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Development of an Electrochemical Surface-Enhanced Raman Spectroscopy (EC-SERS) Aptasensor for Direct Detection of DNA Hybridization

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ABSTRACT

Rapid detection of disease biomarkers at the patient point-of-care is essential to timely and effective treatment. The research described herein focuses on the development of an electrochemical surface-enhanced Raman spectroscopy (EC-SERS) DNA aptasensor capable of direct detection of tuberculosis (TB) DNA. Specifically, a plausible DNA biomarker present in TB patient urine was chosen as the model target for detection. Cost-effective screen printed electrodes (SPEs) modified with silver nanoparticles (AgNP) were used as the aptasensor platform, onto which the aptamer specific for the target DNA was immobilized. Direct detection of the target DNA was demonstrated through the appearance of SERS peaks characteristic for adenine, present only in the target strand. Modulation of the applied potential allowed for a sizeable increase in the observed SERS response and the use of thiol back-filling prevented non-specific adsorption of non-target DNA. To our knowledge, this work represents the first EC-SERS study of an aptasensor for the direct, label-free detection of DNA hybridization. Such a technology paves the way for rapid detection of disease biomarkers at the patient point-of-care.

INTRODUCTION

Considerable research attention is currently focused on finding ways of pre-determining diseases that have a negative impact on human health. In developing nations, thousands of largely preventable deaths occur every year due to diseases that are unable to be diagnosed or treated in a timely manner. One example is tuberculosis (TB), a common, lethal infectious disease caused by the bacterium *Mycobacterium tuberculosis (M.tb.)*.[1] TB is an airborne disease which can easily spread between people through coughing, sneezing, or even talking. Symptoms of an active TB infection may include night sweats, fever, weight loss, coughing up blood or sputum, and chest pain.[1] Currently it is estimated that one third of the worldwide population is infected with latent TB, and active TB results in approximately two million deaths annually.[3-5] In South Africa, the two main methods used for diagnosing TB in rural settings are smear microscopy and culture studies, which typically take between 4-16 weeks to provide a result to the patient, which is problematic since ~40% of patients never return to the clinic for diagnosis or treatment.[3] The Xpert® MTB / RIF nucleic acid amplification test is available only in some centralized laboratories in South Africa; as a result this test does not solve the loss-to-follow-up problem for patients living in more remote areas. Such statistics suggest that a rapid-sensing device should be developed to detect tuberculosis at or near the patient point-of-care (POC) as a way to improve global healthcare. Such a POC device will help to rapidly diagnose a disease or host of diseases wherever the patient is being treated. Some desirable characteristics that such a device should possess include a fast response time, an easily interpreted signal, portability, ruggedness, the ability to detect an analyte at low concentration, use of disposable chips or strips, and finally the device should be cost-effective.[6] In addition, there are many challenges one faces when developing such a device for use in third world settings, such as environmental conditions of extreme

temperature and humidity, limited access to clean water, operator illiteracy, and an unstable or non-existent source of electricity.[5]

Sensor technology has increased rapidly over the past several decades. In particular, sensor technology incorporating nanostructured materials has become increasingly common. Such technologies often take advantage of the unique optical and electronic properties of such materials.[7-9] In addition, new ways of selectively capturing target molecules onto the sensor surface are being developed to help solve issues related to false-positive and negative results due to interfering matrix species. One such sensor technology gaining in popularity are aptamer-based sensors, also referred to as aptasensors.[10-15] Approximately twenty years ago aptamers were independently described by three groups as nucleic acid macromolecules consisting of artificial single-stranded DNA or RNA sequences which are engineered to bind specifically to a variety of targets, including single ions, small molecules, proteins and even whole organisms.[16-20] DNA aptamers typically demonstrate binding affinities in the nanomolar to picomolar range for large molecules, and in the micromolar range for small molecules.[21-23]

Aptamers have been used in analytical and biological applications for diagnostic purposes because they offer a number of advantages over traditional protein-based antibodies.[21] Some of these advantages include the ability to be regenerated after denaturation, easy amplification via PCR, straightforward modification with various functional groups, straightforward engineering and synthesis without the need for animal systems, and in addition aptamers have desirable storage properties, such as excellent temperature and humidity stability.[21] In literature, there have been many studies where aptamers have been used to bind disease-related targets such as IgE, IFN-g, PTPase, cancer cells, and H1N1 influenza virus.[23-26]

