

# Quantitative Electrochemical SERS of Flavin at a Structured Silver Surface

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In situ electrochemical surface enhanced Raman spectra (SERS) for an immobilized monolayer of a flavin analogue (isoalloxazine) at nanostructured silver surfaces are reported. Unique in the present study, the flavin is not directly adsorbed at the Ag surface but is attached through a chemical reaction between cysteamine adsorbed on the Ag surface and methylformylisoalloxazine. Even though the flavin is held away from direct contact with the metal, strong surface enhancements are observed. The nanostructured silver surfaces are produced by electrodeposition through colloidal templates to produce thin ( $<1\ \mu\text{m}$ ) films containing close-packed hexagonal arrays of uniform 900 nm sphere segment voids. The sphere segment void (SSV) structured silver surfaces are shown to be ideally suited to in situ electrochemical SERS studies at 633 nm, giving stable, reproducible surface enhancements at a range of electrode potentials, and we show that the SER spectra are sensitive to subfemtomole quantities of immobilized flavin. Studies of the SER spectra as a function of the electrode potential show clear evidence for the formation of the flavin semiquinone at the electrode surface at cathodic potentials.

## Introduction

Since the first observation and recognition of surface enhanced Raman spectroscopy (SERS) over 30 years ago<sup>1,2</sup> the high sensitivity of SERS has been exploited to study molecules adsorbed at roughened or textured metal surfaces in a wide range of applications.<sup>3–6</sup>

SERS has a number of attributes that make it very attractive for in situ studies of electrode surfaces and electrochemical processes. First, the surface enhancement is highly surface selective, so the technique is uniquely sensitive to molecules adsorbed at, or very close to (within  $\sim 100\ \text{nm}$ ), the electrode surface. Thus, it discriminates against the much larger number of molecules in the bulk solution in favor of the small number at the electrode surface. Second, the Raman cross section for water is low so that SERS can be used to study the electrode surface in aqueous solution. Third, SERS gives information about the molecular structure of the adsorbate and its binding to the electrode surface. Fourth, the resonant Raman intensity directly reflects the oxidation state of a molecule and can be probed in situ during an electrochemical reaction.<sup>7</sup> Finally, the proximity of the metal surface causes quenching of the fluorescence background, which can be a problem in Raman spectroscopy.

The flavin moiety is a biologically important redox group that can participate in both direct two-electron and step by step, consecutive, single-electron oxidation or reduction reactions because of the stability of the intermediate flavin semiquinone. Flavins, including flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and riboflavin (vitamin B), play central roles in many biological electron transfer processes.<sup>8</sup> Reduced flavins can also reduce molecular oxygen either in a two-electron reaction, as in oxidases, to produce hydrogen peroxide or in a four-electron reduction to water, as in monooxygenases. Resonance Raman spectroscopy has been widely used to investigate flavins and flavoproteins<sup>9–13</sup> either under conditions where a suitable quencher such as KI is added to the solution to suppress fluorescence or by using ultraviolet excitation<sup>10</sup> or SERS.<sup>11–13</sup> High-quality spectra from flavoproteins<sup>10</sup> and free flavins<sup>9</sup> were reported using silver colloids, and roughened silver electrodes have been employed to study flavin systems under potential control.<sup>14</sup> In that work, the authors obtained in situ electrochemical SERS and SERRS (surface enhanced resonant Raman spectroscopy) spectra for adsorbed oxidized riboflavin and for the semiquinone radical; however, they did not report any quantitative studies of the spectra.

Although electrochemically roughened Ag or flocculated Ag colloids give good SER(R)S enhancements for flavins, the magnitude of the surface enhancement is generally irreproducible, and therefore the technique is not quantitative. The observed

