Chapter 2
Exploring the Unique Characteristics of LSPR Biosensing

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Abstract  Plasmonic biosensors based on the localized surface plasmon resonance (LSPR) of metal nanoparticles have been developed using both nanoparticle arrays and single nanoparticles. We introduce LSPR biosensing by describing the initial experiments performed using both model systems and disease biomarkers. LSPR shift-enhancement methods, exploitation of the short electromagnetic field decay length, and single nanoparticle sensors are discussed as pathways to further exploit the strengths of LSPR biosensing. Coupling molecular identification to LSPR spectroscopy is a significant aspect of biosensing. Therefore, examples from surface-enhanced Raman spectroscopy and laser desorption ionization mass spectrometry are provided. This chapter highlights examples which emphasize the unique characteristics of LSPR biosensing.

Introduction

In 1998, the first use of localized surface plasmon resonance (LSPR) biosensing was reported by Englebienne [1]. At that time, the Van Duyne group had been utilizing the unique properties of LSPR for over 2 decades, but from within the framework of a different field: that of surface-enhanced Raman spectroscopy (SERS). Beginning with the successful observation and explanation of surface-enhanced Raman

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scattering in 1977 by Van Duyne [2] and Creighton and colleagues [3], our group began fabricating roughened silver surfaces displaying LSPR properties for use in SERS. In 1993, we reported our first use of Ag film over nanosphere (AgFON) surfaces that had more well-defined surface roughness and periodicity [4]. These surfaces were made by depositing over one or more layers of nanoscale polystyrene spheres. Two years later, we began depositing silver films over monolayers of polystyrene spheres and removing the spheres from the substrate post metal deposition, creating arrays of silver nanoprisms on the substrate surface (Fig. 2.1a) [5]. These periodic particle arrays (PPAs) displayed the hallmarks of a plasmonic sensing material: selective wavelength absorption, as evidenced by a distinct maximum in the extinction band in the visible wavelength region, and enhanced electromagnetic fields at the particle surface, as evidenced by both theoretical calculations [6, 7] and experimental observations of enhanced Raman scattering of molecules at the particle surfaces [8].

While Englebienne had demonstrated a new application for plasmonic materials, he did not exploit the shift in extinction maximum for unaggregated colloids. The colorimetric properties of gold colloids were already well known, and had been used previously as a reporter for enzyme immunoassays [9] and DNA
Bioassays performed using solution-phase gold colloids were inherently limited, however, by the finite stability of the colloids themselves. For example, high ionic concentrations lead to colloid aggregation and precipitation, and the basic pH required for biomolecule-colloid attachment can denature proteins. Several research groups, including our own, had developed methods of fabricating surfaces coated with colloidal metal structures that could overcome the limitations of solution-phase colloids, but these were primarily used in SERS applications. In 1999, we began to tune the properties of our film-over-nanosphere (FON) and PPA substrates in order to optimize their optical properties, and we demonstrated the powerful refractive index sensitivity of these surfaces [11, 12]. Three years later, we applied these plasmonic surfaces to the detection of biotin-streptavidin interactions [11], in what was the first quantitative report of \( \Delta \lambda_{\text{max}} \) for LSPR biosensing.

Because proteins have a relatively high refractive index (RI ~ 1.5), probing protein interactions was a natural choice for utilizing the refractive index sensitivity of nanoparticles. In addition, the short electromagnetic (EM) field decay length \( (l_d \sim 5–10 \text{ nm}) \) of localized surface plasmons is on the same size scale as proteins, making biomolecules an ideal sensing target. In an early demonstration of LSPR biosensing using silver PPAs, Haes and Van Duyne demonstrated the quantitative detection of streptavidin-biotin interactions (Fig. 2.1c) [11]. The LSPR extinction maximum \( (\lambda_{\text{max}}) \) shifted to redder wavelengths upon streptavidin (SA) binding, with the magnitude of the shift increasing with SA concentration.

Plotting these shifts as a function of SA concentration and fitting to (2.1)

\[
\frac{\Delta R}{\Delta R_{\text{max}}} = \frac{K_{\text{a,surf}}[\text{SA}]}{1 + K_{\text{a,surf}}[\text{SA}]}
\]  

(2.1)

revealed a binding constant, \( K_{\text{a,surf}} \), of \( 10^{11} \text{ M}^{-1} \) (Fig. 2.2), where \( \Delta R \) is the measured response and \( \Delta R_{\text{max}} \) is the maximum response possible. The maximum response, \( \Delta R_{\text{max}} \), was calculated from (2.2)

\[
\Delta R = m(n_A - n_E) \left[ 1 - \exp \left( -\frac{2d}{l_d} \right) \right]
\]  

(2.2)

in which \( m \) is the refractive index sensitivity, \( n_A \) is the adsorbate refractive index, \( n_E \) is the external environment refractive index (N\(_2\) in this experiment), \( d \) is the adsorbate layer thickness, and \( l_d \) is the decay length thickness. Not only does (2.2) determine the maximum response expected, but it also depicts which experimental factors affect the LSPR \( \lambda_{\text{max}} \) response: refractive index sensitivity of the nanoparticles, change in refractive index, decay length of the nanoparticles, and adsorbate thickness. Based on measurements of the standard deviation in \( \lambda_{\text{max}} \), the smallest detectable wavelength shift for this system was 1.5 nm, providing a limit of detection of less than 1 pM. In subsequent work, Riboh et al. characterized biotin and anti-biotin interactions using the same silver nanoprisim arrays [12]. This work revealed larger shifts for anti-biotin binding than for SA binding, a result that was expected based on the larger size of antibiotin (MW ~ 150 kDa) compared to SA (MW ~ 63 kDa). However, despite larger shifts in \( \lambda_{\text{max}} \), the detection limit for
this system was only ~1 nM due to the weaker binding affinity between biotin and anti-biotin ($K_a = 4.5 \times 10^7 \text{ M}^{-1}$).

