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Rapid aptasensor capable of simply diagnosing prostate cancer

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ABSTRACT

Using guanine (G)-rich DNA aptamer-conjugated 6-carboxyfluorescein (6-FAM) capable of rapidly capturing prostate specific antigen (PSA) in human serum, cost-effective and simple biosensor with guanine chemiluminescence detection was developed for early diagnosis of prostate cancer. Free G-rich DNA aptamer-conjugated 6-FAM emits bright light in guanine chemiluminescence reaction based on the principle of chemiluminescent resonance energy transfer (CRET). However, G-rich DNA aptamer-conjugated 6-FAM bound with PSA cannot emit light because PSA acts as a strong interference in CRET between 6-FAM and high-energy intermediate formed from the reaction of 3,4,5-trimethoxylphenylglyoxal (TMPG) and guanine of G-rich DNA aptamer. A chemiluminescent biosensor, developed using the different properties of G-rich DNA aptamer-conjugated 6-FAM in the absence and presence of PSA in guanine chemiluminescence reaction, was able to quantify trace levels of PSA in human serum within 30 min without time-consuming and complicated procedures (e.g., multiple incubation and washings) required for conventional immunoassays operated with expensive and intractable antibodies. The limit of detection of chemiluminescent biosensor having a wide linear dynamic range (1.9–125 ng/ml) was 1.0 ng/ml. The excellent correlation (R=0.985) between chemiluminescent biosensor and conventional enzyme immunoassay indicates that the accurate, precise, and rapid chemiluminescent biosensor can be applied as a new method for early diagnosis of prostate cancer.

1. Introduction

Numerous research groups and companies around the world have competitively developed and commercialized various devices and kits for the diagnosis and prognosis of cancer, one of the major critical diseases in highly industrialized countries. Unfortunately, most of the devices developed to diagnose various cancers such as prostate and lung cancer are too complicated and expensive to operate without scientific experience and information at outside of hospital and laboratory. For example, enzyme immunoassays (EIAs) with several detections (e.g., colorimeter, chemiluminescence, electrochemical, and fluorescence) capable of quantifying prostate specific antigen (PSA) in human serum were developed to early diagnose and prognose prostate cancer (Acevedo et al., 2002; Lee et al., 2010; Panini et al., 2008; Zheng et al., 2008). Unfortunately, sandwich EIAs using expensive capture antibody and detection antibody-conjugated enzyme (e.g., horseradish peroxidase and alkaline phosphatase) produced from the sacrifice of small animals are complicated and time-consuming even though the method is very sensitive (Acevedo et al., 2002; Lee et al., 2010; Panini et al., 2008; Zheng et al., 2008).

In order to solve critical problems of EIAs, various types of aptamers using single strand DNAs (Chen et al., 2012; Huang et al., 2012) and RNAs (Jeong et al., 2010; Lee and Lee, 2012) were developed in order to capture and sense tumor markers instead of antibodies. The procedures for synthesizing DNA and RNA aptamers are more cost-effective, faster, simpler, and stable than those of antibodies using small animals such as mouse, rat, and sheep. As the additional advantage of aptamer, an aptamer conjugated with fluorescent dye can capture a specific biomarker in a sample as well as detect the concentration of biomarker for the diagnosis of diseases (Chen et al., 2012; Cho et al., 2014; Choi and Lee, 2013; Choi et al., 2010; He et al., 2012; Leung et al., 2011; Ma et al., 2013).

Glow chemiluminescence with the reaction between guanine nucleotides and 3,4,5-trimethoxylphenylglyoxal (TMPG) was observed in aqueous solution containing N,N-dimethyl formamide (DMF) (Kai et al., 1999). Yamasuji reported that fluorescein-5-isothiocyanate (FITC) conjugated with G-rich single strand DNA (ssDNA) can emit bright chemiluminescence based on the principle of chemiluminescence resonance energy transfer (CRET)

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(Yamasuji et al., 2011). Recently, we observed that G-rich ssDNA aptamer-conjugated 6-carboxyfluorescein (6-FAM), instead of FITC, capable of binding thrombin in human serum can emit bright light in guanine chemimiluminescence system (Cho et al., 2014). However, G-rich ssDNA aptamer-conjugated 6-FAM (G-quadruplex-conjugated 6-FAM) transformed in the presence of thrombin cannot emit light. This is because that primary and secondary amines of guanine immobilized due to strong hydrogen bonding between guanines to form G-quadruplex cannot react with TMPG in aqueous solution (Cho et al., 2014).

