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Non-stop aptasensor capable of rapidly monitoring norovirus in a sample

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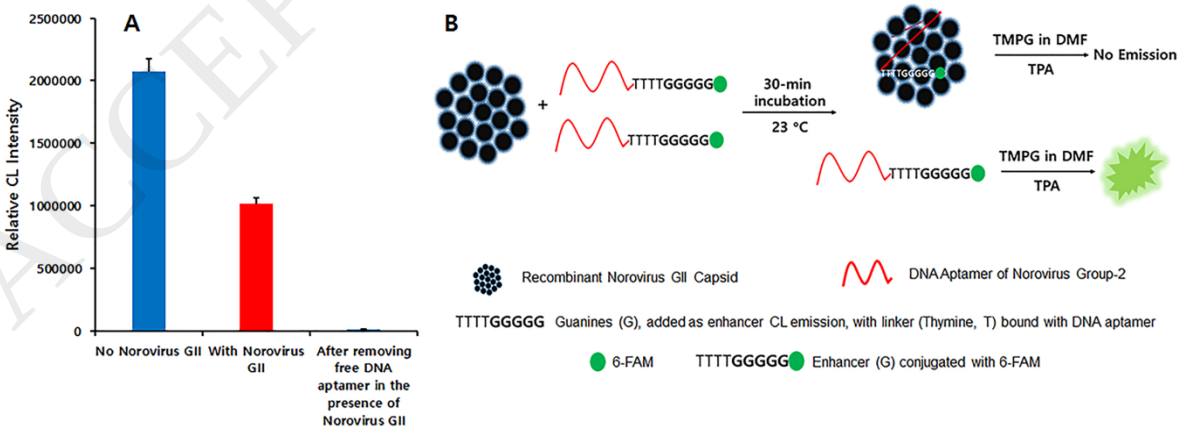
Graphical Abstract



Highlights

- Development of a non-stop aptasensor for the rapid analysis of norovirus
- Understanding of chemiluminescent resonance energy transfer (CRET) between high-energy intermediate formed in guanine chemiluminescence and 6-FAM
- Understanding of CRET of guanine chemiluminescence in the absence and presence of norovius bound with DNA aptamer

Abstract

A cost-effective and simple biosensor was developed for the accurate and rapid monitoring of norovirus GII in a sample. The modified DNA aptamer, 5 guanines linked to conventional DNA aptamer, designed for the research, was able to rapidly capture norovirus GII in tap water as well artificial urine. In addition, the extra guanines of the modified DNA aptamer enhanced the sensitivity of biosensor with guanine chemiluminescence detection based on the principle of intra chemiluminescent resonance transfer (Intra-CRET). This is because additional high-energy intermediates formed from the reaction of extra guanines and 3, 4, 5-trimethoxylphenylglyoxal (TMPG) were able to directly transfer energy to 6-carboxylfluorescein (6-FAM) to emit bright chemiluminescence. The biosensor operated without time-consuming and tedious procedures (e.g., sample pretreatment, long and multiple incubations, washings) was able to accurately quantify trace levels of norovirus GII capsid with excellent specificity and reproducibility. The limit of detection (LOD = 3σ) of the biosensor for norovirus GII capsids in tap water was as low as 80 ng/ml. It is expected that the new technology confirmed while developing the biosensor can be applied to devise alternative biosensors capable of rapidly quantifying various food-borne pathogens in a

sample.

Keywords: Norovirus, Aptasensor, Biosensor, Chemiluminescence**,** Graphene oxide, nanocomposite

1. Introduction

Norovirus is a very contagious virus capable of infecting anyone. The general infection of norovirus usually happens by (1) eating food or drink contaminated with norovirus, (2) touching contaminated objects, or (3) having contact with patients who are infected with norovirus. In addition, a patient suffering from inflammation of the stomach or/and intestines with the infection of a norovirus can be re-infected with a different type of norovirus because there are many types of noroviruses [1].

Centers for Disease Control and Prevention (CDC) have announced the annual average trends of norovirus infection in the United States (http://www.cdc.gov/norovirus/trends-outbreaks.html). For example, (1) in United States there are as many as 19-21 million cases of gastroenteritis with the norovirus infection every year, (2) about 56,000 – 71,000 patients are hospitalized, and (3) about 570 – 800 patients, mostly young children and the elderly, die every year.

