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Probing biomolecular interactions using surface enhanced Raman spectroscopy: Label-free protein detection using a G-quadruplex DNA aptamer**

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Electronic supplementary information:
Materials and methods, figures detailing nanoshell characterisation and figures detailing control SERS spectra available. Please see http://dx.doi.org/10.1039/B920941G

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Abstract

We demonstrate a strategy for label-free protein detection through monitoring the Surface Enhanced Raman Spectrum of an aptamer probe attached to a gold nanoshell. Low limit of detection and minimal non-specific binding show potential for *in-vitro* and *in-vivo* assays.

Introduction

The ability to measure molecular binding events in real time, without labeling, is critically important in aiding our understanding of biological processes.[1] In this paper we demonstrate that the binding between an aptamer and unlabelled protein can be detected by measuring changes in the surface enhanced Raman spectroscopy (SERS) signal of the aptamer caused by conformational changes which arise from formation of an aptamer/protein complex. While Raman spectroscopy has huge potential for probing such biomolecular systems,[2] it has historically suffered from low signal intensities. High laser power can often compensate for low intrinsic signal, but can often lead to photo-damage of the sample. SERS is an alternative Raman technique which uses the plasmon resonance of noble metal particles or nanoscale surface structures to increase the Raman signal from analytes near the surface and therefore requires lower laser power, shorter acquisition times, and is increasingly valuable for measuring Raman spectra from dilute or photosensitive samples. SERS can be used to measure spectra from sensitive biological samples and has been applied in investigations of the live cell intracellular environment.[3] Novel SERS substrates which exhibit high enhancement factors can be designed for specific purposes, e.g. core shell particles, resonant at near IR wavelengths are suitable for biomedical applications[4] and we have recently shown that such particles can be introduced to mouse fibroblast cells in a controllable non-toxic way for the measurement of SERS spectra.[5]

Biomolecular detection has been demonstrated using several approaches which facilitate SERS on nanoscale roughened noble metal surfaces or by using nanoparticles in a "sandwich" format.[7] A few approaches for SERS detection of proteins have used aptamers, which are highly specific oligonucleotide probes[8] that bind target molecules with high specificity. In these cases, Raman active dyes are generally attached to one end of the aptamer and are drawn closer to the surface of the SERS substrate during binding of the target protein leading to a large enhancement of the dye spectrum.[9] Perhaps the best characterized aptamer is the thrombin binding aptamer (TBA) which can be used as an inhibitor of blood coagulation.[10,11] The wealth of characterization data available for this aptamer makes it an ideal candidate to use as a model-system for development of new detection methods. Furthermore, thrombin is an important regulator of the cascade of reactions which lead to blood clot formation. Blood clots cause an estimated 25,000 deaths in the UK per year and recent clinical

guidelines have suggested that evaluation of the risk of clot formation could significantly reduce this toll. A simple, sensitive test of thrombin levels could be a valuable tool in such evaluations.[12]

