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Resonance Raman scattering of catalytic beacons for DNA detection†

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A novel method for analysing the catalytic action of a DNAzyme is reported. Resonance Raman scattering (RRS) is shown to successfully monitor the oxidation of two different peroxidase substrates and has been implemented in an assay for the detection of target DNA, providing a more sensitive method of analysis than current colorimetric techniques.

DNAzymes are functional nucleic acid sequences with specific catalytic activity that have been attracting interest in recent years.[1] An example of catalytic DNA that has been reported is the horseradish-peroxidase (HRP) mimicking DNAzyme formed by guanine rich nucleic acid sequences.[2,3] The peroxidase activity exhibited is the result of the intercalation of the metalloporphyrin haemin into the G-quadruplex structure assembled from guanine rich DNA in the presence of certain cations.[2,4–6] This complex is able to catalyse the H_2O_2-mediated oxidation of peroxidase substrates such as 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS)[1] and 3,3′,5,5′-tetramethylbenzidine (TMB).[3]

The oxidation of TMB and ABTS by an HRP-mimicking DNAzyme has been used in a number of detection assays for various analytes such as metal ions,[7,8] proteins,[9,10] and G-quadruplex ligands.[11] Research has also focussed on the use of DNAzymes for the visual and spectroscopic detection of target DNA.[1,3,12–16] ABTS has been the substrate of choice for many of the detection assays to date.[12–16] However, the green oxidation product produced from interaction with HRP or an HRP-mimicking DNAzyme is a relatively unstable molecule, and often reverts to its colourless form over time.[3] This renders ABTS impractical for reactions that involve longer analysis timescales and as such, there has been a recent move towards TMB as an alternative substrate.

Most ABTS and TMB based DNAzyme assays reported have relied on either UV-Vis spectroscopy or chemiluminescence for detection. In order to obtain the lowest possible detection limits from DNAzyme detection, additional steps such as the polymerase chain reaction (PCR) have been implemented to amplify the target DNA in order to obtain an observable signal.[13] Additionally, in an attempt to move away from PCR based bioanalysis, novel enzymatic methods of signal amplification have been developed, that enable a target or DNAzyme cycling strategy for increased sensitivity.[8,15–19] Whilst these methods are successful in their ability to provide lower detection limits, they introduce additional steps into potentially simple assay formats.

A recent publication by Willner and co-workers utilised fluorescence spectroscopy in an attempt to increase the sensitivity of a DNAzyme based detection scheme without compromising the simplicity.[20] By changing the method of analysis the limit of detection was successfully lowered compared to that previously reported.[12] Fluorescence spectroscopy is a very robust and sensitive technique, however, it is not without limitations, in particular, the broad emission spectra produced can be problematic. Therefore, in the present study we investigate resonance Raman scattering as an alternative method of analysis.

We have previously used resonance Raman analysis of TMB for the successful detection of human tumour necrosis factor α.[21] Additionally, surface enhanced resonance Raman scattering (SERRS) has been used for the detection of human prostate specific antigen (PSA) using ABTS as the substrate.[22] However, both of these detection mechanisms used the enzyme HRP to oxidise the substrate before analysis. As such, Raman scattering, be it resonance or surface enhanced resonance Raman, has not yet been utilised to monitor the oxidative products from TMB and ABTS resulting from the catalytic activity of a G-quadruplex DNAzyme.

Herein, we assess the suitability of the chosen substrates for resonance Raman analysis after interaction with a DNAzyme, and incorporate this method of analysis into a catalytic beacon assay for the detection of target DNA, in this case a synthetic sequence correlating to the *MecA* gene of a methicillin-resistant *Staphylococcus aureus* (MRSA) strain.

Fig. 1 shows the UV-Vis and RRS results obtained for both ABTS and TMB after interaction with PS2.M, a known DNAzyme sequence with HRP-mimicking activity. PS2.M is an 18-base

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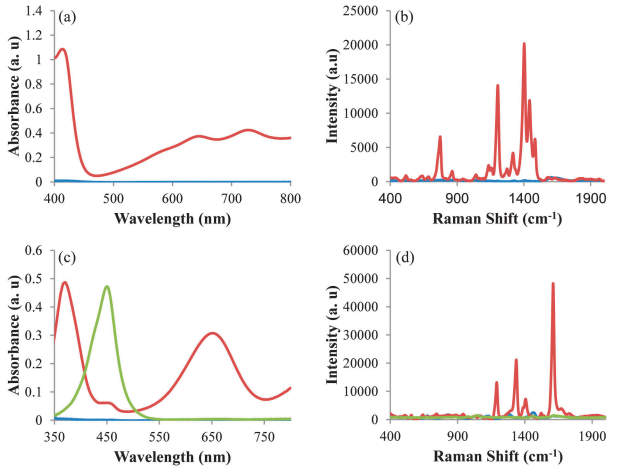