Our recent report (Cho et al., 2014) implies that guanine used to capture a biomarker in a sample cannot react with TMPG to emit bright light. If the hypothesis is correct, relative CL intensity of G-rich ssDNA aptamer emitted in the presence of a biomarker will be lower than that in the absence of a biomarker. Based on the hypothesis described above, we studied whether prostate specific antigen (PSA), a biomarker to diagnose prostate cancer, can be quantified with a biosensor we designed in this research.

2. Experimental

2.1. Chemical and materials

Two different types of PSA aptamers conjugated with 6-carboxyfluorescein (6-FAM) were purchased from Alpha DNA (Montreal, Quebec, Canada).

PSA aptamer 1: 5′-6-FAM-TTT TTA ATT AAA GCT CGC CAT CAA ATA GCT TT-3′

PSA aptamer 2: 5′-TTT TTA ATT AAA GCT CGC CAT CAA ATA GCT GGG GG-6-FAM-3′

G-rich PSA aptamer 2 was modified with original PSA aptamer 1 (Savory et al., 2010). 6-FAM labeled with PSA aptamers was selected based on the research results we previously reported (Cho et al., 2014). PSA was purchased from Sigma-Aldrich (Milwaukee, WI, USA). 3,4,5-trimethoxylphenylglyoxal hydrate (TMPG, 97%) was purchased from Matrix Scientific (Columbia, SC, USA). $FeCl_2$ (99%), $FeCl_3$ (99%), and Tetra-*n*-propylammonium hydroxide (TPA, 40% w/w aqueous solution) were purchased from Alfa Aesar (Ward Hill, MA, USA). COOH functionalized multiwall carbon nanotubes (MWCNT, 20–30 nm) were purchased from Nanomaterial Store (Fremont, CA, 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). The coded patient serum samples containing PSA were obtained from the Washington County Hospital, Hagerstown, MD, USA.

2.2. Procedure

2.2.1. Fabrication of magnetic Fe_3O_4 GO nanoparticles

Fe_3O_4 MWCNTs were prepared based on the method for synthesizing Fe_3O_4 graphene oxide (Cho et al., 2014). $FeCl_2$ (44 mg) and $FeCl_3$ (11 mg) were dissolved and mixed in water (5 ml). MWCNTs (1.1 mg/ml) were prepared in water. The mixture of $FeCl_2$ and $FeCl_3$ (0.5 ml) was mixed with MWCNTs (0.5 ml) in a 1.5-ml centrifuge tube. The microcentrifuge tube inserted into a Micro Centrifuge Tube Thermomixer (Eppendorf) was shaken at 500 rpm 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. Fe_3O_4 MWCNTs formed in the microcentrifuge tube were cooled at room temperature. Fe_3O_4 MWCNTs in the microcentrifuge tube were washed thrice in water using a magnetic separator (Bioclone, Inc.). Fe_3O_4 MWCNTs were stored in a refrigerator to use as a stock solution. Fig. 1 shows images of Fe_3O_4 MWCNTs observed with a JEOL 2100 TEM operating at 200 kV accelerating voltage and a Gatan Ultrascan 1000XP digital camera.

2.2.2. Sequence effect of G-rich ssDNAs in guanine chemiluminescence

In order to study whether guanine chemiluminescence depends on the sequence of G-rich ssDNA, three different types of G-rich ssDNAs (5′-GGG GTT TTG GGG-3′, 5′-TTG GGG TTG GGG-3′, and 5′-GGG GGG GGT TTT-3′) and G-rich ssDNAs conjugated with 6-FAM (5′-6-FAM-GGG GTT TTG GGG-3′, 5′-6-FAM-TTG GGG TTG GGG-3′, and 5′-6-FAM-GGG GGG GGT TTT-3′) were synthesized by Alpha DNA. Each G-rich ssDNA (200 nM) and TPA (0.01 M) was prepared in water. TMPG (1 mM) was prepared in DMF. Each G-rich ssDNA (20 μl) was mixed with TPA (10 μl) in a borosilicate test tube (12×75 mm^2). 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 20 s.

