunable plasmon resonance

We first studied the effect of the inter-particle distance on the optical properties for the conventional square particle (hemispheric NP) array, the circular array and the toroid structure. The distance between hemispheric particles in the circular array was varied by changing the diameter of the whole array (i.e., from 120 nm to 200 nm). The particles were spaced equally for each diameter. Equivalent inter-particle distance was applied to the square periodic array of hemispheric NPs. Figure 3.2 shows the effect of increasing inter-particle distance. All four models consistently showed red-shift of the peak wavelength with decreasing inter-particle distance. The red-shift of peaks with closer particles was also found in dimer LSPR studies.\textsuperscript{13,18} The red-shift of the resonance wavelength of the particles is due to the reduced net dipole power between NPs, which results from the attraction between induced opposite charges accumulating on each end of the particles along the light polarization axis.\textsuperscript{11} More detailed explanation about the SPR red-shift with a reduced inter-particle distance can be found in Chapter 1. The toroid structure from Figure 3.2c also showed a minimal change (a slight blue shift of 2.5 nm) when the diameter of the toroid increased, in contrast to the discrete particle cases (Figure 3.2a (12.5 nm), Figure 3.2b (22.5 nm) and Figure 3.2d (17.5 nm)). Since the toroid structure can be considered as an arrangement of infinitely many hemispheric NPs, there is no localized surface plasmon excited on a discrete NP and hence, almost no spectral change was observed with the variation of the toroid’s diameter. Therefore, the discrete NP-based arrays with LSPR effects (i.e., both circular and square NP arrays) have better flexibility in
tuning the surface plasmon properties by controlling the inter-particle distances than the
toroid structure.

3.3.2 FWHM and Q Factor

The linewidth of the resonance peak was calculated for the square array and the
circular array of NPs on a flat surface and inside a cylindrical hole. Compared to the square
array, the circular arrays of NPs on a flat surface and inside of the hole had relatively
smaller FWHM. The square array had a FWHM of 82.5 nm, whereas the circular arrays on
the flat substrate and inside the hole had a FWHM of 65 nm and 52.5 nm, respectively.
This indicates that the circular arrays show better sensitivity with a higher Q factor; thus, it
is better for an optical sensor than the square arrays.

The lateral in-plane coupling of the LSPR was studied by varying the number of
particles in each circular array in Figure 3.3a. Having four and eight NPs on the sidewall
resulted in a peak position of 470 nm for both, but a circular array with 16 NPs had a red-
shifted peak located at 500 nm. This implies the overall energy was lowered with larger
number of NPs. The linewidth broadening with the 16 NPs was also observed and this is
attributed to extra losses in the metal due to increased surface scattering. The calculated
surface charge distributions associated with the 16 NPs in Figure 3.3b show large effective
dipole moments that align and oscillate in phase. This leads to higher resonant coupling
efficiency and high radiative losses (scattering), thus increasing the linewidth of radiation.
On the other hand, the higher-energy dipolar modes for four and eight NP case in Figure
3.3c and Figure 3.3d showed surface charges that are partially cancelled due to phase-
retardation effects resulting in lower effective dipole moments. The reduced dipole
moment leads to relatively lower resonant coupling efficiency and reduced radiative scattering. Hence, the four and eight NP cases have smaller linewidth compared to the 16 NPs case. In terms of sensor application, having eight hemispheric NPs in an array is preferable due to its smaller FWHM than the 16 hemispheric NP circular array. When we compare the spectrum of the four NP array with that of the eight NP array, the SNR of the four NP case is lower than the latter due to a smaller extinction peak intensity, which is 0.075 compared to the 0.114 extinction value of the eight NP case. Thus, we decided to further study the eight NP circular array for better sensing application.

3.3.3 Multiple layers stacking

Achieving a high SNR of the extinction spectrum is crucial to an actual sensor application. We report that the peak intensity of the circular array is enhanced by vertically stacking circular array layers as shown in Figure 3.4. In Figure 3.4a, the extinction peak wavelength red-shifts from 470 nm with a single layer to 480 nm with triple and quintuple layers of NP array. The triple and quintuple layers have the fixed vertical distance of 100 nm between the centers of each NP array layer. The vertical inter-layer distance effect on the extinction spectra will be explained in Figure 3.4b. In addition, the SNR linearly increases by 4.15-fold for the quintuple layer compared to the single layer, while the FWHM increases only by 1.2-fold. The increase in intensity by stacking multiple layers is due to the induced magnetic field between each layer of NPs. This creates an electric current orthogonal to the magnetic field, resulting in an increase in intensity. Thus, introducing more scattering objects such as silver NPs in the direction of light propagation instead of in the light polarization direction is the key to increasing the signal intensity while retaining most of the optical properties of the original single layer.
Figure 3.4b shows the effect of inter-layer distance for the triple layer case. When the vertical inter-layer’s smallest surface-to-surface distance decreases from 220 nm to 5 nm, the resonance peak at 480 nm blue-shifted to 475 nm. Similarly, the FWHM was decreased by 5 nm or ~10%. Thus, the overall Q factor improved with increased resonance frequency and narrower linewidth of the peak as the inter-layer distance decreased. It was reported that the Q factor of plasmonic resonators is independent of the geometry and dielectric environment of the nanostructure.\textsuperscript{76} In contrast, here we show that the Q factor can be affected by vertically stacking the arrays of nanostructure. The resulting blue-shift of the peak wavelength with vertical coupling is similar to the response of LSPR peak shift with the excitation light polarized orthogonal to the inter-particle axis of linearly arranged two or more NPs.\textsuperscript{11,12} When the incident light is polarized perpendicular to the inter-particle axis, it leads to the accumulation of charges with identical polarity on the opposite sides of the gap. As the identical charges repel each other, the restoring force increases and thus the $\lambda_{\text{LSPR}}$ decreases. On the other hand, the 5 nm blue-shift of the resonance wavelength from the small inter-layer gap distance is a relatively small value when compared to a few tens of nm shift for the in-plane orthogonal coupling between two NPs.\textsuperscript{11} The main reason why resultant response from the NP circular array in nanohole is smaller than the conventional LSPR peak shift driven from two circular NPs with s-polarized light is the non-spherical shape of the NPs on the sidewall. The electric field developed on the hemispheric NP is weaker due to its asymmetry, compared to the perfect spherical NPs. In addition, different interfacial dielectric properties on each end of NP in the direction of light polarization, which are air inside the hole and a high dielectric constant of the substrate, contribute to non-uniform distribution of electric field at each end.
of NP. Therefore, there is less coupling force among electric dipoles, resulting in smaller blue-shift. The circular array in the nanohole, however, did not exhibit broadening of peak or loss in the peak intensity. This indicates that the vertical coupling of the collective resonance mode of particle array effectively confines the field without the loss of Q factor.

We further examined the vertical coupling of the plasmonic mode from each layer by presenting the current density, |J|, and the magnetic field distribution, |H| (Figure 3.4c and Figure 3.4d). The field distributions of two models, small and large inter-layer spacing in the triple layer NP circular arrays, are plotted at each resonance peak wavelength. Figure 3.4c clearly shows the vertical plasmon coupling among the layers, which is absent in Figure 3.4d. The current density and the magnetic field with vertical coupling are no longer uniform across these three circular array layers but concentrated on the top-most layer. The magnetic field is also highly confined in-plane inside the cylindrical hole, which agrees with a higher Q factor for a smaller layer-to-layer separation gap. The charge distribution map (Figure 3.5) shows individual dipole formation on each NP for the model with a large inter-layer displacement; however, single dipole among the three-array stack was formed with a shorter displacement. As larger net electric dipole is built among the layers with shorter inter-layer distance, a larger magnetic field loop across the layers is produced. Similar magnetic field enhancement was reported with nanorod pairs with opposite current directions.77,78

3.3.4 Morphology of NP

The electric field on the NP circular array is mainly confined at the interface between the dielectric cylindrical hole substrate and the silver particle. Figure 3.6a shows
strong electric field (Ez) located at the contact region of the metallic NP with the cylindrical sidewall. A single circular array with eight hemispheric NPs attached inside of the 200 nm cylindrical hole was chosen for this numerical analysis. Since the electric field is mostly confined at the interface between the silver particle and the substrate sidewall, the plasmonic peak wavelength should respond accordingly with the change of the dielectric property of the substrate, which is in contact with the silver NP. The magnitude of the electric field is also dependent on the morphology of the NPs, which in turn defines the contact area.

Figure 3.6b shows the effect of the interfacial area between the NP and the substrate sidewall. The extinction spectra were collected while the substrate hole diameter gradually increased from 85 nm, no NP exposed and existed, to 115 nm, a complete exposure of Ag spherical NP attached on the sidewall. The size of the NP was consistent to be 30 nm in diameter and the center of each NP was fixed in circular arrangement inside the cylindrical hole structure with a refractive index of 1.56 RIU. When the hemispheric NP was exposed to air, a distinguishing extinction peak emerged. The peak wavelength blue-shifted, as a larger portion of the particle was exposed outside of the sidewall, forming less interfacial area. The circular arrangement of perfect spherical NPs had the peak position similar to the Mie scattering peak wavelength (Figure 3.8a).

3.3.5 Sensitivity to environment change

The supporting substrate dielectric property brings additional degree of freedom in tuning the plasmon resonance peak position. The gradual increase of the surrounding refractive index of the dielectric material usually leads the red-shift of the resonance peak
wavelength as single NP study showed.\textsuperscript{79,80} However, the relationship between the plasmonic property and the refractive index of the supporting substrate is scarcely studied. Figure 3.6c shows the map of extinction spectra from eight hemispherical NP circular array by varying surrounding media refractive indices from $n_{med} = 1$ to 1.6 (noted on x-axis) as well as varying the substrate refractive indices from $n_{sub} = 1$ to 1.6. The resonance peak changes from 400 nm to 480 nm when $(n_{sub}, n_{med})$ changes from (1, 1) to (1.6, 1). It should be noted that the circular array of hemispheres does not always exhibit a single resonance peak in the visible range. For example, when the $(n_{sub}, n_{med}) = (1, 1)$, the extinction spectrum becomes similar to the hemisphere’s scattering spectrum (Figure 3.8a) with double peaks due to its asymmetric structure, inducing a multipolar resonance mode.\textsuperscript{81} However, as the substrate refractive index increases ($n_{sub} > 1.4$), the main peak starting from 400 nm red-shifts and it becomes prominent compared to the second peak in the lower wavelength range. Therefore, the substrate dielectric property such as refractive index is one of the key parameters to control the starting resonance peak position and to achieve colorimetric sensing modality.

To utilize the actual model in the real colorimetric sensor application, a linear response of the scattering peak spectra with the media dielectric property change is desired for the direct intuitive calibration and interpretation of the data. The colorimetric responses of the four models listed below to varying surrounding media are depicted in Figure 3.7: (1) the stacked NP circular array on the cylindrical hole sidewall, (2) the circular array on the flat surface, (3) a half toroid structure on the flat surface, and (4) the conventional square array of single NP. The square array (Figure 3.7a), which had a broad peak compared to the circular array and the toroid structure in Fig. 2c, eventually showed double peaks with
high surrounding refractive index. This result agrees well with the experimental result from Y. Lin et al.\textsuperscript{38} for the square nanodot array. Unlike the square array, the toroid structure and the circular array whether on a flat surface or inside the cylindrical hole had the single distinct peak in the visible range even in the high refractive index surrounding medium. The sensitivity, which is the peak shift per RIU, was the greatest for the quintuple circular array with the cylindrical hole structure (128.6 nm/RIU), compared to the square array (106.3 nm/RIU), the circular array on flat substrate (87.5 nm/RIU) and the toroid structure (65.2 nm/RIU).

Among the four models in Figure 3.7, the quintuple stack of eight NP circular arrays on the vertical sidewall (Figure 3.7d) had the highest SNR. The SNR can be further improved by adding more layers on top as depicted in Fig. 4a. In addition, the sensitivity is also the highest for the stacked circular array. Single circular array on the flat substrate (Figure 3.7b) has comparable sensitivity, but its low intensity makes it less attractive for the sensor application.

