

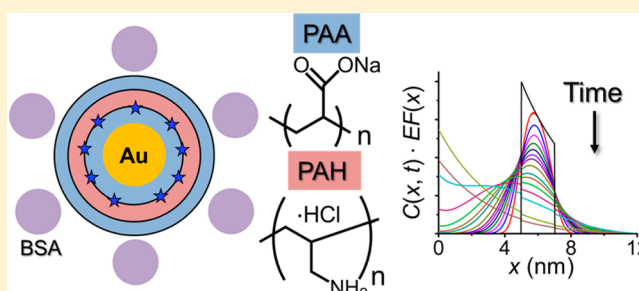
## Observation of Molecular Diffusion in Polyelectrolyte-Wrapped SERS Nanoprobes

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### S Supporting Information

**ABSTRACT:** The popularity of nanotechnology-based sensing technologies has rapidly expanded within the past decade. Surface-enhanced Raman spectroscopy (SERS) is one such technique capable of chemically specific and highly sensitive measurements. The careful preparation of SERS-active nanoprobes is immensely vital for biological applications where nanoprobes are exposed to harsh ionic and protein rich microenvironments. Encapsulation of optical reporter molecules via layer-by-layer (LbL) polyelectrolyte wrapping is an emerging technique that also permits facile modification of surface chemistry and charge. LbL wrapping can be performed within a few hours and does not require the use of organic solvents or hazardous silanes. Nonetheless, the stability of its products requires further characterization and analysis. In this study, Raman-active methylene blue molecules were electrostatically encapsulated within alternating layers of cationic and anionic polyelectrolytes surrounding gold nanospheres. We observed molecular diffusion of methylene blue through polyelectrolyte layers by monitoring the change in SERS intensity over a period of more than 5 weeks. To minimize diffusion and improve the long-term storage stability of our nanoprobes, two additional nanoprobe preparation techniques were performed: thiol coating and cross-linking of the outer polyelectrolyte layer. In both cases, molecular diffusion is significantly diminished.



## INTRODUCTION

Chemical sensing using surface-enhanced Raman spectroscopy (SERS) has recently exploded in popularity as wet chemical nanosynthesis techniques, especially related to surface control, have evolved.<sup>1–3</sup> A technique called layer-by-layer (LbL) polyelectrolyte wrapping has been used in recent years for a diverse set of surface control applications ranging from drug delivery,<sup>4</sup> biomimetic sensors,<sup>5</sup> and biofilms for medical implants.<sup>6</sup> LbL offers multiple advantages in terms of practical sensing including facile, precise, and robust control over nanoparticle surface chemistry. With LbL, it is straightforward to modify the surface chemistry and charge of hydrophilic nanoparticles with the incubation of polyelectrolyte-containing solutions with minimal preparation.<sup>7</sup> Nanoparticles are easily functionalized with reactive moieties and reacted with biomolecules or fluorescent tags for sensing applications. Additionally, LbL-wrapped nanoparticles resist aggregation in both polar and nonpolar solvents through steric effects, making them a valuable tool for improving colloidal nanoparticle stability.<sup>8</sup> LbL wrapping is a major advance in terms of nanomaterial surface control and will continue to be an important tool for biological sensing due to the chemical and optical complexity of the tissue microenvironment.

The incorporation of specific optical reporter molecules into plasmonic nanostructures is necessary to accomplish SERS sensing. Optical reporter molecules typically consist of fluorophores or other heavily conjugated molecules with delocalized  $\pi$ -electrons electrostatically bound near the nanoparticle surface. Encapsulation of reporter molecules is necessary upon exposure to harsh ionic environments or biological systems where the molecules are likely to leach into the environment. Currently, a variety of techniques are employed for this purpose. The most popular technique involves the encapsulation of Raman-active molecules into silica layers grown around metallic nanoparticles. This process is based off the Stöber method in which silica is formed via the condensation of tetraethyl orthosilicate (TEOS) or sodium silicate onto a nanoparticle surface bearing poly(ethylene glycol) (PEG) or silane ligands.<sup>9,10</sup> While silica is effective at molecular encapsulation, its growth is prone to variability, requires organic solvents, and is time-consuming. Furthermore, it is difficult to spatially localize vitrified molecules with respect

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to their distance from the metallic surface, which is a critical factor in determining SERS activity.

