Plasmonic nanostructures for unifying surface enhanced Raman and Infrared Absorption spectroscopy

by

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Abstract

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Plasmon resonances control the electromagnetic near field and far field properties of various metallic nanostructures (e.g. nanoparticles, nanoshells, metallic thin films). The enhanced electromagnetic near field, strongest at the surface of the nanostructures, has been successfully exploited for a variety of surface enhanced spectroscopies. Visible and near-IR surface enhanced Raman spectroscopy (SERS) is an example of such surface enhanced spectroscopic technique that has attracted substantial attention due to its huge enhancement factors ($\sim 10^8-10^9$) and wide range of applications. However, surface enhanced Infrared absorption (SEIRA) spectroscopy, complementary to SERS, has not received nearly the same attention because engineering the necessary strong near fields in the mid-IR is challenging. This thesis is an effort for developing rationally designed Au nanoshell based substrates for SEIRA and for combining SERS and SEIRA to unify the field of surface enhanced vibrational spectroscopy for comprehensive biochemical sensing applications. Specifically, this thesis describes the utilization of interparticle junction hot spots for SEIRA. Aggregates
of near-IR resonant nanoshells with naturally occurring junction hot spots are demonstrated to be excellent SEIRA substrates with high enhancement factors \((10^3-10^4)\). Applications of SEIRA in conjunction with SERS (exploiting near fields from single particle plasmon of nanoshells) is demonstrated for a variety of biologically relevant processes such as adsorption, local orientation and binding of adenine and adeninemonophosphate (AMP) on Au nanoshell surface, intercalation of ibuprofen in hybrid lipid membranes, and lipid transfer/exchange between hybrid lipid bilayers and vesicles. Finally, the random aggregate geometry for SEIRA is elegantly extended into 2D periodic array of nanoshells that truly unifies SERS and SEIRA on a common single substrate by simultaneously enhancing both Raman and Infrared signals in two diverse frequency regimes with high spectral sensitivity.
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Chapter 1. Introduction

1.1. Plasmonics

Plasmonics is a new emerging paradigm that has attracted considerable attention owing to its potential applications for technological miniaturization. Plasmonics, sometimes called as 'light on a wire', primarily deals with manipulating light at sub wavelength scales. It allows the transmission of signals (as electron density distribution rather than photons) at optical frequencies along the surface of a tiny metal wire. Plasmonics is an approach based on using the localized surface plasmon resonances of metal particles to control light below the wavelength limit, down to nanometer length scales. Plasmon, the collective oscillations of conduction band electrons in metal, interacts strongly with light and plays pivotal role in the transmission of signals. When a plasmon resonance is excited, the fields associated with these modes are very significantly enhanced; they are also evanescent or near-field in character, falling exponentially in strength with distance away from the particle. Crucially, this means that the light may be localized into a volume of space only ~10 nm in dimension. Because of this unique ability, there is a lot of interest in using plasmonic structures in applications such as waveguides\textsuperscript{1,2}, chemical and biological sensors\textsuperscript{3-6}, metamaterials\textsuperscript{7}, and photothermal medical devices\textsuperscript{8,9}. Many of these applications rely on the ability to engineer the plasmon to be resonant at a particular wavelength. For instance, a plasmonic structure resonant at standard telecommunications wavelengths (1.3 - 1.6 \mu m) would be useful for waveguiding and interconnect applications whereas a structure resonant in the near-infrared
water window of tissue (~ 800 nm) would be useful for medical applications. Exploiting the strong near fields of nanostructures with plasmon resonance tuned to Visible/near-IR and mid-IR regime would allow enhancements of the traditional Raman and Infrared spectroscopic techniques. This surface enhancement of Raman scattering (SERS) and Infrared absorption (SEIRA) would be beneficial for comprehensive bio-chemical sensing.

The plasmon resonance of a particular structure is highly dependent upon its shape\textsuperscript{10}. Metal nanoshells, which consist of a spherical dielectric core surrounded by a concentric metal shell, are a class of plasmonic nanoparticles that can be engineered to have plasmon resonances at a wide range of wavelengths\textsuperscript{11,12}. The plasmonic properties of these nanostructures are determined by the dimensions of the inner and outer radii of the metallic shell and the dielectric constants of the constituent materials. The strong optical near field of the nanoshells are known to spatially extend ~10 nm or more from the surface.\textsuperscript{13} Spherical metal nanoshells possess two distinct advantages over other tunable asymmetric plasmonic nanostructures such as nanorods\textsuperscript{14,15} or metal triangles\textsuperscript{16}: they are straightforwardly modeled and are easy to fabricate. The concentric spherical geometry of a nanoshell enables its plasmon resonance to be readily predicted by the plasmon hybridization model\textsuperscript{17,18} or Mie scattering theory\textsuperscript{19}. This theoretical framework makes it possible to design nanoparticles with the desired plasmonic properties easily. Once the desired nanoshell geometry has been determined, they can be fabricated in an inexpensive and
simple scalable wet chemistry method. Furthermore, functionalization of Raman active molecules, organic fluorophores, and other molecules of interest can be easily accomplished by utilizing the established conjugation chemistry techniques. Commonly, the strong gold-thiol affinity is exploited to tether the molecules of interest close to the metal surface. Thus, nanoshells with strong near fields in the Visible/near-IR, make efficient substrates for metal enhanced spectroscopy.21-23

A detailed introduction to nanoshell theory, its fabrication and characterization, and its utilization as SERS and SEIRA substrate can be found in Appendix A as nanoshell background information.

1.2. Vibrational and surface enhanced vibrational spectroscopy

Vibrational spectroscopy is a classical analytical spectroscopic technique routinely used to elucidate molecular structures. It probes the periodic oscillations of atoms within a molecule. These oscillations do not occur randomly but in a precisely defined manner. Quantum mechanics dictates that only certain atomic displacements at certain frequencies are allowed. A linear molecule with N atoms has (3N-5) normal modes, and a non-linear molecule has (3N-6) normal modes of vibration. In each normal mode every atom of the molecule oscillates in phase with the same frequency, although with different amplitudes. The frequency of the oscillation, primary observable in vibrational spectroscopy measurements, depends sensitively on the chemical composition of the molecule and its environment. Hence, the frequencies of the normal modes constitute a characteristic signature of the identity of the molecule. The other important
observable in vibrational spectrum is the intensities of the bands, which, unlike the frequencies, are not independent of the method used to probe the vibrational spectrum.

The two main techniques used to acquire vibrational spectra of analyte molecules, Raman and Infrared (IR) spectroscopy, are based on different physical mechanisms and are discussed below.

*The Raman Effect:* When monochromatic electromagnetic radiation of energy content $h\nu$ (UV or Visible or near-IR) irradiates a molecule, the energy can be transmitted, absorbed or scattered by the molecule. The phenomenon of scattering of the light by the molecule is known as Rayleigh scattering which is analogous to Tyndall effect where the radiation is scattered by particles (e.g. smoke or fog). Rayleigh scattering is elastic in nature (*i.e.* no change in the frequency of the incident radiation) and is typically very weak (one out of a thousand photons are scattered). In 1928, C. V. Raman reported inelastic scattering of the incident radiation by the molecule, such that the frequency of the scattered light is shifted by the molecular vibration. In Stokes scattering, the Raman scattered light has lower frequency than the incident light. However, some Raman scattered light can be higher in frequency than the incident light. This can occur when the molecule that is being studied is not in the vibrational ground state. This results in Anti-Stokes Raman scattering. The frequency difference from the incident light reveals the vibrational frequency of the mode of the ground state molecule. A Rayleigh and Raman scattering energy level diagram is presented in Figure 1. Raman spectra may display a variety of
frequency bands with its associated intensities. The probability of scattering induced vibrational transitions, hence Raman band intensity, is governed by how effectively the incident radiation interacts with the molecular vibration. The molecular polarizability (ease of electron cloud deformability) plays an important role in coupling the incident radiation to the molecule in Raman scattering. It can be rigorously proved that for a molecular vibration to be Raman active, the vibration must be accompanied by a change in the molecular polarizability.\textsuperscript{25}

**Infrared spectroscopy:** In infrared spectroscopy, molecules are exposed to a continuum of IR radiation (wavelength spanning from 2.5 to 50 microns range) and those photons that have energies corresponding to the frequencies of the normal modes can be absorbed to excite the respective vibrations. This transfer of energy from the incident light to the molecule is mediated by molecular dipole moment (charge separation) that is generated when molecules are irradiated with incident light. This oscillating dipole can absorb energy from the oscillating electric field of light only if the field oscillates at the same frequency. If the frequencies of light and the vibrations are not the same then this transition is non-resonant with the light – the molecule does not absorb. The absorption intensities of the IR bands are governed by the net change in the dipole moment of the molecule along the vibrational coordinate. In fact, it can be rigorously proved that for a molecular vibration to be IR active, the vibration must be accompanied by a change in the dipole moment.\textsuperscript{25}
Figure 1. Raman scattering and IR absorption process

It is important to note that Raman and IR spectroscopy are physically two different techniques, yet they are unified under the field of vibrational spectroscopy. It is because Raman and IR spectroscopy complement each other in the sense that together they can probe all vibrational modes of the molecule and provide far more detail information about the molecular structure than one technique could do alone. With technological advancement vibrational spectroscopy has now been accepted as a well-established analytical technique for molecular identification.\textsuperscript{26,27}

\textbf{Surface enhanced Raman scattering (SERS):} Raman spectroscopy has numerous advantages as an analytical technique but it is limited as the Raman cross sections of molecules are very small (such as 2.6x10\textsuperscript{-31} cm\textsuperscript{2} sr\textsuperscript{-1} at 514.5 nm for N\textsubscript{2})\textsuperscript{28}. Hence, there has been a lot of research interest in increasing the Raman sensitivity. By placing the analyte molecule or scatterer on or near a
roughened noble-metal substrate, the magnitude of the Raman scattering signal can be significantly enhanced. This technique is known as surface enhanced Raman spectroscopy (SERS) and is well-suited for detecting small amounts of analytes.\textsuperscript{29-34} It is generally accepted that SERS has two important enhancement mechanisms: chemical effect and electromagnetic (EM) effect. The chemical effect, involves the creation of a charge transfer state between the metal and adsorbate molecules.\textsuperscript{35} However, it is the EM effect that provides the main contribution, where the local electromagnetic field close to a roughened noble-metal surface is greatly enhanced due to the excitation of surface plasmon. This enhanced near field interacts with the molecular polarizability resulting in Rayleigh and Stokes shifted Raman scattered light. Since both the excitation field and the Raman scattered field contribute to enhancement, the SERS signal is proportional to the fourth power of the local field.\textsuperscript{36} SERS using isolated, single, colloidal silver and gold nanoparticles have reported maximum electromagnetic field enhancements on the order of $10^6$-$10^7$. However, random aggregates of these colloidal nanoparticles were used to provide the first clear demonstration of single molecule SERS detection.\textsuperscript{34,37}

**Surface enhanced Infrared Absorption (SEIRA):** The identification of SERS fuelled the search for the complimentary surface enhanced infrared absorption spectroscopy as Raman and IR are the two faces of vibrational coin. Combining SERS and SEIRA together into surface-enhanced vibrational spectroscopy (SEVS) provides enhanced scattering and enhanced absorption techniques that could potentially be utilized for comprehensive sensing
applications. SEIRA involves direct mid-IR excitation of molecules on metal structures where the IR absorption, $A$ is proportional to the square of the scalar product of the local $E$ field and dipole moment derivative with respect to vibrational coordinate.\textsuperscript{38,39} Mathematically, conventional gas phase IR absorption can be expressed by the following equation,

$$A = |E \cdot \left( \frac{\partial \mu}{\partial Q} \right)|^2$$

The above equation clearly leads to the well-known surface selection rule of SEIRA spectroscopy: vibrational modes of molecules with a change in dipole moment perpendicular to the substrate surface are preferentially enhanced.\textsuperscript{40} Therefore SEIRA has excellent potential for providing a straightforward signature of the orientation of adsorbate moieties with respect to the surface, valuable information in the elucidation of molecular structure and dynamical processes.

In the first SEIRA spectra reported\textsuperscript{39} in 1980 the enhancement was observed for the impurity (pump oil) rather than the intended target.\textsuperscript{41} More through study of SEIRA was later pursued in Japan and it was only in the 1990s that SEIRA received its share of attention as there were several reports on both the practical and theoretical aspects of SEIRA. The origin of SEIRA, like SERS, is attributed to electromagnetic and chemical effects. The chemical effect in SEIRA is poorly understood and is a generic term used to attribute several molecule-enhanced interactions that may affect the frequency, the shape of the observed IR band or the intensity of the fundamental vibrational mode governed by the partial derivative $\left( \frac{\partial \mu}{\partial Q} \right)$.\textsuperscript{42}
The electromagnetic contribution is attributed to enhanced optical fields that increase the ‘throughput’ on the molecules leading to enhanced absorption. This enhanced local field augmented absorption intensities is plasmon assisted and has been beautifully explained in Osawa's review. Experimentally, enhanced local field in the mid-IR can be achieved using SEIRA substrates with discrete and non-discrete surface plasmons. Examples of the latter type include the traditionally used Au and Ag island films of tunable mass thickness. For metal island films the plasmon absorption band tails well into the mid-IR region and typical SEIRA enhancement factors using these substrates are in the order of $10^2-10^3$. Electromagnetic enhancements are anticipated to be even higher for substrates with geometries specifically designed to produce strong discrete plasmon absorption bands in the mid-IR region. SEIRA substrates with discrete plasmons such as ordered arrays of uniformly sized Ag nanoparticles have recently been reported to produce a mid-IR plasmon response for electromagnetic enhancements. However, the enhancement factor for substrates with discrete plasmon was found to be very similar to that of island film substrates. The most distinct electromagnetic property, the distance dependence of SEIRA, has been demonstrated using Langmuir-Blodgett monolayers and it was found that SEIRA is most efficient within 5 nm of the surface. Effective medium theories have been successfully utilized to account for the electromagnetic enhancement and the asymmetry of bands observed with small molecules. Since, the bulk of the SEIRA work (theoretical and experimental) reported in the literature is recent, it is particularly interesting
to demonstrate the effect itself with different enhancing substrates that are rationally engineered to produce strong mid-IR hotspots eliciting higher electromagnetic enhancements.

1.3. Scope of the thesis

This thesis focuses on rational design and fabrication of Au nanostructures based substrates for surface enhanced Infrared absorption spectroscopy (SEIRA) and to combine SERS and SEIRA together as a comprehensive biochemical sensing modality. The success of nanoshells as excellent SERS substrates has already been demonstrated, where the strong near field of the single particle plasmon of nanoshells, tuned into the visible/near-IR, is exploited. However, the near fields produced from single particle plasmons tuned to the mid-IR are nominal that limits the success of SEIRA. This thesis demonstrates that interparticle junction ‘hot spots’, which can be tuned to mid-IR with strong near fields, can be exploited for fabricating effective substrates for SEIRA with high enhancement factors. Two different nanoshell geometries are demonstrated to be efficient SEIRA substrates: i) Aggregates of near-IR resonant nanoshells, ii) 2D periodic array of nanoshells. The later substrate is shown to truly unify SERS and SEIRA on a common single substrate. Both of these geometries are utilized for demonstrating the application of SERS and SEIRA together for bio-chemical sensing.

Structure of this thesis is as follows: Chapter 2 discusses the rational design and fabrication of nanoshell aggregate based substrate for surface
enhanced Infrared absorption spectroscopy (SEIRA). Naturally occurring junction 'hot spots' in the aggregates of near-IR resonant nanoshells on silicon substrate is exploited for demonstrating SEIRA utilizing several test molecules. For this aggregate geometry, the SEIRA enhancement factor and detection limit is quantified. Chapter 3-5 demonstrate the application of SEIRA (utilizing nanoshell aggregates), in conjunction with SERS (exploiting near fields from single particle plasmon of nanoshells), for a variety of biologically relevant processes such as adsorption of nucleic acid bases, intercalation of ibuprofen in hybrid lipid membranes, and lipid transfer/exchange process. In Chapter 6, the random aggregate geometry for SEIRA is elegantly extended into 2D periodic array of nanoshells that truly unifies SERS and SEIRA on a common single substrate by simultaneously enhancing both Raman and Infrared signals in two diverse frequency regimes with high spectral sensitivity. Chapter 7 describes the overall summary of the thesis and provides future directions.
Chapter 2. Surface enhanced Infrared absorption (SEIRA) spectroscopy on nanoshell aggregate substrates

This work was done in collaboration with F. Le and P. Nordlander.

2.1. Introduction

The dramatic changes observed in the optical properties of molecules when adsorbed on or near structured metal surfaces, such as metallic nanoparticles or metal island films, have stimulated an intense interest in surface enhanced spectroscopies. The best-known example is surface enhanced Raman scattering (SERS). A closely related and complementary surface enhanced spectroscopy, surface enhanced infrared absorption (SEIRA), can also be performed by direct mid-IR excitation of molecules on metal structures. In SEIRA, vibrational modes of molecules with a change in dipole moment perpendicular to the substrate surface are enhanced. Therefore SEIRA has excellent potential for providing a straightforward signature of the orientation of adsorbate moieties with respect to the surface, valuable information in the elucidation of molecular structure and dynamical processes.

Since its discovery, SEIRA has been used in numerous applications in chemical analysis and characterization, and in biochemical sensing. SEIRA enhancement factors are known to depend crucially on the substrate’s properties. For vacuum evaporated metal island films (Ag, Au), the SEIRA enhancement factors have been reported in the range of 100–600. Wet chemically prepared Au island films have shown an enhancement factor of 2000.
For metal island films the plasmon absorption band tails well into the mid-IR region. This property gives rise to enhanced electromagnetic fields, resulting in increased IR absorption by adsorbed molecules. Electromagnetic enhancements are anticipated to be even higher for substrates with geometries specifically designed to produce strong plasmon absorption bands in the mid-IR region. SEIRA substrates have recently been reported on structures designed to produce a mid-IR plasmon response for electromagnetic enhancements, fabricated using relatively complex and costly cleanroom techniques. In this work we report surface enhanced infrared absorption spectroscopy (SEIRA) performed on substrates composed of infrared resonant Au nanoshell aggregates.

Au nanoshells are spherical nanoparticles consisting of a dielectric (silica) core and a metallic (Au) shell whose plasmon resonance is a sensitive function of the relative size of the inner and outer radius of the nanoparticle’s shell layer. The plasmon resonances of nanoshells can be tuned from the visible well into the infrared region. When nanoshells are positioned directly adjacent to each other, they support junction or ‘dimer’ plasmon in direct analogy with Au or Ag nanosphere aggregates or nanosphere arrays. In the closely related field of surface enhanced Raman spectroscopy (SERS) this geometry has proven to be extremely important. In SERS, the ‘hot spots’ formed at the ultra small gaps of colloidal aggregates or between directly adjacent metallic structures provide sufficiently intense electromagnetic fields to make single molecule spectroscopy possible. In the experiments reported here, nanoshells serve as the infrared-resonant analog to the colloidal aggregates used in SERS. Here we
show that infrared ‘hot spots’ formed by nanoshell aggregation provide strong enhancements of the infrared vibrational spectrum for adsorbate molecules, over a broad mid-IR spectral range.

2.2. Experimental

SEIRA substrate preparation

Silica core-gold nanoshell were fabricated using a method previously described\textsuperscript{11}. Electroless plating of metallic Au on the colloidal silica core particles in solution is accomplished by reducing chloroauric acid in the presence of formaldehyde reductant. The nanoshell solution was then centrifuged and redispersed in water. The aqueous solution of freshly prepared concentrated nanoshells was drop-dried onto a silicon wafer, where aggregates were formed upon evaporation of the solvent. The samples were then soaked overnight in 1 mM ethanolic solution of paramercaptoanilide (pMA), the analyte molecule, followed by copious rinsing with ethanol. Adenine samples were prepared in the same manner.