Achieving a high Q factor is another necessary factor in colorimetric sensing. The conventional colloidal colorimetric sensing with single particle induced LSPR has a peak broadening in high refractive index media (Figure 3.8b with single particle Mie scattering numerical analysis result). In air, the single particle based sensing seems to be a better detector with high Q factor, but its FWHM increased by 57.8 % after changing the surrounding refractive index of 1 to 1.6. The circular array, however, had a 34.7 % increase in FWHM with the same refractive index variation. This indicates that the circular array
has better colorimetric property with better consistency in the Q factor compared to the conventional single particle (Mie scattering) based colorimetric sensor.

3.4 Conclusions

The NP circular array addresses one of the strong designs for colorimetric sensing with less noise level. This eliminates the need of solvent, contrary to the conventional colloidal NP colorimetric sensing. It provides a solid template for detecting environmental change. Out-of-plane coupling produced by stacking the circular arrays enables to achieve extinction spectra with stronger peak intensities as well as 4.15-fold increase in SNR with quintuple layer. Higher SNR can be achieved by stacking more layers. In addition, a careful selection of substrate materials as well as precise substrate and NP geometrical modeling is crucial for achieving the single resonance peak in the visible range. The parameters controlling the resonance peak position are the distance between each particle, the refractive indices of the surrounding medium and the substrate, and the morphology of the NP. Stronger in-plane LSPR coupling, which involves smaller cylindrical hole diameter and larger number of particles in one layer, leads to the red-shift of the resonance peak wavelength. Out-of-plane coupling, on the other hand, results in blue-shift of the resonance peak wavelength and decrease in FWHM by 5 nm for triple layer case. Q factor, therefore, was improved by inducing stronger out-of-coupling with shorter inter-layer distance. We additionally report that stacked circular arrays of NPs has advantage in retaining Q factor with less broadening of the resonance peak, compared to Mie scattering based colloidal NP plasmonic sensing platform. Optimizing the colorimetric properties by tuning geometrical and optical parameters studied, the NP circular array as a colorimetric
sensor offers a broad range of color selection or spectrally active region for interesting potential applications.
3.5 Figures

Figure 3.1 Schematic representation of the simulated sidewall NPs on the periodic nanohole in (a) x,y-plane view and (b) tilted perspective view.
Figure 3.2 Extinction spectra with varying inter-particle distance and toroid’s diameter.

Extinction spectra of (a) the rectangular array of NPs spaced equally as the distance between each adjacent NP in circular array with the diameter of 120 nm, 160 nm, and 200 nm, (b) the circular array of NPs on a flat substrate, (c) the toroid metallic ring structure on a flat substrate, and (d) the circular array of NPs attached on the vertical sidewall inside a hole were compared with increasing diameter of array.
Figure 3.3 (a) Extinction spectra from a circular array with 16 NPs, 8 NPs, and 4 NPs. The current density distribution, J, of (b) 16, (c) 8, and (d) 4 NPs on the vertical sidewall at each peak wavelengths are presented.
Figure 3.4 (a) Extinction spectra with different number of layers and (b) with varying distance between each layer. (c, d) The current density (|J|) and the magnetic field (|H|) of the triple circular array stacks with (c) 5 nm and (d) 220 nm edge-to-edge inter-layer distance. All circular arrays has fixed hole diameter of 200 nm.
Figure 3.5 Derivative of electric field, which is proportional to surface charge, of the triple layer stack of circular arrays with eight NPs. Two different edge-to-edge inter-layer distances which are (a) 220 nm and (b) 5 nm are calculated. When the inter-layer distance is 250 nm (layer center-to-center distance), each NP has an individual dipole oscillating out of phase with each other. On the other hand, shorter inter-layer distance produces single dipole formation along three vertically arranged NPs in z-direction. Considering the net dipole strength along three layers, larger electric dipole built along the NPs induces a larger magnetic field loop across the layers. This results in higher in-plane magnetic field in the nanohole as Figure 3.4 shows.
Figure 3.6 The effect of the dielectric property of the substrate and the morphology of the particle on achieving a single resonance peak in the visible light range. (a) The electric field ($|E_z|$) distribution showing the field confinement at the interface between the particle and the substrate at the line crossing two NPs in x-directions. (b) The effect of the morphology of the NP on the extinction spectra by varying the substrate interface position while fixing the particle center position. (c) The extinction spectral response of a single circular array of eight NPs to the variation of the substrate and the surrounding media refractive indices, ranging from 1 to 1.6 respectively.
Figure 3.7 Extinction spectra of four models with the refractive index change of surrounding media: (a) the square array of hemispheric NPs on a flat surface, (b) the circular array on a flat surface, (c) the toroid structure on a flat surface, and (d) the quintuple layer stack of the eight NP circular array on a vertical sidewall of nanoholes. The greatest sensitivity and highest SNR were observed with the quintuple array stack.
Figure 3.8 (a) The scattering cross section of a silver sphere and a hemisphere. Two scattering peaks are present for a silver hemispheric NP, whereas a single narrow peak is achieved by a perfect silver spherical NP with the same diameter of 30 nm. (b) Refractive index dependent Mie scattering spectra of a single nanosphere with the diameter of 30nm. There is broadening of the peak as the refractive index of the surrounding media increases, resulting in lowering Q factor from increased FWHM. The slope of the peak wavelength variation is similar to that of the stacked quintuple layer circular array in the cylindrical hole.
CHAPTER 4

3D ASSEMBLED PLASMONIC NANOPARTICLES ON NANOCUP ARRAYS: ADDITIVE HETEROGENEOUS PLASMON COUPLING INDUCED ENHANCED REFRACTOMETRIC SENSITIVITY

Surface modification of the nanoLCA substrate was performed in order to enhance the refractive index sensitivity of a colorimetric sensor. Self-assembly of the NPs on the nanoLCA substrate resulted in a three-dimensional circular arrays of NPs in each nanocup. The plasmon coupling was observed between the plasmons from NP and the nanocup. The resulting increase in the hot spot densities and the electric field intensity achieved an improved sensitivity. This work is reproduced from \(^{82}\). Copyright 2016 Society of Photo Optical Instrumentation Engineers (SPIE).

4.1 Introduction

The colorimetric detections by plasmonic nanostructures or NPs have attracted huge interest in chemical, biological, and medical fields by its rapid identification of the targets without complex signal transducers.\(^ {14,29,83,84}\) Extensive studies to enhance the sensitivities of the colorimetric refractive index sensors have been reported by varying geometrical parameters of structures or size and shape of particles in order to find optimum structures.\(^ {28,29}\) However, most reports involve expensive fabrication techniques such as electron beam lithography or focused ion beam.\(^ {11}\) Here we demonstrate a method for increasing sensitivity of a colorimetric sensor without using lithography techniques but by introducing a plasmonic coupling between NPs and nanostructures.
The intensity of the localized electric field at hot spots or the density of hot spots determines the sensitivity of a plasmonic response to the surrounding refractive index change in a system.\textsuperscript{10,85} The distance between metallic NPs or nanostructures influences the intensity of the electric field at each hot spot, whereas the numbers and arrangements of plasmons control the density of these hot spots. In order to increase both intensity and density of hot spots, more plasmons are required in a system. Herein, an enhanced sensitivity of a refractive index sensor was achieved by assembling NPs in circle on a nanocup array substrate. A controlled number of NPs in each cup determines the amount of plasmonic resonant wavelength shift with the surrounding refractive index variation.

The sensitivity of a plasmonic nanostructured substrate for a refractive index sensing is fixed by the structure designs and thus, there has been almost no report on how to increase the sensitivity of a given substrate without re-designing the structure. We report for the first time that an additional plasmonic coupling by assembling NPs on a given plasmonic substrate can increase the sensitivity. This offers a chance of boosting performance of existing nanostructured substrates studied by many other researchers and broadening their applications to detecting much smaller amount of target analytes.

When metallic NPs are in close proximity to another metallic nanostructure instead of an identical NP, heterogeneous plasmonic coupling is induced.\textsuperscript{86} The resulting optical properties are mainly governed by how NPs are positioned on the nanostructure.\textsuperscript{87,88} The application of heterogeneous plasmonic coupling between a bottom-up synthesized NP and a top-down fabricated nanostructure has not been explored extensively for refractive index sensing applications, compared to that of a homogenous plasmonic coupling. We
demonstrate a sensitive antigen-antibody binding detection by improving label-free refractive index sensing via heterogeneous plasmonic coupling. The sensitivity enhancement was achieved by forming additional hot spots between the NPs and the nanostructure via plasmonic coupling between them. The resulting larger plasmonic resonant wavelength shift with respect to unit refractive index change achieves detections of small refractive index change induced by the adsorption of target molecules on a sensor surface.

A great advantage of using heterogeneous plasmonic coupling rather than the homogenous plasmonic coupling is that a top-down fabricated plasmonic nanostructure can be utilized as a template during the NP assembly in addition to a basic optical sensing substrate. Unlike identical NP assemblies in two-dimensions or three-dimensions, the template-assisted NP assembly shows great uniformity and controllability in NP arrangements;\textsuperscript{89,90} thus, the mass production of sensors with high uniformity is feasible. In addition, the NP assembly on a template results in a three-dimensional arrangement of NPs along the surface of the template. The three-dimensional arrangement of metallic NPs, especially which involves vertically stacked NP circular arrays in a cylindrical hole in the direction of an incident electromagnetic wave propagation, shows colorimetric properties with a single plasmonic resonance peak in the visible light range.\textsuperscript{72} Attaching NPs on the inner surface of cylindrical holes or cups produces a vertical stack of multiple circular arrays of NPs, accomplishing colorimetric refractive index sensing. The additional optical scattering from the nanocup structure itself results in the heterogeneous plasmonic coupling with these NP arrays and produces strong localized electric field between the NPs.
and the nanostructure surface. The related studies are described in detail in the numerical analysis section.

4.2 Materials and Methods

4.2.1 NP assembly on nanoLCA

NP assembled-nanoLCA (NP-nanoLCA) substrate was fabricated by using the Au nanoLCA structure as a base substrate. 50 nm Au NPs, purchased from Ted Pella, were self-assembled on the nanoLCA substrate in order to induce heterogeneous plasmonic coupling between NPs and the nanoLCA surface. As the NPs are negatively charged due to the citric acid capping ligands on the surface, cysteamine molecule was used as a linker molecule to bond with the nanoLCA surface and the NPs. The cysteamine molecules are positively charged in distilled water, so the NPs can be electrostatically attracted to the self-assembled cysteamine molecules on the nanoLCA. Cysteamine, purchased from Sigma Aldrich, was dissolved in 100% ethanol and the nanoLCA substrate was subsequently immersed in the 10 mM cysteamine solution for 2 hours. After 2 hours of incubation, the substrate was thoroughly rinsed with ethanol and blown dried with nitrogen. The cysteamine treated nanoLCA was immersed in Au NP solution (4.5×10^10 particles/mL) and incubated for 24 hours on a plate shaker (400 rpm).

4.2.2 Optical characterization

Transmission images of the nanoLCA and the NP-nanoLCA were taken by Olympus BX51 upright microscope under the bright field with 5X objective lens. The reflection dark field images were taken by the Zeiss AxioScope A1 microscope with 50X
objective lens and a Zeiss AxioCam MRc color CCD camera. Transmission spectra were measured by Cary 5G UV-Vis spectroscopy.

4.2.3 Numerical analysis

A FDTD simulation was performed by building a nanoLCA structure with the periodic boundary conditions in x- and y-directions. The periodicity was 320 nm. The +z and –z boundaries had perfectly matched layers to avoid any reflection from these boundaries. A 200 nm-diameter and 500 nm-tall dielectric nanocup structure with 85° sidewall angle (to the x-axis) were designed with a refractive index of 1.56 RIU to represent the NOA61 polymer pattern. 9 nm Ti layer was placed over this nanostructure and 90 nm Au film was then put on top of this Ti layer. The sidewall of the nanocup was layered with a 2 nm-thick Ti layer and a 10 nm-thick Au layer. The NP-nanoLCA was built based on this nanoLCA structure with additional NPs at the brim of the nanocup.

