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An engineered one-site aptamer with higher sensitivity for label-free detection of adenosine on graphene oxide

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Abstract

The 27-nucleotide DNA aptamer for adenosine and ATP, originally selected by the Szostak lab in 1995, has been a very popular model system for biosensor development. This unique aptamer has two target binding sites and we recently showed that it is possible to remove either site while the other one still retains binding. From an analytical perspective, tuning the number of binding sites has important implications in modulating sensitivity of the resulting biosensors. In this work, we report that the engineered one-site aptamer showed excellent signaling properties with a 2.6-fold stronger signal intensity and also a 4.2-fold increased detection limit compared to the wild-type two-site aptamer. The aptamer has a hairpin structure and the length of the hairpin stem was systematically varied for the one-site aptamers. Isothermal titration calorimetry and a label-free fluorescence signaling method with graphene oxide and SYBR Green I were respectively used to evaluate binding and sensor performance. While longer stemmed aptamers produced better adenosine binding affinity, the signaling was quite independent of the stem length as long as more than three base pairs were left. This was explained by the higher affinity of binding to GO by the longer aptamers, cancelling out the higher affinity for adenosine binding. This work further confirms the analytical applications of such one-site adenosine aptamers, which are potentially useful for improved ATP imaging and for developing new biosensors.

Introduction

Aptamers are single-stranded nucleic acids that can selectively bind to target molecules.[1-3] Since its discovery, aptamers have been widely used for biosensor development.[4-12] Aptamers binding to small molecules are particularly attractive since they often show even better binding properties than antibodies.[13,14] The 27-nucleotide DNA aptamer for adenosine, AMP and ATP isolated by Huizenga and Szostak in 1995 is an extensively studied model aptamer,[15] and it has been used for measuring ATP in various samples.[16-22] This aptamer is unique in terms of its simultaneous binding of two target molecules, while very few aptamers for small molecules have such a property.[23-25]

We were quite intrigued by the two binding sites and found that binding was still retained even when one of the sites was removed.[26] The resulting one-site aptamer could be shorter with even higher sensitivity since the need of cooperative binding was eliminated. To have a systematic understanding of its analytical performance, we herein explored a series of one-site aptamers and made a comparison with the wild-type two-site aptamer. Our goal was to identify the optimal sensing sequences using a label-free signaling method.

Graphene oxide (GO) has been a unique platform for designing DNA-based biosensors in the past few years.[27-32] GO can adsorb non-structured single-stranded DNA and quench fluorescence.[33-36] By adding the aptamer target, the equilibrium is shifted towards the aptamer leaving the surface and increasing fluorescence. GO also allows the development of label-free sensors by using DNA staining dyes,[37-39] which makes sensor optimization cost-effective. In this work, we systematically studied the signaling of various engineered adenosine aptamers adsorbed onto GO, and our results confirmed one-site aptamers were better than the wild-type two-site aptamer for more sensitive detection.

Materials and Methods

Chemicals. All the DNA samples were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Single layer carboxyl graphene oxide (GO) was purchased from ACS Material (Medford, MA). Sodium chloride, magnesium chloride, 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adenosine, other nucleosides and nucleotides were from Mandel Scientific (Guelph, ON, Canada). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St Louis, USA). 10000× SYBR Green I dye in dimethyl sulfoxide (DMSO) was purchased from Invitrogen (Carlsbad, CA). Milli-Q water was used to prepare all the buffers and solutions.

ITC. Isothermal titration calorimetry (ITC) was performed using a VP-ITC microcalorimeter instrument (MicroCal). All the ITC tests used the following protocols unless otherwise specified. Prior to each measurement, each solution was degassed to remove air bubbles. An aptamer sample (10 μM) in buffer A (20 mM HEPES, pH 7.6, NaCl 100 mM and 2 mM $MgCl_2$) was loaded in a 1.45 ml ITC cell at 15 °C. Adenosine or other nucleosides (280 μL, 0.5mM) in the same buffer were titrated into the cell through a syringe (10 μL each time, except for the first injection of 2 μL). The enthalpy (ΔH) and binding constant (K_a) were obtained through fitting the titration curves to a one-site binding model using the Origin software. The dissociation constant (K_d) values were calculated from $1/K_a$ and $\Delta G = -RT\ ln(K_a)$, where R is the gas constant. ΔS was calculated from $\Delta G = \Delta H - T\Delta S$.

DLS and TEM. The hydrodynamic size and ζ-potential of GO (50 μg/mL) was measured using dynamic light scattering (DLS) on a Malvern Zetasizer Nano ZS90 with a He−Ne laser (633 nm) at 90° collecting optics at 25 °C in the buffer A. Transmission electron microscopy (TEM) was performed on a Philips CM10 transmission electron microscope with an acceleration voltage of

80 kV. The sample was prepared by pipetting a drop of the GO aqueous dispersion (100 μg/mL) onto a 230 mesh copper holy carbon grid and then dried in air for tests.