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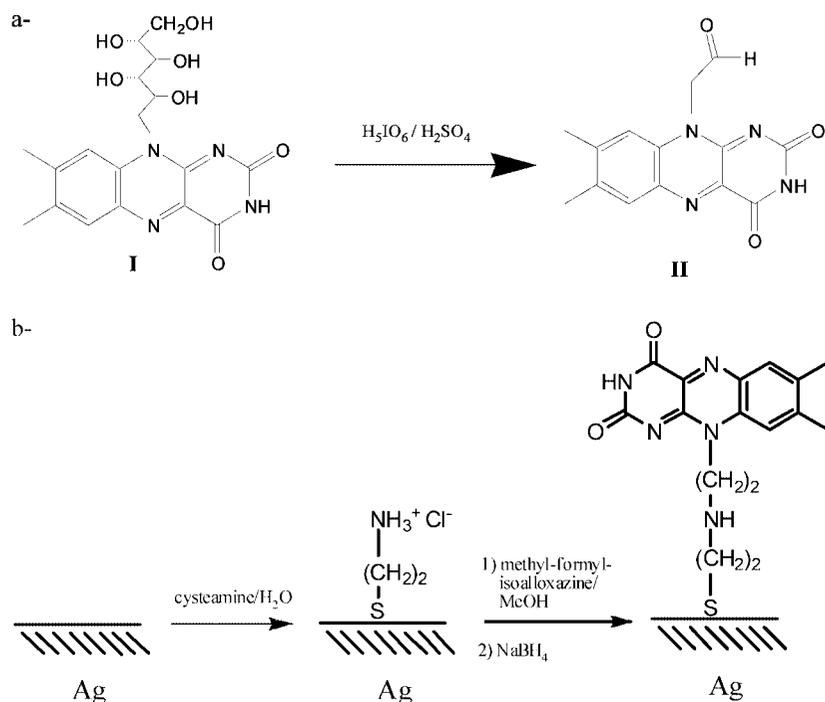
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**Scheme 1. Modification of the Silver Electrode Surface with Flavin by Covalent Coupling of Methylformylisoalloxazine (II) to Adsorbed Cysteamine: (a) Preparation of II and (b) Surface Modification**

enhancement is thought to be due to nanoscale “hot spots” of tightly localized plasmons that produce orders of magnitude local enhancements of the surface electromagnetic field.<sup>15</sup> The presence of this random distribution of hot spots also accounts for the extreme variability of the enhancement from place to place on the surface and the difficulty in reproducibly fabricating the active surface. The surface enhancement is also observed to degrade when cleaning of the surfaces is attempted; presumably because the sharp nanoscale features that localize the plasmons are easily etched or deformed and these changes in the surface lead to changes in the surface enhancement. A recurrent problem that has hampered the application of SERS and SERRS to electrochemistry over the last 30 years has been the irreversible loss in enhancement on cycling to high cathodic potentials.

In view of these limitations, it is desirable to find surfaces that can give more reproducible SERS and SERRS. We recently described a new form of nanostructured metal surface in which the plasmon modes can be engineered with precision<sup>16–19</sup> through control of the film thickness and void diameter. We have shown that for Au and Ag these surfaces show very significant SERS enhancements<sup>20–23</sup> and that the effect can be extended to applications in the near-infrared<sup>21</sup> or to metals such as Pt and

Pd.<sup>24</sup> The plasmons observed on these nanostructured surfaces correspond to localized electromagnetic fields that can be excited by incident light and can be confined strongly in the region of the metal surface. Such surfaces are distinguished from other SERS substrates by their low surface roughness, by their geometric regularity, and by their sphere segment void geometry, which allows confinement of localized plasmons within the cavities. In earlier work, we have shown that these surfaces show excellent reproducibility of SERS enhancement (<10%) from place to place on the sample surface<sup>22,23</sup> and between replicate surfaces.<sup>25</sup> Here we extend our work to show that structured electrodeposition of silver through colloidal templates can be used to produce thin (<1  $\mu\text{m}$ ) films containing close-packed hexagonal arrays of uniform sphere segment voids and that these surfaces show surface enhancement for Raman scattering from flavin molecules adsorbed on them. Unique in the present study, the methylformylisoalloxazine is not directly attached to the Ag surface but is attached through chemical reaction with a previously adsorbed cysteamine on the Ag surface. Because the films we produce do not have high surface roughness and are stable and reproducible, we believe that they will be particularly useful in studies of electrode processes. We have carried out SERS measurements at silver electrodes that allow us to monitor flavin reduction and obtain in situ spectral information at the same time.