A plane wave, propagating in –z direction, was placed above the nanoLCA. An override mesh size was 2 nm around the brim of the nanocup. Transmission spectra and electric field distributions at the plasmon resonance wavelengths were collected from the simulation.

4.2.4 Biotin-streptavidin conjugation on the substrates

The biotinylation of the Au surface on both devices was conducted by using thiolated biotin. The thiolated biotin was purchased from Nanoscience Instruments, Inc. The devices, nanoLCA and NP-nanoLCA, were immersed in 1 mM biotin probe solution in 1X phosphate-buffered saline (PBS) for 24 hours and rinsed. After thoroughly drying with nitrogen flow, the devices were post-treated with 1 mM 6-mercaptop-1-hexanol (MCH)
for 1 hour in order to cover any open space that is not covered by biotin. Streptavidin solution (100 ng/mL) was prepared in 1X PBS and incubated on both nanoLCA and NP-nanoLCA for 1 hour. The nanoLCA and the NP-nanoLCA were rinsed and dried subsequently.

4.3 Results and Discussion

4.3.1 Colorimetric properties of NP-nanoLCA

The nanoLCA substrate transmits green color (Figure 4.1a) in air with a plasmonic resonance peak at 530 nm (black spectrum in Figure 4.1e). There was almost no change in the transmission spectra after the cysteamine treatment to the nanoLCA (red spectrum in Figure 4.1e). After the NP assembly, the overall transmission intensity from the NP-nanoLCA was decreased (Figure 4.1b), but its plasmonic resonant wavelength remained the same as the original nanoLCA (Figure 4.1e). This is because the 50 nm NPs have the resonance wavelength at 535 nm, which is close to 530 nm. The surface of each nanoLCA and NP-nanoLCA was characterized by the SEM (Figure 4.1c and Figure 4.1d). A monolayer of NPs was formed on the nanoLCA surface and circular arrays of NPs were spontaneously developed along the brim and inside of each nanocup.

4.3.2 Bulk refractive index sensing

The spectral responses of the nanoLCA and the NP-nanoLCA were measured while increasing surrounding refractive index on the substrate surface. Sucrose solutions with different concentrations (0, 10, 20, 30, 40, and 50 w/v %) were prepared and dropped on each nanoLCA and NP-nanoLCA substrate. When the concentration of sucrose increased, the plasmonic resonant wavelength red-shifted linearly with increasing dielectric refractive
index (Figure 4.2). The amount of peak shift per unit % concentration change was larger for the NP-nanoLCA by 160.7 % compared to the nanoLCA. Assuming that the spectral resolution is 1 nm, the NP-nanoLCA can detect a sucrose concentration change by 1.96%, whereas the nanoLCA cannot detect below 3.14 % sucrose concentration variation.

4.3.3 Numerical analysis

The nanoLCA and the NP-nanoLCA were numerically analyzed by the FDTD simulations. The NP-nanoLCA was modeled as the nanoLCA with four 50 nm-diameter NPs, which surfaces touched the brim of the nanoLCA. The electric field distributions of the nanoLCA and the NP-nanoLCA at their plasmonic resonant wavelengths are plotted in Figure 4.3. The NP-nanoLCA had strong electric field confined at the interface between the NPs and the brim of the cup (Figure 4.3b). It has been verified that the plasmonic peak wavelength shift is large when the intensity of localized electric field is high;\textsuperscript{91} therefore, the resulting hot spots by attaching NPs on nanoLCA substrate allow a detection of smaller refractive index variations than the bare nanoLCA does. In addition, the existence of strong localized electric field between the NP and the nanoLCA verifies the heterogeneous plasmonic coupling of NPs with the nanoLCA.

As the strong electric field confinement, or a hot spot, is induced when a NP is attached near the brim of the nanocup, larger number of hot spots will result with more NPs assembled on the nanoLCA. We numerically analyzed the effect of the NP numbers on the refractive index sensitivity by adding zero, four and eight NPs along the brim of the nanocup (Figure 4.4). The NP-nanoLCA with eight NPs had the largest sensitivity and the nanoLCA without any NP had the smallest sensitivity. This verifies that the dense hot spot
formation by adding NPs as many as possible along the brim of the nanocup is a key factor for enhancing the sensitivity.

4.3.4 Biosensing: antigen-antibody binding detection

Biotin and (strept)avidin are of the most crucial antibodies and antigens found in living creatures. When biotin molecules are chemisorbed on a sensor surface and a streptavidin molecule binds to a biotin, the local refractive index on the sensing surface increases due to their conjugation. Using this idea, refractive index sensors, nanoLCA and NP-nanoLCA, were tested whether they can detect the formation of biotin-streptavidin complex on the surface.

The optical responses during the biotin, MCH, and streptavidin treatments were characterized by Cary 5G UV-Vis spectrometer. Despite the binding of biotin, MCH and streptavidin, both nanoLCA and NP-nanoLCA showed no shift of the peak position at 530 nm (Figure 4.5a); however, a slight decrease in the transmission peak intensity was observed. Compared to the nanLCA, the NP-nanoLCA showed larger reduction in the transmission peak intensity upon the streptavidin binding. In addition, the NP-nanoLCA had another spectral change upon the binding of streptavidin at the transmission dip at ~650 nm: the dip red-shifted from 646.5 nm to 654.8 nm when streptavidin bound to the surface (Figure 4.5b). The nanoLCA, however, did not show any shift of the transmission dip.

The dip of the transmission represents a plasmonic dark mode. The electric field distributions at the bright mode (i.e., at the transmission peak wavelength) and the dark mode (i.e., at the transmission dip wavelength) were collected from the FDTD simulation.
(Figure 4.6). In order to model the biotin-streptavidin layer, a 10 nm-thick dielectric layer covering the surface of the nanoLCA and the NP-nanoLCA models was imposed in the simulation. The dark mode showed the strong electric field confined only between the NP and the nanocup (Figure 4.6b). The electric field distribution of the plasmonic dark mode verifies that the heterogeneous plasmon coupling between the plasmons of NPs and the nanocup dominates at this dark mode, and this strong electric field helps detect a small refractive index change induced by a molecular adsorption on the sensing surface.

4.4 Conclusions

Additional hot spots, which are resulted from the heterogeneous plasmonic coupling between the NPs and the nanoLCA, improved the sensitivity of the nanoLCA substrate by 1.67-fold. Re-designing the nanostructures was evitable by using the plasmonic NP assembly technique when one wants to increase the sensitivity of a plasmonic resonance peak shift for detecting the surrounding refractive index variation. Self-assembled Au NPs through the electrostatic attraction by a cysteamine monolayer on the Au nanoLCA surface produced 3D vertically stacked circular arrays of NPs inside the nanocups, which exhibits colorimetric properties. The FDTD simulation results proved the stronger electric field development on the nanoLCA surface by assembling NPs in it. As a result, the NP-nanoLCA achieved a detection of a small refractive index increase by the biotin-streptavidin binding. This technique of tuning sensitivity by the NP assembly on a nanostructure is expected to be applicable to and improve many other plasmon-based refractometers.
4.5 Figures

**Figure 4.1** Transmission images of (a) the nanoLCA and (b) the NP-nanoLCA and scanning electron micrographs of (c) the nanoLCA and (d) the NP-nanoLCA. (e) Transmission spectra of the nanoLCA (black solid line), cysteamine treated nanoLCA (red solid line) and the NP-nanoLCA (blue solid line). All spectra were taken in air.
Figure 4.2 Plasmonic resonant wavelength change with increasing sucrose concentration on (a) the nanoLCA and (b) the NP-nanoLCA surface.
Figure 4.3 The electric field ($|E|$) distribution of (a) the nanoLCA and (b) the NP-nanoLCA at the plasmonic resonant wavelengths. The color bar represents the intensity of $|E|$ from 0 to 20.
Figure 4.4 FDTD simulated spectral response with increasing surrounding refractive index.
Figure 4.5 The transmission spectra of the nanoLCA (top) and the NP-nanoLCA (bottom) in the range of (a) 400 nm – 800 nm and (b) 600 nm – 700 nm. Both spectra were taken in air after the biotin-MCH treatment and the streptavidin (100 ng/mL) incubation.
Figure 4.6 FDTD simulated electric field distribution of the NP-nanoLCA (four NPs along the brim of the nanocup). A 10 nm-thick dielectric layer’s refractive index is 1.50 RIU. The field distribution is plotted (a) at the plasmonic bright mode (transmission peak position) and (b) at the plasmonic dark mode (transmission dip position).
CHAPTER 5

OPTOFLUIDIC FLUORESCENCE ENHANCEMENT TUNING ON PLASMONIC NANOCUP ARRAYS

Using a colorimetric substrate, the nanoLCA, the tunable fluorescence enhancement was achieved without changing the underlying plasmonic designs but simply by varying surrounding fluidic refractive indices on the plasmonic surface. The correlation among the radiative decay rate, the quantum efficiency and the lifetime of fluorophores were identified while varying the plasmon resonance wavelengths. This chapter is reproduced with permission from 92. Copyright 2015 American Chemical Society.

5.1 Introduction

Fluorescence detection is one of the most popular methods for identifying the target molecules in a complex biological or chemical environment.93–95 With the advent of MEF,96–98 a reduced volume requirement in a nanoscale regime or single molecular level detection has been achieved.99 MEF has become more promising for overcoming the inherent limitations of organic fluorophores, which are the low quantum yield and low photostability.45–47 With growing interest, many researchers have studied extensively on how to design the plasmonic NPs or substrates for maximizing the enhancement.8,46,48–50,100,101 In addition, the temperature and ionic strength of the solution, all of which subsequently change the conformations or lengths of linker molecules (e.g., deoxyribonucleic acids (DNAs)) between the metal and the fluorophore, were also precisely controlled to study the influence of fluorophore-metal distance on the fluorescence enhancement.102,103
Even though many previous works manipulated the fluidic properties to study the fluorophore-plasmon interaction, the influence of the fluidic refractive index on the fluorescence intensity near plasmons, however, was scarcely studied. Considering the fact that the fluorescence-based detections are mainly conducted in a dynamic and fluidic environment, the effect of environmental factors such as refractive index change onto the MEF response should be studied in detail. Here we examine the effect of dielectric properties on plasmonic and fluorescence response and demonstrate a fluidically tuned fluorescence enhancement on a colorimetric plasmonic substrate.

A systematic study on the fluidically tuned fluorescence is achieved with a colorimetric substrate, which plasmon resonance is strongly influenced by the surrounding refractive index. As the nanoLCA structure, introduced in Chapter 2, shows dynamic plasmon resonance shift with surrounding refractive index change, it is a good substrate candidate for deliberately studying the fluidically tuned fluorescence response. A schematic representation of possible fluorescence response mechanisms to these optical changes is presented in Figure 5.1a. The surrounding refractive indices influence the reflection and scattering efficiencies on the nanoLCA structure surface, which ultimately affect the degree of the fluorescence enhancement. Fine-tuning of the plasmon resonance was achieved by increasing the fluidic refractive indices on the nanoLCA surface. The fluorescence responses were monitored at the same time.
5.2 Materials and Methods

5.2.1 Fluorophores and Oligodeoxynucleotides

Hexachloro-fluorescein (HEX), which is a green emission dye, has the excitation spectral center at 538 nm and the emission spectral center at 555 nm. Sulforhodamine 101 acid chloride (TEX), which is a red emission dye, has its excitation peak at 596 nm and emission peak at 613 nm. Each of these organic fluorophores was covalently bonded at one end of single-stranded deoxyribonucleic acids (ssDNAs) with 18 bases. Dithiol group was attached at the other end of the ssDNAs for the self-assembly of the fluorophores on the Au surface. The oligodeoxynucleotide sequences of ssDNAs are 5’-TEX615 CAG CAA ATG GGC TCC GAC-thiol-3’ (TEX-DNA) and 5’-HEX CAG CAA ATG GGC TCC GAC-thiol-3’ (HEX-DNA). Each strand was high-performance liquid chromatography purified by Integrated DNA Technologies.

