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Aptamer-Based ATP Assay Using a Luminescent Light Switching Complex

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With the increasing applications of nucleic acid aptamers as a new class of molecular recognition probes in bioanalysis and biosensor development, the development of general and simple signaling strategies to transduce aptamer−target binding events to detectable signals is demanding. We have developed a new signaling method based on aptamers and a DNA molecular light switching complex, $[Ru(phen)_2(dppz)]^{2+}$, for sensitive protein detection. In this work, we have demonstrated the applicability of this signaling mechanism to small-molecule detection using ATP as a model target. Our results have shown that upon ATP binding to the folded aptamer where $[Ru(phen)_2(dppz)]^{2+}$ intercalated, the conformational change or distortion of the aptamer is large enough to cause a significant luminescence change of $[Ru(phen)_2(dppz)]^{2+}$. By monitoring the ATP-dependent luminescence intensity change, we have achieved ATP detection with high selectivity and high sensitivity down to 1 nM in homogeneous solution. The method is very simple without the needs for covalently labeling aptamers or using costly enzymes and multistep analysis as other reported fluorescence/luminescence assays for ATP. The successful detection of ATP indicates that using the signaling aptamers with $[Ru(phen)_2(dppz)]^{2+}$ is expected to be a general method for aptamer-based target detection.

Recent years have seen rising interest in the development of aptamers, a new class of single-stranded DNA/RNA molecules selected from synthetic nucleic acid libraries, for molecular recognition and bioanalytical applications.[1−7] The advantages of aptamers such as simple synthesis, easy labeling, good stability, and wide applicability have made them ideal recognition probes for analyte detection. For example, aptamers against proteins have been expected to rival commonly used antibodies in diagnosis and drug development.[8] Since first proposed in 1990, the in vitro SELEX (systematic evolution of ligands by exponential enrichment) process has been recognized as a general method to select aptamers for any target ranging from metal ions over small organic molecules, complex proteins, to entire organisms.[1,2] However, efforts are still being made to develop general strategies to transduce aptamer recognition events to detectable signals, which is the key to realizing their potentials in analytical applications.[3−7]

Recently, we have developed a new method of signaling aptamer binding for aptamer-based protein detection using a molecular light switching complex, $[Ru(phen)_2(dppz)]^{2+}$ (structure shown in Figure 1A).[4] The method takes advantage of the unique property of $[Ru(phen)_2(dppz)]^{2+}$: it has no luminescence in aqueous solution but strong luminescence when intercalating to the nonaqueous pocket of DNA duplex.[9] Although aptamers are single-stranded DNAs or RNAs, they usually fold into special three-dimensional structures through base pairing. This allows the intercalation of $[Ru(phen)_2(dppz)]^{2+}$ to emit luminescence. The binding of a protein to an aptamer changes or distorts the aptamer conformation, leading to a significant protein-dependent luminescence change.[4] Monitoring the luminescence change, we have achieved the protein quantitation in homogeneous solution with high sensitivity and selectivity. The generalizability of the method for protein detection is very promising as demonstrated by the detection of the three tested proteins with their DNA or RNA aptamers. In this work, we further extend this new signaling strategy for small -molecule detection using adenosine triphosphate (ATP) as a model target.

ATP is an important substrate in living organisms.[10,11] Being a major energy currency of the cell, it plays a critical role in the

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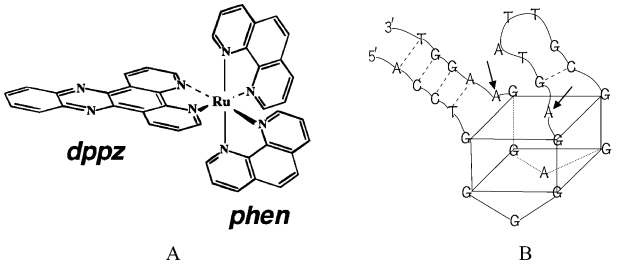