Measurement techniques

Nanoshell aggregate morphology was characterized using scanning electron microscopy (SEM). The optical properties of nanoshell aggregates on glass were characterized using a UV–vis-NIR spectrophotometer (Cary 5000). Nanoshell aggregates on silicon were characterized using a FT-IR (Nexus 670, Thermo Nicolet) equipped with liquid N\textsubscript{2} cooled MCT detector. Unpolarized IR spectroscopy measurements were performed in transmission geometry.
2.3. Results and Discussion

Optical analysis

Fabricated nanoshells have inner and outer radii of \([r_1, r_2] = [190, 210]\) nm with a 6.2% polydispersity in total nanoshell size distribution as determined by quantitative comparison of the UV–visible extinction spectrum with Mie scattering theory, with independent verification using SEM analysis. The SEM image of a nanoshell film (glass substrate), shown as an inset in Fig. 1B, reveals individual nanoshells as well as a large distribution of nanoshell aggregate ‘n-mers’. A visible-near IR extinction spectrum, shown in Figure 2A(i), has a strong, broad band in the IR and sharper bands in the shorter wavelength region. Mie theory calculations, an analytical solution to Maxwell’s equations for the scattering of electromagnetic radiation by spherical particles, were performed to obtain the theoretical extinction spectra. Figure 2A(ii) shows the calculated extinction spectrum for an individual nanoshell. A \([r_1, r_2] = [190, 205]\) nm nanoshell provided the best correspondence to the experimental spectrum, where an effective medium of \(\varepsilon = 1.3\) was used to model the effect of the glass substrate\(^6^9\). The short wavelength peaks at 1369 nm, 798 nm, and 647 nm correspond to the dipole (\(l = 1\)), quadrupole (\(l = 2\)), and octupole (\(l = 3\)) of the individual nanoshell plasmon response. The broad long wavelength peak at 2.4 microns in the experimental extinction spectrum is attributed to the junction plasmons of the nanoshell aggregates. By increasing the refractive index of the substrate, the IR plasmons of the nanoshell aggregate can be further redshifted, increasing the mid-IR plasmon response and the overall spectral range for SEIRA.
enhancement. This is easily accomplished by the use of silicon (instead of glass) substrates. Silicon substrates have excellent optical transmission characteristics in the mid-IR region, and can be described in the context of these experiments as an effective embedding medium\textsuperscript{58}. Figure 2B shows a representative extinction spectrum of nanoshell aggregates deposited on mid-IR transparent silicon substrates. Based on this spectrum, an approximate wavelength range for the nanoshell/Si substrates may be anticipated to be nominally 2.5–8 microns in wavelength (1250–4000 cm\textsuperscript{-1}) spanning a large portion of the chemical fingerprinting range.

**Figure 2.** (A) Experimental extinction spectrum of nanoshell aggregate film on (i) glass substrate and (ii) calculated extinction spectrum of constituent nanoshell, with parameters specified in text. (B) Experimental near-IR – mid-IR extinction spectrum of typical nanoshell aggregate film on silicon shown with the SEM image of the film with a single nanoshell shown in inset.
**SEIRA analysis**

Para-mercaptoaniline (pMA) was chosen as an analyte molecule to study the SEIRA activity of the prepared nanoshell aggregate substrate. Figure 3(ii) shows a characteristic unpolarized transmission SEIRA spectrum of pMA obtained from our substrates. A conventional IR spectrum of neat pMA dispersed in a KBr pellet is also shown in Figure 3(i). For the SEIRA spectrum of Figure 3(ii) it is immediately apparent that excellent signal-to-noise is obtained. Moreover, strong molecular signals are observed over a broad spectral range. This observed enhancement range extends slightly further into the long wavelength region of the infrared than can be inferred solely by the far field extinction spectrum of Fig. 2B, analogous to the SEIRA enhancement observed by previous workers with metal island film substrates. SEIRA measurements on isolated, well-separated nanoshells were performed as a control, however, no molecular signals were observed. A comparison of the SEIRA and the bulk IR spectra show both spectral shifts and intensity differences. The IR band assignments of bulk pMA\textsuperscript{70} are listed as a table in Figure 3. The $b_1$ and $b_2$ modes in the bulk IR spectra are absent in the SEIRA spectra.
Figure 3. (i) Transmission spectra of neat pMA dispersed in KBr and (ii) transmission SEIRA spectra of pMA on nanoshell aggregates with table of peak assignments for normal IR and SEIRA of pMA. ($\delta$, v, and $\pi$ stands for bend, stretch, and wag mode, respectively).

Based on the surface selection rules for the modes observed in the experimental spectra it can be inferred that the pMA molecules are oriented in an upright manner on the nanoparticle surfaces. A high packing density of molecules on the surface and the observation of an Au–S stretching mode at low wavenumbers are also consistent with an upright orientation of the pMA molecules on the nanoparticle surfaces.

To demonstrate the general suitability of this substrate to other adsorbate molecules, we chose adenine, as another test molecule. Lacking a thiol moiety, adenine associates with the Au surface through the affinity its amine groups have for this surface. A SEIRA spectrum was obtained for adenine as shown in Figure 4(ii). A normal IR spectrum of adenine, as shown in Figure 4(i), was also
acquired for comparison purposes. Strong molecular peaks from adenine are seen in the fingerprint region of the SEIRA spectra. In this spectrum, C–H stretching modes in the high wavenumber region are also seen (Figure 4, inset).

![Spectra](image)

**Figure 4.** Transmission spectrum of (i) neat adenine and (ii) SEIRA spectrum of adenine on nanoshell aggregates. Spectra are offset for clarity. Inset shows the high wavenumber SEIRA spectra.

To determine the SEIRA detection limit, SEIRA spectra were acquired for various concentrations of pMA solutions ranging from 1000 to 10 μM (acquired after 3 hours incubation time with the substrate). The SEIRA signal-to-noise ratios (S/N) are plotted for different pMA modes at these concentrations as shown in Figure 5 and the SEIRA detection limit is estimated to be in the range of 1–10 μM for the analyte probed (at 10 μM concentration, the obtainable (S/N) is ~6).
Figure 5. SEIRA signal-to-noise (S/N) ratio of some of the observed vibrational modes of pMA obtained from various concentrations of pMA solution.

The spectral features of the SEIRA spectra typically show the highly asymmetric, Fano-type lineshape characteristic of SEIRA$^{45,49,71,72}$. The degree of lineshape asymmetry has been observed to correlate with greater enhancement factors for substrates consisting of coalescent rather than dispersed particles$^{45}$. Empirical SEIRA signal enhancement factors were determined by comparing the normalized intensity of several of the major IR modes of pMA observable in SEIRA to their corresponding unenhanced IR modes obtained from neat pMA films of known thickness deposited on the silicon wafer. The normalization is performed with respect to the total number of analyte molecules being probed in each case. First, the aggregation statistics of nanoshells on silicon were analyzed using SEM images and particle counting, which provides the number distribution of the various ‘n-mers’ as shown in the histogram plot of Figure 6A. In
order to estimate the SEIRA sensitive 'hot spot' area, finite difference time
domain (FDTD) simulations were performed for nanoshell dimers, trimers and
quadrumers with geometries shown in Figure 6B. In these simulations, hotspot
areas are estimated using an interparticle gap of 4 nm, the smallest gap size
feasible in our simulations (for separations less than 4 nm, staircasing artifacts
are likely to be introduced into the calculations). For dimer, trimer and quadrumer
nanoshells, the total hot spot areas were estimated to be $6 \times 10^4$, $10 \times 10^4$, and
$12 \times 10^4$ nm$^2$, respectively, and extrapolated for higher order n-mers. The total
number of pMA molecules present in the interparticle junctions of the nanoshell
aggregate was then estimated using the total hot spot area contributed by the
individual n-mers, the number density of the individual n-mers in the experimental
nanoshell films, and the reported footprint of pMA molecule on Au (0.3 nm$^2$)$^{73}$.
The total number of molecules probed in the unenhanced reference sample is
calculated using the known molecular density of pMA, film thickness of pMA and
the IR beam spot size. The unenhanced IR intensities were obtained from the
normal IR spectra. The ratios of the normalized SEIRA intensity to that of the
unenhanced intensity yields SEIRA enhancement factors for the various
vibrational modes of pMA in the $10^4$ range as shown in Figure 6C.
Figure 6. (A) Histogram plot of a representative SEIRA substrate characterizing nanoshell aggregation. (B) FDTD simulated local field enhancements for a (a) dimer, (b) trimer, and (c) quadrumer nanoshell aggregate. Each nanoshell, embedded in vacuum, has inner and outer shell dimensions of \([r_1, r_2] = [190, 210] \text{ nm}\) with an interparticle separation distance of 4 nm. The incident direction and polarization of light, scale bar of near field enhancements, and local field maximum are shown. The hot spot sizes in each system are based on an estimation of the surface area on all nanoshells with local electric field enhancement factors >10. (C) Enhancement factors obtained for different IR modes of pMA.
2.4. Conclusions

We have shown that IR-resonant nanoshell aggregates on Si are excellent substrates for SEIRA, producing high-quality spectra across a broad 700–3300 cm\(^{-1}\) range required for vibrational spectroscopy. By detailed modeling and statistical analysis of the infrared ‘hot spots’ formed within nanoshell aggregates, we have shown that large SEIRA enhancement factors, in the \(10^4\) range, are achievable using these substrates. These results open up new opportunities for the development and mainstreaming of SEIRA as a reliable and highly useful technique for molecular spectroscopy, with numerous applications in chemical sensing.
Chapter 3. Adenine- and adenosine monophosphate (AMP)-gold binding interactions studied by surface enhanced Raman and Infrared spectroscopies

This work was done in collaboration with O. Neumann (equal contributor), B. G. Janesko, D. Zhang, S. Lal, A. Barhoumi, and G. E. Scuseria.

3.1. Introduction

Knowledge of the interactions of biomolecules with noble metal surfaces is critically important to our development of functional materials or devices that interface well to living systems. In particular, the biocompatibility, relative chemical inertness, and useful optical properties of gold nanoparticles have permitted the development of many biomolecule-nanoparticle complexes for biomedical applications. Nucleic acids are important biological molecules due to their multiple cellular functions, such as replication, transmission of genetic information, and molecular recognition. These attributes, along with their chemical and thermal stability, has led to the increasing use of DNA in functional sensors and devices. The performance of these types of devices is governed in part by details of the nucleic acid-metal surface interactions, such as the ability of pristine or functionalized DNA to form self-assembled monolayers or superstructures on Au nanoparticle surfaces, or the nonspecific binding of the nucleotide constituents to the Au nanoparticle surface. Of the four nucleic acid constituents, adenine is of particular interest: with its high level of nonspecific
binding on Au nanoparticle surfaces, and its widespread presence in the metabolome in addition to the genome, it is an extremely important molecule with which to study chemisorption to Au nanoparticle surfaces in detail.

Just like their bulk spectroscopy counterparts, Raman and Infrared spectroscopy, surface enhanced Raman scattering (SERS) and surface enhanced infrared absorption (SEIRA) are complementary spectroscopic techniques that can be used for investigating the adsorption of biomolecules on metal surfaces. Vibrational spectroscopy generally provides valuable, detailed information concerning molecular structure and binding of adsorbates to surfaces. Combining SERS and SEIRA provides an opportunity to probe virtually all vibrational modes of a molecule or an adsorbate-substrate complex, due to the complementary selection rules of these spectroscopies. This paper presents a combined surface enhanced vibrational spectroscopic (SERS-SEIRA) study of the adsorption of adenine (A), adenine mono-phosphate (AMP) and poly-adenine (poly-A) (thiolated single stranded DNA of 24 bases) on Au nanoshells at different pH values. Au nanoshells, tunable plasmonic nanoparticles consisting of a dielectric (silica) core and a thin metallic (gold) shell, were used as SERS and SEIRA substrates due to their large and reproducible SERS and SEIRA enhancements which even permit the opportunity to perform both SERS and SEIRA on the same substrate. Combined SERS and SEIRA spectral analysis was performed to provide insight into the preferred orientation of the adenine-based molecules on the Au nanoshell surface. Density functional electronic structure theory calculations were performed for free
adenine and for adenine bound to an Au surface to obtain peak assignments and to confirm the molecular structure and orientation of the molecule on the Au surface.

It is known that adsorbed adenine is multidentate and can display a variety of orientations on different surfaces. This multidentate nature is due to the presence of several possible coordinating sites (nitrogen atoms) (Fig. 1). For example, it is known that adenine can coordinate to surfaces through the exocyclic amino group and/or ring nitrogen atoms. Adenine has been reported to adsorb on noble metal surfaces in a planar or non-planar conformation due to interactions with the surface via N1, N7, external amino group or the purine ring. Studies on the organic reactivity of the adenine molecule have revealed that the N3 and N9 sites are more reactive functionalities than the external amino group. Detailed information on the actual chemical and orientational nature of the adsorbed adenine moiety, which is the central unit that directs the self-assembly of biomolecules such as AMP and poly-adenine, is of fundamental chemical interest.

![Molecular structure of adenine, AMP, and poly-A](image)

**Figure 7.** Molecular structure and atom labeling of adenine, AMP, and poly-Adenine.
3.2. Experimental

A. Materials: Adenine (99%), adenosine 3'-monophosphate from yeast, DTT (1, 4 Dithio -DL-threitol sol. 1M) were purchased from Sigma-Aldrich and employed without further purification. The thiolated ss-DNA, a 24-A base oligonucleotide was obtained from IDT (Integrated DNA Technologies). NaCl (Sigma-Aldrich), K₂HPO₄·3H₂O (Sigma-Aldrich) were used to prepare buffer solutions. The pH of the buffer was adjusted by adding HCl or KOH. The pH measurements were performed with a Fisher Scientific accumet AP63 portable pH meter with an accuracy of 0.01 pH units.

B. Reduction of thiolated ss-DNA: Prior to adsorption, the thiolated ss-DNA [(HS-(CH₂)₆-(A)₂₄] was incubated with 100 mM of DTT (reducing agent) in 100 mM sodium mono-phosphate buffer at pH=7.5. The mixture was incubated at room temperature for 1 hour to allow for complete reduction of the disulfide bonds. The solution was then filtered through a NAP-5 column to remove the excess DTT and the oxidized form of DTT (S₂-(CH₂)₂-(CH-OH)₂). Elution of the column with water was performed to collect the purified ss-DNA in H₂O. The concentration of the ss-DNA stock solution was determined by measuring its absorbance at 260 nm, using a UV-vis-NIR spectrophotometer (Cary 5000).

C. Preparation of SERS and SEIRA substrates: Films of Au nanoshells supported on quartz and silicon were used as substrates for SERS and SEIRA, respectively. Quartz and silicon substrates were cleaned by immersing in
"piranha solution" (H$_2$SO$_4$;H$_2$O$_2$=3:1) for 1 hour followed by copious rinsing with deionized water (18.3 MΩ, Millipore). Note that piranha solution is extremely oxidizing, reacts violently with organics, and should be stored in loosely covered containers to avoid pressure buildup. Silica core-gold nanoshells were fabricated using a method previously described. Nanoshells of inner and outer radii \([r_1, r_2] = [63, 86]\) with a strong dipole plasmon mode at 780 nm were used for the SERS studies. Infrared resonant nanoshells of inner and outer radii of \([r_1, r_2] = [190, 210]\) were fabricated to produce a film of nanoshell aggregates on silicon (by drop drying) with a broad plasmon response in the mid-IR for SEIRA measurements.

For the SERS measurements, nanoshells were immobilized by depositing 100 μL of aqueous nanoshells solution on PVP coated quartz substrate for 3 hours. These substrates were then rinsed with water several times to remove the free-floating nanoshells. These nanoshell films, immobilized on quartz substrates, were incubated overnight in a solution of analyte molecules (adenine (0.1 mM), AMP (0.1 mM), and poly-adenine (100 μM solution). Before acquiring Raman spectra at different pH values, the sample was rinsed several times with the corresponding buffer solution.

For SEIRA measurements, as-prepared infrared resonant nanoshells were incubated overnight in solutions of analyte molecules at a given pH. Solutions of analyte molecules (adenine (0.1 mM) and AMP (0.1 mM)) were prepared at two values of pH: 2.5 and 8.5. The nanoshells, coated with analyte molecules deposited from solutions of different pH, were then drop-dried on a silicon wafer.
Drop-drying results in the formation of films of nanoshell aggregates, which comprise active SEIRA substrates with analyte molecules in the interparticle junctions (electromagnetic ‘hot spots’). The analyte-functionalized nanoshell film was rinsed with the corresponding buffer solution prior to SEIRA measurements.

**D. Measurements:** SERS and Raman spectra were acquired on a Renishaw in Via Raman microscope (Renishaw, United Kingdom) with 785 nm excitation wavelength and 55 µW laser power at the samples. Backscattered light was collected using a 63x water immersion objective lens (Leica, Germany) with a 40 second integration time. SEIRA and IR spectroscopy measurements were performed on dry samples using transmission geometry on a FTIR spectrometer (Nexus 670, Thermo Nicolet) equipped with liquid nitrogen cooled MCT (HgCdTe) detector. The reported FTIR spectra were collected under N₂ environment at 4 cm⁻¹ resolution with the coaddition of 256 scans.

**E. Computational methods:** Gas-phase Raman and IR spectra of protonated and deprotonated adenine, as well as adenine bound to a single Au (+) atom were calculated. While clearly not a quantitative model of the solvated molecule at an Au surface, similar gas-phase cluster models have been successfully used to interpret vibrational frequency shifts in SERS. All calculations were performed with a development version of the Gaussian electronic structure program using the B3LYP density functional.
Calculations on isolated adenine and adenine-H+ used the large Sadlej basis set\textsuperscript{116,117} obtained from the EMSL basis set library.\textsuperscript{114} Calculations on Raman spectra of adenine-Au (+) complexes used the aug-ccpVTZPP\textsuperscript{118} basis set and ECP on Au, and the Sadlej polarized valence triple zeta basis set on other atoms. Calculations on the IR absorbance spectra of isolated adenine used the polarizable continuum model\textsuperscript{119} to simulate aqueous solvation, and assumed that adenine is protonated at N1. All calculations were performed for isolated molecules, and all molecules are fully geometry optimized. The calculated normal modes of adenine are labeled according to the atom labeling shown in Figure 7.

3.3. Results and Discussion

SERS spectra of adenine, adenosine monophosphate, and poly adenine at the following pH values: 2.5; 5.1; 6.5; 8.5 and 11.5 are shown in Figure 8(a-c). All spectra are normalized with respect to the strongest Raman band at 735 cm$^{-1}$ in each spectrum and offset for clarity. These pH values were chosen in order to observe the effects of protonation and deprotonation of the adenine (pKa = 4.1, 9.8)\textsuperscript{120} and the phosphate (pKa = 1.9, 6.8, 12.5)\textsuperscript{121} moieties present in the analyte molecules spectroscopically. For comparison purposes, normal Raman spectra of adenine, polycrystalline AMP, and in solution poly-A are shown (Figure 8(d)). A careful study of these spectra leads to several interesting observations. First, for a given pH, the SERS spectra of adenine, AMP and poly-A are all seen to be very similar to each other. This suggests that all the analyte molecules bind to surface through the same adenine moiety. The SERS spectra of all the
analytes are dominated by the adenine ring breathing mode, which upshifts from 723 cm\(^{-1}\) in normal Raman spectra (Figure 8(d)) to 735 cm\(^{-1}\) in SERS (Figure 8(a-c)). The 735 cm\(^{-1}\) mode in all the SERS spectra (Figure 8(a-c)) is pH-independent. Unlike AMP and poly-A, adenine is devoid of any phosphate and sugar moiety. However, the SERS spectra of AMP and poly-A (Figure 8(b, c)) are similar to the SERS spectra of adenine, showing no evidence of enhanced bands from the phosphate groups. The peaks at 1010 cm\(^{-1}\) and 1099 cm\(^{-1}\) in Figure 8(d), attributed to phosphate and sugar stretching modes respectively\(^{122}\), are observed in the normal Raman spectra of AMP and poly-Adenine but are absent in the corresponding SERS spectra. This could be due to the small Raman cross-section of the phosphate band\(^{123}\). The C-NH\(_2\) in-plane bend at 330 cm\(^{-1}\) (Figure 8(d)), appears at ~326 cm\(^{-1}\) in the SERS spectra (Figure 8(a-c)) and is observed to be insensitive to pH changes. The 1307 and 1337 cm\(^{-1}\) mode in normal Raman spectra (Fig.3) are clearly seen to be shifted to 1323 and 1342 cm\(^{-1}\) in the SERS spectra of the analytes (Figure 8(a-c)). The relative intensities of these bands are observed to change as a function of pH, which is a consequence of adenine protonation.
Figure 8. SERS spectra of (a) adenine, (b) AMP, and (c) and poly-adenine on nanoshell at different pH values of (i) 2.5, (ii) 5.1, (iii) 6.5, (iv) 8.5, and (v) 11.5 with (d) normal Raman spectra of (i) powder adenine, (ii) powder AMP, and (iii) in solution poly-adenine.

