Microfluidic Channels on Nanopatterned Substrates: Monitoring Protein Binding to Lipid Bilayers with Surface-Enhanced Raman Spectroscopy

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Abstract

We used Surface Enhanced Raman Spectroscopy (SERS) to detect binding events between streptavidin and biotinylated lipid bilayers. The binding events took place at the surface between microfluidic channels and anodized aluminum oxide (AAO) with the latter serving as substrates. The bilayers were incorporated in the substrate pores. It was revealed that non-bound molecules were easily washed away and that large suspended cells (Salmonella enterica) are less likely to interfere with the monitoring process: when focusing to the lower surface of the channel, one may resolve mostly the bound molecules.

I. Introduction

Recent developments in microfluidic devices and surface-enhanced spectroscopic methods opened new venues for biosensor technologies. Commercial systems often exploit surface plasmon resonance (SPR) phenomenon to achieve sensitive label-free detection of analyte binding. In such systems, the sensing element consists of a prism, coated with a thin metal film, which is functionalized with desired ligands. The film is typically illuminated from the back by a polarized light under conditions of the total internal reflection [1]. Microfluidic channels enable the flow of analytes on top of the prism and binding events are monitored through shifts in the SPR signal. Such shifts are the result of refractive index changes at the immediate proximity to the metal layer. Typical measurements involve recording of optical intensity values as a function of polarization and wavelength of the incident beam [2]. Other surface plasmon (SP) configurations are based on fiber schemes [2a], Wood anomaly [3] and waveguides [4]. Currently, SPR-based biosensors are widely employed for binding studies of water-soluble proteins and oligonucleotides [5] and, to a lesser degree, in investigations of membrane-protein interactions [6]. While SPR biosensor technology is

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quite mature, there is room for further improvements in several areas including screening of
drug for specific affinity to lipid bilayers and membrane proteins. Additional molecular
fingerprinting, such as offered by Raman spectroscopy, is also highly desirable.

Raman spectroscopy is an established tool to study molecular vibration states. Surface-
enhanced Raman scattering (SERS) is a modification to this technique, which provides
signal amplification for molecules absorbed on rough metallic substrates, e.g.,
electrochemically roughened silver or metallic nanoparticles [7]. Recently, SERS has been
combined with microfluidic technology by packing metallic nanoparticles within
microfluidic channels [8]. However, loading channels with nanoparticles could complicate
the measurements because of nonuniform light scatterings and local analyte density
variations. In addition, an optimal adherence of analyte molecules to nano-metallic particles
often requires prolonged incubation times, thus, limiting the speed at which these devices
operate. While roughing a metallic bottom surface of microfluidic channels (MFC) is a
viable option for some analytes, such surfaces are usually incompatible with lipid bilayer
membranes and pose a threat to their integrity. In addition, concerns may be raised regarding
repeatability of the experiments and uniformity of the lipid bilayer coverage. In contrast, the
flat-face nanostructured aluminum oxide surfaces are bio-compatible and past studies
demonstrated its seamless integration with lipid bilayers [9].

In our approach, the SP wave is carried by ca. 50 nm thin, perforated dielectric layer
of anodic aluminum oxide (AAO) on top of a metallic aluminum (Al) substrate (Figure 1a, c).
Such SP-carrying platform is referred to as a loaded surface waveguide and provides for a
periodic array of ‘hot spots’ at the AAO surface [10]. In a typical configuration SPR
interrogates the molecules in a single optical path. In contrast, by exposing the analyte to
standing SP modes, which are scattered back-and-forth along the substrate surface, the
effective interaction length is increased many times. The face surface of the nano-hole array
is essentially flat as the holes occupy only ca. 5% of the total area (although this parameter
could be varied through a pore enlargement procedure and/or changes in anodization
conditions). It was shown that such substrate configuration does not exert strain on soft
molecules, including DNA [11]. Finally, fabricating these structures on silicon by use of
lithography provides an attractive option for further development of this spectroscopic
platform.