Fig. 1 Oxidation by 0.4 μM DNAzyme of (a) ABTS (blue) and oxidation product (red) monitored by UV-Vis and (b) RRS, and (c) TMB parent diamine (blue), CTC (red) and final diimine (green) monitored by UV-Vis and (d) RRS at an excitation wavelength of 633 nm.

oligonucleotide rich in guanine that forms a unimolecular G-quadruplex structure in the presence of certain cations, namely sodium and potassium. It is known for its catalytic properties when complexed with haemin,[2] and can oxidise ABTS to its one electron oxidation product, as shown in Fig. 1(a). This product is a radical anion (Fig. S1, ESI†), which absorbs at 414 nm. A second less intense peak at 650 nm also exists resulting from the cationic form of the oxidation product. The reaction can be monitored both visually and spectroscopically since an intense green product is produced as the oxidation proceeds. This HRP-mimicking DNAzyme can also be used to initiate the oxidation of TMB which involves two successive one-electron oxidation steps (Fig. S1, ESI†).[3] In the first step, the parent diamine is oxidised to a radical cation, which exists in rapid equilibrium with a charge transfer complex (CTC). These species absorb at 370 nm and 650 nm, respectively, and this intermediate has an intense blue colour.

Under acidic conditions the second oxidation step will proceed, resulting in the final diimine, a yellow product that absorbs at 450 nm (Fig. 1(c)). A Raman excitation wavelength of 633 nm was chosen to be resonant with the CTC of TMB and with the radical cation produced from the oxidation of ABTS. Fig. 1(b) and (d) show the spectra obtained from the resonance Raman scattering of the starting materials and oxidation products for ABTS and TMB. It can be seen that the resonant species are greatly enhanced compared to the off resonance starting solutions of ABTS and TMB and the final product for TMB.

To obtain the greatest catalytic activity and hence resonance Raman signal from the substrates, optimisation experiments were essential. Most importantly, the DNAzyme sequence used was shown to exhibit different G-quadruplex structures in the presence of Na^+ ions compared to K^+ ions, and it has been reported that this structural difference can affect catalytic activity.[2,5,6] Two different buffers were initially trialled for both substrates; 10 mM HEPES/10 mM NaCl, pH 7.1 and 10 mM HEPES/10 mM KCl, pH 7.1. The amount of substrate was also varied and it was determined that the greatest absorption intensities were gained using 200 μL of ABTS in the potassium ion buffer, and 100 μL of TMB in the sodium ion buffer (Fig. S2, ESI†). The time allowed for the oxidation reaction to proceed was also important to assess, in particular for ABTS which can revert back to its colourless form over time. Time studies were carried out using UV-Vis spectroscopy to determine the time taken to gain maximum absorbance. As shown in Fig. S3 (ESI†), TMB produced a maximum absorbance after 3 h and remained stable for up to 16 h, while ABTS reached a maximum after 1 h before the absorbance began to rapidly decrease.

The optimum buffer conditions, along with substrate concentration and analysis time, were used to analyse the primary oxidation reaction and a set of controls for both ABTS and TMB to ensure enhancement in the presence of DNAzyme alone. For both substrates there is significant enhancement in signal when all the components needed for HRP-based activity are present (Fig. S4, ESI†). The DNAzyme sequence on its own affords no peroxidase activity, however haemin has catalytic properties and thus a background signal exists. Since HRP, and most peroxidases utilise heme as their cofactor, haemin is an essential component to ensure catalytic activity from an HRP-mimicking DNAzyme. As an iron porphyrin, it is capable of catalysing peroxidase reactions on its own, though at a much lower rate than haemin-utilising enzymes. Our results show that haemin provides a level of catalytic activity, however to a lesser extent than when complexed with the correct sequence of guanine-rich DNA, which is demonstrated in Fig. S4 (ESI†). As a result of this, the control using nonsense DNA that is not rich in guanine, also provides some signal, however this is purely the result of the catalytic behaviour of haemin.

A study was carried out varying the concentration of DNAzyme and monitoring the intensity of resonance Raman signal obtained for both ABTS and TMB. While ABTS followed a linear relationship and TMB a logarithmic one, an observable concentration of DNAzyme at 10 nM was obtained for both substrates (Fig. 2), which encouraged the use of this method of detection within a catalytic beacon based assay protocol. The detection mechanism used was based on that first reported by Willner and co-workers.[12,20] The group demonstrated the success of a hairpin DNA structure that exposes an HRP-mimicking DNAzyme when a target nucleic acid sequence is present. Only in the presence of target DNA will the catalytic G-quadruplex be allowed to self-assemble and activate the peroxidase function (Fig. 3 inset). The group initially used ABTS as the substrate, monitoring the oxidation both visually and using UV-Vis spectroscopy, gaining a detection limit of 0.2 μM. In a later publication Amplex Red was the chosen substrate, which is oxidised to the fluorescent product Resorufin by the DNAzyme, and using fluorescence spectroscopy the detection limit was lowered to 1 nM.[12,20]

Upon addition of a target sequence of DNA, the beacon will change conformation to favour the more stable duplex between the loop sequence and complement. This will leave the full sequence of DNAzyme free to complex with haemin, mimicking HRP and catalysing the chosen substrate, TMB.

