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Rapid and simple G-quadruplex DNA aptasensor with guanine chemiluminescence detection

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ABSTRACT

Cost-effective and sensitive aptasensor with guanine chemiluminescence detection capable of simply quantifying thrombin in human serum was developed using thrombin aptamer (TBA), one of the G-quadruplex DNA aptamers, without expensive nanoparticles and complicated procedures. Guanines of G-quadruplex TBA-conjugated carboxyfluorescein (6-FAM) bound with thrombin do not react with 3,4,5-trimethoxylphenylglyoxal (TMPG) in the presence of tetra-*n*-propylammonium hydroxide (TPA), whereas guanines of free TBA- and TBA-conjugated 6-FAM immobilized on the surface of graphene oxide rapidly react with TMPG to emit light. Thus, guanine chemiluminescence in 5% human serum with thrombin was lower than that without thrombin when TBA-conjugated 6-FAM was added in two samples and incubated for 20 min. In other words, the brightness of guanine chemiluminescence was quenched due to the formation of G-quadruplex TBA-conjugated 6-FAM bound with thrombin in a sample. High-energy intermediate, capable of emitting dim light by itself, formed from the reaction between guanines of TBA and TMPG in the presence of TPA, transfers energy to 6-FAM to emit bright light based on the principle of chemiluminescence energy transfer (CRET). G-quadruplex TBA aptasensor devised using the rapid interaction between TBA-conjugated 6-FAM and thrombin quantified trace levels of thrombin without complicated procedures. The limit of detection (LOD = background + 3 × standard deviation) of G-quadruplex TBA aptasensor with good linear calibration curve, accuracy, precision, and recovery was as low as 12.3 nM in 5% human serum. Using the technology reported in this research, we expect that various types of G-quadruplex DNA aptasensors capable of specifically sensing a target molecule such as ATP, HIV, ochratoxin, potassium ions, and thrombin can be developed.

1. Introduction

Since 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990), it has been well-known that nucleic acid aptamers selected from a large pool of random sequences, instead of expensive antibodies produced from the sacrifice of small animals, can bind specifically to target molecules in a sample collected from human (Bock et al., 1992; Phan et al., 2005), food (Cruz-Aguado and Penner, 2008), and nature (Ueyama et al., 2002). For example, 15 nt thrombin aptamer (TBA, 5′-GGTTGGTGTGGTTGG-3′) was developed to inhibit thrombin activity capable of converting fibrinogen into clottable fibrin in the hemostasis process (Bock et al., 1992). Studies with circular dichroism (CD) spectra (Pasternak et al., 2011), NMR (Kelly et al., 1996), and X-ray (Kelly et al., 1996; Padmanabhan et al., 1993) were confirmed that the shape of TBA bound with two anion binding recognition exosites of thrombin is G-quadruplex as shown in Fig. 1(a). G-quadruplex was formed with two square planar structures. The square planar structure called a guanine tetrad was formed through hydrogen bonding between four guanines as shown in Fig. 1(b). A number of anti-parallel or parallel G-quadruplex aptamers were developed to bind and quantify a wide variety of target molecules such as ATP (Huizenga and Szostak, 1995), hemin (Travascio et al., 1998), HIVs (Phan et al., 2005), insulin (Yoshida et al., 2009), ochratoxin (Cruz-Aguado and Penner, 2008), potassium ions (Ueyama et al., 2002), Sars-CoV helicase (Shum and Tanner, 2008), and selerostin (Shum et al., 2011) in a sample. Using the characteristics of G-quadruplex DNA aptamers, currently, various types of aptasensors with chemiluminescence (Bi et al., 2010; Freeman et al., 2011; Freeman et al., 2012; Zhou et al., 2012), colorimeter (Zhang et al., 2011), electrochemical (Yuan et al., 2011), or fluorescence (Yuanboonlim et al., 2012) detection have been developed.