LbL encapsulation of reporter molecules is an emerging technique that addresses many crucial aspects in the design of SERS nanoprobes. In this technique, reporter molecules are electrostatically bound to an oppositely charged polyelectrolyte wrapped around the nanoparticle surface. After a short incubation period, additional layers of polyelectrolyte may be wrapped around the nanoparticle, effectively trapping the reporter molecule in a soft template of polymer. LbL wrapping, in contrast to silica coating, is straightforward and reproducible and requires minimal characterization during the wrapping stages. Design flexibility involving factors such as porosity, coating density, and conformation of the bound polyelectrolyte is possible through tuning the pH, salt concentration, molecular weight of the polyelectrolyte, and electrolytic strength. Furthermore, each polyelectrolyte multilayer adds an additional thickness of  $\sim 1.5$  nm, from which it is possible to approximately localize the trapped molecules.<sup>7</sup> We have previously demonstrated this technique,<sup>11,12</sup> illustrating its robustness as a preparation method for SERS nanoprobes.

In this study, we investigated the long-term storage and stability of SERS nanoprobes in the form of polyelectrolyte-wrapped gold nanoparticles. Predicting and understanding the SERS signal intensity over long periods of time under a variety of environmental conditions is essential for the design of SERS nanoprobes intended for biological sensing applications. Numerous reports investigating the formation and control over layer deposition of polyelectrolyte multilayers have yet to explore the signal stability of polyelectrolyte encapsulated reporter molecules. Unlike drug delivery studies with intentionally porous or degradable films, SERS nanoprobes are designed to maintain their structure and chemical signature over an indefinite period of time without noticeable signal loss. We maximized the reproducibility of our measurements and mimicked the optical environmental conditions of a tissue-based measurement by performing all Raman measurements in suspension with near-infrared laser excitation. Near-IR excitation exploits the so-called “optical window” (600–1000 nm) where tissue absorbs less light, increasing penetration depth.<sup>13,14</sup> Suspension-based measurements within a finite path length cuvette ensured proper accounting for the anticipated optical effects between SERS and light extinction in tissue measurements.<sup>11,15</sup> Nanoprobe stability was investigated by storing aliquoted samples at 4 °C, room temperature, and physiological temperature (37 °C) for a period of 5 weeks. SERS measurements were performed periodically during this period to assess changes in signal. To further investigate the stability and lifetime of our nanoprobes, we also studied chemically cross-linked polyelectrolyte layers and thiolated molecules.

## EXPERIMENTAL SECTION

**Materials.** Cetyltrimethylammonium bromide (CTAB, >99%), sodium borohydride ( $\text{NaBH}_4$ , >99.99%),  $\sim 15\,000$  g/mol poly(acrylic acid, sodium salt) (PAA),  $\sim 15\,000$  g/mol poly(allylamine hydrochloride) (PAH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, >98%), ascorbic acid (>99%), 5000 g/mol methyl ether poly(ethylene glycol) thiol (mPEG-SH), glutaraldehyde (EM grade 8% in  $\text{H}_2\text{O}$ ), bovine serum albumin (BSA, >96%), and methylene blue (MB, >82%) were purchased from Sigma-Aldrich and used without further purification. All glassware were cleaned with aqua regia (3:1  $\text{HCl}:\text{HNO}_3$ ) and rinsed multiple times with 18 M  $\Omega\cdot\text{cm}$  water.

**Characterization.** Holey carbon transmission electron micrograph (TEM) sample grids were purchased from Pacific Grid-Tech. Samples were prepared by drying 10  $\mu\text{L}$  of solution onto each grid. The size distribution was verified by examining at least 100 particles per grid using ImageJ analysis software. The  $\zeta$ -potential measurements were performed on a Brookhaven ZetaPALS instrument. Electronic absorption spectra were recorded with a GE GeneQuant 1300 spectrophotometer. Centrifugation was performed using a Thermo Scientific Sorvall Legend X1 centrifuge with a swinging bucket configuration.

**Raman Measurements.** Raman spectra were measured using a Horiba LabRAM Confocal Raman microscope with the laser line configured to an excitation wavelength of 785 nm. All measurements were performed in solution with a 1 cm path length quartz cuvette and an incident power of  $\sim 14$  mW in reflection. Integration time was varied between 10 and 20 s, depending on the concentration of individual nanoparticle solutions, with a spectral resolution of  $10\text{ cm}^{-1}$ . Samples were stored at 4 °C, room temperature ( $\sim 22$  °C), or 37 °C. All samples were allowed to equilibrate to room temperature before Raman measurements were performed.

