In Vitro Selection in Serum: RNA-Cleaving DNAzymes for Measuring Ca^{2+} and Mg^{2+}

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Abstract

RNA-cleaving DNAzymes have been attempted as in vivo analytical probes and gene silencing reagents over the last two decades. Despite progresses already achieved, concerns still exist regarding the activity of DNAzymes in biological fluids. An example is the low activity of the 10-23 DNAzyme in intracellular Mg^{2+} concentrations. To obtain DNAzymes that work optimally in biological samples, we herein report the first DNAzyme in vitro selection in undiluted human blood serum. The selection starts with a large DNA library containing 50 random nucleotides, and sequences that can be cleaved in serum were isolated and amplified. After deep sequencing analysis, 80% of the final library are a variant of the 8-17 DNAzyme (named 17EV1). The main difference between 17E and 17EV1 is a single mutation at the N_{12} position of the catalytic core. 17EV1 is ~6-fold faster in serum than 17E, since 17EV1 is preferentially activated by Ca^{2+} and serum is rich in Ca^{2+} over Mg^{2+}. On the other hand, 17E has a similar activity with Ca^{2+} or Mg^{2+}. With this observation, a method for measuring the Ca^{2+}/Mg^{2+} ratio was developed by combining the 17E and 17EV1 DNAzymes. This study demonstrates the feasibility of selecting DNAzymes in biological fluids and will facilitate the application of DNAzymes in bioanalytical chemistry and gene therapy.

Keywords: SELEX; DNAzymes; RNA; metal ions; biosensors; serum; aptamers

DNA-based catalysts (DNAzymes) have found important analytical applications for metal detection in the last two decades.[1] In particular, DNAzymes for RNA cleavage were used most often, which is attributable to their excellent metal specificity, catalytic activity, programmability, and stability.[2-5] While most studies were focused on environmental metal analysis, in recent years we see a growing trend of using DNAzymes for biological samples, such as serum,[6, 7] cells,[8-14] and cell extracts.[15]

Ca^{2+} and Mg^{2+} are the most abundant metal ions in biological fluids, and they are critical in cell signaling, protein folding, and catalysis. Therefore, their detection has been a long-standing topic of bioanalytical chemistry.[16-18] However, using DNAzymes for their analysis was rarely explored.[6, 7] DNAzymes might offer better metal selectivity, and using multiple DNAzymes, both ions might be detected simultaneously. In fact, using Mg^{2+}-dependent DNAzymes for cleaving viral RNA has been proposed since 1994.[19-21] In vitro DNAzyme selection using Ca^{2+} was also attempted.[22] A lot of excitements were brought to the field by Santoro and Joyce in 1997, reporting two efficient DNAzymes that cleave full RNA with Mg^{2+}.[20] In particular, the 10-23 DNAzyme was studied for intracellular RNA cleavage because of its excellent substrate generality.[11] The 8-17 DNAzyme was researched more as a model biosensor.[2]

However, concerns have been recently raised regarding the in vivo activity of these DNAzymes.[23] For example, the 10-23 DNAzyme is hardly active with the physiological concentration of Mg^{2+} (<1 mM).[24, 25] In contrast, most of their biochemical assays were carried out with 10-50 mM Mg^{2+}. For both analytical and biomedical applications, obtaining highly active DNAzymes in physiological conditions is critical.

While many DNAzyme selections were carried out previously, most were performed in clean buffers with well-defined chemical compositions. We reason that improvement might be achieved by performing in vitro selections directly in target biological fluids. This idea has been used to obtain aptamers for cells and tissues under physiological and native conditions.[26, 27] The closest examples for DNAzymes are probably the selections performed in bacterial cultures.[28, 29] We recently demonstrated that DNAzymes and their substrates have sufficient stability in serum.[6] This might be related to DNA binding proteins in serum, which inhibit the activity of nucleases.[30] This study also encouraged us to perform selections in serum. In this work, our primary goal was to test the feasibility of selecting DNAzymes in undiluted human blood serum. The secondary goal is to use the selected DNAzymes for the analysis of Ca^{2+} and Mg^{2+}.

Materials and methods

Chemicals. The DNA samples for in vitro selection were from Integrated DNA Technologies (Coralville, IA), and the other DNAs were from Eurofins (Huntsville, AL). See Table S1 for the sequences and modifications of DNA. T4-DNA ligase, dNTP mix, Taq DNA polymerase and DNA ladder were from New England Biolabs. SsoFast EvaGreen supermix was from Bio-Rad. Tris(hydroxymethyl) aminomethane (Tris) and sodium chloride were from Mandel Scientific (Guelph, ON, Canada). All metal salts were from Sigma-Aldrich with the highest available purity. Human AB serum was from Mediatech (Manassas, VA, USA).