5.2.2 Immobilization of DNAs on the nanoLCA substrate

After fabricating the Au nanoLCA substrate 0.4 µL of ssDNAs in immobilization buffer solution was dropped on the device surface. The immobilization buffer consists of 100 mM Tris-HCl, 500 mM NaCl and 100 mM Tris(2-carboxyethyl)phosphine, a disulfide reducing agent. The device with both HEX-DNA and TEX-DNA solution droplets was incubated in 37 °C for 4 hours.

5.2.3 Fluorescence imaging and spectral measurement

The fluorescence images were taken by an Olympus BX51 upright microscope with 5X objective lens. The excitation light and the fluorescence from the sample passed
through tetramethylrhodamine (TRITC) filter that has an excitation window centered at 541 nm and an emission window centered at 572 nm.

The fluorescence spectra were taken with Horiba confocal Raman instrument with 532 nm laser light source. Since the fluorescence signal, in general, is much stronger than the Raman signal from the molecular vibrational modes, fluorescence emission spectra could be obtained with this equipment.

5.2.4 FDTD simulations

The simulations were performed by the commercial FDTD software from Lumerical Solutions, Inc. The same geometrical information from the SEM image of the nanoLCA structure was implemented in the FDTD model. The underlying substrate dielectric constant was 1.56 RIU, which is the refractive index of NOA 61. On top of the plastic substrate, 9 nm Ti and 90 nm Au, using built-in materials database with Ti from Palik and Au from Johnson and Christy, were added. The periodic boundary condition was applied in x- and y- direction with a periodicity of 350 nm. An override mesh size of 2 nm was implemented on the nanocup regions. A single dipole source, polarized in x-direction, was placed 10 nm apart in z-direction from the top Au surface of the nanocup. Here the dipole source was used to represent a fluorophore. A three-dimensional monitor surrounding the dipole source was to measure the dipole power and the other three-dimensional monitor enclosing the nanocup structure was to measure the power affected by the nanostructure, which is considered as the radiative decay rate. The non-radiative decay rate was calculated by the dipole power subtracted by the radiative decay rate and the final quantum efficiency was calculated by the radiative decay rate divided by the
dipole power (the sum of the radiative decay rate and the non-radiative decay rate). The method of calculations was also described in Lumerical website.

5.2.5 Lifetime measurement

The fluorescence lifetime was measured by Fluorescence Lifetime Imaging (FLIM) system, called Alba FCS built by ISS (Champaign, IL), in Beckman Institute, University of Illinois at Urbana-Champaign. Multiphoton excitation mode was used so that it can excite both HEX and TEX in one system. 470 nm diode laser is used to provide photons and lifetime imaging was conducted with 60X objective lens.

5.3 Results and discussion

5.3.1 Fluorescence response on the nanoLCA with surrounding refractive index change

The reflected light from the device surface made up of Au is capable of re-exciting fluorophores. The reflection intensity at 532 nm from the colorimetric nanoLCA substrate was measured while changing the surrounding refractive indices (Figure 5.1c). 532 nm is chosen, since it is the excitation wavelength of the fluorescence. The intensity increased by 24.1 % in 0 % glycerol (water, 1.333 RIU) and by 37.3 % in 100 % glycerol (1.474 RIU), compared to that in air (1 RIU) (please see the inset of Figure 5.1d). If the excitation process is more critical than the emission process, fluorescence emission will be enhanced with increasing refractive index due to a higher chance of excitation with stronger reflected light intensity.

The scattering peak position of the nanoLCA, shown in Figure 5.1e, red-shifted with increasing refractive index: 530 nm (air), 592 nm (0 % glycerol), 598 nm (20 %
glycerol), 607 nm (50 % glycerol), and 628 nm (100 % glycerol). Since the electric field confinement and the scattering efficiency are maximized at the plasmon resonance peak position, a selective enhancement of the fluorescence emission at that wavelength is expected. This increase in the spontaneous emission rate is caused by the enhanced radiative decay rate. In addition to the fluorescence intensity change, a shape alteration of fluorescence emission curves at the resonance wavelength is also reported. Thus, the shape of the HEX emission spectra will be changed on its right tail with the red-shift of the plasmonic scattering peak. On the other hand, there will be smaller spectral shape change for TEX compared to HEX, as there is smaller deviation between the TEX emission peak position (613 nm) and the plasmonic scattering peak positions (from 592 nm to 628 nm) under the surrounding refractive indices tested in this paper.

The increase in absorbance upon the fluorophore immobilization is an indication of the additional energy exchange between the plasmons and the fluorophores by the light beam path length change. As Figure 5.2 shows, the absorbance dip of the nanoLCA without the fluorophores on the surface changed from 530 nm in air (Figure 5.2a and Figure 5.2b) to 592 nm in water (Figure 5.2c and Figure 5.2d). Here the dip in absorbance is exactly the same as the peak of the transmission or the scattering spectra. Since the refractive index also increases by the chemisorption of the ssDNAs (i.e., HEX-DNA and TEX-DNA) on the surface, the red-shifts of the absorbance dip both in air and in water were observed. At the same time, the absorbance intensity increased upon the immobilization of HEX-DNAs and TEX-DNAs on the device surface. The increment of the absorbance intensity was larger in water compared to that in air. This indicates that the energy exchange between the plasmon and the fluorophore is more efficient in water. The
main reason for the larger energy exchange from plasmon to fluorophores is because of the overlap between the SPR wavelength (i.e., absorbance dip of the nanoLCA) and the absorbance peak of the fluorophores. The photons scattered by the plasmon from the nanoLCA surface is re-absorbable by the nearby fluorophores so that the absorbance value increases with this overlap.

Continuous surrounding media variation on the nanoLCA structure was accomplished by using a microfluidic channel, which has a single inlet and an outlet. The obvious enhancement of the fluorescence intensity was observed with the alternate flows of air and water in the microfluidic channel. The reflection and the fluorescence images were taken with a fixed field of view under the microscope. The fluorescence intensity instantly increased when water was on the device surface (Figure 5.3a and Figure 5.3b). The overall reflection color also changed from orange color in air to green color in water (Figure 5.3c and Figure 5.3d). This shows that the light reflected from the nanoLCA in water has more green-portion of light compared to that in air, which ultimately provides a better chance of re-exciting both HEX and TEX dyes.

Alternately flowing air and water in the microfluidic channel resulted in the dynamic fluorescence intensity variations. The total of seven cycles of air and water flows on the device had a consistent HEX and TEX fluorescence intensity variation. The average image brightness variation with air and water flows was plotted in Figure 5.3e. The image brightness was calculated by the HSV image analysis of each fluorescence image and the value (V) from HSV results, which represents the brightness of the image, was plotted. The HEX and TEX fluorescence intensities increased by 4.83-fold and 2.08-fold on average,
respectively. There are two main reasons for the larger increment of the brightness from HEX compared to that from TEX: (1) the TRITC excitation band overlaps well with HEX absorbance spectrum but does not cover the main absorbance peak of TEX, which is at 596 nm, and (2) the TRITC emission band only covers the left-tail of the TEX emission spectrum. Nevertheless, the constant response of the brightness to the surrounding media change was observed for both HEX and TEX.

We compared the fluorescence response of HEX and TEX assembled on the 90 nm-thick Au thin film to confirm that the colorimetric property of the nanoLCA structure is the key factor in achieving the tunable fluorescence enhancement by controlling the surrounding refractive index (Figure 5.4). Since the Au thin film does not have the resonance wavelength shift with the surrounding refractive index change under the direct illumination of the white light, there was no reflection color change despite the refractive index increase on top of the film (Figure 5.4a – Figure 5.4d). Even though the exposure time was remained the same, the fluorescence intensities from Au thin films (Figure 5.4e – Figure 5.4h) were much smaller compared to the fluorescence signals from the nanoLCA structures (Figure 5.4m – Figure 5.4p). This verifies again that the colorimetric properties from nanoLCA structures are critical in achieving the optofluiddically tuned fluorescence enhancement.

The fluorescence spectral responses of HEX (Figure 5.5a) and TEX (Figure 5.5b) immobilized on the nanoLCA surface were measured with a variety of glycerol solution concentrations of 0 (v/v) % (water), 20 %, 50 %, 80 % and 100 %. Both fluorescence emission spectra were measured with 532 nm excitation laser and each curve is the average
spectrum of five measurements from different regions. The emission intensity variations of HEX and TEX showed noticeably different trends with increasing glycerol concentrations: HEX had the 6.93-fold increase in peak intensity with 20 % glycerol, but TEX had 7.12-fold increase with 80 % glycerol from those measured in air (1 RIU).

As shown in Figure 5.5c, the spectral shape of the HEX fluorescence emission curve, which has a main peak position at 555 nm and a shoulder peak ranging from 600 nm to 625 nm, changed as the media refractive index increased from 1 RIU to 1.474 RIU (100 % glycerol). When fluorescence spectra were normalized, the relative shoulder peak where the plasmonic scattering peak wavelength positioned was enhanced with increasing media refractive index. For instance, when the nanoLCA substrate was under 0 % glycerol (water), the scattering peak was at 592 nm and the normalized fluorescence intensity at this wavelength was enhanced by 1.24-fold from the intensity in air (blue arrow in Figure 5.5c). When the concentration of glycerol increased further, the normalized fluorescence intensity at larger wavelength region (with a red arrow) was enhanced. The intensity increase at the shoulder peak compared to the main peak indicates a spectral shape change from a positive correlation between the plasmonic effect and the fluorescence emission.

The relative fluorescence intensity ratio of the shoulder peak to the main peak was calculated by the integrated area under the shoulder peak divided by the main peak area with 25 nm bandwidth (Figure 5.6). The ratio increased linearly with the increase in glycerol concentration and 1.73-fold increase in ratio was achieved with the media change from air to 100 % glycerol. Although the degree of spectral shape change here is not as large as the total spectral shape change studied by M. Ringler et al.$^{57}$ or the fluorescence
emission peak position shift,\textsuperscript{107,108} this certainly confirms that the plasmon-fluorophore interaction results in fluorescence emission spectral shape alterations as well as the overall intensity variations depending on where the plasmonic scattering wavelength locates.

The biggest difference from the previous works on MEF is that the variation in the spectral shape and intensity is driven not by varying the plasmonic nanostructure’s geometrical factors but by shifting the plasmon resonance with surrounding refractive index increase on the same plasmonic system. The electric field confinement dramatically changes with the inter-particle distance or the aspect ratio of NP,\textsuperscript{46,55,109,110} however, simply varying the refractive index on the surface of the nanostructure does not significantly change the degrees of the electric field confinement or the scattering efficiency at each shifted resonance wavelength with the same plasmonic mode. These factors may explain why there was a relatively smaller spectral shape change with the shift of the plasmon resonance associated with the refractive index change. Despite less dramatic changes in spectral shapes, this finding still corroborates the idea of the plasmonic effects on the fluorescence intensity enhancement as well as the spectral shape alteration from the plasmon resonance tuning by the surrounding fluidic refractive index.

The integrated fluorescence area under each HEX and TEX spectra in Figure 5.5d behaved similarly to the peak intensity change. Although both HEX and TEX were immobilized through the same ssDNAs with the same base sequences and lengths, the refractive index at which each fluorophore had maximum enhancement in fluorescence emission was distinctly different. The studies on the radiative decay rate and the quantum
efficiency are required, since these directly influence onto the fluorescence emission enhancement.

On the other hand, the different fluorescence emission intensity trends between HEX and TEX are not consistent with the trends of the reflection intensity variation at 532 nm shown in Figure 5.1d, which is related to the excitation process of the fluorescence. Both HEX and TEX experience the same degree of the enhancement of the reflected light intensity, but HEX did not have the maximum intensity with 100 % glycerol. This indicates that the fluorescence intensity is mainly affected by the plasmonic scattering effect, which influences the emission process.

