Detecting oxidation and reduction of Cytochrome *c*

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ABSTRACT

The surface coverage of cytochrome c (Cyt c) on a gold surface modified with carboxylic acid terminated thiol monolayer is investigated in this paper with the ultimate goal of monitoring biochemical reactions of proteins with surface enhanced resonance Raman spectroscopy (SERRS) in microfluidic devices. The surface coverage of Cyt c on a gold surface as a model system for Au nanoparticle is investigated here through cyclic voltammetry (CV). This helps to establish the number of proteins per surface area under given solution conditions and hence the number SERRS-active species on the particle surface. With controlled surface coverage of a metal particle, the ratio between oxizied and reduced Cyt c will be monitored by observing a single metal nanoparticle under flow with SERRS. Observing the nanoparticles in flow with excitation in resonance with the electronic transition energy of the protein provides the possibility of constructing a model system for monitoring biomolecules in real-time.

Keywords: Cyt c, surface coverage, nanoparticle, SERRS, CV

1 INTRODUCTION

Surface enhanced Raman spectroscopy (SERS) is a powerful tool used for observing molecules and their structural properties, due to its ability to enhance the otherwise weak Raman scattering signals on a roughened metal surface or nanoparticles. The enhancement comes as a result of the increased electric field felt on the molecule as the result of excitation of localized surface plasmons in the metal surface. The signal can be further enhanced by matching the excitation frequency with the electronic transisition of the analyte, a condition known as surface enhanced resonance Raman spectroscopy (SERRS). Through these signal improvements, SER(R)S has been used in many studies for biomolecule monitoring [1-2]. Figure 1 shows an illustration of a metal nanoparticle used for protein monitoring with SER(R)S.

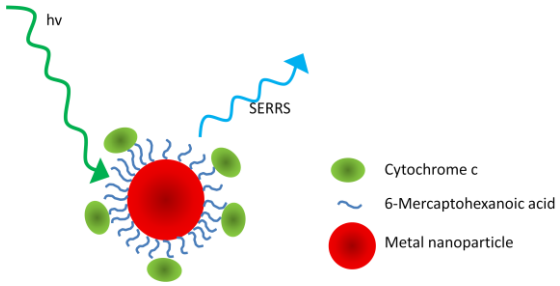

Figure 1 : Illustration depicting the use of SERRS to monitor Cyt c on metal nanoparticles. A Mercaptohexanoic acid self-assembled monolayer electrostaticically bins the cytochrome c.

Single metallic nanoparticle detection has recently been demonstrated in flow-based systems where the time limitations associated with diffusion-limited approaches has been removed [3-4] effectively improving nanoparticle flux through the probe volume. Correlation spectroscopy has been shown as a powerful tool to characterize single metallic nanoparticles. By measuring the time-resolved intensity fluctuations the average rotational and translational characteristics of the nanoparticles can be obtained and related to the nanoparticle physical properties [5-6]. Cecchini et al. has recently shown the ability to size nanoparticles in flow using a modified autocorrelation function [7].

Here we build upon previous work [7], and aim not only detect the presence nanoparticles, but also the state of the active layer, which is composed of reduced and oxidized cytochrome c (Cyt c). Cyt c was chosen since the protein is very stable and well-characterized both structurally and electrochemically [8-11]. Additionally, Cyt c has also been well studiedby Resonance Raman and SERRS in solution and on roughened metallic surfaces [12-13]. Using a protein, rather than a small-molecule SER(R)S marker, will also help to fathom the potential of single-particle SER(R)S in flow for biological systems and biomedical diagnostics.

The shift in absorbance maximum between fully oxided Cyt c (Fe^{3+}) (530 nm) and fully reduced Cyt c (Fe^{2+}) (520 nm) can be used as a detection point to detemine the state of the protein at given solution conditions. With the exception of the soret band at 410 nm for both states [14] the reduced absorbance of the reduced Cyt c is expected to produce a

less intense Raman singal The absorbance maximum of Cyt c coincides well with the surface plasmon resonance of 40 nm gold nanoparticles who have an extinction maximum at 535 nm [15].

Surface coverage of proteins have been examined in previous studies with CV [16] from integrating peaks withinthe voltammograms. This method has been adapted here to examine the surface coverage of Cyt c on the gold electrode. Using the conditions from these experiments, nanoparticles will be modified with Cyt c with controlled surface coverage. These particles will be examined with flow-based SERRS detection under various concentrations of reducing agents.

Herein, this paper introduces results relating to the surface coverage of Cyt c on gold electrodes. These results willl be used to control the surface coverage on metal electrodes. Detection of Cyt c on a single metal nanoparticle under flow with SERRS will be performed employing these results and the ratio between oxidized states and reduced states of the protein on single metal nanoparticle will be monitored in real-time.