***In vitro* selection**. In vitro selection was performed following a previously published protocol with slight modifications.[31] Briefly, the initial DNA pool was prepared by ligating Lib-FAM-N_{50} (0.2 nmol) and Lib-rA (0.3 nmol) with splint DNA (0.3 nmol) using T4 ligase by following the

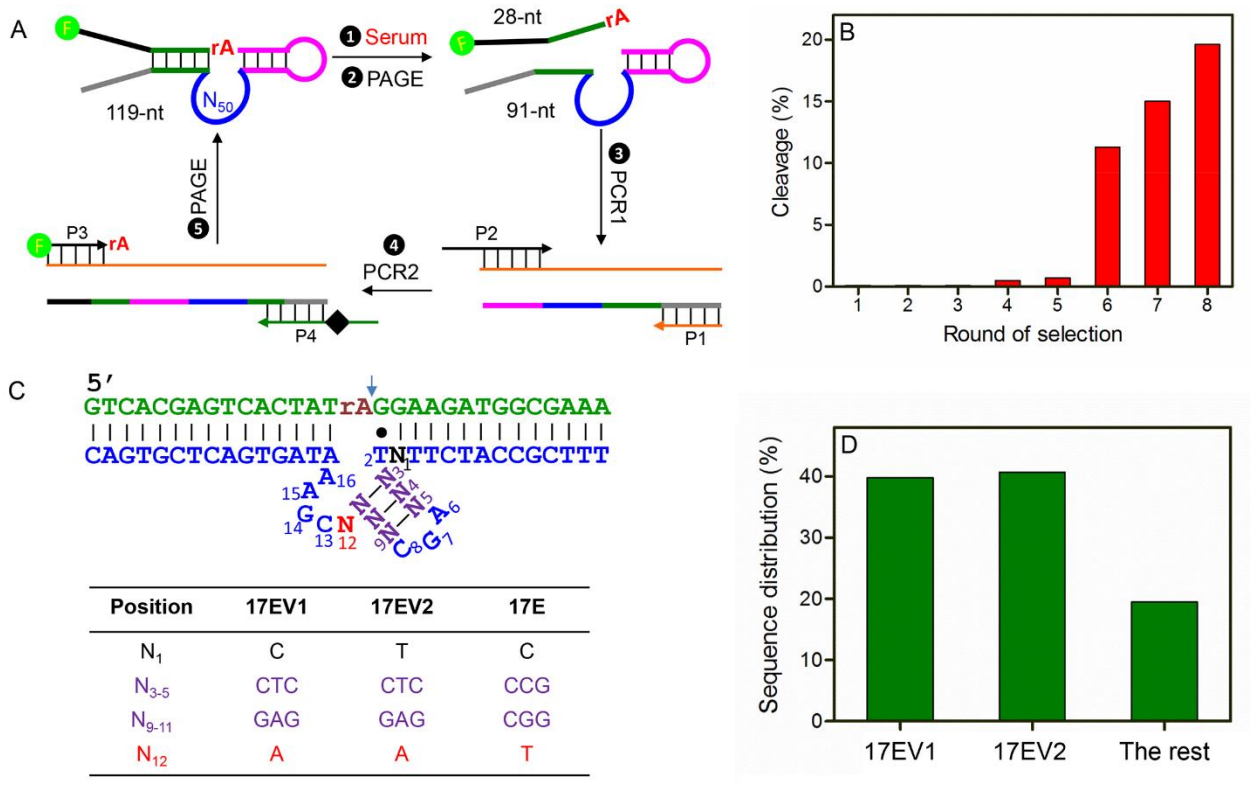
vendor's protocol (see Table S1 for the sequence of DNA). After denaturing polyacrylamide gel electrophoresis (dPAGE) purification, this library was dissolved in 60 μL buffer A (50 mM MES, pH 6.0, 25 mM NaCl) with a DNA concentration of ~2.5 μM. For the first round of selection, 2 μL of the DNA library was added to 10 μL human AB serum. The incubation time for each round is summarized in Table S2. The reaction was then quenched with 8 M urea with heating at 80 °C for 3 min. The cleaved DNA sequences were separated by 10% dPAGE, desalted using a Sep-Pak column, and vacuum dried for PCR following reported previously conditions.[31] The dried products were dissolved using human serum to initiate next round of selection. The round 8 cleavage products were analyzed using deep sequencing. For the negative selections to eliminate the 17E DNAzyme, the PCR products were treated with a metal mixture containing 2.5 mM Ca^{2+} and 1 mM Mg^{2+} (selection A) or 10 μM Pb^{2+} (selection B). The uncleaved DNA was then harvested for the positive serum selection.

