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Detection of Non-Nucleic Acid Targets with an Unmodified Aptamer and a Fluorogenic Competitor

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Abstract

Aptamers are oligonucleotides that can bind to various non-nucleic acid targets, ranging from proteins to small molecules, with a specificity and affinity comparable to that of antibodies. Most aptamer-based detection strategies require modification on the aptamer, which could lead to a significant loss in its affinity and specificity to the target. Here we reported a generic strategy to design aptamer-based optical probes. An unmodified aptamer specific to the target and a fluorogenic competitor complementary to the aptamer are utilized for target recognition and signal generation, respectively. The competitor is a hairpin oligonucleotide with a fluorophore attached on one end and a quencher attached on the other. When no target is present, the competitor binds to the aptamer. However, when the target is introduced, the competitor will be displaced from the aptamer by the target, thus resulting in a target-specific decrease in fluorescence signal. Successful application of this strategy to different types of targets (small molecules and proteins) as well as different types of aptamers (DNA and RNA) has been demonstrated. Furthermore, a thermodynamics-based prediction model was established to further rationalize the optimization process. Due to its rapidness and simplicity, this aptamer-based detection strategy holds great promise in high throughput applications.

INTRODUCTION

Aptamers are oligonucleotides that can bind tightly and specifically to various molecular targets ranging from proteins to small molecules.¹ Aptamers are usually isolated from combinatorial oligonucleotide libraries by the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) process,^{2–4} but natural aptamers also exist.^{5–6} In terms of the affinity and specificity to the molecular target, aptamers are comparable to antibodies. Moreover, due to the nucleic acid nature of aptamers, they offer certain advantages over antibodies, such as low cost, high stability, and easy to label. This makes aptamers promising candidates for applications where antibodies are routinely used.^{7, 8} Many different strategies to design aptamer-based optical probes have been developed. In order to generate the target-specific optical readout, most strategies require either sequence alteration^{9–11} or labeling^{12–19} of aptamers. Implementation of these strategies often involves a laborious aptamer-specific optimization process because even slight modifications on aptamers could lead to a significant loss in their affinity and specificity.^{20–22} Recently a few label-free design strategies, which utilize either photoactive polymer²³ or DNA-intercalating dyes^{24–26} to report the aptamer-

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target recognition event, have been demonstrated. Although no modification on aptamers is necessary in these strategies, their multiplex and *in situ* detection capabilities are limited.

Therefore, we recently developed a generic design strategy for aptamer-based optical probes, which doesn't require any modification on aptamers and is also suitable for multiplex or *in situ* detection.²⁷ In this design strategy (Figure 1), an unmodified aptamer specific to the target and a competitor complementary to the aptamer serve as the molecular recognition and signal transduction element, respectively. The competitor is a molecular beacon²⁸ oligonucleotide, which consists a loop complementary to the aptamer and two complementary stems. A fluorophore is attached to one stem and a corresponding quencher is attached on the other stem. In the absence of the target, the aptamer will bind to the competitor, which keeps the competitor in the "open" configuration where the fluorophore is away from the quencher, thus emitting a strong fluorescence. In the presence of the target, the aptamer will bind to the target, which allows the competitor to assume the "closed" configuration where the fluorophore is in the close proximity to the quencher, therefore leading to a decrease in the fluorescence. Recently, a strategy that utilizes an unmodified aptamer, a molecular beacon with the sequence of the loop same to that of the aptamer, and a short DNA scrambled sequence complementary to both the aptamer and the molecular beacon loop, was reported.²⁹ Because of the similarity between this signal-on strategy and our signal-off one, both strategies have some shared advantages over most other existing ones. On one hand, compared to the strategies requiring modifications on the aptamer,^{9–19} these two strategies eliminate the lengthy optimization process to preserve the affinity and specificity of the aptamer. On the other hand, compared to the label-free strategies where the reporting molecules lack the selectivity against specific aptamers,^{23–26} these two strategies are suitable for the multiplexing detection because different reporting molecules can be conjugated to the molecular beacons specific to the different aptamers.