Unfortunately, there is no commercially available medicine to specifically treat patients with norovirus illness. This is because the viral infection such as norovirus infection cannot be

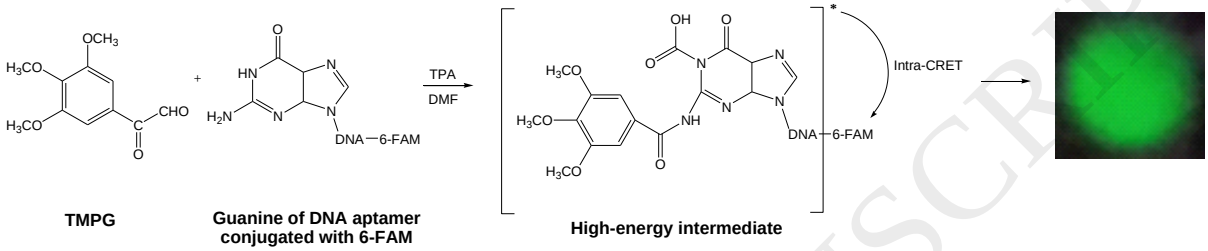
treated with antibiotics capable of treating a bacterial infection. Thus, in order to rapidly detect the appearance of norovirus, it is necessary to develop a highly sensitive biosensor to rapidly detect norovirus in food and drink as well as to early diagnose norovirus infection so that patients can be treated in a cost-effective manner. In addition, the method and cost to operate the biosensor should be simple and low, respectively.

Noroviruses, one of the family *Caliciviridae*, are a group of non-enveloped single-stranded positive-sense RNA (7.7 kb in length) viruses. Noroviruses are genetically classified into 7 geno-groups (GI to GVII) [2]. The majority of norovirus illnesses in human comes from GI and GII virus. In addition, GIV viruses are active as the cause of epidemic and sporadic diseases. The outbreak (89 %) of GII viruses is predominant, whereas GI viruses cause about 11 % of the outbreaks [3]. In particular, GII.4, a single genotype of GII, is widely detected as the cause of norovirus diseases in the world [4-6].

Currently, multiple analytical methods are applied for the detection of norovirus in laboratories even though they have several disadvantages. For example, the analytical method using an expensive electron microscopy operated by a trained inspector is insensitive even though the testing time is rapid [7, 8]. Time consuming enzyme immunoassay has only 37 ~ 76 % sensitivity even though the method has high specificity and high throughput [9]. The real-time polymerase chain reaction (RT-PCR) operated with complicated and time-consuming procedures (analytical time: 3 ~ 6 hours) has reduced clinical specificity [8, 9].

Recently, DNA aptamers, capable of capturing and detecting human norovirus strains in a sample, were developed using the systematic evolution of ligands by exponential enrichment (SELEX). The effectiveness of the low-cost DNA aptamers is similar to that of expensive antibodies applied in enzyme immunoassay for the quantification of norovirus [10]. DNA aptamers are able to sense GII genotype of norovirus with good selectivity. In addition, they

are stable in ambient condition, whereas antibodies should be stored in a freezer or refrigerator. Thus, using the advantages of DNA aptamer, it is possible to develop a cost-effective biosensor with high throughput and good selectivity if a highly sensitive optical sensor is applied as a detection method of the biosensor.



Scheme 1. Reaction pathway of guanine chemiluminescence

Chemiluminescence, UV-visible absorbance and fluorescence are widely applied as an optical sensor of an analytical system. It is well-known that chemiluminescence is more sensitive than UV-visible absorbance and fluorescence because the background noise of the former is much lower than those of the latter [11, 12]. Guanine chemiluminescence shown in Scheme 1 is applied as a highly sensitive optical sensor designed based on the role of DNA aptamer [13-16]. The high-energy intermediate shown in Scheme 1 is formed from the reaction of 3,4,5-trimethoxylphenylglyoxal (TMPG) and guanine of single strand DNA in the presence of tetra-n-propylammonium hydroxide (TPA) and dimethylformamide (DMF). TPA in this reaction acts as a base and DMF provide O_2 in the reaction shown in Scheme 1[13, 17, 18]. Thus, Scheme 1 indicates that the guanine of DNA aptamer can bind with a specific target marker in a sample as well as react with TMPG to produce the high-energy intermediate. The high-energy intermediate shown in Scheme 1 can transfer energy to fluorescent dye (e.g., fluorescein, 6-FAM) conjugated with DNA aptamer based on the principle of intra chemiluminescent resonance energy transfer (Intra-CRET) [13-16]. With the rapid Intra-

CRET, fluorescent dye can emit bright light as shown in Scheme 1.