**Nanosphere Synthesis.** Gold nanospheres were first prepared by synthesizing seed using a modified protocol intended for nanocube synthesis.<sup>16</sup> Under vigorous magnetic stirring 0.25 mL of  $\text{HAuCl}_4$  (0.01 M) and 7.5 mL of CTAB (0.1 M) were mixed. To this solution, 0.6 mL of freshly prepared, ice-cold (0.01 M)  $\text{NaBH}_4$  was added. The solution immediately turned from yellow to light brown. The seed was kept at room temperature for a minimum of 1 h to fully hydrolyze any remaining  $\text{NaBH}_4$ . Gold nanospheres were synthesized in 40 mL batches consisting of 6.4 mL of CTAB (0.1 M), 0.8 mL of  $\text{HAuCl}_4$  (0.01 M), and 32 mL of  $\text{H}_2\text{O}$ . To this solution, 3.8 mL of ascorbic acid (0.1 M) was added and turned the solution colorless. The seed was diluted 2 $\times$ , and 20  $\mu\text{L}$  was added. The solution slowly turned pink and after 30 min turned red. Centrifugation was performed twice at 5000g for 60 min to remove excess surfactant.

**Polyelectrolyte Wrapping of Gold Nanospheres.** Aqueous stock solutions of PAA and PAH were prepared at a concentration of 10 mg/mL (containing 1 mM NaCl). To 30 mL of gold nanospheres, at the as-synthesized concentration, 6 mL of PAA or PAH stock was added along with 3 mL of 10 mM NaCl. To the first layer of PAA, 500  $\mu\text{L}$  of 1 mM methylene blue (dissolved in methanol) was added and allowed to complex for 1 h. After each step, the nanoparticle solutions were centrifuged at 3000g for 1 h to remove excess reagents. The BSA layer was formed by adding 500  $\mu\text{L}$  of 1 wt % BSA to the suspension and allowing it to react for 2 h at room temperature. This corresponds to a  $\sim 2000\times$  molar excess of BSA to nanoparticles. Immediately afterward, the solution was dialyzed in 4 L of water with a 100 000 g/mol membrane for 48 h. The water was changed multiple times to ensure complete removal of unbound reagents. A highly concentrated stock solution was made such that aliquots were taken and diluted into the appropriate buffer for each experiment. PAA and PAH layers have weak Raman scattering cross sections as compared to methylene blue (Figure S1). All samples were stored in 15 mL polypropylene conical tubes pretreated with 1 wt % BSA solution to minimize sticking of nanoparticles to the tube. Measurements were performed in triplicate and each solution was adjusted to a concentration of 0.15 nM.

**Thiol Coating of Gold Nanospheres.** To 40 mL of as-synthesized gold nanospheres, 5 mL of 1 mM mPEG-SH was added dropwise and under sonication. Immediately following mPEG-SH addition 2 mL of 1 mM DTNB was added. Note that the DTNB was adjusted to a pH 7–7.4 to facilitate water solubility. The solution was allowed to complex overnight and then centrifuged at 4200g for 1 h. The supernatant was discarded, and the pellet was resuspended to 3 mL and 500  $\mu\text{L}$  of 1 wt % BSA was added. Dialysis with a 100 000 g/mol membrane was performed in 4 L of water over 48 h.

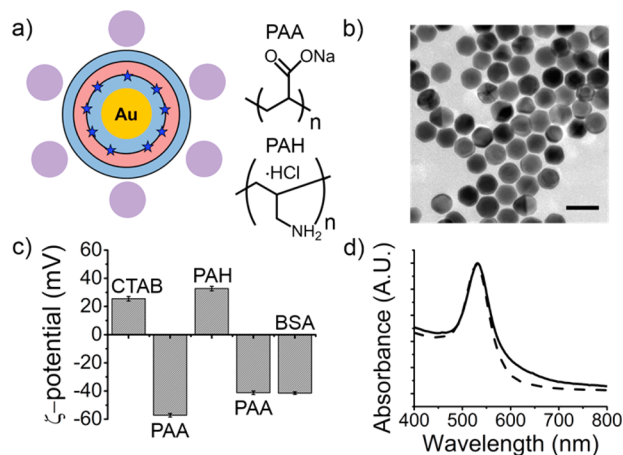
**PAH Cross-Linking.** Twice purified gold nanospheres were diluted to a volume of 30 mL. 6 mL of PAA solution was added along with 3 mL of 10 mM NaCl and allowed to sit for 1 h. The solution was then centrifuged at 4800g for 1 h. Following purification of PAA, 1 mL of 750  $\mu\text{M}$  of methylene blue (in methanol) was added. After 1 h, the solution was centrifuged at 4800g for 1 h to remove excess dye. The

solution was suspended in 30 mL of H<sub>2</sub>O, and 6 mL of PAH stock was added with 3 mL of 10 mM NaCl. Finally the solution was centrifuged again at 4000g for 1 h and resuspended in H<sub>2</sub>O to obtain an optical density of 4. 1 mL of 8% glutaraldehyde was added and allowed to react for 2 h at 4 °C. Purification was performed against a 3500 g/mol dialysis membrane for 48 h in 4 L of water to remove excess reagents. Raman measurements were performed on aliquots of this solution at a concentration of 0.25 nM.