Several disease-specific biomarkers are present in a TB patient's blood, urine, and sputum which can be used for rapid diagnosis. One of these biomarkers is the IS6110 DNA fragment which has been previously reported to be observable in TB patient urine; a portion of this biomarker is used in the present study.[27] The IS6110 fragment belongs to the IS3 family, and it is considered to be the most abundant insertion sequence (IS) found in the *M.Tb*. complex family.[27-29] In this work, a small fragment of the IS6110 sequence was used as the target biomarker and detected directly via hybridization to a specific probe sequence. There are many techniques that are used to detect DNA hybridization with the aid of a label molecule[30-33], however, these methods typically have low sensitivity, require significant method development, and most importantly the target affinity of the DNA probes may be reduced due to labelling with florescent or electroactive species.[34] As a result, label-free DNA detection methods are highly sought after. Some methods that have been used successfully for label-free DNA hybridization include voltammetry, electrochemical impedance spectroscopy, amperometry and Raman spectroscopy.[35-36]

Raman spectroscopy has been widely explored for detecting biological analytes due to its simplicity, the fact that almost no sample preparation is required, small sample requirement, non-destructive nature, rapid analysis time, and portable instrumentation.[37-39] As opposed to other types of vibrational spectroscopy such as infrared spectroscopy, Raman scattering is weak for water, glass, carbon dioxide and alcohols, making it ideal for studying biological molecules in aqueous environments.[39] A significant limitation of Raman spectroscopy, however, is that the Raman scattering is relatively weak since only approximately one in one million photons undergo this type of inelastic scattering.[40] However, this problem can be overcome by using surface-enhanced Raman spectroscopy (SERS). In 1974, a significant enhancement in the Raman signal of pyridine molecules was observed when present on roughened silver, and over time this signal enhancement, which is understood to occur through both electromagnetic and chemical mechanisms, was described as SERS.[41-42] SERS can be coupled with electrochemistry (EC-SERS) to detect the signal of the analyte present on the metal surface at a chosen applied voltage. Electrochemical SERS can be useful in detecting the signal of the analyte in a biologically relevant environment, to observe how molecules change conformation and / or orientation at different applied voltages, and also to observe the electrochemical stability of certain analytes.[38]

Previous SERS-based DNA studies have demonstrated hybridization of DNA with the aid of Raman labels such as methylene blue, rhodamine B, Texas Red, Cy3, and Cy5.[43-45] In some cases, label-free SERS detection of DNA hybridization is difficult to achieve because the signal of both strands will consist of the four DNA bases: adenine, guanine, cytosine and thymine. Also, it may be difficult to monitor or detect DNA hybridization at low concentration.[46] In literature, however, label-free SERS detection of DNA hybridization was successfully monitored by replacing adenine bases within the target sequence with its isomer (2-aminopurine) which provides a different signal, thus directly confirming hybridization.[47] While studies have been conducted which explore the effect of applied potential on DNA sequences via SERS [36,48-49], there has not, as of yet, been any studies reported in the literature which employ EC-SERS to investigate direct target binding to an aptamer. Bartlett et al. recently reported a label-free detection method for DNA hybridization using electrochemical SERS[33], however this was an indirect detection strategy employing leucomethylene blue as a Raman reporter.

The goal of this research was therefore to develop an EC-SERS based aptasensor capable of *directly* detecting TB DNA, without the need for costly labels or Raman reporter molecules. In the current work, the probe – target DNA pair was chosen such that the target strand, a sequence contained within the IS6110 fragment, contained adenine, while the probe strand did not. The probe strand was modified with a 5' thiol to allow for immobilization onto the AgNP surface and a complete self-assembled monolayer was prepared by thiol back-filling in order to reduce non-specific macromolecular adsorption onto the SERS substrate. Incubation with the target strand allowed for direct monitoring of DNA hybridization in synthetic urine via the appearance of the adenine marker bands. Application of an applied voltage provided a significant improvement in signal, demonstrating that this EC-SERS aptasensor shows promise for the detection of disease biomarkers.

EXPERIMENTAL

All glassware was cleaned in an acid bath of 95-98% ACS grade sulfuric acid for several hours and rinsed thoroughly with Milli-Q ultra-pure water (>18.2 MΩ•cm) prior to use. The same Milli-Q water was used to prepare all solutions. DNA bases (adenine ≥99%, thymine ≥99%, cytosine ≥99%, and guanine 98%), DNA nucleotides (dAMP 98-100%, dTMP ≥99%, dCMP ≥98%, dGMP ≥99%), sodium fluoride (99.99%), and 12-mercaptododecanoic acid (12-MDA) (99%) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Monobasic potassium phosphate (KH_2PO_4, 98-100%) was purchased from ACP Chemicals (Montreal, QC, Canada). Dibasic potassium phosphate (K_2HPO_4, 98-100%) was purchased from Anachemica (Montreal, QC, Canada). Synthetic urine was purchased from Ricca Chemical Company (Pocomoke city, MD, USA).

