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In Situ Surface-Enhanced Raman Spectroelectrochemical Analysis System with a Hemin Modified Nanostructured Gold Surface

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S Supporting Information

ABSTRACT: An integrated surface-enhanced Raman scattering (SERS) spectroelectrochemical (SEC) analysis system is presented that combines a small volume microfluidic sample chamber (<100 μ L) with a compact three-electrode configuration for in situ surface-enhanced Raman spectroelectrochemistry. The SEC system includes a nanostructured Au surface that serves dual roles as the electrochemical working electrode (WE) and SERS substrate, a microfabricated Pt counter electrode (CE), and an external Ag/AgCl reference electrode (RE). The nanostructured Au WE enables highly sensitive in situ SERS spectroscopy through large and reproducible SERS enhancements, which eliminates the need for resonant wavelength matching of the laser excitation source with the electronic absorption of the target molecule. The new SEC analysis system has the merits of wide applicability to target molecules, small sample volume, and a low detection limit. We demonstrate in situ SERS spectroelectrochemistry measurements of the metalloporphyrin hemin showing shifts of the iron oxidation marker band ν_4 with the nanostructured Au working electrode under precise potential control.

Spectroelectrochemistry combines conventional electrochemistry with optical spectroscopy, which provides a more complete description of chemically driven electron transfer processes and redox events. In situ Raman spectroelectrochemistry is particularly important for understanding electrode processes at the interface of a working electrode (WE) and sample solution.¹ With the WE under potential control, electrochemistry can provide thermodynamic and kinetic information on chemical processes, while in situ Raman spectroscopy provides molecular vibrational information indicative of structure and conformation. The discovery of surface-enhanced Raman scattering (SERS) on nanostructured metal surfaces, which is due to the large enhancement of the inelastic scattering cross-section of a molecule at the metal surface, has enabled the determination of structural information on adsorbed molecules at very low concentrations using Raman spectroscopy.^{2–4} The combination of surface-enhanced Raman spectroscopy and electrochemistry has been shown to be a powerful tool to monitor in situ structural changes of surface adsorbates or reaction intermediates.^{5–9} The application of spectroelectrochemistry for the investigation of redox processes is of great interest, especially when the processes can be triggered electrochemically through precise control of the WE potential.

Resonance Raman spectroscopy and in situ surface-enhanced Raman spectroelectrochemistry have been applied extensively

to redox-active proteins, heme proteins, and metalloporphyrins.^{10–19} However, in most cases, surface-enhanced resonance Raman scattering (SERRS), excited with high laser powers, is required in order to resolve small quantities of adsorbed species on the WE.^{10–17} SERRS requires that the laser source wavelength is resonant with the surface plasmon resonance wavelength of the nanostructured metal surface as well as the electronic absorption band of the molecule of interest; thus, SERRS spectroelectrochemistry is most often performed on Ag nanostructures, which are prone to oxidation in aqueous solutions. The SERRS requirement limits in situ Raman spectroelectrochemistry to molecules with absorption wavelengths in the visible spectrum, and the high laser powers can affect thermally and photochemically sensitive redox systems in an uncontrolled manner.

In this Technical Note, we present an in situ SERS spectroelectrochemical (SEC) analysis system that is composed of a microfluidic sample chamber integrated with a compact three-electrode configuration. The WE and counter electrode (CE) are microfabricated on a silicon substrate that is directly bonded to the microfluidic chamber. The WE is a SERS-active

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nanostructured Au surface that has been recently reported by our research group.²⁰ The nanostructured Au WE surfaces provide reproducible SERS enhancements on the order of 10^8 that can be precisely tuned to the incident laser wavelength. Therefore, resonance Raman excitation is no longer required to achieve a reasonable signal-to-noise ratio, which makes possible the use of a Au WE. Additionally, the small sample volume microfluidic chamber is especially suitable for the analysis of hazardous or costly samples.