Activity assays. To test DNAzyme activity in serum, FAM-labeled substrate (10 μM, FAM-Sub) and enzyme (20 μM, 17E or its variants) were annealed in buffer B (140 mM NaCl, 50 mM Tris, pH 7.5) to form DNAzyme complexes (See Table S1 for each DNA sequence). Then, 1 μL of the annealed complex was added to 9 μL serum to initiate the cleavage reaction. The reaction was quenched by transferring 2.5 μL of the reaction mixture to 14 μL urea (8 M) followed by heating at 80 °C for 3 min. The cleaved products were separated using 15% dPAGE gel and quantified by a ChemiDoc MP imaging system (Bio-Rad). To measure DNAzyme activity in buffer, the procedure was the same, except that dilution was made using buffer B instead of serum, and the reaction was initiated by adding divalent metal ions.

Results and Discussion

In vitro selection of DNAzymes in serum. So far, all known DNAzymes were obtained via in vitro selection. To select DNAzymes in serum, we used a protocol similar to the normal selections,[31] except that undiluted human blood serum was used to induce cleavage without additional buffer or metal ions added. The selection library contained 50 random nucleotides, and this random region was positioned near the rA (ribo-adenine) by two short base-paired duplexes (Figure 1A). Note this rA is the only RNA nucleotide in this DNA library, serving as the cleavage site since RNA has much lower stability compared to DNA. See Figure S1 for the full library sequence. After added serum, active sequences that underwent self-cleavage were separated from the uncleaved DNA using gel electrophoresis, and amplified by two steps of PCR. The cleavage yield at each selection round was monitored (Figure 1B) and a significant jump was achieved at round 6. The round 8 library was deep sequenced, yielding 65,559 sequences.

Interestingly, the library was dominated by two vary similar variants of the 8-17 DNAzyme (denoted as 17EV1 and 17EV2, respectively). Figure 1C shows the general secondary structure of the 8-17 DNAzyme. 17EV1 and 17VE2 differ only by a single nucleotide at the N_1 position, which is for substrate binding. Therefore, they are essentially the same enzyme. Statistical analysis revealed that 17EV1 and 17EV2 are about equally populated (each ~40% of the final library, Figure 1D). For the remaining 20%, we tested 18 representative sequences in their trans-cleaving forms (Table S1, New1-18), but none was active (Figure S2). If we disregard the difference between 17EV1 and 17EV2, they differ from 17E only in two aspects: 1) the base composition in the 3 base-pair stem (in purple, Figure 1C), and 2) the N_{12} position (in red).



Position	17EV1	17EV2	17E
N_1	C	T	C
N_{3-5}	CTC	CTC	CCG
N_{9-11}	GAG	GAG	CGG
N_{12}	A	A	T

Figure 1. (A) A scheme of an in vitro selection cycle in serum. The cleavage step (step 1) is carried out by dissolve the DNA library in serum followed by incubation. The cleaved library is harvested using PAGE and amplified by two steps of PCR. The 'F' label in P3 denotes for a carboxyfluorescein (FAM) fluorophore. The black diamond in P4 is a polymer spacer to stop polymerase extension so that this strand is longer and can be removed after PAGE at step 5. (B) The yield of cleavage for each selection round. (C) The general trans-cleaving structure of the 17E DNAzyme. The difference between 17E and 17EV1/17EV2 are tabulated. The arrowhead indicates the cleavage site. (D) Sequence distribution in the final library.

We recently reported activity of the 17E DNAzyme in undiluted serum.[6] Therefore, it is not surprising that variants of 17E were selected here. In fact, 17E variants have been isolated

from many previous selections using different metal ions,[20, 22, 32-34] which is attributed to its small size, tolerance to mutation, and high activity.[35, 36] Unlike previous selections done in clean buffers, serum contains many other chemicals (e.g. proteins, nucleic acids, and small molecules). From our results, these chemicals did not play a major role in directing the selection outcome using the current library design.