MATERIALS AND METHOD

Probe design

In order for the strategy in Figure 1 to be successful, binding between the competitor and the aptamer should be strong enough so that formation of the competitor-aptamer complex will be more favorable than that of the competitor self-structure in the absence of the target, but not too strong otherwise the competitor will not be able to be displaced from the aptamer by the target. Therefore, the sequence of the competitor must be carefully selected. Instead of a pure trial-and-error experimental approach, a more rational approach is taken in designing the competitor sequence. A thermodynamic model is established to verify that a design is thermodynamically feasible before the experimental test. The similar approach has been used in a few studies to optimize the probe design for nucleic acid detections.^{30, 31} In our thermodynamic model, various reactions, including the formation of the aptamer-target complex, the aptamer-competitor complex, the self-structure and self-dimer of both aptamer and competitor, are considered.

where A is the aptamer, AA is the self-dimer of the aptamer, A_h is the self-structure of the aptamer, T is the target, A_hT is the aptamer/target complex, C is the competitor, AC is the aptamer/competitor duplex, CC is the self-dimer of the competitor, and C_h is the self-structure of the competitor.

The equilibrium equations for the above reactions are as follows:

$$\frac{[A] \cdot [A]}{[AA]} = K_{A/A} \quad (7)$$

$$\frac{[A]}{[A_h]} = K_{A,h} \quad (8)$$

$$\frac{[A_h] \cdot [T]}{[A_hT]} = K_{A/T} \quad (9)$$

$$\frac{[A] \cdot [C]}{[AC]} = K_{A/C} \quad (10)$$

$$\frac{[C] \cdot [C]}{[CC]} = K_{C/C} \quad (11)$$

$$\frac{[C]}{[C_h]} = K_{C,h} \quad (12)$$

where $[X]$ is the concentration of X ($X = A, AA, A_h, T, A_hT, C, AC, CC, C_h$) at equilibrium and $K_{A/A}, K_{A,h}, K_{A/T}, K_{A/C}, K_{C/C}, K_{C,h}$ are dissociation constants of $AA, A_h, A_hT, AC, CC, C_h$ respectively.

The mass balance equations of A , T , and C are given by:

$$[A] + 2[AA] + [A_h] + [A_hT] + [AC] = [A]_T \quad (13)$$

$$[T] + [A_hT] = [T]_T \quad (14)$$

$$[C] + [AC] + 2[CC] + [C_h] = [T]_T \quad (15)$$

where $[X]_T$ is the total concentration of X ($X = A, T, C$). The (7) to (15) forms a closed system of equations about $[A]$, $[AA]$, $[A_h]$, $[T]$, $[A_hT]$, $[C]$, $[AC]$, $[CC]$, and $[C_h]$.

Except $K_{A/T}$ which is found from the literature, other dissociation constants are determined from:

$$K_{A/A} = e^{\frac{\Delta G_{A/A}}{RT}} \quad (16)$$

$$K_{A_h} = e^{\frac{\Delta G_{A_h}}{RT}} \quad (17)$$

$$K_{A/C} = e^{\frac{\Delta G_{A/C}}{RT}} \quad (18)$$

$$K_{C/C} = e^{\frac{\Delta G_{C/C}}{RT}} \quad (19)$$

$$K_{C_h} = e^{\frac{\Delta G_{C_h}}{RT}} \quad (20)$$

where R is the universal gas constant ($8.314 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), T is the absolute temperature in Kelvin, and $\Delta G_{A/A}$, ΔG_{A_h} , $\Delta G_{A/C}$, $\Delta G_{C/C}$, ΔG_{C_h} are the change of Gibbs free energy during the formation of AA , A_h , AC , CC , C_h . These Gibbs free energy changes can be calculated based on the Nearest Neighbor (NN) model³² with tools such as RNAstructure^{33, 34} and mfold^{35, 36}. Once all the constants are determined, the performance of a competitor design can be predicted by numerically solving the set of equations (7) to (15) with Matlab (MathWorks, Natick, MA).

The above thermodynamic model helps to determine whether a certain design of the competitor, when displaced by the target, will transit from the “open” configuration to the “closed” configuration. To generate a dramatic change in the optical readout accompanying the transition, the following non-thermodynamic factors are also considered in designing the competitor.