Although biotin-streptavidin and biotin-anti-biotin interactions provide an ideal system for biosensor development due to their well-studied interactions and predictable binding affinities, these systems have limited utility in real-world sensing applications. In a compelling demonstration of the potential of LSPR sensors to be used as clinical diagnostic devices for disease detection, Haes et al. detected an oligomeric form of the Alzheimer’s disease biomarker amyloid beta, known as amyloid-beta derived diffusible ligands (ADDLs) [13]. Using a sandwich assay format analogous to an enzyme-linked immunosorbent assay (ELISA), the binding of synthetic ADDLs to an antibody-functionalized silver nanoprism array was detected by redshifts in the nanoprism $\lambda_{\text{max}}$, the magnitude of which depended on ADDL concentration. The concentration of synthetic ADDLs exposed to the sensor was varied from $10^{-15}$ to $10^{-5} \text{ M}$ in order to characterize the sensor response and determine affinity constants. Two affinity constants emerged with values on the order of $10^8$ and $10^{12} \text{ M}^{-1}$, with variations most likely due to size-dependent differences in epitope recognition. Finally, the LSPR sensor was used to detect ADDLs in clinical samples from both living and deceased patients. Although the total number of samples evaluated was too small to define sensor accuracy, a comparison between diseased samples and age matched controls revealed significant differences in sensor response. In an assay for the presence of ADDLs in cerebral spinal fluid (CSF), the Alzheimer’s sample induced a total $\lambda_{\text{max}}$ shift of 33.9 nm, compared to a shift of 7.2 nm for the aged-matched control. Although additional studies are needed to prove that the LSPR sensor can meet standards

![Normalized LSPR shift, $\Delta R/\Delta R_{\text{max}}$, vs. [SA] response curve for the specific binding of SA to a biotinylated Ag nanobiosensor. The normalized experimental LSPR responses (solid circles) were calculated by subtracting $R_{\text{layer 1}}$ for the biotinylated Ag nanobiosensor from $R_{\text{layer 2}}$ after exposure to SA and dividing by $\Delta R_{\text{max}}$. All extinction measurements were collected in a N$_2$ environment. Reprinted with permission from ref. [11]. Copyright 2002 American Chemical Society](image_url)
required for clinical detection of Alzheimer’s disease, this study demonstrated the potential for LSPR assays to fill unmet needs in disease detection.

Finally, as the field of LSPR biosensing continues to grow, it is important to ask: what advantages do we gain by using nanoparticle-based sensors? As the predecessor to LSPR, surface plasmon resonance (SPR) sensors serve as a point of comparison. The introduction chapters of the book outlined the differences in these two sensing modalities, including the large differences in EM field decay length at the sensor surface. These differences in the EM field decay length between LSPR and SPR surfaces create an important advantage for LSPR sensing when detecting nanoscale analytes. As the name suggests, the LSPR response is dominated by events happening within a highly localized (~5–10 nm) region at the nanoparticle surface. Binding of nanoscale analytes, such as proteins, induces large shifts in the LSPR $\lambda_{\text{max}}$, while refractive index changes in the bulk media do not. Work by Yonzon et al. demonstrated the impact of these sensing volume differences in a study describing the binding of Concanavalin A to saccharide monolayers [14]. SPR and LSPR sensors functionalized with either mannose or galactose were exposed to micromolar concentrations of the lectin-binding protein Concanavalin A. During the dissociation phase, the SPR signal decreased by 60%, while the LSPR signal decreased by only 14% (Fig. 2.3). The larger signal decrease for the SPR sensor can be explained by the fact that a larger fraction of the SPR response arises from bulk media changes due to its relatively long EM field decay length. In fact, it was shown that by changing the aspect ratio of the silver

**Fig. 2.3** Real-time response of sugar-functionalized sensor as 19 μM of Concanavalin A was injected in the cell following buffer injection. (a) Mannose-functionalized SPR sensor, (b) galactose-functionalized SPR sensor, (c) mannose-functionalized Ag nanosensor, and (d) galactose-functionalized Ag nanosensor. The points are the experimental data. The *solid line* for the SPR measurement is composed of straight line segments connecting the experimental data. The *solid line* in LSPR measurement is a first-order adsorption kinetics fit to the data and should only be interpreted as a guide to the eye. Reprinted with permission from ref. [14]. Copyright 2004 American Chemical Society
nanoprisms of the LSPR sensor, the EM field decay length can be tuned to allow more or less detection of bulk media changes \([6, 15]\). For applications in which the analyte is small (\(<10\) nm) and binding occurs close to the substrate surface, LSPR provides improved sensitivity and spatial resolution compared to its SPR counterpart. That sensitivity, coupled with the vast variety of plasmonic nanostructures and surface chemistries available for use in LSPR sensing, make nanoparticles an ideal sensing substrate for a multitude of systems. The following sections of this chapter will outline how the unique properties of LSPR sensors can be exploited to achieve even greater sensitivity and used in conjunction with other analytical techniques to achieve molecular identification.

**Improving the Limit of Detection of LSPR Sensors**

*Methods to Enhance the LSPR Shift*

The introduction discussed the first experiments in LSPR biosensing that were based on the local refractive index change induced by biomolecules adsorbing close to the nanoparticle surface. The change in the local dielectric due to molecule adsorption was measured by a shift in the LSPR \(\lambda_{\text{max}}\). An ideal biosensor is both specific and sensitive to the target analyte. While experiments continually aim to quantify and improve biosensor specificity, sensitivity is often inherent to the specific biosensor system. Therefore, new methods are examined to increase the biosensor sensitivity; that is, increase the \(\lambda_{\text{max}}\) shift from the same amount of analyte.