Glow chemiluminescence with the reaction between guanine nucleotides and 3,4,5-trimethoxylphenylglyoxal (TMPG) was observed

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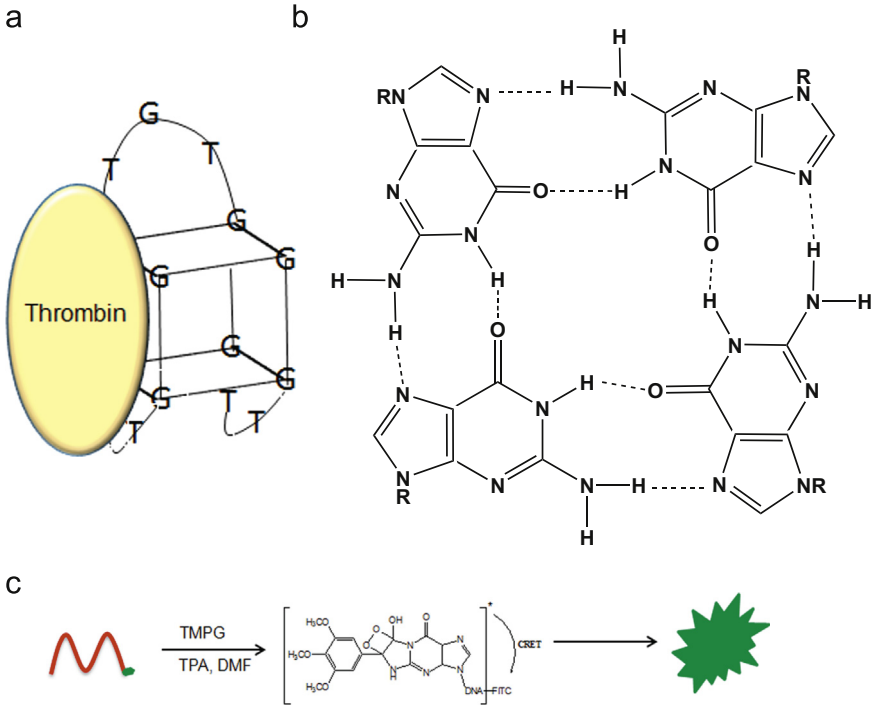

Fig. 1. (a) G-quadruplex TBA aptamer bound with thrombin, (b) G-tetrad structure of guanines in G-quadruplex TBA, and (c) CRET between high-energy intermediate and FITC in guanine chemiluminescence.

(Kai et al., 1999). Recently, Yamasuji et al. (2011) observed bright chemiluminescence emitted in the presence of fluorescein-5-isothiocyanate (FITC) conjugated with G-rich single strand DNA. Based on the principle of chemiluminescence resonance energy transfer (CRET) as shown in Fig. 1(c), FITC excited by dim chemiluminescence generated from the reaction between G-rich single strand DNA and TMPG emitted bright green light (Yamasuji et al., 2011). The results indicate that it is possible to develop highly sensitive biosensors with guanine chemiluminescence detection.

Fig. 1 indicates that TBA, G-rich single strand DNA, can be transformed to G-quadruplex in the presence of thrombin as well as react with TMPG to emit glow chemiluminescence. Using the combination of two apparently distinctive roles of TBA, it is possible to develop G-quadruplex DNA aptasensor with guanine chemiluminescence detection capable of sensing thrombin in a sample. Based on the hypothesis, cost-effective, rapid, and simple G-quadruplex aptasensor with highly sensitive guanine chemiluminescence detection was developed in this study.

2. Experimental

2.1. Chemical and materials

Thrombin aptamer (TBA) and TBA (5′-6-FAM-GGTTGGTGTGGTTGG-3′) conjugated with 6-carboxyfluorescein (6-FAM) were purchased from Alpha DNA (Montreal, Quebec, Canada). Thrombin was purchased from Sigma-Aldrich (100 UN, Milwaukee, WI, USA). 3,4,5-trimethoxylphenylglyoxal hydrate (TMPG, 97%) was purchased from Matrix Scientific (Columbia, SC, USA). $FeCl_2$ (99%), and $FeCl_3$ (99%), Tetra-*n*-propylammonium hydroxide (TPA, 40% w/w aqueous solution) was purchased from Alfa Aesar (Ward Hill, MA, USA). Graphene oxide (GO, 5.5 mg/ml, Flake size: 0.5–5 μm) was purchased from Graphene Supermarket (Calverton, NY, USA). N,N-Dimethylformamide (DMF) and deionized water purchased from EMD (Billerica, MA, USA). Phosphate buffer solution (1.0 M, pH 7.0) was purchased from Teknova (Holliser, CA, USA). 0 calibrator (human serum) was purchased from Monobind, Inc. (Lake Forest, CA, USA).