Figure 1. Structures of $[Ru(phen)_2dppz]^{2+}$ (A) and the ATP aptamer (B)

regulation of cellular metabolism and biochemical pathways in cell physiology. It has also been used as an indicator for cell viability and cell injury. Therefore, the determination of ATP is essential in biochemical study as well as clinic diagnosis. Methods based on chromatography, fluorometry, bioluminescence, chemiluminescence, and electrochemical biosensors have been developed for ATP detection.[11−12] The fluorescence/luminescence assays have the advantage of sensitive ATP analysis in homogeneous solution without the need for target separation. However, both bioluminescence and chemiluminescence methods involve chemical reactions among multiple components; especially the enzymes used in bioluminescence are costly and unstable. Ellington et al. recently designed a signaling aptamer that could be used for ATP detection.[6] They labeled a fluorescent dye adjacent to the binding site of a DNA or RNA aptamer against ATP and observed ATP-dependent fluorescence signal change due to the change of the microenvironment of the fluorophore. The lowest ATP concentration used in their report was 10 μM, which is higher than the detection limits of the common ATP assays.[11,12] With our new aptamer signaling strategy based on the molecular light switch, ATP has been detected in physiological buffer with high selectivity and sensitivity down to 20 nM. A 1 nM ATP detection limit has been achieved under an optimized buffer condition. The method is simple and does not need the labeling of aptamers. Our results have demonstrated that the binding of small molecules is able to change aptamer conformation and thus the luminescence of $[Ru(phen)_2(dppz)]^{2+}$. Therefore, using signaling aptamers with the luminescent light switching complex is expected to be a general method for aptamer-based target detection.

EXPERIMENTAL SECTION

Materials. The 27-nt ATP aptamer used in the experiment,[6,14] 5′-ACCTG GGGGA GTATT GCGGA GGAAG GT-3′, its complementary strand, 5′-ACCTT CCTCC GCAAT ACTCC CCCAG GT-3′, and the thrombin DNA aptamer, 5′-GGTTG GTGTG GTTGG-3′, were all synthesized by SBS Genetech Co. (Beijing, China). ATP, UTP, GTP, and CTP were purchased from Sigma (St. Louis, MO). $[Ru(phen)_2dppz]^{2+}$ was synthesized as in ref 13. The physiological buffer used in the experiment consisted of 20 mM Tris-HCl (pH 7.6), 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Milli-Q purified water (18.2 MΩ) was used to prepare all the solutions.

Instrumentation. Luminescence measurements were performed on a Fluorolog-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ). All experiments were carried out at room temperature. The sample cell was a 1-mL quartz cuvette. The luminescence intensity was monitored by exciting the sample at 450 nm and measuring the emission at 610 nm. The slits for both excitation and emission were set at 12 nm. The fitting of the experimental data was accomplished using the software Origin 6.0.

RESULTS AND DISSCUSSIONS

ATP-Dependent Aptamer/$[Ru(phen)_2(dppz)]^{2+}$ Luminescence Change. A 27-nt DNA aptamer against ATP selected by Huizenga and Szostak[14] was used in this work. It is known that although aptamers are single-stranded DNAs or RNAs, their folded three-dimensional structures are critical for the recognition of their specific targets. As shown in Figure 1B, the DNA aptamer against ATP has two stacked G-quartets and two short double-helix stems. The proposed binding site for ATP is above the upper quartet and between the two adenosine residues that are adjacent to the quartet (shown by the two arrows).[6,14] In the luminescence measurement, while the luminescence intensity of the $[Ru(phen)_2(dppz)]^{2+}$ solution was low, it increased greatly (~15 times) after the addition of the ATP aptamer. This indicated that $[Ru(phen)_2(dppz)]^{2+}$ did intercalate into the folded aptamer efficiently. We have found that the ratio of the concentrations of $[Ru(phen)_2(dppz)]^{2+}$ to the aptamer was ~6:1 when the maximum luminescence intensity was reached. In our later experiments for ATP detection, the concentration ratio of 20 was used to ensure the saturation of $[Ru(phen)_2(dppz)]^{2+}$ intercalation. When 1 μM ATP was added, the luminescence intensity of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ decreased markedly (~50% decrease).