The LSPR \(\lambda_{\text{max}}\) shifts are dependent on the nanoparticle sensitivity and the size of the bound molecules. Nanoparticle sensitivity can be experimentally determined by systematically changing the refractive index environment—for example, by adding successive layers of a dielectric material or changing the ambient solvent—and plotting \(\lambda_{\text{max}}\) shifts as a function of a refractive index. A linear fit to the data will reveal the nanoparticle sensitivity factor, \(m\), which is particular to nanoparticle composition, size, and morphology. The linear relationship is described in (2.3),

\[
\Delta \lambda_{\text{max}} = m \Delta n
\]  

(2.3)

where \(n\) is the refractive index. In general, nanoparticles with a higher aspect ratio are more sensitive to the local dielectric environment \([6, 15]\). The shift is also roughly proportional to the adsorbate molecule mass \([6, 7, 15]\). As such, a protein is expected to produce a significantly larger LSPR \(\lambda_{\text{max}}\) shift than a small molecule. However, in the last several years, methods have been developed to detect unexpectedly large shifts from molecules binding to the metal nanoparticle \([16–18]\). These large shifts are the result of a chromophore coupling to the LSPR of a nanoparticle and have also been observed from small molecules binding to protein receptors on metal nanoparticles. A different way to enhance the \(\lambda_{\text{max}}\) shift is to use plasmonic labels such as gold nanoparticles \([11]\). This section discusses in detail the methods to increase the LSPR \(\lambda_{\text{max}}\) shift using both resonant molecules and plasmonic labels.
Resonant Molecules

The interaction between the molecular resonance of a chromophore and the plasmon resonance of a nanoparticle was examined using several different chromophores [16]. A monolayer of non-resonant molecules would be expected to yield a shift consistent with (2.2). However, when a monolayer of chromophores resonant with the LSPR was probed, shifts were significantly larger or smaller than expected and were highly dependent on the overlap of the chromophore absorbance and plasmon resonance.

Work by Haes et al. examined the interaction between the molecular resonance of 
\[\text{[2,3,7,8,12,13,17,18-octakis(propyl)porphyrizinato]magnesium (II)}\,\text{(MgPz)}\]
and the plasmon resonance of Ag nanoparticles [16]. As depicted in Fig. 2.4, the experiments demonstrated that the shift was highly dependent on the amount of overlap between the molecular absorbance of MgPz and the LSPR. In Fig. 2.4, the \(\lambda_{\text{max}}\) shift due to the MgPz monolayer was plotted as a function of initial LSPR \(\lambda_{\text{max}}\) with the molecular absorbance of MgPz overlaid. It was observed that when the absorbance maximum of MgPz directly overlapped with the LSPR \(\lambda_{\text{max}}\), only a 2 nm redshift was observed. However, when the LSPR \(\lambda_{\text{max}}\) was slightly redder than the molecular absorbance peak, a large redshift of \(\approx 60\) nm was observed. These resonant cases contrast drastically with the non-resonant conditions (where there is little overlap between the molecular chromophore resonance and Ag nanoparticle plasmon resonance), in which \(\approx 20\) nm redshifts were observed. It was determined that the \(\lambda_{\text{max}}\) shift observed from a monolayer of MgPz tracked with the real component of the wavelength-dependent refractive index, which was extracted by the Kramers-Kronig transformation. (Note that the molecular absorbance spectrum is representative of the imaginary component of the refractive index.) The measured shift was largest when the real part of
the wavelength-dependent refractive index of the dye was greatest. These experiments demonstrated the LSPR shift enhancement that is possible by using a molecule resonant with the LSPR, a significant result considering many biological targets containing chromophores.

To extend the chromophore-plasmon research to a dye used in many SERS experiments (especially single-molecule SERS), Zhao et al. used rhodamine 6G (R6G) to probe the chromophore-plasmon interaction in a similar fashion to the MgPz work. The LSPR shift due to a monolayer of R6G was plotted as a function of initial LSPR wavelength along with the absorbance spectrum of R6G (Fig. 2.5). It is significant to note that three maxima were observed in the LSPR shift, whereas only two maxima are apparent in the R6G absorbance spectrum. Contrasting with results from the MgPz work, the LSPR shift from R6G did not track with only the real component of the dielectric (extracted by the Kramers-Kronig transformation). To probe the unique spectral features, the absorbance was measured using different R6G concentrations on a Ag surface and it was determined that three different forms of R6G were present: H-dimers, J-dimers, and monomers, each with slightly different absorbances. The three maxima of the LSPR wavelength shift in Fig. 2.5 were attributed to the three forms of R6G present on the Ag nanoparticle surface. Therefore, it was concluded that the LSPR wavelength shifts tracked with the dielectric constants (real and imaginary) of the three forms of dimers. These findings demonstrated that LSPR spectroscopy could provide information on electronic structural changes of adsorbates.

The work based on chromophores coupled to Ag nanoparticles motivated the study by Zhao et al. to examine the shift observed when the small molecule camphor (molecular weight = 152.25 g mol$^{-1}$) bound to the protein cytochrome...
The absorption spectrum maximum of CYP101 changes from 417 to 391 nm upon camphor binding. The peak shift is due to camphor replacing the water coordinated to the Fe$^{3+}$ of CYP101, causing the spin state of the heme iron to change from low to high spin [19–21].

To probe the effect of the molecular absorbance change of CYP101-Fe$^{3+}$ on the LSPR of nanoparticles, CYP101-Fe$^{3+}$ was covalently attached to a carboxylic acid-terminated SAM on the Ag nanoparticles via EDC coupling chemistry. Camphor was then added to the CYP101-functionalized nanoparticles. The LSPR $\lambda_{\text{max}}$ shift was measured after CYP101 bound to the SAM-Ag nanoparticle surface ($\Delta\lambda_1$) and after camphor binding to CYP101 ($\Delta\lambda_2$). Shifts were measured relative to the initial $\lambda_{\text{max}}$ of the SAM-functionalized Ag nanoparticles ($\lambda_{\text{max,SAM}}$) and the experiment was repeated for multiple Ag nanoparticle samples with varying LSPR $\lambda_{\text{max}}$ to probe the interaction of overlapping molecular and plasmon resonances. The results, depicted in Fig. 2.6, demonstrated the shifts were highly dependent on the LSPR $\lambda_{\text{max,SAM}}$ relative to the molecular absorbance of CYP101. When the plasmon was slightly red-shifted from the CYP101 absorbance, a large shift of over 60 nm was observed due to CYP101 binding. As the LSPR $\lambda_{\text{max}}$ moved farther from the CYP101 absorption, the red-shift ($\Delta\lambda_1$) decreased dramatically. When camphor bound to the CYP101, a blueshift was observed ($\Delta\lambda_2$).
When the LSPR $\lambda_{\text{max, SAM}}$ was close to the CYP101 absorbance, a large blueshift (nearly 40 nm) occurred. The $\Delta\lambda_2$ decreased as the plasmon resonance was tuned farther from the molecular absorbance; however, a blueshift was still observed.