Sensor preparation and test. Different DNA aptamers (100 nM) were respectively mixed with an excess concentration of GO (100 μg/mL) at 25 °C in the buffer A for 1 h to make sure all the DNA was adsorbed. No free DNA was left in the buffer solution (confirmed using UV spectroscopy after centrifugation). The sensors were freshly prepared before each test. The sensor signaling kinetics were monitored on a Varian Eclipse fluorescence spectrometer with excitation at 490 nm and emission at 520 nm using 4 μM (2×) SYBR Green (SG) dye at 25 °C in buffer A. After monitoring the background for 10 min, different concentrations of adenosine or other nucleosides and nucleotides were added, followed by another ~40 min of monitoring. Multiple samples of the free sensors without adenosine were measured to calculate the standard deviation of the background variation (σ). Then the initial slope of the calibration curve was calculated to determine the limit of detection (3σ /slope). To determine the sensor performance in biological samples, fresh fetal bovine serum (FBS, 1% v/v) was spiked with different concentrations of adenosine. The sensor was prepared and tested in the same way as in buffer.

Results and Discussion

Engineered adenosine aptamers with different binding sites. The secondary structure of the wild-type adenosine aptamer is shown in Figure 1A. This aptamer is named Apt2, where the '2' describes its binding to two adenosine molecules based on its NMR structure.[25] To facilitate discussion, each nucleotide in the aptamer is numbered. The two adenosine binding pockets are relatively symmetric (the red colored 'A_1' and 'A_2' denote for the target adenosine). We recently found that binding was still retained by deleting a site or adding more sites, as long as the

binding pocket could stably form.[26] For example, by inserting an extra cytosine base and fixing the G5·A23 mismatch, a one-site aptamer is produced (Figure 1C).

Analytically, cooperative binding of multiple analytes can produce a sigmoidal switch-like response, while the sensitivity at low analyte concentrations is usually low.[40] Therefore, the one-site aptamers are interesting since they might be more sensitive. Our main focus in this study is to systematically study the new one-site aptamers for better analytical applications. The folded aptamer has a stem-loop structure. After removing a binding site, the main parameter for optimization is the length of the aptamer stem structure. We systematically varied the number of base pairs (bp) from 8 to 0 (Figure 1B-G). The aptamers with shorter arms may have different binding affinities to GO and to adenosine, thus affecting the analytical performance. For comparison, we also designed two aptamers with even more binding sites (Apt3 and Apt4, Figure 1H-I).

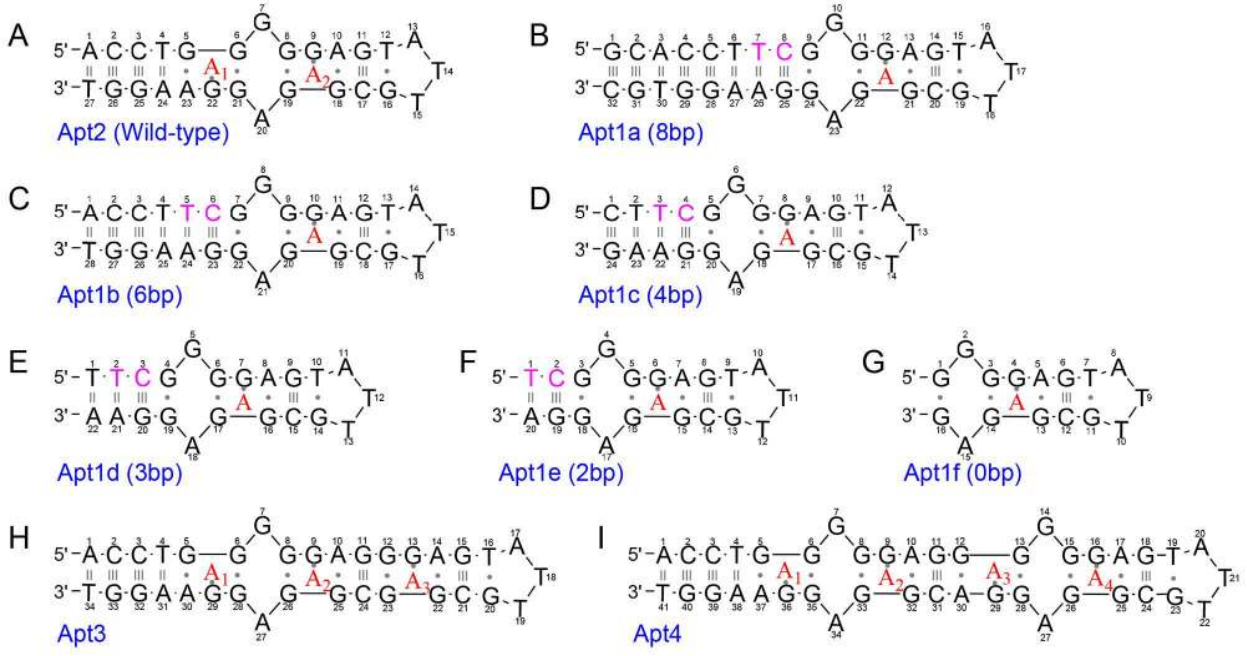

Figure 1. The secondary structures of (A) the wild-type adenosine aptamers (Apt2) with two adenosine binding sites, (B–G) the engineered one-site aptamers with different stem lengths, and the extended aptamers with (H) 3 and (I) 4 adenosine binding sites. The wild-type aptamer has two binding sites (A_1 and A_2). The red colored 'A' represents the bound adenosine. The nucleotides in pink were introduced to remove adenosine binding sites. Each nucleotide in the aptamers is numbered.