1. The distance between the fluorophore and the quencher when the competitor is in the “open” configuration. Because the three aptamers used in our study (Table 1) have a relatively weak affinity to their targets, only the competitor with shorter loop or longer stems will satisfy the thermodynamic consideration. In this case, when the competitor binds to the aptamer, the fluorophore could still be brought close to the quencher by

the long flexible stems. Therefore, the shared-stem molecular beacon competitors, where along with the loop, one stem is also complementary to the aptamer, are designed. An alternative way to increase the distance between the fluorophore and the quencher in the “open” competitor is to add a stretch of irrelevant nucleotides in the loop of the competitor, such as one design of competitor C2 in Table 1.

2. Position of the fluorophore on the competitor. The fluorescence of the conjugated fluorophore is sensitive to the local environment. To prevent the fluorophore to be quenched by guanine³⁷, the fluorophore should not be attached to the stem with a terminal guanine. To minimize the effect of the aptamer structure on the fluorophore³⁸ when the competitor hybridizes with the aptamer, attachment of the fluorophore to the non-shared stem is usually preferable.

Experimental details

Material—All oligonucleotides used in the experiments (Table 1) were custom-made by Integrated DNA Technologies (Coralville, IA). Upon received, the oligonucleotides were resuspended in nuclease-free water and the resulting 100 μ M oligonucleotide stock solutions were then stored in aliquots at -20°C until used in the experiments. If not specified, all the other reagents were purchased from Sigma-Aldrich (St Louise, MO). The concentration of ATP, GTP, and human α -thrombin was determined by the standard absorbance assay in a quartz cuvette with self-masking black walls (Starna Cells, Atascadero, CA) on a UV/Visible spectrophotometer (Agilent Technologies, Santa Clara, CA).

Detection—Before adding the sample, the aptamer and the competitor was pre-annealed. For thrombin detection, the pre-annealing was performed in 5 mM KCl, 1 mM MgCl_2 , 0.1 mM EDTA, 10 mM Tris, pH 8.0 by heating at 45°C for 5 min. For ATP detection, the pre-annealing was performed in 300 mM NaCl, 5 mM MgCl_2 , 0.1 mM EDTA, 10 mM Tris, pH 8.0 by heating at 65°C for 5 min. Following the addition of the sample, the solution was incubated at room temperature for 30 min and then the fluorescence intensity was recorded on a microplate reader (Molecular Devices, Sunnyvale, CA) with excitation at 490 nm and emission at 520 nm. All the experiments were performed in triplicates.

RESULTS AND DISCUSSION

Detection of thrombin and ATP

To demonstrate the feasibility, the design strategy was first applied to detect thrombin, a key serine protease that has been linked to the prevalent diseases including cardiovascular diseases⁴² and cancers⁴³. Generally speaking, detection with a low-affinity recognition element tends to be more difficult than that with a high-affinity recognition element. However, in order to examine whether our design strategy works for the more challenging cases, we purposely chose the 15nt anti-thrombin DNA aptamer³⁹, whose affinity is 20~50 fold lower than that of another anti-thrombin DNA aptamer⁴⁴. With the help of the thermodynamic model, a 17nt competitor oligo with 6-carboxyfluorescein at 5' and Iowa Black FQ quencher at 3' was designed and tested. In the experiment, an equal molar ratio of the aptamer and the competitor was first mixed and pre-annealed, followed by addition of the samples containing different concentrations of thrombin. Figure 2 shows the fluorescence intensity observed after the samples were incubated with the probes for half an hour. Compared to the sample that contains no thrombin, the samples with thrombin has a lower fluorescence intensity, which indicates that thrombin displaces the competitor from the aptamer, thus allowing the competitor to take the quenched hairpin configuration. The higher the concentration of the thrombin, the lower the fluorescence intensity. This concentration-dependent decrease in the fluorescence intensity can be used to quantify the thrombin in unknown samples. To examine the specificity, the

nonspecific protein bovine serum albumin (BSA) was introduced. Compared to the specific target thrombin at the same concentration, the nonspecific BSA results in only a nominal decrease of fluorescence.

To examine the applicability of our design strategy for non-protein targets, we then applied it to detect the small molecule ATP. Two anti-ATP aptamers were used in our study. One is a 27nt DNA aptamer⁴⁰ and another is a 40nt RNA aptamer⁴¹. Competitors were designed for the two aptamers, respectively. Figure 3 shows the experimental results of using the two aptamers and their corresponding competitors to detect the ATP. Both aptamer-competitor pairs were able to detect ATP with high sensitivity and specificity. This demonstrated the general applicability of the design strategy in Figure 1 to different types of targets (protein and non-protein) as well as different types of aptamers (DNA and RNA).