Initial experiments on the use of AAO matrices for SERS of biological sample have been
recently described [12]. Here we expand on those and outline several new features brought
by the microfluidic technology. Specifically: (1) Raman signals of aggregated molecules or,
large biological species are less significant due to the short decaying distance of SP fields
and the small – ca. 20 nm - diameter of the pores. We provide an example of such size
selection in the experiments for the bacterium Salmonella enterica. (2) Imbedded bilayers
retain many biophysical properties of unsupported membranes and their leaflet surfaces
could be made assessable to solvent molecules [13]. (3) Microfluidic channels (MFC)
improve the throughput of such studies: (a) the MFC ensure the solvency of molecules
throughout the experiments; (b) improvements in the coupling between the laser beam and
substrate are obtained through lensing by the solvent filled channels.
II. Experiment and Methods

Chemicals and biochemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless indicated otherwise. Synthetic zwitterionic phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and biotinylated 16:0 Biotinyl Cap PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl), sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL) as chloroform solutions and stored in a freezer at −80 °C prior to use. 0.3 mg of Streptavidin (Sigma-Aldrich) was dissolved in a 0.5 mL of water to yield 10^{-5} M concentration and administered into the microfluidic channels by a syringe.

Nanoporous substrates

A nanoporous layer of hexagonally packed holes of ca. 20 nm in diameter and about 50 nm deep was prepared by anodizing a high purity (99.999+) Al foil (250 μm thick) as described in [12]. The oxide layer was left to reside on the foil (see Figure 1b, c).

Biotinylated multilamellar lipid vesicles were prepared by mixing chloroform solutions of DMPC and Biotinyl Cap PE in 20:1 or in 200:1 molar ratio. Subsequently, chloroform was removed by a rotary evaporator yielding a thin lipid film on the surface of a round bottom flask. Residual chloroform was removed overnight by keeping the flask open to a liquid nitrogen trap and a vacuum pump. Multilamellar vesicles were formed by adding 50 mM Hepes buffer, pH 7.0, and cycling the flask for at least ten times between liquid nitrogen at −197 °C (77 K) and a water bath at 30 °C. The final concentration of lipids in aqueous media was 150 mg ml^{-1}. In order to minimize the lipid residue outside the holes we gently wiped the samples with a wet Q-tip and washed it with water.

Unlabeled multilamellar lipid vesicles (without biotin) were prepared from DMPC using the same procedure but without adding Biotinyl Cap PE. The final concentration of lipids in aqueous media was 30 mg ml^{-1}. This solution was administered to the AAO substrate at 30 °C which is well above the main phase transition temperature of DMPC (about 23 °C). In order to minimize the lipid residue outside the holes we gently wiped the samples with a wet Q-tip and washed it with water.

Salmonella enterica (Newport) was grown in a Luria-Bertani (LB) medium (1-1: 10 g tryptone, 5 g yeast extract, 5 g NaCl) at 37 °C for 16 hrs. Cells were harvested by centrifugation (14,000 × g, 15 min), washed with distilled water twice, and fixed in 70% ethanol in water (v/v). Fixed cells were stored at −20 °C until further use [14].

Microfluidic channels were fabricated from polydimethylsiloxane (PDMS) using a photoresist pattern on silicon as a master template [15]. The PDMS prepolymer was deposited onto the surface and let cure at 60 °C. The flexible layer was then removed and pressed against the AAO substrate or glass as a control. The channel dimensions were: height − 70 μm, width −1000 μm, and length − 1 cm. A narrower channel (500 μm wide, 5 mm long) led to the main channel. The overall height of the PDMS film was 2 mm. The Raman signals from the microfluidic channels were collected directly through the transparent PDMS film using an extended microscope objective.
The Raman system was set in a confocal arrangement (Figure 1b). A 10 mW power of an Ar ion laser at 514.5 nm and TM polarization (the electric field of the incident beam has a component oscillating perpendicularly to the substrate’s surface) was focused by an ULWD ×50 microscope objective through the transparent PDMS film to a 2 μm spot on the AAO surface within a microfluidic channel. In some experiments the vertical focus point was moved above the AAO surface in order to assess free (unbound) molecules in the channel. A 75 cm long spectrometer equipped with a 1200 g/mm grating and a TE cooled CCD array was used to assess the scattered light. The sample was rotated and tilted to achieve resonance conditions. After administering the analytes into the channel the flow was stopped and the Raman spectra were acquired by averaging five 10 s scans, thus, taking less than 1 min in total. In some experiments the flow was stopped for prolonged periods of time to assess effects of long-term exposure (over 10 minutes). No long term effects were found as the PDMS enclosure prevented solvent evaporation and, thus, any changes in the analyte concentration. We note that while high concentration of DMPC may lead to several layers within the nano hole [13] the Raman spectra is mainly collected from the hole-channel surface due to the evanescent nature of the SP modes [10]. Unexpected lensing effect from MFC led to an increase of the optical throughput.