2.2.3. Quantification of PSA antigen using biosensor with guanine chemiluminescence detection

8 standards (0–125 ng/ml) and samples containing different concentration of PSA were prepared in 10% human serum. 250 nM

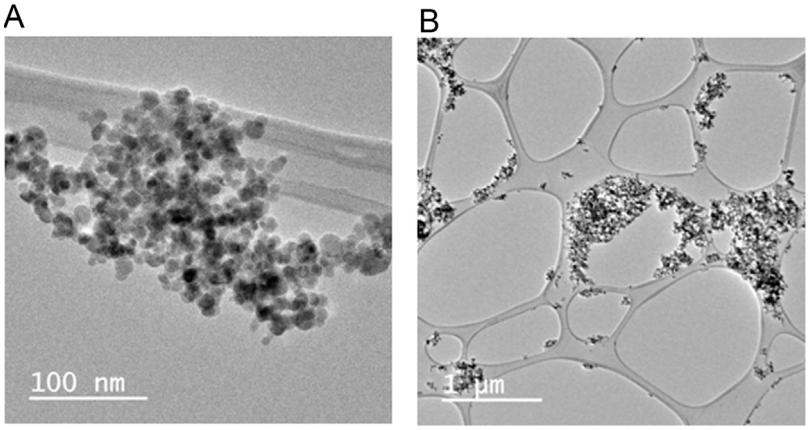

Fig. 1. Images (a and b) of Fe_3O_4 MWCNT taken with TEM under two different focusing ranges.

PSA aptamer conjugated with 6-FAM and 10 mM TPA were prepared in water. 4 mM TMPG was prepared in DMF. The mixture of PSA aptamer conjugated with 6-FAM (100 μl) and standard or sample (100 μl) was incubated for 30 min at room temperature. After the incubation, the mixture (20 μl) and TPA (10 μl) were inserted into a borosilicate test tube. The concentration of PSA in a sample was determined with the measurement of CL emission using the luminometer described in Section 2.2.3.

3. Results and discussion

3.1. Sequence effect of ssDNAs in guanine chemiluminescence

High-energy intermediate formed from the reaction of guanine and TMPG can emit light by itself as well as transfer energy to fluorescent dye labeled with the ssDNA due to CRET. Interaction between fluorescent dye and high-energy intermediate formed from the reaction of guanine and TMPG is similar to that between fluorescent dye and high-energy intermediate from peroxyoxalate chemiluminescence reaction (Augusto et al., 2013; Lee et al., 2003; Maruyama et al., 2013; Park et al., 2011) even though the former is generated by the intra-CRET (Cho et al., 2014), whereas the latter is observed by the inter-CRET.

As shown in Fig. 2, CL emission of 5′-GGGGTTTTGGGG-3′ was the brightest, whereas ssDNA (5′-GGGGGGGGTTTT-3′) emits relatively dim light. The results indicate that relative CL intensity of the latter was decreased due to the self-quenching observed in the presence of excess emission material (guanine) in narrow space. In addition, relative CL intensity of 5′-TTGGGGTTGGGG was lower than that of 5′-GGGGTTTTGGGG. The results indicate that thymine next to guanine acts as interference in guanine chemiluminescence.

Fig. 2 shows that relative CL intensity of ssDNA conjugated with 6-FAM is much stronger than that of ssDNA not conjugated with 6-FAM due to the intra-CRET between 6-FAM and high-energy intermediate formed from the reaction of guanine and TMPG in the presence of TPA and DMF (Cho et al., 2014). However, the order of relative CL intensity of ssDNAs conjugated with 6-FAM was determined like the results observed in guanine chemiluminescence of ssDNAs not conjugated with 6-FAM. These results indicate that intra-CRET is dependent on the sequence of ssDNA aptamer.

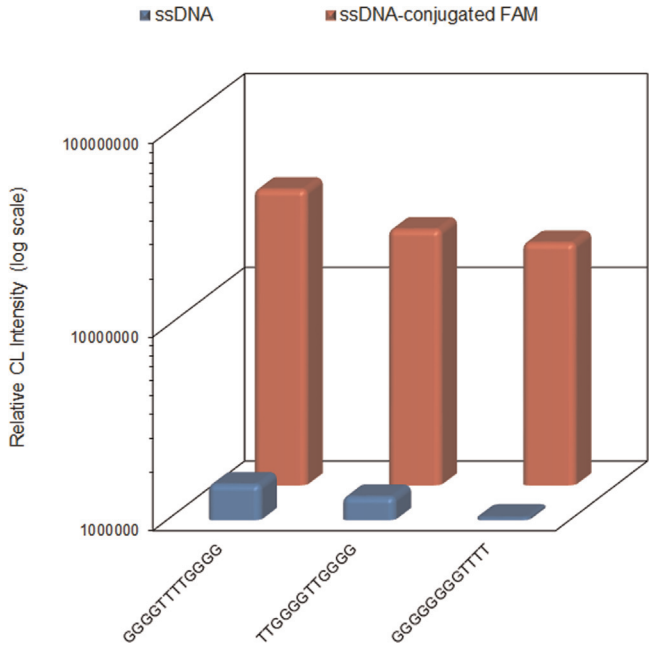

Fig. 2. Sequence effect of ssDNAs in guanine chemiluminescence. Error range (standard deviation) of each value was 3–6%.