Binding thermodynamics measured by ITC. To confirm binding, we first measured the binding affinity of the aptamers using isothermal titration calorimetry (ITC). ITC is a label-free technique providing rich thermodynamic information for aptamer binding.[41-43] Our previous work has measured the wild-type two-site aptamer (Apt2) and demonstrated that the one-site mutant has a similar binding affinity.[26] Here we measured the effect of the number of base pairs in the aptamer stem (Figure 2). In these experiments, adenosine was gradually titrated into the aptamer solution, and the downward spikes indicate heat release and binding. The aptamers Apt1a to Apt1d (Figure 2A-D) with more than 3 bp in the stem showed a significant heat release indicating strong binding. However, further shortening the aptamer (e.g. Apt1e and Apt1f) to 2 or 0 bp nearly fully inhibited binding (Figure 2E and F). By observing the trend, it appears that a longer stem resulted in a tighter binding of adenosine.

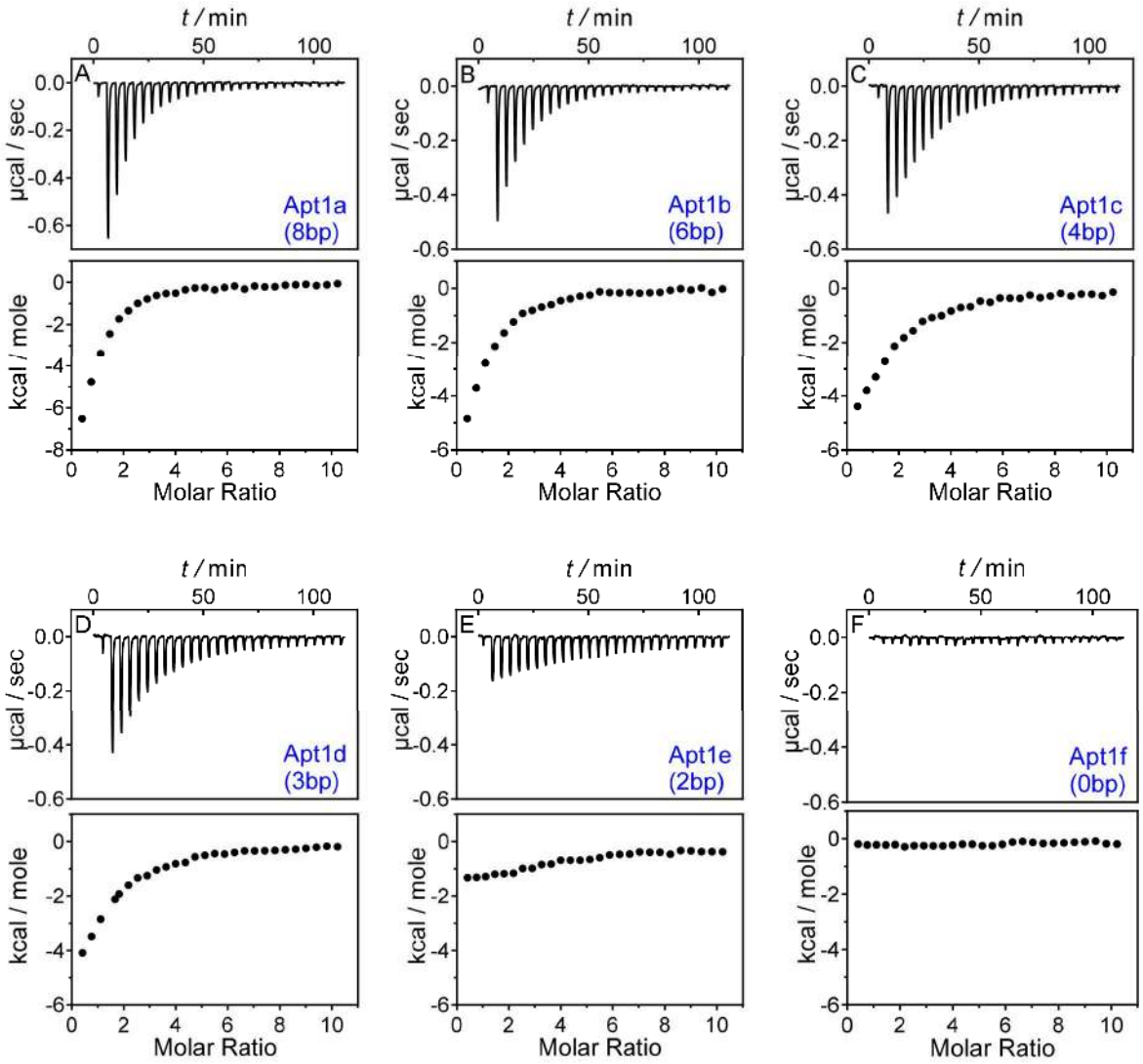

Figure 2. ITC traced and integrated the heat of the one-site aptamers with various base pairs in the stem region of the aptamers. In each figure, the top panel is the original thermogram and the bottom panel is the integrated heat.