**Finite Element Method Calculations.** Finite element method calculations were performed using COMSOL Multiphysics v4.3b with the RF and Chemical Reaction Engineering modules. Diffusion of methylene blue through polyelectrolyte layers was modeled with an impermeable boundary around a gold core of diameter 40 nm. A spherical shell 5 nm thick coated the gold core to represent the CTAB and PAA layer. Methylene blue was assumed to have a 2 nm thick layer with a uniform distribution equating to 3000 molecules based off our previously experimental work.<sup>12</sup> A 5 nm shell surrounded the methylene blue layer to account for the PAA/PAH/BSA layers.

## RESULTS AND DISCUSSION

SERS nanoprobe were fabricated by wrapping alternating layers of weakly anionic poly(acrylic acid, sodium salt) (PAA) and cationic poly(allylamine hydrochloride) (PAH) around gold nanospheres stabilized with cetyltrimethylammonium bromide (CTAB) surfactant. As shown in Figure 1a, cationic



**Figure 1.** (a) Schematic of prepared gold nanospheres with alternating layers of PAA (light blue shell), PAH (red shell), and methylene blue (blue stars). To prevent aggregation in highly ionic solutions, BSA (purple circles) was bound to the outer layer. (b) Transmission electron micrograph of gold nanospheres. Scale bar: 50 nm. (c) Representative  $\zeta$ -potential for each surface coating in the synthesis. (d) Electronic absorption spectra of CTAB-stabilized gold nanospheres (dotted line) compared to polyelectrolyte-wrapped nanospheres (solid line).

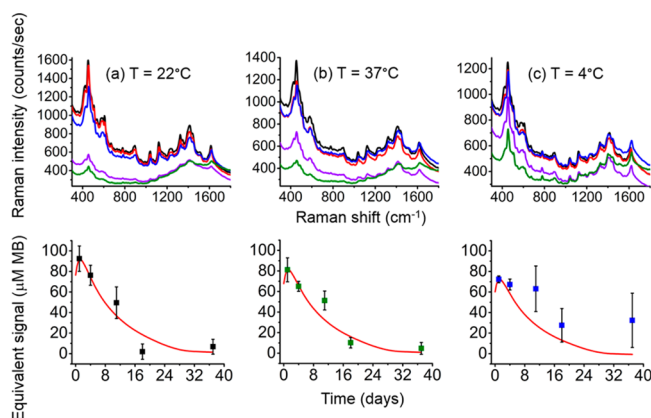
optical reporter molecules (methylene blue) were encapsulated electrostatically between the first layer of PAA and subsequent layers of polyelectrolyte and bovine serum albumin (BSA). Protein-induced flocculation was prevented by wrapping a final layer of PAA around the nanostructure before the addition of BSA. Bioconjugation studies typically use BSA to quench excess reactive sites on antibody-conjugated nanoparticles as well as to prevent nonspecific binding to “sticky” cell receptors *in vitro*. Here, BSA serves dual purposes; first, BSA stabilizes nanoparticles in highly ionic solutions such as phosphate buffered saline, which is a common buffer used in cell culture and mimics the environment found in tissue. Second, all nanoparticles exposed to whole blood or serum will immediately

develop a protein corona in which proteins dynamically associate and dissociate from the surface. Researchers continue to intensely study the effects of the protein corona both *in vitro* and *in vivo* as it can have significant unavoidable consequences on biocompatibility, renal clearance, and targeting capabilities.<sup>17</sup> The synthesized nanostructures were characterized with transmission electron microscopy (TEM) and  $\zeta$ -potential (Figure 1b,c). At room temperature all samples displayed stable electronic absorption spectra for >5 weeks (Figure 1d). This robust method provides some design guidance in terms of the spatial localization of the reporter molecules. We anticipate that the reporter molecules will be located approximately 4 nm from the metallic surface.<sup>7</sup>

