DNAzyme Sensor for the Detection of Metal Ions using Resistive Pulse Sensing

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DNAzymes are DNA based catalysts that can undergo cleavage upon binding of the target analyte. The cleavage reaction is highly specific, and DNAzymes exist for a wide range of metal ions. The change of structure upon binding of a specific metal ion has given rise to many sensing strategies, but few exist with nanopore sensors. Resistive Pulse Sensing, RPS, is a platform that has emerged in recent years capable of identifying changes in DNA structure and sequence. Here we develop the use of DNAzymes with RPS technologies for the detection of Ca^{2+} ions in solution. Ca^{2+} plays an important role in biological processes, critical for cell signaling, protein folding and catalysis. Extreme concentrations of Ca^{2+} within drinking water have also been linked to problems with corrosion, scaling and the taste of water. Using DNAzyme functionalised nanocarriers and RPS, it was possible to follow the Ca^{2+} ions binding to the DNAzyme. The binding of Ca^{2+} caused a conformation change in the DNAzyme which was monitored as a change in translocation speed. By following the changes to the translocation speed, it is hypothesised that RPS can verify the changes in structure. In addition, the assay allowed the quantification of Ca^{2+} between 1 – 9 μM , and due to its catalytic nature, increasing incubation time from 30 to 90 minutes allowed lower detection limits, down to 0.3 μM . We demonstrate that the speed changes are specific to Ca^{2+} in the presence of other metal ions, and we can quantify Ca^{2+} in tap and pond water samples.

Calcium (Ca^{2+}) is one of the most abundant metals in the human body, making up to 2%wt of total human body weight, and plays a fundamental role in biological processes including secondary messengers critical for cell signaling, protein folding and catalysis.¹⁻⁴ It has also been demonstrated how Ca^{2+} ions are important within the environment as very high or very low concentrations within drinking water have been linked to problems with corrosion, scaling and the poor taste.⁵ It also represents a hazard to the environment due to its high environmental mobility and bioavailability.⁶ When unregulated, high concentrations of Ca^{2+} can cause various diseases such as hypercalcaemia,⁷ and heart disease,⁸ while low levels can lead to deficiencies such as osteoporosis,⁹ and muscle and nerve tightening.¹⁰ Many different methods have been developed for the detection of Ca^{2+} ions in solutions, such as ion selective electrodes,¹¹ flame atomic absorption spectrometry,¹² fluorescence,¹ and colorimetric methods.¹³

There are many recognition elements that bind to Ca^{2+} with high specificity which have been integrated into various sensing platforms, such as Ca^{2+} binding proteins,^{7,14,15} magnetic nanoparticles,¹⁶ and glycosphingolipids.¹⁷ A new category of recognition ligands to heavy metals include DNA aptamers and DNAzymes. DNAzymes are DNA-based catalysts; all known DNAzymes have been selected through *in vitro* selection.^{8,18,19} There are many DNAzymes that require divalent metal ions such as Pb^{2+} ,²⁰⁻²² Zn^{2+} ,^{23,24} Cu^{2+} ,^{25,26} and UO_2^{2+} ,^{27,28} for activity. DNAzymes have several advantages compared to enzymes commonly used within biosensing, they can easily be grafted onto nanoparticles, are more stable at ambient conditions and can amplify detection due to their catalytic nature.²⁹ DNAzymes have been widely used as they have simple reaction conditions and significant changes in structure.³⁰ Previous work has utilised fluorescence sensing to monitor interactions between the target metal ion and DNAzyme.^{3,31,32}

Resistive pulse sensing (RPS), is a sensing technology capable of probing changes in DNA structure.^{33,34} RPS has a broad range of application including material characterisation,³⁵⁻³⁸ quantification of DNA/peptide analyte interactions,^{39,40} and biosensing.⁴¹⁻⁴³ RPS measures speeds of nanocarriers as they translocate a nanopore and through changes in nanocarrier speed are able to infer carrier binding with target analyte.⁴⁴⁻⁴⁶