To test whether it is possible to eliminate 17E, we also performed extensive negative selections (Figure S3). In one attempt, the library was incubated with Pb^{2+} for negative selection (sequences cleaved with Pb^{2+} were discarded), since Pb^{2+} is very efficient in cleaving 17E.[37] In another attempt, a mixture of Ca^{2+} and Mg^{2+} was used for negative selection. In some rounds, up to four consecutive negative selections were performed before the positive serum selection. However, the resulting DNAzymes were still mainly the 17E variants (data not shown). Therefore, this study adds another example supporting the isolation of 17E under physiological conditions. It does not mean 17E is the only solution for activity in serum, but because of its properties mentioned above, it is easy to dominate the library. It might be possible to avoid 17E by using other cleavage junctions,[38] or by labeling fluorophore/quencher near the cleavage junction.[39]

Activity of the 17E variants in serum. To rationalize our selection results, we tested the activity of these two variants in serum. Both 17EV1 and 17EV2 achieved a similar cleavage rate of 0.14 min^{-1} and 0.15 min^{-1}, respectively (Figure 2A). This is expected since they differ only at the non-essential N_1 position. In contrast, the 17E DNAzyme was 6-fold slower (0.025 min^{-1}). We previously reported 0.04 min^{-1} for 17E in serum,[12] and this slight variation is attributed to a lower DNAzyme concentration used here. This activity assay also partially explains the appearance of 17EV1 and 17EV2 instead of 17E. Although we did not isolate a completely new

DNAzyme, our selection has improved the enzyme activity by 6-fold in the serum. This study also supports the feasibility of in vitro DNAzyme selection in serum.

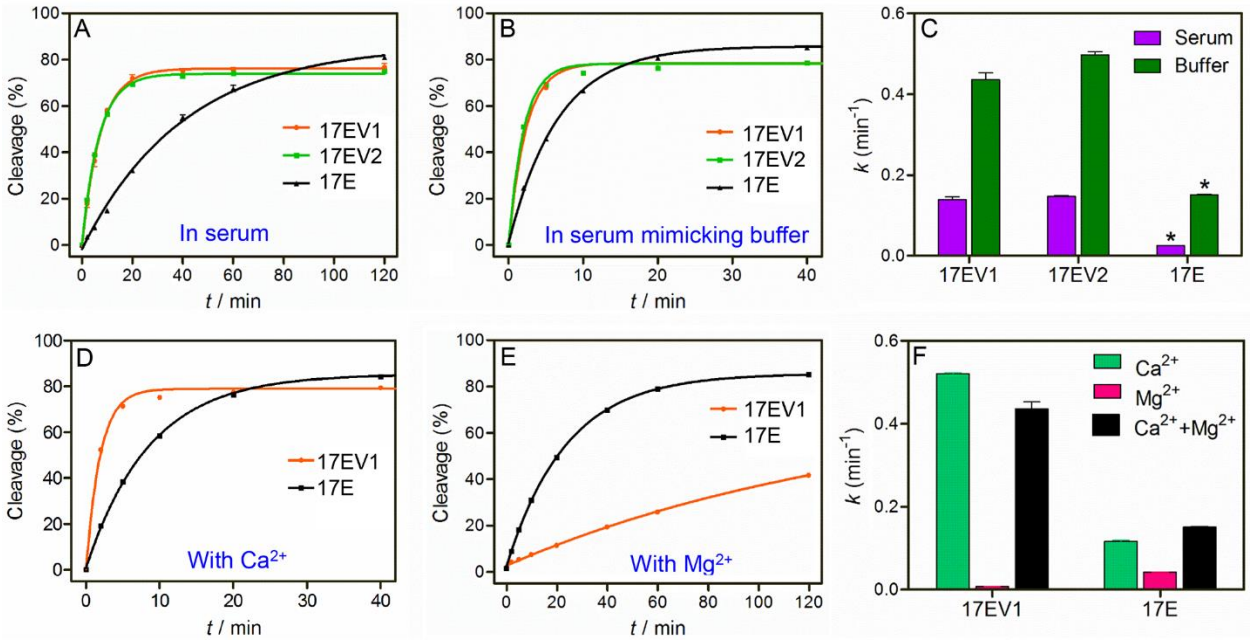


Figure 2. Kinetics of substrate cleavage by the three DNAzymes in (A) serum and (B) buffer (2.5 mM Ca^{2+}, 1.1 mM Mg^{2+}, 50 mM pH 7.5 Tris). (C) Cleavage rates of the three DNAzymes in serum and the buffer. Cleavage kinetics of the 17EV1 and 17E DNAzymes in presence of (D) 2.5 mM Ca^{2+} or (E) 1.1 mM Mg^{2+}. (F) Cleavage rate comparison of 17EV1 and 17E in presence of 2.5 mM Ca^{2+}, 1.1 mM Mg^{2+} or 2.5 mM Ca^{2+} plus 1.1 mM Mg^{2+}.