If camphor did not affect the electronic state of CYP101, a small redshift would have been expected, given an increase in the local refractive index. However, the camphor altered the molecular absorbance of the CYP101 when it bound, specifically shifting the absorbance to bluer wavelengths and therefore blue-shifting the LSPR $\lambda_{\text{max}}$ [17]. This report was significant, as a small molecule binding to a protein may not have been a large enough refractive index change to induce a visible LSPR shift.

### Plasmonic Labels

While the $\lambda_{\text{max}}$ shift enhancement due to resonant interactions between chromophoric molecules and nanoparticle plasmons is dramatic, this signal enhancement technique is limited to dyes and biomolecules with specific absorption properties. To observe the same type of signal enhancement from non-chromophoric molecules, it was necessary to develop an alternative technique. Inspired by previous work on SPR sensors [22–24], we decided to explore the properties of nanoparticle-labeled biomolecules. In 1993 Buckle et al. described the first reported use of gold nanoparticle-labeled proteins for SPR response enhancement [24]. Human serum albumin (HSA) proteins were electrostatically conjugated to gold colloids, and then exposed to an SPR surface functionalized with anti-HSA antibodies. The detection limit for this assay was improved 1,000-fold compared to the nanoparticle-free assay.

Due to the very short (5–10 nm) decay length of LSPR sensors, large sensitivity losses arise when detection platforms utilize longer surface ligands or molecules, such as in the sandwich-style ELISA, that place the detecting molecule far from the nanoparticle surface and outside the sensing volume. We therefore hypothesized that nanoparticle-labeled detection molecules could have a large impact on the LSPR sensor $\lambda_{\text{max}}$ shift by increasing the analyte mass close to the nanoparticle surface. To test this hypothesis, we used the well-studied biotin/anti-biotin binding pair. Biotin was covalently immobilized on a Ag PPA surface, and subsequently exposed to anti-biotin that had been electrostatically conjugated to 20 nm Au colloids. As expected, the $\lambda_{\text{max}}$ shift was enhanced, with the degree of enhancement varying with the concentration of anti-biotin-nanoparticle (AB-NP) conjugate (Fig. 2.7) [25]. The enhancement peaked near 400%, at a concentration close to the $K_D$ of the biotin/anti-biotin interaction.

Based on previous theoretical [26–28] and experimental [26, 28–30] observations, we attributed the enhancement to two sources: (1) the larger refractive index of nanoparticle-labeled antibodies compared to un-labeled antibodies, and (2) plasmonic coupling between the Ag PPA and the Au colloid label. In contrast to a simple dielectric label (such as a glass bead), Au nanoparticle labels have the potential to induce much larger signal enhancements due to plasmonic coupling.
The existence of plasmonic coupling is evident in this study by the increase in full width half max (FWHM) of the LSPR sensor extinction peak that occurs upon AB-NP binding. The FWHM broadening is not observed for un-labeled anti-biotin. By optimizing both nanoparticle substrate and nanoparticle label in order to achieve the greatest plasmon overlap, we believe that even larger shift enhancements than what were reported in this study can be realized.

A fortuitous by-product of the antibody-nanoparticle conjugation was an increase not only in $\lambda_{\text{max}}$ shift, but also in binding affinity between biotin and anti-biotin. This increase can be attributed to the antibody polyvalency gained upon nanoparticle conjugation. Based on surface area calculations, each gold colloid is functionalized with between 8 and 30 antibodies, depending on antibody orientation. This means that each binding event between the biotinylated surface and the gold colloids is likely mediated by more than one antibody. It is well known that...

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**Fig. 2.7** Experiment schematic and LSPR spectra. (a) Biotin is covalently linked to the nanoparticle surface using EDC coupling agent, and anti-biotin labeled gold nanoparticles are subsequently exposed to the surface. LSPR spectra are collected before and after each step. (b) LSPR spectra before (solid black) and after (dashed blue) binding of native anti-biotin, showing a $\Delta\lambda_{\text{max}}$ of 11 nm. (c) LSPR spectra before (solid black) and after (dashed red) binding of anti-biotin labeled nanoparticles, showing a $\Delta\lambda_{\text{max}}$ of 42.7 nm. Adapted and reprinted with permission from ref. [25]. Copyright 2011 American Chemical Society
polyvalent antibodies bind to antigens with higher affinity compared to antibody monomers. Because biosensor sensitivity is inherently dependent on the strength of the binding interaction between surface and analyte, the improved affinity induced by nanoparticle-antibody conjugation offers an additional source of sensitivity for the plasmonic label-enhanced LSPR sensor.

**Exploiting the Short EM Decay of LSPR**

As mentioned briefly in the introduction, one of the chief advantages of LSPR sensors is the very short decay length of the EM field at the nanoparticle surface. This short decay length is an inherent property to LSPR, and arises due to the confined nature of the plasmons in a nanoscale material [31]. As a result of this short decay length, LSPR sensors are most sensitive to refractive index changes occurring within 5–10 nm of the nanoparticle surface [6, 7]. This highly localized sensitivity confers huge advantages when the analyte in question is on the nanoscale, or when refractive index changes occur very close to the nanoparticle surface. In this section we discuss the advantages of LSPR in detecting nanoscale materials utilizing protein conformational studies by Hall et al. as the prime example [32]. Additionally, the short EM field decay length of LSPR can even be advantageous for gaseous analytes incorporated into a partition layer.

**Protein Conformational Changes**

Biological and materials research often requires tools that can characterize dynamic changes in protein conformation and immobilized molecules. Techniques based on NMR [33], FRET [34], and X-ray scattering [35] are widely used. SPR sensors have been used to detect conformational changes in proteins, but due to the large sensing volume at the SPR surface, detection generally requires that the protein be immobilized in 3D dextran layers, where observed signals are convoluted by matrix effects [36]. LSPR sensors have been used to detect lipid membrane formation over a hole-array substrate [37] and solution-phase colloid aggregation induced by protein denaturation [38]. LSPR-based approaches are advantageous because, in addition to highly localized detection capabilities, they provide an intense signal that does not bleach and allow non-destructive measurements over long periods of time. Limits of detection of LSPR sensors have been reduced in recent years by the development of instrumentation capable of accurately measuring spectral shifts of only hundredths of nanometers [32, 39]. Chapter 11 of this book highlights some of the advances that have been made in LSPR instruments, advances that contributed to our ability to detect small wavelength shifts in response to conformational changes.