3.2. Comparison between PSA aptamer 1 and PSA aptamer 2

As shown in Section 2.1, PSA aptamer 1 composed of 32 bases has only 3 guanines capable of reacting with TMPG to emit light. In addition, 6-FAM conjugated with PSA aptamer 1 is next to thymine as well as is too far from guanine. Thus, it is possible that PSA aptamer 1 emits dim light in guanine chemiluminescence. In order to solve the problem, we modified PSA aptamer 1 into PSA aptamer 2 based on the results of Fig. 2.

Fig. 3(a) indicates that relative CL intensity of PSA aptamer 2 having additional 5 guanines is about 53-fold stronger than that of PSA aptamer 1 in the absence and presence of PSA antigen. Also, Fig. 3(a) shows that relative CL intensity(CL_0) of PSA aptamer in the absence of PSA is higher than that (CL_{PSA}) in the presence of PSA antigen. The result implies that free PSA aptamer can emit light, whereas PSA aptamer bound with PSA cannot emit light or emit dim light. The ratio (0.44 ± 0.02) of CL_{PSA}/CL_0 using PSA aptamer 2 is lower than that (0.78 ± 0.03) using PSA aptamer 1. It indicates that the binding of PSA and PSA aptamer 2 is faster than that of PSA aptamer 1 and PSA under this condition. Also, these results indicate that the sensitivity of biosensor using PSA aptamer 2 is better than that using PSA 1. Based on the results shown in Fig. 3(a), we selected PSA aptamer 2, as a capture and detection aptamer, to develop a cost-effective and rapid biosensor, capable of quantifying PSA in a sample without expensive and intractable antibodies (e.g., capture and detection antibodies) and time-consuming procedures such as multiple washings.

In order to confirm whether PSA aptamer-bound PSA emits light, we fabricated Fe_3O_4 MWCNTs to remove free PSA aptamer, using the π–π stacking interaction between free aptamer and micro- or nano-particles (Cho et al., 2014; Choi et al., 2013; Park et al., 2013; Sheng et al., 2011; Yang et al., 2011) such as graphene oxide and MWCNTs, in aqueous solution based on the procedure shown in Fig. 3(b). First, free PSA aptamers were added in a sample containing PSA. Then, the mixture was incubated to form PSA aptamer bound with PSA for 30 min at 37 °C. With the addition of Fe_3O_4 MWCNTs in the solution, free PSA aptamers were rapidly immobilized on the surface of Fe_3O_4 MWCNTs at room temperature (21 ± 2 °C). Free PSA aptamers bound with Fe_3O_4 MWCNTs were removed with a separator. Finally, relative CL intensity of the remaining solution containing PSA aptamer bound with PSA was measured using guanine chemiluminescence detection system.

As shown in Fig. 3(b), the relative CL intensity in the presence of PSA aptamer 1 (or 2) bound with PSA was the same as the background in the absence of PSA aptamer bound with PSA. The confirmation indicates that primary and secondary amines of guanine existing in PSA aptamer 1 were used to bind PSA antigen before adding TMPG and TPA.

We proposed that PSA aptamer 2 bound with PSA might emit light because 5 more extra guanines, added into the PSA aptamer 1 to enhance chemiluminescence (see Fig. 3(a)), of PSA aptamer 2 bound with PSA can react with TMPG to form high-energy intermediate capable of transferring energy to 6-FAM due to the intra-CRET. However, no emission in the presence of PSA aptamer 2 bound with PSA was observed as shown in Fig. 3(b). The result indicates that PSA, relatively larger biomolecule (protein) than aptamer, bound with PSA aptamer 2 acts as a strong quencher under this condition. For example, it is possible that PSA bound with PSA aptamer 2 may act as a strong absorber of light emitted in guanine chemiluminescence reaction and/or as a strong inhibitor of guanine chemiluminescence reaction.