Quantification of surface enhancement is often a source of confusion and contention. A major source of contention originates from the mechanism of surface enhancement itself. Molecules obeying Raman selection rules exhibit enhancement through a combination of the commonly attributed “chemical” and “electromagnetic” enhancement effects of SERS. The electromagnetic enhancement effect is generally regarded as the dominant mechanism, although this remains an active area of research.<sup>18,19</sup> Quantification is further confused by the mechanics of calculating enhancement factors. Recently, Le Ru and Etchegoin discussed mathematical and interpretative errors made in the first two reports on single-molecule SERS where enhancement factors (EFs) of  $10^{14}$  were reported.<sup>18</sup> The propagation of these errors in comparison to fluorescence cross sections resulted in numerous subsequent reports claiming similar results. In many of these studies, the EF was empirically determined from the following equation  $EF = (N_{\text{Raman}} I_{\text{SERS}} / N_{\text{SERS}} I_{\text{Raman}})$ , where  $N_{\text{Raman}}$  and  $I_{\text{Raman}}$  are the number of molecules in the focal volume and its corresponding spontaneous Raman intensity. Likewise,  $N_{\text{SERS}}$  and  $I_{\text{SERS}}$  correspond to the number of probed molecules bound to the nanoparticles and their corresponding SERS intensity. We approach the quantification of SERS enhancement in a slightly different manner.<sup>12</sup> Aqueous MB standards were prepared ranging in concentration from 5 to 80  $\mu\text{M}$ . A linear calibration curve was developed (Figure S2). All data were quantified at the  $\nu(\text{C}-\text{C})$  ring stretching vibration at  $1616\text{ cm}^{-1}$ .<sup>20</sup> Based on the calibration curve and SERS intensity of each sample (normalized to incident laser power), an equivalent spontaneous Raman signal may be calculated. The Raman equivalent signal corresponds to the equivalent concentration of MB molecules required to produce the same spontaneous Raman signal intensity as the probed solution. With this technique no assumptions of molecular coverage are necessary.

Using a spontaneous Raman quantification technique, we investigated the signal stability of the nanoprobe described in Figure 1 over the course of 5 weeks. Triplicate aliquots of solutions were stored at room temperature ( $\sim 22\text{ }^{\circ}\text{C}$ ),  $37\text{ }^{\circ}\text{C}$ , and  $4\text{ }^{\circ}\text{C}$ . As shown in Figure 2, the spectral features tend to change and decay as time progresses. Samples stored at  $4\text{ }^{\circ}\text{C}$ , while having the largest variation in signal, maintained their spectral shape better than samples stored at warmer temperatures. Intuitively, the diffusion of molecules through polyelectrolyte layers is expected to be linearly related to temperature, i.e., analogous to the Stokes–Einstein relationship. After 5 weeks of storage, several samples were centrifuged to concentrate the nanoparticles into pellets. We calculated that a 3 mL solution of 0.15 nM nanoparticles should release approximately 5  $\mu\text{M}$  of reporter molecules. The supernatant was extracted, and we found that it did not contain a



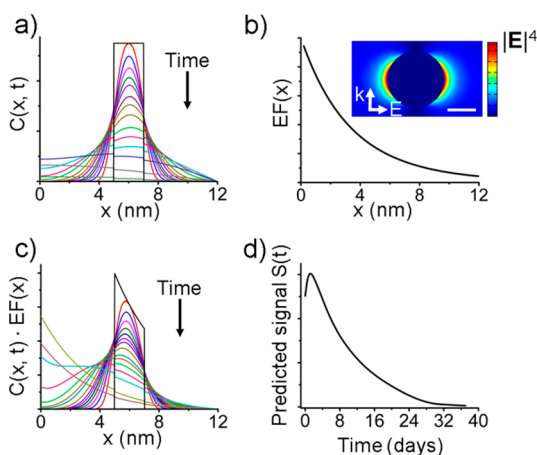


**Figure 2.** Top: SERS spectra of LbL-wrapped nanospheres: day 1, black; day 4, red; day 11, blue; day 18, purple; day 37, green. Samples were stored at (a) 22, (b) 37, and (c) 4 °C. Bottom: predicted signal intensity (red line) and the experimental spontaneous Raman equivalent signal quantified at the 1616 cm<sup>-1</sup> band. All samples were normalized to 0.15 nM.

micromolar quantity of dye molecules, indicating that a fraction of the methylene blue molecules were trapped away from the metallic surface but not free in solution. To understand this process and determine if diffusion can be ascribed as a major cause of the loss of signal, we carried out modeling. The first part of our model involves the temporal prediction of the diffusion of the reporter molecules through the polyelectrolyte layers. The second part relates the concentration to the electromagnetic enhancement factor, thereby producing a total predicted intensity.