The intracellular calcium-binding protein calmodulin (CaM) was used to illustrate the sensitivity of LSPR sensors to surface-adsorbate conformation. Calmodulin
regulates cellular signaling pathways via calcium ion-dependent switching between open and closed conformational states. A recombinant cutinase-calmodulin-cutinase (CutCaMCut) construct was used to flexibly tether CaM to the surface [40] (Fig. 2.8a). Binding of CutCaMCut to the LSPR sensor was accompanied by a 3.6 nm redshift in the sensor $\lambda_{\text{max}}$, with a standard deviation in $\lambda_{\text{max}}$ of only $4 \times 10^{-3}$ nm (Fig. 2.8b). Thus, the signal:noise ratio for this immobilization was over 900.

Exposure of the CutCaMCut functionalized LSPR surface to 2 mM CaCl$_2$ resulted in a $\lambda_{\text{max}}$ redshift of 2.2 nm. This redshift was reversed upon chelation of the calcium ions using 2 mM ethylene glycol tetraacetic acid (EGTA). The $\lambda_{\text{max}}$ shifts induced by calcium binding and unbinding were reproducible over several cycles, with a standard deviation in $\Delta \lambda_{\text{max}}$ of 0.18 nm (Fig. 2.9b). To verify that the changes in $\lambda_{\text{max}}$ were a result of protein conformation, two controls were performed using a sensor with no surface bound protein and a sensor functionalized with a non-calcium sensitive cutinase-cutinase (CutCut) construct. Both controls exhibited only small reversible $\Delta \lambda_{\text{max}}$ changes which were opposite in trend to what was observed in the presence of calmodulin, demonstrating that the conformational changes in calmodulin were the source of the observed spectral shifts.

Finally, the ability to monitor $\lambda_{\text{max}}$ in real-time enabled the determination of opening and closing rates for the calmodulin conformational change (Fig. 2.9c). First-order exponential fits to the real-time $\lambda_{\text{max}}$ shifts using (2.4),

$$
\lambda_{\text{max}} = \lambda_{\text{max}(0)} + A \exp(-t/\tau) + Bt
$$

(2.4)
where $\lambda_{\text{max}(0)}$ is the initial $\lambda_{\text{max}}$ value, $\tau$ is the rate in seconds, and $Bt$ is a correction factor for linear baseline drift, revealed an opening rate of $0.034 \text{ s}^{-1}$ and a closing rate of $\sim 0.127 \text{ s}^{-1}$. These rates are slower than reported rates in solution, which we attributed to steric interactions between proteins due to their immobilized density on the surface, as well as the added mass from the second cutinase moiety in the construct. Although we expect differences between solution measurements and measurements made on a surface, the demonstrated ability to measure rates of binding and conformational change is nonetheless significant because the rates of protein-mediated reactions play an integral role in regulating cellular pathways, and disruptions to these rates can lead to diseased states. Indeed, the surface measurements described in this study may offer a good model for observing the rates of proteins that are anchored in vivo, such as lipid membrane receptors and channels. In addition, the results from this study demonstrated a novel means to reversibly switch a plasmon signal using ionic concentration changes, and pave the way for more creative use of biomolecules in the development of small-molecule and ion sensors.

**Fig. 2.9** (a) Schematic representation of the reversible conformational changes of CutCaMCut immobilized on the LSPR nanosensor. (b) Plot of changes in the extinction maximum (1 Hz collection) of the sensor as the buffer is cycled between 2 mM CaCl$_2$ and 2 mM EGTA, a calcium chelator. (c) Closeup of one CaCl$_2$/EGTA cycle, with an exponential fit of $\lambda_{\text{max}} = \lambda_{\text{max}(0)} + A \exp\left(-t/\tau\right) + Bt$ (black) to the experimental data (red), where $\tau_{\text{open}} = 30 \text{ s}$, $\tau_{\text{close}} = 8 \text{ s}$, and $|B| < 0.0013 \text{ nm s}^{-1}$. Reprinted with permission from ref. [32]. Copyright 2008 American Chemical Society
Gas Sensing

Typical biomarker detection focuses on solution phase analytes; however, volatile biomarkers in the gaseous phase have been investigated for certain diseases, like lung cancer [41]. Therefore, it is significant to expand traditional solution phase detection schemes to gaseous media. Traditional SPR spectroscopy has been used for gas sensing due to the high sensitivity to bulk refractive index changes [42, 43]. Recent work in the Van Duyne lab has demonstrated that gas sensing is feasible using a high-resolution, low noise LSPR spectrometer. For example, N₂ and He have similar refractive indices, differing by only 2.62 × 10⁻⁴ refractive index units. However, it was demonstrated that even a small change in bulk refractive index was detectable using LSPR spectroscopy [44, 45]. More recently, Kreno et al. expanded LSPR gas sensing experiments to CO₂ and SF₆ gases [46]. Figure 2.10a depicts the λ_max as a function of time as the environment is switched between N₂ and CO₂ (gray) and N₂ and SF₆ (black). To increase the selectivity and sensitivity of the LSPR sensor, thin partition layers are required. The Ag nanoparticle substrate was functionalized with a metal-organic framework (MOF) such that select gaseous analytes of interest would be close to the nanoparticle surface, and therefore detected. Figure 2.10b compares the LSPR response of bare Ag nanoparticles (gray) to MOF-functionalized Ag nanoparticles (black) while switching between N₂ and CO₂. The MOF-functionalized nanoparticles demonstrated selectivity of CO₂ over SF₆ [46]. Since the EM field decay length of nanoparticles is only several nanometers, the environment beyond the partition layer is not detected. Therefore, the short EM field decay length utilized in LSPR spectroscopy is advantageous for gas sensing using a thin, selective partition layer, like a MOF.
Single-Nanoparticle Sensors: Toward the Ultimate Detection Limit