The peak shifts of SERS bands from the corresponding normal Raman bands are consistent with "chemical effects" of adenine interaction with the metal nanoshell surface and are in accordance with the SERS results reported for adenine adsorbed on silver colloid, silver electrode and silver island films. These observed similarities between the SERS spectra of A, AMP, and poly-A
along with a dominant adenine ring breathing mode suggest that all these molecules bind to the Au surface in a very similar way.

Adenine is known to be protonated at nitrogen N1 (Figure 7) with a pK$_a$ of 4.1$^{120}$. This results in pH dependence of the SERS spectra of A, AMP, poly-A (Figure 8). The observed pH dependence of the SERS spectra is induced by buffer exchange and was found to be reversible under our experimental conditions. Changes in the SERS band intensity and shift of peak positions of adenine with pH are presented in Figure 9 below. Variation of pH most significantly affects the relative intensities of the 1323 cm$^{-1}$ and 1342 cm$^{-1}$ features (highlighted by * in Figure 8), assigned to a summation of two different vibration modes. As the pH is changed from 2.5 to 11.5, the 1342 cm$^{-1}$ mode is seen to gradually intensify relative to the 1323 cm$^{-1}$ mode in the SERS spectra of A, AMP, and poly-A. This highly reproducible observation deserves special attention and is discussed below.

**Figure 9.** The relative SERS intensity (solid line) and peak positions (dashed line) of adenine bands [970 cm$^{-1}$ (top) and 620 cm$^{-1}$ (bottom)] as a function of pH. The 1323 cm$^{-1}$ band indicates a similar trend as shown by the 970 cm$^{-1}$ band, while the bands at 1401, 1380, 1273, 1140 cm$^{-1}$ show the trend as shown by the 670 cm$^{-1}$ band.
The study of the pH dependence of in solution normal Raman spectra of adenine and AMP, as shown in Figure 10, confirms that the observed pH-dependent differences in the relative intensities of the 1323 and 1342 cm\(^{-1}\) modes in the SERS spectra are due to the protonation of the adenine moiety. While the signals are weaker for normal Raman studies in solution (Figure 10), the intensity ratio of the 1307 and 1337 cm\(^{-1}\) peaks clearly show the same pH dependence as the SERS spectra in Figure 8. The change in peak intensities as function of pH in the normal Raman solution spectra is very similar to that observed in the SERS spectra. This strongly suggests that the pH-dependence of SERS is due to adenine protonation, with perhaps a small contribution from conformational changes brought about by the differences in orientation of the anchoring group at different pH.

![Figure 10](image)

**Figure 10.** Normal Raman spectra of adenine at pH=8.5; 0.85 and AMP at pH=8.5.

Surface-enhanced infrared absorption (SEIRA) studies were performed to confirm the local structure and orientation of the analyte molecules on metal
surface. SEIRA spectra of adenine and AMP deposited on nanoshells from solutions of pH 2.5 and 8.5 are shown in Figure 11. For comparison purpose, normal IR spectra of adenine and AMP (dispersed in KBr pellet) are shown in Figure 12. It is important to note that normal IR spectra of solid adenine is well known to have the effects of H bonding interactions that lead to downshifting of the NH\textsubscript{2} stretch modes (3440-3450, 3555-3570 cm\textsuperscript{-1}) and upshifting of the NH\textsubscript{2} scissor mode (1625-1640 cm\textsuperscript{-1}).\textsuperscript{89,124,125}

![Figure 11. SEIRA spectra of (a) adenine and (b) AMP molecules deposited on nanoshells from solutions of pH (i) 2.5 and (ii) 8.5 with the high wavenumber region shown as an inset. Spectra are offset for clarity.](image-url)

The SEIRA spectra of adenine deposited from solution at two different pHs are observed to be similar to each other. They all are dominated by the in-plane symmetric NH\textsubscript{2} scissor mode\textsuperscript{89} at ~1620-1640 cm\textsuperscript{-1} (a weak mode in the corresponding SERS spectra) and various ring modes. High wavenumber SEIRA spectra of adenine at both pH levels show the presence of the NH\textsubscript{2} symmetric stretch mode at 3290 cm\textsuperscript{-1}. However, the in-plane symmetric NH\textsubscript{2} scissor mode
presents a notable difference in its peak position. This peak appears at 1625 cm$^{-1}$ (Figure 11a, (ii)) and at 1640 cm$^{-1}$ (Figure 11a, (i)) in the SEIRA spectra of adenine deposited from solutions of pH 8.5 and 2.5, respectively. This blueshift of the peak position (15 cm$^{-1}$) is due to the effect of a neighboring positive charge (protonation at N1) on the NH$_2$ group.$^{126}$ The intensity of the 1286 cm$^{-1}$ peak is lower than 1310 cm$^{-1}$ peak in the SEIRA spectra of protonated adenine. However, the opposite holds true in the SEIRA spectra of deprotonated adenine. These subtle differences in the ring modes are secondary effects of protonation and are likely due to changes in the electronic distribution within the adenine ring caused by the protonation/deprotonation of the adenine molecule.

SEIRA vibrational analysis, based on the surface selection rule (vibrational modes that have a dynamic dipole moment directed normal to the metal surface are preferentially enhanced)$^{38}$, provides detailed information on the orientation and the probable binding sites of adenine on the Au nanoshell surface. The SEIRA spectra of adenine as shown in Figure 11a are dominated by the in-plane symmetric NH$_2$ scissor mode at around 1620-1640 cm$^{-1}$. The presence of this strong bending mode precludes a flat orientation of the exocyclic amino group. The relative intensity of this scissor mode to that of the purine mode (1595 cm$^{-1}$)$^{126}$ in the SEIRA spectra of adenine is higher than in the normal IR spectra, suggesting that the molecule is preferentially aligned with C-NH$_2$ along the surface normal. Other vibrational modes of the exocyclic amino group are the symmetric and antisymmetric NH$_2$ stretches$^{89}$ that appear in the high wavenumber region at 3296 and 3355 cm$^{-1}$ respectively in the normal IR
The high wavenumber SEIRA spectra of adenine (Figure 11a, inset) confirm the absence of the NH2 asymmetric stretch mode and the presence of a NH2 symmetric stretch mode at 3290 cm⁻¹. This provides additional evidence that the NH2 group is largely upright to the surface. Various in-plane ring modes (1058, 1286, 1310 cm⁻¹) are also observed in the SEIRA spectra. The presence of these in-plane ring modes (band assignments provided in Table S1 at the end of this chapter) strongly suggest the ring plane is not lying flat but is largely inclined from the surface with the C6-NH2 bond aligned almost normal to the surface. This evidence suggests that adenine molecules bind to the metal surface through N3 and/or N9 sites, in agreement with the established fact that N3 and N9 sites are more reactive functionalities than the external amino group of adenine.

Figure 12. Normal IR spectra of (i) adenine and (ii) AMP dispersed in KBr pellet in the low and high wavenumber (inset) region. Spectra offset for clarity.
The experimental SEIRA spectra of AMP deposited on nanoshells from solutions at different pH are also similar to each other. The presence of the sugar and phosphate groups give rise to the appearance of new bands in the 900-1200 cm\(^{-1}\) region\textsuperscript{126,127}. The peak intensity ratio at 1640 and 1595 cm\(^{-1}\) is seen to decrease to almost unity with increasing pH. The 984 cm\(^{-1}\) peak, attributed to symmetric stretching of the deprotonated terminal phosphate\textsuperscript{126} is clearly observed under basic conditions and grows less intense under acidic conditions. These subtle differences in the pH-dependent SEIRA of AMP may be partially attributed to the effects of protonation, in addition to pH-dependent AMP-nanoshell surface interactions.

In the SEIRA spectra of AMP deposited from solution of pH=8.5 (Figure 11b), the 1640 cm\(^{-1}\) peak (NH\(_2\) scissor mode) and the 1595 cm\(^{-1}\) peak (purine mode) are of almost equal intensity, in contrast with the case of adenine, where the 1645 cm\(^{-1}\) peak is stronger than the 1595 cm\(^{-1}\) peak. This indicates that the ring of AMP, as opposed to adenine, orients itself with a wider variety of conformations. Hence, it is likely that the exocyclic amino group and/or other available ring nitrogen of AMP interacts with and coordinates to the metal surface. The ring modes in the SEIRA spectra of AMP and adenine show subtle differences. For example, the 1286 cm\(^{-1}\) peak, which appears strongly in the SEIRA spectra of adenine, is very weak in the case of AMP. The relative intensities of the ring modes also vary. For example, in the SEIRA spectra of
AMP, the 1372 and 1343 cm\(^{-1}\) peak are of equal intensity, but for adenine, the intensity of 1372 cm\(^{-1}\) peak is higher than the 1343 cm\(^{-1}\) peak.

The observed differences in the various modes (NH\(_2\) scissor and ring modes) between adenine and AMP SEIRA spectra are likely a consequence of differences in the manner that adenine and AMP bind to the metal surface. Unlike the adenine case, the in-plane NH\(_2\) scissor mode does not dominate the SEIRA spectra of AMP. As discussed above, some of the ring modes that appear strongly in the SEIRA of adenine are weak in the SEIRA of AMP. These observations may indicate that the external amino group along with ring nitrogens can interact with the metal surface, leading to a different orientation of the ring of AMP as compared to adenine. For AMP, the exocyclic amino group, N3 and N1 are the only available possible coordinating sites since the most reactive N9 position is already blocked by the ribose sugar. Due to the relative positioning of these coordinating sites on the ring of AMP, it is anticipated that the tilt angle and orientation of the ring can be quite different when bonded through the exocyclic amino group and/or N3 and/or N1. The binding of adenine and AMP with different coordinating sites can potentially give rise to differences in the tilt angles of adenine and AMP on the Au surface.

Adenine adsorbed to metal surfaces has been extensively investigated, both experimentally and theoretically, over the past few decades. Here we review past work on adenine adsorption, and confirm our assignments of the adenine
SERS and SEIRA spectra using these results and our ab initio electronic structure calculations.

Previous studies of adenine at metal surfaces have proposed three main binding modes: flat “face-on” adsorption by the adenine π system, “end-on” coordination to a ring nitrogen, and binding via the external amine group. Kögline and coworkers\textsuperscript{98} measured SERS of adenine, adenosine, and AMP at a roughened silver electrode. Like us, they found significant similarities between the adenine and AMP SERS spectra, and concluded that the spectra were dominated by adenine ring vibrations. Watanabe and coworkers\textsuperscript{105} argued based on SERS experiments that adenine binds end-on at nitrogen N7. (Atom labels are shown in Figure 7.) Suh and Moskovits\textsuperscript{99} measured SERS of nucleic acid bases on silver sol. They assigned adenine’s intense 739 cm\textsuperscript{-1} band to an unspecified NH\textsubscript{2} deformation, and concluded based on the absence of a 3080 cm\textsuperscript{-1} C-H stretch that adenine was bound flat to the surface. Otto and coworkers\textsuperscript{106} also measured adenine SERS at a roughened Ag electrode. They concluded that the ring breathing mode at 732 cm\textsuperscript{-1} was the most enhanced. They also suggested that several enhanced bands involved vibrations of the external amine group, and speculated that this group bound to Ag. Kim and coworkers\textsuperscript{97} reported a significant concentration dependence of adenine SERS, and assigned the spectral changes to a change between face-on adsorption at low concentration, and end-on adsorption at nitrogen N1 at high concentration. Itoh and coworkers\textsuperscript{128} measured voltage- and pH-dependent SERS of 9-methyladenine on Ag electrodes. They suggested a nearly flat N1-protonated
geometry at high potential, with a deprotonated end-on orientation at more negative potentials.

Giese and McNaughton\textsuperscript{102} (henceforth GM) performed a very extensive analysis of adenine Raman and SERS spectra, combining isotopic substitution experiments with density functional theory (DFT) calculations. Based largely on an analysis of relative SERS intensities, they concluded that adenine adsorbs end-on to a variety of silver SERS substrates via nitrogen N7 and the external NH\textsubscript{2} group. McNutt and coworkers\textsuperscript{89} studied adenine on Cu(110) in ultra high vacuum (UHV) using reflection absorption infrared spectroscopy (RAIRS). They found that the spectra were dominated by the NH\textsubscript{2} scissors and in-plane ring modes, similar to our SEIRA spectra, and concluded that adenine tautomerized to coordinate with the surface at N3 and N9. Yamada and coworkers\textsuperscript{95} studied DNA bases on Cu(110) in UHV using infrared reflection absorption spectroscopy (IRAS). They found a very low signal at submonolayer coverages, and concluded that adenine adsorbed face-on. Higher coverages gave spectra dominated by the NH\textsubscript{2} scissors mode, suggested to be a second layer of tilted adenine. A recent temperature programmed desorption study by Östblom and coworkers\textsuperscript{94} of adenine at polycrystalline Au films suggested multiple energetically distinct binding motifs, with the strongest bonding assigned to adsorption by the external NH\textsubscript{2} group.

Theoretical studies of adenine SERS have so far provided only semiquantitative agreement with experiment. The most notable study to date is by Jensen,\textsuperscript{129} who simulated adenine's surface enhanced Raman and Raman
optical activity spectra by modeling adenine bound to a large Au$_{20}$ cluster via N7 and the external amine. These very sophisticated time-dependent DFT calculations did not reproduce typical experimental SERS spectra, predicting a small intensity for the ring breathing mode that tends to dominate adenine SERS. This points to the difficulty of quantitatively reproducing experimental SERS spectra, and validates our focus on simple semiquantitative calculations (see below).

In the remainder of this section, we interpret our adenine SERS and SEIRA spectra and assign putative conformations for the adenine-Au interaction. The spectral interpretations are based largely on the assignments by GM, buttressed by additional ab initio calculations and information from the low-pH spectra. Comparison between experimental and calculated Raman spectra is shown below in Figure 13. For completeness, calculated vibrational spectra for adenine, protonated adenine, and adenine-Au$^+$ complexes with Au at nitrogens N3 and N7, is shown at the end of this chapter (Table 4 - 7).
Figure 13. (i) Calculated B3LYP/Sadlej vibrational Raman spectrum of adenine, and (ii) experimental powder Raman spectrum of adenine. Calculated Raman activities are broadened by Lorentzians of width 10 cm$^{-1}$, intensities are in arbitrary units.

Because the SERS and SEIRA of adenine, AMP, and poly-adenine are so similar, we feel confident in assuming that the adsorption geometries are also similar, and thus focus on assigning the adsorption geometry of adenine itself. We also argue that these very strong, and reproducible adenine SERS and SEIRA signals would not occur for disordered adenine weakly bound to the nanoshells, such that the "overlayer" model proposed by Kawai and coworkers does not apply here.\textsuperscript{95} Figure 14 compares the experimental adenine powder Raman spectrum with the experimental adenine SERS obtained at pH 8.5 and 2.5. Peaks $< 1350$ cm$^{-1}$ tend to be shifted up in frequency, while peaks $> 1350$ cm$^{-1}$ are generally shifted down in frequency. GM suggested, based on an argument of Muniz-Miranda and coworkers,\textsuperscript{130,131} that this is due to a competition between the electronic and steric effects of end-on adenine-Au binding at the heterocyclic ring nitrogens. Low frequency peaks are shifted to higher
frequencies due to steric hindrance from the adjacent Au surface, while high frequency peaks are shifted to lower frequencies due to adenine → Au electron donation and adenine bond softening. Comparison of the high-pH and low-pH SERS shows that this effect is less pronounced at low pH, suggesting that protonated adenine binds less strongly. This is consistent with reduced adenine → Au electron donation from protonated, positively charged adenine. The low- and high-pH SERS spectra are otherwise rather similar, ruling out any dramatic pH-induced conformational change.

Figure 14. (i) Experimental powder Raman spectrum of adenine, and experimental SERS spectrum of adenine at (ii) pH 2.5 and (iii) 8.5. Intensities are in arbitrary units. Starred peaks are dominated by motions of the external amine group.

The adenine SEIRA spectra in Figure 11a are dominated by the NH$_2$ scissors mode at 1625 cm$^{-1}$. Based on the SEIRA surface selection rule, the C6-N10 bond must be aligned close to the surface normal. Like other workers, our SERS spectra are dominated by adenine ring vibrations including the 737 cm$^{-1}$ ring breathing, suggesting that the adenine ring is nearly perpendicular to the Au
surface. These two observations rule out the possibility of a "flat" adsorption geometry, though the adenine ring plane may still be somewhat tilted from the surface normal. Further evidence for a slightly tilted adsorption geometry comes from the weak SERS peaks at 565, 682 and 787 cm$^{-1}$, assigned by GM to out-of-plane adenine vibrational modes. (The peak at 565 cm$^{-1}$ might also be a shifted version of the 536 cm$^{-1}$ ring deformation.)

Based on this analysis, adenine is most likely bound to Au by one of the ring nitrogens N1, N3, N7, or N9, with possible additional contributions from the external amine. While adenine itself could adsorb at nitrogen N9 via tautomerism (as suggested by McNutt and coworkers$^{89}$), this binding mode is not possible for AMP and poly-adenine, and is thus ruled out. Binding at N1 is also unlikely, as this is the only nitrogen with a pK$_a$ above 1.0,$^{132}$ suggesting that the observed pH dependence can only occur if adenine is protonated at N1. Binding at N3 is somewhat unlikely for AMP and poly-A, due to steric hindrance from the substituents bound at N9. GM argued that adenine binds via N7 and the external NH$_2$ group. However, we have some questions about this assignment. Figure 14 shows that the C6-NH$_2$ bend at 330 cm$^{-1}$, the NH$_2$ rock at 1025 cm$^{-1}$, and the NH$_2$ rock at 1234 cm$^{-1}$ (marked by "**") are not strongly shifted in the low-pH or high-pH SERS spectra, in contrast to the substantial SERS shifts seen for the adenine ring modes. To us, this suggests that the NH$_2$ group does not contact the surface. Further evidence for this conclusion comes from the NH$_2$ scissors mode in the high-pH SEIRA, whose position at 1625 cm$^{-1}$ is only slightly shifted from the 1618 cm$^{-1}$ seen in matrix isolation IR.$^{124}$ We suggest that adenine and its
derivatives bind the Au surface exclusively at nitrogen N7, with the C6-NH$_2$ bond aligned near the surface normal. Binding at N3 is also possible. Figure 15 gives a schematic of our two proposed adenine-Au binding modes.