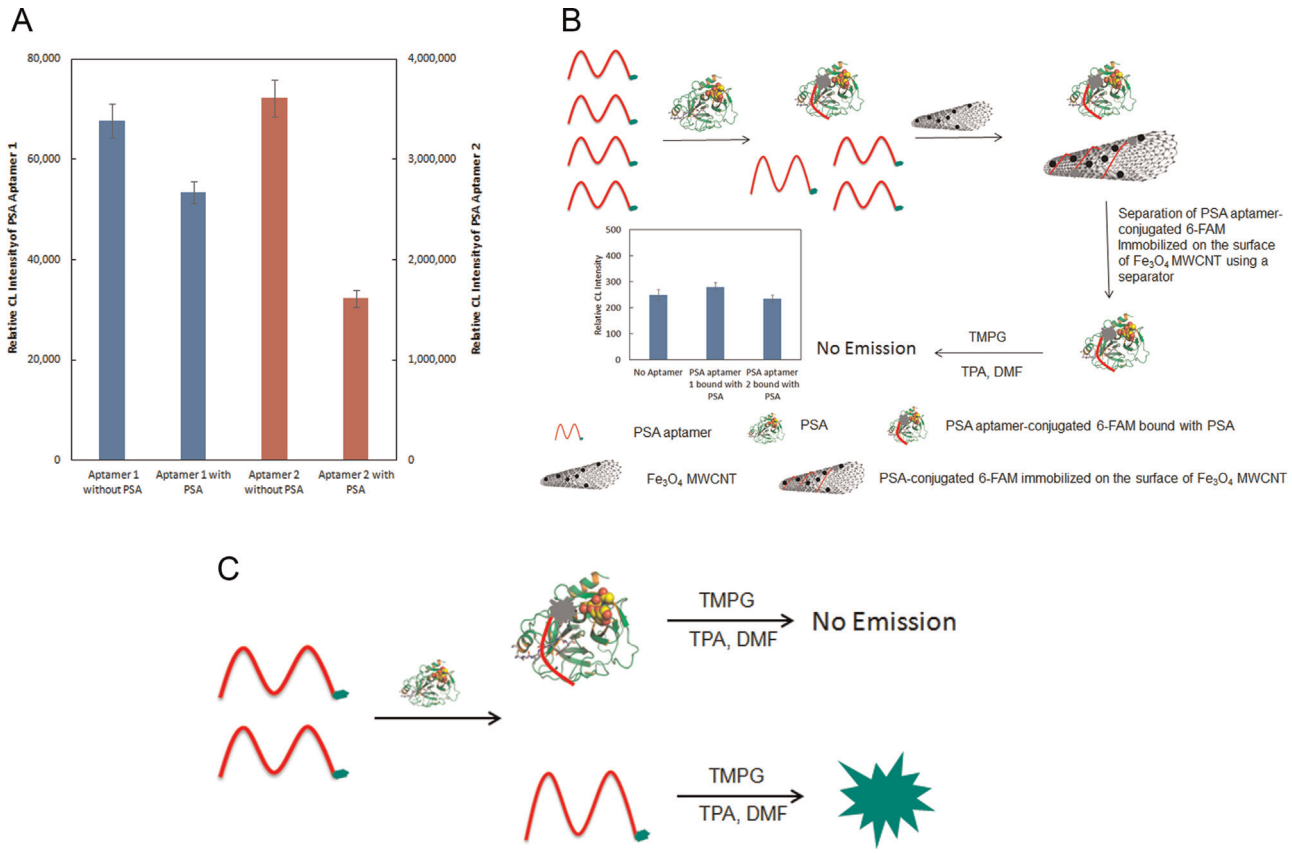

Fig. 3. (a) Comparison between PSA aptamer 1 and PSA aptamer 2, capable of capturing PSA, in guanine chemiluminescence detection system. Condition: [PSA aptamer 1 and 2]=200 nM in water, [PSA antigen]=250 ng/ml in 10% human serum, [TPA]=0.01 M in water, [TMPG]=1 mM in DMF, Incubation time: 20 min, Integration time to measure relative CL intensity: 20 s. (b) Procedure to measure CL emission of PSA aptamer 2 bound with PSA antigen in guanine chemiluminescence reaction. (c) Possible mechanism for the quantification of PSA antigen using chemiluminescent biosensor with PSA aptamer-conjugated 6-FAM.

Based on the results shown in Fig. 3(a) and (b), PSA aptamer 2, strong emitter and rapid capture of PSA, was selected to develop a chemiluminescent biosensor capable of rapidly quantifying PSA antigen in a sample using the simple procedure shown in Fig. 3(c).

