SERS Detection of Reduction-Oxidation Processes in DNA-Bound Methylthioninium Chloride on Gold Coated Substrates

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ABSTRACT

We developed a conjugate nano-biological system interfacing plasmonic gold nanostructures with thiolated single-stranded DNA carrying an important reduction-oxidation indicator, methylthioninium chloride, also known as methylene blue. Using surface-enhanced Raman spectroscopy, we have detected characteristic bands of DNA-bound immobilized methylene blue in sub-monolayer quantities. We also have detected reversible reduction-oxidation of methylene blue during laser excitation of the samples at neutral pH, in the absense of electrodes or chemical agents.

Keywords: Nanoplasmonics, Nanobioconjugates, SERS

1 INTRODUCTION

Nano-biological systems interfacing solid nano-structured surfaces with biological compounds such as oligonucleotides or proteins are highly regarded as enabling materials for biosensing and biocatalysis applications [1,2]. In particular nanostructures of noble metals such as gold or silver, when exposed to light, exhibit a phenomenon known as surface plasmon resonance. Surface plasmons are oscillations of electron density at a surface of a metal. When a proper metal nanostructure (plasmonic substrate) is exposed to light, very efficient absorption of incoming photons is possible, resulting in a buildup of localized high-energy regions, or "hot-spots" [3]. Energy of hot-spots may be used in a variety of ways, such as for example inducing of reduction-oxidation responses in biological materials [4,5]. Furthermore, plasmonic hot-spots are also known to enhance inelastic Raman scattering of light by materials located nearby [6,7]. The surface-enhanced Raman spectroscopy (SERS) allows capturing unique vibrational fingerprints of materials in sub-monolayer quantities, and as such it is highly regarded as a method of ultra-sensitive and selective biodetection [7,8]. Combining energy harvesting from light, photo-catalytic stimulation of materials, and ultrasensitive SERS characterization of molecular events in the same design promises numerous transformative applications [8-10].

In this work, we have developed a nano-biological system interfacing plasmonic gold nanostructures with thiolated single-stranded DNA carrying an important reduction-oxidation indicator, methylthioninium chloride $C_{16}H_{18}ClN_3S$, also known as methylene blue (MB). Using surface-enhanced Raman spectroscopy (SERS), we have demonstrated the detection of immobilized DNA-bound methylene blue. We also have observed reversible reduction-oxidation of DNA-bound MB in the course of a 523 nm laser excitation of the samples.

2 METHODS

Plasmonic nanostructures were created by magnetronic deposition of thin (15 nm) gold coating on glass sliides using an Emitech K550X sputter system with a deposition rate of 10 nm/min. Prior to the Au coating, a thorough cleaning of glass substrates was done. The glass substrates were first washed with acetone and DI water; then they were taken to sonication bath for acetone sonication for 5 mins. After the sonication, each sample was nitrogen-dried. Finally, the substrates were RF plasma-etched using a Jupiter III (Nordson March) instrument. Etching was performed for 1 min with a mixture of Ar and H_2 followed by 30 sec of H_2 etching.

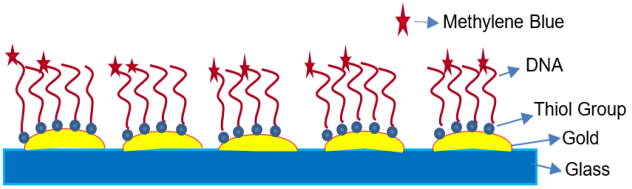

Fig 1. Scheme of DNA functionalized, gold-coated plasmonic substrate.

After cleaning and Au deposition, the substrates were biofunctionalized as schematically shown in Fig. 1. For this purpose, thiolated DNA strands 5'-GGTTTGGAGGGGC CA-3' were used. Lyophilized DNA was purchased from Sigma-Aldrich. An 1 μM DNA solution was prepared with Tris-EDTA buffer at a pH of 7. The DNA solution was kept at 5°C. To biofunctionalize the samples, a 100 µl drop of DNA solution was deposited onto the gold-coated substrates. Next, a 100 µl drop of the MB solution (4.7 mM, Sigma-Aldrich) was added onto the same spot, and the samples were incubated for 24 h at 5°C. The incubation was done in humid environment to avoid drying of the samples. Fig. 2 illustrates the functionalization process. It has been

expected that during incubation, DNA strands attach to the gold coating through the thiol groups [11-13], whereas MB binds to guanine bases of DNA [14]. For comparison, control samples were also prepared by incubating similar gold-coated glass substrates with the MB solution alone in the absence of DNA.