Activity of 17E variants in buffer. To rationalize the rate difference of the 17E variants in serum, we also measured their activity in buffer. We prepared buffers to mimic the serum metal composition, containing 142 mM NaCl, 2.5 mM Ca^{2+} and 1.1 mM Mg^{2+}.[6] Similarly, no significant difference was observed in the activity of 17EV1 (0.44 min^{-1}) and 17EV2 (0.50 min^{-1}) (Figure 2B, C), but both variants were ~3-fold faster in the buffer than in serum. The slower rate

in serum might be caused by DNA binding proteins in serum (e.g. some proteins can bind guanine-rich DNA),[6, 40] or by proteins sequestering metal ions.

Since the above buffer contained both Ca^{2+} and Mg^{2+}, we next studied each metal ion individually to dissect the effect. Since 17EV1 and 17EV2 are quite similar, only 17EV1 was studied. In the presence of 2.5 mM Ca^{2+}, a rate of 0.52 min^{-1} was observed for 17EV1, 4.3-fold faster than 17E (0.12 min^{-1}, Figure 2D), consistent with the result in serum. For comparison, 17EV1 was 0.007 min^{-1} in 1.1 mM Mg^{2+}, 6-fold slower than 17E (Figure 2E). This result is very interesting; the mutation from T_{12} to A_{12} in the catalytic core inverted the selectivity for Ca^{2+} and Mg^{2+}. Mutating T_{12} to A_{12} increases the Ca^{2+} activity but decreases the Mg^{2+} activity. Serum has a higher Ca^{2+} concentration and thus the DNAzyme activity in serum is dominated by Ca^{2+}. This difference might provide a way for measuring both metal ions using a ratiometric method.

Since 17E is active with many divalent metal ions, an interesting question is the activity difference caused by this N_{12} position using other metal ions. It was previously reported that with this position being an adenine, the activity is optimal with Cd^{2+}, Zn^{2+} and Mn^{2+}.[34] We measured the activity of 17E and 17EV1 in the presence of Pb^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Fe^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+} and Ce^{3+}. Aside from Ca^{2+}, only Cd^{2+} and Pb^{2+} showed better activity for 17EV1 than 17E, while the activity with Sr^{2+}, Ba^{2+}, Cu^{2+}, and Ce^{3+} were too low to compare (Figure S4). Earlier studies pointed out that the N_{12} position plays two crucial roles in 17E.[41] First, it acts as spacer between the short stem and the conserved C_{13} to ensure that C_{13} is at the most adequate position to carry out the catalytic function. In addition, N_{12} acts as a chaperon to correct folding of the DNAzyme.[41] Charge density, ionic radius and coordination pattern of difference metal ions may contribute to the observed difference.

To understand the contribution of each metal to activity, we then compared the DNAzymes activity in the presence of Ca^{2+}, Mg^{2+} and the mixture of Ca^{2+} plus Mg^{2+} (Figure 2F). For 17EV1, the fastest cleavage was achieved with 2.5 mM Ca^{2+} alone. After adding Mg^{2+}, the cleavage rate even reduced slightly. This is explained the competition from the catalytically less capable Mg^{2+} (slower by 75-fold). Since Mg^{2+} has a higher charge density, Mg^{2+} competes favorably with Ca^{2+} for the metal binding site and decreases the rate. For 17E, on the other hand, Ca^{2+} and Mg^{2+} display an additive effect on activity. Although the cleavage rate for Mg^{2+} is lower than Ca^{2+}, the difference is quite small (Ca^{2+} is only 2.8-fold faster). Since the apparent K_d of 17E for Mg^{2+} and Ca^{2+} is 15 mM and 8 mM, respectively,[42] at low mM metal concentration, they both contribute to cleavage activity.