There are several novel and important features to the work reported here. First this is the first full paper describing the application of these sphere segment void substrates to electrochemical SERS measurements. The only other publication to date is a brief communication<sup>23</sup> that presents preliminary data on adsorbed pyridine on gold. The present paper demonstrates for the first time quantitative measurements at a range of potentials using a redox molecule that is covalently attached to the electrode surface. This is possible because of the stable and reproducible

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SERS enhancement at these surfaces. Second, the work reports for the first time SERS spectra for flavin covalently attached to the electrode surface through a linker. This linkage has been well characterized in earlier work<sup>26</sup> and it has been shown that the flavin is held away from the metal surface. Our spectroelectrochemical studies show clear evidence for the formation of the semiflavin form during the reduction of the attached flavin and confirm that the spectral intensities are stable both with time and changes in electrode potential. This is in contrast to earlier work on the SERS of flavin that was restricted to studies of the adsorbed molecule either without potential control or, at best, at fixed potential where no attempt was made to analyze the potential dependence of the spectra.<sup>9,11–14</sup>

### Experimental Section

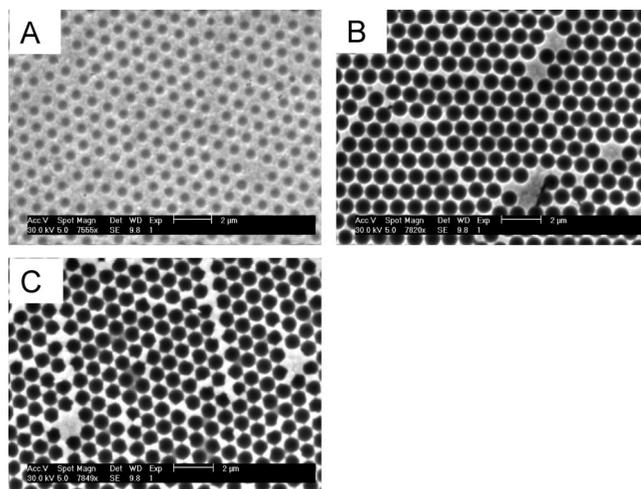
The structured silver substrates were prepared by templated electrodeposition of silver. One millimeter thick glass microscope slides were coated with a 10 nm thick chromium adhesion layer and a 200 nm thick gold layer in a vacuum evaporator. The gold-coated slides were cleaned in 2-propanol (Analytical grade, Fisher Scientific) in an ultrasonic bath (CE, Ultrawave Limited, Cardiff, UK) for 90 min and then chemically modified by soaking for 3 days in a 10 mM solution of cysteamine (Aldrich) in ethanol (HPLC grade, Rathburn). Templates of close-packed monodisperse polystyrene latex spheres (diameter 900 nm, Duke Scientific, 1 wt % aqueous suspension) one sphere thick were assembled on the modified gold coated slides by capillary evaporation in a thin layer cell following the method described in our earlier publication.<sup>27</sup>

Silver was electroplated through the templates from a cyanide-free plating bath (Technic, Lektrachem Ltd., Warwickshire, UK). The electrodeposition was carried out potentiostatically at  $-0.125$  V vs saturated mercury/mercurous sulfate (MSE) in a thermostated cell at 25 °C using a conventional three-electrode system controlled by an Autolab PGSTAT30. The template-coated gold substrate was the working electrode with a large area platinum gauze as the counter electrode and a homemade (MSE) reference electrode. These conditions led to the deposition of smooth silver films. Following electrodeposition, the latex spheres were removed by dissolving in dimethylformamide (DMF, Analytical reagent grade, Fisher Scientific) to leave an ordered array of interconnected sphere segment voids. The thickness of the silver film was controlled by controlling the total charge passed.

A Philips XL30 environmental scanning electron microscope (ESEM) was used to image the structured metal films. All Raman spectra were recorded using a Renishaw Raman 2000 system using a 633 nm HeNe laser with 5  $\mu$ m diameter spot size and 3 mW power using a single 10 s accumulation, unless otherwise stated, at a spectral resolution of 2  $\text{cm}^{-1}$ .

The flavin group was attached to the silver surface using a method previously described in the literature;<sup>26</sup> see Scheme 1. The structured silver electrodes were first modified with cysteamine by soaking in a 0.01 M solution in ethanol for 2 h and then rinsed with ethanol. Methylformylisoalloxazine (**II**) was prepared by periodic acid oxidation of riboflavin (**I**) following a reported procedure.<sup>28,29</sup> The modified silver electrodes were immersed in 5 mL of a methanol solution containing 8 mg of **II** and 50  $\mu$ L of triethylamine for 12–15 h in the dark. Excess potassium borohydride was then added to reduce the Schiff base and the solution was cooled in an ice bath and stirred for 1 h at room temperature. Finally, the electrode was copiously rinsed with water and dried in a jet of nitrogen.