III. Theoretical Considerations

Surface Plasmon Resonance Conditions

In our experiments, the AAO layer provides a subwavelength periodic hole array that couples the incident light to the SP mode. In order to achieve the optimal coupling and a sustainment of the standing surface wave, the sample was slightly tilted at an angle \( \theta \) and rotated in-plane (at an angle \( \phi \)) with respect to the incident beam (Figure 1B) to achieve the SP resonance condition [16]:

\[
\sin(\theta) = \frac{\lambda_0}{a} \sqrt{\left(\frac{4}{3}\right)(q_1^2 - q_1 q_2 + q_2^2) - n_{\text{eff}}} \quad (1)
\]

where, \( \lambda_0/a \) is the ratio between the optical wavelength and the structural pitch (\( a=90 \text{ nm} \) in our case). Parameters \( q_1 \) and \( q_2 \) are inversely proportional to the number of the sub-planes in the Brillion zone along each of the reciprocal vectors: \( q_i = 1/m_i \) where \( m_i \) is an integer. The effective refractive index, \( n_{\text{eff}} \), characterizes the loaded surface plasmonic guide and is typically on the order of unity (\( n_{\text{eff}}=1.02 \) for aluminum/air interface). A loaded guide means that the metal is coated with a very thin dielectric layer (50 nm thick aluminum oxide). The optimal coupling conditions ensure formation of a standing wave in the SP mode that is scattered back-and-forth along the substrate surface.

Example

The scattering Stokes’ Raman line of 1600 cm\(^{-1} \) is translated to a wavelength of 557 nm if the molecule is pumped with the 514.5 nm line of an Ar laser. A good coupling between this scattering wavelength in air and the periodic structure may be made with the \( q_1=1/7 \) and \( q_2=-1/7 \) planes. The optimal tilt angle for this wavelength is \( \theta=0^\circ \) (normal incidence). Based on simulations, the best choice for the pitch-to-wavelength ratio is \( 1/4 \). The wavelength in water...
is 1.33-fold shorter than that in air; also, $n_{\text{eff}}=\left(\varepsilon _{\text{W}}\varepsilon _{\text{Al}}/(\varepsilon _{\text{W}}+\varepsilon _{\text{Al}})\right)^{1/2}$, with $\varepsilon _{\text{W}}, \varepsilon _{\text{Al}}$ the permittivity of water and aluminum, respectively; $n_{\text{eff}}=1.35$. Thus, for $\lambda _{0}=557$ nm ($\lambda _{W}=419$ nm), resonance conditions at $\theta \approx 0^\circ$ are obtained with $q_1=1/4$, $q_2=0$ and $q_1=1/7$, $q_2=-1/7$.

### Origin of Signal Enhancement and Procedures for Data Processing

Nonlinear polarization, $P_S$, of the scattered beam as a result of a pump field $E_P$ is given by [17]:

$$P_S^{(3)}=3\varepsilon_0 \chi_R |E_P|^2 E_S,$$

where, $\chi_R$ is the Raman susceptibility and $E_S$ is the scattered (Stokes or anti-Stokes) field.