Neglecting the effects of concentration due to the low loading and charge, diffusion through polyelectrolyte layers was assumed to be constant and modeled using Fick's second law of diffusion (Figure 3a):

$$\frac{\partial C(x, t)}{\partial t} = D \frac{\partial^2 C(x, t)}{\partial x^2} \quad (1)$$



**Figure 3.** (a) Concentration profile as a function of time with a diffusion coefficient of  $D = 10^{-18}$  cm<sup>2</sup>/s. (b) Calculated electromagnetic enhancement factor (EF) for a 40 nm gold nanosphere as a function of distance from the surface. Inset: gold nanosphere illustrating a dipolar plasmon resonance (scale bar 20 nm). (c) Calculated profile of EF and concentration. (d) Predicted signal intensity  $S(t)$ .

where  $D$  is the diffusion coefficient and  $C$  is the concentration as a function of position,  $x$ , and time,  $t$ . Equation 1 was solved using the finite element method with an impermeable boundary condition at the surface of the gold core, a uniform distribution of methylene blue, and an enforced concentration of zero at the edge of the BSA–water interface. The latter boundary condition is justified as diffusion within the solution would be much faster than diffusion in the polyelectrolyte layers. Mathematically, the boundary conditions may be stated as

$$\frac{\partial C(x, t)}{\partial x} = 0, \quad x \leq 0 \text{ nm} \quad (2)$$

$$C(x, t) = C_0, \quad 5 \text{ nm} \leq x \leq 7 \text{ nm}, t \leq 0 \quad (3)$$

$$C(x, t) = 0, \quad x \geq 12 \text{ nm} \quad (4)$$

where  $C_0$  corresponds to the initial distribution of molecules uniformly positioned within a 2 nm thick layer. Furthermore, the model conserved flux across these three regions:  $\partial C_1 / \partial x = \partial C_2 / \partial x$  and  $\partial C_2 / \partial x = \partial C_3 / \partial x$  with a fixed coefficient  $k = C_1 / C_2 = C_2 / C_3 = 0.9$ . The diffusion coefficient was determined by simulating concentration versus time with a range of values from  $D = 10^{-16}$  to  $6 \times 10^{-18}$  cm<sup>2</sup>/s (Figure S4). After an approximate match was identified, the simulated signal was compared to the experimental data (Figure 2) to further optimize the result. We found that a diffusion coefficient of  $D = 10^{-18}$  cm<sup>2</sup>/s provided the most appropriate fit. It should be noted that the value of  $D$  is calculated under the assumption of specific boundary conditions, i.e., zero concentration at the particle boundary. It is likely that some methylene blue is trapped within the particle and at its surface, resulting in a smaller concentration gradient than the one assumed in our model that would lead to a lower value of  $D$  being estimated. Under these constraints, the value of  $D$  should be used to impute a loss of signal rather than a loss of concentration.

The second portion of our model incorporates the ability of a plasmonic nanoparticle to surface-enhance the chemical signature of nearby molecules from the metallic surface. For a spherical plasmonic nanosphere, a dipolar evanescent electric field is generated when illuminated with a plane wave. The local electric field  $E_{\text{loc}}$  dictates the degree of enhancement approximately by  $|E_{\text{loc}}|^4$ . Under the assumption of a quasi-static electric field, a nanoparticle's enhancement factor (EF) decays as  $\text{EF} \propto 1/(a + d)^{12}$ , where  $a$  is the radius of the nanoparticle and  $d$  is the distance of the molecule from the surface (Figure 3b).<sup>21</sup> Thus, the signal relies heavily on the position of the molecule. The product of enhancement and concentration is shown in Figure 3c as a function of time. The predicted signal,  $S(t)$ , was calculated by the following (Figure 3d):

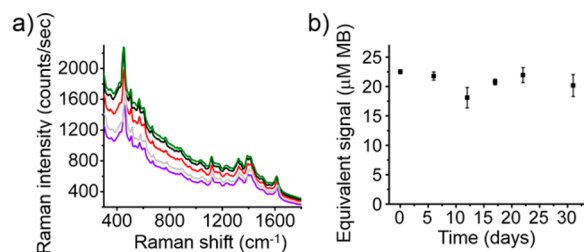
$$S(t) = \int \text{EF}(x) C(x, t) dx \quad (5)$$