To confirm the ATP-dependent luminescence signal decrease, a 15-nt DNA aptamer against thrombin was used for a control experiment.[15] The thrombin aptamer has two stacked G-quartets similar to the ATP aptamer, but it has no double-helix stem. The luminescence of the thrombin aptamer with $[Ru(phen)_2(dppz)]^{2+}$ is much lower than that of the ATP aptamer, indicating the intercalation of $[Ru(phen)_2(dppz)]^{2+}$ to the G-quartets is weak. As ATP could not bind with the thrombin aptamer, the addition of 1 μM ATP to the thrombin DNA aptamer/$[Ru(phen)_2(dppz)]^{2+}$ solution resulted in no luminescence intensity change. Another control experiment was performed by adding ATP to a mixture of $[Ru(phen)_2(dppz)]^{2+}$ and the duplex DNA formed by the ATP aptamer and its complementary ssDNA. Although the luminescence of the $[Ru(phen)_2(dppz)]^{2+}$/dsDNA was high, its signal was essentially the same in the absence or presence of ATP (data not shown). All these data supported the idea that the binding of ATP is indeed the reason for the luminescence intensity decrease of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$.

The above results demonstrated that the reported protein signaling mechanism[4] based on aptamer and the molecular light switching complex can be also applied to the signaling of small target molecules such as ATP. In aqueous solution, $[Ru(phen)_2(dppz)]^{2+}$ has no luminescence as its triplet metal-to-ligand charge-transfer excited state is effectively quenched by hydrogen binding between water and the phenazine nitrogen of the ligand.[9] When

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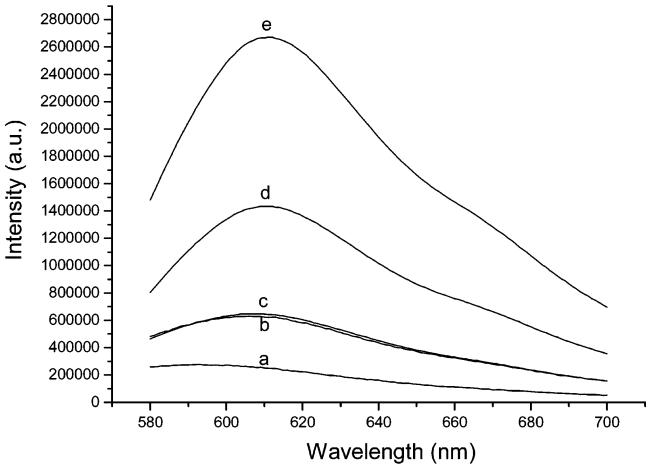

Figure 2. Luminescence emission spectra of $[Ru(phen)_2(dppz)]^{2+}$ (a); the mixture of $[Ru(phen)_2(dppz)]^{2+}$, the thrombin DNA aptamer, and ATP (b); the mixture of $[Ru(phen)_2(dppz)]^{2+}$ and the thrombin DNA aptamer (c); the mixture of $[Ru(phen)_2(dppz)]^{2+}$, the ATP aptamer, and ATP (d); the mixture of $[Ru(phen)_2(dppz)]^{2+}$ and the ATP aptamer in the physiological buffer (e). The concentrations of the ATP aptamer and thrombin aptamer were all 0.1 μM. The concentrations of $[Ru(phen)_2(dppz)]^{2+}$ and ATP were 2 and 1 μM, respectively.

it binds to the ATP aptamer, the intercalation between the ligand and base pairs of the folded aptamer protects the phenazine nitrogen from water, resulting in an intense luminescence emission. Since the luminescence of $[Ru(phen)_2(dppz)]^{2+}$ is remarkably sensitive to its local environment, the induced aptamer conformational change/distortion and the blocking of $[Ru(phen)_2(dppz)]^{2+}$ intercalation result in a significant ATP-dependent luminescence change. In our previous reports, the luminescence intensity decreases caused by the proteins were 35−50%.[4] Therefore, the luminescence decrease brought by the binding of small target molecule ATP is comparable to that by the macromolecule proteins. This enables a sensitive aptamer-based detection of small molecules using the aptamer/ $[Ru(phen)_2(dppz)]^{2+}$ strategy.