Fig. 3 shows the results obtained from incorporating resonance Raman analysis into the catalytic beacon assay protocol using TMB as the substrate, as this was shown to provide better

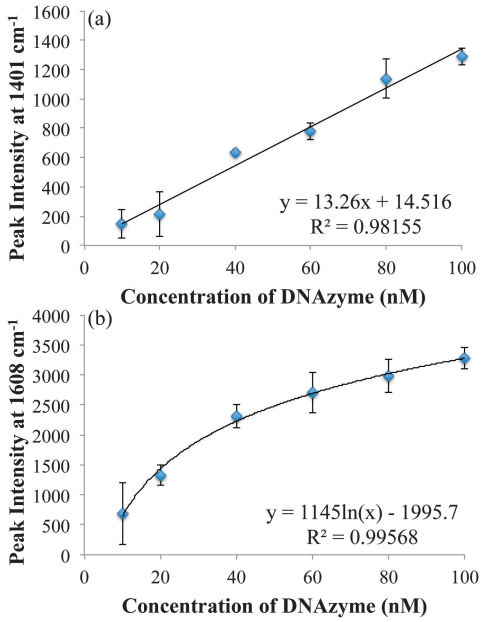

Fig. 2 Concentration studies of DNAzyme activity using (a) 100 μL ABTS in 10 mM HEPES/10 mM KCl, pH 7.1 buffer and (b) 200 μL TMB in 10 mM HEPES/10 mM NaCl, pH 7.1 buffer. 1 h and 3.5 h reaction time was used, respectively, with 0.1 μM haemin and varying concentrations of DNAzyme. Peak heights at 1401 cm^{-1} and 1608 cm^{-1} were analysed by subtracting the background haemin signal from each data point. Error bars represent one standard deviation resulting from 5 replicate sample and 5 scans of each using an excitation wavelength of 633 nm.

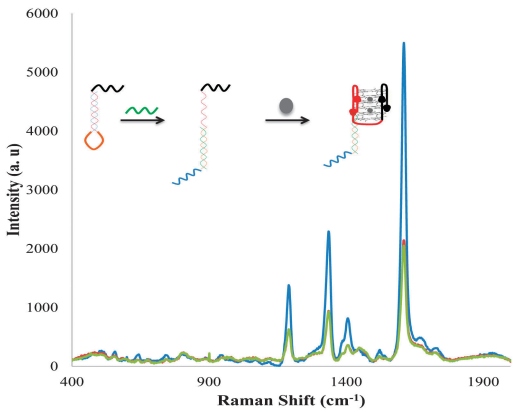

Fig. 3 Detection of 1 μM target by 0.1 μM beacon and haemin using 50 μL TMB in 10 mM HEPES/10 mM NaCl, pH 7.1 buffer after 3.5 h using an excitation wavelength of 633 nm. The blue spectrum represents the presence of target DNA, while green and red represent the no target and nonsense controls, respectively. Inset shows assay schematic. In the absence of target, part of the DNAzyme (red) is locked by a complementary region of DNA (blue) in the stem of the beacon. When the target (green) is present the complementary loop region (orange) hybridises, opening the beacon and allowing the full DNAzyme (black and red) to form and exhibit catalytic activity.

discrimination than ABTS. The amount of substrate was decreased to prevent over oxidation and hence a loss of signal. In the presence of target DNA the main peaks for TMB are enhanced compared to the signal intensities obtained in the absence of target, and with a sequence of DNA non-complementary to the loop region of the beacon. When the target is present the DNAzyme is free to oxidise the TMB to its CTC, which is resonant with the laser excitation wavelength of 633 nm. The signal seen from the control samples that contain no target or a nonsense sequence of DNA is purely the result of the oxidation capabilities of haemin. This protocol was used to detect 10 nM concentration of DNA (Fig. S5, ESI†). Additionally, a 99 base sequence of post-PCR DNA was used within the catalytic beacon assay to successfully detect a clinically relevant synthetic target correlating to the *MecA* gene of MRSA (Fig. S6, ESI†).

The oxidation products of both TMB and ABTS have been successfully analysed by resonance Raman scattering after interaction with a DNAzyme. RRS was also used to detect target DNA using a DNAzyme based assay protocol. To the best of our knowledge this is the first reported case of the use of resonance Raman spectroscopy for detecting oxidation by an HRP-mimicking DNAzyme, and this technique should be considered an alternative method when using DNAzymes along with peroxidase substrates for the sensitive detection of analytes.

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