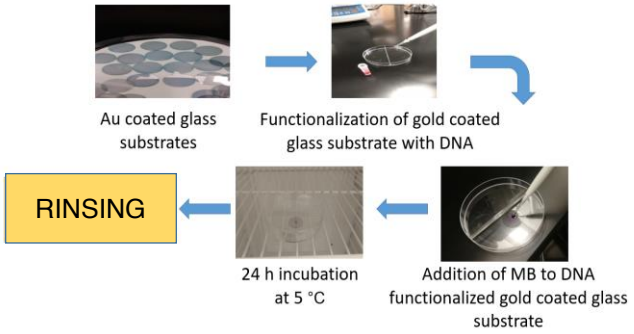

Fig 2. Steps of substrate bio-functionalization with DNA and methylene blue.

After the incubation, the samples were thoroughly rinsed in Tris-EDTA buffer with pH 7, and characterized using a Horiba LabRAM HR800 Evolution Raman micro-spectroscope. For benchmarking purposes, Raman spectra of the MB solution were also collected immediately after deposition on the substrates without addition of DNA, incubation and rinsing. A 523 nm excitation wavelength, a nominal power of 50 mW, and a 10X magnification were used for the acquisition of Raman and SERS spectra.

3 RESULTS

Fig. 3 shows a typical benchmark Raman spectrum of methylene blue solution. To prepare this sample, a 100 μL drop of the MB solution was deposited onto a gold-coated glass slide, and Raman characterization was performed immediately without incubation or rinsing. The spectrum exhibits pronounced bands that we have attributed to stretching of C=C bond of MB's aromatic ring at 1625 cm^{-1}; vibrations of C=N bonds and CH_3 groups between 1398 cm^{-1} and 1470 cm^{-1}; and C-N-C skeletal bending at 501 cm^{-1} and 446 cm^{-1} [15,16].

Next, the same amount of MB solution was incubated on gold-coated glass substrates for 24h at 5°C. Then, the samples were rinsed in the Tris-EDTA buffer and their Raman spectra collected. An example spectrum is shown in Fig. 4. It can be seen that in the absence of DNA functionalization, the spectrum does not exhibit Raman bands attributable to MB.

Fig. 5 shows a SERS spectrum of DNA-functionalized substartes incubated with the addition of MB. In striking contrast with DNA-free experiments, the DNA-functionalized samples exhibited pronounced Raman bands of methylene blue, including a strong peak at approximately1623 cm^{-1}.

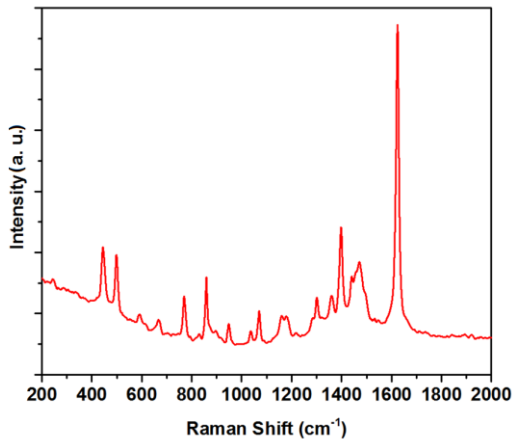

Fig 3. Raman spectrum of methylene blue solution on gold coated glass substrate without incubation.

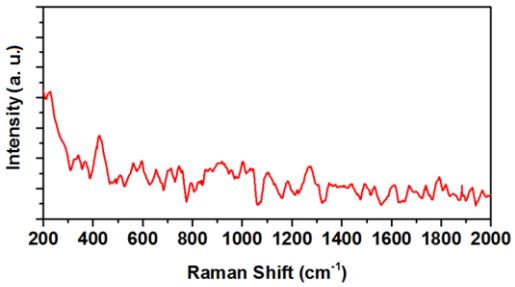

Fig 4. Raman spectrum of rinsed gold-coated glass substrate after a 24 h incubation with methylene blue in the absence of DNA functionalization.