High Selectivity of the Method. We have tested the selectivity of the signaling aptamer for ATP by comparing the luminescence signal changes of the aptamer/ $[Ru(phen)_2(dppz)]^{2+}$ brought by ATP and other three ATP analogues, UTP, CTP, and GTP. The only difference in the structure of these four molecules is the nucleobase attached to ribose. When 0.2 μM ATP, UTP, CTP, and GTP were added respectively to the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ solution with 0.1 μM aptamer, only ATP caused a marked luminescence reduction (28% decrease). The other three analogues failed to cause significant changes in luminescence (3% for UTP, 0.8% for GTP, and 0.3% for CTP). Increasing the concentrations of the three ATP analogues did not result in obvious signal changes either. This selectivity is as high as that of the ATP signaling method in the previous report where the same DNA aptamer was used but labeled with fluorescein.[6] The inherent specificity of the aptamer toward its target ATP is kept in this aptamer/$[Ru(phen)_2(dppz)]^{2+}$ signaling complex.

High Sensitivity for ATP Quantitation. Titration of ATP was carried out to determine the sensitivity of the method for ATP detection. During the titration, different amounts of ATP were introduced to the ATP aptamer/ $[Ru(phen)_2(dppz)]^{2+}$ in physiological buffer. The typical response curve (Figure 3A) showed a monotonic decrease of the luminescence intensity following the increase of ATP until a plateau was reached. When 100 nM ATP aptamer was used, there was a good linear relationship between the luminescence decrease and ATP concentration from 0 to 100 nM with the correlation coefficient of 0.998 (Figure 3B). the detection limit for ATP was experimentally determined to be 20 nM based on a signal/noise ratio of >3. Although the dynamic range of the method is limited (~1 order), the sensitivity is comparable or better than that in the reported ATP assays (1 nM−10 μM).[6,11,12] This detection limit was also achieved in the presence of 100 nM dsDNA (formed by the ATP aptamer and its complementary ssDNA) when a higher concentration of $[Ru(phen)_2(dppz)]^{2+}$ was used to ensure the saturation of the intercalating dye to the aptamer. Therefore, the high sensitivity of the method is not affected by the background dsDNAs. Moreover, our method is simple without the need for aptamer labeling or target separation.

As the ATP aptamer has two ATP binding sites,[6,14] we consider the following two equilibriums in the titration experiment:

$$A + B \overset{K_1}{\rightleftharpoons} AB_1 \qquad K_1 = \frac{[AB_1]}{[A][B]} \tag{1}$$

$$AB_1 + B \overset{K_2}{\rightleftharpoons} AB_2 \qquad K_2 = \frac{[AB_2]}{[AB_1][B]} \tag{2}$$

Where A stands for the ATP aptamer/$[Ru(phen)_2(dppz)]^{2+}$ complex in the presence of an extra amount of $[Ru(phen)_2(dppz)]^{2+}$. B stands for ATP. AB_1 and AB_2 stand for the complexes of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ binding with one or two ATP molecules, respectively. K_1 and K_2 are the association constants for the formation of AB_1 and AB_2.

Then, the equations showing the dependence of the luminescence change (z) of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ with the added ATP concentration (c) can be deduced (details in Supporting Information):

$$z = \frac{(1-f_1)K_1 n}{K_2 + K_1 n + K_1 n^2} + \frac{(1-f_2)K_1 n^2}{K_2 + K_1 n + K_1 n^2} \tag{3}$$

$$n^3 + (1 - K_2 c + 2aK_2)n^2 + \left(\frac{K_2}{K_1} - K_2 c + aK_2\right)n - \frac{cK_2^2}{K_1} = 0 \tag{4}$$

where a is the initial concentration of the ATP aptamer, f_1 is the ratio of the molar luminescence intensities of AB_1 to A, and f_2 is the ratio of the molar luminescence intensities of AB_2 to A. n is the ratio of concentrations of AB_2 to AB_1.