Nanoparticle Synthesis and Characterization:

Citrate-reduced silver colloids were prepared using a modified Lee and Meisel method[50] and this procedure is described in detail elsewhere.[38] Briefly, 0.09 g of silver nitrate (Sigma Aldrich, 99.999%) was added to ~500 mL Millipore water and brought to a vigorous boil, and then 10.0 mL of a 1% w/w trisodium citrate (ACP Chemicals, ≥98%) was directly added to the boiling solution. After 30 minutes, the reaction mixture was removed from heat and allowed to cool. After cooling, a 1.0 mL aliquot of the colloidal nanoparticle suspension was centrifuged 10 times for 15 minutes at 3600 rpm (VWR, Galaxy 16) in order to concentrate and aggregate the colloids. Each time, ~0.9 mL of the supernatant was carefully removed, a fresh 1.0 mL aliquot of the colloidal suspension was added, and the procedure was repeated. UV-vis (Varian Cary 50 Bio UV-visible spectrophotometer) and transmission electron microscopy (TEM) (FEI Tecnai 12 electron microscope with an operating voltage of 80 kV) characterization of the prepared Ag NPs is provided in the supporting information in Figure S-1.

Screen Printed Electrode Modification:

Once centrifugation was complete, the AgNPs were deposited onto the working electrode of commercially available screen printed electrodes (SPEs). The carbon SPEs (15 mm x 61mm x 0.36 mm) were purchased from Pine Research Instrumentation (Durham, NC, USA) and consisted of a silver/silver chloride (Ag/AgCl) reference electrode, a carbon counter electrode, and a carbon working electrode. These SPEs were functionalized by depositing three 5 µL layers of the concentrated silver nanoparticle (AgNP) suspension onto the working electrode surface (5 x 4 mm, rectangular carbon surface). The electrodes were left to dry completely after deposition of each AgNP layer, and the final layer was allowed to dry completely prior to use. All electrolyte solutions were purged with argon (99.999%, Praxair Canada Inc., Ontario, Canada) prior to measurement.

DNA Aptamers (Probe, Target, scrambled Target):

All DNA solutions were prepared in fresh pH 7.4 phosphate buffer prepared in house. The DNA sequences used in this project included: ***probe*** (5'-/5ThioMC6-D/TCC TGG GCT GGC GGG TCG CTT CC-3'), ***target*** (5'-GGA AGC GAC CCG CCA GCC CAG GA-3'), and ***scrambled target*** (5'-ACC GAG CCA GGC AGC CAG GGC AC-3'). All DNA fragments were synthesized by Integrated DNA Technologies, Coralville, Iowa, USA. The probe and target were used for the hybridization studies, whereas the non-complementary scrambled target strand was used as a control to investigate non-specific interactions. A certain amount of phosphate buffer was added to each vial to make up a chosen concentration of each DNA aptamer. The exact amount of phosphate buffer added, as well as the final concentration of each solution was: probe (230 µl, 2.08 mM), target (230 µl, 4.16 mM), scrambled target (230 µl, 2.28 mM). Each vial was vortex mixed well to ensure that the DNA was completely dissolved in the phosphate buffer, and the tubes were then stored at 4 ºC prior to use.

Functionalization of Electrode and Hybridization Protocol:

The AgNP electrodes were typically prepared several days in advance of conducting experiments. Approximately 10 µL of the DNA aptamer (probe) solution was deposited on the AgNP surface and the electrode was left to dry in air. The surface was then rinsed gently with Milli-Q water to remove any excess unbound DNA aptamer. The electrode was then immersed in a 1.0 mM ethanolic solution of 12-MDA for 2 hours and subsequently rinsed with ethanol to remove any excess 12-MDA that was not adsorbed onto the surface. After leaving the functionalized electrode to dry, 10 µL of the stock solution of either the complementary strand or non-complementary strand was then added to the AgNP electrode and left to dry in air. The electrode was then rinsed gently with Milli-Q water, and left to dry in air prior to conducting the EC-SERS studies. In addition, dilutions of the target stock solutions were made in order to determine the limit of detection for this EC-SERS aptasensor.