2.2. Procedure

2.2.1. Interaction between TBA and GO in guanine chemiluminescence

100 nM TBA or TBA conjugated with 6-FAM was prepared in water. GO (110 μg/ml) was prepared in phosphate buffer (5 mM, pH 7). TPA (0.01 M) was prepared in water. TMPG (2 mM) was prepared in DMF. TBA solution (100 μl) was mixed with GO solution (100 μl) in a 1.5-ml microcentrifuge tube. The mixture was incubated for 10 min. After the incubation, the mixture (20 μl) was mixed with TPA (10 μl) in a borosilicate test tube (12 × 75 mm). The test tube was inserted into the sample holder of luminometer with two dispensers (Lumat LB 9507, Berthold Technologies). After touching the start button of the luminometer, the test tube was moved into the detection area. Then, TMPG (200 μl) was injected using the dispenser of luminomer. Finally, light emitted in the test tube was measured for a certain time (e.g., 25, 50, 100 s).

2.2.2. Fabrication of magnetic Fe_3O_4 GO nanoparticles

Magnetic Fe_3O_4 GO nanoparticles were prepared with the modification of method to fabricate magnetic Fe_3O_4 GO/polystyrene nanocomposite (Kassaee et al., 2011). $FeCl_2$ (0.44 mg) and $FeCl_3$ (0.11 mg) were dissolved and mixed in water (5 ml). GO (1.1 mg/ml) was prepared in water. The mixture of $FeCl_2$ and $FeCl_3$ (0.5 ml) was mixed with GO (0.5 ml) in a 1.5 ml-centrifuge tube. The microcentrifuge tube inserted into a Micro Centrifuge Tube Thermomixer (Eppendorf) was shaked for 10 s at 85 °C. Then, ammonium hydroxide (20 μl, 30%) was dispensed into the microcentrifuge. The microcentrifuge tube in the themomixer was shaken at 1400 rpm for 50 min at 85 °C. Magnetic Fe_3O_4 GO nanoparticles formed in the microcentrifuge tube were cooled at room temperature. Magnetic Fe_3O_4 GO nanoparticles in the microcentrifuge tube were washed 3 times in water using a magnetic separator (Bioclone, Inc.).

Magnetic Fe_3O_4 GO nanoparticles as a stock solution were stored in a refrigerator. We obtained images of magnetic Fe_3O_4 GO nanoparticles with a JEOL 2100 TEM operating at 200 kV accelerating voltage and a Gatan Ultrascan 1000XP digital camera.

2.2.3. Removal of TBA not bound with thrombin using magnetic Fe_3O_4 GO nanoparticles

8 different concentrations of thrombin (0–8 μg/ml) were prepared in water. 200 nM TBA conjugated with 6-FAM was prepared in water. 0.01 M TPA was prepared in water. 2 mM TMPG was prepared in DMF. The stock solution of magnetic Fe_3O_4 GO nanoparticles was diluted 10 times in water. Each solution containing a certain concentration of thrombin (100 μl) was mixed with TBA conjugated with 6-FAM (100 μl) in a 1.5-ml microcentrifuge tube. Then, the mixture was incubated for 20 min at room temperature (21 ± 2 °C). After the incubation, 10-time diluted magnetic Fe_3O_4 GO nanoparticles were inserted into the microcentrifuge tube. The mixture was incubated for 1 min to remove TBA not bound with thrombin based on the π-π stacking interaction between TBA and magnetic Fe_3O_4 GO nanoparticles. Then, magnetic Fe_3O_4 GO bound with TBA was removed from the solution using the magnetic separator. The solution containing only TBA bound with thrombin (20 μl) was mixed with TPA (10 μl) in a borosilicate test tube. Finally, light emitted in the test tube was measured for 20 s using the luminometer after adding TMPG (200 μl) in the test tube using the dispenser of luminometer.

2.2.4. Quantification of thrombin with TBA without nanoparticles capable of removing TBA not bound thrombin in a sample

8 standards (0–8 μg/ml) to quantify thrombin in a sample were prepared in 5% human serum in water. 250 nM TBA conjugated with 6-FAM and 0.01 M TPA were prepared in water. 2 mM TMPG was prepared in DMF. The mixture of TBA-conjugated 6-FAM (100 μl) and standard or sample (100 μl) was incubated for 20 min at room temperature (21 ± 2 °C). After the incubation, the mixture (20 μl) and TPA (10 μl) were inserted into a borosilicate test tube. Light immediately emitted with the addition of TMPG (200 μl) using the dispenser of the luminometer was measured for 20 s at room temperature.