By fitting the experiment data in Figure 3A to eqs 3 and 4 using the nonlinear least-squares fitting, the calculated two dissociation constants ($1/K_1$,$1/K_2$) of ATP to the aptamer were 0.1 and 0.2 μM (the fitting correlation coefficient is 0.99; Supporting Information). Comparing the derived relative molar luminescence intensities of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ with one or two ATP molecules (f_1, 85%; f_2, 32%), it showed that when the second ATP bound, more luminescence decrease was obtained.

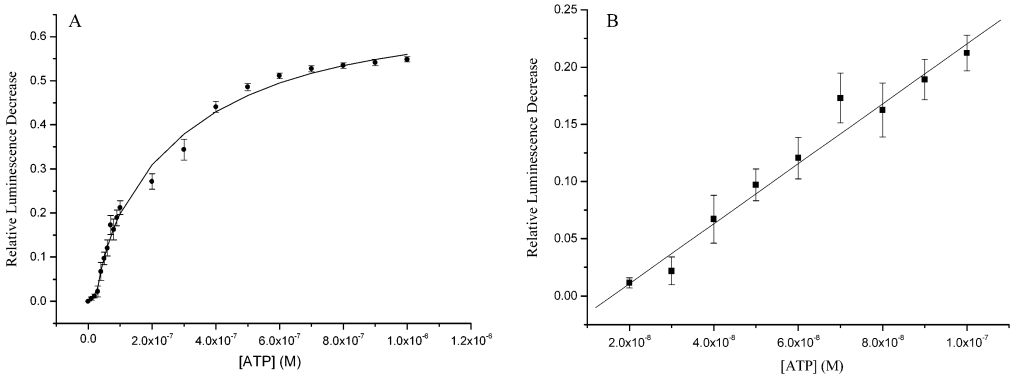

Figure 3. (A).Titration of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ by ATP in the concentration range of 0−1 μM. The circles with the error bars were the experimental data averaged by four parallel experiments, and the line was the fitting curve. (B).The linear relationship of the luminescence change of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ and ATP concentration ranging from 0 to 0.1 μM. The concentrations of the ATP aptamer and $[Ru(phen)_2(dppz)]^{2+}$ were 0.1 and 2 μM, respectively.

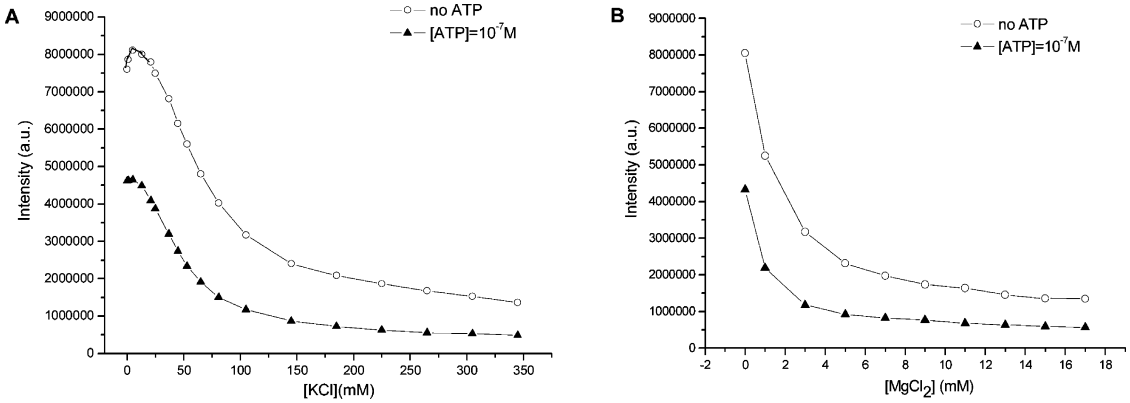

Figure 4. Effect of metal ion on the luminescence of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ with and without ATP in the Tris-HCl buffer (20 mM, pH 7.6). The concentrations of the ATP aptamer, ATP, and $[Ru(phen)_2(dppz)]^{2+}$ were 0.1, 0.1, and 2 μM, respectively.