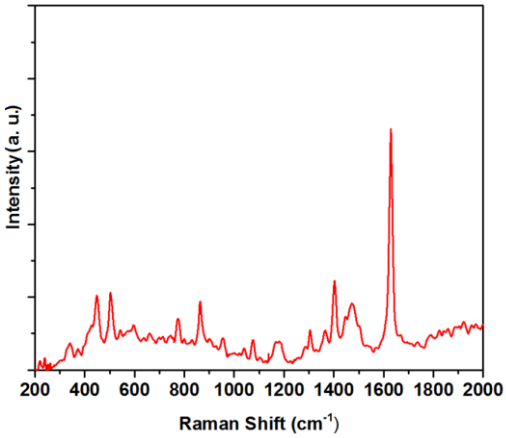

Fig 5. SERS spectrum of DNA-functionalized, MB-loaded gold coated glass substrate after a 24 h incubation and rinsing.

Next, we have investigated the evolution of SERS spectra of DNA-functionalized and MB-loaded samples after 20 min, 40 min, 60 min, 80 min, 100 min, 120 min, and 140 min of exposure to a 523 nm laser excitation. As it can be seen from Fig 6, the Raman bands have decayed with time. After 100 min of exposure to the laser, Raman

fingerprint of MB has completely disappeared. Then, we added 20 μL of Tris-EDTA buffer (pH 7) on the same substrate, and continued the laser exposure. Remarkably, this resulted in a recovery of MB's Raman fingerprint. After 160 min, pronounced Raman bands characteristic of MB have been detected again. Continuing the exposure beyond this point caused the Raman bands diappearing again, in a similar reversible fashion.

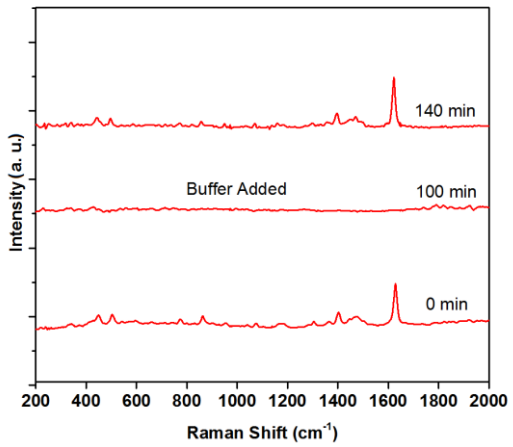

Fig 6. SERS spectra of DNA-functionalized, MB-loaded gold-coated glass substrate at the beginning of a 523 nm laser exposure (bottom); after 100 min of the exposure (middle); and after 140 min of the exposure following the addition of Tris-EDTA buffer (top). A vertical offset was applied to the spectra for clearer presentation.

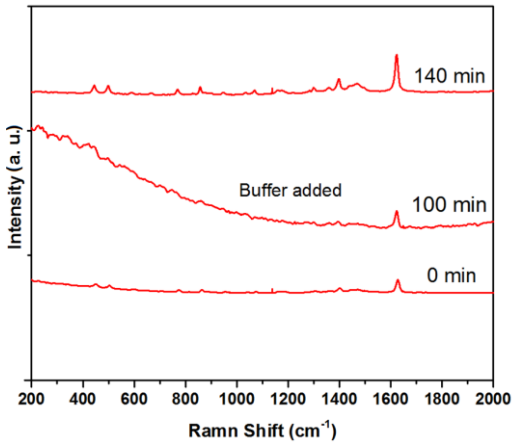

Fig 7. Raman spectra of a drop of MB solution on a bare uncoated glass slide at the beginning of laser exposure (bottom); after 100 min of the exposure (middle); and after 140 min of the exposure following the addition of Tris-EDTA buffer (top). A vertical offset was applied to the spectra for clearer presentation.

For the sake of comparison, we also have deposited a 0.5 μL drop of the MB solution onto a cleaned glass slide without gold coating and DNA functionalization. This control sample was immediately exposed to the 532 nm laser excitation, and the entire experiment was repeated as described above. As Fig. 7 illustrates, in this case a characteristic Raman band, centerd at approxomately 1629 cm^{-1}, has remained after 100 min of exposure. Subsequent addition of 20 μL of Tris-EDTA buffer did not change the Raman fingerprint.