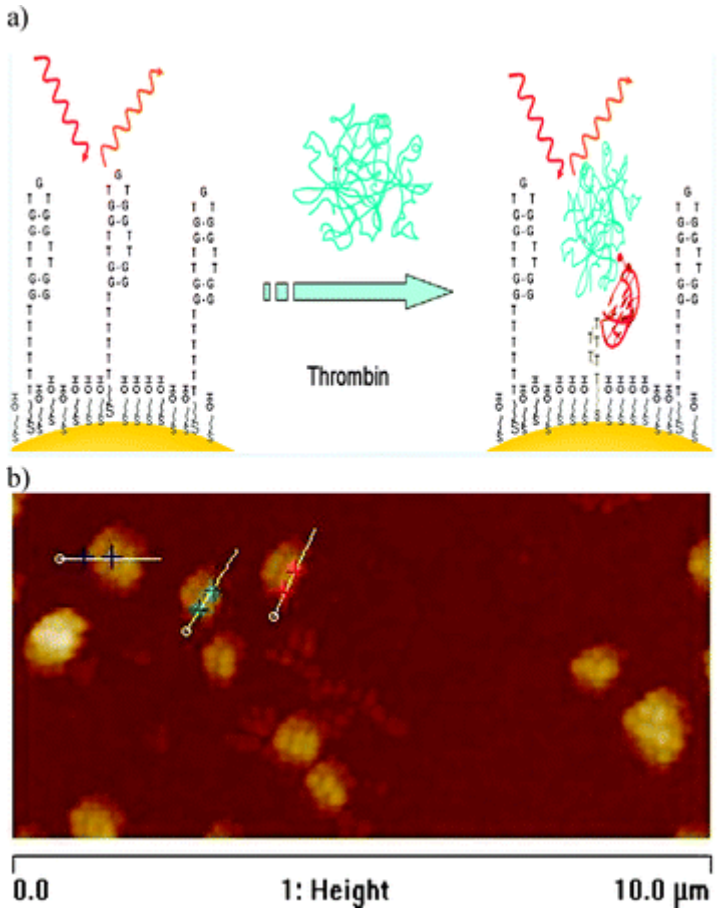


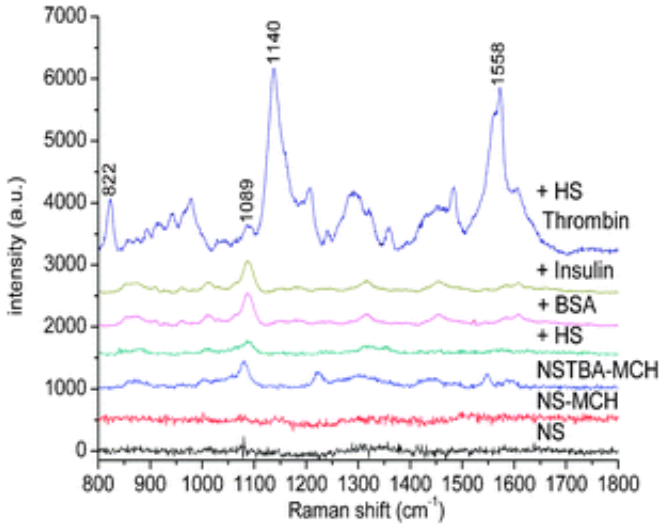
Figure 1. a) Sketch shows the assembled mixed monolayer of prehybridized TBA oligonucleotide and MCH and the model for the detection. b) AFM image of NS aggregates used for SERS in this work. The clusters shown consist of two self-assembled layers with a size of ca. 0.5 µm.

Since aptamer-protein binding often leads to a change in the aptamer structure, we speculate that we could use changes in the SERS spectrum of the aptamer to signal protein binding. Furthermore, since the vibrational spectra of proteins are distinct from those of nucleic acids, we might expect to see a difference between the spectrum of the aptamer and the spectrum of the aptamer-protein complex. Here we report the results of an investigation into whether we can directly view either the structural change of TBA on interaction with thrombin, or see new peaks in the sensor spectrum as a result of protein binding (Figure 1a). The system we used consisted of commercially available 152±6 nm (as determined by SEM)[5] diameter gold nanoshells with a maximum absorbance at 780 nm (Auroshell™, Nanospectra Inc., US) aggregates attached to amino-silane functionalised glass slides (UV-Vis

spectrum in Figure S1).[13] Aggregation on the glass-surface leads to ca. 0.5 µm particle islands consisting of aggregates such that the particles are separated from each other by about 0-25 nm according to AFM measurements (Fig. S2). Figure 1b shows the aggregates in an area where measurements were conducted.

The oligonucleotides were conjugated covalently onto the gold surface by a 5'-C6-thiol linker.[14] Non-specific binding of DNA or proteins to the gold surface was reduced by blocking any unreacted surface sites with a self-assembled monolayer of mercapto-hexanol (MCH)[15] (details in supporting info).

