Supporting Information: Label-Free *In Situ* Detection of Individual Macromolecular Assemblies by Surface Enhanced Raman Scattering

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SERS substrate preparation: SERS substrates were prepared by thermal evaporation of silver (Ag, Sigma-Aldrich, 99.999%) onto an anodized aluminum oxide (AAO) filter (Whatman Anodisc 13) with 0.1μm pores. Filters were cleaned in an argon plasma for 5 minutes prior to deposition of metal onto one side of the filter. Silver was deposited at 1 Å/second to a thickness between 500 and 1000nm. Variations in silver thickness do not contribute to variations in enhancement. Substrates were stored under vacuum to prevent contamination and oxidation.

Flow cell assembly: Prepared films were epoxied to a microaqueduct slide for assembly of the flow cell (Bioptechs FCS3). A standard 0.1mm thick gasket with a 1mm channel was cut to accommodate the presence of the film in the chamber. A diagram of this configuration is shown in Fig. S1. With the chamber assembled, the AAO filter could be removed by flowing 100mL of 0.5M NaOH through the chamber.

Solution Preparation: Solutions of 1 wt% dipalmitoylphosphatidylcholine (DPPC) (Avanti Polar Lipids) were prepared by suspending 10mg of lipid into 1 mL of nanopure water. Uniform vesicles were formed by hydrating the lipids with five cycles of heating (65°C), vortexing, and cooling, then passed through a 1μm filter (Whatman, Nuclepore) thirteen times. Polystyrene bead (0.1 μm, Sigma-Aldrich) solutions were prepared at 1.5 wt% in

nanopure water (18.2 MΩ cm). Solutions of 1,2-di-(4-pyridyl)-ethylene (BPE, Sigma-Aldrich, >98%) for limit of detection studies were prepared in ethanol.

Raman measurements: Raman spectroscopy was performed using a 17mW HeNe laser at 632.8nm (Thor Labs) or a 62mW solid-state laser at 531.5nm (Innovative Photonic Solutions). The laser output was filtered with an appropriate laser-line filter (Semrock), half-wave plate (Thor Labs) and polarizer (Thor Labs). The beam was expanded with a 5x beam expander prior to passing through a thin film polarizer (ArcOptix) and reflected off of the appropriate notch filter (Edmund Optics). The beam was coupled into an Olympus BX-51 microscope through a camera port where a 90/10 beam splitter (Chroma) directed the beam to the objective lens. Raman backscatter was collected using the same objective (Olympus 60x, 1.2NA water immersion cover glass corrected) and transmitted through the notch filter into a 50 μm optical fiber. The fiber was coupled to an Andor SR i303 spectrograph with 300, 600, and 1200 groove/mm gratings and either a Newton DU970N-BV EMCCD or iDus 420BV CCD camera. The excitation power was attenuated by rotating the half-wave plate relative to the polarizer to a power of 1mW.

Spectra were collected in kinetic series of 50-1000 acquisitions of 25 or 50ms. For LOD experiments with BPE, 10 acquisitions were taken for 0.5s each. A polystyrene reference spectrum was collected in 100 acquisitions for 30s each with 632nm excitation, while the DPPC reference was collected in 10 acquisitions of 20s each with 532nm excitation.

The flow cell was positioned in the microscope with a stage adapter (Bioptechs). Solutions were pumped through PTFE tubing (Zeus) into the cell via peristaltic pump with

silicone C-flex tubing. Prior to spectral acquisition for macromolecule assemblies, solutions were allowed to circulate through the chamber and tubing for 10 minutes. For limit of detection experiments, acquisitions were performed within one minute of the introduction of each solution.

Sandwich Configuration: To increase the probability of observing assemblies, a separate experimental configuration was adopted. In this configuration, an enhancing film was epoxied to a microscope slide. The AAO filter was then removed through an overnight soak in 0.1M NaOH, leaving only the SERS film. Substrates were rinsed with nanopure H_2O and a drop of PS bead solution (10 wt%) was then placed on the film, followed by a coverglass.