3.3. Study of main variables to develop biosensor capable of sensing PSA

3.3.1. Concentration effect of PSA aptamer 2

Fig. 4(a) shows that 250 nM PSA aptamer 2 conjugated with 6-FAM is the optimum concentration for the quantification of PSA using guanine chemiluminescence detection. CL_{PSA}/CL_0 in the presence of higher concentration than 250 nM PSA aptamer 2 was higher than that in the presence of 250 nM PSA aptamer. This is because free PSA aptamer 2 remaining after the interaction of PSA aptamer 2 and PSA antigen (125 nM) under the former condition is higher than that in the latter condition. The results indicate that the concentration of 250 nM PSA aptamer 2 is enough to bind with PSA antigen.

Also, Fig. 4(a) shows that CL_{PSA}/CL_0 in the presence of lower concentration than 250 nM PSA aptamer 2 was higher than that in the presence of 250 nM PSA aptamer. This is because PSA aptamer 2 lower than 250 nM is not enough to bind with PSA during the limited incubation time (30 min).

3.3.2. Concentration effect of TMPG

As shown in Fig. 4(b), the optimum concentration of TMPG for the quantification of PSA using guanine chemiluminescence detection is 4 mM. CL_{PSA}/CL_0 in the presence of lower TMPG than 4 mM is higher than that in the presence of 4 mM. This is because lower TMPG concentration than 4 mM is not enough to react with 250 nM PSA aptamer 2 in the absence of PSA antigen.

3.3.3. Determination of incubation time to quantify PSA antigen

Fig. 4(c) indicates that the optimum incubation time for the quantification of PSA antigen using the chemiluminescent biosensor is 30 min under this condition. The relative CL intensity measured after 1-h incubation was the same as that after 30-min incubation within acceptable error range (5%) because PSA (125 ng/ml) is enough to interact with PSA aptamer 2 (250 nM) with the incubation of 30-min incubation. Thus, all samples were incubated with PSA aptamer 2 (250 nM) for 30 min to develop a biosensor capable of quantifying trace levels of PSA in a sample.

3.4. Quantification of PSA antigen

Fig. 5(a) and (b) shows that trace levels of PSA in a sample can be quantified with the wide calibration curve (1.9–125 ng/ml) within 30 min without time consuming and complicated procedures. The limit of detection (LOD=CL intensity measured in the absence of PSA antigen – 3 × standard deviation) of the new biosensor determined using Fig. 5(a) and(b) was 1 ng/ml. Fig. 5 (a) and (b) indicates that guanine chemiluminescent biosensor can be applied to early diagnose prostate cancer because the cut-off value of PSA regulated for the diagnosis of prostate cancer is 4 ng/ml (Nicoll, 2007). However, LOD of the cost-effective and simple biosensor developed in this research is higher than those of