Thermodynamics-assisted optimization

On one hand, this design strategy greatly simplifies the optimization process by utilizing an unmodified aptamer for target recognition and a molecular beacon competitor for signal transduction. On the other hand, in this design strategy, both target recognition and signal transduction is a thermodynamics-driven process, and therefore it is possible to further rationalize the optimization process with a thermodynamics-based prediction model.

Before using the thermodynamic model represented by Equations (7)–(15) to guide the optimization process, its validity and accuracy was first evaluated. Figure 4A compares the experimental result and the prediction result for the anti-ATP DNA aptamer-competitor C1 pair. To facilitate the comparison, the experimental data is presented in terms of normalized fluorescence change $1 - I/I_{[T]=0}$, where I is the fluorescence intensity in the presence of the target and $I_{[T]=0}$ is the fluorescence intensity in the absence of the target, and the predication data is presented in terms of normalized change of the competitor & aptamer complex (AC) concentration $1 - [AC]/[AC]_{[T]=0}$, where $[AC]$ is the concentration of AC in the presence of the target and $[AC]_{[T]=0}$ is the concentration of AC in the absence of the target. It can be seen that the prediction result doesn't completely agree with the experimental one and however the shape of the experimental and predication curves are similar. This implies the prediction model is at least qualitatively valid.

Considering the qualitative similarity between the experimental and numerical curves, the inaccuracy of the constants used in the predication could be an important reason for the discrepancy between the experimental result and the prediction. Therefore, the dependence of the predication result on the thermodynamic constants was investigated (Figure 4B). There are six constants involved in the prediction model Equations (7)–(15): the dissociation constants of the aptamer/target complex ($K_{A/T}$) and the Gibbs free energy change during the formation of the aptamer/competitor complex ($\Delta G_{A/C}$), the self-structure of the competitor ($\Delta G_{C,h}$), the self-dimer of the aptamer ($\Delta G_{A,h}$), the self-dimer of the competitor ($\Delta G_{C/C}$), and the self-dimer of the aptamer ($\Delta G_{A/A}$). For the anti-ATP DNA aptamer, the $K_{A/T}$ was reported to be $6 \pm 3 \mu\text{M}$.⁴⁰ Replacing the $K_{A/T}$ used in the original predication $6 \mu\text{M}$ (black curve) by $3 \mu\text{M}$ (blue curve), the difference between the predication and the experiment is decreased by ~50%. The Gibbs free energy changes ΔG estimated based on the Nearest Neighbor (NN) model are generally quite accurate. Estimation from different online tools, such as RNAstructure and mfold, could be slightly different in some cases. However, even a slight difference in ΔG could lead to a significant change of the predication result because each 1 kcal/mol difference in the ΔG will cause a ~5.4 time change in the corresponding dissociation constant. ΔG for different reactions has different effects on the predication result. As shown in Figure 4B, a 10% decrease in the magnitude of $\Delta G_{A/C}$ (red curve) leads to a dramatic change of the prediction results compared to the original prediction (black curve); nevertheless, a 10% increase in the magnitude of $\Delta G_{C/C}$ (magenta curve, which is overlapped with the black curve) and $\Delta G_{A/A}$ (data not shown)

causes almost no change. Therefore, when overestimated or underestimated, the constants that have a significant effect on the prediction results, including $\Delta G_{C,h}$, $\Delta G_{A/C}$, $\Delta G_{A,h}$, $K_{A/T}$, are more likely to be responsible for the discrepancy between the experimental result and the prediction. For $\Delta G_{A,h}$ of the aptamer self-structure, only the secondary structure of the aptamer was considered here based on the assumption that the secondary structure is much more stable than other high-order structures. This could result in a significant underestimation of $\Delta G_{A,h}$ because the well-defined 3D structure of the aptamer, which is critical for the aptamer to recognize the specific target with a high affinity,⁴⁵ could be more stable than the secondary structure. Currently, an accurate estimation on the ΔG for the high-order structure of the aptamer can only be experimentally determined.⁴⁶ Although it is possible to further improve the accuracy of the predication model by considering the factors discussed in the above, the simple yet still semi-quantitative predication model presented here is sufficient for the purpose of guiding the optimization.