For quantitative analysis, we integrated the heat of each titration (Figure 2, lower panels) to directly obtain the reaction enthalpy (ΔH), binding stoichiometry (n) and dissociation constant (K_d), which allowed further calculation of ΔG and ΔS.[44-46] We first compared the aptamers'

binding affinity using the K_d values as a function of the number of base pairs in the stem (Figure 3A). The Apt1a aptamer with the longest stem (8 bp) showed the lowest K_d (7.1 ± 1.2 μM) indicating the tightest binding. As the base pairs reduced from 8 to 0 (Apt1b to Apt1e), the aptamers' binding affinity gradually decreased. We reason that longer stems provide a more stable aptamer structure and result in tighter binding. All the aptamers with more than 3 bp (Apt1a to Apt1d) retained a high binding affinity (K_d ~ 7.1 to 19.2 μM). However, the Apt1e with only two base pairs showed very poor binding (K_d > 250 μM) and the even shorter Apt1f lost all its binding activity. All the thermodynamic parameters are tabulated in Table S1 (Supplementary Data).

We also compared the enthalpy changes (ΔH) and entropy changes (ΔS) in these aptamers. All the binding aptamers released heat (Figure 3B), but showed decreased entropy change (Figure 3C) upon binding to adenosine. The trend of enthalpy change matched well with that of in K_d. Therefore, binding was mainly driven by enthalpy.[47,48] From these results we concluded that all the one-site aptamers with more than 3 bp can bind adenosine, and longer stems resulted in tighter binding. The Apt1d with 3 base pairs is the limit that has the shortest sequences while still retaining a relatively high affinity.

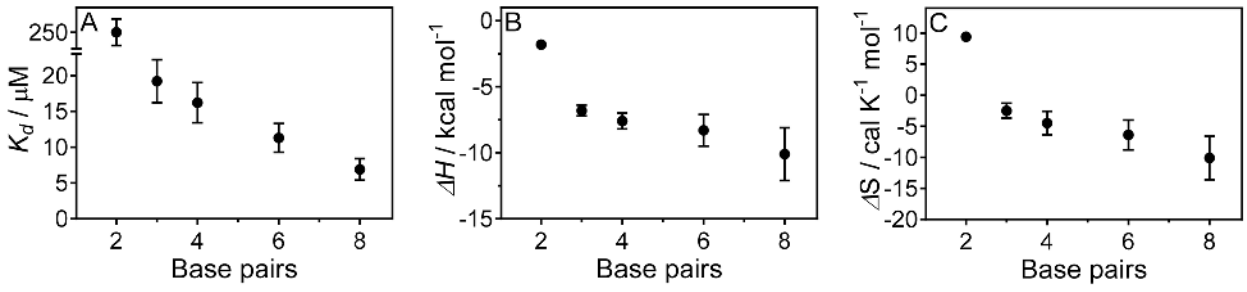

Figure 3. The (A) dissociation constant (K_d), (B) enthalpy change (ΔH), and (C) entropy change (ΔS) of the one-site adenosine aptamers as a function of the number of base pairs in the stem region.

Sensitivity as a function of binding sites. After measuring the binding of our engineered one-site aptamers, we next used them for designing biosensors. Graphene oxide (GO) was used as a platform for DNA aptamer adsorption.[33-35] The GO sheets were negative charged (ζ-potential: -42.1 ± 5.6 mV) with an average size around 820 nm measured by DLS and TEM (Figure S1 and S2). By adding adenosine, the aptamers can bind the target molecule and the equilibrium is shifted towards desorption from GO (Figure 4A).[31,49] To detect the signal, we used SYBR Green I to stain the released DNA aptamers for fluorescence output.[50,51] This label-free method is attractive for its simplicity and cost-effectiveness.[52-56]

The sensor performance was evaluated by measuring the signaling kinetics. To ensure all the DNA aptamers were adsorbed, extra GO (100 μg/mL) was used for DNA adsorption in a high salt buffer condition (buffer A: 20 mM HEPES, pH 7.6, 100 mM NaCl, 2 mM $MgCl_2$).[34] Full adsorption was confirmed by UV-vis spectroscopy. Using the wild-type aptamer (Apt2) as an example, we monitored the background fluorescence of the aptamer/GO complex mixed with SG for 10 min and the signal remained stable (Figure 4C). Then 1 mM adenosine was quickly added (the arrowhead), and an immediate fluorescence increase was observed. It took around 10 min for the fluorescence signal to reach saturation and then the signal remained stable for a long time, indicating that the system reached equilibrium.