The Peracchi group reported that the 8-17 DNAzyme and most of its variants are more active in Ca^{2+} (by 10 to 20-fold) than in Mg^{2+}.[43] In our work, the rate of 17EV1 was 75-fold faster for Ca^{2+}, and this is due to a higher Ca^{2+} (2.5 mM) used than Mg^{2+} (1.1 mM). In addition, Peracchi also found that the relative activity between Ca^{2+} and Mg^{2+} was influenced by nucleotides from N_{12} to N_{15} (Figure 1C). The best preferential activation by Ca^{2+} was obtained when these positions are ACGA, which is consistent with our results. They further demonstrated that the N_{12} position plays an important role in metal specificity.[44] For example, changing T_{12} to other nucleotides has only a moderate effect on the Ca^{2+}-dependent activity, while the Mg^{2+}-dependent activity is more affected. We also observed a significant influence of N_{12} on metal activity and selectivity (Figure S4). The new information we obtained from this study includes the use of mixed Ca^{2+} and Mg^{2+}, and the test of a broader range of divalent metal ions for the N_{12} position.

In addition to their difference at the N_{12} position, their stem sequence (Figure 1C in purple) is also different. To ensure our above observations are indeed due to the N_{12} position, we made a single A12T mutation to 17EV1. This change has suppressed the Ca^{2+} activity but promoted the Mg^{2+} activity (Figure 3A). A similar observation was made based on 17E (Figure 3B). With A_{12}, both DNAzymes displayed better selectivity for Ca^{2+} over Mg^{2+}. Therefore, the base pair composition in the stem is less important for metal selectivity, and the key is the N_{12} position. A previous report indicated that a three-G/C pair in the stem promotes activity due to higher stem stability.[44] However, a different result was observed by the Li group, where sequences with different number of G/C pairs had comparable activity.[33] Similar conclusions were also made by Kasprowicz *et al* in a recent report.[34] With our observed metal preference in these 17E variants, we next aimed to test the feasibility of using these two related DNAzymes for the analysis of Ca^{2+} and Mg^{2+}, which are two important physiological metal ions.

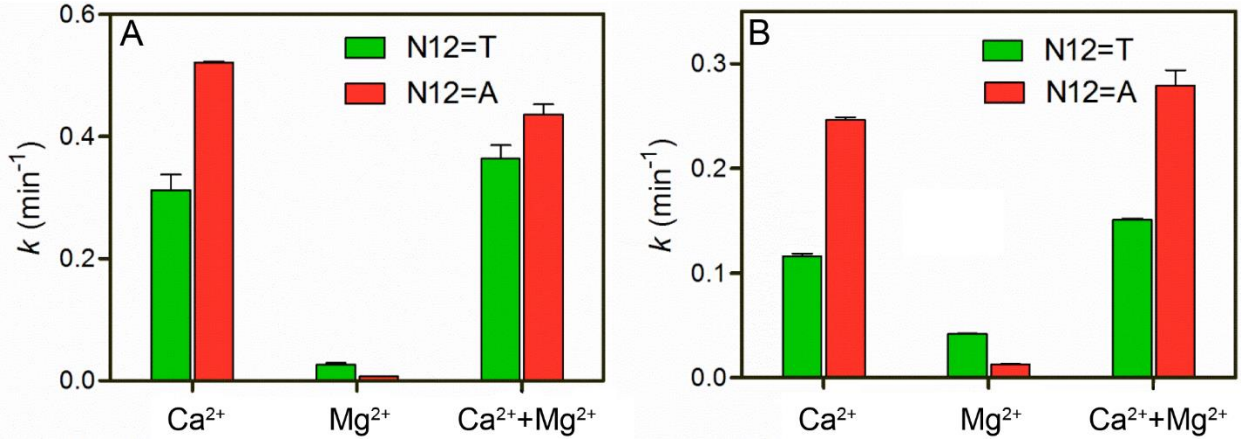


Figure 3. Cleavage rates of (A) the 17EV1 and (B) the 17E DNAzymes in presence of 2.5 mM Ca^{2+}, 1.1 mM Mg^{2+}, or 2.5 mM Ca^{2+} plus 1.1 mM Mg^{2+} with the N_{12} position being an adenine or thymine. In this study, the red and green bars in each figure differ only at the N_{12} position. Note that 17EV1 and 17E are also different in the stem region.

Ca^{2+}/Mg^{2+} ratio measurement. Mg^{2+} and Ca^{2+} are two important and most abundant divalent cations in serum and they play crucial roles in a variety of biochemical processes. For example, Ca^{2+} participates in neurotransmitter releasing, gene transcription, and stimuli-mediated hormone secretion.[45] Fluctuation of Ca^{2+} concentration is considered to be a barometer for diseases such as hyperparathyroidism.[46] Mg^{2+} is important for signal transduction, protection against hypertension and blood vessel spasm.[47] Various methods have been developed to measure Mg^{2+} and Ca^{2+}.[16-18] The concentrations of these two metal ions are quite different in different parts of the body. For instance, the concentration of Mg^{2+} is comparable in serum (~1.1 mM) or in cells (~1.6 mM). However, serum Ca^{2+} is about 2.5 mM while intracellular Ca^{2+} is only a trace amount (< 1 μM). Since both probes are active with both metals, it is difficult to measure their absolute concentrations. As a first step, we test the feasibility of measuring the Ca^{2+}/Mg^{2+} concentration ratio, which is ~2.3 in serum and close to zero intracellularly. Therefore, this ratio can span >1000-fold in different samples.