2 EXPERIMENTAL SECTION

2.1 Chemicals and Materials

Equine heart cytochrome c with a molecular weight of 12384 basis was purchased from Sigma and purified with PD10 columns from GE Healthcare before use. 6-Mercaptohexanoic acid (MHA) was purchased from Aldrich and used as received. Ethanol (≥ 99.7 % ,VWR) was used as solvent for self assembled monolayer deposition on gold electrode. A 11 mM, pH 6.7 potassium phosphate buffer was prepared using potassium phosphate monobasic and potassium phosphate dibasic all purchased from Sigma-Aldrich. Potassium sulfate purchased from Fluka (6 mM) was added as supporting electrolyte to the buffer. Custom made electrochemical cells were used for cyclic voltammetry measurements. Gold electrodes, shaped to have a facet with a geometric area of 9.731 mm^2 and (111) single crystallinity was previously prepared by the group according to Clavillier's method [17] and was used as working electrode. Silver/silver-chloride electrode (SCHOTT®) was used as reference electrode and platinum wire as counter electrode. CH potentiostat was used for all CV measurements.

2.2 Method

MHA solutions (in ethanol) of 7.23 μM were prepared in glass vials for monolayer deposition on gold electrodes. It has been previously discovered that carboxylic acid terminated alkanethiol self-assembled monolayer (SAM) are excellent candidates for immobilizing Cyt c electrostatically as the protein maintains its native structure [18]. 6-Mercaptohexanoic acid (MHA) is used here as the SAM on gold electrodes and metal nanoparticles. Gold electrodes were placed in the oven at 850 °C overnight and cooled slowly. They were flame annealed (using with hydrogen gas) and immediatley immersed into deionized water and placed into the previously prepared MHA solution. The electrodes were left in the solution overnight to allow the MHA molecules to assemble with order on the surface.

The electrochemical cell was cleaned prior to use with 20 % nitric acid solution at 225 °C for 2 hours. The cell was then rinsed with deionized water 3 times in an ultrasonic bath for 3 to 5 minutes each. All glassware were cleaned using the same procedure.

CV measurements were conducted in the haning-meniscus configuration. Silver/silver-chloride reference electrode was rinsed thoroughly with deionized water, and placed into the electrochemical cell with 10 mL of phosphate buffer. The latinum wire counter electrode was flame annelaed, immersed into deionized water, and then directly placed into the cell. The buffer was degased thoroughly for 20 minutes with argon gas and a constant gas flow above the solution was maintained throughout the experiment.

The concentration of the Cyt c in solution was increased step by step by injecting Cyt c directly into the cell for each CV measurement for adsorption isotherm characterization. The concentration of Cyt c after each step was confirmed by obtaining UV-Vis spectra of the solution from the cell after obtaining cyclic voltammograms.

3 RESULTS

Cyclic voltammograms of Cyt c immobilized on MHA were recorded according to the concentration of Cyt c. Measurements were taken at a scan rate of 0.1 V/s and sampling interval of 0.001 V after 10 minute equilibration time. The voltammograms were reproducible for very low concentrations with distinct peaks. However, during the course of Cyt c addition the voltammograms became unstable and oxidation and reduction peaks broaden as the concentration reached 0.07 μM. Figure 2 shows the cyclic voltammograms of Cyt c immobilized on MHA-gold electrodes at concentrations of 0.01 μM to 0.05 μM. Figure 3 shows higher concentrations ofof 0.07 μM to 0.14 μM.

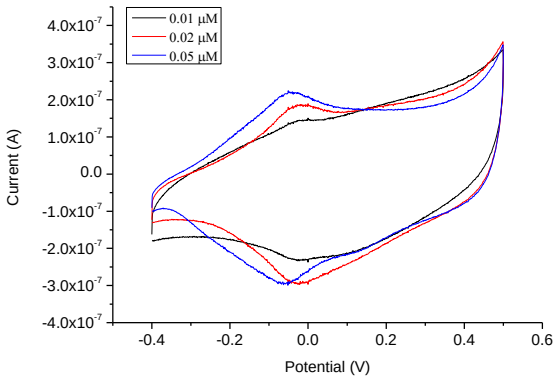

Figure 2 : Cyclic voltammograms of Cyt c immobilized on MHA-gold electrode at low concentrations (0.01 to 0.05 µM) of Cyt c after 10 minutes of equilibration

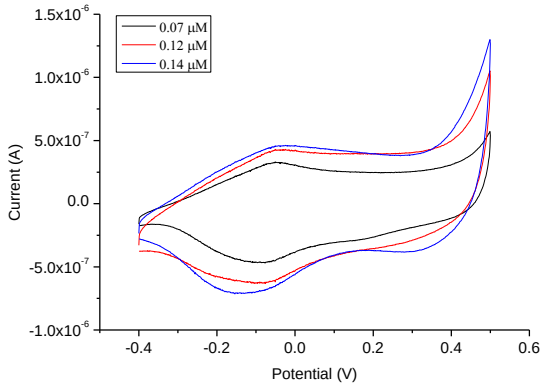

Figure 3 : Cyclic voltammograms of Cyt c immoblized on MHA-gold electrode at higher concentrations (0.07 to 1.7 µM) of Cyt c after 10 minutes of equilibration

The concentration was confirmed for each step with the UV-Vis spectrum. The absorbance of the soret band (410 nm) and the absorption coefficient of Cyt c (1.06×10^5 $M^{-1}cm^{-1}$) was used to calculate the concentration using Beer-Lambert's Law.