5.3.2 Quantum Efficiency, Radiative Decay Rate and Lifetime

Since the quantum efficiency is directly proportional to the integrated fluorescence emission area, a study on the quantum efficiency variations with surrounding refractive index change is necessary. From a FDTD simulation with a dipole placed on the top Au surface of the nanoLCA structure, the quantum efficiency and the radiative decay rate curves (Figure 5.7a and Figure 5.7c, respectively) changed with increasing surrounding refractive index. The radiative decay rate peak position slightly differs from that of the quantum efficiency, because the quantum efficiency calculation includes the non-radiative decay rate term. Even though there is difference in peak positions of the quantum efficiency and the radiative decay rate of each fluorophore, we note that each of the radiative decay rate and the quantum efficiency exhibits different dispersion curves depending on the surrounding refractive index. These trends differ from conventional radiative decay rate responses from free fluorophores, which were reported to be simply
proportional to the square of the refractive index.\textsuperscript{111,112} The whole spectral shape changes as well as the peak shifts in both radiative decay rate and quantum efficiency curves suggest that the nearby plasmonic effect dominantly governs the radiative decay rate and the quantum efficiency over the intrinsic rule with the surrounding refractive index.

The average quantum efficiency (Figure 5.7b) and the average radiative decay rate (Figure 5.7d) were calculated under each fluorophore’s emission band: from 550 nm to 575 nm for HEX and from 595 nm to 620 nm for TEX. The maximized average quantum efficiency of each HEX and TEX was observed in the surrounding media of 1.35 RIU and 1.49 RIU, respectively. The average radiative decay rate curve, on the other hand, exhibited a peak at 1.42 RIU for HEX and a peak at 1.55 RIU for TEX. We note that the actual integrated fluorescence emission area for both HEX and TEX, shown in Figure 5.5d, followed the same trend in the calculated average quantum efficiency curve on the nanoLCA structure, since the refractive index of 20 % glycerol is 1.363 RIU and that of 80 % glycerol is 1.448 RIU. This agreement in both theoretical and experimental results confirms that the fluorescence emission enhancement can be selective depending on how well the fluorescence emission band matches with the resonance wavelength. In addition, the refractive index of the sensing media containing the fluorophore should be considered for maximizing the enhancement factor.

The lifetime of a fluorophore is inversely proportional to the sum of the radiative decay rate and the non-radiative decay rate. The fluorophores near the metallic NPs or the nanostructures achieve higher photostability by a reduced lifetime due to an increase in the radiative decay rate.\textsuperscript{45,52,99,113–115} The lifetimes of free HEX and TEX in solution state and
those immobilized on the nanoLCA were measured by the FLIM system in different solutions. The mean lifetime of free HEXs in solution, without nanoLCA substrate, was 4.48 ns (Figure 5.8a) and that of free TEXs was 5.15 ns (Figure 5.8b). When both fluorophores were attached to the Au nanostructures, both lifetimes were dramatically reduced: the lifetimes of HEX on the nanocup structure in air, water (0 % glycerol) and 100 % glycerol were 0.62 ns, 0.65 ns and 0.47 ns, respectively (Figure 5.8c) and the lifetimes of TEX were 0.59 ns, 0.63 ns and 0.42 ns, respectively (Figure 5.8d). Maximum of 9.58-fold decrease for HEX and 12.16-fold decrease for TEX in lifetimes were observed by using the plasmonic nanoLCA substrate. This implies that the radiative decay rate of free fluorophores in solution state can be at least 9.58-fold smaller for HEX and 12.16-fold smaller for TEX, compared to those attached on the plasmonic colorimetric substrate.

5.4 Conclusions

The investigations on the main factors for the optofluidically tuned fluorescence enhancement are presented here with the reflection and scattering spectral responses of the nanoLCA to the surrounding refractive index change. The gradual red-shift of the plasmon resonance wavelength by the increase in surrounding refractive index resulted in varying fluorescence intensities. The optimum enhancement condition was different for different fluorophores. HEX, a green fluorescence dye, achieved 6.93-fold increase by the surrounding refractive index change from 1 RIU (air) to 1.36 RIU (20 (v/v) % glycerol) and TEX, a red fluorescence dye, achieved 7.12-fold increase by the refractive index change from 1 RIU (air) to 1.45 RIU (80 % glycerol). This agrees well with the theoretical quantum efficiency and radiative decay rate calculations, which showed the distinctive concave parabolic curves for different fluorophores with their own emission bands. This
behavior is remarkably different from the intrinsic properties of the quantum efficiency and the radiative decay rate, which typically increase proportionally with the square of the refractive index. On the colorimetric plasmonic substrate, the plasmon resonance is the major player in determining the degree of the fluorescence enhancement and the refractive index is mainly considered as a rudder that controls the plasmon resonance wavelength position. The lifetime reductions by 9.58-fold for HEX and by 12.16-fold for TEX on the nanoLCA compared to the solution states suggest that the plasmonic effects of the nanoLCA facilitate the energy relaxation in the emission process, resulting in higher fluorescence intensities.

Understanding optofluidic fluorescence enhancement will benefit current fluorescence-based bio-sensing and monitoring system. Since target molecules or proteins are often tagged with fluorophores, a local binding event of other drug or biological molecules to the tagged protein can be detected by the fluorescence intensity variations, which is indirectly driven by the plasmon resonance peak shift due to a local refractive index change from binding. Multiple tagging of different fluorophores that are sensitive to a specific range of local refractive index of interest, which depends on binding types or surrounding media, will enable multiplexed bio-detections in one pot.
5.5 Figures

Figure 5.1 The optical properties of the nanoLCA substrate. (a) A schematic representation of the fluorescence enhancement on the nanoLCA is presented. The nanocup array polymer substrate is in grey, gold deposited on the substrate is in yellow, and two different refractive indexed media are in blue and purple color. Green and red point sources are HEX and TEX dye, respectively. In different media, reflected light intensity (hv\text{ref}) and scattered light intensity (hv\text{scat}) differ despite the same excitation light intensity (hv\text{ex}), resulting in different fluorescence emission intensities (hv\text{em}). (b) The SEM image shows the nanoLCAs (top view). (c) The reflection spectra of the nanoLCA measured with a spectrometer changes with different refractive indexed surrounding media.
(Figure 5.1 cont.)

(d, inset) The reflection values at 532 nm are plotted with surrounding media which are air, 0 % glycerol and 100 % glycerol. (e) The scattering peak position shifts to red with increasing concentrations of glycerol.
Figure 5.2 The absorbance curves change with the immobilization of (a, c) HEX and (b, d) TEX on the nanoLCA surface. (a) and (b) show the absorbance changes measured in air and (c) and (d) show the changes measured in water on the device surface. Blue dash-dotted curves in (c) and (d) are the normalized absorbance spectra of free HEX and TEX in solution state. The dip of the absorbance, which represents the plasmon resonance, red-shifts upon the fluorophore assembly (▼).
Figure 5.3 The alternate flow of air and water in the microfluidic channel on the nanoLCA substrate induces instant changes of fluorescence intensity from the fluorophores. The fluorescence images with (a) air and (b) water and the reflection images with the same surrounding media (c and d) are presented (scale bar: 2 mm). The red emission is from TEX and the green emission is from HEX. (e) The dynamic fluorescence image brightness response with alternating flow of air (A) and water (W) is demonstrated by HSV analysis.
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Figure 5.5 The fluorescence emission spectra measured with 532 nm excitation laser are presented with different refractive indices. The emission spectra from (a) HEX and that from (b) TEX show different intensity variations despite the same concentration change of the glycerol. (c) The normalized HEX spectra (upper graph) show relative intensities increasing at the shoulder peak, as the scattering peak red-shifts (bottom graph). (d) The integrated fluorescence emission curves of HEX and TEX show their maxima at different refractive indices. HEX has maximum integrated fluorescence intensity with 20 % glycerol, but TEX has it with 80 % glycerol.
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Figure 5.8 The lifetimes of HEX and TEX (a, b) in solution state and (c, d) on the nanocup structure are measured by FLIM. Both lifetimes of HEX and TEX decreased dramatically on the nanocup structure.
CHAPTER 6

ABSORBANCE AMPLIFICATION USING CHROMOPHORE-NANOPLASMON COUPLING FOR ULTRASENSITIVE PROTEIN QUANTIFICATION

In this Chapter, the enhancement of the absorbance near the nanoLCA substrate is demonstrated. Strong scattering electric fields at the plasmon resonance wavelength provide extra photons for dyes (or chromophores) to absorb near the surface of the nanoLCA. Many immunoassays use dyes in order to colorimetrically detect the amount of target analytes in a solution, using absorbance as a standard metric; thus, adding the nanoLCA to the immunoassay-based bio-analyte detection greatly improves the conventional limit-of-detection and the sensitivity. This chapter is reproduced with permission from 116. Copyright 2015 American Chemical Society.

6.1 Introduction

Plasmonic effects induced by nanoscale metallic particles or nanostructures produce a local field enhancement that can offer chemical,69,117 optical,50,118 or photothermal119,120 signal amplification. Although extensive studies exist describing this unique optical effects of nanoscale metals on nearby molecules such as fluorophores,55,56,59,121 the interactions between plasmons and chromophores (without fluorescence) have been scarcely investigated for achieving absorbance enhancement. Since most colorimetric immunoassays involve chromophores during chemical reactions with target analytes,122,123 plasmon-assisted amplification of absorbance, which is a
standard metric for these assays, can potentially bring huge impact on current colorimetric biomolecular or chemical detection for improving their sensitivity and the LOD.

Colorimetric detection is widely used for protein quantification,\textsuperscript{124} bacteria sensing,\textsuperscript{125} drug molecule identification,\textsuperscript{126} cell viability testing,\textsuperscript{127} and environmental monitoring\textsuperscript{128} due to its associated high specificity and rapid intuitive interpretation. Conventional colorimetric immunoassays have shown great performance at defined concentration regions, typically in the µM – mM range;\textsuperscript{129,130} nonetheless, in order to achieve early diagnosis of diseases, lower concentration sensing (e.g., in or below the nM range) is now in high demand. In addition, as most colorimetric assays utilize microplates (e.g., 96-microwells) that require sample volumes greater than 100 µL, their use gets discouraged for bio-molecular detection that involves limited sample volumes and high sensitivity needs.

Conventional efforts to achieve a low LOD for protein concentrations include the use of enzyme-assisted chemistry,\textsuperscript{131} electrochemistry\textsuperscript{132} and optical ring resonators.\textsuperscript{133} Despite these developments, a requirement of specialized equipment and a lack in cost-effectiveness discourage their immediate use; furthermore, a universal sensing platform that enhances absorbance for a variety of assays has not yet been developed. We propose a versatile device that does not rely on specific chemical reactions to enhance absorbance but, instead, utilizes optical signal amplification by plasmonic effect. A modified microplate sensing platform that incorporates a plasmonic substrate on the bottom of the well achieves enhancement of absorbance values and sensitivities even for lower sample volumes and extends platform application to a variety of colorimetric assays. We intend to discuss the
energy transfer mechanism between chromophores and plasmons and to apply this principle to protein quantification by using a conventional colorimetric Bradford assay at a higher sensitivity and a lower LOD.

6.2 Materials and Methods

6.2.1 Chemicals

In order to characterize the absorbance response of different colors on the nanoLCA, three different colored chromophores approved by the Food and Drug Administration were purchased from McCormick: Fd&C Red 40 (or 2-naphthalenesulfonic acid), Fd&C Yellow 5 (or tartrazine) and Fd&C Blue 1 (or erioglaucine). Coomassie Bradford assay for total protein quantification and bovine serum albumin (BSA) were purchased from Life Technologies. The assay was left at room temperature before use.