This study has provided a pair of 17E-based DNAzyme probes for such an application. To achieve ratiometric measurement, we chose 17E and 17EV1 since they show an opposite trend with Ca^{2+} and Mg^{2+}. 17E is one of the most frequently used DNAzymes for Pb^{2+} detection.[48-51] However, most biological samples do not contain free Pb^{2+} or other transition metal ions, leaving Mg^{2+} and Ca^{2+} as the most relevant metals for activating the DNAzymes.

The activity of these two DNAzymes was first tested in present of the individual metal ions. Since for both DNAzymes, Ca^{2+} induced faster cleavage than Mg^{2+}, and at the same Ca^{2+} concentration, 17EV1 is about 4-fold faster than 17E (Figure 2F), we used 20 min reaction time

for 17E while only 5 min for 17EV1 to ensure that cleavage yield is linearly proportional to Ca^{2+} concentration. Since the rate with Mg^{2+} is slower, for these time points with low mM Mg^{2+}, the Mg^{2+} signal would not saturate the DNAzyme probes.

Using these time points, Mg^{2+} and Ca^{2+} concentration-dependent cleavage was measured for both DNAzymes. When Mg^{2+} was tested, the data were fitted with a linear regression using equation A (Figure 4A). In this case, 17E had a more sensitive response to Mg^{2+} concentration, with a slope of 61%/mM Mg^{2+}, which is 22-fold higher than that of 17EV1. On the other hand, the cleavage yield as a function of Ca^{2+} concentration was best fitted with a nonlinear equation B (Figure 4B). This is again due to the faster cleavage rates with Ca^{2+}. Both 17EV1 and 17E showed a similar cleavage trend with increasing Ca^{2+} concentration (note their different reaction time).

Based on the above observations, it seems feasible to determine the Ca^{2+}/Mg^{2+} concentration ratio by using both DNAzymes. In our experimental conditions, since Ca^{2+} contributes similarly to cleavage of both 17E and 17EV1, the cleavage difference between 17E and 17EV1 is thus proportional to the fraction of Mg^{2+}. With a higher content of Mg^{2+}, a larger difference is expected (Figure 4A). To confirm this hypothesis, we test these two DNAzyme at various total metal concentrations, and at each concentration we tested different Ca^{2+}/Mg^{2+} ratios (Figure 4C). Indeed, regardless of the total metal concentration, an increased 17EV1/17E cleavage ratio was observed with increasing Ca^{2+} percentage, and all these curves converge to the same line. We calculated the fitting parameters for each line (Table S3), from which we obtained the averaged values for the final fitting equation.