All solutions were freshly prepared using reagent-grade water (18 M $\Omega$  cm) from a Whatman RO80 system coupled to a Whatman "Still Plus" system.



**Figure 1.** Scanning electron micrographs of structured SSV silver films produced using 900 nm diameter template spheres with thicknesses of (A) 150 nm, (B) 430 nm, and (C) 700 nm.

### Results and Discussion

Structured sphere segment void (SSV) silver surfaces were prepared by electrodeposition through close-packed monolayers of 900 nm polystyrene spheres assembled onto evaporated gold substrates. The thickness of the electrodeposited film was controlled by varying the charge passed during electrodeposition. After deposition, the polystyrene template was removed to leave the thin structured films containing a regular hexagonal array of uniform segment sphere voids. These SSV surfaces are strongly colored, the precise color depending on the viewing angle, void diameter, and film thickness.<sup>19</sup> Figure 1 shows typical SEM images of films of different thicknesses. The micrographs show that the spherical segment voids left after the removal of the polystyrene spheres are smooth and uniform. The pore mouth diameter and the topography of the spherical cavities changes as expected with the film thickness.

The flavin moiety was attached to the SSV silver surface by first modifying it with cysteamine and then reacting with **II**; see Scheme 1. Cysteamine linking layers have been widely used to bind proteins to metal surfaces without denaturation.<sup>30,31</sup> Figure 2 shows the SERS spectrum of cysteamine adsorbed on a structured silver surface (thickness 540 nm, 900 nm template sphere diameter). The band positions in the spectrum are in good agreement with those in the literature for cysteamine on roughened silver substrates.<sup>32–34</sup> Two strong Raman bands attributed to the C–S stretching vibration of adsorbed cysteamine molecules are observed. The band at 638  $\text{cm}^{-1}$  is characteristic for a gauche conformer of S–C–C chain, whereas the band at 729  $\text{cm}^{-1}$  is typical of a trans conformer.<sup>33,35,36</sup> The strong bands at 930 and 1010  $\text{cm}^{-1}$  are attributed to the C–C stretching vibration probably being coupled to the C–N stretching vibration.<sup>37</sup> The relative ratio of gauche to trans conformations on the silver surface is known to vary with the incubation time, electrolyte composition, and solution pH.<sup>32,33</sup> Our results, with a significant proportion of gauche isomers present after 2 h incubation time, are consistent with the literature.

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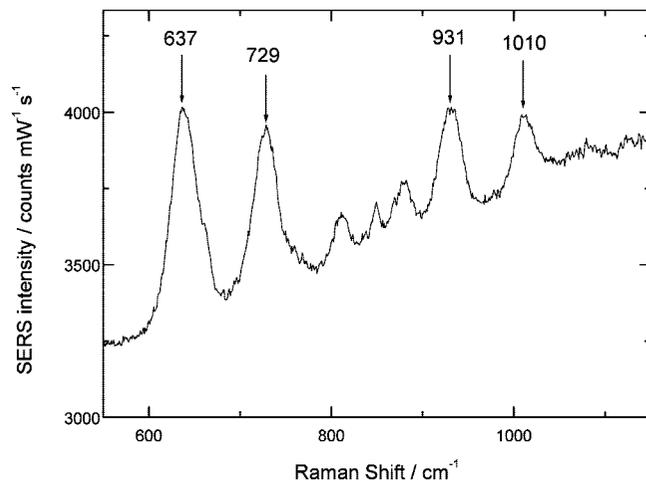
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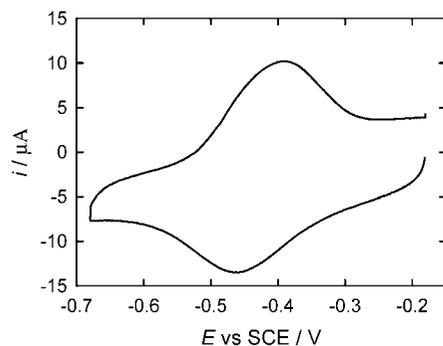
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**Figure 2.** SERS spectrum of cysteamine adsorbed on an SSV silver film produced using template spheres of 900 nm diameter and 540 nm thickness. The spectrum was taken using a 633 nm HeNe laser (3 mW power, single 10 s accumulation).