The integration of DNAzymes onto nanocarriers presents a new method of analysis, as the cleavage of the DNAzymes could be identified and characterised. RPS has demonstrated the ability to measure speed changes between double and single stranded DNA.³³ There have also been other studies which have utilised DNAzymes with a-hemolysin and solid-state nanopores.⁴⁷⁻⁴⁹

Here we present the use of a DNAzyme with RPS to detect Ca^{2+} ions in solutions. Using RPS's ability to identify changes in structure we were able to measure the cleavage of the DNAzyme by Ca^{2+} as previously reported,^{3,50,51} and take this further by infer the rearrange of the DNAzyme structure post cleavage. The catalytic nature of the DNAzyme allowed for the assay limits of detection to be tuned dependent on incubation times, as well as allowing quantification of Ca^{2+} when incubation times are kept constant. We were also able to demonstrate our assay is specific to Ca^{2+} observing no interferences from a mix of divalent ions. Finally, we showed the ability of our assay to be deployed in real environmental samples through the quantification of Ca^{2+} in both tap and pond water samples.

Figure 1. (a) Schematic of a particle traversing the RPS device and the signal produced. Blockade magnitude (ΔI_p), and full width half-maximum (FWHM) are shown. (bi) Schematic of the DNAzyme once both strands have bound together, the substrate strand (blue) and the enzyme strand (orange), (bii) binding with two Ca^{2+} ions and cleaving, (biii) rearrangement of DNAzyme post cleavage. (c) Example of a baseline current and blockade events caused by the carriers translocating the pore from 10 to 30 s, inset showing 17 to 21 s and two example pulses.

Table 1: Table of the two different DNAzyme strand sequences and lengths.

	DNA sequence	
Substrate strand	[Bt _n]GTCACGAGTCACTATRAGGAAG ATGGCGAAA	31 mer
Enzyme Strand	GCCATCTTTTCTCACAGCGTACTCG CTAAGGTTGTTAGTGACTCGTGAC	49 mer

Experimental

Chemicals and reagents

Calcium(II) chloride and potassium chloride were purchased from Fisher Scientific, UK. Carboxylated polystyrene particles were purchased from Bangs Laboratories US, denoted as CPC200 (mode diameter 210 nm, measured concentration 1×10^{12} particles mL^{-1}). Nanopores were purchased from Izon Science Ltd NZ. Reagents were prepared in deionised water (Elga PureLab), with a resistance of 15 M Ω cm. 2-(N-Morpholino)ethanesulfonic acid (MES) and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Lithium Chloride, nickel sulphate hexahydrate, iron chloride, magnesium chloride, and TWEEN 20 were purchased from Sigma-Aldrich, UK. Streptavidin-modified magnetic particles, 120 nm, were purchased from Ademtech, France.

Custom DNA oligonucleotides

Two custom DNA oligonucleotides were purchased from Sigma-Aldrich, in lyophilised form, purified using reverse-phase cartridge purification by the manufacturer. The two oligonucleotides ordered were; GCCATCTTTTCTCACAGCGTACTCGCTAAGGTTGTTAGTGACTCGTGAC (enzyme strand) and biotin-TCACGAGTCACTATRAGGAAGATGGCGAAA (substrate strand), they were diluted to 100 μM stock solutions using deionised water.

Particle preparation

The DNAzyme complex was first formed by heating the substrate strand, 2.5 μL , with the enzyme strand, 5 μL , in 50 mM MES buffer (pH 6 with 25 mM LiCl) with 0.05 % TWEEN 20 added, at 70 °C for 2 min followed by cooling. Once cool, streptavidin modified particles were added to the solution and left to bind for 30 min. Once bound the particles were placed on a MagRack (Life Sciences) until a clear cluster of particles was seen, the buffer was then removed and replaced with 10 mM HEPES, pH 7.6, 50 mM LiCl, buffer with 0.05% TWEEN 20 added.