The G-quadruplex, is an important feature of the aptamer/protein complex of the TBA oligonucleotide and human α thrombin and is stabilized by cations such as K^+ or Mg^{2+}. The buffer used in this study consists of 10 mM K^+ and Mg^{2+} and 140 mM NaCl at a pH of 7.5 (supporting info). During interaction with the protein, the aptamer adopts a stacked G-quadruplex structure forming a "rocking chair" like shape.[11] Representative Raman spectra of NS, NS covered with mercaptohexanol, aptamer conjugated-NS with mercaptohexanol blocking layer (NSTBA) in binding buffer; NSTBA after 30 min incubation with different control samples [bovine serum albumin (BSA) 10 µM, human serum (HS) 1% and insulin 0.6 µM] and after incubation with target protein thrombin in binding buffer are shown in Figure 2. Raman measurements were carried out using a 785 nm laser exciting with power of 0.03±0.02 mW and acquisition times of 10 seconds (details in supporting info) on a Renishaw InVia Raman microscope. All measurements were made using a 50X water immersion objective in buffered solutions. In the case of NS and NS-MCH, the spectra show no detectable signal. The spectra of the completed NSTBA sensor show only weak signals with a prominent and stable feature at 1089 cm^{-1} assignable to the DNA O-P-O backbone stretch. Spectra of the sensor incubated with non-target proteins show weak signals similar to the background spectrum of the sensing platform.



← ***Figure 2.*** Spectral changes caused by human α-thrombin (10 nM) binding to NSTBA sensor. The dominant peaks can be assigned to DNA vibrational modes (see text). Controls with BSA (10 mg/ml), insulin 600 nM and 1% human serum show no non-specific protein binding. Lower three spectra show spectra of NS, NS plus blocking agent (mercaptohexanol) and the fully assembled NSTBA sensor. Spectra are averages of 10 acquisitions.

Only incubation of the cognate target protein leads to a measurable change in the spectrum of the NSTBA sensor. Spectra measured when the sensor was incubated with 10 nM human thrombin show increases in signal at 822 cm^{-1}, 1140 cm^{-1} and 1558 cm^{-1}. These can be assigned to the combined *C2′-endo* and C3′*-endo* modes of the 2'-deoxyribose sugars, the C-O-C stretch at 1140 cm^{-1} and guanine ring modes at 1558 cm^{-1}. At 1480 cm^{-1} we observe another feature with lower intensity which can also be assigned to a guanine ring mode.[16] Further signal increases can be assigned to vibrational modes of protein, such as the amide III backbone vibration at 1220 cm^{-1}, CH_2 stretching mode at 1440-1460 cm^{-1} or tyrosine aromatic ring vibrations at 1610 cm^{-1}.[17] An experiment using a control oligonucleotide lacking specific thrombin binding capability did not show any changes on thrombin incubation (Figure S3). Further SERS measurements of thrombin, guanosine- and thymidine-triphosphate on NS (Figure S4) confirm these spectral assignments. Since TBA is known to adopt a G-quadruplex conformation when it binds to thrombin, we speculate that the strong spectral changes detailed above are caused by the switch from a relatively unstructured oligo to the G-quadruplex structure. This suggests that formation of a G-quadruplex positions key nucleotides closer to the gold surface and in a better position to interact with surface plasmons. In previous studies by Halas *et al.*[18] it was shown that the hybridization of oligonucleotides leads to changes in SERS spectra. The same authors have also described the correlation between dsDNA orientation and SERS signal intensity and found signal increases depending on the angle between the molecule and the surface.[19]

In order to further investigate the spectral features attributable to G-quadruplex formation we used an oligonucleotide which has been specifically selected for G-quadruplex formation.[20] The SERS spectrum shows peaks at 820 cm^{-1}, 1132 cm^{-1}, 1480 cm^{-1} and 1560 cm^{-1}. These peaks can be assigned in a similar way to the prominent peaks of the TBA/thrombin complex with the features at 1480 cm^{-1} and 1560 cm^{-1} exhibiting altered intensity ratios (Figure 3). These slight differences in intensity are to be expected between different macromolecules since their relative orientations to the surface may differ. We also investigated the temperature dependent behavior of this control oligonucleotide to demonstrate the correlation between SERS signal and secondary structure formation. A melting experiment shown in Figure 3 demonstrates that the spectral features which we attribute to the G-quadruplex structure can be reversibly eliminated and regained through temperature cycling.