Using this method, we then compared the sensor performance of the aptamers with different numbers of binding sites (Figure 4B-E). These aptamers included the one-site (Apt1c), two-site (Apt2), three-site (Apt3) and four-site (Apt4), and all these four aptamers had the same stem length (4 bp, see the structures in Figure 1). Among them, the engineered one-site aptamer had the best performance (Figure 4B). It showed a similar background as the wild-type aptamer, while the final signal was more than two-fold higher.

Since each aptamer has a different length, they may have a different final fluorescence after binding adenosine. To understand the effect of DNA length, we also measured the fluorescence intensity of the free aptamers without GO (Figure 4F). While SG is strongly fluorescent upon binding to double-stranded DNA, it is also fluorescent with a high concentration of single-stranded DNA.[50] The three- and four- sites aptamers (Apt3 and Apt4) had a slightly higher fluorescence likely due to their longer sequences than the Apt1c and Apt2 (Figure 1). After adding adenosine, the relative fluorescence increase of each aptamer was around 20-30%. Therefore, without GO, SG is not very effective in sensing the binding of adenosine.

With each free aptamers' intensity (F_0, Figure 4F the red bars) and their maximum released fluorescence (F, Figure 4B-E), we normalized each aptamers' fluorescent signaling (F/F_0, Figure 4G). This ratio has taken the fluorescence difference due to DNA length change into consideration, and thus is a pure indication of the relative amount of DNA released from GO due to binding to adenosine. Among the different aptamers, the one-site aptamer Apt1c had the highest ratio at approximately 0.76 (i.e. 76% aptamer desorbed from GO), which was more than 2.5-fold higher than the multiple-site aptamers (Figure 4G).

It is known that all the binding sites have a similar binding affinity,[26] and thus the higher sensitivity of the one-site aptamer on GO was due to its fewer binding sites. Note the signaling

efficiency was quite similar for the free aptamers (without GO) (Figure 4F). GO improved the signaling for all the aptamers and the most improvement was seen with the one-site aptamer. Since an aptamer needs to form a full binding complex to leave the GO surface, the multi-site aptamers are more difficult to satisfy this requirement and thus they require higher adenosine concentrations.