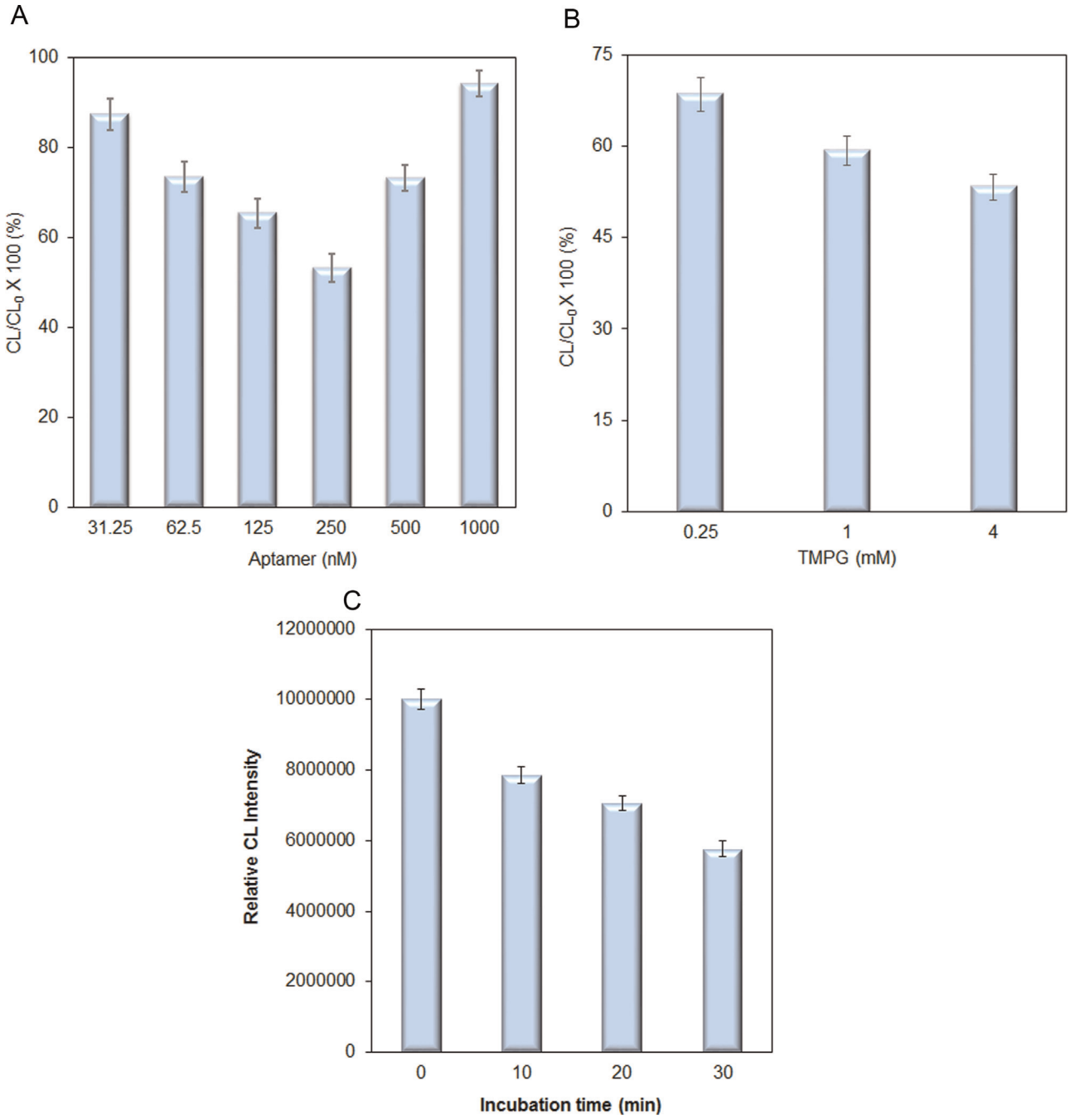

Fig. 4. (a) Concentration effect of PSA aptamer 2 in guanine chemiluminescence. Condition: incubation time: 30 min, measuring time: 20 s, [TPA]=0.01 M, [TMPG]=4 mM, [PSA antigen]=125 nM. (b) Concentration effect of TMPG in guanine chemiluminescence. Condition: incubation time: 30 min, measuring time: 20 s, [TPA]=0.01 M, [PSA aptamer 2]=250 nM, [PSA antigen]=125 nM. (c) Effect of incubation time for the quantification of PSA antigen using guanine chemiluminescence detection. Condition: measuring time: 20 s, [PSA aptamer 2]=250 nM, [TPA]=0.01 M, [TMPG]=4 mM, [PSA antigen]=125 ng/ml.

complicated and time-consuming enzyme immunoassays with chemiluminescence detection (Lee et al., 2010; Zheng et al., 2008).

Fig. 5(c) shows the calibration curve for the quantification of PSA in human serum using 1.1′-oxalyldiimidazole chemiluminescent enzyme immunoassay (ODI-CLEIA) (Choi et al., 2010; Chong et al., 2012; Chong et al., 2013; Lee et al., 2010). ODI-CLEIA reported as a highly sensitive CLEIA can quantify PSA antigen with excellent accuracy, precision, and reproducibility (Lee et al., 2010). As shown in Fig. 5(d), we studied the correlation between guanine chemiluminescent biosensor and ODI-CLEIA. Based on the excellent results shown in Fig. 5(d), we expect that guanine chemiluminescent biosensor can be applied to diagnose prostate cancer.

As shown in Table 1, the range of recovery of guanine chemiluminescent biosensor was 93.6–107.3. The results indicate that guanine chemiluminescent biosesensor can be applied to rapidly quantify or monitor PSA antigen in human serum with acceptable accuracy.

4. Conclusions

With understanding of properties of ssDNA PSA aptamer in the absence and presence of PSA in guanine chemiluminescence reaction, we developed for the first time a rapid and simple