Instrumentation:

Two different Raman instruments were used throughout this project. The majority of the experiments were conducted using a DeltaNu benchtop dispersive Raman spectrometer equipped with 785 nm laser (Intevac Photonics, Santa Clara, USA). The spectrometer, with a resolution is 5 cm^{-1}, is equipped with an air-cooled CCD detector and an optics extension tube. Sample acquisition times ranged from 30-60 seconds at laser powers between 22.3-55.9 mW. This portable EC-SERS system has been previously reported by our group; the schematic of the set-up is shown in Figure S-2.[38] The self-assembled monolayer (SAM) studies using 12-MDA were conducted using a DXR Smart Raman spectrometer equipped with a 780 nm laser (Thermo Fisher Scientific, Mississauga, ON, Canada). The spectrometer resolution is 3 cm^{-1} and it also is equipped with an air-cooled CCD detector. Origin 8.1 was used for the spectral processing and data analysis (OriginLab Corporation, Northampton, MA, USA). All Raman spectra were normalized for both laser power and acquisition time for ease of comparison. Both Raman spectrometers were coupled to a Pine Research Instrumentation portable USB Wavenow potentiostat/galvanostat (Durham, NC, USA) to perform electrochemical measurements such as cyclic voltammetry (CV) and EC-SERS. The CV parameters were the same for all experiments; initial and upper potentials were set to 0.0 V vs Ag/AgCl, the lower potential was -1.0 V vs Ag/AgCl, and the sweep rate was 50 mV/s. For EC-SERS, the applied potential ranged from 0.0 V to -1.0 V vs Ag/AgCl in increments of 0.1 V for a time interval of 60 seconds. All potentials are vs. Ag/AgCl unless otherwise stated.

RESULTS AND DISCUSSION

Initial studies consisted of the detection of the individual DNA bases and nucleotides using EC-SERS before performing studies with the DNA aptamer. These studies were helpful for the later spectral interpretation of the DNA aptamer. The normal Raman (powder) signal was collected for each of the four DNA bases. As shown in Figure 1, each DNA base gave a unique spectral profile and each gave a strong peak between 600 and 800 cm^{-1} which is indicative of ring breathing vibrations.[51] In addition, there are several other strong peaks that can be used to distinguish these bases from one another; for example, guanine (650, 1266 cm^{-1}), adenine (723, 1334 cm^{-1}) cytosine (790, 1278 cm^{-1}), and thymine (1371, 1673 cm^{-1}); these values are consistent with literature values.[37,51]

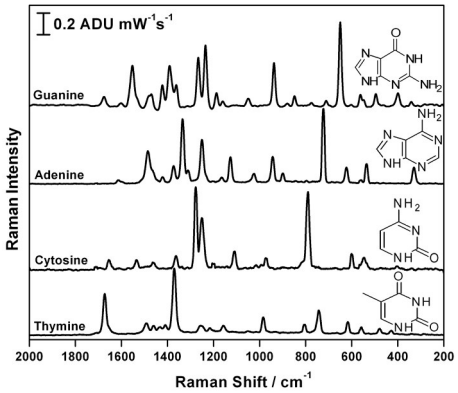

Figure 1: Normal Raman spectra (powder) for the four DNA bases: guanine, adenine, cytosine and thymine. All spectra were collected at medium power (22.3 mW) for a time interval of 30 seconds using 785 nm excitation.

As mentioned previously, normal Raman spectroscopy exhibits poor sensitivity for the detection of analytes in solution at low concentration. Due to this limitation, the previous measurements were done on pure powder; however SERS was used in order to detect these analytes at low concentration in solution. EC-SERS was performed in 1.0 mM DNA base solutions that were prepared in 0.1 M NaF as a supporting electrolyte. A modified screen printed electrode (SPE) was immersed in this solution and a potential was applied in 100 mV steps in the cathodic direction (0.0 to -1.0 V) and also in the anodic direction (-1.0 to 0.0 V). From Figure 2, it is apparent that the adenine signal increased by ~ 10 fold between open circuit potential (OCP) and -0.8V, indicating that the behaviour of the DNA bases at the metal/solution interface is influenced by application of a voltage. A similar result was observed for the other three DNA bases.