This indicated that a larger conformation change of the ATP aptamer took place when two ATPs bound. It is worth noting that the two dissociation constants we calculated were different from what Ellington et al. reported for the fluorescein-labeled aptamer (K_{d1}, 30 μM; K_{d2}, 53 μM).[6] The possible reason is that we monitored the luminescence change of the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ complex instead of the aptamer alone. Therefore, K_1 and K_2 show the affinity of ATP to the aptamer/$[Ru(phen)_2(dppz)]^{2+}$ complex. Moreover, the difference in the intercalation of $[Ru(phen)_2(dppz)]^{2+}$ to the two double-helix stems of the aptamer resulted in its different effects on the two ATP binding sites, which are close to the stems.

Effect of the Ionic Strength of the Solution. It is known that the affinities of both $[Ru(phen)_2dppz]^{2+}$ to dsDNA and an aptamer to its target molecule may depend on buffer salt concentration.[3,9] We have investigated the effect of metal cations on ATP detection. Since the luminescence change (the luminescence intensity before ATP binding minus that after binding) was used for ATP detection, the effect of metal ion concentration on both aptamer/$[Ru(phen)_2dppz]^{2+}$ and aptamer/$[Ru(phen)_2dppz]^{2+}$ with ATP were studied.

As shown in Figure 4, the effects of the two metal ions on the luminescence of the aptamer/$[Ru(phen)_2dppz]^{2+}$ were different. The luminescence of the aptamer/$[Ru(phen)_2dppz]^{2+}$ always decreased with the increase of Mg^{2+} concentration. However, with the increase of K^+ concentration, the luminescence of aptamer/$[Ru(phen)_2dppz]^{2+}$ first increased slightly and then decreased significantly at higher K^+ concentration (>21 mM). Since metal ions are expected to reduce the electrostatic force between the negatively charged DNA bone and positively charged $[Ru(phen)_2dppz]^{2+}$, they usually have a negative effect on the intercalation of $[Ru(phen)_2dppz]^{2+}$ to the aptamer. However, monovalent cations are known to embed into or between the planes of G-quartets for the stacking of two or more G-quartets. Therefore, a small amount of K^+ is benefit for the stabilization of the aptamer's folding structure, resulting in better $[Ru(phen)_2(dppz)]^{2+}$ intercalation, thus stronger luminescence. On the other hand, with the increase of either monovalent K^+ or divalent Mg^{2+} concentration, the luminescence of the binding complex of aptamer/$[Ru(phen)_2dppz]^{2+}$ with ATP decreased.

Considering the overall effect of the metal ions, the maximum luminescence change for the ATP assay occurred at an optimized K^+ concentration (21 mM) without any Mg^{2+}. Under this condition (Tris-HCl 20 mM, pH 7.6 and KCl 21mM), the detection limit of ATP could be improved to 1 nM based on the signal/noise ratio of >3 (data not shown). This is comparable to the best ATP detection limits in the previous reports.[11] However, to demonstrate the capability of the aptamer probe for direct ATP detection in biological samples, the nanomolar detection capability in physiological buffer might be more important.

CONCLUSION

In this work, we have developed a new ATP detection method based on the aptamer and the molecular light switching complex $[Ru(phen)_2(dppz)]^{2+}$. The method takes advantage of the sensitive luminescence intensity change when ATP binds to the folded aptamer where $[Ru(phen)_2(dppz)]^{2+}$ intercalated. The ATP-dependent luminescence change is large enough for ATP detection in homogeneous solution with high sensitivity. The method is simple, label-free, and highly selective. The signaling aptamer with $[Ru(phen)_2(dppz)]^{2+}$ has been previously developed for macromolecule protein detection. This work has demonstrated that the binding of small molecules also enables a significant luminescence change of $[Ru(phen)_2(dppz)]^{2+}$. Therefore, using signaling aptamers with $[Ru(phen)_2(dppz)]^{2+}$ as a general method for aptamer-based target detection is promising. The developed signaling strategy is expected to promote the exploitation and application of aptamers in biochemical and biomedical studies.

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SUPPORTING INFORMATION AVAILABLE

The deduction of the equation showing the luminescence change as the function of the ATP concentration, and the results of the experiment data fitting. This material is available free of charge via the Internet at http://pubs.acs.org.

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