Reporter molecules are predicted to diffuse both toward and away from the metallic core. As a result, signal intensity is bolstered by molecules diffusing closer to the nanoparticle surface. The diffusion coefficient was empirically determined from our data. In a previous study, Chung and Rubner experimentally determined the diffusion coefficient for methylene blue in alternating layers of PAA and PAH on substrates to be on the order of  $10^{-14}$ – $10^{-16}$  cm<sup>2</sup>/s, depending on pH and buffer conditions.<sup>22</sup> Klitzing and Möhwald recorded a diffusion coefficient on the order of  $10^{-15}$  cm<sup>2</sup>/s for the diffusion of rhodamine through polyelectrolyte films.<sup>23</sup> In our

case, the diffusion coefficient was smaller by 2–3 orders of magnitude. Numerous factors could contribute to the discrepancy including the presence of a negatively charged outer BSA layer, ionic strength, buffer conditions, or the binding affinity of polyelectrolyte layers around gold nanoparticles versus glass substrates. It should also be noted that there have been some reports describing the non-Fickian behavior of small molecule diffusion through polyelectrolytes as a result of swelling and electrostatic effects.<sup>24,25</sup>

Typically, the design of SERS nanoprobe using encapsulation methods seek to minimize diffusion. While simple encapsulation in a multilayer system can be a facile route, it results in a limited shelf life due to these effects. Hence, we explored a strategy to reduce diffusion by gelling the outermost polymer layer and imparting much greater stability to the nanoprobe's SERS intensity over long periods of time.

Chemical cross-linking was investigated as a method for minimizing undesirable diffusion of optical reporter molecules (Figure 4). An amine reactive cross-linker (glutaraldehyde) was



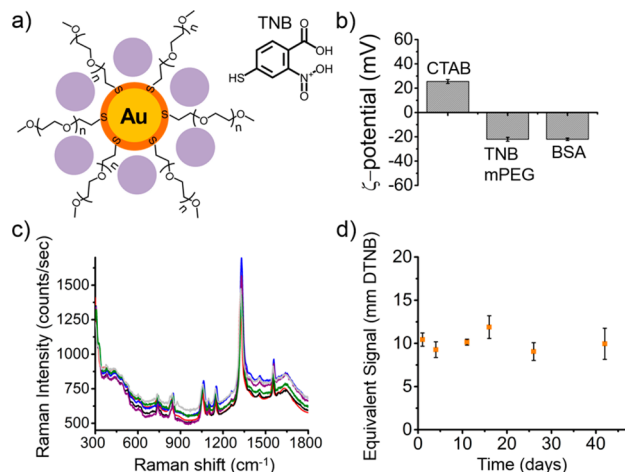
**Figure 4.** Methylene blue molecules were electrostatically encapsulated between PAA and PAH layers. Aliphatic amines from the PAH layer were chemically cross-linked and stored at 4 °C to help prevent diffusion. (a) SERS spectra of glutaraldehyde cross-linked nanoparticles. Day 1, black; day 6, red; day 12, blue; day 17, purple; day 22, green; day 31, gray. (b) Quantification of SERS intensity in terms of equivalent spontaneous Raman signal of methylene blue.

added in molar excess to a solution consisting of LbL-encapsulated nanostructures (PAA + MB + PAH). Glutaraldehyde cross-linked available aliphatic amines on the terminal PAH layer. We observed that after a 2 h incubation period LbL glutaraldehyde nanoparticles were stable and did not show signs of aggregation as measured by electronic absorption spectroscopy. In Figure 4, we show that cross-linked nanoparticles are significantly less susceptible to diffusion-dominated signal loss. Furthermore, cross-linked samples stored at 4 °C were more stable than samples stored at room temperature (Figure S5).

Cross-linking of the outer polyelectrolyte layer likely improves the outer shell stability of the nanoparticle and decreases pore size such that the reporter molecules do not as readily diffuse. DLS was used to verify the size distribution after multiple weeks of storage (Figure S6). The porosity and conformation of polyelectrolyte layers bound to gold nanoparticles are difficult to ascertain due to inherent variability in individual nanoparticles and the sheer quantity of particles in solution. To date, polyelectrolyte-based studies have focused on the characterization of polyelectrolyte multilayer thin films bound to substrates. Researchers have shown results demonstrating that changes in a solution's pH can affect porosity. In fact, when washing with acidic solutions (pH = 2.4), the porosity of PAA/PAH films is greatly increased.<sup>26</sup> It has also been demonstrated that exposure of PAA/PAH films to pure

water can increase the surface roughness of bound polyelectrolytes.<sup>27</sup> By exploiting tunability in porosity, it may also be possible to design versatile drug delivery systems. In addition to porosity, the diffusive properties of polyelectrolyte-wrapped nanoparticles has led to their use as drug delivery platforms,<sup>4,25</sup> where control of the diffusion of small molecules is desirable. Our experimental and theoretical results indicate diffusive-like behavior in polyelectrolyte multilayers, which is necessary for tuning the release profile in terms of drug delivery and is, of course, likely to be crucial in the design of multifunctional theranostic particles.