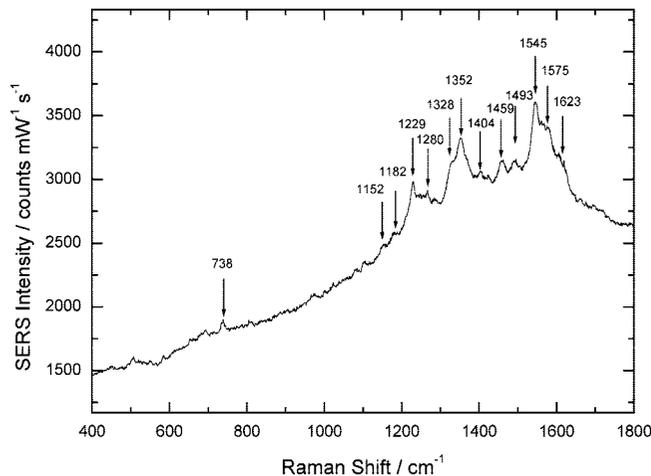
**Figure 3.** Cyclic voltammogram for flavin immobilized on the structured silver electrode used to record the SER spectrum shown in Figure 2 (Tris buffer, pH 7, scan rate of 50 mV/s).

The method of attaching flavin to the electrode surface used here has previously been used by one of the authors to modify a gold electrode surface.<sup>26</sup> In that work, the resulting flavin modified Au surfaces were investigated in detail using FTIR, AES, and STM. Those studies showed that the cysteamine molecules were adsorbed at on top sites of Au(111) with a separation of 0.5 nm and that after coupling the flavin groups were oriented perpendicular to the surface with a 2 nm separation between molecules. As a result of the contrast observed in the STM profiles between areas only covered by unreacted cysteamine and those covered by flavin, it was possible to see that the flavin molecules were 0.7 nm above the chemisorbed cysteamine layer. Thus we assume that a similar situation will apply on our sculpted silver surfaces with a fraction of the cysteamines being modified with flavin and with the flavin oriented perpendicular to the surface.

Figure 3 shows a cyclic voltammogram of the flavin-modified silver electrode recorded in Tris buffer at pH 7. In the voltammogram, we can clearly see oxidation and reduction of the isoalloxazine groups attached to the electrode surface. The voltammetry is characteristic for a surface-bound redox group and corresponds to a  $2\text{H}^+ + 2\text{e}^-$  redox process



The midpeak potential at pH 7 is  $-0.42$  V, and at  $50 \text{ mV s}^{-1}$  the peak separation is 0.06 V. The position of the peaks and the peak separation agree with those reported by Calvo et al. for the same system on gold once one corrects for the difference in



**Figure 4.** SER spectrum of flavin immobilized on an SSV silver film produced using template spheres of 900 nm diameter and thickness 540 nm. The spectrum was taken using a 633 nm HeNe laser (3 mW power, single 10 s accumulation).

reference electrodes used.<sup>26</sup> Integration of the area under the redox peak yields an average charge from which the surface coverage of the bound flavin can be calculated assuming it is a two-electron reaction. The surface coverage was found to be  $5.5 \times 10^{-10} \text{ mol cm}^{-2}$  based on the real surface area of the electrode. This is comparable to previous work where values between  $5.0 \times 10^{-10}$  and  $1.5 \times 10^{-10} \text{ mol cm}^{-2}$  have been reported,<sup>38,39</sup> but somewhat higher than that in Calvo's original work.<sup>26</sup>