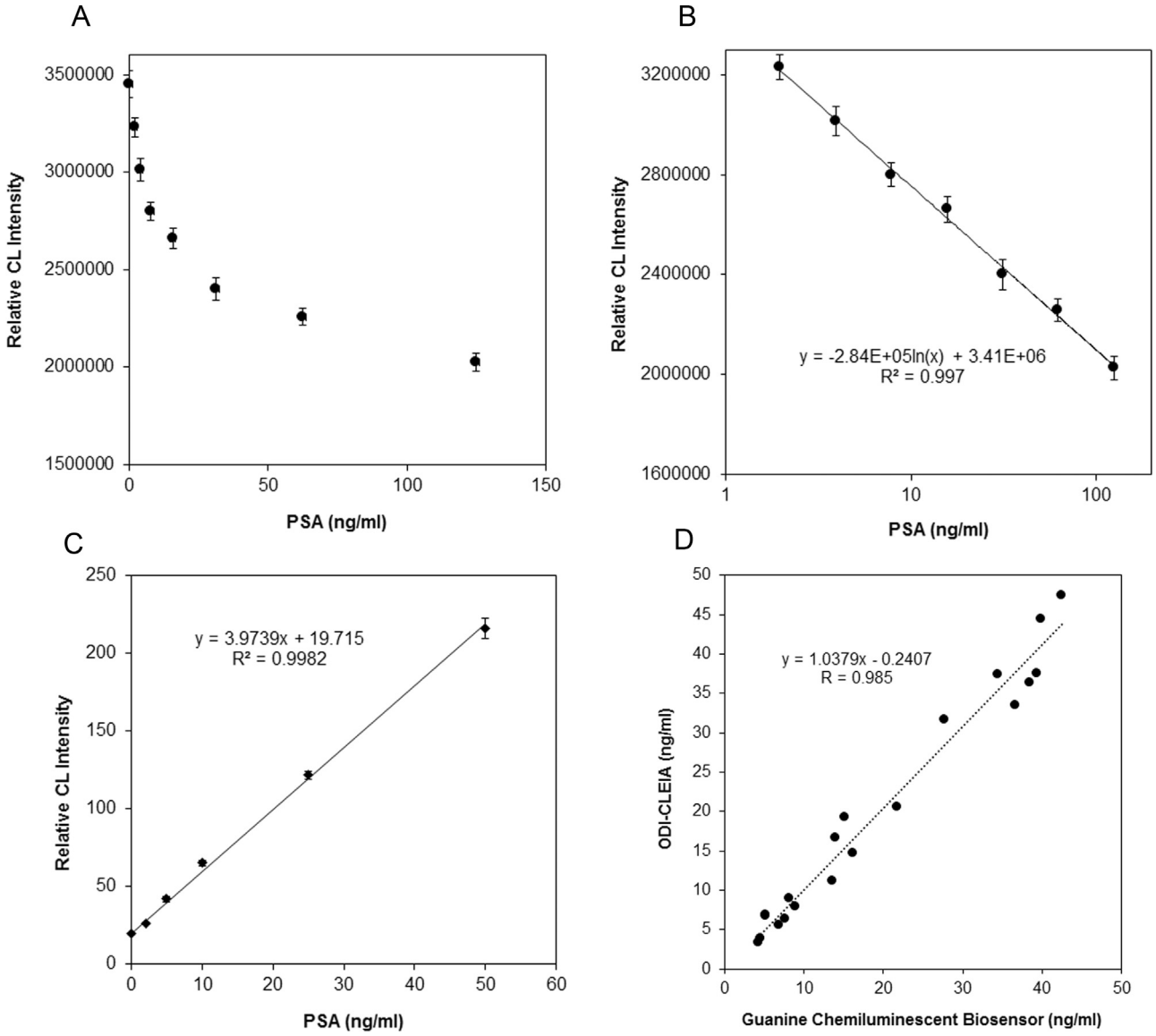

Fig. 5. (a and b) Calibration curve for the quantification of PSA antigen using guanine chemiluminescent biosensor. (c) Calibration curve for the quantification of PSA antigen using 1.1′-oxalyldiimidazole chemiluminescent enzyme immunoassay (ODI-CLEIA). (d) Correlation between guanine chemiluminescent biosensor and ODI-CLEIA.

Table 1
Recovery test ($n=5$) of guanine chemiluminescent biosensor.

Sample (ng/ml)	Spiked (ng/ml)	Calculated (ng/ml)	Measured (ng/ml)	Recovery (%)
10	5	7.5	7.2	96.0
10	20	15	16.1	107.3
10	40	25	23.4	93.6

guanine chemiluminescent biosensor capable of early diagnosing prostate cancer with good accuracy, precision, and reproducibility. Also, guanine chemiluminescent biosensor is cost-effective and stable because it is operated without expensive and intractable materials such as nanoparticles and antibodies. We expect that the method developed in this research can be applied to early diagnose other cancers as well as various human diseases. In addition, it is possible that scientists and engineers of various research fields including biochemistry, clinical chemistry, food safety, homeland security, and toxicology will be interested in the method.

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