While the previous discussion has focused on nanoprobe that were electrostatically trapped by LbL assembly, another route to effective SERS sensing is by way of passivating optical reporter molecules at the surface of the nanoparticle. Thiolated molecules were used to investigate the stability of nondiffusive, covalently bound Raman molecules. Thiol has a strong affinity for metals such as gold and forms a covalent gold–thiolate bond.<sup>28</sup> To investigate signal stability, we used Ellman's reagent or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), which has a strong symmetric NO<sub>2</sub> stretch at 1333 cm<sup>-1</sup>.<sup>29</sup> Upon reduction, the disulfide bond readily cleaves into two 5-thionitrobenzoic acid (TNB) molecules. Nanoprobes were prepared with a mixed layer of methyl ether poly(ethylene glycol) thiol (mPEG-SH) and TNB (Figure 5a). A layer of BSA was



**Figure 5.** (a) Schematic of 5-thionitrobenzoic acid (orange shell) and methyl ether poly(ethylene glycol) thiol (TNB-PEG) coated nanoparticles with adsorbed BSA (purple spheres). (b)  $\zeta$ -potential measurements of each synthetic step. (c) Representative Raman spectra of TNB-PEG coated nanoparticle: day 1, black; day 4, red; day 11, blue; day 16, purple; day 26, green; day 42, gray. (d) Spontaneous Raman equivalent of TNB-PEG nanoparticles in terms of mM of DTNB.

adsorbed to the outermost layer to mimic the polyelectrolyte configuration. The  $\zeta$ -potential measurements verified that each synthetic step was successful (Figure 5b).

Samples were prepared in triplicate and stored at identical temperatures as the polyelectrolyte-wrapped nanoparticles. Figure 5c shows representative Raman spectra (normalized to laser power) of TNB-PEG coated nanoparticles stored for more than 5 weeks at room temperature. Throughout the storage period the spectral features exhibit only slight variations. Spontaneous Raman equivalents (Figure 5d) were computed using a spontaneous Raman calibration curve of aqueous DTNB (ranging from 5 to 15 mM) with an adjusted pH of 7

(Figure S3). Because of the strength of the gold–thiolate bond, TNB-PEG coated nanoparticles were less susceptible to signal loss over time. As expected, the storage considerations for thiolated Raman reporters are less crucial than LbL nanostructures.

## CONCLUSION

Careful consideration of electrostatically encapsulated optical reporter molecules for SERS nanoprobe is critical. In this study, we investigated the signal stability of SERS nanoprobe using polyelectrolyte LbL wrapping to encapsulate reporter molecules. We found that polyelectrolyte-wrapped samples stored at colder temperatures (4 °C) are more likely to maintain their spectral signature. Samples stored at room temperature and 37 °C were more likely to exhibit strong diffusion effects over the course of 5 weeks. A diffusion coefficient of  $10^{-18}$  cm<sup>2</sup>/s was derived by fitting our experimental data to a diffusion/electromagnetic enhancement model. To overcome signal degradation of SERS nanoprobe, chemical cross-linking of a polyelectrolyte-wrapped nanoparticle is sufficient. We find that storage of cross-linked samples at 4 °C is best for long-term usage. Alternatively, stronger covalent gold–thiolate bonds also prevent reporter molecule diffusion; however, thiolated molecules are typically limited in availability and expensive.

## ASSOCIATED CONTENT

### Supporting Information

Spontaneous Raman and SERS spectra of bound polyelectrolytes; calibration curve of Raman reporter molecules methylene blue and DTNB; simulated concentration versus time calculations with varying diffusion coefficients; spontaneous Raman equivalent data of cross-linked PAA + MB + PAH nanoparticles at room temperature; DLS data of CTAB stabilized nanoparticles, polyelectrolyte wrapped nanoparticles, and glutaraldehyde cross-linked nanoparticles after >5 weeks of storage. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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