6.2.2 Plasmonic substrate fabrication

The nanoLCA substrate was employed to study the interaction between the surface plasmons and the chromophores. It was fabricated by the same method described in Chapter 2. SEM images in top-down (Figure 6.1a) and cross-sectional view (Figure 6.1b) show the NPs formed on the sidewalls of nanocups after the deposition of 9 nm Ti and 90 nm Au.

6.2.3 Modified nanoLCA-microplate sensing platform

The nanoLCA device can be cut to a desirable size, such as that of a microwell, without damaging its nanostructures (Figure 6.1c), because it is fabricated on a UV curable polymer (NOA 61). This enables a versatile sensing platform by using microplates; the existing conventional bench-top microplate reader can be utilized. Absorbance values
presented here were measured by BioTek Synergy HTX microplate reader. We verified that the absorbance curve of nanoLCA under water, which was obtained using this sensing platform, is identical to the results from the Cary 5G spectrometer (Figure 6.2). This proves the validity of the nanoLCA-microplate platform as a reliable sensing tool comparable to conventional UV-visible spectroscopy.

6.3 Results and Discussion

The nanoLCA can sense changes in refractive index due to its extraordinary optical transmission properties, similar to nanohole arrays.\textsuperscript{26,134} When one replaces air (1 RIU) on the device’s superstrate with water (1.33 RIU), the plasmon resonance wavelength of 530 nm, which corresponds to the dip in absorbance, shifts to 600 nm (Figure 6.1d). The transmission color (inset) also changes from green in air to orange in water. This sensitive response to changes in surrounding refractive indices is attributed to the circularly arranged NPs in the cup area.\textsuperscript{72}

The probability of light interacting with the molecules of a solution contained within a microwell increases when there is an efficient scatterer, such as a plasmonic surface, on the bottom of the microwell. Chromophores, which absorb light at a specific wavelength, experience an increased electromagnetic field intensity resulting from the enhanced scattered light and localized electric field near the nanocup area. Since the light scattering from the surface of the metal maximizes at the plasmon resonance wavelength,\textsuperscript{46} absorbance increases when the plasmon resonance wavelength matches the absorbance peak wavelength of the chromophores.
We performed absorbance measurements on various concentrations of three different colorants: Fd&C Red 40, Fd&C Yellow 5 and Fd&C Blue 1. The absorbance peak wavelength of each dye is as follows: 524 nm for Fd&C Red 40, 423 nm for Fd&C Yellow 5 and 627 nm for Fd&C Blue 1. Using the original solution packaged by McCormick, we prepared four different concentrations of each dye solution using dilution factors of $10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$. The microplate reader was used to measure absorbance for each 100 µL dye solution with and without nanoLCA devices attached to the bottom of the microwells. There is almost an identical increase in absorbance for both Fd&C Red 40 and Fd&C Yellow 5 when comparing the absorbance values of wells with devices to the ones without devices (Figure 6.3a – b). On the other hand, the absorbance of Fd&C Blue 1 is enhanced in the well with the device compared to the one without the device (Figure 6.3c).

The wavelength of maximum absorbance enhancement for Fd&C Blue 1 was not at the dye’s own absorbance peak position but at the plasmon resonance position: 600 nm (Figure 6.4). This indicates that the absorbance enhancement occurs due to the presence of plasmons. After light is absorbed by the plasmons, it scatters out from the metallic surface at 600 nm. Nearby chromophores absorb the scattered light so that there is much less transmission through the plasmonic surface at this resonance wavelength. Here a plasmon and a chromophore act as an energy donor and an acceptor, similar to Förster resonant energy transfer. Therefore, matching the plasmon resonance wavelength with a chromophore’s absorbance peak wavelength is critical to achieving absorbance enhancement, because it facilitates the energy transfer between plasmons and dye molecules. The schematic representation of this mechanism is shown in Figure 6.3d.
The quantification of ultralow numbers of proteins, unattainable by conventional protein quantification assays, is feasible with the absorbance enhancement of the nanoLCA. To meet the condition for absorbance enhancement, which requires an overlapping of the dye’s absorbance peak wavelength and the plasmonic wavelength of the nanoLCA device, we chose the Bradford assay; it utilizes Coomassie Brilliant Blue G-250 dye that has an absorbance peak at 595 nm. The conventional Bradford assay is reported to have a LOD as low as 20 – 100 µg mL⁻¹ of BSA on a microplate.\textsuperscript{129,136,137} Here we demonstrate how the integration of the nanoLCA device helps surpass that limit. Solutions composed of the following were added to one row of microwells that included the nanoLCA device and to another row of microwells without the device: 5 µL of BSA solution in concentrations ranging from 0 – 1000 µg mL⁻¹ mixed with 250 µL of the standard Bradford assay solution, resulting in a total volume of 255 µL. Please note that any future reference to the phrase “total solution” refers to a solution consisting of BSA and Bradford assay. After moderately shaking the microplate for 2 minutes, absorbance was measured using the microplate reader.

On additional experiments, we utilized 50 µL rather than 255 µL of the total solution. We assumed that a similar degree of absorbance enhancement would occur on the nanoLCA when using a reduced solution volume as long as the concentration of chromophores in the solution remained the same near the surface of the nanoLCA. If the same number of dye molecules are present in the evanescence field decay region, the absorbance enhancement should be the same. The evanescence field decay length of the nanoLCA is approximately 177 nm from the surface (Figure 6.5).\textsuperscript{138}
We firstly examined the effect of the nanoLCA on the absorbance from the reaction between the Bradford assay and the BSAs, of which concentrations are below the LOD of stand-alone Bradford assay (i.e., 20 µg mL⁻¹). With a total solution volume of 255 µL, absorbance values resulted from the BSA concentrations of 0, 2.5, 5 and 10 µg mL⁻¹ in the microwells without the device followed a random trend with increasing protein concentration (Figure 6.6a, inset); the BSA concentrations below the LOD were not detectable as expected. On the other hand, absorbance values of wells containing the nanoLCA devices showed a positive correlation with the BSA concentration, particularly for the values read at the plasmon resonance wavelength (Figure 6.6b). When the total solution volume was reduced to 50 µL, the absorbance measured in wells containing the nanoLCA still increased proportionally with the BSA concentration (Figure 6.6d). This indicates that the enhancement factor is mostly governed by the near-field interaction between chromophores and plasmons. In contrast, the wells without the device showed no distinguishable order in absorbance value for different protein concentrations (Figure 6.6c).

Figure 6.7 shows the LOD and the sensitivity for various BSA concentrations in microwells with and without the device. Figure 6.7a and Figure 6.7c show the absorbance value at 595 nm from the total solution of 255 µL. The results from the 50 µL total solution are shown in Figure 6.7b and Figure 6.7d. Two different BSA concentration ranges were used to calculate the sensitivities for: (1) a higher BSA concentration regime, i.e., 10 – 100 µg mL⁻¹ (Figure 6.7a – b), and (2) a lower BSA concentration regime, i.e., 0 – 10 µg mL⁻¹ (Figure 6.7c – d), which is below the LOD of the Bradford assay.
The LOD without the nanoLCA device is 20 µg mL\(^{-1}\) with the total solution volume of 255 µL (Figure 6.7a), but it becomes 100 µg mL\(^{-1}\) when the total solution volume is 50 µL (Figure 6.7b). The 5.1-fold decrease in solution volume produces a 5-fold increase in the lowest detectable concentration. The Beer-Lambert law explains this effect. It states that absorbance is proportional to beam path length, solution concentration, and molar extinction coefficient. With a 5.1-fold reduction in solution volume, the beam path length decreases proportionally, causing the absorbance value to decrease accordingly; thus, a conventional colorimetric microplate-based measurement requires a significant quantity of light absorbers (dye molecules) for reliable sensing of a reduced total solution volume. On the contrary, the LOD remained unchanged (i.e., 0.5 µg mL\(^{-1}\)) on the well containing the nanoLCA device despite a reduction in total solution volume. The nanoLCA with a total solution volume of 50 µL thus resulted in a LOD enhancement factor (EF) of 200 when compared to the LOD achieved without the nanoLCA.

Achieving a high sensitivity along with a low LOD is critical for sensing applications. The sensitivity is defined as the relative absorbance change from the 0 µg mL\(^{-1}\) divided by the change in BSA concentration, represented as the slope of the linearly fitted regression lines. Each sensitivity value for the two different BSA concentration ranges is presented in Table 6.1. When using a 50 µL total solution volume, sensitivity values obtained without the device were indeterminate for concentrations within the 0 – 100 µg mL\(^{-1}\) range, since it was below the LOD for microwells without the device (Figure 6.7b). The nanoLCA, however, not only detects BSA concentrations below 100 µg mL\(^{-1}\) but also has high sensitivity with much lower BSA concentrations of 0 – 10 µg mL\(^{-1}\) (Figure 6.7d). Comparing the slopes of the green lines for the high BSA concentration
ranges in Figure 6.7a and Figure 6.7b, the sensitivity of the device decreased 2.75-fold for the 50 µL total solution. This is contrary to the sensitivity drop of 11.74-fold for wells without devices.

We achieved the highest sensitivity when detecting a low concentration (i.e., 0 – 10 µg mL⁻¹) and using a 50 µL total solution volume on the nanoLCA. The sensitivity increased 1.22-fold when compared to the sensitivity achieved with a 255 µL total solution volume (Figure 6.7c and Figure 6.7d). The increase in sensitivity implies definite absorbance enhancement caused by the plasmonic effect on the device surface, as there are fewer chromophores in the reduced total solution volume. When smaller concentrations of chromophores result from the protein-assay reactions, more photons reach the plasmonic surface and get involved in the plasmon-chromophore interactions. Therefore, absorbance enhancement becomes dominant with smaller dye concentrations in the solution, and the sensitivity remains unchanged as long as dye concentration stays the same within the plasmon-active region.

The nanoLCA has shown consistent performance with reduced sample volume and low concentrations. This indicates that the nanoLCA is capable of detecting a much smaller number of target molecules with the same sensitivity by further reducing the sample volume. When we calculated the weight (or the molar number) of BSA molecules detected by the sensing platform at the lowest limit, the smallest detectable weight of BSA was 0.49 ng (7.37 fmol) for a 50 µL total solution volume. The smallest detectable weight of BSA without the device was 98.04 ng (1.48 pmol); thus, the EF of 200 in terms of the lowest detectable number of targets was achieved using the nanoLCA. The detectable
number of proteins can further be reduced to 29.4 pg (442 amol) by utilizing 1536-microplate wells with a 3 µL total solution volume.

Absorbance enhancement by the nanoLCA device is applicable to many other bio-assays that have absorbance peak positions matching the resonance wavelength. By varying the periodicity of the arrays, the cup diameter, the depth of the cup and/or the thickness of the Au (or Ag) deposited onto the nanoLCA pattern, one can tune the resonance wavelength, enabling extensive bio-sensing applications.