Measurements of single nanoparticles have several benefits to ensemble-averaged measurements. First, the single nanoparticles are readily implemented in multiplexed detection schemes since each unique nanoparticle possesses a distinguishable LSPR maximum. A second advantage is that the absolute detection limit (number of analyte molecules per nanoparticle) is significantly reduced. A third advantage of single-nanoparticle spectroscopy is the ability to measure the LSPR spectrum of individual particles with a high signal-to-noise ratio [47]. Additionally, single nanoparticles can be used for in vivo studies. These advantages provide motivation to examine the environmental dependence of single nanoparticle LSPR [47–49]. In this section, experiments utilizing resonant Rayleigh scattering spectroscopy to measure the LSPR $\lambda_{\text{max}}$ of single Ag nanoparticles are described. The first part details the response of the $\lambda_{\text{max}}$ of individual Ag nanoparticles to varying solvent environments, self-assembled monolayer formation, and protein adsorption [47, 49]. The second half describes a high-throughput wide-field single nanoparticle spectroscopy method. The wide-field method demonstrates the ability to not only measure $\sim10^2$ single nanoparticles simultaneously, but also measure the single-particle trajectory and scattering spectra of moving single Ag nanoparticles [50].

Single Nanoparticle Sensing

McFarland and Van Duyne [47] and Raschke et al. [48], independently reported the first sensing experiments with single nanoparticles. A dark-field scattering image of single Ag nanoparticles is depicted in Fig. 2.11a. McFarland and Van Duyne demonstrated sensitivity to SAM formation with multiple alkanethiols. Citrate-reduced Ag colloids were exposed to a hexadecanethiol (HDT) solution and a HDT monolayer formed on the Ag surface, displacing the citrate ions. These single-nanoparticle studies exhibited the adsorption of fewer than 60,000 HDT molecules on a single Ag nanoparticle resulting in a LSPR shift of 40.7 nm (Fig. 2.11b), thus demonstrating low zeptomole sensitivity [47].

The bulk refractive index sensitivity of individual citrate-reduced Ag colloids was measured by exposing the nanoparticles to various solvent environments and single nanoparticles with three different shapes were used to illustrate the effect of nanoparticle shape on refractive index sensitivity [47]. The nanoparticle shapes were inferred from TEM imaging [51], LSPR line shape, LSPR $\lambda_{\text{max}}$, and LSPR polarization dependence. Similar to the results from ensemble-averaged measurements, the LSPR $\lambda_{\text{max}}$ shifted to longer wavelengths as the refractive index of the solvent was increased (Fig. 2.11c). The particle with the largest aspect ratio (a rod-shaped particle) exhibited the highest refractive index sensitivity (235 nm RIU$^{-1}$), consistent with predictions from Mie theory. The refractive index sensitivity decreased with decreasing particle aspect ratio and the spherical nanoparticle exhibited a refractive index sensitivity of 161 nm RIU$^{-1}$ [47]. Additionally, the LSPR $\lambda_{\text{max}}$ response was monitored in real-time as an octanethiol SAM
The response exhibits first-order kinetics with a rate constant of 0.0167 s$^{-1}$, demonstrating that single-nanoparticle sensing platforms provide the same advantageous real-time behavior as nanoparticle arrays and SPR sensors [47].

The feasibility of single Ag nanoparticles for biosensing was demonstrated using the streptavidin-biotin system. The single Ag nanoparticles were functionalized with a mixed monolayer with carboxyl terminal groups, and amine-functionalized biotin was covalently attached to the nanoparticle via amide bond formation.
The biotinylated Ag nanoparticles were incubated in 10 nM streptavidin, rinsed, and dried in N₂. The λ_max of the nanoparticle shifted +12.7 nm as a result of the streptavidin binding to the biotinylated Ag surface (Fig. 2.12). This shift is approximated to result from less than 700 streptavidin molecules binding to the single Ag nanoparticle [49]. Similarly, Raschke et al. demonstrated the use of single biotinylated-Au nanoparticles to detect streptavidin binding [48].

Single-nanoparticle sensing has proven to be an effective method for detecting small amounts of molecules. However, it has been an under-exploited area of research because it is an extremely low-throughput method, as only one particle can be measured at a time. Additionally, it has been a challenge to measure the LSPR spectra of moving single nanoparticles. In the next part of this section, a high-throughput wide-field apparatus is described, in which moving particles can be measured and characterized.

**Wide-Field Single Nanoparticle Imaging and Spectroscopy**

Typical methods of measuring single nanoparticle LSPR spectra require the nanoparticle to be isolated in a narrow field of view determined by the slit width of the spectrometer [47, 52, 53]. This method is inefficient for collecting multiple single nanoparticle spectra and limits experiments to particles that are immobile. Spatial tracking of moving single particles has been of interest to the biological community due to the new information it provides on the organization of cell membranes, particle movement on cell surfaces, and the effects of the external cell environment [54–60]. Single particle tracking is used to determine the diffusion coefficients of individual particles, allowing for modes of motion inside cells to be characterized. The work reported here describes results obtained from a wide-field LSPR imaging apparatus which was used to simultaneously track and collect scattering spectra from hundreds of mobile nanoparticles.
The wide-field imaging method utilized dark-field microscopy and an LCTF to measure the resonant Rayleigh scattered light from multiple nanoparticles. The scattered light was sent through an LCTF, which has a continuously tunable transmission from 400 to 720 nm, to a CCD detector (Fig. 2.13). A wide-field intensity image was obtained from light scattered by multiple nanoparticles at each specified wavelength and the intensity of the scattering was integrated as a function of wavelength to construct single nanoparticle spectra. This wide-field method allowed for hundreds of single nanoparticle spectra to be measured simultaneously, providing a plasmon distribution of the specific nanoparticle sample [50].