The number of Cyt c electrostatically bound to MHA-gold electrode was calculated by integrating the oxidation and reduction peak area. The peak area is proportional to the amount of charge injected into or extracted from the adsorbed Cyt c layer, and since Cyt c undergoes one electron redox reactions, the number of proteins can be directly calculated using Eq. 1.

$$Fe^{3+} + e^- \leftrightarrow Fe^{2+} \quad (1)$$

The coverage vs. concentration data can be fitted to a Langmuir isotherm shownn in Eq. 2 indicating that under the conditions used, the protein forms a monolayer and that protein/protein interactions on the surface are negligible (fig. 4).

$$\theta = \frac{\alpha C}{1 + \alpha C} \quad (2)$$

where θ is the surface coverage, C the concentration, and α a constant representing the ratio of adsorption to disorption rate constants [19].

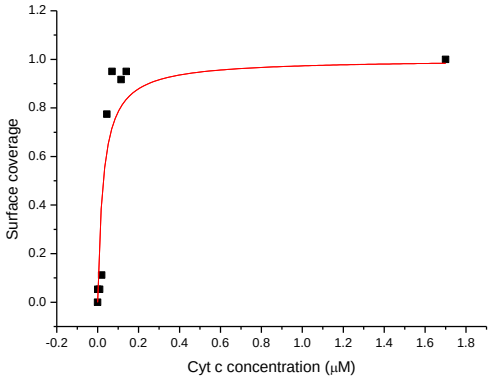

Figure 4 : MHA-gold electrode surface coverage according to Cyt c concentration. The surface coverage of the gold electrode is shown by the black data points. The red solid line represents the fitted langmuir isoterm with an α value of 36.3 with a standard error of 13.9

4 DISCUSSION

From the cyclic voltammograms the expected number of Cyt c that would be immoblized on the MHA-gold nanoparticle of a specific size can be calculated, provided that the adsorption characteristics of the plane Au surface and the (curved) Au particle are comparable. Figure 5 shows the number of expected Cyt c on a 40 nm sized gold nanoparticle.

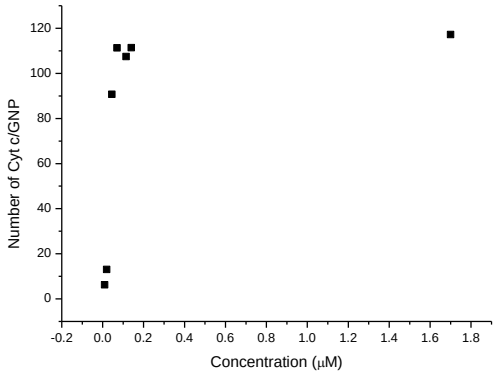

Figure 5 : Expected number of Cyt c on a 40 nm sized gold nanoparticle

The number of proteins expected to be bound to the gold nanoparticle surface will range from 6 at the lowest concentration (0.01 μM) and 117 at full coverage (1.7 μM).

In the course of obtaining all cyclic voltammmograms, a trend was repeatedly observed. As shown in figures 2 and 3, the characteristic peak of Cyt c broadened as the concentration increased. In addition to this peak broadening it can also be seen that the capacitive current also increased. The increase in capacitive current could be due to the increase in dielectric constant of the adsorbed layer. According to previous studies, the dielectric constant is increased as a protein disrupts the monolayer [20]. Therefore, it is thought that the adsorbing Cyt c perturbs the SAM and allows water and ions to penetrate into the layer resulting in the increase in capacitive current. The cyclic voltammogram at 1.7 μM was in fact taken after immersing the gold electrode in the Cyt c solution for 24 hours, allowing the proteins to arrange with order on the MHA monolayer.

5 CONCLUSION

The monitoring of biochemical reactions in different solution conditions is thought to be possible when performed with SER(R)S under flow. As a model system, the ratio of reduced and oxidized Cyt c immobilized on metal nanoparticles will be examined with SER(R)S using controlled protein surface coverage of the nanoparticle. Characterization of surface coverage has been examined here with CV and the expected quantity of the immobilized protein at various concentrations on a nanoparticle were calculated. This adsorption isotherm can be used in the future to controllably immobilize Cyt c on gold nanoparticles . The Raman signals obtained from could then be used to monitor the biochemical reaction of Cyt c in real-time, high throughput environment.

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