Langmuir Regression Analysis

The number of molecules necessary for a detectable signal was determined using Langmuir linear regression.[1] Experimentally, we performed a concentration study using solutions of 1,2-di-(4-pyridyl)-ethylene (BPE) (Sigma-Aldrich, >98%) in ethanol with concentrations varying from 200 μM to 2 nM. The SERS signal was obtained within one minute of injecting BPE into the solution cell. Shown in Figure S3 A are representative spectra for different solution concentrations. The spectrum of the molecule is clearly evident at 200 nM, but is not distinguishable at 20 nM. The pyridyl groups are expected to interact with the surface, enabling Langmuir regression analysis of coverage. Because the SERS signal arises almost exclusively from the surface monolayer, at low concentrations the observed signal should be proportional to coverage.

The obtained spectra were fit using a Lorentz lineshape to determine the peak areas associated with the strong modes at 1200, 1606, and 1636 cm-1. (Fig. S4.) The peak areas

determined as a function of concentration are listed in Table S1. At the low concentrations used, sub-monolayer coverage is expected and should be reasonably characterized by a Langmuir isotherm:

$$\Gamma = \Gamma_{MAX} \frac{K \times c}{1 + K \times c} \quad \text{(Eq. S1)}$$

Where Γ is coverage (Peak area), K is the equilibrium coefficient, and c is the concentration. Rearrangement of Eq. S1 yields:

$$\frac{c}{\Gamma} = \frac{c}{\Gamma_{MAX}} + \frac{1}{K \times \Gamma_{MAX}} \quad \text{(Eq. S2)}$$

Linear regression was performed by plotting c/Γ vs. c and determining the slope, which corresponds to $1/\Gamma_{MAX}$. For all three peaks, the value of $1/\Gamma_{MAX}$ was approximately 4×10^{-7}.

The ratio of the signal at 200nM relative to the signal determined for full coverage indicates the 200 nM spectrum arises from 0.25 of a monolayer. The lack of signal at 20 nM may arise for chemical equilibrium in solution, where there may be no molecules on the surface.

Calculation of Signal to Noise: The signal to noise ratio was calculated for 25 or 50ms and 1 s co-added spectra by comparison to a longer co-added spectrum. A background signal was established by averaging the signal obtained at a frequency of interest over 40 (DPPC) or 80 (PS) spectra, or 2s of spectral data, respectively. This average is represented by □. From this calculation, a standard deviation of the signal at the frequency of interest (□) over the same time frame was established. This deviation was used in lieu of other deviations as it allowed for direct comparison of the 25 or 50ms and 1s co-added spectra,

and provides an accurate measurement of the noise sources in the experiment near the frequency of interest.[2]

With these two parameters in place, the signal to noise relation was calculated using Equation S3:

$$\frac{S}{N} = \frac{(S - B)}{\sigma} \quad \text{(Eq. S3)}$$

where S is either the signal at the frequency of interest in either the 25 or 50ms or the average signal at the frequency of interest of the 1s co-added spectrum.

While this calculation leads to relatively low estimates of the signal to noise ratio than one might expect from the spectral data (4.4 for DPPC, and 3.0 for PS), it shows that the signal to noise ratio is sufficient to confirm the presence of the specified molecules in the enhancing region of the flow channel. Representative spectra are shown in Fig. S5.

Table S1. Peak fitting of results from spectra of 1,2-di-(4-pyridyl)-ethylene (BPE)

Concentration of BPE (M)	Peak area (1200)	Peak area (1606)	Peak area (1636)
2E-7	5.37e+05	7.03e+05	4.70e+05
2E-6	8.80e+05	1.7901e+06	1.43e+06
2E-5	1.8e+06	2.3e+06	1.95e+06
2E-4	2.05e+06	2.91e+06	2.33e+06

Table S2. Langmuir Linear Regression results

Peak frequency (cm-1)	Slope	Intercept
1200	$4.83 \pm 0.04 \times 10^{-7}$	$9.9 \pm 4 \times 10^{-13}$
1606	$3.40 \pm 0.05 \times 10^{-7}$	$8.5 \pm 5 \times 10^{-13}$
1636	$4.25 \pm 0.05 \times 10^{-7}$	$8.1 \pm 5 \times 10^{-13}$