6.4 Conclusions

In conclusion, we have shown that a plasmonic nanoLCA substrate serves as an effective absorbance amplifier when integrated with a conventional microplate. Using the modified microplate with the plasmonic absorbance enhancer on the well-bottom, ultralow protein quantification was achieved. The energy transfer between plasmons and chromophores occurs when the plasmon resonance wavelength matches the chromophore’s absorbance peak wavelength. The near-field coupling between the plasmons and dye molecules helps reduce the sample volume requirement (e.g., by hundreds of microliters) by ~5-fold. When using the Bradford assay during BSA detection, the nanoLCA device achieves a consistent LOD of 0.5 µg mL$^{-1}$ regardless of the total solution volume, resulting in an EF of 200 compared to stand-alone Bradford assays. The consistent LOD despite the reduction in solution volume suggests the capability of the nanoLCA device to detect a much smaller number of proteins, potentially detecting as few as hundreds of molecules or even a single molecule. Given the nanoLCA sensor’s high sensitivity even with low sample concentrations, its low sample volume requirement, and its cost-saving integration
with existing benchtop microplate readers, it may have broad applications in early clinical diagnoses.
6.5 Figures

Figure 6.1 SEM images of the nanoLCA device (a) in a top-down view and (b) in a cross-sectional view. (c) Photograph of the microplate-nanoLCA sensing platform. (d) Absorbance spectra of the nanoLCA device in air and in water. Insets are the transmission images of the nanoLCA. (e) Schematic representation of absorbance enhancement from nanoLCA device (shown by an atomic force micrograph). Dye molecules used are the Coomassie Brilliant Blue and the red arrows indicate the scattered field from the nanoLCA.
Figure 6.2 Comparison between absorbance spectrum measurements from microplate reader (BioTek Synergy HTX) and conventional spectrometer (Cary 5G) corresponding to nanoLCA device with water on surface. Absorbance measured by microplate reader is almost identical to that measured by the spectrometer. This verifies the validity of the nanoLCA device – 96-microwell sensing platform as an optical characterization tool.
Figure 6.3 Measured absorbance values of (a) 100 µL Fd&C Red 40, (b) 100 µL Fd&C Yellow 5 and (c) 100 µL Fd&C Blue 1 with and without nanoLCA device attached to microplate. Absorbance values plotted at each maximized absorbance peak wavelength. (d) Schematic representation of energy diagram to show energy transfer mechanisms between plasmon and dye molecule.
**Figure 6.4** Absorbance spectra associated with 100 µL Fd&C Blue 1. Measurements were taken in wells (a) without devices and (b) with devices.
Figure 6.5 Numerical calculation of the evanescent field decay length from the nanoLCA structure using the finite-difference time-domain methods. (a) Electric field distribution at a resonance wavelength with a surrounding media of 1.40 RIU. (b) Electric field intensity with respect to z-direction. Estimated evanescent field decay length ($\Delta \delta$) is 177.26 nm.
Figure 6.6 Absorbance spectra from BSA-Bradford assay reaction in wells (a, c) without nanoLCA device and (b, d) with nanoLCA device. Two different total solution volumes of (a, b) 255 µL and (c, d) 50 µL were tested. Each curve was resulted from different BSA concentration: 0 µg mL⁻¹ (black solid lines), 2.5 µg mL⁻¹ (red dashed lines), 5 µg mL⁻¹ (blue dotted lines) and 10 µg mL⁻¹ (pink dash-dotted lines). Insets in (a) and (c) show a random trend of absorbance at 595 nm with increasing BSA concentration [µg mL⁻¹] (x-axis) for wells without the device.
Figure 6.7 Relative absorbance changes with increasing BSA concentration with and without nanoLCA device. Change in absorbance values at 595 nm with total solution volume of (a, c) 255 μL and (b, d) 50 μL presented. (a) and (b) were plotted with BSA concentration range of (0, 100) [μg mL\(^{-1}\)] and (c) and (d) plotted with range of (0, 10) [μg mL\(^{-1}\)]. Slopes of green lines in (a) and (b) represent sensitivities of device in BSA concentration range of (10, 100) [μg mL\(^{-1}\)]. Slopes of blue lines in (c) and (d) are sensitivities in lower BSA concentration range of (0.5, 10) [μg mL\(^{-1}\)]. Gray dashed lines are where detection is not possible.
Table 6.1 Set of sensitivity values (ΔA [a.u.]/Δc [µg mL⁻¹]) for different total solution volumes and BSA concentration ranges. The sensitivity of microwells without the nanoLCA for the range of 10 – 1000 µg mL⁻¹ dropped by 11.74-fold when total solution volume decreased from 255 µL to 50 µL. On the other hand, the sensitivity of microwells with the nanoLCA for the same concentration range had a 2.75-fold decrease. For the range of 0.5 – 10 µg mL⁻¹, sensitivity with the nanoLCA increased by 1.22-fold with the same total solution volume change.

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CHAPTER 7
HOT-SPOT ENGINEERING FOR SERS ON A NANOPARTICLE-NANOCUP ARRAY HYBRID STRUCTURE

In this chapter, enhanced SERS performance is demonstrated by using the previously discussed NP-nanoLCA structure. The NP-nanoLCA structure shows strong localized electric field confinement between the NP and the nanocup. The resulting “hot spots” enhance the SERS performance of the nanoLCA. The control over the number of NPs assembled on the nanoLCA substrate reveals the strong relationship between the density of NPs and the SERS enhancement factor. This chapter will be reproduced as an article for a submission to a peer-reviewed journal with a co-author, Te-Wei Chang, and the corresponding author, Gang Logan Liu*.

7.1 Introduction

The signal amplification without losing SNR is critical in sensing and detection. Raman scattering, which indicates unique molecular fingerprints (e.g., the molecular vibrational states), is one of extremely weak signals compared to the fluorescence; thus, the surface enhanced Raman scattering has gained huge attention for the last decades. The advances in SERS technologies have accomplished the detection of cancer biomarkers, glucose, infection related disease biomarkers, drugs or even pesticide residues on fruits. The advantage of using SERS in molecular detection is its direct quantification of target analytes without optical labels (e.g., fluorophores and chromophores) even in complex media.
Many SERS substrates were fabricated with Ag rather than Au. As Chapter 1 described, Au has more damping of plasmon oscillation by having interband transition in the visible light range. Ag, however, is highly unstable when the target analytes are in high electrolyte concentrations. Ag is easily oxidized and highly reactive in a high salt concentration condition compared to Au, so the application of Ag SERS substrate is relatively limited. Therefore, plasmonic SERS substrates, consisted of relatively more stable materials, are in demand. Here improving the SERS performance of Au plasmonic substrate is demonstrated by hot-spot engineering. By adding NPs on the plasmonic substrate, more plasmons are introduced in the sensing surface and the resulting plasmon coupling between the plasmons of NP and nanostructure induces strong electric field confinement; consequently, the SERS is enhanced by the strong hot-spot formation.

7.2 Materials and Methods

7.2.1 Fabrication of NanoLCA and NP-nanoLCA

As Chapter 2 and Chapter 4 described, the nanoLCA and the NP-nanoLCA were firstly fabricated as a base Au plasmonic substrate. The NP-nanoLCA was then prepared by assembling the cysteamine SAM on the Au nanoLCA surface and incubating Au NP solution (2.0×10^{11} particles/mL) for 24 hours.

7.2.2 Raman measurements

The probe molecules used in the experiments were rhodamine 6G (R6G) and trans-1,2-bis(4-bipyridyl)ethylene (BPE). Each chemical was purchased from Sigma-Aldrich. 2 µL of 10 nM, 100 nM, 1 µM, 10 µM and 100 µM R6G were dropped on the plasmonic substrates. Prior to the Raman measurement, the solvent was evaporated. A monolayer of
BPE was prepared to measure its Raman signal. Incubation of 5 mM BPE solution (in ethanol) on the devices for 24 hours and washing and drying the devices produced the BPE SAM.

The Raman signals of these probe molecules were detected by Horiba confocal Raman instrument with 632 nm laser source. The laser power was 120 µW. In order to test the influence of the surrounding refractive index on the SERS substrate, Raman spectra was measured both in air and in water on the nanoLCA. The same measurement condition was used for collecting Raman spectra of the same target molecules on the NP-nanoLCA.

### 7.2.3 FDTD simulations

The same nanoLCA and NP-nanoLCA models used in Chapter 3 were employed here. The NP-nanoLCA models were the nanoLCA structures with four, five, six, seven and eight 30 nm NPs in each nanocup. The electric field distributions in 1 RIU and 1.33 RIU were collected at each resonance wavelength.

### 7.3 Results and Discussion

The Raman spectra of 10 µM R6G were measured on the nanoLCA and the NP-nanoLCA with the integration time of 1 sec (Figure 7.1a). This 1 sec of integration time is relatively small compared to other SERS reports (e.g., from 10 sec to 30 sec are commonly used for SERS measurements with a mW range laser power). When the NPs are assembled on the nanoLCA structure, the intensity of each characteristic R6G Raman peak (i.e., 607.2, 770, 1177, 1308, 1357, 1504, 1572, 1644 cm\(^{-1}\)) was increased. The peak at 1357 cm\(^{-1}\), which represents the vibrational mode of aromatic C-C stretching, was used
for calculating the enhancement factor. The maximum of 54.74-fold peak intensity increment was observed from the NP-nanoLCA when compared to the nanoLCA.

Considering the nanoLCA’s colorimetric properties, or its plasmon resonance wavelength shift with increasing surrounding refractive indices, we estimated that the measurement in air (1 RIU) and in water (1.33 RIU) would result in different enhancement factors. As the plasmon resonance wavelengths of the nanoLCA are 530 nm and 596 nm in air and in water, respectively, the measurement in water results in the resonance wavelength closer to the laser excitation wavelength (632 nm). As Figure 7.1b shows, the Raman intensity measured in water was much larger than those in air (Figure 7.1b). We confirmed that both nanoLCA and NP-nanoLCA had the additional SERS enhancement in the wet-state. The nanoLCA had an average of a 2.16-fold and the NP-nanoLCA had a 2.17-fold of enhancement. Similar SERS enhancement was observed for nanoLCA and NP-nanoLCA, because the resonance peak positions of the nanoLCA and the NP-nanoLCA in water were similar (i.e., 596 nm and 599 nm for nanoLCA and NP-nanoLCA).

At the plasmon resonance wavelength, the scattering electric field is strong at the metallic surface. The SERS is thus more enhanced when the resonance wavelength is closer to the excitation laser wavelength. The SERS enhancement by matching a plasmon resonance wavelength with the excitation laser wavelength has been reported.\textsuperscript{148,149} Most reports achieved the plasmon resonance matching by designing a new SERS substrate; however, the use of colorimetric plasmonic substrate could avoid the laborious and time-consuming extra substrate fabrications. The plasmon resonance of a colorimetric sensor is easily tuned by controlling the surrounding refractive index.\textsuperscript{150} Furthermore, the
enhancement of the SERS in the wet-state implies that the nanoLCA or the NP-nanoLCA is more useful for biosensing, which typically involves buffer solutions, electrolytes, or cell culture media. As the direct target analysis without drying the solvent is more favorable for those biomedical applications, colorimetric nanoLCA or NP-nanoLCA can be a great SERS substrate choice.

R6G Raman spectra with increasing concentrations were measured on the NP-nanoLCA in water (Figure 7.1c). The lowest detectable concentration was 100 nM. As we put 2 µL R6G solution (100 nM) over the area of 1 mm radius, the density of 63.66 fmol/mm² is obtained. When we consider the actual number of R6G molecules under the laser spot size of 6.06 µm with 20X objective lens, the Raman signal was collected from 1.836 attomol R6G. Therefore, the NP-nanoLCA serves as a good SERS platform that can detect down to attomol range. When we consider the number of R6G molecules per nanocup (the periodicity is 350 nm), approximately 4,696 R6G molecules per nanocup were detected on the NP-nanoLCA.

R6G Raman spectra from 20 different spots over the 1 mm × 1 mm region were measured from the NP-nanoLCA surface (Figure 7.1d). Despite the semi-randomly assembled NPs on the nanoLCA structure, uniform SERS over a large area was obtained. When we consider the laser spot size, which is approximately 6 µm, the Raman signals are collected from at least 280 nanocups. This avoids random SERS signals measured from nanocups with different NP numbers, showing average SERS performance over these NP-nanoLCA ensembles. As the uniform SERS performance over a large area is another important requirement for a good SERS substrate, the NP-assembled nanoLCA is
confirmed to be a good SERS substrate candidate. This implies that the NP assembly is indeed a cost-effective and useful technique for improving SERS properties of a given nanostructure and is applicable to many other existing SERS substrates.