The scattered field from a film of thickness $t$ may be written directly as:

$$E_S(t)=E_S(0)\exp(i3\omega_S t \chi_R I_P(0)t/2\varepsilon_0 c^2 n_S n_P),$$

where $I_P(0)$ is the incident pump intensity, $\omega_S$ is the scattered radial frequency, and $n_S$ and $n_P$ are the refractive indices of the sample (structure and molecule) at the pump and scattered wavelengths, respectively. Linearizing Eq. 3 for small scattered intensity values, the signal-to-noise ratio (SNR) may be written as:

$$\text{SNR} \approx I_S/I_N \approx 1-\text{Im}\{\chi_R\} I_P(0)t/\varepsilon_0 c^2 n_S n_P$$

If we assume that the optical noise intensity $I_N$ (mainly from coherent linear substrate scatterings) is the seed for the Raman process, then $I_S \approx I_S(0)$. Note the strong effect of the refractive index at either the pump, or, the scattered wavelengths (or both) as resonance conditions are obtained with either $n_S$ or $n_P$ approaching zero (Eq. (4)). Also note that for a given input pump intensity $I_P(0)$ and molecular susceptibility $\chi_R$ the change in the SNR value and, thus, the enhancement factor only depends on the effective refraction index under resonance conditions. The enhancement of the Raman signal with such perforated platforms may be experimentally validated by tilting the samples towards and away from the resonance conditions as such procedures should produce some variations in the Raman peak intensities [10,12].

It is worth noting that the spectral background, as well as the signal, vary through coherent light scatterings. Such light scatterings are expected to translate into spectral signals due to imperfect beam collimation and finite apertures for the lens and the spectrometer’s grating. Light scatterings are the main reason for the background spectra. Without a priori knowledge of the origin of this spectral background one cannot simply subtract it through fitting and other post-processing data techniques because these background counts represent signals in the frequency domain (in contrast to truly random effect, i.e., white noise). The contribution of the substrate itself (MFC filled with only the solvent) may be assessed separately as a function of the substrate orientation. The contribution of a MFC filled with only the solvent may be subtracted from the Raman spectra since it is uncorrelated with the molecular signal.
Summarizing, we conduct experiments and process the data as follows: the analyte solution is introduced into a MFC formed on a perforated dielectric substrate. The MFC assembly is tilted and rotated such that the laser and scattered beams are coupled to the SP modes of the substrate (see also Figure 1a). At that point, the signal is maximized and the spectral background is minimized. Consequently, a background signal is obtained for MFC on the same substrate but filled with only the solvent (i.e., without the analyte) and under the same tilt and in-plane rotation angles. This background signal is subtracted from the Raman data for the analyte. The remaining noise count (presumably from coherent light scattering) is low if we couple to standing SP waves (Figure 2, \(\theta=0^\circ\)). If this component of the noise is white, the noise equivalent power (NEP) is constant. Otherwise, the frequency dependent background is taken as, \(I_N\). Signal-to-noise ratio (SNR) is evaluated by taking the ratio of the Raman peak count to some background value away from any identifiable Raman peak. Note that the detector noise has already been subtracted from the data (known as up/down integration): the detector noise is independently assessed by taking the data from the sample under dark conditions. Also, at issue here is a comparison between samples for which the launching conditions are varying. SNR comparison between experiments made for various samples according to this data processing protocol is more credible since it directly accounts for all light scattering processes - something that is not conveyed by a more typical data processing approach of subtracting an arbitrary background from the spectra.

**IV. Results and Discussion**

Figure 2 compares Raman spectra for an aqueous streptavidin solution within MFCs on either flat glass or AAO substrates. The sample on flat glass lacks identifiable streptavidin Raman peaks as illustrated in the Figure: low concentration of \(10^{-5}\) M and small MFC cross section are insufficient for detection. In contrast, the AAO-lined MFC reveals the known Raman spectra of streptavidin [20] as a result of interaction with SP waves close [19] and at resonance with the periodic structure [21]. These SP waves are confined to distances smaller than 100 nm from the AAO/channel surface [22]. For the SNR analysis let us consider the background value at 1500 cm\(^{-1}\), although the level at 1300 cm\(^{-1}\) may suit our analysis even further. The best results were obtained at normal incidence, \(\theta=0^\circ\), in good agreement with the theoretical considerations discussed above; yielding SNR~7. The SNR value for \(\theta=6^\circ\) is only SNR=1.5 wheras no measurable signal for streptavidin solution on glass was detected (i.e., SNR \(\leq 1\)).