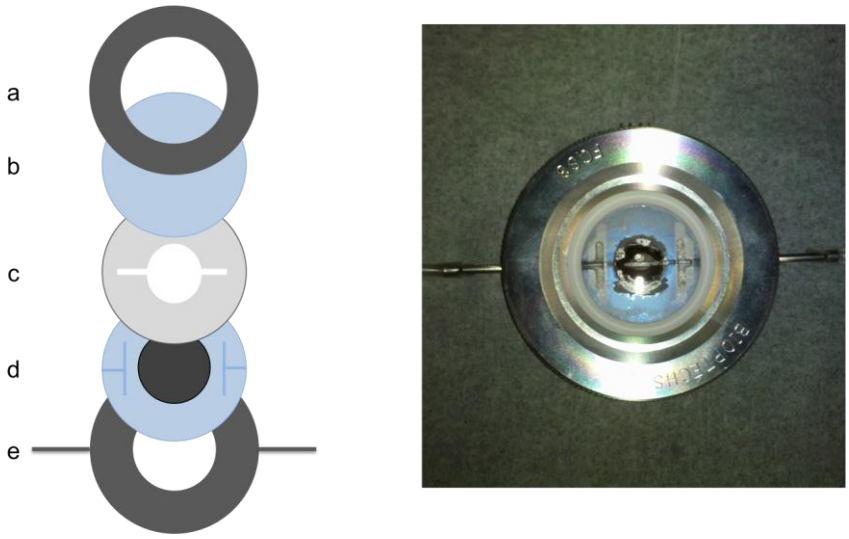

Figure S1: Diagram and photo of flow cell configuration, showing the cut gasket (c) and SERS film epoxied to a micro-aqueduct slide (d).