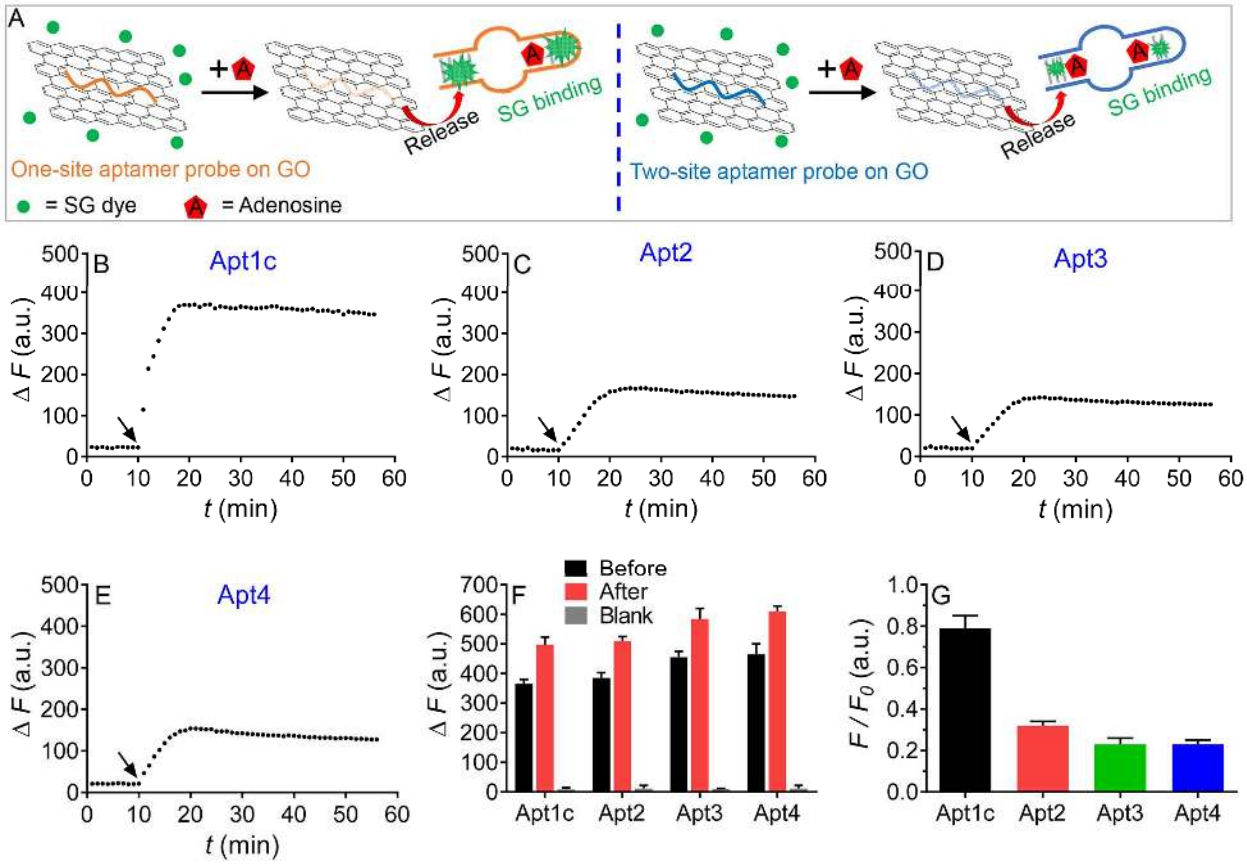

Figure 4. (A) Schemes of the designed sensors with label-free fluorescence signaling, showing the one-site aptamer is more sensitive. Fluorescence signaling kinetics of the adenosine aptamers with different numbers of binding sites: (B) Apt1c, (C) Apt2, (D) Apt3, and (E) Apt4 released from GO (100 μg/mL) tested at 25 °C in the buffer A. 1 mM of adenosine were added in 10 min (pointed by the arrowheads). (F) Fluorescence intensities of the free aptamers stained by SG before and after adding 1 mM adenosine. The Blank control was the intensity of SG with

adenosine but without any DNA. (G) The normalized fluorescence ratio of the aptamers with GO over that without GO (F/F_0). The chemical meaning is the fraction of aptamers released from the GO surface. All the aptamers were100 nM, and the SG was 4 μM (2×).

Sensitivity as a function of stem length. After confirming that the one-site aptamer had the best performance, we then further studied the effect of the stem length. We tested a series of one-site aptamers with various base pairs (0 to 8 bps, see Figure 1) in the same way. We first measured their signaling kinetics. The Apt1a with the longest 8 bp stem showed the highest final fluorescence intensity (Figure 5A). By shortening the stem, the final intensities gradually became lower (Figure 5B-F). This difference may be related to either the aptamers' binding affinities according to the ITC results, or the fluorescence of the free aptamers after stained by SG, or both. To identify the reason, we measured the signaling of the free aptamers (Figure 6A). It is very interesting to note that the fluorescence of the free aptamers was roughly linearly correlated with the length of the aptamers. This is not surprising since longer DNA produces more signals with SG staining. Adenosine barely induced any fluorescence change for the very short 0 and 2 bp aptamers, while the signal increase by adenosine was similar (around 20%) for all the longer aptamers.

We then calculated the fluorescence ratios with and without GO as we did above (F/F_0, Figure 6B) which is roughly the fraction of aptamer released from GO. Among the aptamers, the ones with more than 3 bp all had a high and similar efficiency of ~0.81, indicating their similar and excellent sensor performance (Figure 6B). We reason that longer aptamers bind adenosine more tightly according to ITC but they also bind to GO more tightly. Therefore, these two factors cancelled out with each other, leading to a similar desorption ratio. From the signaling standpoint

in the current system, the aptamers with a longer stem are still better due to the very low background fluorescence. The aptamers with less than 2 bp showed a much lower fraction of release which was consistent with their loss of adenosine binding from our ITC data (Figure 3A).

When comparing with two- and multiple-site aptamers, all the one site aptamers with more than 3 bp had better sensing performance with an efficiency of ~ 0.8 (Figure 6B), which is more than two-fold higher than that of the two- and multiple-site aptamers (~0.3, Figure 4G). From these tests, we concluded that all the one-site aptamers with more than 3 bp in the stem had better biosensor performance than the wild-type two-site aptamer.