Figure 3 demonstrates that streptavidin within MFCs, formed on top of either bare AAO or AAO impregnated with DMPC (though, without biotin), can be easily washed away. This indicates that streptavidin is not absorbed on the alumina surface, or, becomes trapped in the nanopores in any measurable quantity even after the deposition with lipid bilayer. Note that the curve for obtained after the streptavidin washing (Figure 3a) is essentially flat implying that our data processing protocol succeeded in accounting for the light scattering effects from the substrate. A lower SNR for the AAO/DMPC platforms reported here is explained by the reuse of substrate for repeated experiments and a possible partial clogging of some of the pores with DMPC, resulting in a less effective substrate.
Monitoring analyte binding to lipid membranes by SERS is presented in Figure 4. Such binding events mimic targeted drug delivery. The lipid bilayer membranes deposited onto the AAO pores were composed of DMPC doped with biotinylated lipids. The latter exhibits high affinity to streptavidin. As expected, the Raman spectra from streptavidin bound to biotinylated lipid membranes are very similar to that from the non-biotinylated substrate (Figure 3). The binding event occurs within a few seconds as no changes in the Raman spectra were observed after the first signal was acquired. These and previous experiments imply that the nano-holes did not prevent diffusion of streptavidin through the DMPC membrane yet, we note that the obtained Raman signals are mostly due to molecules residing at the AAO hole-channel interface. The Raman signature of streptavidin persisted even after washing the channels with water. While the Raman intensities before and after the wash were similar, a reduced background level is observed after the wash. This implies that the coherent scatterings from suspended proteins have been effectively eliminated from the data. In addition to demonstrating SERS for monitoring analyte binding events, these data confirm the strong adhesion of biotinylated lipid bilayers to the upper portion of the pores in AAO substrates.

The Raman spectra of *Salmonella enterica* introduced into PDMS channels are shown in Figure 5a. Similar to the streptavidin experiments, no signal was detected for MFC on glass (not shown). As the AAO-based platform was tilted and rotated the signal has improved and some peaks appeared as a result of resonance coupling. This signal is attributed to cells residing at close proximity to the AAO surface as evident by the sensitivity of the curves to tilt and rotations of the substrate. On the other hand, the relatively small SNR is due to the large size of the bacterium, extending a few decaying lengths away from the AAO surface. The decaying length of the SP waves is smaller than 100 nm.

Many biological species portray similar Raman peaks and questions are often raised to the merit of ‘clean’ experiments where only one bio species is tested at the time. In contrast, practical biosensors should be capable of detecting analyte(s) in a mixture of several relevant bio-species. As a step in this direction we have conducted the following sequential experiment: Firstly, a MFC was placed on the AAO substrate lined with biotynilated lipid bilayers. We administered $10^{-5}$ M streptavidin in water to the MFC (Figure 5b, trace 1) similarly to the experiments summarized in Figure 4. We emphasize that since no signal may be obtained from similar MFC on glass, the resultant Raman spectra is due to streptavidin bound to the biotinylated lipid bilayer membranes stabilized on the AAO surface. Secondly, *Salmonella eneterica* was added to the channel and a complex spectrum was observed (Figure 5b, curve 2a). Such noisy spectrum is a result of multiple spectral lines and, in particular, the ~1410 cm$^{-1}$ C-H stretching lines attributed to both species. One may hypothesize that smaller molecules will have a larger impact on the signal (*i.e.*, larger SNR) if the optical beam is focused in the vertical direction, such that it interrogates small distances away from the AAO surface. Indeed, when the laser beam was focused at AAO-channel interface, the Raman signature of mainly bound streptavidin (evidenced by the two main streptavidin peaks) reappeared (Figure 5b, curve 2b). Finally, the channel was washed with water to reveal only the spectra of bound streptavidin (Figure 5b, curve 3). This serves as a further proof that only a thin layer next to AAO surface is responsible for the amplified
Raman signals. We note that one should not directly compare our data with the SERS peaks from *Salmonella enterica* reported in the literature because the latter method amplifies the signals from bacteria directly absorbed on rough surfaces of metal colloids. Such treatment may result in Raman peak shifts [11] whereas our data were obtained without adhesion of *Salmonella enterica* to the surface.