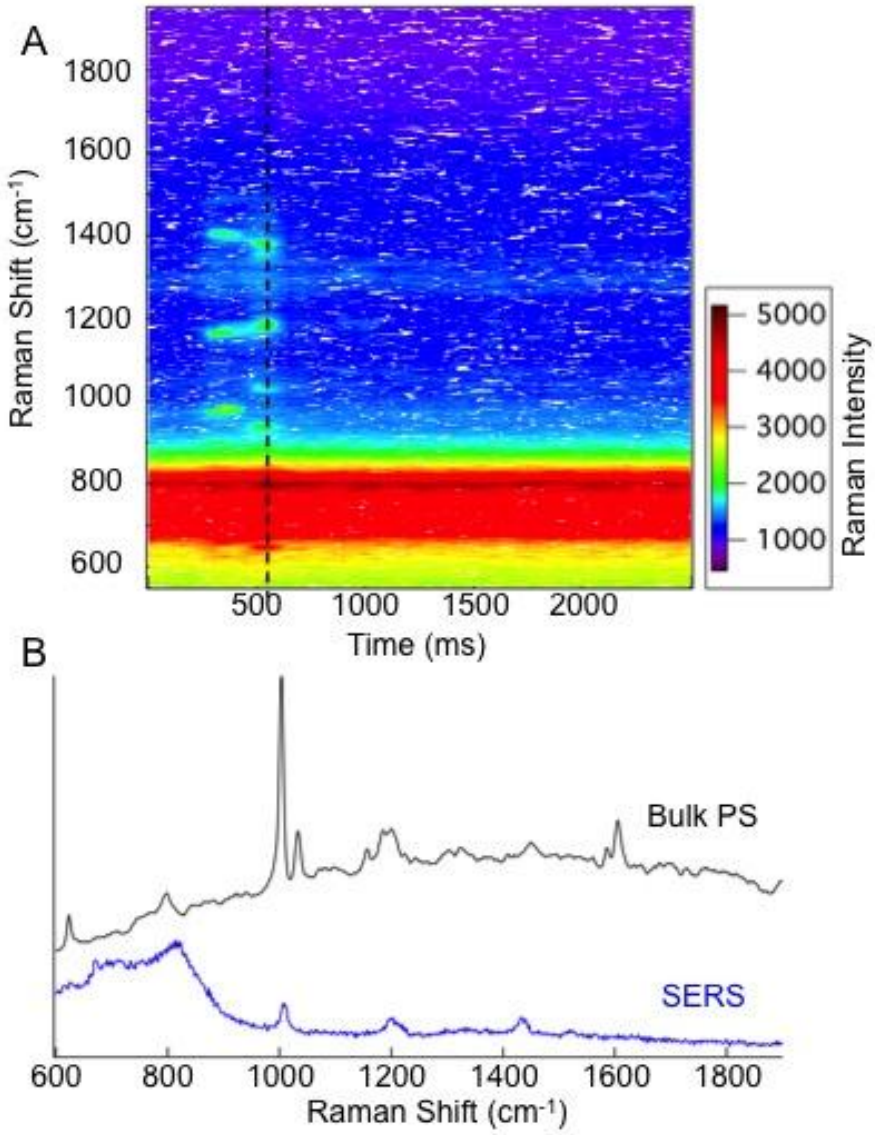

Figure S2. Map of Raman intensity vs. time for 0.1 μ m polystyrene beads. Panel B shows a comparison of a bulk polystyrene Raman spectrum (black) and the spectrum 50ms spectrum at t= 550ms (blue). The reference spectrum has been scaled for clarity.