![Figure 15. Schematic of proposed adenine-Au binding modes, through ring nitrogens N3 (left) or N7 (right).](image)

As further evidence for this assignment, Table I below presents calculations on adenine bound to a single Au$^+$ atom. GM and others have stated that the SERS spectra of adenine on Ag is qualitatively quite similar to Raman of adenine-Ag$^+$ complexes,$^{102}$ thus we assume that this is also a reasonable first order model of the energetics. Binding is strongest to ring nitrogens N1 and N3, somewhat weaker to N7, and quite a bit weaker to the external NH$_2$ group. While this model system is rather crude, it indicates that our suggested possible binding sites (N3 and N7) are both energetically reasonable.
Table 1. Calculated binding energy (kcal/mol) between adenine and Au$^+$

<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Energy (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>79.2</td>
</tr>
<tr>
<td>N3</td>
<td>79.5</td>
</tr>
<tr>
<td>N7</td>
<td>73.5</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>64.7</td>
</tr>
</tbody>
</table>

Table II further demonstrates that the low-pH adenine SERS and SEIRA involve protonation at nitrogen N1. The table presents gas-phase B3LYP/6-31G(d,p) calculations on neutral adenine, and adenine-H$^+$ protonated at the N1, N3, or N7 ring nitrogens. As expected, protonation at N1 is energetically most favorable. Moreover, the C-NH$_2$ bend, ring breathing, and NH$_2$ rock vibrational modes are shifted very little by N1 protonation, while the NH$_2$ scissors mode is dramatically shifted to higher frequencies. This is consistent with the differences between low-pH and high-pH SERS and SEIRA spectra.

Table III illustrates why adenine binding at nitrogen N7 is somewhat inconsistent with the NH$_2$ modes marked with "*" in Figure 8. The table shows calculated vibrational frequencies of these modes for isolated adenine and adenine bound to Au$^+$ at the N1, N3, N7 ring nitrogens or the external NH$_2$ group. Details are as in Table I. Au$^+$ binding at N3 yields relatively small shifts of these NH$_2$ modes, and binding at N7, N3, and (especially) the external NH$_2$ group produces rather significant frequency shifts. While none of these
calculations perfectly reproduce experiment, they demonstrate that N3 binding is consistent with the experimental spectra.

Table 2. Calculated relative energy (kcal/mol) and selected vibrational frequencies (cm\(^{-1}\)) of neutral adenine and adenine protonated at the N1, N3, and N7 ring nitrogens.

<table>
<thead>
<tr>
<th>Species</th>
<th>Energy</th>
<th>C-NH(_2) bend</th>
<th>Ring breathing</th>
<th>NH(_2) rock</th>
<th>NH(_2) scissor</th>
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<tr>
<td>Neutral</td>
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<td>275</td>
<td>728</td>
<td>1020</td>
<td>1674</td>
</tr>
<tr>
<td>H(^+) on N1</td>
<td>-239</td>
<td>276</td>
<td>724</td>
<td>1004</td>
<td>1732</td>
</tr>
<tr>
<td>H(^+) on N3</td>
<td>-238</td>
<td>278</td>
<td>721</td>
<td>999</td>
<td>1712</td>
</tr>
<tr>
<td>H(^+) on N7</td>
<td>-231</td>
<td>295</td>
<td>724</td>
<td>1003</td>
<td>1701</td>
</tr>
</tbody>
</table>

Table 3. Calculated frequencies (cm\(^{-1}\)) of the vibrational modes marked with "**" in Figure 14, for four adenine-Au\(^+\) complexes.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>C6-NH(_2) bend</th>
<th>NH(_2) rock</th>
<th>NH(_2) rock</th>
<th>NH(_2) scissor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>276</td>
<td>1017</td>
<td>1246</td>
<td>1661</td>
</tr>
<tr>
<td>Adenine-Au(^+), N1</td>
<td>292</td>
<td>1016</td>
<td>1224</td>
<td>1687</td>
</tr>
<tr>
<td>Adenine-Au(^+), N3</td>
<td>284</td>
<td>1012</td>
<td>1228</td>
<td>1686</td>
</tr>
<tr>
<td>Adenine-Au(^+), N7</td>
<td>322</td>
<td>1016</td>
<td>1234</td>
<td>1671</td>
</tr>
<tr>
<td>Adenine-Au(^+), NH(_2)</td>
<td>235</td>
<td>758</td>
<td>1271</td>
<td>1617</td>
</tr>
</tbody>
</table>

Figure 16 illustrates that the observed pH dependence of the adenine SEIRA spectra in
Figure 11 is consistent with the effects of adenine protonation. The figure shows the calculated IR absorbance spectra of adenine and protonated adenine. The calculated spectra clearly show that the intense NH\textsubscript{2} scissors mode shifts to higher frequency upon protonation. Protonation also makes the NH\textsubscript{2} scissors mode more intense relative to the purine ring mode observed at 1595 cm\textsuperscript{-1}. The NH\textsubscript{2} stretch modes and the N9-H stretch calculated in the 3400-3700 cm\textsuperscript{-1} range shift to slightly lower frequencies upon protonation, and become slightly less intense. All of these effects are consistent with the experimental adenine SEIRA in Figure 11a. As mentioned previously, the antisymmetric NH\textsubscript{2} stretch calculated at \sim 3630 cm\textsuperscript{-1} appears absent in the experimental SEIRA spectra, providing further evidence that the C6-NH\textsubscript{2} bond is aligned near the surface normal. The C-H stretches around 3000 cm\textsuperscript{-1} are calculated to have rather low IR intensities. As mentioned previously, the SEIRA of AMP show some differences from the adenine SEIRA, which may be consistent with a different binding motif for AMP.

![Figure 16](image-url)

**Figure 16.** Calculated IR absorbance spectra of (i) adenine and (ii) adenine-H\textsuperscript{+}. Asterisks **"** denote the NH\textsubscript{2} scissors mode, other details are in the text.
3.4. Conclusions

Surface-enhanced Raman spectra (SERS) of adenine, adenosine monophosphate (AMP), and poly-adenine adsorbed on Au-nanoshells in aqueous solution of different pH values have been investigated to study the pH-induced conformational changes of the analyte adsorbate molecules. The pH dependent SERS spectra were found to be reversible under experimental conditions. The SERS spectra of the analytes were found to be very similar to each other, suggesting that all the analytes bind to the surface in very similar way. Normal modes obtained from electronic structure theory calculations at DFT level for Au-adenine complexes along with the existing literature on adenine binding were used to assign the observed SERS bands of adenine. The local structure and orientation of adenine and AMP molecules on an Au nanoshell substrate surface was confirmed using surface-enhanced infrared absorption (SEIRA) spectroscopy. The effect of protonation of the adenine moiety can clearly be observed in the acquired SERS and SEIRA spectra. Spectral analysis indicates that the ring planes of both adenine and AMP are not lying flat but have upright orientation with a small angle from the surface normal. Experimental results suggest that adenine adsorbs "end-on" to Au surface with the C6-NH2 bond almost aligned normal to the surface, while AMP binds through N3 and/or the external NH2 group. DFT calculations for adenine support our experimental SERS-SEIRA spectra and provide insight to the local binding geometry. Adenine binding "end-on" through N3 with C6-NH2 bond aligned near the surface normal,
as shown in the left schematic of Fig 15, is proposed as the plausible orientation on Au nanoshell surface.
Table 4. Calculated gas-phase B3LYP/Sadlej and experimental frequencies of adenine.

<table>
<thead>
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<th>Expt</th>
<th>GM</th>
<th>Current Plane</th>
<th>Assignment</th>
</tr>
</thead>
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<tr>
<td>3730</td>
<td>in</td>
<td>str NH(_2) (antisymmetric)</td>
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</tr>
<tr>
<td>3644</td>
<td>in</td>
<td>str N9-H</td>
<td></td>
</tr>
<tr>
<td>3595</td>
<td>in</td>
<td>str NH(_3) (symmetric)</td>
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</tr>
<tr>
<td>3226</td>
<td>in</td>
<td>str C8-H</td>
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</tr>
<tr>
<td>3154</td>
<td>in</td>
<td>str C2-H</td>
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</tr>
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<td>1674</td>
<td>1665</td>
<td>1661</td>
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</tr>
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<td>1643</td>
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</tr>
<tr>
<td>1597</td>
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<td>1606</td>
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<td>1483</td>
<td>1524</td>
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</tr>
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<td>1373</td>
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<td>1246</td>
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<td>897</td>
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</tr>
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<td>877</td>
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</tr>
<tr>
<td>797</td>
<td>805</td>
<td>827</td>
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<td>725</td>
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</tr>
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<td>239</td>
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</tr>
<tr>
<td>133</td>
<td>166</td>
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<td>out</td>
</tr>
</tbody>
</table>

*Experimental values from Ref. 102 \(^{b}\) B3LYP/6-311++G(d,p) values from Ref. 102; \(^{c}\) Bend, bending; breath, breathing; def, deformation; rock, rocking; sciss, scissoring; str, stretching; wag, wagging; R5, five membered ring; R6, six membered ring.*
Table 5. Calculated gas-phase B3LYP/Sadlej vibrational frequencies of adenine-

$H^+$, protonation at nitrogen N1, details as in Table 4.

<table>
<thead>
<tr>
<th>Mode</th>
<th>Freq (cm$^{-1}$)</th>
<th>Plane</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'</td>
<td>3692</td>
<td>in str NH$_2$ (antisymmetric)</td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>3603</td>
<td>in str N9-H</td>
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<td>219</td>
<td>out Butterfly</td>
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<td>36</td>
<td>174</td>
<td>out tors molecule, wag C6-NH$_2$</td>
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Table 6. Calculated gas-phase B3LYP/Sadlej/aug-cc-pVTZ-PP vibrational frequencies of adenine-Au\textsuperscript{+}, with Au bound at nitrogen N3, details as in Table 4.

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<th>Assignment</th>
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<tr>
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<td>3619</td>
<td>in</td>
<td>str N9-H</td>
</tr>
<tr>
<td>3'</td>
<td>3571</td>
<td>in</td>
<td>str NH\textsubscript{2} (symmetric)</td>
</tr>
<tr>
<td>4'</td>
<td>3239</td>
<td>in</td>
<td>str C8-H</td>
</tr>
<tr>
<td>5'</td>
<td>3192</td>
<td>in</td>
<td>str C2-H</td>
</tr>
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<td>1686</td>
<td>in</td>
<td>str C6-N10, C5-C6</td>
</tr>
<tr>
<td>2</td>
<td>1628</td>
<td>in</td>
<td>str N3-C4, N1-C2, N7-C8, bend N9-H</td>
</tr>
<tr>
<td>3</td>
<td>1618</td>
<td>in</td>
<td>sciss NH\textsubscript{2}</td>
</tr>
<tr>
<td>4</td>
<td>1529</td>
<td>in</td>
<td>str N7-C8, bend C8-H, C2-H, sciss NH\textsubscript{2}</td>
</tr>
<tr>
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<td>in</td>
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<tr>
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<td>1497</td>
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</tr>
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<tr>
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<td>Ring breathing</td>
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<tr>
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<td>692</td>
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<td>170</td>
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<td>R5, R6 torsion</td>
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<td>115</td>
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<td>64</td>
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Table 7. Calculated gas-phase B3LYP/Sadlej/aug-cc-pVTZ-PP vibrational frequencies of adenine-Au⁺, with Au bound at nitrogen N7, details as in Table 4.

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<tr>
<td>36</td>
<td>96</td>
<td>in</td>
<td>N7-Au rock</td>
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<tr>
<td>37</td>
<td>34</td>
<td>out</td>
<td>N7-Au wag</td>
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Chapter 4. Interactions of ibuprofen with hybrid lipid bilayers probed by complementary surface-enhanced vibrational spectroscopies

This work was done in collaboration with C. S. Levin (equal contributor), B. G. Janesko, G. E. Scuseria, R. M. Raphael.

4.1. Introduction

The interactions of amphiphilic molecules, such as nonsteroidal anti-inflammatory drug (NSAIDs), with cellular membranes are biologically important. The clinical use of NSAIDs for their analgesic, antipyretic and anti-inflammatory properties is extremely widespread; however, these compounds cause serious side effects such as gastrointestinal bleeding and peptic ulcer disease. It has been postulated that these side effects occur due to the interaction of amphiphiles with phospholipid layers covering the gastrointestinal mucosa. In support of this hypothesis, salicylate has been shown to interact with and change the physicochemical properties of lipid membranes in a way that enhances the formation of membrane pores. Ibuprofen, one of the most heavily prescribed NSAIDs, is known to partition into synthetic and biological membranes and cause changes in the permeability, fluidity, mechanical, and structural properties of the membrane. It has been shown using optical-trapping confocal Raman microscopy that ibuprofen causes a high level of
disorganization in lipid phosphatidylcholine (PC) acyl chains in a dose-dependent manner.\textsuperscript{139}

Recently there has been growing interest in probing biological membrane function by coupling the properties of nanostructured materials with lipid membranes or membrane mimics.\textsuperscript{140-143} Hybrid lipid bilayers provide a very important materials system for this approach. They typically consist of an alkanethiol self-assembled monolayer (SAM)\textsuperscript{144}, bound to a noble metal substrate, with an associated outer layer of phosphatidylcholine (PC) lipids.\textsuperscript{145} Phospholipids with phosphocholine headgroups are known to exist in high abundance within the plasma and intracellular membranes of eukaryotic cells.\textsuperscript{146} In a hybrid lipid bilayer, the phospholipid layer assembles onto the SAM with its hydrophobic tail groups directly adjacent to the alkane chains of the SAM, forming a structural analog to the natural lipid bilayer structures found in biological systems.\textsuperscript{147} Hybrid bilayers are a good composite system for investigation because they mimic the composition of biological membranes\textsuperscript{148} with the additional advantages that they are robust, stable, and can be dried and rehydrated.\textsuperscript{149} Additionally, they can be fabricated to cover large surface areas, are easy to form through self-assembly techniques, and have been characterized by a variety of analytical methods.\textsuperscript{150}

In this work, hybrid bilayers are assembled onto Au nanoshells, whose plasmon-derived properties allow them to serve as strongly enhancing substrates for both surface enhanced vibrational spectroscopies,\textsuperscript{93} surface enhanced Raman scattering (SERS) and surface enhanced infrared absorption
spectroscopy (SEIRA). Au nanoshells are spherical nanoparticles consisting of a dielectric (silica) core and a thin metallic (gold) shell. Nanoshells have the unique property that their plasmon resonance can be tuned across a broad region of the optical spectrum by varying the relative dimensions of the core and shell layers of the nanoparticle. Surface enhanced spectroscopies require large local electromagnetic fields at the substrate surface, which are an important characteristic of plasmon-resonant nanoparticles. With nanoshells, the large local electromagnetic field can be resonantly engineered in a frequency range of choice by controlling the internal nanoshell geometry, and by forming small aggregates or arrays. For SERS, the single particle plasmon resonance of the substrate is tuned near the frequency of the excitation laser, while for SEIRA, the local fields in the interparticle junctions must be intense across the entire infrared wavelength range over which the molecules will be probed. SERS and SEIRA enhancements observed with nanoshells as the plasmonic substrate are both strong and highly reproducible. By utilizing geometries with closely adjacent nanoshells, both Raman and infrared spectroscopies have been shown to be enhanced simultaneously on the same nanoshell-based substrate.

Due to the complementary nature of these vibrational spectroscopies, the combination of both SERS and SEIRA provides a uniquely information-rich method for probing molecular systems at the nanoscale. Specifically, ibuprofen's aromatic ring structure is known to have strong Raman-active modes whereas the polar headgroups of the lipid layer have strong IR-active vibrational modes. By utilizing both SERS and SEIRA, we can spectroscopically monitor both
Raman and IR active functional groups of both the intercalant molecules and the hybrid bilayer. These two combined spectroscopies could be potentially used to chemically differentiate between similar analogs of an analyte molecule with high specificity.

Two different effects are thought to contribute to the total enhancement in both SERS and SEIRA: electromagnetic and chemical effects. The electromagnetic contribution consists of the enhancement associated with the local surface plasmon excitation\textsuperscript{155} while the chemical contribution is due to the electronic coupling interactions between the adsorbate molecules and the substrate\textsuperscript{156}. These interactions lead to the observed changes in frequency of Stokes modes in SERS when compared to unenhanced Raman spectroscopy.\textsuperscript{157}

It is interesting to note that in addition to their relevance as a biological mimic, hybrid bilayers and their intercalant species also provide a simple approach for isolating electromagnetic from chemical effects in SERS\textsuperscript{158-160}, since the bilayer-intercalated molecules are adjacent to, but not directly interacting with, the substrate surface.

A schematic diagram of the nanoparticle-hybrid bilayer complex is shown in Figure 17. The sample geometries consist of (A) ibuprofenate adsorbed onto bare nanoshells for SERS and (B) ibuprofen intercalated in hybrid bilayers for SERS (where the single particle plasmons are utilized), and (C) ibuprofen intercalated in hybrid bilayers on nanoshell aggregates for SEIRA. (Ibuprofenate adsorbed onto nanoshell aggregates for SEIRA is not shown for simplicity).
4.2. Experimental

Au nanoshells of core radius 60 nm with shell thickness 20 nm and core radius 190 nm with shell thickness 35 nm were fabricated for SERS and SEIRA measurements respectively, according to previously described protocols. The core and shell dimensions of the nanoshells used for SERS were adjusted so that the plasmon resonance provided a near-field enhancement maximum near 785 nm in water, to provide enhancement at the 785 nm pump laser wavelength. The dimensions of the nanoshells used for SEIRA measurements were adjusted to provide a plasmon resonance absorbance maximum in air in the mid-infrared (mid-IR) region: the full SEIRA bandwidth originates from both plasmon-resonant and lightning rod effects. The nanoshell dimensions were then confirmed by scanning electron microscopy (SEM).

The formation of a SAM on Au nanoshells requires the nanoshells to be dispersed in an ethanolic solution of dodecanethiol. The aqueous solutions of
\([r_1, r_2] = [60, 80] \) nm and \([r_1, r_2] = [190, 125] \) nm nanoshells were centrifuged at 350 RCF for 30 minutes and 90 RCF for 20 minutes, respectively. The particles were then resuspended in absolute ethanol (200 proof, AAPER Alcohol and Chemical Co.).

Alkanethiol monolayers were first prepared on the nanoshells by separately making a solution of 10 mM 1-dodecanethiol (Aldrich \( \geq 98\% \)) in absolute ethanol. This was diluted to 30 \( \mu \)M solutions with the Au nanoshells in ethanol, and allowed to incubate overnight in the dark for covalent attachment. 30 \( \mu \)M solutions were chosen based on the nanoshell surface area and concentration, and based on the size of the dodecanethiol molecule\(^{161}\) to provide monolayer coverage in ten times excess. After incubation, the nanoshells had settled and the supernatant was removed, so that the nanoshells were redispersed in fresh ethanol and any unreacted thiol was removed from solution. The dodecanethiol functionalized nanoshells were then allowed to dry completely.

For in solution measurements, an initial stock solution of ibuprofen sodium salt was prepared according to Du et al.\(^{162}\) by dissolving ibuprofen (Sigma, USA) in sodium hydroxide (1N, Fisher Scientific) and then adjusting the pH with hydrochloric acid (1N, Fisher Scientific). Since the pK_<sub>a</sub> of ibuprofen is about 4.6,\(^{163}\) the molecule is predominantly in the ionized form under basic conditions. For the unenhanced powder measurements, the ibuprofen sodium salt utilized was purchased from Sigma.
Lipid solutions of either dimyristoylphosphatidylcholine (DMPC) or 1,2-Dimyristoyl-D54-sn-Glycerol-3-Phosphocholine-1,1,2,2-D4-N,N,N-trimethyl-D9 (DMPC-D54) (Avanti Polar Lipids, Inc., Alabaster, AL) were prepared by solubilizing the lipids in isopropyl alcohol (Fisher Scientific) (50 μL per 2 μmol of lipid). The DMPC-D54 lipids and ibuprofen stock solution were added to alkanethiol functionalized nanoshells to give a final concentration of 100 μM DMPC lipids and final concentrations of 0.01 mM, 0.1 mM, 1 mM, 10 mM, and 100 mM of ibuprofen respectively. The solutions were placed in an ultrasonicator bath (VWR Model 150D) for 30 minutes at 30 °C, above the main phase transition temperature of the DMPC lipid \( T_m = 24 \, ^\circ C \) and DMPC-D54 lipid \( T_m = 18.7 \, ^\circ C \) where the lipid is in the liquid crystalline phase and the hydrocarbon chains are more compressible and fluid. All measurements were then performed at room temperature around 22 °C.