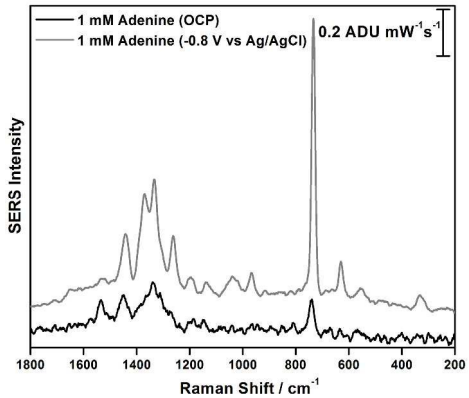

Figure 2: SERS signal of 1.0 mM adenine at OCP (black curve), and at -0.8V (grey curve). At OCP, the signal was collected at medium-high power (46.5 mW) for a time interval of 20 seconds, and at -0.8 V, it was collected at the same power for a time interval of 60 seconds using 785 nm. Both spectra are normalized for both laser power and exposure time.

Collecting the signal for these four DNA bases was very useful as an initial step towards characterizing the DNA aptamer. The four DNA nucleotides used in this research were 2'-deoxyadenosine 5'-monophosphate (dAMP), 2'-deoxycytidine 5'-monophosphate (dCMP), thymidine 5'-monophosphate (dTMP), and 2'-deoxyguanosine 5'-monophosphate (dGMP). As shown in Figure S-3, the normal Raman signal (powder) was collected for these DNA nucleotides and it is apparent that the signal was much weaker than that which was observed for the DNA bases. According to the literature, DNA nucleotides are poor Raman scatterers due to the presence of the weakly scattering ribose sugar and phosphate groups, which have the effect of diluting the observed signal.[52] However, by using EC-SERS this problem was overcome. As shown in Figure S-4, a clear signal for a 1.0 mM dAMP solution was collected at negative voltages; the signal was mostly dominated by the adenine base. Similar results were obtained for the other three nucleotides, where the signal was mostly dominated by the respective base.

It was important to test if EC-SERS would be able to detect the presence of all four nucleotides in one solution, since according to the literature dAMP tends to dominate the signal over the other nucleotides which could be problematic for the development of a useful DNA-based SERS sensor.[47,53] To test this, a 1.0 mM mixture of each of the four nucleotides was prepared. From Figure S-5, it is apparent that all the nucleotides were detected in the mixture solution, and adenine did not completely dominate the signal. The four nucleotides were easily distinguished from one another in the mixture solution; for example, adenine (732, 1328 cm^{-1}), guanine (683, 1481 cm^{-1}), cytosine (789, 1636 cm^{-1}), and thymine (792, 1650 cm^{-1}) could all be easily identified via their respective marker bands. By conducting these studies, it was concluded that EC-SERS can be used to detect the DNA nucleotides present in DNA aptamers and the signal should not be dominated by any particular nucleotide.

The DNA aptamer investigated in this work, referred to as the *probe,* contained 23 DNA bases, as outlined in the materials section. This sequence, as mentioned previously, is complementary to a section of DNA found in the IS6110 sequence of TB DNA. Adenine was absent in the probe sequence in order to give an unambiguous response when hybridization occurred, since the complementary strand (*target*) did contain adenine. The probe was chemically synthesized to contain a thiol and a six-carbon spacer at the 5' end to facilitate immobilization. Cyclic voltammetry (CV) was performed to ensure that the working electrode was functioning properly and also to confirm that a DNA monolayer was present on the electrode surface. Figure 3 shows the comparison of the CV data for the unmodified and modified AgNP SPE surface; it is apparent that the current is significantly reduced for the electrode modified with the probe, suggesting that a stable monolayer was formed on the surface of the electrode.

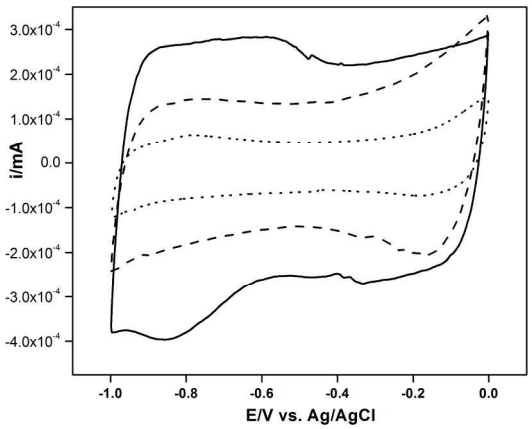

Figure 3: Cyclic voltammogram of a bare AgNP electrode in 0.1 M NaF (solid line), an electrode containing a monolayer of probe + 12-MDA (dashed line), and an electrode containing a monolayer of probe + target + 12-MDA (dotted line). Scan rate: 50 mV/s.