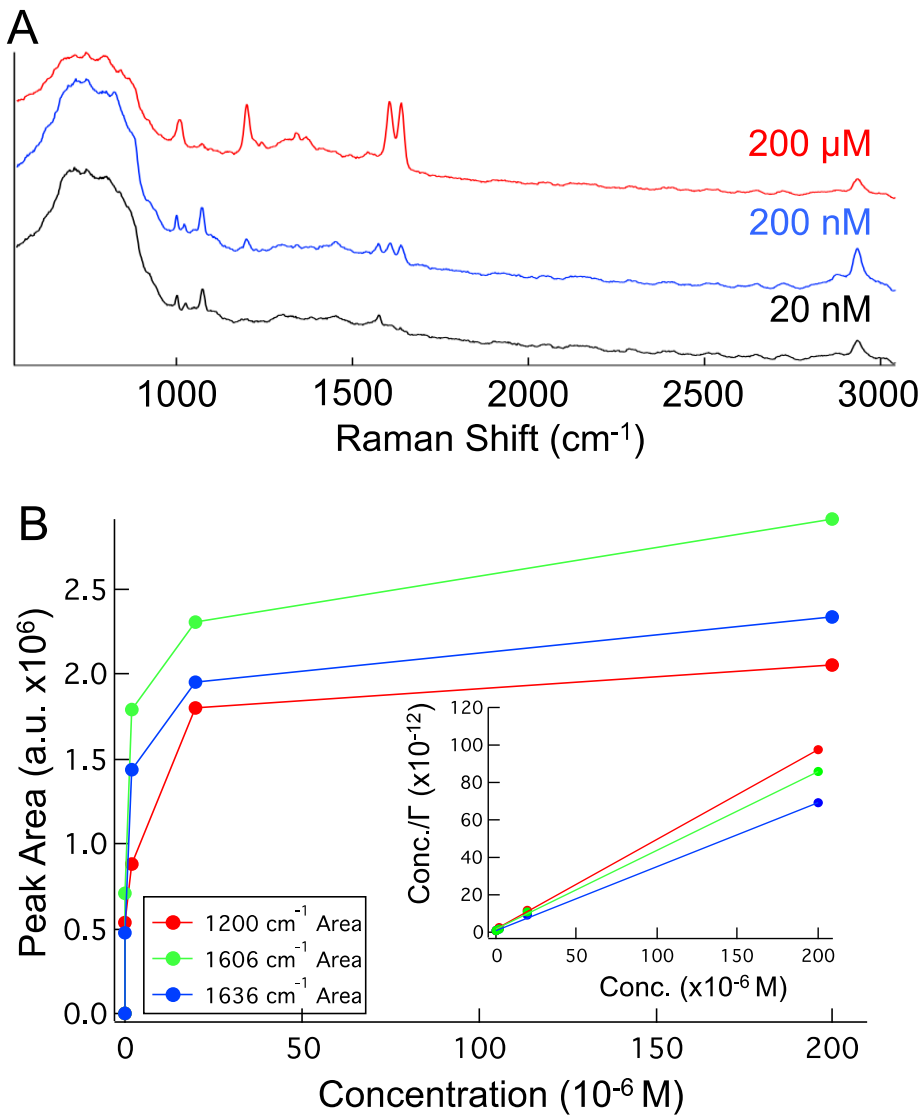

Figure S3. A) SERS spectra are plotted from solution concentrations of 20nM (black), 200 nM (blue), and 200 µM (red). B) Plot of peak area vs. concentration of 1,2-di-(4-pyridyl)-ethylene in ethanol is shown. The inset shows the results of the Langmuir linear regression, which predicts a signal of 0.8x1012 for a full monolayer coverage.

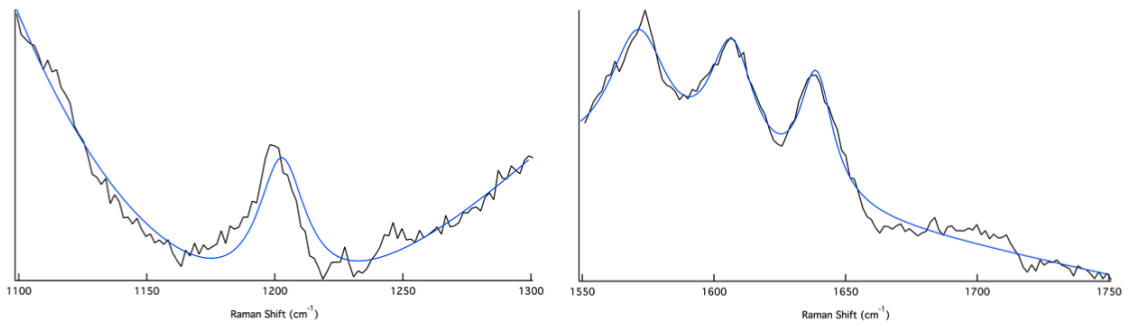

Figure S4: Peak fits (blue) to raw data (black) of 200nm BPE spectra. Fits were used to determine peak areas at 1200 cm^{-1} (left) and 1606 and 1636 (right) in BPE limit of detection experiments.

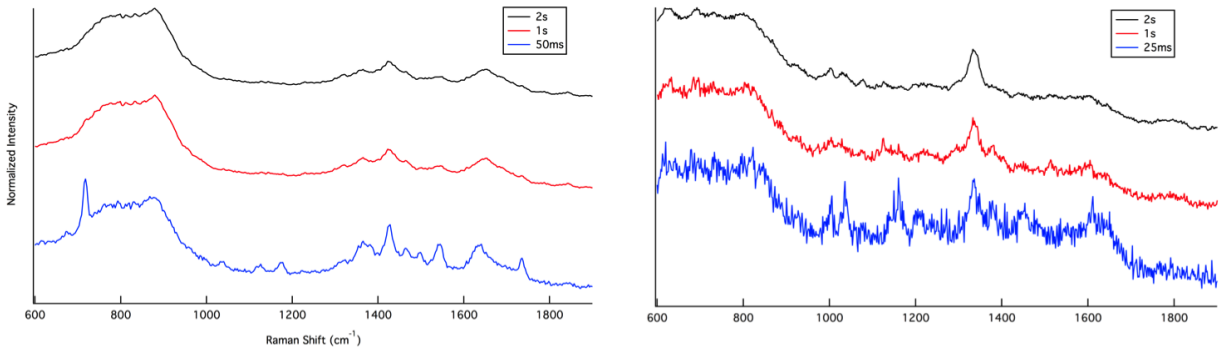

Figure S5: Comparison of the signal to noise in an individual 50ms of 25ms spectrum (blue) to 1s (red) and 2s (black) co-added spectra for 1.0 um DPPC vesicles (left) and 0.1 um polystyrene beads (right). Spectra are scaled and offset for clarity.

References:

1. I. Langmuir, *J. Am. Chem. Soc.*, 1916, **38** (11), 2211-22295.

2. R. L. McCreery, *Raman Spectroscopy for Chemical Analysis*, John Wiley and Sons, Inc., New York, NY, 2000, 49-52.