EXPERIMENTAL SECTION

Reagents. 4-Mercaptopyridine (MPy), dimethyl sulfoxide (DMSO), NaH_2PO_4 , Na_2HPO_4 , 30% H_2O_2 , and concentrated H_2SO_4 are analytical grade and purchased from Sigma-Aldrich (Munich, Germany). Hemin (H651-9, Frontier Scientific, USA) was used without further purification. Water was purified by a Maxima Ultrapure water system (ELGA, High Wycombe, Bucks, UK).

SERS WE Modification. Electrochemical measurements were performed on a conventional Au disk electrode, while the nanostructured Au electrode was used for the in situ SEC measurements. The conventional Au disk electrode (1.6 mm diameter) was polished (Micromesh microcloth, grade 3200) with alumina slurry (0.05 μm diameter particles) and followed by sonication in water, ethanol, and water, each for 1 min. The Au electrode was then immersed in a 3:1 piranha solution (H_2SO_4 /30% H_2O_2) and washed thoroughly with deionized water and dried with N_2 . (CAUTION: "Piranha" solution reacts violently with organic materials; it must be handled with extreme care.) Following the cleaning step, the Au electrode was immersed in a 1 mM MPy in ethanol solution for 4 h, followed by 1 min sonication in ethanol, to form the self-assembled monolayer (SAM) on the Au surface. After drying the MPy modified Au electrode with N_2 , it is immersed in a hemin solution (1 mM in DMSO) for 18 h. Before use, the hemin modified electrode (MPy/hemin) was sonicated for 1 min in DMSO and water, respectively. The nanostructured Au WE was prepared and modified with the MPy/hemin layer using a similar protocol.

Electrochemical Instruments and Measurements. Electrochemical measurements were performed with a potentiostat (VSP 200, Bio-Logic SAS, France). A batch cell system with conventional three-electrode configuration consisting of the conventional Au WE (1.6 mm diameter, MF-2014, Bioanalytical Systems, Inc.) and Pt wire (MW-4130, Bioanalytical Systems, Inc.) CE was used for control experiments. All potentials were measured against an Ag/AgCl reference electrode (RE) (MF-2079, BaSi). A 25 mM sodium phosphate buffer (pH 7.0) was used as the supporting electrolyte. The SEC-chip was clamped and bonded to a custom-made printed circuit board and spring-loaded Cu clips provided the electrical connection from the microfabricated Pt electrodes to the Cu trace on the circuit board. Conventional coaxial cables and connectors were used to connect the SEC-cell to the potentiostat.

Raman Scattering Measurement Instrumentation. A confocal Raman microscope system (alpha300R, Witech GmbH) was used for the Raman scattering measurements, which is composed of a TE-cooled charge coupled device (DU970P-BV, Andor Technology, Belfast, Northern Ireland), UHTS300 spectrometer (f/4 300 mm FL; grating: 600 lines mm^{-1}), fiber-coupled confocal configuration with 50 μm core diameter, and fiber coupled laser excitation through a $\lambda/2$

rotator plate for polarization control and laser focusing with the microscope objective. The spectral resolution of the spectrometer is $\pm 3 \text{ cm}^{-1}$. Elastically scattered light is removed with an edge filter. A He–Ne (1.96 eV/632.8 nm) laser source in a backscatter configuration was focused on the surfaces using a 40 $\times$ /0.8 NA dipping microscope objective (Nikon, Fluor).

SERS Measurements. The surface plasmon resonance energy was tuned to the laser excitation energy (to maximize the SERS enhancement) by controlling the pitch and nanogap width (Figure S1, Supporting Information) through controlling the nanostructured Au layer thickness, which was verified with reflectance measurements. The SERS spectra of the MPy and MPy/hemin modified nanostructured Au surfaces were measured in aqueous sodium phosphate buffer with a laser power of 0.2 mW (the laser power was measured at the entrance of the microscope objective). The integration time for the MPy modified electrode measurements was 3 s, and the integration time for the MPy/hemin modified electrode measurements was 10 s. Spatial imaging was performed over a $5 \times 5 \mu\text{m}^2$ area.