In Figure 5, the prediction model was used to compare the two designs (C1 and C2) of the competitor for the anti-ATP DNA aptamer. C1 and C2 differ in both $\Delta G_{A/C}$ (-22.2 kcal/mol vs. -16.8 kcal/mol) and $\Delta G_{C,h}$ (-7.5 kcal/mol vs. -4.6 kcal/mol). Therefore, the stability of the competitor/aptamer complex and the competitor self-structure is higher for C1 than C2. Figure 5A shows the normalized change in the concentration of the aptamer/competitor complex [AC] when varying concentration of the target is present. The normalized [AC] change reflects the normalized fluorescence change. The saturation is reached at the lower target concentration with C2, which indicates that the C2 has a smaller dynamic range but a higher sensitivity compared to C1. In addition to the normalized [AC] change, the absolute level of [AC], which reflects the fluorescence intensity, is also an important consideration when comparing different designs. Generally, the higher the fluorescence intensity, the higher the signal-to-noise ratio of the fluorescence measurements. As shown in Figure 5B, use of the design C2 is expected to lead to a poorer signal-to-noise ratio due to the lower fluorescence intensity. To verify whether the comparison made between C1 and C2 with the prediction model is valid, both C1 and C2 were tested in the experiment. As shown in Figure 5C, the normalized fluorescence change is significantly higher at the low target concentration range with C2 than with C1, which indicates that limit of detection (LOD) with C2 is better than with C1. As implied by the error bar in Figure 5C, the variation of fluorescence measurements has a larger effect on C2, which is due to the relatively weaker fluorescence intensity (Figure 5D). The observation in the experiment generally agrees with the predication and this proves that optimization of the competitor design based on the thermodynamic predication is valid.

Furthermore, the thermodynamics-based predication model can also be used to optimize the experimental conditions. One of such examples was shown in Figure 6. The predication shows that the lower the concentration of the aptamer and the competitor, the lower the absolute [AC] (Figure 6B) but the higher the normalized [AC] change (Figure 6A). The same trends were observed in the experiment (Figure 6C, 6D). Therefore, the predication model can be used to find out the optimal concentration of the aptamer and the competitor for a certain circumstance. Besides the concentration of the aptamer and the competitor, other experimental conditions that can be reflected in the thermodynamic-based predication model, such as temperature and buffer composition, can also be optimized in a similar way.

CONCLUSION

We developed a generic design strategy to detect non-nucleic acid targets with an unmodified aptamer and a fluorogenic competitor, which was partially complementary to the aptamer. First of all, compared to most of design strategies where extensive optimization is necessary to preserve the affinity and specificity of the modified aptamer, elimination of modification on aptamers greatly simplifies the implementation of the design strategy. This has been

demonstrated with different types of non-nucleic acid targets (small molecules and proteins), but also with different types of aptamers (DNA and RNA). Secondly, compared to the label-free design strategies which utilize non-selective dye to generate the optical output and thus suffer from limited applicability in multiplex detection, the current strategy allows different dyes to be used to monitor different targets and has the capability to simultaneously detect multiple targets. Thirdly, the thermodynamic-based predication model enables the rational optimization on not only the probe design, but also the experimental conditions.

Due to the rapidness and simplicity in developing and performing the detection assay, the design strategy holds promising potential in many high throughput applications. For a particular application, the design strategy should be optimized to satisfy the corresponding requirements on various aspects such as sensitivity, specificity, and detection time. If a high sensitivity is required, the sensitivity could be improved by using high-affinity aptamer, and/or changing the sequence and the dye-quencher pair of the competitor, and/or optimizing the probe concentration and the buffer composition. If a high specificity is needed, the major sources of non-specificity in the detection environment should be carefully identified and minimized. If a fast detection is desired, optimization could be carried out on binding kinetics between the aptamer and the competitor (e.g. tune the length and GC contents of loop and stems in the molecular beacon competitor) and/or the binding kinetics between the aptamer and the target (e.g. manipulate the length of incubation time during *in vitro* selection cycle).

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Figure 1.