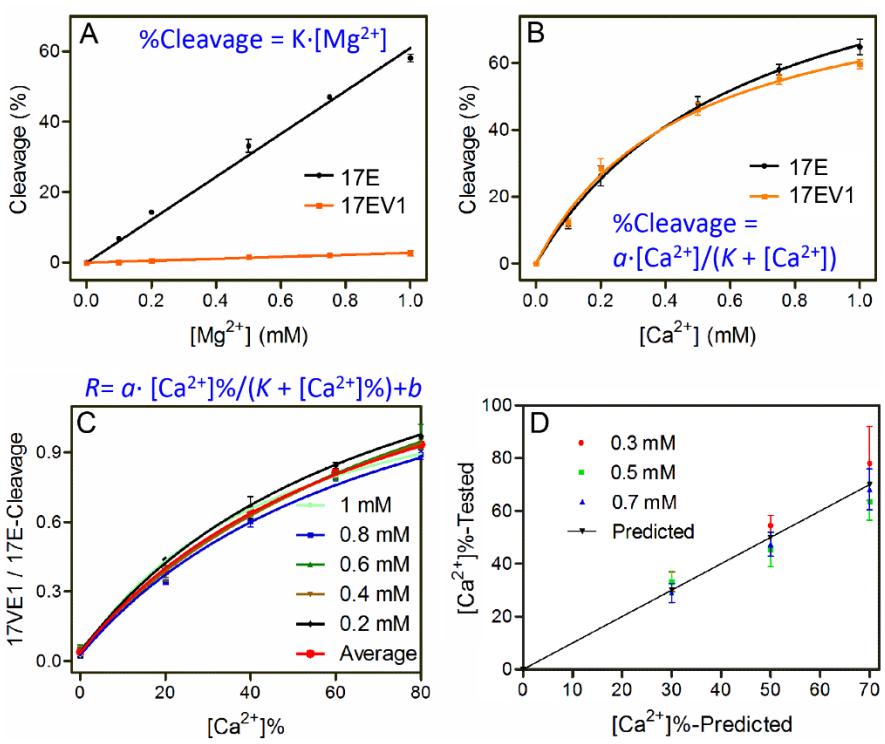


Figure 4. Fraction of substrate cleavage by 17E and 17EV1 with various concentrations of (A) Mg^{2+} and (B) Ca^{2+}. The reaction time here was 20 min for 17E and 5 min for 17EV1. (C) Sensor calibration curves for Ca^{2+} molar percentage at various total metal ion concentrations. The overlapping of these curves indicates that the signal is insensitive to the total metal concentration but only to the fraction of Ca^{2+} in the mixture of Ca^{2+} and Mg^{2+}. (D) Detection of Ca^{2+} percentage in various metal concentrations and a comparison with the theoretical results (the solid line). Each fitting equation is shown and all the parameters are in Table S3.

Finally, to validate this method, we prepared a series of solutions with different total metal ion concentrations (0.3 mM, 0.5 mM and 0.7 mM, respectively). At each concentration, three Ca^{2+} percentage were prepared (30%, 50% and 70%). Then, both DNAzymes were tested with these solutions to calculate the 17EV1/17E cleavage ratios. Using the equation in Figure 4C,

the Ca^{2+} molar percentages were calculated and compared with the theoretical results (Figure 4D, the solid line). Within the error range, all the Ca^{2+} molar percentages analyzed by our method is on the solid line, indicating this method is suitable for the concentration ratio measurement. If this ratio indicates that the Ca^{2+} concentration is comparable or even higher than the Mg^{2+} concentration, we can estimate the absolute Ca^{2+} concentration from the 17EV1 cleavage yield since its Mg^{2+} response is very low. However, this estimation only works when the cleavage by Ca^{2+} is dominating. For example, it would not work for intracellular conditions.

Overall, this pair of DNAzymes have demonstrated the possibility of measuring the ratio of these two important metal ions, but it is still far from ideal. For example, 17EV1 has a large difference between Ca^{2+} and Mg^{2+}, while for 17E, this difference is quite small under our measurement condition. This has limited the accuracy and range of measurement. We only tested between 0.2-1 mM metal ions. By extending the incubation time, it might be possible to wider the measurement range to lower metal concentrations. In addition, it would be highly desirable to have enzymes specific for only one of the metals, which may allow us to quantify the absolute concentration instead of just the ratio. We obtained the current DNAzymes from *in vitro* selection in serum. We reason that a few strategies might help us to achieve better metal selectivity. For example, the library design can be changed (e.g. using different cleavage junction to avoid 17E).[38] In addition, different selection conditions might be used (e.g. involving organic solvents),[52] from which we might obtain new DNAzymes to fulfill this goal.

Conclusions

In summary, we carried out the first *in vitro* DNAzyme selection experiments in undiluted human blood serum. While we did not obtain completely new DNAzymes, the selection

experiment was a success, indicating the feasibility of DNA selection in biological fluids even though the library contains an RNA linkage. The resulting RNA-cleaving DNAzyme is a variant of 17E and it has nearly 6-fold higher activity in the presence of Ca^{2+} than the originally tested 17E in serum.[6] From the practical application standpoint, this study has also broadened the applicability of DNAzyme-based biosensor. Two active mutants were used and they display an opposite metal preference, allowing measurement of Ca^{2+} and Mg^{2+} concentration ratios, which has never been demonstrated before in the DNAzyme field. Currently, the detection was based on DNAzyme cleavage, which is irreversible. The fact that DNA can tell the difference between Ca^{2+} and Mg^{2+}, two very similar metal ions, will inspire related work based on other sensing mechanisms. For example, DNA folding-based sensors might be developed to achieve reversible and faster detection of these important ions.

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Supporting Information Available: The following files are available free of charge.

DNA sequences; in vitro selection conditions; negative selections; activity of other potential DNAzymes; and activity of 17EV1 and 17E with various metal ions. (PDF)

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