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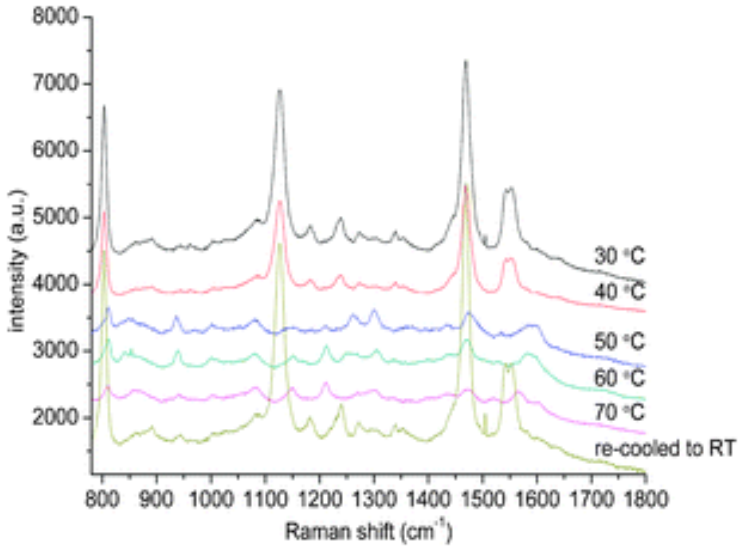

Figure 3. To show the relationship between the oligonucleotide secondary structure and SERS signal we increased the temperature and observed the change in Raman spectrum. Spectra show averages of 20 spectra with acquisition times of 10 sec. Temperatures were increased in 10 °C steps from 30 °C up to 70 °C and the intensity of the marker bands decreased in intensity. Cooling back to RT after the 70 °C measurement gave spectra with the original high intensity signals and we therefore suggest that the oligo refolds to its original structure at low temperature.

To study the reproducibility and the re-usability of the sensor, successive washing steps were performed with buffer which disrupts the aptamer-protein interaction (details in supporting information). Washing led to the same low intensity signal which we measured prior to protein incubation. On reincubation with thrombin (10 nM), the characteristic SERS spectrum of the aptamer/protein complex returned. This cycle was repeated and the SERS changes shown to be reproducible (Figure 4a). This figure also shows a comparison of the control measurements with the binding signal. Furthermore, we observe the same spectral changes when incubating the sensor with thrombin doped into human serum (Figure 4a) confirming that we can observe this specific biomolecular interaction even in a complex biological matrix (the data represented in figure 4a are shown as a standard histogram with error bars in figure S5). Using this approach, we can reliably detect thrombin down to 0.1 fM (Figure 4b) as experimentally determined by using thrombin concentrations between 500 yM and 50 μM.