The hybrid bilayer phase behavior was characterized using the fluorescent probe Laurdan, following the techniques of Parassis et al., Bagatolli et al. and Zhou et al. For these measurements, an appropriate amount of hybrid bilayers formed with either DMPC-D54 or DMPC were mixed with a methanolic solution of Laurdan (Sigma Aldrich) to achieve a lipid/probe ratio of 300:1. The emission spectra between 400 nm and 600 nm were obtained at a fixed excitation chosen between 320 nm and 390 nm.

SERS substrates consisted of fused quartz (Technical Glass Products, Inc., Painesville Twp., OH), while SEIRA substrates consisted of silicon (Sumco Oregon Corp., Salem, OR). Both substrates were coated with poly(4-
vinylpyridine) (PVP). Substrates of this composition combine the advantages of colloidal suspensions and the stability of solid substrates. Cut fused quartz or silicon substrates were first treated with a piranha solution (a concentrated solution of sulfuric acid and hydrogen peroxide) for two hours followed by rinsing with ethanol and drying with nitrogen. Films of poly(4-vinylpyridine) (Aldrich Chemical Company) were deposited by immersing in dilute (0.1%) solutions in absolute ethanol (AAPER Alcohol, Shelbyville, KY) for two hours followed by rinsing with ethanol, drying with nitrogen and were then allowed to cure overnight. The hybrid bilayer ibuprofen solutions were drop dried on the functionalized fused quartz or silicon substrates and then examined with either an inVia Raman microscope (Renishaw) with a 63x water immersion objective after rehydrating the sample at the appropriate pH, or a normal incidence transmission FTIR system (Thermo Nicolet) in air using a liquid nitrogen cooled MCT detector (4 cm⁻¹ resolution, 256 scans). All IR and SEIRA spectra were background corrected using the Omni software package. Water purified by a Milli-Q water system was used throughout the experiments. The transmission electron microscopy (TEM) images presented in Figure 2 were obtained with a JEM 2010 Cryo-TEM. Excitation generalized polarization spectra were obtained using a JOBIN YVON UV-vis Fluorolog.

4.3. Results and Discussion

Representative TEM images for both a bare nanoshell and a hybrid bilayer functionalized nanoshell are shown in Figure 18A and 18B, respectively.
Additional evidence for the formation of hybrid bilayers on nanoshells is demonstrated by the optical image (Figure 18C), which shows nanoparticle solubility at various stages of hybrid bilayer formation. Unfunctionalized nanoshells easily disperse in aqueous solvent (Figure 18C, i), however, nanoshells functionalized with an alkanethiol are hydrophobic and preferentially disperse in an organic carbon disulfide phase, rather than a water phase (Figure 18C, ii). Once a lipid layer self-assembles atop of the alkanethiol, the nanoshells are readily dispersed back into water (Figure 18C, iii).

**Figure 18.** TEM images of (A) a bare Au nanoshell and (B) hybrid bilayer functionalized Au nanoshells deposited on TEM grids (as shown by the line and contrast in B). (C) Optical images of Au nanoshells (i) dispersed in water, (ii) functionalized with an alkanethiol (which preferentially disperse in an organic carbon disulfide layer on the bottom, rather than the top water layer), and (iii) functionalized with a hybrid lipid bilayer dispersed in water.
A phase-sensitive fluorescent probe, Laurdan, was utilized to examine possible hybrid bilayer gel-phase characteristics at both pH 3 and 10. Excitation generalized polarization is defined as

\[ GP_{\text{ex}} = \frac{I_{444} - I_{484}}{I_{444} + I_{484}} \]

where \( I_{444} \) and \( I_{484} \) are fluorescence intensities at 444 and 484 nm, respectively. The excitation generalized polarization spectra (GP_{ex}) can be utilized to indicate phase behavior below, near, and above the main phase transition temperatures. The GP_{ex} spectra for hybrid bilayers with both deuterated DMPC-D54 and nondeuterated DMPC lipids are presented in Figure 19. Both deuterated and nondeuterated systems were probed because it has been experimentally observed that deuterating the lipid can lead to an approximate 4-5 degree decrease in the main phase transition temperature. For hybrid bilayers formed using nondeuterated DMPC, the GP_{ex} spectra show no appreciable tilt either downward or upward, indicating that they are not affected by the excitation wavelength. This evidence suggests that hybrid bilayers formed with nondeuterated DMPC are below the main phase transition temperature and have gel phase characteristics under the employed experimental conditions at both pH 3 and 10. For the deuterated hybrid bilayers, however, a slight downward tilt of the GP_{ex} spectra is observed for both pH values. Since GP_{ex} spectra show a downward tilt above the main phase transition temperature, it is reasonable to conclude that under these experimental conditions the deuterated hybrid bilayers reside in a liquid phase. These
conclusions are in accord with the established result that the $T_m$ for a deuterated lipid is lower than its nondeuterated analog.

![GPex spectra](image)

**Figure 19.** The excitation generalized polarization (GPex) spectra of hybrid bilayers on nanoshells (HBL) and deuterated hybrid bilayers on nanoshells (HBL-D54) with Laurdan at pH 3 and 10.

Figure 20 shows the unenhanced Raman and IR spectra of sodium ibuprofenate along with the SERS and SEIRA spectra of ibuprofenate on bare nanoshells. The enhanced Raman and infrared (IR) spectra of sodium ibuprofenate differ from their respective unenhanced spectra, revealing significant chemical interaction between the molecule and the Au nanoshell surface (Fig. 20). The major peaks in the SERS spectrum (Fig. 20A, iii) are attributable to the sodium ibuprofenate molecule and indicate its interaction with the surface. The intense low wavenumber peak at 249 cm$^{-1}$ has been assigned to the adsorption of ibuprofenate onto the Au nanoshell surface via the COO$^-$ moiety, and arises from the CO$_2$-Au vibration. Several peaks appear to be shifted in the SERS spectrum with respect to the unenhanced Raman spectrum. For example, the in-plane ring deformation at $\sim$637 cm$^{-1}$ in the unenhanced
spectrum shifts to $\sim 663 \text{ cm}^{-1}$ in the SERS spectrum. The peaks at 1381 and $1579 \text{ cm}^{-1}$ in the SERS spectrum can be attributed to the symmetric and asymmetric stretching vibrations of the $\text{COO}^-$ group.\textsuperscript{175} The presence of the peak at 1703 cm$^{-1}$ in the SERS spectrum on bare nanoshells is assigned to the C=O stretch. The appearance of these three peaks allows us to infer that not all of the carboxylate groups of ibuprofen are in an ionized state. It is reasonable that both protonated and deprotonated forms of the molecule may be present, since the aqueous nanoshell solution (pH $\approx 5.2$) to which the ibuprofenate was added was near the pKa of ibuprofen. Additionally, the $\sim 1185 \text{ cm}^{-1}$ and $\sim 1610 \text{ cm}^{-1}$ peaks are relatively intense in the unenhanced Raman but not in the SERS spectrum, which may be due to conformational variability of the ibuprofenate as it is adsorbed onto the Au nanoshell surface.

Although ibuprofen is a commonly prescribed analgesic, there have been few spectroscopic studies of this molecule in the literature,\textsuperscript{174-176} and none reported for sodium ibuprofenate. Also, ibuprofen exhibits a strong IR spectral dependence on its degree of solvation and local environment.\textsuperscript{177-181} The SEIRA spectra (Fig. 20B, ii) shows several shifted and enhanced modes of ibuprofenate compared to its unenhanced spectrum. The various in-plane ring modes assigned by Jubert et al.\textsuperscript{174} and Gordijo et al.\textsuperscript{175} (at 847, 1090, 1365, 1466, and $1510 \text{ cm}^{-1}$) seen in the SEIRA spectra are shifted from their corresponding peak positions in the normal IR spectra. The significantly different spectra obtained for the unenhanced and enhanced Raman and IR cases indicate that both chemical
interactions and electromagnetic effects are contributing to both SERS and SEIRA in these studies.

**Figure 20.** Raman spectra (A) of (i) ibuprofen sodium salt powder (50x objective, 25.5 mW laser power), (ii) sodium ibuprofenate in aqueous solution (2 M, 63x objective, 40.6 mW laser power), and (iii) surface enhanced Raman spectrum of sodium ibuprofenate (50 mM, 63x objective, 0.144 mW) adsorbed onto Au nanoshells. IR spectra (B) of (i) ibuprofen sodium salt powder and (ii) surface enhanced IR spectrum of sodium ibuprofenate (50 mM) adsorbed onto Au nanoshells (inset shows high frequency regime). (Spectra offset for clarity).
Hybrid bilayer-coated nanoshell substrates using a deuterated lipid (DMPC-D54) were exposed to a range of concentrations of ibuprofen at two different pH values to study its intercalation into the hybrid bilayers using SERS (Fig. 21A,B). The pH values three and ten were chosen so that ibuprofen remains predominantly in either the protonated or deprotonated form. Figure 21A,B provides spectral evidence for the deuterated lipid where there are additional peaks that occur due to C-D stretches in the 2000 cm$^{-1}$ to 2200 cm$^{-1}$ region. These peaks reveal that the lipid component of the hybrid bilayer is present near the nanoshell surface and allow for spectral segregation of different aspects of the hybrid bilayer system under study. By increasing the ibuprofen concentration at both pH values (Fig. 21A,B (ii-vii)), several ring modes of the molecule at 803, 1185, 1205 and 1610 cm$^{-1}$ in the SERS spectra were observed to increase in intensity, confirming its presence in the lipid bilayer. Figure 21C shows the gradual increase of ibuprofen partitioning into the bilayers for both pH values obtained by monitoring the normalized SERS intensity of the strongest mode at 1610 cm$^{-1}$. The increase in intensity as a function of ibuprofen loading concentration displays an isotherm-like response. Interestingly, at low pH when the ibuprofen molecule is predominately protonated, the signal from ibuprofen is stronger, allowing for better detection than at high pH. The stronger ibuprofen signal intensity at low pH may result from a change in the hydrophobicity of the molecule. In the protonated form, ibuprofen is anticipated to be more hydrophobic in nature than in the dissociated form. Due to the hydrophobic effect, a decrease in pH may allow for more ibuprofen molecules to intercalate.
into the hybrid bilayer, increasing their local concentration and in turn, allowing for the ibuprofen molecules to get closer to the hydrophobic acyl chains.

Ibuprofen itself is a known surfactant and has a critical micelle concentration (CMC), above which it can exist in an aggregate, rather than monomeric form. The interactions between ibuprofen and lipids and its toxicity depend on the aggregation pattern of ibuprofen. High concentrations above the CMC for ibuprofen can damage the integrity of lipid bilayers. While electrochemical studies, such as cyclic voltammetry and impedance spectroscopy, can act as a direct way to examine membrane permeability and integrity, similar studies to characterize pore formation for this system are difficult. The system presented here is unique in that it is the first demonstration of hybrid bilayers on a nanoparticle surface, but the small size of the nanoparticles and the lack of an electrically conductive continuous film of nanoparticles hinder their use as electrode probe, as finite interparticle separations are imperative for large spectroscopic enhancements. However, the observed gradual decrease in the C-D stretch intensity as a function of increasing ibuprofen concentration (shown in the SERS spectra in Figure 21A,B) may provide an indirect indication that hybrid bilayer is being disrupted by the presence of ibuprofen.

Al-Saidan et al. has reported a CMC value of 0.83 mM for ibuprofen solutions prepared in aqueous 0.2 M disodium hydrogen phosphate. The 0.1 mM concentration detected at both pH 3 and 10 in the SERS measurements (Figure 21) is below the CMC of ibuprofen, and therefore, provides
physiologically relevant information on the interaction of ibuprofen and hybrid lipid bilayers in a liquid-crystalline phase, characteristic to that of biologically functional cell membranes\(^{185}\).

Figure 21. Raman spectra of (i) ibuprofen in aqueous solution at (A) pH 10 and (B) pH 3 and SERS spectra of hybrid bilayer functionalized nanoshells with deuterated DMPC as a function of ibuprofen concentration: (ii) 0 mM, (iii) 0.01 mM, (iv) 0.1 mM, (v) 1 mM, (vi) 10 mM, and (vii) 100 mM at (A) pH 10 and (B) pH 3. The dashed lines indicate the peaks from clearly identifiable modes of the ibuprofen, indicating its presence in the hybrid bilayer (spectra offset for clarity). (C) Normalized SERS intensity of ibuprofen ring mode (11610-1585/11434-1388) as a function of ibuprofen concentration in hybrid bilayers at pH 10 (●) and pH 3 (■) with best-fit Langmuir isotherm (lines). The fitting equation and parameters used are \(y = \frac{ax}{(1 + bx)(1 - c)}\), where at low pH \(a = 1.64 \pm 0.22\), \(b = 1.53 \pm 0.87\), and \(c = 0.42 \pm 0.21\) and at high pH \(a = 0.57 \pm 0.09\), \(b = 0.42 \pm 0.12\), and \(c = 0.48 \pm 0.11\).
Unlike the SERS spectra of ibuprofenate on bare nanoshells (Figure 20A (iii)), the SERS spectra of intercalated ibuprofen exhibit similar peak positions to the unenhanced Raman spectrum of ibuprofen (Figure 21A,B (i)). For example, the 1610 cm\(^{-1}\) mode of ibuprofen, which was not a strong spectral feature in the SERS spectrum of ibuprofenate on nanoshells (Fig. 20A (iii)) is clearly visible in the SERS spectrum of the intercalated ibuprofenate (Fig. 21 (ii-vii)). Conversely, the spectral features in the 1165-1230 cm\(^{-1}\) region are intense in the unenhanced Raman (Fig. 21A,B i)) and intercalated SERS spectra (Fig. 21A,B (ii-vii)), but not in the SERS spectrum of ibuprofenate on bare nanoshells (Fig. 20A (iii)). The lack of significant spectral shifts of the ibuprofenate modes in the case of hybrid bilayer intercalation indicates negligible chemical interactions between the ibuprofenate and the nanoshell surface. However, the molecule is nonetheless in close proximity to the nanoshell surface, since strong SERS enhancement is observed.

To test whether the Raman scattering cross section of isolated ibuprofen can account solely for the observed signal strength under these experimental conditions (1.18 mW, 60 seconds integration, 100 mM, Figure 22, C) the Raman spectra were acquired in solution. The Raman spectrum of 100 mM ibuprofen without nanoshells shows negligible signal, indicating the presence of significant SERS enhancements when nanoshells are utilized as substrates (Fig. 21A,B (ii-vii)).
Figure 22. (A) Unenhanced Raman spectra of deuterated DMPC powder (25.5 mW power). (B) In solution unenhanced Raman spectra of ibuprofen at pH 10 and pH 3 (25.5 mW power, normalized and offset for clarity). (C) In solution unenhanced Raman spectrum of 100 mM ibuprofen at 1.18 mW and 60 second integration time (parameters equivalent to SERS measurements obtained in Figure 4). This demonstrates that electromagnetic enhancement is responsible for the observed SERS signal strength. (D) SERS spectra acquired sequentially at the same sample spot for hybrid bilayer functionalized nanoshells with deuterated DMPC and 1 mM ibuprofen for pH values cycled between 3 and 10 (spectra offset for clarity).

SEIRA provides a means to spectrally observe the effects of ibuprofen intercalation on the DMPC lipid portion (chemical structure shown in Fig. 23A) of the hybrid bilayer structure. Figure 23B (i) shows the normal incidence transmission SEIRA spectrum of hybrid bilayers on Au nanoshells. The IR absorption peaks of the hybrid bilayer are identifiable at various frequencies and
are listed in Table 8. The methyl symmetric and asymmetric bending modes at 1375 cm\(^{-1}\) and 1462 cm\(^{-1}\), characterizing the aliphatic part of the hybrid bilayer, are all easily observable. The zwitterionic, polar headgroup of the hybrid bilayer also presents characteristic peaks (see Table 8). The symmetric and asymmetric PO\(_2^-\) stretches, the symmetric and asymmetric N(CH\(_3\))\(_3^+\) stretches, and the phosphate skeletal vibration (CO-P-O-C stretch) are also all clearly observable in the SEIRA spectrum. The asymmetric CO-O-C stretch and the ester carbonyls (primary and secondary) of the lipid appear as strong features.\(^{182,186-188}\)

Figure 23B (ii, iii) shows the normal incidence transmission SEIRA spectra of ibuprofen intercalated into hybrid bilayers at ibuprofen loading concentrations of 200 mM and 500 mM, respectively. In these two spectra one can observe significant differences between the SEIRA spectra of hybrid bilayers (Fig. 23B (i)) and ibuprofen intercalated bilayers (Fig. 23B (ii, iii)). The SEIRA spectra of intercalated hybrid bilayers have molecular peaks from the hybrid bilayer with additional distinct peaks attributable to the ibuprofen intercalant (Table 9). Various ring modes of ibuprofen are clearly seen at 847, 1361, 1400, and 1510 cm\(^{-1}\). These modes grow in intensity as the concentration of the ibuprofen loading solution is increased. An asymmetric CO\(_2^-\) stretch is observable at 1582 cm\(^{-1}\) and a carbonyl peak around 1730-1740 cm\(^{-1}\). The presence of the asymmetric carboxylate peak can be solely attributed to the deprotonated form of ibuprofen, since the lipid itself has a ketone rather than a carboxylate moiety. The carbonyl stretching peak, however, can be attributed to both the protonated form of ibuprofen and the lipid structure. Therefore, the presence of a protonated form
of ibuprofen in the hybrid bilayers cannot be ruled out in addition to a deprotonated form interacting with the lipid. It is expected that the presence of ibuprofen would cause changes in the packing and ordering of DMPC molecules. In fact, it causes several peaks of the hybrid bilayer system to shift when compared to the case of nonintercalated bilayers. The presence of ibuprofen is observed to primarily affect the peaks arising from the acyl linkage (CO-O) and the polar phosphocholine headgroup of the hybrid bilayer. Symmetric and asymmetric CO-C stretches have undergone a shift in the peak position from 1067 to 1057 cm\(^{-1}\) and from 1175 to 1167 cm\(^{-1}\), respectively. The ester carbonyl group develops a more prominent double peak structure in the intercalated system, and the symmetric phosphate stretching mode band also undergoes a slight but readily observable broadening. These spectral changes may arise from alterations in the local chemical environment of the lipid molecules when ibuprofen is present. For example, ibuprofen may change the conformational freedom of the lipid headgroups and/or disrupt the spatial packing of the lipids. Since the observed spectral peak shifts appear primarily for the functional groups in the polar headgroup and the backbone portion of the lipid, these results indicate that the interaction of predominantly ionized ibuprofen in hybrid bilayers takes place primarily at the zwitterionic polar headgroup and acyl chain region of the lipid.
Figure 23. (A) Chemical structure of 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) highlighting the phosphate and acyl linkage groups. (B) SEIRA spectra for (i) hybrid bilayers (30 μM dodecanethiol with 100 μM DMPC lipids), (ii) hybrid bilayers with 200 mM, and (iii) hybrid bilayers with 500 mM ibuprofenate loading solution. The stars indicate the peaks from clearly identifiable modes of the ibuprofenate, indicating its presence in the hybrid bilayer. The lines indicate DMPC modes (spectra are offset for clarity).