As shown in Figure 4, EC-SERS was performed after the probe was deposited on the AgNP surface, as described in the experimental

section. At open circuit potential (OCP), where no voltage is being applied, the SERS signal was mostly due to citrate that was present on the AgNPs; citrate typically has dominant peaks at ~1390 and ~930 cm^{-1} which are assigned to $\upsilon_s(COO^-)$ and $\upsilon(C\text{-}COO^-)$, respectively.[54] As the potential was stepped in the negative (cathodic) direction, the metal surface charge becomes increasingly less positive (potential of zero charge for Ag(poly) = -0.95 V vs Ag/AgCl)[55], which causes the negatively charged citrate molecules to desorb from the surface, resulting in a decrease in the observed citrate signal. At -0.5 V, some peaks appear which are indicative of the probe and as the potential was made more negative the probe signal further increased. At -1.0 V, the signal was due only to probe, indicating that citrate is no longer present on the AgNP surface. For example, peaks at 790, 1257 and 1577 cm^{-1} are due to cytosine, thymine, and guanine; respectively.[37] A peak around 687 cm^{-1} was also present which is due to the C-S stretch, further proof that the probe was indeed present on the surface.[26,56] A detailed SERS peak analysis for both the probe and target sequences is included in the supporting information as Table S-1. The remainder of the results shown in this work will only show the EC-SERS signal at -1.0 V since the best signal for the probe was consistently observed at this potential. In order to test if the probe formed an electrochemically stable monolayer on the AgNP surface, the potential was stepped back in the positive (anodic) direction. By looking at Figure S-6, it is apparent that the probe signal was indeed stable, which indicates that the probe was strongly adsorbed onto the AgNP surface. Application of increasingly less negative potentials during the anodic scan resulted in a slight signal increase.

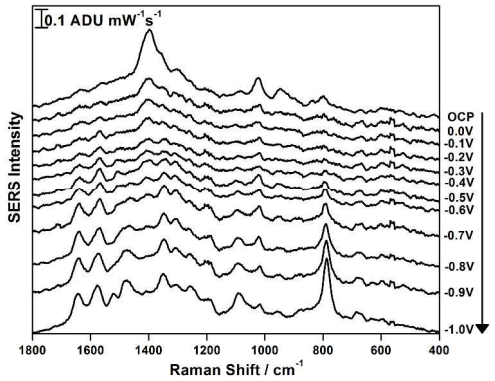

Figure 4: EC-SERS cathodic signal of probe immobilized on a AgNP electrode, measured at medium-high power (46.5 mW) for a time interval of 60 seconds using 785 nm excitation.

A problem that may arise with this DNA aptasensor is non-specific binding of non-target analytes onto the SERS substrate; more specifically non-target DNA in this case. Another issue that could arise is adsorption of the aptamers in a horizontal rather than vertical conformation, or a surface-induced denaturation of the DNA; both of these situations may prevent the target from binding properly to the probe. In order to test the extent that non-specific binding between non-target DNA and the SERS substrate could occur, EC-SERS was performed using the complimentary strand to the probe, referred to as the target, in the absence of the probe. The target sequence has the same number of nucleotides as the probe but it does not contain a thiolated end. In theory, since this DNA strand is not thiolated, it should not adsorb strongly onto the SERS substrate, and therefore no signal should be detected. However, the spectrum in Figure 5b strongly indicates that non-specific binding is indeed occurring; adenine peaks were clearly present at 730 and 1328 cm^{-1}. The likely mode of adsorption in this case is via the nitrogen-rich DNA bases. [57] Also, it seems that the signal of the complementary strand is mostly due to the adenine moiety which suggests that adenine could in fact be dominating the signal of the DNA strand in this case. This issue of non-specific binding of non-target DNA will be dealt with in the next few sections.

EC-SERS spectra for both the probe and target were obtained independently (Figures 5a and b, respectively); however the goal was to perform hybridization studies between these two sequences. Figure 5c shows the EC-SERS spectrum resulting from the hybridization study and it is apparent that the adenine peaks were present at 730 and 1330 cm^{-1} which indicates the presence of the target since adenine is only found in the target strand. This could indicate that the hybridization study was successful between target and probe; however, as seen earlier with the non-specific binding between the target strand and the SERS substrate, a control study was needed.