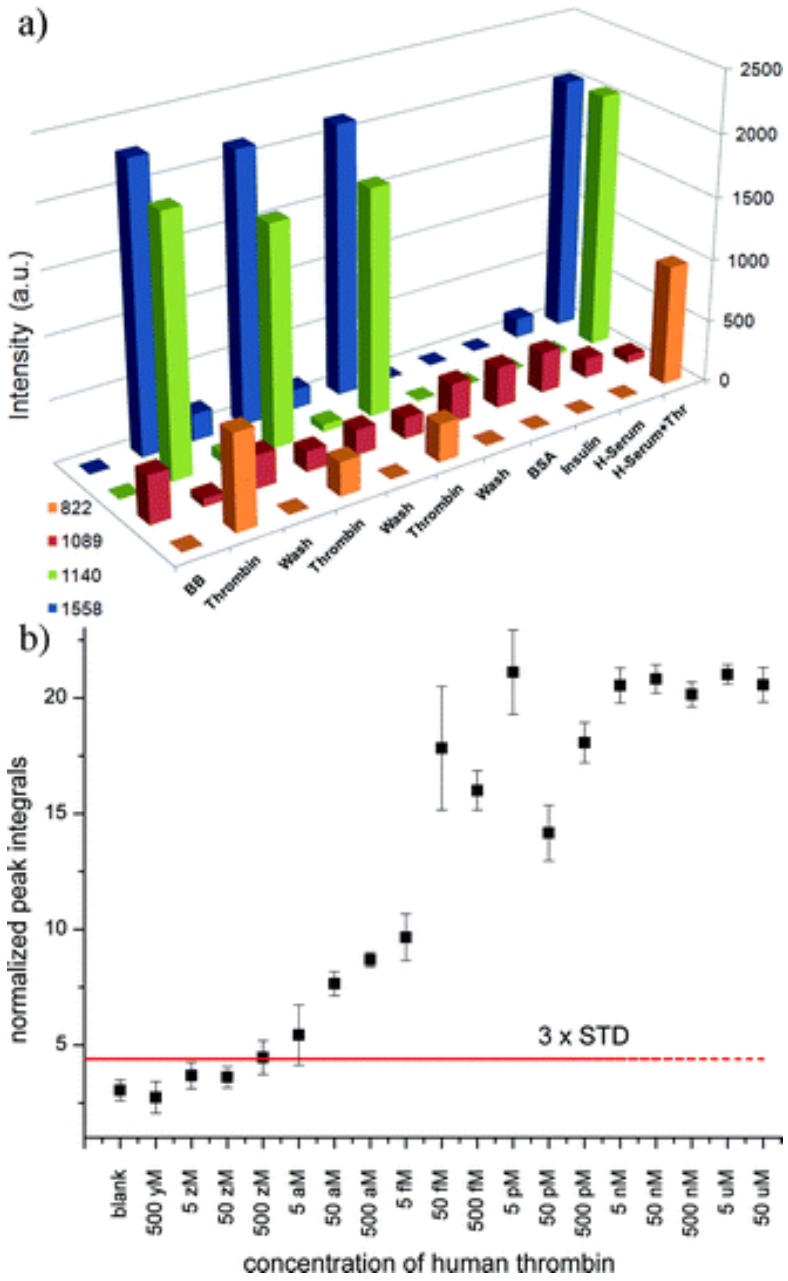

Figure 4. a) Washing and target incubation cycles of NSTBA with 10 nM thrombin. Graph shows SERS intensities of 3 peaks associated with conformational switching and one at 1089 cm-1 which is used as a control peak. Peak intensities are highly reproducibly at every incubation cycle. A further incubation in which 10 nM thrombin was dissolved in a 1% solution of human serum produced similar peak intensities. Control experiments incubating only with bovine serum albumin, insulin and human serum do not show any increased signals compared to the washed sensor. b) The limit of detection was determined by integrating the peaks at 820 cm^{-1}, 1140 cm^{-1} and 1558 cm^{-1} of 10 spectra and normalized to the integral of the constant peak at 1089 cm^{-1}. Errorbars represent a single standard deviation. The red line indicates the blank measurement plus 3 standard deviations. The datapoint at 50 aM is the first over the limit of detection threshold and we state a confident limit of detection of 0.1 fM.

We have shown an easy and versatile approach for the label-free study of biomolecular interactions. Our example demonstrates sensitive, specific protein detection using the G-quadruplex TBA with NS aggregates as substrate. The advantage of our approach is that the detection is independent of a separate Raman reporter and opens the door to various sensor applications such as the multiplexed detection of proteins in an array. Since we have recently demonstrated the utility of nanoshells for intracellular SERS[5] we expect that the combination of these two discoveries will enable the study of biomoelecular interactions in the cytoplasm of eukaryotic cells.

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