Not only did each intensity frame have wavelength information, but it also had time information, since the LCTF was scanned with a specific time interval. This feature made it possible to measure moving particles. The location of each particle at a given time in the wide-field image was known, allowing diffusional dynamics of moving particles to be characterized (Fig. 2.14). Figure 2.14a, b display the LSPR scattering spectra and the single-particle trajectories, respectively, of three single Ag nanoparticles. The mean square displacement \( \langle r^2 \rangle \) was calculated from the nanoparticle trajectories and plotted as a function of time lag, \( t \) (Fig. 2.14c) demonstrating a linear relationship. For Brownian diffusion in two dimensions, the relationship between \( \langle r^2 \rangle \) and \( t \) is expected to be linear with the slope equal to \( 4D \), where \( D \) is the diffusion coefficient. From the linear fit in Fig. 2.14c, particles 1, 2, and 3 were determined to have diffusion coefficients of \( 1.33 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1} \), \( 8.75 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1} \), and \( 5.73 \times 10^{11} \text{ cm}^2 \text{ s}^{-1} \), respectively. Using the Stokes-Einstein relationship, the nanoparticles were found to
Fig. 2.14 Characterization of three single moving nanoparticles. (a) The normalized LSPR scattering spectra of particles 1, 2, and 3 with $\lambda_{\text{max}}$ of 524, 627, and 689 nm, respectively. The scattering spectra were obtained while the particles were moving according to the trajectories in (b). (b) Two-dimensional trajectories of particles 1, 2, 3. The insets show a magnification of the trajectories at 200, 400, and 500% for particles 1, 2, and 3, respectively. (c) The mean square displacement, $\langle r^2 \rangle$, is plotted as a function of the time lag, $t$, for particles 1, 2, and 3 and fit with a linear regression. Adapted and reprinted with permission from ref. [50]. Copyright 2009 American Chemical Society.
have sizes of 177 nm, 269 nm, and 410 nm for particles 1, 2, and 3, respectively. Using this method, the particle size was approximated within a factor of ~2–3 of the actual nanoparticle size of 80–100 nm [61].

Although the sizes of the Ag nanoprisms were larger than expected by a factor of ~2–4, this work demonstrated the first time both spectra and diffusion coefficients of single nanoparticles were correlated in real-time. Wide-field LSPR imaging and spectroscopy has proven to be an effective and efficient method for obtaining many single nanoparticle spectra for both stationary and moving particles. With high throughput methods and particle tracking capabilities, it is feasible that LSPR spectroscopy can reach the ultimate level of detection, single molecule.

**Coupling Molecular Identification to LSPR Spectroscopy**

LSPR spectroscopy is a sensitive method for measuring the local refractive index changes surrounding noble metal nanoparticles. The LSPR spectrum shifts when adsorbates bind close to the nanoparticle, allowing for the quantitative determination of adsorbate concentrations. LSPR spectroscopy methods obtain specificity from nanoparticle functionalization with molecular recognition elements like antibodies, SAMs, or other specific interactions. However, LSPR spectroscopy methods are not well-suited to identify unknown analytes. Therefore, the versatility of LSPR assays can be improved by coupling with molecular identification techniques such as SERS and laser desorption ionization mass spectrometry.

**Surface-Enhanced Raman Spectroscopy**

Light scattering by molecules can occur elastically (i.e., Rayleigh) or inelastically (i.e., Raman). Raman scattered photons are shifted in frequency from the incident photons by the energy difference necessary to excite a vibrational mode, a process known as normal Raman scattering (NRS). Raman scattering spectrum is unique to a molecule and can be viewed as a molecular “fingerprint.” For example, the dye rhodamine 6G (R6G-d₀) and its isotopologue R6G-d₄ (which is identical to R6G-d₀ with the exception of four deuterium atoms in place of hydrogen atoms) have unique Raman spectra [62]. However, Raman scattering is a weak effect. When molecules are in proximity to a plasmonic nanoparticle, the enhanced local electromagnetic field increases the Raman scattering by a factor of $10^6$–$10^8$, known as surface-enhanced Raman scattering [2, 8]. The field enhancement results from Raman excitation and emission coupling with the nanoparticle LSPR. The enhancement is greatest when the LSPR $\lambda_{\text{max}}$ falls between the excitation wavelength and the wavelength of the scattered photon [8]. Therefore the complementary nature of LSPR and SERS makes them ideal techniques for biomolecular recognition and detection. SERS substrates can be fabricated with a
specific LSPR so that the enhancement is maximized. This section discusses the detection of glucose and calcium dipicolinate (CaDPA) using SERS-based sensors.

A critical aspect in the management of diabetes is the quantitative, real-time detection of glucose. Lyandres and Shah et al. demonstrated the feasibility of a SERS-based glucose sensor using Ag FON substrates [63]. For the development of a SERS glucose sensor, a partition layer on the metal nanoparticle surface was necessary. The partition layer increased the affinity of glucose for the sensor surface, and allowed glucose to bind reversibly in order to accurately reflect fluctuations in glucose levels. The partition layer consisted of a mixed monolayer of decanethiol (DT) and mercaptohexanol (MH), which possessed both hydrophobic and hydrophilic properties making it well-suited for in vivo use [63]. The SERS glucose sensor provided stable glucose level measurements in bovine plasma over 10 days. To establish the reversibility of the sensor, the DT/MH AgFON was exposed to cycles of water and 100 mM glucose solutions. Difference spectra demonstrated partitioning and departitioning of glucose into and out of the DT/MH SAM (Fig. 2.15). Recently, the glucose SERS sensor was successfully transitioned
from in vitro models to in vivo testing in the Sprague–Dawley rat [64]. The in vivo studies were compared to a commercial glucose sensor (Ascensia ELITE) and both were successful in monitoring glucose concentration fluctuations with similar trends.

SERS-based sensors have also been used for biological warfare agent detection. In addition to being specific and sensitive, the SERS biowarfare agent sensor must exhibit a fast response time. Work by Zhang et al. demonstrated sensitive and rapid detection of CaDPA, a biomarker for anthrax, using a portable Raman spectrometer [65]. Extracted from *Bacillus subtilis* spores, (a harmless stimulant of *Bacillus anthracis*), the CaDPA was drop coated on bare AgFON surfaces, which were optimized for 750 nm laser excitation. The intensity of the characteristic 1,020 cm\(^{-1}\) CaDPA peak increased linearly with spore concentration initially, then saturated at higher concentrations. The LOD was determined to be 2,600 spores, which is below the anthrax infectious dose of 10\(^4\) spores. When the AgFON substrates were functionalized with alumina, fabricated by atomic layer deposition (ALD), the LOD for the spores was improved to 1,400 spores [66]. Alumina has a strong affinity for carboxylate groups, making the alumina-AgFON an ideal candidate for *Bacillus* spore detection using CaDPA.