SEC Chip Fabrication. For detailed information about the fabrication of the SEC chip and the nanostructured gold working electrode, refer to Figure S1 (Supporting Information).

RESULTS AND DISCUSSION

The in situ SERS SEC system consists of a SEC-cell optically interfaced to the Raman microscope and electrically interfaced to a potentiostat, as shown in Figure 1a. The SEC-cell is

Figure 1. In situ SERS SEC analysis system. (a) Schematic representation of the Raman SEC analysis system, (b) SEC-cell composed of a SEC-chip directly bonded to a small-volume microfluidic sample chamber with an optical interface to a microscope objective, (c) SEC-cell consisting of a Pt CE and a nanostructured Au WE patterned on the silicon SEC-chip and an external Ag/AgCl RE, and (d) representative SEM image of the nanostructured Au WE.

composed of an on-chip nanostructured Au WE and microfabricated Pt CE, directly bonded to the microfluidic sample chamber that is optically interfaced to the Raman spectrometer via a dipping microscope objective (Figure 1b). An external Ag/AgCl wire RE is inserted directly into the microfluidic chamber (Figure 1c). A representative scanning electron microscopy (SEM) image of a nanostructured Au WE surface is shown in Figure 1d. The peak surface plasmon resonance wavelength of the nanostructured Au WE is tuned to coincide with the He–Ne laser excitation wavelength of 632.8 nm to ensure an optimal SERS enhancement.¹⁹ Additionally, a thin Ti

adhesion layer is deposited prior to the Au deposition to prevent delamination during WE modification (see the Supporting Information). The nanostructured Au WE is first modified with the 4-mercaptopyridine (MPy) SAM. Hemin is subsequently added to the MPy surface, which was previously reported to form a coordinative bond between the iron core of the heme and the nitrogen of the MPy SAM.^{14,21,22}

The electrochemical characteristics of the hemin modified electrode were first investigated on a conventional Au disk electrode and subsequently reproduced on the nanostructured Au working electrode. Cyclic voltammetry (CV) was performed with 5 mM $\text{Fe}(\text{CN})_4^{3-/4-}$ in the supporting electrolyte. The CV from an unmodified Au disk WE shows a pair of redox peaks corresponding to the $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox transitions (blue trace, Figure 2a). After incubation with MPy and hemin, the current decreased significantly (black and red traces, Figure 2a), thus demonstrating that hemin is immobilized on the MPy

modified WE. In Figure 2b, the CV of a MPy/hemin modified Au disk WE in argon-saturated sodium phosphate buffer with pH 7.0 (red trace) shows a well-defined, quasi-reversible redox couple (vs Ag/AgCl), which is characteristic of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox couple of hemin.²³ The CV characteristics from the MPy/hemin modified nanostructured Au WE is shown in Figure 2c, where the anodic and cathodic peak currents increase linearly for scan rates in the range of 3–10 V s^{-1} , thus indicating that the redox reaction of the immobilized hemin is a surface-controlled process,²⁴ which further supports immobilization of hemin on the nanostructured Au WE. The surface coverage of hemin is estimated to be $\Gamma \approx 9 \times 10^{-12} \text{ mol cm}^{-2}$ (see the Supporting Information).