4 DISCUSSIONS

The results presented in Figs. 4 and 5 indicate that in the absence of DNA functionalization, methylene blue does not attach to gold-coated substrates strongly enough to remain on the surface after rinsing. However in the presence of DNA, pronounced SERS signal of MB indicates its presence on the surface after rinsing.

We expect that during incubation, DNA binds to the surface of gold through the mediation of thiol groups [11-13], whereas MB binds to guanine bases of DNA [14], which plays a role of surface-immobilized linker. During Raman characterization of incubated and rinsed samples, DNA-bound MB molecules are expected to be located in close proximity of the gold coating. This is consistent with a red shift of approximately 23 nm observed in SERS spectra from these samples, in comparison with Raman spectra collected from a drop of MB solution on bare un-coated glass slides (Fig.8). Scattering spectra are known to be red-shifted in a proximity of gold nanostructures due to a change of local refractive index [6].

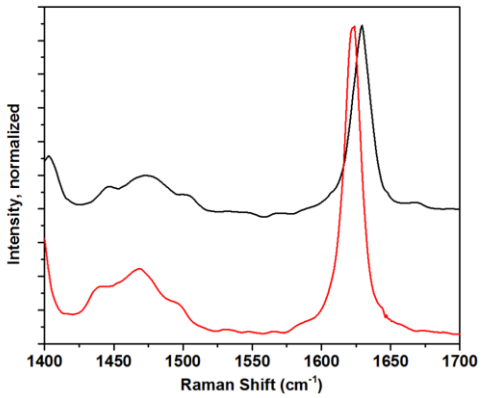

Fig 8. Fragments of SERS/Raman spectra from MB-loaded, DNA functionalized gold-coated glass substrate (red) and from MB solution on bare glass slide (black). An offset was applied to the spectra for clearer presentation.

Remarkably, after rinsing of the incubated samples, MB-loaded, surface-immobilized DNA is supposed to be present in a monolayer quantity or less. Therefore, pronounced Raman fingerprints such as shown in Fig. 5 correspond to a very small quantity of MB molecules, indicating significant SERS enhancement in our samples.

A well-known redox indicator, MB can adopt oxidized or reduced forms, which differ in charge and protonation state [17]. In water or buffer solution at neutral or acidic pH conditions MB would adopt an oxidized form, which produces a characteristic Raman spectrum such as shown in Figs. 3 and 7. As our results indicate, similar fingerprints

are also observed in SERS spectra from MB-loaded, substrate-immobilized DNA after relatively short exposures to laser excitation (Figs. 5 and 6). However more extensive exposures to the laser light in the presence of the buffer have resulted in disappearance of the Raman fingerprint (Fig.6). Degradation of Raman bands of MB has been attributed to its reduction, as it was demonstrated by electrochemical stimulation of DNA-bound MB [18]. The reduction of MB has been reversed by placing a drop of fresh Tris-EDTA buffer (pH 7) on the surface of the sample. Continued laser exposure followed by addition of the buffer has resulted in repeating reduction-oxidation cycles.

Importantly, the observed reversible reduction-oxidation transformations of MB occurred at neutral pH in the absence of electrodes or chemical agents other than the buffer. However, the presence of gold coating has been critical for these transformations. As Fig. 7 indicates, exposure to laser excitation did not induce a reduction of MB solution on a bare un-coated glass slide. We conclude that energy of plasmonic hot-spots generated by laser excitation in the gold coating is required for the reversible transformation of MB to occur.

5 CONCLUSIONS

We have successfully prepared plasmonic gold-coated substrates, and functionalized these substrates with thiolated single-stranded DNA carrying a reduction-oxidation indicator, methylene blue. MB is hypothetically attached to guanine bases of DNA. Surface-enhanced Raman spectroscopy enabled by plasmonic hots-pots from gold coating allowed us to detect pronounced bands of MB immobilized on the substrates through the mediation of DNA. Exposure to laser excitation induced a gradual disappearence of MB's Raman bands, indicating that MB has been reduced. Remarkably, this reduction has occurred in the absense of electrodes or chemical agents. Addition of fresh buffer at neutral pH has reverted the reduction. Such bio-conjugate architectures may be useful to conduct future research on reduction-oxidation processes involving DNA-bound compounds on nanostructured plasmonic substrates.

6 ACKNOWLEDGMENTS

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