3. Results and discussion

3.1. Interaction between TBA and GO in guanine chemiluminescence

It is well-known that DNA aptamers such as TBA are immobilized on the surface of GO due to the π–π stacking interaction of both materials (Xing et al., 2012). In addition, DNA aptamer conjugated with fluorescent dye immobilized on the surface of GO cannot emit fluorescence because of fluorescence resonance energy transfer (FRET) between DNA aptamer conjugated with fluorescent dye and GO (Xing et al., 2012). As a proof, we confirmed that TBA-conjugated 6-FAM immobilized on the surface of GO cannot emit fluorescence using microplate reader with fluorescence detection.

Using guanine chemiluminescence detection, we measured relative CL intensities emitted from TBA-conjugated 6-FAM and TBA-conjugaed 6-FAM immobilized on the surface of GO at three different time intervals (e.g., 25, 50, and 100 s). As shown in Fig. 2 (a), TBA-conjugated 6-FAM as well as TBA-conjugated 6-FAM immobilized on the surface of GO emit strong and glow chemiluminescent light with the addition of TPA and TMPG. Fig. 2(a) shows that the ratio between relative CL intensity of TBA-conjugated 6-FAM and relative CL intensity of TBA-conjugated 6-FAM immobilized on the surface of GO was dependent on the measuring time of emission. The ratio determined with 100 s measurement was the smallest. The results shown in Fig. 2(a) indicate that G-rich TBAs immobilized on the surface of GO began to slowly react with TMPG. G-rich TBAs reacted with TMPG, which are not DNA aptamers, rapidly detached from GO. Then, they emitted strong CL emission through the chemiluminescence resonance energy transfer (CRET) from a high-energy intermediate, formed from the reaction between guanine of TBA and TMPG, to 6-FAM (see Fig. S1). The possible CRET mechanism in guanine chemiluminescence reaction may be similar to chemically initiated electron exchange luminescence (CIEEL) mechanism of peroxyoxalate chemiluminescence (Lee et al., 2002a, 2002b; Maruyama et al., 2013; Park et al., 2011). In conclusion, Fig. 2(a) indicates that it is impossible to develop a DNA aptasensor with guanine chemiluminescence detection using GO.

3.2. Role of magnetic Fe_3O_4 GO nanoparticles in guanine chemiluminescence

In order to solve the problem shown in Fig. 2(a), we fabricated magnetic Fe_3O_4 GO nanoparticles (see Fig. 2(b)–(d)) to completely remove free TBA-conjugated 6-FAM in aqueous solution containing G-quadruplex TBA-conjugated 6-FAM bound with thrombin that does not interact with magnetic Fe_3O_4 GO nanoparticles.

Fig. 2 (c) and (d) shows that Fe_3O_4 composited on the surface of GO nanoparticles. Fig. 2(c) and (e) show that the difference between pure GO and magnetic Fe_3O_4 GO nanoparticle. Magnetic Fe_3O_4 GO nanoparticles, instead of GO, were added in solution containing only TBA-conjugated 6-FAM without thrombin. Then, TBA-conjugated 6-FAM immobilized on the surface of magnetic Fe_3O_4 GO nanoparticles was removed in the solution using the magnetic separator shown in Fig. 2(b). Thus, no CL emission was observed in the solution not containing TBA-conjugate 6-FAM.

With the fabrication of magnetic Fe_3O_4 GO nanoparticles, it is possible to develop a guanine-chemiluminescent aptasensor capable of quantifying thrombin in human serum because magnetic Fe_3O_4 GO nanoparticles can completely remove free TBA-conjugated 6-FAM that does not bind with thrombin. As shown in Fig. 2(f), however, no CL emission was observed in solution containing only complexes of G-quadruplex TBA bound with thrombin. The result shown in Fig. 2(f) indicates that guanines folded to form G-quardruplex TBA with the hydrogen bonding between guanine and guanine (see Fig. 1(a) and (b)) do not have primary and secondary amines capable of reacting with TMPG based on the mechanism shown in Fig. 1(c). In conclusion, it is impossible to develop a G-quardruplex DNA aptasensor with guanine chemiluminescence detection using magnetic Fe_3O_4 GO nanoparticles due to the mechanism shown in Fig. S2 even though magnetic Fe_3O_4 GO nanoparticles can completely remove free TBA-conjugated 6-FAM remaining in the solution after the reaction to form G-quadruplex TBA-conjugated 6-FAM bound with thrombin.