RPS set up

A qNano (Izon Science Ltd, NZ) was used to complete all the measurements for this study. A qNano uses data capturing software (Izon Control Suite v3.3) to record the particles as they traverse the pore. The lower fluid cell contained 80 μL of KCl solution and the upper fluid cell contained 40 μL sample solution. After each measurement was taken the nanopore was cleaned by first rinsing the upper fluid cell with background buffer before the buffer was removed and replaced multiple times each time a different pressure or vacuum was applied. The nanopore stretch was also varied wider and narrower; this was done until there weren't any residual particles observed in the system ensuring no cross contamination between the samples. The qNano was operated with a positive bias, i.e. the positive electrode in the lower fluid cell and the negative electrode in the upper fluid cell, so the particles traverse the pore towards the positive electrode unless otherwise stated. For all experiments an NP200 nanopore was used able to analyse particles from 85-500 nm, more than 200 particles were measured for each sample. To account for any manufacturing variation between pores the baseline current was kept constant throughout all experiments, and the stretch was slightly changed to ensure this, with

max 10% difference in baseline between sample runs. Each pore was first characterised using the calibration particles so day-to-day differences could also be accounted for.

Saturation point testing

Particles were functionalised with different amounts of the DNA substrate strand added to determine the saturation point. Heating the substrate and enzymes strands together formed the DNAzyme before different amounts were added to the particles to determine DNAzyme forming and saturation point. Finally, post heating of the two DNA strands they were left to cool in different places before being added to particles to determine best cooling practise.

Binding time

DNAzyme particles were functionalised as above and Ca^{2+} was added to make 1 μM solutions. The samples were placed on a rotary wheel and at 5, 10, 20 and 30 min an aliquot was taken out and vortexed before being analysed.

Metal ion interferences

Metal ion stocks were prepared from MgCl_2 , CrCl_3 and FeCl_3 . These were added to DNAzyme functionalised particles to give 3 μM solutions, a mix was also prepared which included Ca^{2+} . Samples were left on a rotary wheel for 30 min before being vortexed and analysed.

Environmental water samples

Water samples were collected from a tap, and an outdoor pond, DNAzyme particles were functionalised as above and added to the water samples. The samples were also spiked with Ca^{2+} to give a final concentration of 3 μM . They were then left on a rotary wheel for 30 min before being magnetically separated and resuspended in buffer before analysis.

Particle speed

Particle speed through the pore was calculated from the pulse width. As particles translocate the nanopore they produce a pulse, the maximum magnitude of this pulse is recorded as $T_{1.0}$ as this magnitude decreases multiple time points are recorded correlating to $T_{0.9}$, $T_{0.8}$, $T_{0.7}$ etc. here we use the reciprocal of the value at $T_{0.5}$ (width of the pulse at 50% peak height) to determine relative particle speed. These values were then normalised to either calibration particles (CPC200s) or blank peptide functionalised particles ran on the same day, using the same nanopore and experimental conditions, normalisation was done by running multiple calibration runs, getting an average setting this as 1 then dividing the sample data by this to get a ratio. This was done to account for any differences in measured speed between different pore and different days.

Results and Discussion

Within RPS experiments each translocation of a carrier through the nanopore produces a pulse, figure 1a. The magnitude of the pulse, known as the pulse magnitude, ΔI_p , is related to the volume of the carrier, and the width or full width half maximum, *FWHM*, of the pulse relates to its velocity.³³ In the absence of convection, the velocity of the carrier can be proportional to the surface charge or zeta potential of the carrier, assuming electro osmosis remains constant.^{33,52} Thus RPS allows the quantity and structure of the DNA on the nanocarrier to be analysed.³³

The DNA sequences of the two DNA strands are shown in Table 1, the two strands are labelled *substrate* and *enzymes*. The structure of the Ca^{2+} DNAzyme was previously reported by Yu et al. after systematic mutation to identify the optimal sequence, this is shown schematically in Figure 1bi.³ The substrate strand has a single RNA linkage (rA) that operates as the cleavage site. Upon binding with two Ca^{2+} ions,⁵¹ the substrate strand cleaves and rearranges changing from dsDNA to ssDNA, figure 1bii.^{3,4} RPS can differentiate from dsDNA and ssDNA through changes in nanocarrier translocation speed.³³

Previous work from our group has shown the technique is cable of differentiating between the location and position of dsDNA on a mixed ss/dsDNA strand.³³

Figure 2: (a) Determining the concentration of substrate strand DNA required to saturate the nanocarriers through changes in carrier speed. (b) Determining if the DNAzyme complex had formed post heating through the differing saturation points of the substrate strand vs DNAzyme complex.