The SERS spectra measured from the MPy/hemin modified nanostructured Au WE using the new in situ SERS SEC analysis system are shown in Figure 3. Figure 3a shows a schematic of the MPy/hemin modified Au WE. The SERS vibration bands of the MPy modified nanostructured Au WE are shown in the spectrum of Figure 3b, which are observed at $6a(a_1)$, $\nu(\text{C-S}) + \beta(\text{C-C})$ (700 cm^{-1}); $10b(b_1)$, $\gamma(\text{C-H})$ (778 cm^{-1}); $1(a_1)$, $\beta(\text{C-C-C})$ (1000 cm^{-1}); $18a(a_1)$, $\beta(\text{C-H})$ (1036 cm^{-1}); $12(a_1)$, $\beta(\text{C-C-C}) + \nu(\text{C-S})$ (1094 cm^{-1}); $9a(a_1)$, $\beta(\text{C-H})$ (1210 cm^{-1}); $3(b_2)$, $\beta(\text{C-H})$ (1274 cm^{-1}); $19a(a_1)$, $\nu(\text{C=C/C=N})$ (1492 cm^{-1}); $8b(b_2)$, $\nu(\text{C-C})$ (1577 cm^{-1}); and $8a(a_1)$, $\nu(\text{C-C})$ (1608 cm^{-1}), and are consistent with previous assignments.²⁵ The labels γ , β , and ν indicate out-of-plane bending, in-plane bending, and stretching modes, respectively. The spatially averaged enhancement factor of the Au nanostructured surface is in the range of 10^7 – 10^8 .^{20,26} The signal-to-noise ratio (SNR) is defined as $\text{SNR} = \bar{S}/\sigma$, where $\bar{S}$ is the average band intensity and σ is the amplitude of the noise signal in the region of the band.²⁷ For the $1(a_1)$ vibration mode, $\text{SNR}_{1(a_1)} \approx 12$, and for the $12(a_1)$ vibration mode, $\text{SNR}_{12(a_1)} \approx 22$.

The SERS spectra measured from the MPy/hemin modified nanostructured Au WE are shown in Figures 3c,d and 4. Since the primary scope of this Technical Note is the development of the SEC analysis system, we provide a preliminary analysis of the measured SERS spectra of the MPy/hemin modified WE. Figure 3c,d depicts SERS spectra for WE potentials $E = -0.2 \text{ V}$ and $E = -0.5 \text{ V}$ (vs Ag/AgCl), respectively. Many of the MPy vibration bands are observed in the SERS spectra, such as 700 cm^{-1} ($6a(a_1)$), 1000 cm^{-1} ($1(a_1)$), and 1094 cm^{-1} ($12(a_1)$). Certain vibrational modes of the porphyrin ring of hemin have been observed to shift in frequency consistent with the oxidation state, axial ligation, or coordination, and spin state of the central iron atom.^{11–15,28,29} The $\nu(\text{C-C})$ and $\nu(\text{C-N})$ stretch vibrations of the porphyrin ring are typically observed to shift in frequency according to the iron oxidation state, which occur in the marker band range of 1300 – 1700 cm^{-1} . The ν_4 (A_{1g}) vibration mode is due mainly to C–N stretch vibrations of the pyrrole subunits that are sensitive to electron transfer in the π^* orbital of the porphyrin ring³⁰ and is an indicator of the iron oxidation state. The ν_4 mode is observed in the frequency range of 1368 – 1377 cm^{-1} for ferric (Fe^{3+}) hemin and in the range of 1344 – 1364 cm^{-1} for ferrous (Fe^{2+}) hemin.^{18,31} Figure 4 shows the corresponding SERS spectra in the marker band range. For an applied potential of $E = -0.2 \text{ V}$ (Figure 4a), two bands can be resolved at 1345 and 1366 cm^{-1} . The $\nu_{4-1} = 1345 \text{ cm}^{-1}$ band lies in the ferrous state range with 81% of the integrated counts, and the $\nu_{4-2} = 1366 \text{ cm}^{-1}$ band lies in the ferric state range, within the experimental error of the

Figure 2. Cyclic voltammograms from conventional and nanostructured Au WEs. (a) Unmodified (blue), MPy modified (black), and MPy/hemin modified (red) conventional Au WE measured in 5 mM $\text{Fe}(\text{CN})_4^{3-/4-}$ (scan rate 100 mV/s), (b) MPy modified (black) and MPy/hemin modified (red) conventional Au WE in 25 mM sodium phosphate buffer, pH 7.0 (argon saturated, scan rate 1 V/s), (c) MPy/hemin modified nanostructured Au WE in 25 mM sodium phosphate buffer (pH 7.0, argon saturated), with scan rates in the range of 3 – 10 V s^{-1} (inner to outer traces). Inset: plots of redox peak currents versus scan rate; blue trace: oxidation peaks; red trace: reduction peaks.