3.3. Development of G-quadruplex DNA aptasensor with guanine chemiluminescence detection without any nanoparticles

Based on the results shown in Fig.2, we confirmed that it is difficult to develop a G-quadruplex TBA aptasensor with guanine chemiluminescence detection using nanoparticles (e.g., GO, magnetic Fe_3O_4 GO) capable of immobilizing TBA through π–π stacking interaction between nanoparticle and TBA. However, Fig. 2 also shows a positive possibility that thrombin can be quantified with only TBA-conjugated 6-FAM without any nanoparticles. This is because (1) TBA-conjugated 6-FAM not bound with thrombin can emit bright light in guanine chemiluminescence system (see Fig. 2 (a)) and (2) G-quadruplex TBA-bound thrombin cannot emit light in guanine chemiluminescence system (see Fig. 2(f)). These results indicate that relative CL intensity of guanine chemiluminescence in

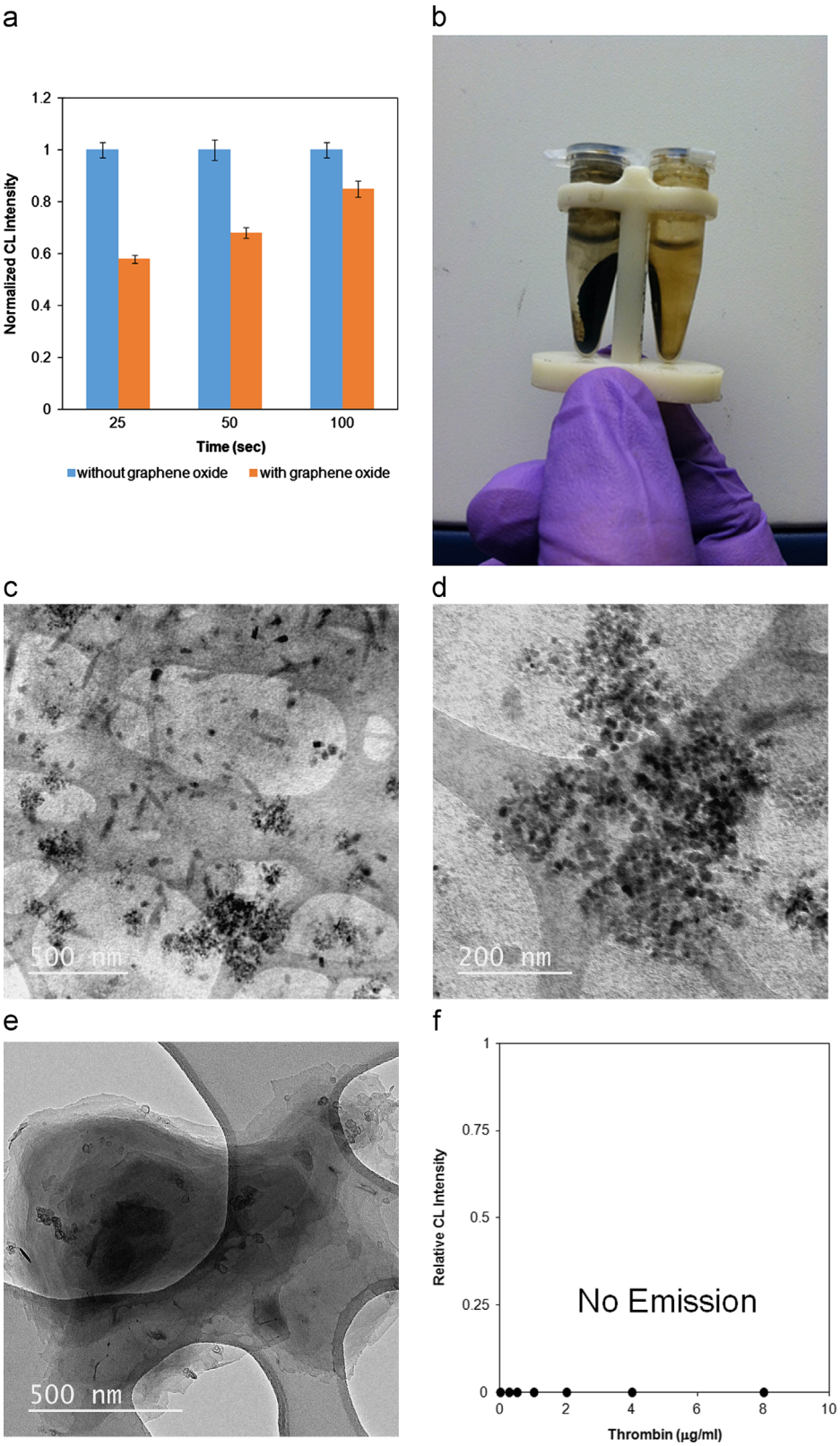

Fig. 2. (a) Comparison of guanine chemiluminescence emitted from individual TBA-conjugated 6-FAM and TBA-conjugated 6-FAM immobilized on the surface of GO, (b) separation of magnetic Fe_3O_4 GO nanoparticles using a magnetic separator, (c) and (d) images of magnetic Fe_3O_4 GO nanoparticle taken with a TEM, (e) Image of pure GO taken with a TEM, and (f) CL emission of G-quadruplex TBA bound with thrombin after removing free TBA-conjugated 6-FAM using magnetic Fe_3O_4 GO nanoparticles.

the presence of a certain concentration of TBA-conjugated 6-FAM is dependent on the concentration of thrombin. In other words, relative CL intensity of guanine chemiluminescence is quenched with the increase of thrombin concentration in a sample due to the increase of G-quadruplex TBA-bound thrombin concentration. Based on the hypothesis, G-quadruplex TBA aptasensor with guanine chemiluminescence capable of sensing thrombin in human serum was developed with additional research as shown below.

3.3.1. Effect of human serum in guanine chemiluminescence

In order to study the effect of human serum in guanine chemiluminescence, 1 μM TBA (100 μl) or 1 μM TBA-conjugated 6-FAM (100 μl) in water was added in each (100 μl) of three different solutions as shown in Table S1. Relative CL intensity of TBA-conjugated 6-FAM was about 244-fold higher than that of TBA in water. Also, the former was about 229-fold higher than the latter in 10% human serum. Relative CL intensity of TBA-conjugated 6-FAM in water was slightly higher (about 1.16-fold) than that in 10% human serum. In the case of 100% human serum, CL emission of TBA was not observed. Also, relative CL intensity of TBA-conjugated 6-FAM in 100% human serum was much lower than those in water and 10% human serum. Table S1 indicates that it is difficult to develop a highly sensitive G-quadruplex TBA aptasensor without diluting human serum because human serum contains interferences capable of reducing guanine chemiluminescence.