In order to perform a control study, a non-complementary strand was used, referred to as the *scrambled target*. This DNA strand has the same number of adenines as the complementary strand but the sequence is scrambled such that the nucleotides will not be able to base-pair with the probe. Clearly, hybridization between the probe and the scrambled target should not occur because they are not complementary; that being said, however; if adenine peaks are present in the EC-SERS spectra this would indicate that non-specific binding is indeed occurring. By looking at Figure 5d, it is apparent that the adenine peaks were indeed present, thus strongly suggesting non-specific binding was occurring. This non-specific binding can be problematic for developing the aptasensor because it will lead to false-positive results. Therefore, in order to reduce or prevent this problem, thiol backfilling was explored. During thiol backfilling, a thiol molecule is introduced to the surface after immobilization of the aptamers in order to fill in the spaces between the aptamers. Thiol chemistry provides a broad range of thiol lengths and different head groups which can be useful for different applications.[57] The thiol in this case will act as a spacer between the probe strands immobilized on the SERS substrate, reducing the likelihood of surface-induced denaturation and filling any unoccupied surface spaces in order to eliminate or reduce any non-specific surface adsorption that might occur for non-target analytes.

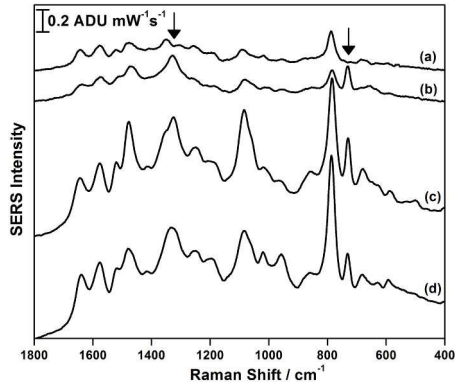

Figure 5: SERS signal comparison between a) probe, b) target c) probe + target, d) probe + scrambled target; all recorded at -1.0V, measured at medium-high (46.5 mW) power for a time interval of 60 seconds. Arrows indicate peaks specific to adenine.

There are several conditions that must be met in order to choose an appropriate thiol to use for this aptasensor. For example, the thiol should be stable on the surface over a significant period of time (> 6 months), should have the ability to displace citrate molecules, and most importantly it should not displace the aptamer. In literature, 12-mercaptododecanoic acid (12-MDA) is routinely used to form self-assembled monolayers on electrodes for sensing applications.[57-59]

Therefore, 12-MDA was selected as the thiol for this aptasensor platform. EC-SERS was first performed on 12-MDA by incubating a bare AgNP electrode in 1.0 mM 12-MDA solution for two hours, followed by careful rinsing. Figure S-7 shows the EC-SERS measured for the AgNP electrode functionalized with the monolayer of 12-MDA in 0.1 M NaF; looking at the OCP spectrum it is apparent that the citrate signal was readily displaced. As the potential was stepped in the cathodic direction, the signal increased but then started to decrease at -0.7 V. The peaks at 704, 1100, and 1435 cm^{-1} are indicative of the thiol CH_2 rocking, C-S stretching, and CH_2 deformation vibrations, respectively.[60-61] EC-SERS was performed on the same electrode after 2, 6, and 10 months to test the stability of the 12-MDA monolayer over time, as shown in Figure S-8. It was observed that the signal of 12-MDA is stable for a long period of time, and most of the peaks that were present on the first day were still present with similar intensity several months later (Figure S-8). These studies suggested that 12-MDA is indeed a good thiol spacer candidate for this aptasensor, and it was therefore used for the subsequent hybridization studies.

The hybridization study between the probe and target was repeated in the presence of 12-MDA as the thiol spacer. It is apparent from the spectrum shown in Figure 6a that the adenine peaks were present. This shows that the 12-MDA is unable to displace the probe that was already immobilized on the surface, and that hybridization could still be monitored. Also, the CV of this experiment shown in Figure 3 indicates that a stable monolayer had formed. A similar spectroscopic result could be obtained for the same sensor platform even two years later, although with some reduction in intensity (Figure S-9). Such a finding illustrates the long term stability of these substrates. The same experiment was then repeated using the non-complementary strand (*scrambled target*) in order to monitor the effect of thiol backfilling on non-specific binding. As shown in Figure 6b, the adenine peaks were not present which suggests that thiol backfilling is indeed required to reduce / eliminate non-specific binding. This finding was consistent with what has been suggested in literature for other systems.[57]

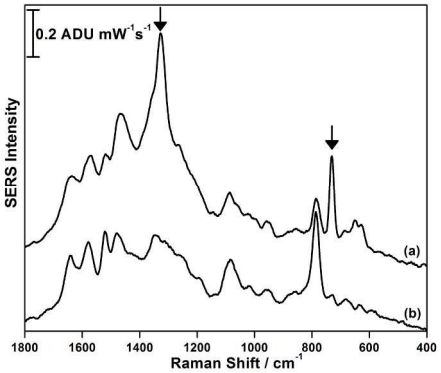

Figure 6: SERS comparison at -1.0 V for a) probe + target b) probe + scrambled target; both with 12-MDA used as a back-filled spacer. Measured at medium-high power (46.5 mW) for a time interval of 60 seconds using 785 nm excitation.