Table 8. IR Peak Assignments for Hybrid Bilayers on Au Nanoshells

<table>
<thead>
<tr>
<th>Wavenumbers (cm⁻¹)</th>
<th>Band assignment</th>
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<tr>
<td>1740, 1730</td>
<td>C=O stretch (primary, secondary)</td>
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<tr>
<td>1462</td>
<td>CH₂ scissor, Methyl asymmetric bend</td>
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<tr>
<td>1375</td>
<td>Methyl symmetric bend</td>
</tr>
<tr>
<td>1236</td>
<td>Asymmetric PO₂⁻ stretch</td>
</tr>
<tr>
<td>1175</td>
<td>Asymmetric CO-O-C stretch</td>
</tr>
<tr>
<td>1094</td>
<td>Symmetric PO₂⁻ stretch</td>
</tr>
<tr>
<td>1067</td>
<td>CO-P-O-C stretch</td>
</tr>
<tr>
<td>966</td>
<td>Asymmetric N(CH₃)₂⁺ stretch</td>
</tr>
<tr>
<td>920</td>
<td>Symmetric N(CH₃)₂⁺ stretch</td>
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<td>871</td>
<td>Methyl rock</td>
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<tr>
<td>812</td>
<td>CH₂ rock-twist</td>
</tr>
<tr>
<td>714</td>
<td>C-S stretch, Methylene rocking-twisting</td>
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</table>
Table 9. IR Peak Assignments for Ibuprofen\textsuperscript{174,175}

<table>
<thead>
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<th>Wavenumbers (cm\textsuperscript{-1})</th>
<th>Band assignment</th>
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<tr>
<td>847, 1361, 1400</td>
<td>C4-C6 ring stretch, in plane</td>
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<td>CH ring bend, CH bend</td>
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<td>1510, 1549</td>
<td>Ring vibration</td>
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<td>1582</td>
<td>Asymmetric CO\textsubscript{2} stretch</td>
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In summary, based on the following observations we can conclude that ibuprofen is most likely interacting with the lipid portion of the bilayer rather than the alkanethiol layer. First, the SERS spectra (Fig. 21A (ii-vii)) indicate that with increasing ibuprofen concentration in the hybrid bilayer, the trans carbon-sulfur stretch ($\nu$(C-S)$_T$) at 710 cm\textsuperscript{-1} remains significantly stronger than the gauche carbon-sulfur stretch ($\nu$(C-S)$_G$) at 638 cm\textsuperscript{-1}. Second, the low-wavenumber peak at 324 cm\textsuperscript{-1}, which corresponds to coupling between the gold-sulfur stretch and the longitudinal acoustic modes of the alkane chain, does not shift in frequency with increasing ibuprofen concentration.\textsuperscript{189} Ibuprofen intercalation into the alkanethiol layer would disorder the alkane chain packing, a disorder that would likely shift the longitudinal acoustic mode frequencies\textsuperscript{190}. These aforementioned observations indicate that the underlying alkanethiol layer remains ordered and largely unperturbed. Third, as discussed earlier, the SEIRA data (Fig. 23B) show significant differences in the peak positions of the lipid headgroup and acyl chain when predominately ionized ibuprofen is present in the bilayers, indicating that ibuprofenate is affecting the outer leaflet of the hybrid bilayer with a greater impact on the headgroup portion of lipid. Fourth, since the lipid headgroup is zwitterionic, when the ibuprofen is in an ionized form, it is certainly plausible for there to be electrostatic interactions between the ibuprofenate and the DMPC
near the headgroup. And finally, while the SEIRA data suggests that it is the predominantly ionized state which shows an association with the headgroup and acyl region, the SERS data indicates that the hydrophobic effect dominates for intercalation of the protonated form. Together, these two experimental findings provide a more complete picture of ibuprofen interacting with and intercalating in hybrid lipid bilayers.

4.4. Conclusions

We have reported a study of ibuprofen intercalation into a hybrid bilayer structure, a membrane mimic system, using surface enhanced vibrational spectroscopies. The spectral features of ibuprofen appearing in the pH dependent SERS spectra indicate incorporation of the analyte into the bilayer. Stronger SERS signals of ibuprofen are observed at low pH, where hydrophobicity of the molecule plays a dominant role in its intercalation. Shifts in the lipid peak positions upon ibuprofen intercalation in the SEIRA spectra reveal that the headgroup portion of the lipid structure has been affected, indicating that predominantly deprotonated form interacts near the interfacial region of the hybrid bilayer. The spectroscopic results combined from SERS and SEIRA studies provide chemical insight into the nature of ibuprofen-lipid interactions and have clinical importance in understanding the effects of NSAIDs on the integrity and permeability of the gastric mucosal membrane. The plasmonic nanostructures utilized in these studies are applicable for spectroscopic investigation of other biologically relevant phenomena in membrane mimics, such
as the effect of cholesterol on membrane fluidity, the role of glycolipids in membrane structure, and the binding of peripheral membrane proteins.
Chapter 5. Real time monitoring of lipid transfer between vesicles and hybrid bilayers on Au nanoshells using surface enhanced Raman scattering (SERS)

This work was done in collaboration with C. S. Levin.

5.1. Introduction

Recently there has been a renewed interest in understanding the dynamic properties of lipids, including lipid exchange/transfer processes, since lipids, an integral constituent of cell membranes, frequently appear to act as messengers that trigger important metabolic events. Lipid transfer by nonspecific forces, along with specific lipid transfer proteins, play a vital role in a myriad of biological processes, such as cell signaling, antimicrobial defense, lipid absorption during digestion, and parasitic invasion of erythrocytes. With insight into the thermodynamics of lipid mixing and the kinetics of lipid exchange/transfer, these processes can be used for effective tracking of lipid metabolism and for studying membrane organization and biogenesis by incorporating reporter lipids into biological membranes of interest.

Processes at cellular membranes usually take place in a very complex environment; therefore it is particularly enlightening to examine the interaction of two simple model lipid membranes to understand the mechanisms and dynamics of membrane interaction and lipid exchange/transfer. Among the plethora of synthetic membranes explored, supported lipid bilayers (SLBs) and lipid vesicles
are appropriate mimics for investigating interacting membranes, due to their ease of preparation and study.\textsuperscript{150} Assessing lipid transfer dynamics between vesicles and SLBs can shed light on the subtle aspects of membrane architecture and provide new perspectives for SLB modification and asymmetric SLB formation.\textsuperscript{194-198} SLBs such as phospholipid-nanoparticle composites have recently being proposed as nonviral vectors for drug delivery across cell membranes,\textsuperscript{199} calling for further study of the interactions between these composite materials and cellular membranes. Recently, we have successfully demonstrated the fabrication and functionality of hybrid bilayers (HBLs) assembled on Au nanoshells as a biomembrane mimic.\textsuperscript{200} HBLs on nanoshells constitute a special class of SLBs typically consisting of an alkanethiol self-assembled monolayer bound to a noble metal substrate, with an associated outer layer of lipids. Nanoshells, acting as an underlying metal support, are spherical plasmonic nanoparticles consisting of a silica core and a thin, gold shell that can support highly tunable plasmon resonances from visible to mid-IR. The optical excitation of plasmons at desired wavelengths generates a strongly enhanced local electromagnetic field close to the metal surface (within 10 nm) that has been successfully exploited for surface enhanced spectroscopies with high spectral reproducibility.\textsuperscript{93} Hence, HBLs are good composite materials that can simultaneously serve as robust biomembrane mimics and strongly enhancing substrates for surface enhanced spectroscopy.

The kinetics of the exchange/transfer of lipids between membranes has been studied using various methods such as fluorescence, calorimetry, and light
However, these methods either require special probes (fluorescent tags, labeled lipid) or lack sensitivity; for example, a large lipid fraction (>10%) must be transferred in order to detect exchange/transfer. Hence, there has been great research interest in developing significantly more sensitive techniques to overcome these limitations.\cite{194,197,205} Surface enhanced Raman scattering (SERS), a powerful spectroscopic method that permits the identification and detection of chemicals at very low concentrations\cite{32}, is an ideal probe and well suited for studying the exchange/transfer process. In this study, we have investigated the exchange/transfer of lipids between hybrid bilayers of DMPC on nanoshells (acceptor) and small unilamellar vesicles of deuterated DMPC (donor). We probed changes in the chemical structure of the HBL using SERS, addressing the three possible outcomes of interaction of the donor lipid vesicles with acceptor HBL. They are: (I) no lipid exchange/transfer, (II) formation of a new bilayer on top of the existing HBL, and (III) partial and/or complete exchange/transfer of lipid into the outer leaflet of the HBL (Fig. 24). Our experimental observations provide conclusive, real-time, spectroscopic evidence for case (III), i.e., partial and/or complete exchange/transfer of lipids into the outer leaflet of the HBL.
Figure 24. Schematic diagram illustrating the possible outcomes of lipid exchange when DMPC HBL on Au nanoshells are exposed to a solution of deuterated DMPC vesicles: (I) no lipid transfer, (II) formation of a second bilayer on top of the existing HBL, and (III) partial and/or complete transfer of lipid into the outer leaflet.

5.2. Experimental

For the present study, small unilamellar vesicles (SUV) of deuterated DMPC, typically measuring 85-100 nm were fabricated using a published protocol\(^{197}\). SERS-active Au nanoshells were fabricated by following a previously published protocol\(^{11}\). A HBL of alkanethiol-DMPC lipids was fabricated on nanoshells following a recently reported method\(^{200}\). Briefly, the aqueous solutions of nanoshells were centrifuged at 350 RCF for 30 min. The particles were then resuspended in absolute ethanol. Alkanethiol monolayers were first prepared on
the nanoshells by separately making a solution of 10 mM 1-dodecanethiol in absolute ethanol. This was diluted to 30 μM solutions with the Au nanoshells in ethanol and allowed to incubate overnight in the dark for covalent attachment. Thirty micromolar solutions were chosen based on the nanoshell surface area and concentration and based on the size of the dodecanethiol molecule to provide monolayer coverage in ten times excess. After incubation, the nanoshells had settled and the supernatant was removed so that the nanoshells were redispersed in fresh ethanol and any unreacted thiol was removed from solution. The dodecanethiol functionalized nanoshells were then allowed to dry completely. Lipid solutions of either 1,2-dimyristoyl-sn-glycerol-3-phosphocholine (DMPC) or 1,2-dimyristoyl-D54-sn-glycerol-3-phosphocholine (D-DMPC) were prepared by solubilizing the lipids in isopropyl alcohol (50 μL per 2 μmol of lipid). The DMPC, D-DMPC lipids were added to alkanethiol functionalized nanoshells to give a final concentration of 100 μM DMPC lipids. The solutions were placed in an ultrasonicator bath (VWR Model 150D) for 30 min at 30 °C, above the main phase transition temperature of the DMPC lipid ($T_m$) 24 °C) and DMPC-D54 lipid ($T_m$) 18.7 °C) where the lipid is in the liquid crystalline phase and the hydrocarbon chains are more compressible and fluid. All measurements were then performed at room temperature around 23 °C. SERS substrates consisted of fused quartz coated with poly(4-vinylpyridine) (PVP). Cut quartz substrates were first treated with a piranha solution (a concentrated solution of sulfuric acid and hydrogen peroxide) for two hours followed by rinsing with ethanol and drying with nitrogen. Films of poly(4-vinylpyridine) were deposited by immersing in dilute
(0.1%) solutions in absolute ethanol for two hours followed by rinsing with ethanol, drying with nitrogen, and then were allowed to cure overnight. The fabricated hybrid bilayer-encased nanoshells, deposited on quartz, were then incubated (for times ranging from 10 to 400 min.) with vesicles of D-DMPC. SERS measurements were performed at room temperature (~23 °C) after washing off the free vesicles. The hybrid bilayer phase behavior was characterized using the fluorescent probe Laurdan, following the excitation generalized polarization technique of Bagatolli et al. For these measurements, an appropriate amount of hybrid bilayers formed with either DMPC or D-DMPC were mixed with an ethanolic solution of Laurdan to achieve a lipid/probe ratio of 300:1. The emission spectra between 400 and 600 nm were obtained at a fixed excitation chosen between 320 and 390 nm. Excitation generalized polarization spectra were obtained using a JOBIN YVON UV-vis Fluorolog.

5.3. Results and Discussion

Fabricated SERS-active Au nanoshells have inner and outer radii of [r1, r2] = [63, 85] nm as confirmed by the optical extinction spectra and SEM analysis (Figure 25A). SERS spectra of the three different systems investigated were acquired: (a) hybrid bilayers formed with DMPC (HBL DMPC), (b) hybrid bilayers formed with DMPC that have undergone exposure to D-DMPC vesicles (HBL DMPC + D-DMPC) for two hours, and (c) hybrid bilayers formed with D-DMPC lipid (HBL D-DMPC) (Fig. 26). SERS spectra collected on these systems are highly reproducible (Figure 25B-D), a prerequisite for performing any quantitative
spectral analysis. Systems (a) and (c) are effectively two control systems that provide a reference ("starting and end points") for comparison to the interacting system (b). There are important and readily observable spectral differences that appear in the SERS spectra of (b) when compared to (a) and (c). This clearly implies that the acceptor HBL and the donor vesicles interact with each other, ruling out case (I). Closer examination of the SERS spectra of system (a) reveals the absence of the C-D stretching mode in the 2000-2200 cm\(^{-1}\) region since, system (a) lacks any deuterated lipids while system (c), having deuterated HBL, has a clearly observable C-D stretching peak (Fig. 26). Notably, the interacting system (b) also reveals the presence of the C-D stretching mode. For the interacting system (b), these observations clearly indicate a close association of the deuterated lipids from the vesicles with the HBL, suggesting the state of this system is likely (II) and/or (III). In order to distinguish between scenarios (II) and (III), we examined the change of the CH content of the HBLs by monitoring the intensity of C-H stretch mode (2850 cm\(^{-1}\)) normalized to the C-S stretch mode (710 cm\(^{-1}\)). It is important to note that for case (II), the presence of an additional bilayer of deuterated lipids on top of the existing HBL of DMPC will not result in a net decrease of the CH content. For (III), the insertion of deuterated lipids in the outer leaflet of the HBL that may occur upon DMPC replacement would result in a net decrease of CH content. The normalized C-H intensities calculated for the three systems from the SERS spectra are plotted (Figure 26, inset). The decrease in CH content values clearly rules out (II) and favors (III), where there is partial and/or complete exchange/transfer of lipids. The CH content for system
(b) lies between that of systems (a) and (c), indicating a partial exchange of lipids. This would also be consistent with the fact that the HBL was exposed to the vesicles for a time period of only two hours duration.

Figure 25. (A) Extinction spectra of silica core-Au nanoshells in water (i) experimental and (ii) Mie theory calculated (parameters \([r_1, r_2]=[63, 84]\) nm, aqueous medium). The insets show a schematic drawing of nanoshell with inner and outer radii \([r_1, r_2]\) and SEM image of fabricated nanoshells. SERS spectral reproducibility for the systems (B) HBL DMPC, (C) HBL D-DMPC, and (D) HBL DMPC + D-DMPC.
Figure 26. SERS spectra of (a) HBL formed with DMPC, (b) HBL DMPC incubated (2 hours) with deuterated DMPC vesicles, and (c) HBL formed with D-DMPC. The decrease in $I_{CH}/I_{CS}$ (2850 and 710 cm$^{-1}$, respectively) for the three different systems, as shown in inset, clearly demonstrates exchange/transfer.

To investigate the extent of the exchange/transfer process and gain insight into the dynamics of the interacting system, kinetic studies were performed by varying the incubation time of the HBL with the donor vesicle. Plotting the intensity of the normalized C-H stretching mode versus incubation time yields the kinetics of the exchange/transfer process (Fig. 27 (A)). The data support a first order exponential fit to the kinetic data points, yielding a rate constant for loading of the outer leaflet with deuterated lipids, of $K = 1.3 \times 10^{-4}$ s$^{-1}$. Previous studies have reported biexponential kinetics for the exchange/transfer process of lipids between DMPC vesicles and D-DMPC SLBs on a plate of Si ATR crystal.$^{196}$ This observed difference is likely due to the lack of a large disparity in the sizes of the interacting systems used in this study (average size...
of HBL is 170 nm, compared to 85-100 nm for vesicles). Moreover, the HBLs here have an inner leaflet of covalently attached alkanethiols, which is different than bilayers on a planar surface attached by electrostatic forces. In fact, it has been reported that lipid exchange between small sonicated vesicles of DMPC and D-DMPC that are very similar in size and chemical composition follow first order kinetics.\textsuperscript{191,205} Theoretical modeling, along with further experiments to quantify activation energy for the exchange process, are required to clarify the mechanisms of lipid exchange/transfer between HBLs and vesicles of comparable dimensions. The extent of exchange/transfer was calculated to be \( \sim 76\% \) at the end of 7 hours, using the CH content of systems (a) and (c) (obtainable from Fig. 26 inset) and at the time point where progress of the exchange/transfer process saturates, i.e., the kinetic data point at 400 min. (Fig. 27 A). A reasonable scheme of events that may occur during the kinetics study of exchange/transfer is shown in Fig. 27, which is consistent with the observed decrease of CH content, implying a partial exchange/transfer of lipids.

We also characterized the molecular structure of the three systems using Laurdan as a phase-sensitive fluorescent dye, since the phase in which the lipids reside is critically important for exchange/transfer process. The excitation generalized polarization spectra (GP\textsubscript{ex}) as defined by

\[
GP_{ex} = \left( \frac{I_{444} - I_{484}}{I_{444} + I_{484}} \right)
\]

where \( I_{444} \) and \( I_{484} \) are fluorescence intensities at 444 and 484 nm, respectively, and can be utilized to indicate phase behavior below, near, and above the main phase transition temperature.\textsuperscript{168} The GP\textsubscript{ex} spectra for system (a)
HBL DMPC, (b) HBL DMPC incubated (6 hrs.) with deuterated vesicles, and (c) HBL D-DMPC, are presented in Figure 27 B. For HBL DMPC (a), the GP$_{ex}$ spectra show no appreciable tilt, indicating that the system has gel phase characteristics at room temperature. For HBL D-DMPC (c), the observed slight downward tilt of the GP$_{ex}$ spectra indicates that the system resides in a liquid phase. These observations agree with the fact that a deuterated lipid has ~ 4-5 degree decrease in its main phase transition temperature.\textsuperscript{200,206} Interestingly, the GP$_{ex}$ spectrum of the interacting system (b) is seen to be very similar to that of system (c). The observed similarity indicates that HBL DMPC incubated with deuterated vesicles exists in a liquid phase and is a result of a change in the chemical composition of HBLs due to the exchange/transfer process.
Figure 27. (A) Kinetics of the transfer of deuterated lipids from vesicles to HBLs as obtained by monitoring the change in $I_{CH}/I_{CS}$. The line is a first order exponential fit to the data points. Accompanied is a schematic of the plausible changes in HBL composition. (B) The excitation generalized polarization (GP$_{ex}$) spectra of (a) HBL DMPC, (b) HBL DMPC incubated (6 hrs.) with deuterated DMPC vesicles, and (c) HBL D-DMPC.

5.4. Conclusions

In summary, we have utilized DMPC hybrid bilayers on Au nanoshells and small unilamellar vesicles of deuterated DMPC to examine the interactions and exchange/transfer of lipids using SERS. The exchange/transfer process kinetics, studied in real time, is determined to be first order and provides conclusive evidence for partial exchange/transfer of lipids. The kinetics data provide a quantitative estimate of the extent of the exchange/transfer process. Generalized polarization spectra suggest that exchange/transfer of lipids cause changes in the phase transition behavior. The presented hybrid bilayer system with inbuilt SERS sensing capability and the experimentally observed kinetic findings can be explored further both for mechanistic insight into membrane interactions and for modification of SLBs. Theoretical modeling, along with future experiments, are required to clarify the mechanisms of lipid exchange/transfer between these interacting membrane systems.
Chapter 6. Plasmonic nanoshell arrays combine surface-enhanced vibrational spectroscopies on a single substrate

This work was done in collaboration with H. Wang.