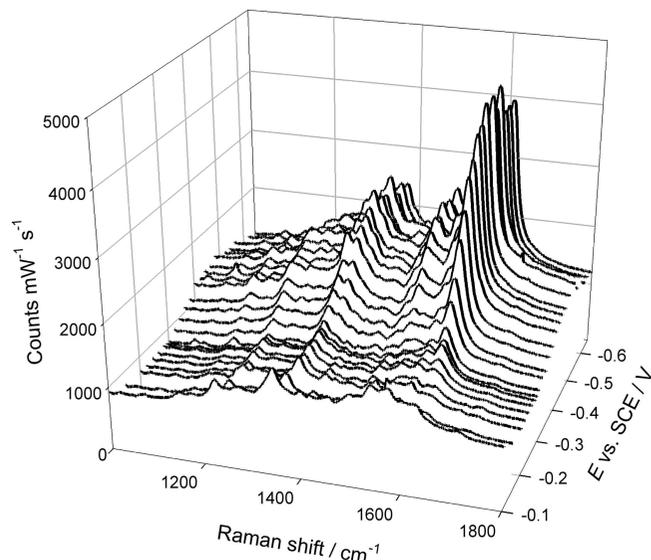
The SERS spectrum for the flavin-modified silver surfaces is shown in Figure 4. The spectrum was recorded using a 633 nm HeNe laser with a spot size of  $5 \mu\text{m}$ . Thus, on the basis of the electrochemically measured surface coverage and the laser spot size, the spectrum comes from  $\sim 6.5 \times 10^7$  molecules ( $\sim 0.1$  fmol) on the surface. There is good agreement between all 12 bands in the spectrum between 1152 and 1623  $\text{cm}^{-1}$  and the published UV resonance Raman spectra<sup>10,40</sup> and SERS spectra of flavin.<sup>14</sup> It is interesting to note that we find strong surface enhancement in the Raman spectrum even though, as we know from the earlier STM studies,<sup>26</sup> the flavin ring is not in direct contact with the silver surface but is held away from the surface by the linker. This indicates that direct contact between the flavin ring and the metal surface is not necessary for surface enhancement, in contrast to the conclusions of some of the earlier work on the SER(R)S of flavin.<sup>9,11–13</sup> The observed enhancement of the in-plane modes is consistent with the perpendicular orientation of the isoalloxazine ring found in the earlier STM and FTIR study.<sup>26</sup> The weak band at  $738 \text{ cm}^{-1}$  in the spectrum is consistent with the trans conformation of the S–C–C in the cysteamine linker.

Figure 5 shows the in situ electrochemical SERS spectra for attached flavin recorded at several different potentials starting from  $-0.1$  V, where the flavin is in the oxidized state, and moving to  $-0.65$  V, where the flavin is reduced. It is clear from the figure that the progress of the electrochemical reduction is associated with a change in the spectra. Comparing the spectra at  $-0.1$  and  $-0.65$  V (Figure 6), we can see that on reduction strong new peaks appear at 1608, 1520, and 1372  $\text{cm}^{-1}$ . On reversing the scan and returning the potential to  $-0.1$  V, we recover the original

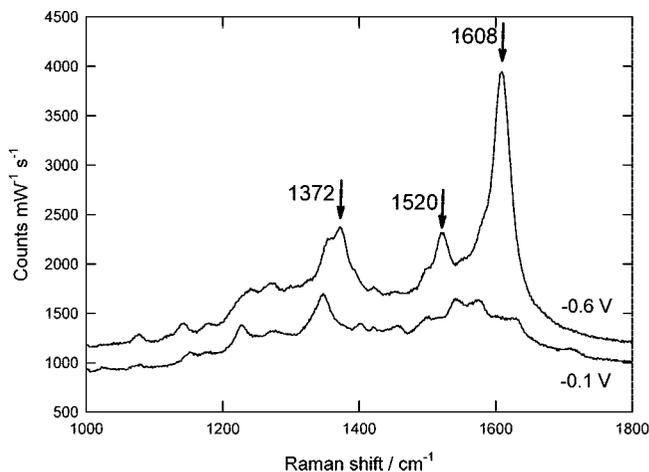
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**Figure 5.** In situ electrochemical SER spectra for flavin immobilized on an SSV silver film produced using template spheres of 900 nm diameter and 540 nm thickness with a He–Ne laser (633 nm, single 10 s accumulation, 3 mW laser power, recorded in Tris buffer, pH 7). The sample was purged with N<sub>2</sub> for 20 min at the beginning of the experiment and then for 1 min between each measurement.



**Figure 6.** SER spectra of immobilized flavin recorded at  $-0.1$  and  $-0.6$  V vs SCE.

spectrum of the oxidized flavin, showing that the process is chemically reversible and that there is not significant laser damage of the attached flavin during the experiment. In order to analyze the potential dependence of the spectra, we plot the peak height at  $1608\text{ cm}^{-1}$  corrected for background as a function of potential (Figure 7). The plot has a sigmoidal shape, reaching a plateau at negative potentials. Modeling the data using the appropriate expression derived from the Nernst equation in the presence of intermolecular interactions between the molecules,<sup>41–43</sup>