Table 1 shows that relative CL intensity measured in the presence of thrombin is lower than that in the absence of thrombin. The result is a proof that G-quadruplex TBA aptasensor with guanine chemiluminescence without any nanoparticles can be developed based on the cost-effective and simple procedures as shown in Fig. 3. Table 1 also indicates that the quenching ratio of guanine chemiluminescence is dependent on the concentration of human serum in water. The quenching ratio in water was larger than those in human serums diluted with water. The results shown in Table 1 indicate that the interaction between TBA-conjugated 6-FAM and thombin in pure in the absence of water human serum is faster than that in the presence of human serum. Based on the results of Table 1, we have used 5% human serum diluted with water to develop more sensitive G-quadruplex TBA aptasensor for the quantification of thrombin in real samples such as serums collected from patients.

3.3.2. Concentration effect of TBA-conjugated 6-FAM

As shown in Fig. 4(a), the quenching ratio of guanine chemiluminescence was dependent on the concentration of TBA-conjugated 6-FAM. The quenching ratio in the presence of 250 nM TBA-conjugated 6-FAM was larger than that in the presence of higher or lower TBA-conjugated 6-FAM than 250 nM because (1) relative CL intensity of higher TBA-conjugated 6-FAM than 250 nM in the absence of thrombin was decreased due to the self-quenching as shown in Figs. 4(b) and (2) lower TBA-conjugated 6-FAM than 250 nM was not enough to bind thrombin (8 μg/ml) within the limited incubation time (20 min). Based on the results of Fig. 4, we selected 250 nM TBA-conjugated 6-FAM for developing G-quadruplex TBA aptasensor with guanine chemiluminescence detection.

Table 1
Relative CL intensities in the absence and presence of thrombin in water and three different human serums at room temperature ($n=3$).

Thrombin (μg/ml)	Water	10% Human serum	5% Human serum	2.5% Human serum
0	1.07×10^6	9.44×10^5	9.83×10^5	9.95×10^5
8.0	3.91×10^5	8.25×10^5	5.53×10^5	5.26×10^5
CL_0/CL_8	0.37 ± 0.01	0.87 ± 0.02	0.56 ± 0.02	0.53 ± 0.02

Condition: [TBA-conjugated 6-FAM]=200 nM in water, [TPA]=20 mM in water, and [TMPG]=2 mM in DMF.