Here the hypothesis was that the binding of Ca^{2+} ions to the DNA would cause a change in the tertiary structure that could be followed using RPS. The substrate strand binds to the nanocarrier via the biotin-avidin interaction. To confirm the immobilisation of the DNAzyme on the carrier, the saturation concentration of substrate strand DNA was first measured to be 120 nM, figure 2a. Note that more DNA may attach to the carrier's surface but the signal does not change above this concentration. Due to the double stranded nature of the DNAzyme and the lower packing density of dsDNA, it was expected a lower concentration of DNAzyme would be required to cover the same surface area. This was measured as a decrease in saturation point from 120 to 60 nM, figure 2b. It is important to note that the binding of the DNAzyme to the carriers did not result in a change in the pulse magnitude

indicating changes in nanocarrier speed were down to differences in carrier charge, not aggregation or disaggregation, figure S1. Alternative methods to ensure a high grafting density of the dsDNA on the carrier used different cooling cycles to ensure the DNAzyme was in its correct structure, the data is shown in figure S2, showing slight differences between different cooling methods with slow cooling showing the fastest speed indicating more DNAzyme has been immobilised on the surface.

Figure 3: (a) Measuring the change in DNAzyme functionalised nanocarrier velocity versus different incubation times with Ca^{2+} ions, ai, blank DNAzyme complex on the bead, aii, cleavage of the DNAzyme from dsDNA to ssDNA and, aiii, DNAzyme rearranging. Samples were ran in triplicate, more than 200 particles were measured each time. Error bars are one standard deviation from the mean

The Ca^{2+} DNAzyme was chosen as it has been shown to aid the detection Ca^{2+} in solutions using fluorescence spectroscopy.³ Once the Ca^{2+} binds to the DNAzyme complex, a section of 10 base pairs of DNA is cleaved, Figure 1bi and bii. If the reported mechanism is correct, it was hypothesised that as the DNA cleaves, changing from dsDNA to ssDNA, it would result in the decrease in nanocarrier translocation speeds.³³

This theory was tested by monitoring the DNAzyme modified nanocarriers speeds at different time points after incubation with Ca^{2+} . As can be seen in figure 3aii, after the initial 5 minutes we measured a significant decrease in nanocarrier speed, from 1 to 0.84 ms^{-1} , before a rapid increase and plateauing after 10 minutes to 1.23 ms^{-1} . We postulated that this increase in speed was due to the DNAzyme rearranging post cleavage by Ca^{2+} , as the most stable form of the enzyme strand is extended, figure 1biii,⁵³ this increase in DNA length would increase particle speed significantly, and correlates to previous studies.³³ This increase in speed is due to the increase in distance from particle surface allowing the overhanging DNA to coil/unfold thereby reducing the number of counter ions shielding the DNA which in turn increases the particle charge density, figure 3aiii. The speed of nanocarriers with different hybridisations in the presence/absence of Ca^{2+} is shown in figure S3. This demonstrated how the observed increase in nanocarrier speed is specific to our DNAzyme hybridised nanocarriers.

Figure 4: (a) Relative particle speeds normalised to blank DNAzyme functionalised particles, versus varying amounts of Ca^{2+} ions added from 1 – 9 μM , measured at 30 mins. (c) 0.3 μM Ca^{2+} ions added, incubated from 30 to 150 minutes. Samples were ran in triplicate, more than 200 particles were measured each time. Error bars are one standard deviation from the mean.