6.1. Introduction

The collective oscillations of free electrons in metallic materials-known as surface plasmons-have properties determined by the structure of the metal, and can ultimately be tailored to impart new optically-induced functionalities into materials for specific practical uses. For sub wavelength metallic structures, the geometry of the structure controls the resonance frequencies of its surface plasmons. These structured metallic materials, in turn, provide new and unique methods for manipulating light, giving rise to a whole set of new properties and applications.\textsuperscript{207-212} The enhancement by a nearby metallic structure of molecular spectroscopies, such as Raman scattering, infrared absorption, UV/Vis absorption, and fluorescence, is a particularly interesting and useful phenomenon.\textsuperscript{3,32,213-218} The optical excitation of plasmon resonances supported by metallic nanostructures gives rise to strongly enhanced electromagnetic fields at their surfaces,\textsuperscript{10} which are largely responsible for the observed spectroscopic enhancements. Enhancements of Raman spectroscopy and infrared absorption spectroscopy are of particular interest due to the usefulness of these methods for elucidating molecular structures.\textsuperscript{219} Because of their virtually complementary
selection rules, it would, in fact, be far preferable to use these two spectroscopies in combination with each other when possible, since together they can provide a complete "chemical fingerprint" for the identification of unknown molecules.

The past decade has witnessed a dramatic resurgence of interest in surface-enhanced spectroscopies, fueled largely by the remarkable discovery that single-molecule sensitivity in surface-enhanced Raman spectroscopy (SERS) indeed appears achievable for molecules dispersed within random aggregates of metallic nanoparticles. This enormous Raman enhancement has since been identified as arising from molecules positioned in the junctions between directly adjacent nanoparticles, a geometry which gives rise to huge field intensities between the two particles when illuminated, a configuration also known as a "hot spot". In contrast to SERS, surface-enhanced infrared absorption (SEIRA) spectroscopy has not received nearly the same attention. This is primarily for two reasons: compared to SERS, more modest enhancements are anticipated, since SEIRA depends quadratically on the local field, not quartically as does SERS; moreover, the excitation of large local fields on metallic substrates across the broad range of mid-infrared frequencies needed for SEIRA has not previously been achieved.

The plasmon hybridization picture, an analogy between interacting plasmons and the wave function hybridization of molecular orbital theory, provides a simple conceptual framework for the design of plasmon resonant structures that can be useful as substrates for surface-enhanced spectroscopies. For example, directly adjacent pairs of nanoparticles can be regarded as
plasmonic “dimers”, where excitation of hybridized “bonding” dimer plasmons gives rise to the strong interparticle field enhancement that results in the large SERS enhancements of the hot-spot geometry. Ordered arrays of metallic nanospheres with sub-10-nm interparticle gaps have been shown to provide SERS enhancements by the same mechanism. Metallic nanoparticles with geometrically tunable plasmon resonances, such as metallic nanoshells, can also serve as optimized substrates for SERS when the Raman excitation laser wavelength is within the linewidth of plasmon resonance of the individual nanoparticles. For metallic nanoshells, the field enhancement essentially arises from strongly interacting plasmons on the inner and outer surfaces of the metallic shell layer. The integrated SERS enhancement of an individual nanoshell approaches that observed for hot spots of adjacent solid metallic nanoparticle pairs.

Herein we report a specifically designed, sub wavelength structured metallic substrate that simultaneously enhances two complementary vibrational spectroscopies, namely, Raman scattering and infrared absorption spectroscopy, by introducing plasmon resonances in the two diverse frequency regions required for both spectroscopies. Our strategy is based on the assembly of near-infrared-resonant nanoshells into a 2D periodic array with sub-10-nm interparticle gaps. The resulting nanoshell array is a unique structure which has hot spots in the interparticle junctions that enhance SERS at near-infrared wavelengths, and simultaneously provide broadband mid-infrared hot spots for SEIRA at precisely the same locations on the substrates.
6.2. Experimental

The Au nanoshells used in the present work were fabricated by following a previously reported wet-chemistry method.\textsuperscript{11} The as fabricated nanoshells were further purified by dialysis. In a typical procedure, 40 mL of aqueous nanoshell solution was transferred into a regenerated-cellulose membrane dialysis bag (Spectra-Por, molecular weight cutoff (MWCO) 6000–8000). The bag was then suspended in a reservoir of Milli-Q water (Millipore, Billerica, MA) and gently stirred overnight. The dialyzed nanoshells were collected by centrifugation and redispersed in 25 mm aqueous CTAB (Sigma-Aldrich) solution for 1 h. The CTAB-capped nanoshells were then collected by centrifugation and redispersed in Milli-Q water to form colloidal solutions with desired particle concentrations. The assembly of the nanoshells into close-packed arrays was accomplished by depositing a droplet of colloidal solution (40 µL) onto a substrate surface (silicon wafer, glass slide, ITO glass, or TEM grid) and allowing it to dry undisturbed under ambient conditions. The pattern morphologies of the nanoshell arrays were dependent on the particle concentration of the nanoshell solutions. At very low particle concentrations, only isolated small monolayer domains of arrays with short-range order could be formed on the substrates. Increasing the particle concentration resulted in an increase in the domain size as the initially separated domains began to coalesce with each other to form continuous long-range-ordered monolayers. The optimum concentration for the fabrication of long-range-ordered monolayer arrays is $2.40 \times 10^9$ particles per milliliter for the
nanoshells with inner and outer radii of $R_1=150$ nm and $R_2=172$ nm, respectively. Further increase in the particle concentration resulted in the formation of double-layer, triple-layer, and eventually multilayer arrays. The submonolayers of isolated nanoshells were prepared by immobilizing the nanoshells onto poly(4-vinylpyridine)-functionalized glass by following a previously reported protocol.\textsuperscript{21}

Optical extinction spectra were obtained with a Cary 5000 UV/ Vis/NIR spectrophotometer. SEM measurements were performed on a Phillips FEI XL-30 environmental scanning electron microscope. TEM images were obtained with a JEOL JEM-2010 transmission electron microscope. The samples for SERS and SEIRA measurements were prepared by evaporating 10 mL of a 20 mm solution of pMA in ethanol on the surface of the nanoshell arrays or isolated nanoshells. Raman spectra were obtained with a Renishaw micro- Raman spectrometer using a 785 nm excitation laser (250 mW), linefocus mode (beam size $4 \times 60$ mm), $50 \times$ objective, 0.05% laser power, and 10-s acquisition time. The laser power focused on the samples was measured to be 0.035 mW when the $50 \times$ objective and 0.05% laser power were used. SEIRA measurements were performed with an FTIR microscope (Nexus 670, Thermo Nicolet) equipped with a liquid-nitrogen-cooled MCT (HgCdTe) detector. The SEIRA spectra were obtained from the coaddition of 256 scans with 4-cm$^{-1}$ resolution in reflection mode.
6.3. Results and Discussion

The major steps involved in the fabrication of Au nanoshell arrays are illustrated schematically in Figure 28a. Au nanoshells were fabricated by following a previously reported seed-mediated electroless plating method and then purified by dialysis. The dialyzed nanoshells were functionalized with the surfactant cetyltrimethyl ammonium bromide (CTAB) and subsequently redispersed in water to form colloidal solutions with desired particle concentrations. Applying droplets of nanoshell solution to a substrate and allowing the solvent to evaporate under ambient conditions resulted in the formation of hexagonally packed nanoshell arrays, which maintain an interparticle spacing established by the bilayers of CTAB that surround each nanoshell as the interparticle spacer. The nanoshells organize into hexagonally close packed (hcp) structures with typical domain sizes ranging from several tens of micrometers to over two hundred micrometers (Figure 28b–d). CTAB plays a critical role both in the self-assembly of these arrays as well as in defining the local electromagnetic properties of the nanoshell array structures.

The CTAB molecules form bilayer structures on the surface of Au nanoparticles, which result in a net positive charge on the nanoparticle surfaces and provide a net repulsive interaction between the nanoparticles to prevent random disordered aggregation during solvent evaporation. Control experiments with unfunctionalized nanoshells only resulted in the formation of disordered aggregates. The CTAB bilayers also define the spacing between neighboring nanoshells and result in an average interparticle spacing determined
Figure 28. a) Fabrication of Au nanoshell arrays. b, c) SEM images of nanoshell arrays formed by drying 40 mL of aqueous solution of CTAB-capped Au nanoshells on silicon wafers. The core radius of the nanoshells is 150±12 nm, and the shell thickness is 22±1 nm. d) TEM image of the nanoshell arrays formed on a TEM grid.

to be about 8 nm, consistent with the reported thickness of approximately 4.4 nm for a CTAB bilayer. These sub-10-nm gaps are instrumental for inducing the
strong plasmon coupling that results in the specific electromagnetic properties of this array geometry.

Figure 29 compares the optical extinction spectra of nanoshell arrays with the spectra of the isolated and dispersed constituent nanoshells. These measurements were performed on nanoshell monolayer arrays formed on glass slides with unpolarized light at normal incidence. Nanoshell arrays have two distinct plasmon resonances: a narrow visible or near-infrared band at frequencies corresponding closely to those of the isolated nanoshell plasmons, and in addition, a broad feature extending from the near-infrared well into the mid-infrared region of the spectrum. The two plasmon bands observed for the nanoshell arrays arise from the plasmon interactions between neighboring nanoshells in the arrays.

How the individual nanoshell plasmons interact with each other to form hybridized nanoshell array plasmons can be understood by applying the plasmon hybridization model to multinanoparticle systems. When nanoshells are closepacked into an array structure, the dipolar plasmons of the individual nanoshells strongly intermix to form a hybridized plasmon band, evolved from the dipolar plasmon mode, which disperses strongly to lower energies corresponding to infrared wavelengths. The individual nanoshell quadrupole resonances, on the other hand, also intermix, but only weakly, do not disperse upon hybridization, and give rise to a plasmon band in the near-infrared that appears quite similar in both wavelength and lineshape to the dipole plasmon of the individual nanoshell particles.
Figure 29. Normal-incidence extinction spectra of monolayer nanoshell arrays (solid curves) and submonolayers of isolated nanoshells (dashed curved) supported on glass slides. a) Au nanoshells with 150 nm core radius and 22 nm shell thickness, b) Au nanoshells with 100 nm core radius and 25 nm shell thickness, and c) Au nanoshells with 60 nm core radius and 20 nm shell thickness.

In addition to the new features arising in the far-field extinction spectra, the interparticle plasmon coupling in the nanoshell arrays produces intense near-field
enhancements at the junctions between neighboring nanoshells, and creates uniform periodic densities of hot spots for surface-enhanced spectroscopies.

Theoretical simulations of the near-field properties of nanoshell arrays by using the finite difference time domain (FDTD) method$^{231,232}$ indicate that the localized field enhancements ($|E|/|E_0|$) inside interparticle junctions are approximately 30 both in the near infrared and over a broad range (ca. 2–8 μm in wavelength) in the mid-infrared. Such large field enhancements in both near- and mid-infrared are achievable only when the interparticle spacing is within the sub-10-nm range. In comparison, isolated nanoshells provide much weaker field enhancements, with maximum enhancements of approximately fivefold for nanoshell plasmons in the near-infrared and negligible field enhancements in the mid-infrared. The nanoshell array structure turns out to be uniquely suited as an integrated SERS-SEIRA substrate, by providing hot spots in the interparticle junctions that enhance both SERS at near-infrared wavelengths and SEIRA at mid-infrared wavelengths. It is this property that enables the observation of large, reproducible SERS and SEIRA enhancements on the same substrate.

The nanoshell arrays were evaluated as SERS-SEIRA substrates by using a nonresonant adsorbate molecule, namely, para-mercaptoaniline (pMA), which is ideal for spectroscopic quantification on these substrates because it displaces CTAB on Au surfaces and is known to form self-assembled monolayers (SAMs) on Au surfaces with a known packing density.$^{64,73}$ Figure 30a shows the SERS spectrum of CTAB ($\lambda_{exc}=785$ nm) on the surface of the as-fabricated nanoshell
Figure 30. SERS performance of the nanoshell arrays. SERS spectrum of a) CTAB and b) pMA on nanoshell (150nm core radius, 22 nm shell thickness) monolayer arrays. c) SERS spectrum of pMA on the isolated nanoshells (150 nm core radius, 22 nm shell thickness). The coverage of the submonolayer of isolated nanoshells was determined to be 212 ± 15 particles in each beam spot according to the SEM images. d) Empirical SERS enhancement factor of pMA adsorbed on nanoshell arrays and the isolated nanoshells.
arrays, with a strong characteristic feature at 189 cm$^{-1}$ corresponding to the Au-Br mode. On complete displacement of the CTAB molecules, the Au-Br mode and all the SERS modes of CTAB disappear, while the SERS modes of pMA and the Au-S bond at 390 cm$^{-1}$, which become evident in the SERS spectrum (Figure 30b), indicate the formation of saturated SAMs of pMA on the nanoshell array surfaces (detailed peak assignments in Table 10 at the end of the chapter). The SERS spectra of pMA on the nanoshell arrays are highly reproducible at different sites on a substrate with a standard deviation of less than 10%. For comparison, the SERS spectrum of saturated pMA SAMs on the surface of isolated nanoshells is also shown in Figure 30c. Empirical signal enhancement factors were determined by comparing ratios of the intensity of SERS modes to the corresponding unenhanced signals from neat pMA films of known thickness. Both the SERS and normal Raman intensities are normalized by the number of molecules being probed. As presented in Figure 30d, the empirical enhancement factors of pMA on the nanoshell arrays are on the order of $10^8$–$10^9$, 10 to 20 times larger than what is achievable on isolated nanoshells. The SERS enhancements obtained on these nanoshell arrays are of the same order of magnitude as those achieved on solid Au nanosphere arrays. These measurements represent the overall array response, not the localized enhancements inside the interparticle junctions. Finite-difference time-domain (FDTD) calculations for arrays of smooth nanoshells show that the maximum local $(|E|/|E_0|)^4$ enhancement factor in the high-field junctions of these arrays is on the order of $10^6$, which enables these substrates to approach zeptomolar
molecular detection limits. This is a conservative number, sensitive to further enhancements by closer internanoparticle spacings and localized asperities on the surface of individual nanostructures.

The broad plasmon of the nanoshell array in the mid-infrared region enhances SEIRA quite well. Figure 31a shows a normal IR spectrum of neat pMA film (0.60 mm thick) supported on a silicon wafer and a typical SEIRA spectrum of SAMs of pMA formed on the nanoshell array surfaces (detailed peak assignments in Table 11 at the end of the chapter). Utilizing the nanoshell arrays as SEIRA substrates enables the acquisition of high-quality SEIRA spectra of pMA across much of the IR fingerprinting region, and enhances several characteristic vibrational modes that correspond to most of the normal IR features of the molecule. The most striking difference between SEIRA and normal IR spectra is the highly asymmetric, characteristic Fano-type lineshape of SEIRA, which is believed to be due to the interaction between the molecular vibrations and the electronic excitations in the metallic substrates.\textsuperscript{45,71,72,234,235}

The SEIRA enhancement factors were determined by directly comparing the ratios of the normalized intensity of SEIRA modes to the corresponding unenhanced IR signals from neat pMA films, in precisely the same manner that we determined the SERS enhancements on the same substrate. The SEIRA enhancement factors of each of the observed IR modes of pMA are shown in Figure 31b. For this substrate and measurement approach, the SEIRA enhancement factors of pMA on the nanoshell arrays for all observed modes are on the order of $10^4$. We have also performed SEIRA measurements on pMA
SAMs on a planar Au surface and isolated nanoshells. However, no signals were observed, as neither the planar Au surface nor isolated nanoshells can provide the large local field enhancements associated with the broadband mid-infrared plasmons. The remarkably large enhancement factors and high reproducibility of the SEIRA spectra obtained on these substrates open new opportunities for the broader development of SEIRA as a reliable and reproducible spectroscopic method.

Figure 31. SEIRA performance of the nanoshell arrays. a) Normal IR spectrum of pure pMA and SEIRA spectrum of pMA SAMs on the nanoshell arrays. b) SEIRA enhancement factors of different IR modes calculated on the basis of the experimental spectra.
6.4. Conclusions

The nanoshell array substrates reported herein provide a new, multifunctional platform for chemical sensing applications by enhancing both RS and IRA spectroscopy. Integrating SERS and SEIRA on a single substrate will enable the identification of unknown molecules by combining both surface-enhanced vibrational spectroscopies, allowing more detailed investigations of molecular structure, orientation, and conformation, as well as adsorbate–substrate and adsorbate–adsorbate interactions. This substrate geometry also provides a system for detailed and highly reproducible correlations between surface-field properties and spectroscopic enhancements, which should enhance our ability to unravel the complex mechanisms involved in surface enhanced spectroscopic processes.
Table 10. Raman peak assignments of pMA

<table>
<thead>
<tr>
<th>Normal Raman (cm$^{-1}$)</th>
<th>SERS (cm$^{-1}$)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1594</td>
<td>1585</td>
<td>CC stretch</td>
</tr>
<tr>
<td>1479</td>
<td>1487</td>
<td>CC stretch + CH bend</td>
</tr>
<tr>
<td></td>
<td>1429</td>
<td>CC stretch + CH bend</td>
</tr>
<tr>
<td></td>
<td>1384</td>
<td>CH bend + CC stretch</td>
</tr>
<tr>
<td>1304</td>
<td>1311</td>
<td>CC stretch + CH bend</td>
</tr>
<tr>
<td>1204</td>
<td>1179</td>
<td>CH bend</td>
</tr>
<tr>
<td>1168</td>
<td>1142</td>
<td>CH bend</td>
</tr>
<tr>
<td>1088</td>
<td>1076</td>
<td>CS stretch</td>
</tr>
<tr>
<td>1004</td>
<td>1005</td>
<td>CCC bend + CC bend</td>
</tr>
<tr>
<td>817</td>
<td>814</td>
<td>CH wag</td>
</tr>
<tr>
<td>631</td>
<td>635</td>
<td>CCC bend</td>
</tr>
<tr>
<td></td>
<td>390</td>
<td>Au-S stretch</td>
</tr>
</tbody>
</table>
Table 11. IR peak assignments of pMA

<table>
<thead>
<tr>
<th>Normal IR (cm⁻¹)</th>
<th>SEIRA (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1628</td>
<td>1612</td>
<td>NH bend</td>
</tr>
<tr>
<td>1590</td>
<td>1587</td>
<td>CC stretch</td>
</tr>
<tr>
<td>1495</td>
<td>1490</td>
<td>CC stretch + CH bend</td>
</tr>
<tr>
<td>1422</td>
<td>1419</td>
<td>CC stretch + CH bend</td>
</tr>
<tr>
<td>1177</td>
<td>1172</td>
<td>CH bend</td>
</tr>
<tr>
<td>1125</td>
<td>1118</td>
<td>CH bend</td>
</tr>
<tr>
<td>1086</td>
<td>1084</td>
<td>CS stretch</td>
</tr>
<tr>
<td>822</td>
<td>819</td>
<td>CH wag</td>
</tr>
</tbody>
</table>
Chapter 7. Summary

Vibrational spectroscopy (Raman and IR) has long been documented as a classical analytical technique for chemical assaying and molecular structure elucidation. With the advancement of plasmonics, it was soon realized that the strong near fields (generated by the excitation of plasmon resonances in metal nanostructures) could be exploited for metal-enhanced spectroscopy. Surface-enhanced Raman scattering (SERS) was demonstrated as the first example of metal enhanced spectroscopic techniques. The discovery of SERS spurred the quest for its complimentary branch: surface-enhanced Infrared absorption (SEIRA) spectroscopy and it was not long ago (1980) that SEIRA was demonstrated. Combined together, SERS and SEIRA constitute the unified field of surface enhanced vibrational spectroscopy (SEVS), counterpart of the traditional vibrational spectroscopy. SERS has attracted substantial research attention due to its huge enhancement factors ($\sim 10^8-10^9$) and wide range of applications. However, SEIRA has not received nearly the same attention because engineering the necessary strong near-fields in the mid-IR is challenging.

This thesis summarizes how Au nanoshell based substrates could be designed and fabricated for SEIRA and for combining SERS and SEIRA to unify the field of surface enhanced vibrational spectroscopy for comprehensive biochemical sensing applications. Silica-Au nanoshells were chosen as potential substrates for SEIRA and SERS-SEIRA due to their well-controlled and
reproducible geometry as well as their ability to generate localized and strong
electromagnetic 'hot spots' near the nanoshell surface at a given wavelength
range of interest.