BPE Raman spectra were measured on each nanoLCA and NP-nanoLCA substrate. Figure 7.2a and Figure 7.2b show the Raman spectra measured in air and in water, respectively. Similar to the R6G results, further SERS enhancements by 2.24-fold for nanoLCA and 2.43-fold for NP-nanoLCA were achieved in water when compared to those collected in air. In addition, the NP-nanoLCA consistently resulted in larger Raman enhancement than the nanoLCA did; an average of 18.05-fold larger BPE Raman peak intensity was observed on the NP-nanoLCA when compared to the results from the nanoLCA. Although the enhancement factor of the NP-nanoLCA with respect to the nanoLCA for detecting BPE was smaller than R6G detection results, the NP assembly on the nanoLCA surface certainly improved the SERS performance. We speculate that the pre-coated cysteamine SAM on the NP-nanoLCA surface is the main reason why the enhancement was smaller for the BPE than the R6G detection. During the BPE immobilization, the exchange of BPE with cysteamine occurs on the surface of NP-nanoLCA. As the regular nanoLCA does not have cysteamine SAM, less dense BPE molecules are adsorbed on the NP-nanoLCA than on the nanoLCA; thus, smaller number of BPE was detected on the NP-nanoLCA. This implies that the actual SERS enhancement by the NP-nanoLCA will be greater than 18.05-fold when comparing the nanoLCA’s SERS performance for detecting the same number of BPE molecules.
The numerical analysis to identify the electric field distributions on the nanoLCA and the NP-nanoLCA was performed using the FDTD simulation (Figure 7.3). The overall electric field intensity from the results from the surrounding refractive index of 1.0 RIU (Figure 7.3a) was smaller than those results collected in 1.33 RIU (Figure 7.3b). For instance, the maximum electric field intensity of the NP-nanoLCA with eight NPs in the 1.33 RIU was 2.87-fold larger than the one in 1.0 RIU. The same comparison for the NP-nanoLCA with four NPs resulted in the 2.34-fold larger electric field intensity in water than in air. The larger electric field confinement or stronger hot-spot intensity in 1.33 RIU than in 1.0 RIU explains why better SERS performance was observed in wet-state than in dry-state for detecting both R6G and BPE. In addition to the electric field distribution, the smaller gap between the laser excitation wavelength (632 nm) and the plasmon resonance wavelength in water (close to 600 nm) supports larger SERS signal in wet-state.

7.4 Conclusions

A simple and cost-effective method for improving SERS on a colorimetric plasmonic substrate was accomplished by the self-assembly of NPs along the surface of nanoLCA structure. Larger hot-spot density by adding NPs on the nanoLCA substrate demonstrated improved SERS performance. The maximum of 54.74-fold Raman intensity enhancement was observed for R6G detection on the NP-nanoLCA compared to the original nanoLCA substrate. Additional improvement of the SERS performance for the detections of R6G and BPE was achieved by tuning the plasmon resonance wavelength closer to the laser excitation wavelength. The colorimetric properties of these substrates facilitated the plasmon resonance tuning through the control over the surrounding media’s refractive indices.
Figure 7.1 (a) Raman spectra of 10 μM R6G on the nanoLCA (black curve) and the NP-nanoLCA (red curve), taken with 1 sec integration time and in air. (b) Raman spectra of 100 μM R6G on the NP-nanoLCA taken in air and in water. (c) Raman spectra of R6G in different concentrations, taken on NP-nanoLCA in water. (d) Raman spectra of R6G at 20 different spots on the NP-nanoLCA substrate.
Figure 7.2 Raman spectra of BPE on NP-nanoLCA (red curve) and nanoLCA (black curve), (a) taken in air and (b) taken in water.
Figure 7.3 The FDTD simulated electric fields (|E|) with increasing number of NPs assembled inside the nanocup. The electric field distributions are collected in the surrounding refractive index of (a) 1 RIU (air) and (b) 1.33 RIU (water).
CHAPTER 8
SUMMARY AND FUTURE WORKS

8.1 Summary

The plasmonic effects on fluorescence, absorbance and Raman scattering were studied on nanocup array structures (i.e., nanoLCA substrate), which show the extraordinary optical transmission phenomena. The unique colorimetric properties of the nanoLCA structure enabled one to easily tune the plasmon resonance wavelength by controlling surrounding medias’ refractive indices. Subsequent changes in the resonance condition influenced the optical interactions among the surface plasmons and the nearby molecules. The most dominant parameter for molecular optical signal enhancement was the larger scattering cross-section of the nanoLCA by facilitating the absorption or the emission of photons associated with the energy relaxation within the molecular electronic states. When a fluorophore was in close proximity to the nanoLCA, the fluorescence enhancement was maximized when the resonance wavelength was located at the fluorescence emission band. The absorbance amplification was achieved when the resonance wavelength matched the absorbance peak of a chromophore. Raman scattering was enhanced when the resonance wavelength was close to the laser excitation wavelength. All of these molecular optical signal amplifications were achieved without re-constructing the plasmonic nanostructures; a simple yet effective fine-tuning of the resonance wavelength through a gradual increase of the surrounding refractive indices allowed understanding of these signal amplification mechanisms.
The sensitivity improvement through the surface plasmon coupling effect was achieved by self-assembling plasmonic NPs on the plasmonic nanoLCA. As a result of the NP self-assembly, three-dimensional circular arrays of NPs along the sidewall of nanocup were formed. Control over the amount of NPs assembled in each nanocup tuned the sensitivity of the label-free colorimetric chemical and bio-sensing. In addition to the refractive index sensing, Raman scattering was enhanced through the dense hot-spot formation.

A brief summary of each chapter is as follows:

• Chapter 2: A plasmonic nanocup array structure showed colorimetric properties with a single prominent extraordinary optical transmission peak in the visible light range. The FDTD simulations proved that the presence of the sidewall metallic layer on a nanocup array structure is critical for achieving a single transmission peak, rather than multiple peaks, in the visible light range. Similar optical properties to the Lycurgus cup were observed by showing the green transmission and the orange reflection in air. The resonance wavelength linearly shifted with increasing surrounding refractive index.

• Chapter 3: The circular arrangement of hemispheric silver NPs achieved colorimetric properties while retaining the FWHM of the plasmon resonance peak in a broad range of surrounding media refractive indices (i.e., from 1.0 RIU to 1.6 RIU). The vertical out-of-plane arrangement of each NP circular array in a nanohole enhanced the SNR. High electric field confinement at the interface between the NPs and the supporting substrate indicated that both substrate material’s dielectric function and morphology of the NP influence the resonance conditions. The circular arrays of NPs arranged in the nanoholes
achieved the largest surface plasmonic sensitivity to the refractive index change with the largest SNR among metallic NP square arrays, toroid structure, circular arrays on a flat surface, and circular arrays arranged in nanoholes.

- Chapter 4: Hybrid NP and nanocup array structure showed a better refractive index sensitivity than the regular nanocup array (i.e., nanoLCA) structure. Additional heterogeneous surface plasmon coupling between the NP and the nanocup resulted in strong localized electric field confined between the NP and the nanocup. The number density of hot-spot, induced by this heterogeneous surface plasmon coupling, increased proportionally with the number of NPs assembled at the brim of the nanocup. The sensitivities for the bulk refractive index sensing and the antigen-antibody (i.e., biotin-streptavidin) conjugation detection were improved when the NPs were densely assembled on the nanoLCA substrate. The limit-of-detection was also improved by at least 10-fold in comparison to the regular nanoLCA.

- Chapter 5: The optofluidic fluorescence enhancement tuning by using a colorimetric nanoplasmonic substrate (i.e., nanoLCA) was accomplished. Two fluorophores, each of which was assembled by using a thiolated ssDNA, showed different optimal fluorescence enhancement conditions on the same plasmonic nanoLCA substrate. The different fluorescence emission enhancement trends of these fluorophores under the same plasmon resonance shift, associated with the surrounding refractive index change, verified the importance of matching the resonance wavelength with each fluorescence emission band in order to maximize the enhancement factor. When the fluorescence was enhanced, the photostability and the quantum yield were improved. As the local refractive index
change affects the plasmon resonance condition and eventually the fluorescence enhancement factor, the optofluidic fluorescence enhancement will be useful for detecting local binding of other drug or biological molecules on a fluorescently tagged object in complex media.

• Chapter 6: High throughput quantitative sensing using a limited sample volume was accomplished by a modified 96-microwell plate with the nanoLCA substrates on the well-bottom. The nanoLCA detected 200-fold smaller protein (i.e., BSA) concentrations with 5.1-fold smaller sample volume than the stand-alone regular protein bioassay (i.e., Bradford assay). The plasmons excited on the nanoLCA served as an energy donor to the chromophores that are in close proximity to the nanoLCA surface. This near-field plasmon – chromophore interaction was accomplished when the plasmon resonance was located at the wavelength where the chromophores absorbed photons the most.

• Chapter 7: A simple yet scalable and cost-effective method of improving the SERS was demonstrated without re-designing a new substrate. Self-assembling 3D circular arrays of NPs on the gold plasmonic substrate (nanoLCA) achieved enhanced Raman scattering by 54.7-fold for detecting small concentration of R6G and BPE on the sensing surface, compared to the regular nanocup array substrate. Tuning the plasmon resonance wavelength to match the laser excitation wavelength further boosted the SERS by approximately 2.2-fold. The numerical calculation of the electric field confirmed larger hot-spot intensity in water than in air and higher hot-spot density with more NPs assembled on the substrate.
8.2 Future works

This dissertation has primarily focused on the colorimetric sensing of a nanoplasmonic substrate and its effect on the optical signal amplifications such as fluorescence, absorbance and Raman scattering. Fluorophores and chromophores are the most commonly used optical labels to tag the actual disease-related targets for diagnoses in the clinics or labs. These optical labels’ signal enhancement will bring huge impact on the current diagnostic techniques by improving their sensitivity and the limit-of-detection. The colorimetric sensing and the Raman detections, on the other hand, allow label-free detections with high sensitivity; therefore, they are particularly useful in a resource-constrained environment. Although improved sensitivity and smaller LOD are demonstrated with several fluorophores and chromophores in the present work, the practical applications to the actual disease biomarkers or toxic analytes detections have not been demonstrated. Therefore, future efforts should focus on the detections of disease-related biomarkers or toxic materials in the actual patients’ blood or urine samples using the plasmonic colorimetric sensor (i.e., nanoLCA substrate). Collaborations with clinics or hospitals for testing the samples from patients will be required to extend the use of plasmonic sensors for broad and practical applications.

A single device, the nanoLCA, can simultaneously enhance a variety of optical signals from molecules as long as the plasmon resonance is well matched with the targets’ optical characteristic band; therefore, a multiplexed detection of fluorophore and chromophore tags and Raman scattering in one-pot will save tremendous amount of detection time and cost by detecting multiple targets in a single field of view on the sensor surface. In a form of microarrays, different surface functionalization of capture molecules
or proteins on a different local region is required. The use of microcontact printing will allow patterning arrays of self-assembled monolayers of capture molecules. Writing an algorithm, which automatically analyzes any changes in the absorbance, transmission, fluorescence and Raman signal from different sensing areas in the array, will facilitate the multiplex sensing with the multispectral imaging system.

In addition to the nanoLCA, new designs of plasmonic structures with colorimetric properties are required for a better sensitivity to the refractive index change and larger enhancement of fluorescence, absorbance and Raman scattering. Although the NP assembly technique improved the refractometric sensitivity and the SERS performance of the given nanocup array substrate without re-designing the structures, the resulting sensitivity is still smaller than those of the state-of-the-art plasmonic sensors. Therefore, applying the same technique to other plasmonic substrates can help reaching unprecedented sensitivity and optical signal enhancement factors. On the other hand, the reduction in the transmission intensity upon the NP assembly on the nanoLCA requires stronger light source intensity in order to have enough optical transmission signals. If one can increase the EOT intensity by designing new plasmonic structures, it will further save energy during the detections.

Lastly, I would like to highlight the merit of the colorimetric technique: its convenience, user-friendliness and cost-effectiveness can benefit end-users by making a user-friendly portable analytic tool without bulky signal transducers. I hope the optical colorimetric sensors based on plasmonics become one’s most accessible biomedical or chemical sensors integrated with widely used smart-phones or portable personal electronics
(e.g., watch and tablet). Equipped with image analysis software, the next optical “smart-sensors” will greatly change the current health diagnostic or environment monitoring system.
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