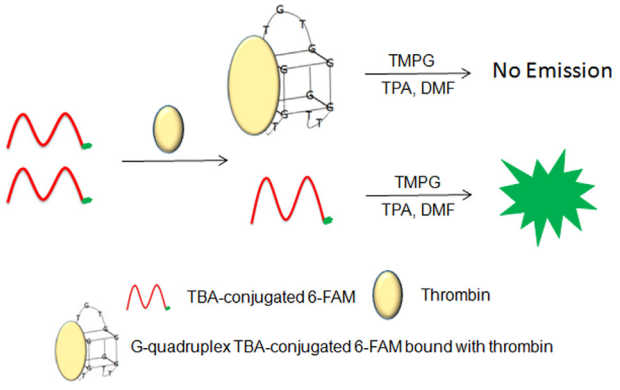

Fig. 3. Principle of G-quadruplex TBA aptasensor with guanine chemiluminescence detection.

3.3.3. Concentration effect of TPA and TMPG

In order to study the effect of TPA concentration used as a catalyst in guanine chemiluminescence reaction, four different concentrations of TPA (2.5, 5, 10, and 20 mM) were prepared in water. 2 mM TMPG in DMF and 200 nM TPA-conjugated 6-FAM in water were prepared for the research. The reaction of guanine chemiluminescence in the presence of 20 mM TPA was faster than those in the presence of other TPA concentrations. In addition, relative CL intensity integrated for 20 s in the presence of 20 mM TPA was the highest. Thus, we selected 20 mM TPA to develop G-quadruplex TBA aptasensor.

We prepared four different concentrations of TMPG (0.25, 0.5, 1, and 2 mM) in DMF to study the effect of TMPG concentration in guanine chemiluminescence. 20 mM TPA and 200 nM TBA-conjugated 6-FAM in water were prepared for the research. Relative CL intensity of guanine chemiluminescence was enhanced with the increase of TMPG concentration. We selected 2 mM TMPG for the development of G-quadruplex TBA aptasensor because the relative CL intensity measured in the presence of 2 mM TMPG was the highest.

3.3.4. G-quadruplex TBA aptasensor capable of quantifying thrombin in human serum

Based on the preliminary experimental results described above, we developed G-quadruplex TBA aptasensor with guanine chemiluminescence detection for the quantification of thrombin in human serum. Fig. 5(a) shows that relative CL intensity of G-quadruplex TBA aptasensor operated based on the procedure shown in Fig. 3 was exponentially decreased with the increase of thrombin concentration. Relative CL intensity and quenching ratio in 5% human serum were smaller than those in water. Also, the linear dynamic range (0.5–8 μg/ml) in 5% human serum was slightly narrow than that (0.25–8 μg/ml) in water as shown in Fig. 5(b). This is because 5% human serum contains interferences capable of reducing the sensitivity of G-quadruplex TBA aptasensor. Normal range of thrombin in human blood is 50–100 μg/ml (2.5–5 μg/ml in 5% human blood). Thus, we expected that G-quadruplex TBA aptasensor developed in this research can be applied to quantify thrombin in human blood. The limit of detection (LOD= background + 3 × standard deviation) of G-quadruplex TBA aptasensor to quantify thrombin in 5% human serum was 0.4 μg/ml (12.3 nM). The background of G-quadruplex TBA aptasensor was the reciprocal of relative CL intensity measured in the absence of thrombin. LOD of G-quadruplex. TBA aptasensor developed in this research was similar to or lower than those of more complicated aptasensors (Goda and Miyahara, 2013; Jalit et al., 2013; Li et al., 2013) reported recently as shown in Table S2. Accuracy, precision, and reproducibility of G-quadruplex TBA aptasensor with guanine chemiluminescence detection were studied with three different samples in 5% human serum at room temperature. As shown in Table S3 the results were good within the acceptable error range.