All studies were initially performed in 0.1 M NaF as a supporting electrolyte; in later studies however synthetic urine was used as an example of a biologically relevant electrolyte. This is important since the eventual goal of this device is for use in a clinical setting and the target DNA biomarker is secreted into urine. As shown in Figures 7a and b, it is evident that the EC-SERS signal in synthetic urine was a bit weaker than the study performed in 0.1 M NaF; nonetheless the adenine peaks were still clearly present. The signal-to-noise ratio was lower than for the study conducted in 0.1 M NaF and this could be due to the chloride ions present in the synthetic urine which tend to form a strong covalent bond with the silver surface; this can lead to displacement of surface adsorbed molecules, including perhaps the aptamer. This suggests that for optimal device performance removal of chloride from patient urine samples prior to analysis may be beneficial for more sensitive detection.

Throughout the course of these studies, a detection limit of 280 μg mL^{-1} was obtained for the target DNA strand. Below this concentration, no observable peaks for adenine could be ascertained. Currently, PCR analysis of DNA has reported detection limits ranging from 2.5 μg mL^{-1} to 0.5 μg mL^{-1}, and this can be reduced considerably with the use of special dyes such as Hoechst 33342 and SYBR® Green.[62-63] While the EC-SERS method for detection of DNA presented herein lacks the sensitivity of modern PCR technology, it does provide numerous advantages, including lower cost per patient, portability and improved selectivity. Improvement of the detection limit in this case is in part dependent on the nature of the SERS substrate, and studies are now underway which will allow for optimization of the SERS enhancement through careful design of the nanostructured surface.

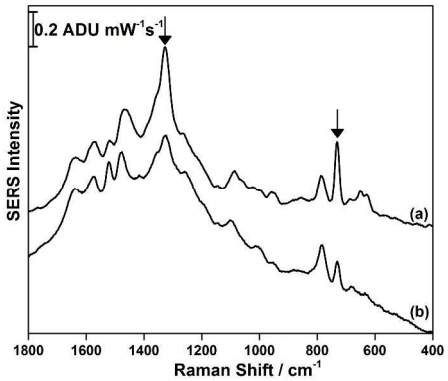

Figure 7: SERS comparison at -1.0V for probe + target +12-MDA conducted in a) 0.1 M NaF and b) synthetic urine, both collected at medium-high power (46.5 mW) for a time interval of 60 seconds using 785 nm excitation. Arrows indicate peaks due to adenine.

Conclusions

Electrochemical Surface-Enhanced Raman spectroscopy (EC-SERS) shows excellent promise for the detection of biological analytes such as DNA bases, nucleotides, and DNA aptamers in aqueous media. A unique spectral profile was observed for each of the four bases and nucleotides which were easily distinguished from one other. It was observed that application of a voltage had a considerable enhancing effect on the observed signal, likely due to the potential-induced desorption of adsorbed citrate molecules. Each nucleotide was easily characterized in a mixture solution, which indicates that adenine does not dominate the signal in an electrochemical environment when all nucleotides are present. EC-SERS was used to detect three different DNA sequences (probe, complementary strand (target), non-complementary strand (scrambled target)), which showed that even non-thiolated DNA sequences were strongly adsorbed on the SERS substrate. Hybridization studies were successfully conducted between the probe and its target with the aid of 12-MDA acting as a spacer

molecule. The spacer prevented non-specific binding of non-target DNA and showed excellent signal stability over a significant period of time. These experiments were also conducted in synthetic urine, a more realistic electrolyte for detecting urine DNA biomarkers; however, the signal was weaker, most likely due to the presence of chloride. This work demonstrates the first example of *electrochemical*-SERS as a tool for the *direct*, label-free detection of DNA hybridization; this aptasensor technology could eventually be implemented for the detection of a variety of disease biomarkers at the patient point-of-care.

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Notes and references

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