V. Conclusions

In conclusion, we have used MFC on nano-patterned platforms for monitoring binding events of analytes to lipid bilayer membranes by Raman spectroscopy. These nano plasmonic platforms were made of periodically perforated dielectric substrates on metal; a standing surface plasmon mode was obtained for interrogating optical beams at normal incidence to the substrate surface. The largest Raman signal enhancement was obtained for proteins at close proximity to the substrate surface. Incorporation of binding groups at the substrate’s pores (biotinylated lipid bilayers) substantially enhanced the interaction between protein in the analyte (streptavidin) and the target (biotinylated lipid bilayers). Without them, the analyte could be easily washed away. Noise in such optical experiments was attributed to suspended unbound species. With our platforms, however, one may obtain selective spectra of suspended and bound molecules.

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References

11. When compared with ordinary Raman signals, SERS peaks may differ by 10 cm$^{-1}$; these variations are sometimes attributed to charge transfer between molecules and metal and ‘active SERS lines’. One also could argue that these are the result of molecular strain. In contrast, one can show that at resonance our perforated substrates yield the same within 1 cm$^{-1}$ Raman peak position as obtained with the ordinary Raman (OR) (Banerjee, A., Li, R., and Grebel, H. Nanotechnology, 2009, 20, 295502). More on comparison between OR and SERS may be found in Sanchez-Cortes S, Berenguel RM, Madejón A, Pérez-Méndez M. Biomacromolecules. 2002;

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Figure 1.
Experimental configuration. The sample is rotated and tilted so that the laser and scattered lights were coupled to SP standing modes. (b) Micro-fluidic channel (MFC) on AAO (yellow). The latter is resting on Al support (blue). Arrow points to the fluid channel and the direction of the flow. (c) SEM image of the nanoporous AAO substrate. The hole diameter was 20 nm and the array pitch was 90 nm. One may avoid tilting the sample by designing the array pitch such that interrogation is made at normal incidence.
Figure 2.
Raman spectra of $10^{-5}$ M streptavidin in water. The PDMS microfluidic channel was placed on either glass or, tilted and rotated AAO substrates. The background of water filled MFC, tilted at the respective angle was subtracted from the curves except for the data for glass. The best data was obtained for $\theta=0^\circ$ (solid black curve). Characteristic signatures of streptavidin at 1410 cm$^{-1}$ are assigned to C-H stretching of $\delta$CH$_2$, $\delta$CH$_3$. The 1228 cm$^{-1}$ peak is assigned to N-H stretching of the $\beta$-sheet backbone amide bonds [20].
Figure 3.
(a) Raman spectra of $10^{-5}$ M streptavidin injected into MFC on AAO substrate. The data was taken at normal incidence (tilt angle $\theta=0^\circ$). (a) Bare AAO substrate and (b) AAO substrate impregnated with DMPC. The streptavidin could be easily washed away from either substrate as noted by the flat curves after wash. The background spectrum of AAO with only DMPC was subtracted from each data.
Figure 4.
Binding experiments: streptavidin attached to biotinylated DMPC before and after wash with water. The SNR is similar for both curves.
Figure 5.
(a) Raman spectra of *Salmonella enterica* introduced into MFC on AAO substrates. The signal has improved as the platform was tilted and rotated, yet, the SNR is small. The best sample orientation was at $\theta=0^\circ$ (lower curve). Peak assignment: $1232 \text{ cm}^{-1}$ is assigned to $\delta_{\text{C-H}}$, $\rho_{\text{CH}_3}$; $1414, 1465, 1498 \text{ cm}^{-1}$ are assigned to $\delta_{\text{CH}_2}$, $\delta_{\text{as CH}_3}$ [23]; (b) Sequenced experiment at $\theta=0^\circ$: 1) Raman spectra of streptavidin, bound to biotinylated DMPC. 2a) The combined spectra when *Salmonella enterica* was added to the channel. 2b) Spectra of streptavidin with *Salmonella enterica* when focusing onto the AAO surface of the channel. 3) After washing off the channel with water. The background of MFC filled with only water was subtracted from all data.