Figure 3. SERS spectra of a nanostructured Au WE under potential control in 25 mM sodium phosphate buffer. (a) Schematic of the MPy/hemin modified Au surface, (b) SERS spectrum of MPy modified Au surface, (c) SERS spectrum of MPy/hemin modified Au surface with $E = -0.2$ V, (d) SERS spectrum of MPy/hemin modified Au surface with $E = -0.5$ V. Blue traces: raw data; red traces: modeled data; green traces: modeled background (the vibration bands are modeled with a Lorentzian band shape after subtraction of the background using a cubic polynomial function).

Figure 4. SERS spectra from MPy/hemin modified nanostructured Au WE in the marker band region (background corrected). (a) Electrode potential $E = -0.2$ V. (b) Electrode potential $E = -0.5$ V. Raw data: blue traces; modeled data: red traces.

measurement system, with 19% of the integrated counts, which indicates the partial oxidation of the iron cores on the surface.¹⁸ For an applied potential of $E = -0.5$ V (Figure 4b), a single vibration band is observed at $\nu_4 \approx 1349$ cm^{-1} , which lies in the reduced ferrous state range, thus indicating reduction of the iron cores on the surface (see Table S2 in the Supporting Information for a tabulated list of the observed Raman bands).

The vibration modes ν_2 (A_{1g}), ν_3 (A_{1g}), and ν_{10} (B_{1g}), which are mainly due to the $\nu(\text{C}-\text{C})$ stretch vibrations, are typical indicators of the coordination and spin state of the $\text{Fe}^{2+/3+}$ ions in hemin. The bands at 1570 cm^{-1} (Figure 4a, $E = -0.2$ V) and 1574 cm^{-1} (Figure 4b, $E = -0.5$ V) are most likely associated with the ν_2 mode of hemin, which corresponds well to previous assignments of five-coordinated ferric heme with high spin state (5cHS).³² The bands at 1619 cm^{-1} (Figure 4a, $E = -0.2$ V) and 1607 cm^{-1} (Figure 4b, $E = -0.5$ V) are likely associated with the ν_{10} (B_{1g}) mode of ferrous hemin, which also correspond well to previous assignments of 5cHS heme.³² It is important to

note that the ν_2 and ν_{10} modes overlap the MPy ring stretch $\nu(\text{C}-\text{C})$ modes $8b(b_2)$ at 1577 cm^{-1} and $8a(a_1)$ at 1608 cm^{-1} (Figure 3b), respectively. The broad vibration modes at 1445 cm^{-1} (Figure 4a, $E = -0.2$ V) and 1447 cm^{-1} (Figure 4a, $E = -0.2$ V) may be due to a combination of the in-plane ring stretching mode of MPy $19b(b_2)$ near 1450 cm^{-1} ²⁵ and the ν_3 (A_{1g}) mode near 1495 cm^{-1} of hemin but requires further investigation before definitive assignment.³² The bands at 1392 and 1387 cm^{-1} are not currently assigned but were previously observed in SERS spectra of hemin adsorbed on Ag surfaces in the presence of pyridine.³³

From the estimation of the hemin surface density from the electrochemical measurements, approximately 5×10^4 hemin molecules on average are in the collection volume of the SERS measurement. On the basis of the SNR of the measurement, we estimate the limit of detection in the range of 2000–5000 hemin molecules.

CONCLUSIONS

In conclusion, we present a new in situ SERS spectroelectrochemical analysis platform that is composed of a small volume sample chamber and a nanostructured Au working electrode, which allows for the simultaneous SERS and electrochemical investigation of any modified electrode surface with very low laser power. The nanostructured Au working electrode has a large and reproducible SERS enhancement, which enables highly sensitive surface-enhanced Raman spectroscopy without resonant excitation of the molecule of interest, thus simplifying the measurement apparatus and expanding the applicability to a wider range of target molecules.

ASSOCIATED CONTENT

Supporting Information

Figures S1–S3 and Tables S1 and S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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