Schematic representation of a design strategy for aptamer-based optical probes. An unmodified aptamer specific to the target and a competitor complementary to the aptamer are used. The competitor is a hairpin oligonucleotide with a fluorophore attached on one end and a quencher attached on the other. When there is no target, the competitor binds to the aptamer. However, upon introduction of the target, the competitor is displaced from the aptamer by the target, thus leading to a target-specific decrease in the fluorescence signal.

Figure 2.

Detection of human α -thrombin based on an anti-thrombin DNA aptamer. Experiments were carried out with 0.8 μ M competitor and aptamer in the buffer containing 5 mM KCl, 1 mM MgCl_2 , 0.1 mM EDTA and 10 mM Tris (pH 8.0).

Figure 3. Detection of ATP: (A) Based on an anti-ATP DNA, (B) Based on an anti-ATP RNA aptamer. Detection was performed in with 0.2 μM competitor/aptamer (300 mM NaCl, 5 mM MgCl_2 , 0.1 mM EDTA, 10 mM Tris, pH 8.0). The competitor design C1 was used in (A).

Figure 4. Validity of the thermodynamic model: (A) Comparison of the experimental result and the thermodynamic prediction, (B) Effects of the constants on the thermodynamic prediction. 0.2 μM of the anti-ATP DNA aptamer and the competitor C1 was used in both experiments and predictions.

Figure 5. Effects of the competitor design: Experimental ((A), (B)) and Prediction results ((C), (D)). 0.2 μM of the anti-ATP DNA aptamer and the competitor C1 or C2 was used in both experiments and predictions.

Figure 6.
Effects of the probe concentration: Experimental ((A), (B)) and Prediction results ((C), (D)).
The anti-ATP RNA aptamer and the competitor were used in both experiments and predictions.

Table 1

Sequences of the aptamer and the competitor

Target	Aptamer Type	K _D	Aptamer Sequence (5' → 3')	Competitor Sequence (5' → 3')
Thrombin	DNA	25~200 nM ³⁹	GGT TGG TGT GGT TGG	/6-FAM/ <u>TGG TGT</u> CAA CCA <u>CAC CA</u> /IabFQ/
ATP	DNA	6±3 μM ⁴⁰	ACC TGG GGG AGT ATT GCG GAG GAA GGT	C1: /IabFQ/ <u>ACC TGG GAA</u> TAC TCC <u>CCC AGG T</u> /6-FAM/ C2: /IabFQ/ <u>ACC TTC</u> CTC CGG TTA <u>GAA GGT</u> /6-FAM/
ATP	RNA	0.7 μM ⁴¹	GGG UUG GGA AGA AAC UGU GGC ACU UCG GUG CCA GCA ACC C	/6-FAM/ <u>TGC CAC</u> AGT TTC TTC CCA <u>ATG GCA</u> /IabFQ/

¹ IabFQ: Iowa Black FQ dark quencher, 6-FAM: 6-carboxyfluorescein.

² Underlined normal fonts represent the non-shared stem, underlined italics fonts represent the shared stem, and strikethrough bold fonts represent the part of the loop that is not complementary to the aptamer.

Table 2

Constants used for the predications in Figure 4B

	$\Delta G_{A/C}$ (kcal/mol)	$\Delta G_{C/h}$ (kcal/mol)	$K_{A/T}$ (μM)	$\Delta G_{A/h}$ (kcal/mol)	$\Delta G_{C/C}$ (kcal/mol)	$\Delta G_{C/C}$ (kcal/mol)
Original	-22.2	-7.5	6	-1.8	-15.5	-5.6
$\Delta G_{A/C}$, -10%	-19.98	-7.5	6	-1.8	-15.5	-5.6
$\Delta G_{C/h}$, +10%	-22.2	-8.25	6	-1.8	-15.5	-5.6
$K_{A/T}$, -50%	-22.2	-7.5	3	-1.8	-15.5	-5.6
$\Delta G_{A/h}$, +10%	-22.2	-7.5	6	-1.98	-15.5	-5.6
$\Delta G_{C/C}$, +10%	-22.2	-7.5	6	-1.8	-17.05	-5.6

¹ The constants different from that used in the original predication are highlighted with bold italics.

² ΔG used in the original predication was estimated with mfold under the following conditions: $T = 25^{\circ}C$, $[C]T = [A]T = 0.2\mu M$, $[Na^{+}] = 300mM$, $[Mg^{2+}] = 5mM$