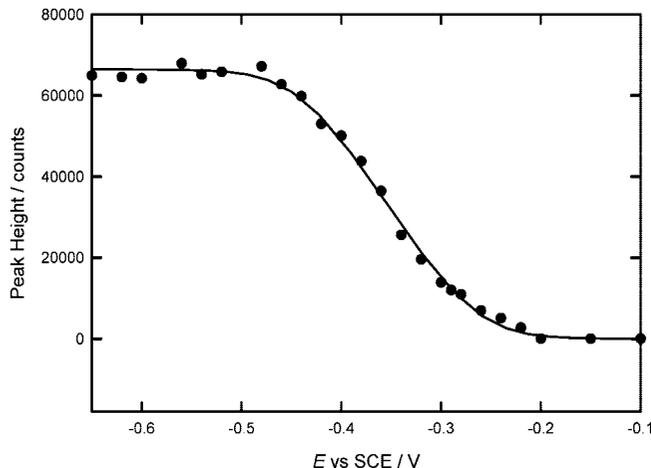
$$E = E' + \frac{RT}{nF} \left[ \ln \left( \frac{x}{1-x} \right) + G(0.5-x) \right] \quad (2)$$

where  $x$  is the fraction of molecules in the oxidized form, gives  $E' = -0.354\text{ V}$  and  $G = -3.4$ . The  $G$  value in eq 2 describes the activity effects caused by interactions between the molecules

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**Figure 7.** Plot of the background-corrected SER intensity for the band at  $1608\text{ cm}^{-1}$  as a function of the electrode potential. The solid curve is calculated from eq 2) with  $G = -3.4$  and  $E' = -0.354\text{ V}$  vs SCE.

in the layer. The negative value for  $G$  indicates that the interactions between the molecules are repulsive, causing broadening of the wave; this is consistent with the broadness of the voltammetric peaks for the immobilized flavin seen in Figure 3. It is noticeable that the value of  $E'$  obtained by fitting the spectral data is significantly less negative than the midpeak potential for the voltammetry of  $-0.42\text{ V}$  from Figure 3. Comparison of the spectra recorded at  $-0.65\text{ V}$  with the Raman spectrum of fully reduced flavin<sup>44</sup> and the SERRS of the semiquinone form of the flavin<sup>14</sup> from the literature shows that the three strong bands at  $1608$ ,  $1520$ , and  $1372\text{ cm}^{-1}$  seen in our spectra at  $-0.65\text{ V}$  correspond to bands in the flavin semiquinone spectrum. This assignment is also consistent with the value of  $E'$  obtained by fitting the intensity potential data. The neutral semiquinone form is blue in color and absorbs in the visible at  $633\text{ nm}$ .<sup>14</sup> We can therefore expect the Raman spectrum for this species to show both surface enhancement and resonance enhancement and, as we have shown in earlier work on our structured substrates, these two enhancements are multiplicative.<sup>25</sup> Consequently, we expect to see a very strong SERRS signal from the flavin semiquinone form. This is consistent with the data in Figure 5, where we see the band at  $1608\text{ cm}^{-1}$  assigned to the semiquinone form appearing around  $70\text{ mV}$  anodic of the midpeak potential of the bound flavin. In principle, the semiquinone form should disappear at higher cathodic potentials, as it is converted to the fully reduced flavin. We do not see this in our present experiments and we speculate that this is because of the presence of residual dissolved oxygen in the cell, which will lead to some oxidation of the reduced flavin and hence a steady state concentration of the semiquinone form. Further evidence for this can be seen in the voltammetry in Figure 3, where there is a clear offset in the baseline current at more cathodic potentials consistent with a slow catalytic oxidation of oxygen.

## Conclusions

In this study we have shown that the templated electrodeposition technique can be used to produce structured Ag films that give SERS active substrates with significant signal enhancements. The structured silver surfaces are smooth and the surface enhancement is stable with time and on potential cycling, making these structures ideal for quantitative electrochemical SERS

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studies. On the basis of the voltammetry, we have shown that the technique is highly sensitive and that we can obtain good quality spectra with subfemtomole sensitivity.

We have reported the first SERS studies for covalently attached flavin and shown, contrary to statements in the literature, that direct contact between the flavin ring and the metal is not necessary for SERS. For the attached flavin, we find that we can reproducibly cycle the system between the oxidized and reduced forms. In the

oxidized state at  $-0.1$  V vs SCE, the spectra are in excellent agreement with the reported Raman spectra for flavin. On reduction, we find clear evidence for the formation of the semiquinone form of the flavin, and we have shown that the changes in the spectra with potential can be analyzed quantitatively to obtain estimates of the redox potential and interaction parameter.

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