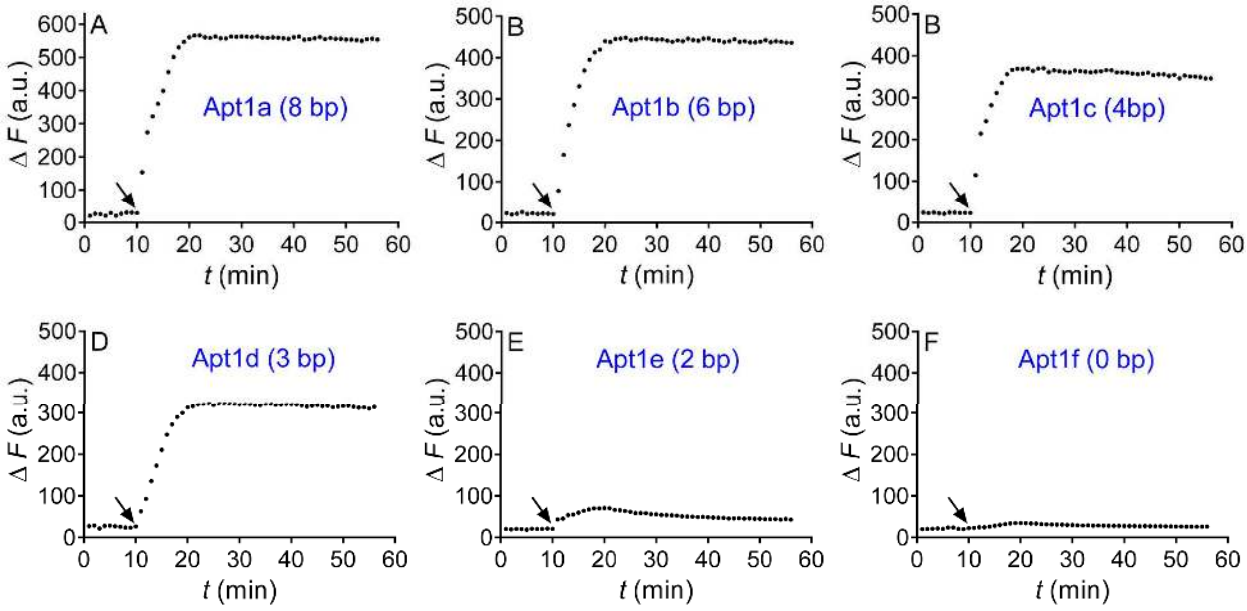

Figure 5. Fluorescence signaling kinetics of one-site adenosine aptamers with various stem lengths adsorbed on GO (100 μg/mL) tested at 25 °C in the buffer A. 1 mM of adenosine were added at 10 min. 4 μM SG was used in all the tests.

Highly sensitive label-free detection. Since the one-site aptamers with 3-8 bp stems all showed a similar performance and shorter aptamers are more cost effective, we next chose the Apt1d with 3 bp for biosensor tests. Compared to the wild-type 27 nucleotide aptamer, this 3 bp aptamer has only 22 nucleotides. To quantitatively evaluate the sensor performance, we gradually titrated adenosine to the Apt1d sensor and a comparison was also made with the wild-type Apt2 sensor (Figure 6C). Both sensors followed a linear growth trend initially, with a slope of 235 ± 19 (a.u./mM) for Apt1d, and 56 ± 6 (a.u./mM) for Apt2. Then, we calculated the limit of detection (LOD) of Apt1d to be 12.8 μM adenosine, which was 4.2-fold better than that of Apt2 with (53.9 μM, Figure 6C). The LOD was calculated based on the adenosine concentration at which the signal was three times higher than background variation.

We also tested the specificity of the two sensors by adding AMP, ATP and other nucleosides such as guanosine (G), cytidine (C), thymidine (T) (Figure 6D). Regardless of AMP or ATP, the sensor with the one-site Apt1d aptamer showed stronger intensity than that with the two-site Apt2 aptamer. Overall, adenosine had the highest sensitivity among these tested derivatives, and this is consistent with the literature reports.[15,57,58] On the other hand, both sensors showed almost no response to other nucleosides confirming excellent specificity for adenosine and its derivatives (Figure 6D).

Aptamer/GO based sensors used for ATP/adenosine detection have been a widely studied model system. We summarized different sensing methods in recent years in the Table S2. Aptamers with a covalently labeled fluorophore is one of most used strategies. While it is easy to operate, label-free sensors have also been explored for cost-effectiveness. Such covalent sensors have a detection limit of a few micromolar. With signal amplification methods, such as using exonuclease or other enzymes, much lower detection limits of low nanomolar was possible.

However, such sensing methods take longer time and are more difficult to control. Label-free sensors are around 10 μM detect limit. Adenosine in normal cells are approximately 0.3 μM and elevate to 1.2 μM in response to cellular damage. Most adenosine are converted to adenosine triphosphate (ATP) which has an abundant concentration of 1–10 mM.[59] This aptamer is known to bind adenosine, AMP and ATP with quite similar K_d's. Therefore, it is likely useful for ATP measurement for intracellular applications. We here used adenosine as a more cost-effective alternative. The main goal of this work is to study the analytical performance of the shortened one-site aptamer, which from our side-by-side comparison, was more sensitive than the original two-site aptamer.