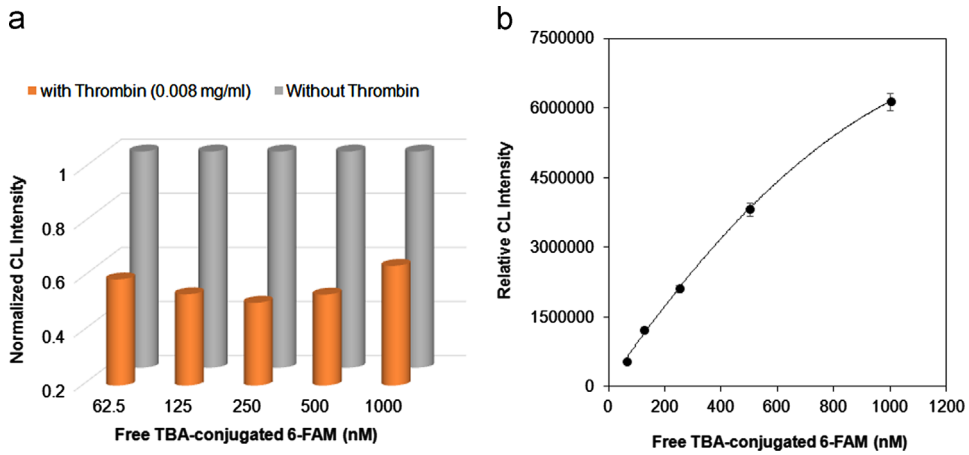

Fig. 4. (a) Concentration effect of TBA-conjugated 6-FAM in guanine chemiluminescence for the quantification of thrombin in 5% human serum, (b) relative CL intensity of TBA-conjugated 6-FAM in the absence of thrombin in 5% human serum. Condition: [TPA]=20 mM in water, and [TMPG]=2 mM in DMF.

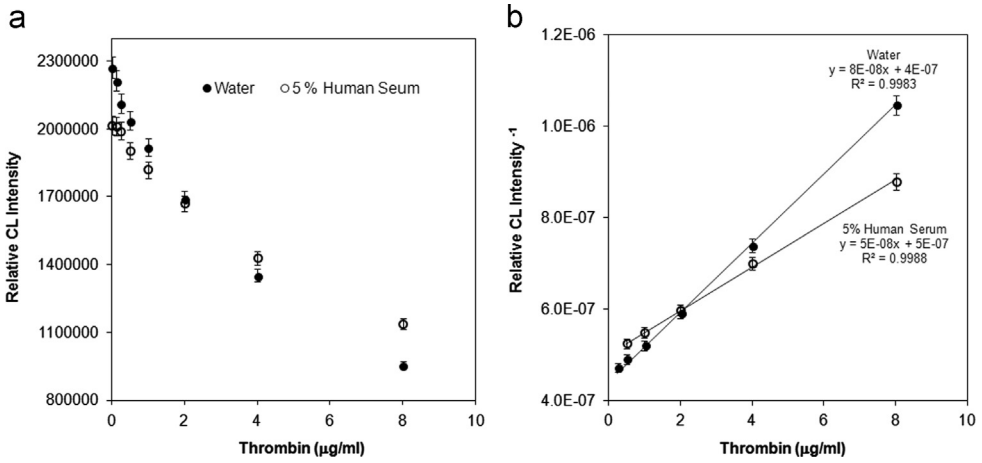

Fig. 5. (a) Quantification of thrombin in water and 5% human serum, (b) calibration curves for the quantification of thrombin in water and 5% human serum. Condition: [TBA-conjugated 6-FAM]=200 nM in water, [TPA]=20 mM in water, and [TMPG]=2 mM in DMF.

4. Conclusion

We reported in this paper for the first time that G-quadruplex TBA bound with thrombin cannot emit light in guanine chemiluminescence system due to lack of chemical reaction between guanines of G-quadruplex TBA bound with thrombin and TMPG, whereas free TBA-conjugated 6-FAM rapidly react with TMPG to form high energy intermediate capable of transferring energy to 6-FAM, which can emit bright light. Based on the concept, we developed a cost-effective, rapid and simple G-quadruplex TBA aptasensor with highly sensitive guanine chemiluminescence detection without expensive and intractable nanoparticles, including magnetic Fe_3O_4 GO nanoparticles, and complicated procedures. In conclusion, the results observed in this research indicate that various types of G-quadruplex DNA aptasensors capable of specifically sensing a target molecule such as ATP, HIV, ochratoxin, potassium ions, and thrombin can be developed. Thus, G-quadruplex DNA aptasensor with guanine chemiluminescence detection can be applied in various research areas such as bio-analytical chemistry, clinical chemistry, food safety, and toxicology.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2013.09.017.

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