**Laser Desorption Ionization Mass Spectrometry**

Mass spectrometry (MS) is a highly utilized analytical technique that allows the identification of unknown analytes via their unique mass or mass fragmentation pattern [67]. Analysis first requires that the sample be ionized to create a mobile charged species, and the mass/charge ratio is subsequently measured by a detector. Variations in ionization techniques allow the analysis of a wide range of analytes, from gases to small molecules to proteins. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS is a technique that was developed to allow intact detection of very large molecules such as proteins. In this technique, the analyte is embedded in a small-molecule matrix and ionized using a laser source. The matrix absorbs at or near the incident laser wavelength, assisting in energy transfer to the analyte (desorption), as well as ionizing the analyte via proton transfer. MALDI-TOF MS functions for analytes over a mass range from 500 Da to upwards of 250,000 Da, and is widely used in the biological community to study protein composition [68]. As an analytical tool, it provides information that complements LSPR bioassays.

Anker et al. demonstrated the first combination of LSPR and MALDI-TOF MS (Fig. 2.16) [69]. LSPR bioassays and MALDI-TOF MS analysis were performed serially in order to first detect, and then identify ADDLs, a biomarker for Alzheimer’s disease. It was shown that an anti-ADDL detection antibody could specifically detect ADDLs adsorbed to the LSPR sensor, and that subsequent MALDI-TOF MS could identify ADDLs by the characteristic mass peak at 4,516 Da. A matrix was spotted directly onto the LSPR sensor post-assay to
facilitate ionization, with no further sample preparation required (Fig. 2.17a). Real-time data collection during the LSPR assays (Fig. 2.17b) allowed for the determination of kinetic rates of binding interactions. In addition to demonstrating the ease and utility of combined LSPR-MS analysis, this study identified a modified form of the ADDLs monomer, with a mass peak shifted by 16 Da (Fig. 2.17c). It was proposed that this second mass peak resulted from a post-translational modification to the ADDLs protein, which may be correlated with ADDLs toxicity. Although this investigation reported the detection of purified, synthetic analytes,
applications to clinical diagnostics can be inferred. LSPR sensors can be patterned with arrays of antibodies to allow multiplexed detection and quantification of multiple biomarkers in a single sample. MS can subsequently verify the identity of the biomarkers immobilized on the antibody array, as well as identify structural modifications which may not be distinguishable in an immunoassay, but which are important indicators of disease progression. Thus, the combination of LSPR bioassays with mass spectrometry provides new information about biological systems that cannot easily be obtained using any other single technique.

**Raman Tags**

An additional way to couple LSPR spectroscopy with molecular information is to use SERS-nanotags. SERS-nanotags are typically composed of a plasmonic nanoparticle coated with a Raman-active molecule. Wustholz et al. characterized single SERS-nanotags by correlating the nanotag structure with both the LSPR spectrum and SER spectrum [70]. Raman tags have been used for in vivo tumor targeting and detection [71] as well as multiplexed imaging in living mice [72]. Additionally, SERS nanotags have been used for sensing applications in SERS immunoassays. The SERS immunoassays are based on the sandwich-type assay utilizing antibodies, with the SERS-nanotag attached to the detection antibody. Both the SER spectrum and the LSPR of the sample are measured and the SER spectrum intensity can be quantitatively correlated to the analyte concentration [73]. When used in a similar fashion to the Au plasmonic labels (AB-NPs) described in section “Plasmonic Labels,” an enhanced LSPR shift will likely be observed, but an additional detection method will be available by examining the SER spectra of the nanotags. Ultimately, by coupling molecular identification methods with LSPR spectroscopy, more specific and sensitive biosensing platforms can be developed.

**Future Directions**

The field of nanotechnology has been rapidly expanding for over a decade, and devices that utilize the LSPR property of nanoparticles will continue to be the focus of numerous fields of research. As demand for these devices grows, it will become increasingly important to have a thorough understanding of structure–property relationships. Despite significant gains in the theoretical understanding of LSPR, we are far from having a comprehensive and predictive model for how structure affects LSPR properties. Mock et al. was among the first to report a systematic study of the effects of size and shape on the spectral features of single Ag nanoparticles by combining TEM imaging and optical spectroscopy [51]. Since then, research has continued to focus on understanding how nanoparticle features, such as tip sharpness, rounding, size, and anisotropy, give rise to specific spectral
features, like peak position, line shape, and line width [70, 74–76]. The ultimate goal of structural-spectral correlation experiments is to develop a structure-plasmonic function correlation in order to drive rational nanoparticle design. For example, in sensing and SERS applications, it is important to know what specific structure is the most sensitive to the external environment or has the most enhanced EM field. With the continuation of correlated structural-spectral studies, these questions can be answered.

In addition to rationally designed nanostructures, the successful commercialization of plasmonic sensors will require the development of scalable fabrication techniques and fast, user-friendly readout capabilities. Parallel fabrication strategies, such as Dip Nanolithography [77], wet chemical syntheses, photolithography, and templated syntheses [78] create highly reproducible nanoscale structures over large areas and should translate well to industrial settings. On the user end, utility will be improved by designing devices with a multiplexed array format, where readout is performed serially using an automated device, or in parallel via imaging technologies. As diagnostics, nanoparticles have been used as imaging contrast agents, force probes, colorimetric or electrochemical detectors, and optical signal-enhancing agents. On the therapeutics side, nanoparticles are utilized to enhance drug bioavailability, tag cancerous cells for subsequent therapy, or act as targeted drug delivery vectors [28, 79–82]. The development of techniques for nanoparticle tracking within cells has enabled researchers to gain insight into the dynamic behavior of cellular components [83]. Further advances in the field of nanotechnology will continue to impact the utility of plasmonic devices as medical diagnostic and therapeutic devices, and may one day enable us to address major challenges in medicine.

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