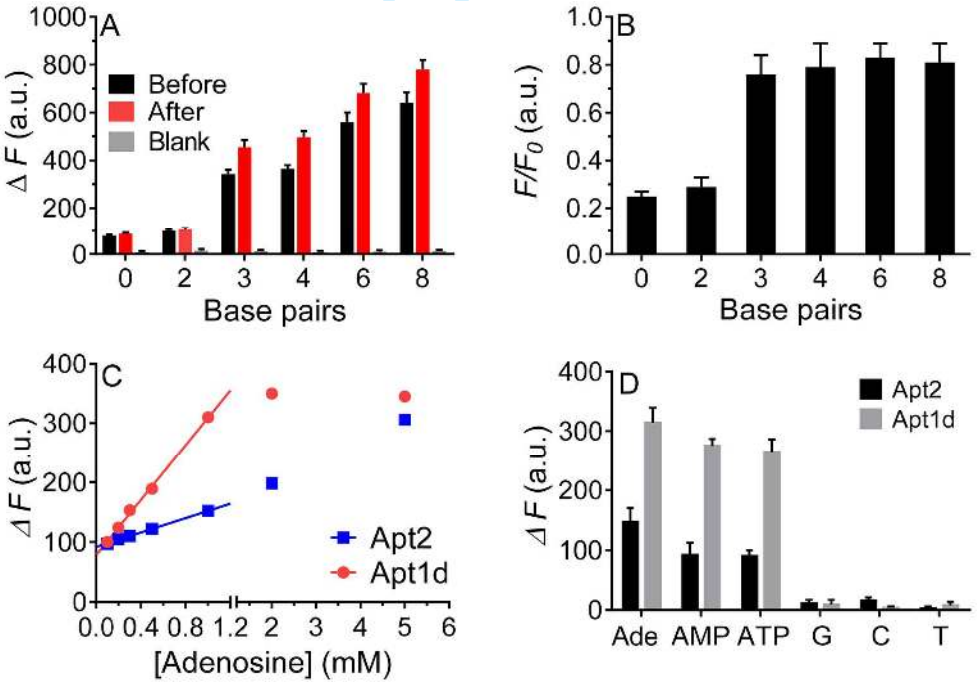

Figure 6. (A) Fluorescence intensity of different free aptamers binding in the presence of SG before and after adding 1 mM adenosine. The blank control is the intensity of SG with adenosine but without aptamers. (B) The fluorescent signaling efficiency (F/F_0) of the one-site aptamers

with different base pairs released from GO. (C) The fluorescence intensity of the two sensors using Apt2 and Apt1d after 40 min as a function of adenosine concentration. (D) Fluorescence intensity of the two sensors using Apt2 and Apt1d (one site, 3 bps) by adding 1 mM of different nucleotide or nucleosides measured in 40 min. All the used aptamers were 100 nM. 4 μM SG was used in all the tests at 25 °C in the buffer A.

Detection of adenosine in serum samples. To test whether this sensor is capable of measuring adenosine in biological samples, we then applied the Apt1d-GO sensor in the presence of fetal bovine serum (FBS). Three serum samples with different concentrations of spiked adenosine (0.1, 0.2 and 0.5 mM) were tested (all within the linear calibration range in Figure 6C). The sensor showed weak fluorescence in the adenosine-free sample (Figure 7A, black curve) but strong intensity in adenosine-containing samples (Figure 7A), which confirmed the sensor performance in serum. With the calibration curve, the adenosine in these samples were measured (Figure 7B, grey bars), which were all close to the added concentrations (Figure 7B, black bars) with an average recovery rate of 93.6 %.

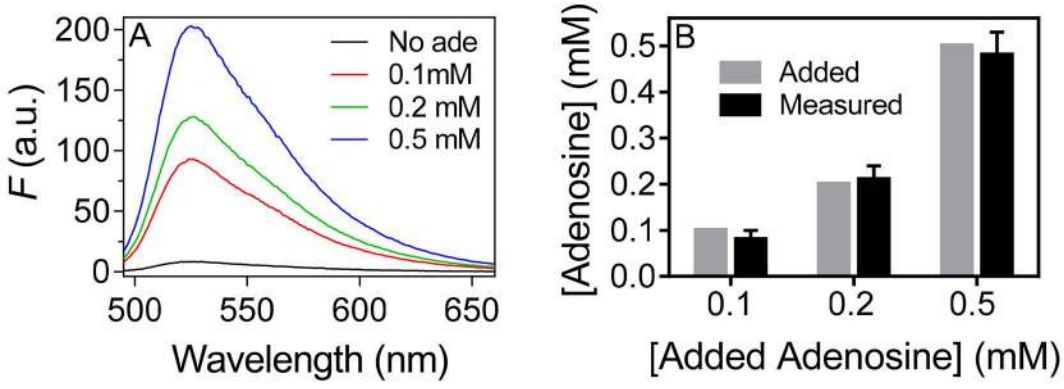

Figure 7. (A) Fluorescence emission spectra of Apt1d-GO sensors tested in adenosine-free (No ade), and a few adenosine containing (0.1-0.5 mM) serum samples (1%). (B) The concentration of adenosine added in three serum samples and measured by the GO-Apt1d sensors.

Conclusions

In this study, we focused our attention on our newly discovered one-site aptamers binding adenosine. The original adenosine aptamer has two binding sites, which is quite unique among the in vitro selected small molecule binding aptamers. By rationally designing the sequence, we removed one of the binding sites and studied the one-site aptamer using ITC and a label-free fluorescence signaling method. The one-site aptamer, although it has a similar binding affinity, showed much better sensitivity on the GO sensing platform. We further measured the effect of the length of the stem region of the one-site aptamer, and found that aptamers with more than 3 base pairs showed similar sensitivity. This allowed us to shorten the original 27-mer aptamer to 22-mer, while still retaining a high sensitivity (4.2-fold lower LOD than the sensor with the two-site aptamer). Furthermore, the sensor is also capable of measuring adenosine in biological samples with a recovery rate of 93.6 %. This work has provided a series of aptamers based on this highly important model adenosine aptamer system for biosensor development, and it has demonstrated the relationship between the number of binding sites, sensitivity, and signal intensity.

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