Figure 4 demonstrates that the change in nanocarrier speed is proportional to the concentration of Ca^{2+} present in solution. The amount of DNAzyme hybridised onto the nanocarrier surface was kept constant, only the Ca^{2+} concentration was varied, incubation times remained the same. As shown in Figure 4a, as the concentration of Ca^{2+} increases, the speed of the nanocarriers increases. This constant speed is stable over a large concentration range, from 3 μM , 1.23 ms^{-1} , to 3000 μM , 1.27 ms^{-1} , demonstrating the end point of the reaction, figure S4. Due to the catalytic nature of DNAzymes we also tested a lower concentration of Ca^{2+} ions, 0.3 μM , with varying incubation times from 30 to 150 minutes. As shown in Figure 4b, as incubation times increases so did the nanocarrier speed until after 90 minutes when the speed becomes constant, 1.25 ms^{-1} and 1.23 ms^{-1} for 90 and 150 minutes respectively. We also expect that with shorter incubation times it would be possible to measure higher concentrations. No aggregation was seen, and the speed increase was reproducible over different nanocarriers batches figure S5 and figure S6.

Figure 5: (a) Relative speed of the DNAzyme functionalised particles when incubated with Ca^{2+} , Mg^{2+} , Fe^{3+} , Ni^{2+} and in a mix. All metals were present at 3 μM . (b) Environmental water samples, blank (orange), and spiked with 3 μM Ca^{2+} (green). Relative particle speeds normalised to blank DNAzyme functionalised particles, ran on the same day under the same conditions. Samples were ran in triplicate, more than 200 particles were measured each time. Error bars are one standard deviation from the mean.

To demonstrate the selectivity of our assay was dependent upon the Ca^{2+} binding to the DNAzyme, we incubated our DNAzyme functionalised particles with different metal ions. As the previous study had reported some binding with Mg^{2+} ,⁵¹ so we tested Mg^{2+} at 3 μM incubated for 30 minutes, and 5 mM tested every hour over 6 hours, S7, to confirm there was no interferences with our assay. As seen in Figure 5a, there was no inferences measured from any of the metals tested at 3 μM , and when present in a mix with Ca the measured speed was within the expected range. Although some slight binding was overserved with 5 mM Mg^{2+} , this was only measured after 4 hours and not to a comparable speed as with Ca^{2+} present at 3 μM , 1.09 vs. 1.21 ms^{-1} respectively.

Finally, to demonstrate the applicability of our assay in more complex sample matrices, we collected water samples from a tap in the laboratory and from a local pond on campus. To each sample we added DNAzyme functionalised particles, incubated for 30 minutes before magnetically separating them and resuspending in buffer to analyse, we also incubated the nanocarriers in water samples spiked with 3 μM Ca^{2+} . As shown in Figure 5b, the nanocarriers increased significantly in speed when compared to the blanks, indicating the ability of our assay to work in these different matrices, without experiencing any interference from other analytes present.

Conclusion

Here we present RPS technologies inferring structure changes in the DNAzyme through differences in nanocarrier speed, enabling quantification of Ca^{2+} in solution. The cleavage of DNA post binding of the Ca^{2+} to the DNAzyme is measured through the increase in nanocarrier speed as it traverses the nanopore. The method works across a large concentration range, tunable through incubation times, and can work in environmental samples. The assay from nanocarrier functionalisation, incubation extraction and quantification in under an hour.

Associated Content

Supporting Information

Differences in blockade magnitudes of nanocarriers with the substrate strand and DNAzyme complex. Comparison of different cooling methods to form the DNAzyme complex. Comparison of CPC200, 15T DNA and DNAzyme particle speed with and without Ca^{2+} present. DNAzyme nanocarriers in the presence of 3 to 3000 μM Ca^{2+} . Differences between different batches of nanocarriers with and without 3 μM Ca^{2+} present. Frequency vs blockade magnitudes in the presence of Ca^{2+} . DNAzyme functionalised nanocarriers incubated with 5 mM Mg^{2+} from 0 to 6 hours.

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