This dissertation documents how near-IR resonant Au nanoshells, when
allowed to aggregate produces electromagnetic hot spots in the mid –IR, could
be utilized as an efficient SEIRA substrate. SEIRA enhancement factor and
detection limit of such a nanoshell aggregate substrate is quantified using pMA
as a test analyte molecule. Application of the SEIRA active substrate, in
conjunction with SERS substrate, is demonstrated to probe select biophysical
processes such as: 1) Binding and orientation of adenine and
adenosinonemonophosphate (AMP) on Au nanoshell surface, 2) Intercalation and
interaction of Ibuprofen drug molecules with hybrid lipid bilayer on nanoshell, and
3) Exchange/transfer of lipids between hybrid lipid bilayer on nanoshell and small
unilamellar vesicles (SUV) of comparable dimensions.

Ab initio calculation and surface selection rule were applied to rationalize
the pH dependent SERS-SEIRA spectra of adenine and
adenosinonemonophosphate (AMP) adsorbed on Au nanoshells. The effect of
protonation of adenine and AMP is readily observable in experimental SERS-
SEIRA spectra. Experimental and theoretical analysis indicates that the ring
planes of both adenine and AMP are not lying flat but have upright orientation
with a small angle from the surface normal. Theoretical calculations also
suggests that the reactive ring nitrogen (N3) is the local binding site for adenine
while for AMP the binding site is very likely to be N3 and/or external NH₂ group.
Spectral evidences from experiments on intercalation of Ibuprofen into hybrid lipid bilayer reveals that molecular hydrophobicity, as tuned by changing the solution pH, plays a vital role in the process of intercalation. The observed shifts in the peak positions of lipid head group portion suggest that the intercalated hybrid lipid bilayers have reduced conformational freedom with a possible change in the lipid packing/structure. Specific interactions between the tail part and/or head part of the hybrid lipid bilayer with the neutral and ionized form of Ibuprofen intercalant respectively, is proposed to be a crucial factor for the intercalation process.

The process of exchange/transfer of lipids is demonstrated to be probed directly (without utilizing any labeled or fluorescently tagged lipids) and sensitively using hybrid lipid bilayers and SERS. The kinetics of the exchange/transfer process is found to be first order in nature yielding a quantitative estimate of rate constant and extent of the exchange/transfer process. Change in the lipid phase resulting from the change in the chemical composition of the lipid due to exchange/transfer process is successfully probed using fluorescence measurements.

Nanoshell aggregate SEIRA substrate is extended into highly periodic and ordered nanoshell array structures that truly combine SERS and SEIRA together on a common substrate. The nanoshell array structures present a narrow vis/NIR mode and a broad MIR mode that is demonstrated to be exploited for SERS and SEIRA respectively with high enhancement factors and spectral sensitivity.
Future directions for this work are many, ranging from developing new plasmonic substrate geometries that cooperatively optimize SERS and SEIRA enhancements to inventing new instrumentation for rapid, combined SERS and SEIRA detection. As our understanding and ability to control various nanoscale parameters improves, we will increase our capability to generate plasmonic devices designed to elicit a strong spectroscopic response. Advanced fundamental understanding of interparticle plasmonic coupling between complex nanostructures could benefit successful practical applications.

More immediate SEIRA related research projects include design and fabrication of SEIRA substrates such as single layer square array of rectangular metallic blocks. Such a substrate can be fabricated using electron beam lithography. Fabricating such a substrate that has electromagnetic hot spot originating in the mid-IR due to lightning rod effect rather than plasmonic near filed enhancement will be useful in providing us a better understanding of the nature of the origin of the electromagnetic enhancement in SEIRA.

Temperature controlled SERS measurements above and below the lipid main phase transition temperature may show spectral differences in analyte partitioning into hybrid lipid bilayers. Temperature and lipid phases are two crucial factors that are anticipated to have a profound effect on the kinetics of the lipid transfer process.

The optical properties of interacting complex nanostructures such as Au septamers (schematic shown in Figure 32) are of practical importance for LSPR sensing and electromagnetically induced transparency (EIT). The plasmonic
coupling between the central particle and the six outer particle of the septamer structure, leads to an interesting optical spectra. The calculated optical spectrum is characterized by a tunable Fano dip that is highly susceptible to its immediate surrounding dielectric environment.\textsuperscript{236} It is also predicted that the Fano dip is very sensitive to the incidence angle of excitation.\textsuperscript{237} Experimentally realizing these optical properties is an active area of research that needs further attention. These Au septamer nanostructures, with a variety of dimensions supported on different solid surfaces (Si/SiO\textsubscript{2}, ITO-glass), has been controllably fabricated utilizing electron-beam lithography (Figure 33). Experimentally characterizing the optical properties for its successful application as LSPR sensor and ‘tilt’ sensor constitutes a major future work for such an interacting system.

\textbf{Figure 32.} Schematic representation of normal incidence excitation of Au septamer with constituting cylinders of diameter d, height h and inter-cylinder spacing g.

Interactions in complex nanostructures and in between molecules and materials form the basis for a number of applications in the scientific field such as biochemical sensing for environment, defense and security. The desire to acquire fundamental knowledge on these interactions has always driven chemists, biophysicists, material scientists, and theorists. Our current understanding on
material – molecule interaction and nanostructure coupling can be intelligently utilized for the benefit and progress of the emerging field of plasmonics.

**Figure 33.** SEM images of Au septamers fabricated utilizing e-beam lithography on Si/SiO₂ and ITO-glass substrate.
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Appendix

Appendix A. Nanoshell Background Information

Noble metal nanoparticles are known for their sensing applications such as surface enhanced Raman scattering (SERS), surface plasmon resonance sensing (SPR) and surface enhanced fluorescence spectroscopy. Nanoshells, consisting of a spherical silica core surrounded by a thin metallic shell (Fig. 32), are special class of nanoparticles that can be utilized for these sensing applications by engineering its plasmon resonance to the desired wavelength. The remarkable tunability of the plasmon resonance of nanoshells (from visible to mid-IR) is governed by the geometry of the nanoshells (r1/r2) and the dielectric constants of the core, shell, and the medium material. The basis for this tunability can be understood in terms of the plasmon hybridization model.17,18 The full electromagnetic behavior of the nanoshells can then be calculated using Mie scattering theory.19 These theoretical models provide guidelines by which nanoshells with specifically chosen plasmonic properties and hence with specific applications can be designed and fabricated.

Figure 34. Schematic of a silica core-gold nanoshell of inner and outer radius r1 and r2 respectively.
**Plasmon Hybridization**

The Plasmon hybridization model is a very powerful and intuitive approach that has been utilized to gain insight and predict plasmonic properties of many plasmonic nanostructures including nanoshells.\textsuperscript{17,58} This model is conceptually inspired by the well-known molecular orbital theory where the atomic orbitals interact/hybridize to form the molecular orbitals. In the Plasmon hybridization theory, the plasmon resonance of a complex structure is derived from the interaction/hybridization of the plasmon resonances of the constituting simpler structures. In the case of a nanoshell, the nanostructure can be decomposed into a metallic sphere and a spherical cavity inside a bulk metal (Fig. 33).

![Diagram of Plasmon Hybridization of gold nanoshell](image)

**Figure 35.** Plasmon Hybridization of gold nanoshell.

The plasmon resonances of a sphere and cavity are given by

$$\omega_{s,j} = \omega_{s} \sqrt{\frac{l}{2l + 1}}$$

and

$$\omega_{c,j} = \omega_{c} \sqrt{\frac{l + 1}{2l + 1}}$$
where $\omega_B$ corresponds to bulk plasma frequency of metal and $l$ is the angular momentum. Hybridization of these two individual plasmon resonances results in two plasmon resonances of nanoshell for each $l$

$$\omega_{l\pm}^2 = \frac{\omega_B^2}{2} \left[ 1 \pm \frac{1}{2l+1} \left( 1 + 4l(l+1) \left( \frac{r_1}{r_2} \right)^{2l+1} \right)^{1/2} \right]$$

where $r_1$ and $r_2$ represents the inner and outer radius of nanoshell, respectively, $\omega_{l\pm}$ is the antisymmetric nanoshell plasmon mode that lacks an infinite dipole moment, and $\omega_{l\pm}$ is the optically observable symmetric nanoshell plasmon mode that has finite dipole moment. From the above equation, it is clear that the inherent tunability of plasmon resonance of nanoshell is achieved by the ratio $(r_1/r_2)$. A large value of $(r_1/r_2)$ corresponds to a thin metallic shell, and a very strong interaction between the sphere and cavity plasmons. This strong interaction causes a large redshift of the symmetric nanoshell plasmon compared to the sphere resonance. On the other hand, a small value of $(r_1/r_2)$ will lead to weak interaction, resulting in a symmetric nanoshell plasmon weakly redshifted from the sphere plasmon resonance.

**Nanoshell Fabrication and Characterization**

Gold-silica nanoshells are fabricated using previously published simple wet chemistry synthesis protocol developed by Oldenburg et al.\textsuperscript{11,23} The schematic steps involved in the fabrication process of nanoshell is shown in Figure 34. Silica cores (~120 nm diameter) utilized for fabricating nanoshells for SERS measurements were purchased from Precision Colloids, LLC (Cartersville,
Silica cores of desired size can also be fabricated using the stöber synthesis method. This synthesis involves the hydrolysis of tetraethylorthosilicate (TEOS) in the presence of ammonium hydroxide ($\text{NH}_4\text{OH}$) in ethanol. Changing the relative concentrations of TEOS and $\text{NH}_4\text{OH}$ results in the formation of silica particles with different sizes. The fabricated silica particles are chemically functionalized with aminopropyltriethoxysilane (APTES) to facilitate the binding of gold shell. The amount of APTES added amounted to 10X the amount necessary to create a monolayer of the silane on the particle surfaces.

The gold shell is grown on the silica cores via seed-mediated electroless plating. First, small gold colloids ($r < 2 \text{ nm}$), fabricated following the method of Duff, et al$^{238}$, are attached to the amine-terminated silica surfaces. Gold was electrolessly plated onto the colloidal nucleation sites from a dilute solution of chloroauric acid ($\text{HAuCl}_4$) and potassium carbonate using formaldehyde as a
reductant. The thickness of the resulting shell is controlled by the relative amounts of the silica core and the Au salt solutions (Fig. 35). If the amount of Au in the solution is insufficient, the extinction spectrum of the resultant particles will be broad, and resemble the spectrum of aggregated gold colloids. When a complete shell is formed, the plasmon band narrows significantly. A continuous Au shell of thickness between 15 nm and 30 nm can generally be fabricated successfully.

Figure 37. Extinction spectra of nanoshell growth with different proportions of silica core and plating gold solution.

Fabrication of Nanoshell Based SERS Substrate
SERS substrates consisted of nanoshells deposited onto either quartz slides or silicon wafer supports (Addison Engineering, Inc.) coated with poly(4-vinlypyridine) (PVP) or functionalized with either (3-aminopropyl)triethoxysilane (APTES,) for attachment of the nanoparticles to the support. Before any coating or functionalization was performed, the slides/wafers were cleaned in a piranha solution (7:3 v/v 98% H_2SO_4: 30% H_2O_2), rinsed with milliQ water, followed by rinsing with 200 proof ethanol and dried in a stream of N_2. Typically, nanoshells were designed to elicit a SERS response on individual nanoshell surfaces. The nanoshell core and shell dimensions were adjusted so that the plasmon resonance provided a far-field absorbance maximum near 785 nm in water, to provide SERS enhancement at the 785 nm pump laser wavelength. For solution SERS measurements, Au nanoshells were fabricated with a 63 nm silica core radius and approximate 20 nm shell thickness The internal and overall dimensions of the nanoparticles were confirmed using UV-visible extinction measurements, comparison with Mie scattering theory, and scanning electron microscopy (SEM).

Fabrication of Nanoshell Aggregate Based SEIRA Substrate

SEIRA substrates consisted of nanoshells deposited onto silicon wafer supports (Addison Engineering, Inc.) coated with poly(4-vinlypyridine) (PVP) for attachment of the nanoparticles to the support. Before any coating or functionalization was performed, the wafers were cleaned in a piranha solution (7:3 v/v 98% H_2SO_4: 30% H_2O_2), rinsed with milliQ water, followed by rinsing with
200 proof ethanol and dried in a stream of N\textsubscript{2}. Typically, nanoshells with mid-IR response were fabricated with a 190 nm silica core radius and approximate 35 nm shell thickness. The SEIRA active nanoshells were prepared electrolessly using formaldehyde as a reactant. The as prepared nanoshells were centrifuged at 80 rcf for redispersing in water. The internal and overall dimensions of the nanoparticles were confirmed using UV-visible extinction measurements, comparison with Mie scattering theory, and scanning electron microscopy (SEM). The prepared nanoshells were then incubated with desired analyte and then recentrifuged to wash off excess analyte molecule. These nanoshells functionalized with analyte molecules were then drop dried on PVP coated silicon wafer for realizing interparticle junction hot spots in the mid-IR.

Appendix B. Electron-beam lithography on Si/SiO\textsubscript{2} and ITO-glass

ESEM (JEOL-6500) Lithography Protocol

Substrate Selection
1. Silicon can be purchased from the following companies:
   - Virigina Semiconductor: www.virginiasemi.com
   - Montco Silicon: http://www.silicon-wafers.com/
   - Silicon Valley Microelectronics: www.svmsi.com

2. ITO coated glass substrates can be purchased from Delta Technologies, Limited. Stillwater, MN 55082-1234. (Part no. CG-61IN-S107, ITO coated on one surface, \( R_s = 15-25 \text{ ohm} \))

3. Cleaved Wafers/slides should be no larger than 8x8 mm
Cleaning

1. Remove large dust particles using dry N₂.
   1) Silicon: Put acetone (A18-4) on lint-free Texwipe swabs, clean surface of Si under microscope.
   2) ITO: Please note that cleaning ITO slides is done ONLY by sonicating (for 10 seconds, lowest power setting on the bath sonicator) the slide submerged in acetone in a petridish. Do not clean the surface with cotton swabs or Texwipe swabs.

2. Rinse with isopropyl alcohol 2 Propanol (IPA)
3. Blow dry with nitrogen
4. Plasma clean (Natelson Lab). Put samples on a microscope slide. Use oxygen plasma.
   1) Hold cover over opening with all valves closed
   2) Power on vacuum (floor power switch)
   3) Power on gauge (on top of plasma cleaner)
   4) Open Oxygen Valve so that the gauge (units? Pressure??) reads between 200-300.
   5) Power on the plasma cleaner then turn on the pump, both switches are on the bottom front of the plasmas cleaner
   6) When ready set cleaner to “Low” for one minute.
   7) If done correctly you should see an orange-purplish tinge inside the cleaner.
   8) WARNING Hold onto to vacuum cover before powering off
   9) Power Off: Turn off cleaner, close oxygen valve power off cleaner (front 2 switches), power off gauge. THEN, holding the vacuum cover power off vacuum (floor power switch). Vent the vacuum hold and also cover to fall off of the front opening.
10) Place wafers in container & seal with parafilm to minimize exposure to moisture.

Spin & Bake

1. Pre-set hot plate to 180C (takes ~15 minutes to stabilize)
2. Use PMMA (495 wt, A4) available in drawer to right side of spinner
3. Settings:
   a. Speed – 3000 RPM
   b. Time – 40 seconds
   c. Accel – 3
4. Replace the carbon tape by scrapping it off with a razor blade. Reason: PMMA is a solvent for the tape.
5. Scrape the surface of the carbon tape with a razor blade to make it easier to remove the chip later
6. Cover chip with resist
7. Start spin within 10 seconds of applying resist
8. Gently remove chip from chuck with razor blade
9. Transfer chip to hotplate
10. Bake at 180°C for 90 seconds (Si wafer), 120 seconds (ITO slide)
    a. This minimizes the gassiness of the PMMA which reduces the particles
       that are emitted in the vacuum chamber, making it faster to lower the
       pressure of the working chamber of the e-beam microscope.
11. Cover with Petri dish, but make sure that dish hangs slightly over hotplate
    edge so that fumes can escape.

Sample Preparation
1. Deposit two small drops of 2-3.5 μm silver powder (Sigma Aldrich), wetted in
   IPA, on opposite sides of the chip. This will allow the microscope to focus on
   the otherwise featureless photoresist surface.

E-beam Lithography
1. Mount sample to the provided lithography sample holder with Au standard &
   Faraday Cup. The Au standard should be pointed toward the SEM operator
   for the FEI e-SEM.
2. Start ESEM as normal up to the point where you would turn on the beam, but
   don't turn the beam on yet.
3. Move to (35,13) Natelson holder or (35,15) for Halas holder, so that when you
   turn on the beam you will be positioned at the Au standard.
4. Turn on electric beam banker, set to external
5. Turn on beam
6. Set to 30kV
7. Focus, set to 7mm WD (using NPGS), manually set Z-height and then correct
   wobble & stigmatism
8. Move to Faraday cup which is to the left/right of the Au std for the
   Natelson/Halas (,) /(28,20) holder
9. Zoom into the center of the cup with highest magnification (x500,000) and set
   in spot mode to read the current
   a. Set spot size to select desired current
      i. Spot size 1.9 is ~ 41pA
      ii. Spot size 5.8 is ~ 4.15nA
10. Find corners of the sample & query the location using NPGS command line
    tools. Use magnetic blanker when traveling across the chip to avoid exposing
    chip.
    a. pg_6500 @
11. Find silver colloid on the side of the wafer
12. Record “snap shot” silver dust position in Stage Map feature of JEOL 6500 to
    quickly return to silver dust on either side of the chip.
13. On 1st silver dust site move to the particle closest toward the center and
    focus.
a. Reset WD to 7mm again with NPGS  
b. Focus with Z-axis, fine focus, wobble & stigmatism.  
c. Query focus lens value after focusing (should be around 178.176)  
i. pg_6500 ol?

14. Move to 2nd silver dust site and focus using ONLY fine focus knob  
a. Query focus lens value of 2nd site. Average focus of 2 dust focus sites to give you the focus lens value of the center.

15. Calculate where to put pattern & correct focus lens for the middle of the sample  
a. Set focus lens value of the center (chg # below with your value)  
i. pg_6500 ol,<178.176>

16. Blank beam and move to center of the chip as calculated from corner position values

17. Make focusing (contamination) dots to further improve focus.  
a. Move to a location away from where you wish to place your pattern  
b. pg_6500 abs,xpos,ypos,3 (positions are in nm)  
c. Set highest magnification (JEOL: x500,000)  
d. Set to spot mode  
e. Unblank beam  
f. Wait for current to drop ~15%  
g. VERY QUICKLY!!!  
h. Focus using fine & stigmators  
i. Blank beam when you have seen a round dot  
j. Repeat as needed

18. To run a pattern  
a. With magnetic blanker on, move into position  
b. Select “Run Runfile”

Developing  
1. Set chip in Pyrex dish of MIBK:IPA (1:3) for 40 seconds (silicon) and 60 seconds for ITO. Gently agitate.  
2. Remove PROMPTLY!!! VERY IMPORTANT  
3. Immediately rinse in dish of isopropyl  
4. Image in optical microscope to check  
5. Place under UV lamp (Natelson Lab) for 5 minutes

E-beam Metal Evaporation  
1. 10 angstrom titanium adhesion layer. 200 angstrom Au layer with a 1 Angstrom/sec deposition rate to avoid baking the photoresist too much to permit lift-off
Lift-off
1. Place wafer in beaker of acetone. Gently agitate.
2. Metal layer on top of PMMA will remove with the PMMA
3. If metal layer does not completely remove place beaker of acetone in a sonicator bath at low power. Sonicate for 10 seconds at a time until metal has been removed
   a. Sonicating for too long will damage metal of patterns