Using the advantages of DNA aptamer applied to develop an aptasensor with guanine chemiluminescence detection, we designed and developed a highly sensitive aptasensor capable of rapidly capturing and accurately analyzing norovirus in a sample. Details are described in this paper.

2. Experimental

2.1 Chemical and materials

Conventional DNA aptamer [10] conjugated with 6-FAM (5'-6-FAM-CATCTGT GTGAAGAC TATATGGCGCTCACATATTTCTTTC-3') and modified DNA aptamer conjugated with 6-FAM 5'-6-FAM-GGGGGTTTTCATCTGTGTGAAGACTATATGGCG CTCACATATTTCTTTC-3') provided from Alpha DNA (Montreal, Quebec, Canada) were used to develop a biosensor capable of sensing GII norovirus. The modified DNA aptamer was designed based on the previous reports [13, 14, 19]. 5 additional guanines of the modified DNA aptamer act as an enhancer to generate bright guanine chemiluminescence. Also, 4 thymines were used as a linker between the conventional DNA aptamer and the enhancer. Non-toxic norovirus GII capsid recombinant (a positive sense RNA virus with 7.5kb nucleotides, encoding a major structural protein VP1 with 58~60 kDa) purchased from PROSPEC Protein Specialist (EAST Brunswick, NJ, USA) was used as a biomarker of GII norovirus because conventional DNA aptamer can bind with norovirus GII capsid recombinant as well as GII noroviruses (e.g., GII.2, GII4) [20]. 3,4,5-trimethoxylphenylglyoxal hydrate (TMPG, 97 %) was purchased from Matrix Scientific (Columbia, SC, USA). $FeCl_2$ (99 %), $FeCl_3$ (99 %), Tetra-*n*-propylammonium hydroxide (TPA, 40 % w/w aqueous solution), deionized H_2O (HPLC grade) were purchased from Alfa

Aesar (Ward Hill, MA, USA). N,N-Dimethylformamide (DMF) was purchased from EMD (Billerica, MA, USA). Various types of buffer solutions (pH 7.0 – 8.5) were purchased from Teknova (Holliser, CA, USA). Graphene oxide (GO) was purchased from Graphene Supermarket (Calverton, NY, USA).

2.2 Fabrication of graphene oxide/iron nanocomposites and its application

Graphene oxide/iron nanocomposites (Fe_3O_4 GO nanoparticles) were fabricated based on the previous report [21, 22]. $FeCl_2$ (25 mg) and $FeCl_3$ (100 mg) were dissolved and mixed in water (10 ml). GO (1 mg/ml) was prepared in water. The mixture of $FeCl_2$ and $FeCl_3$ (0.5 ml) was mixed with GO (0.5ml) in a 1.5ml-centrifugetube. The tube inserted into a Micro Centrifuge Tube Thermo-mixer (Eppendorf) was shaken for 10 seconds at 85°C. Then, ammonium hydroxide (NH_4OH, 20 μl, 30%) was dispensed into the tube. The microcentrifuge tube in the themomixer was shaken at 500 rpm for 50 minutes at 85 °C. Fe_3O_4 GO nanoparticles formed in the microcentrifuge tube were cooled at room temperature (21 ± 2 °C). Fe_3O_4 GO nanoparticles in the microcentrifugetube were washed 3 times in water using a magnetic separator (Bioclone,Inc.). We confirmed the structure of Fe_3O_4 GO nanoparticle using a Gatan Ultrascan 1000XP digital camera and a JEOL 2100 TEM operating at 200kV accelerated voltage as shown in Fig. S1. Fe_3O_4 GO nanoparticles as a stock solution were stored in a refrigerator (2 - 8°C). Free DNA aptamer was immobilized on the surface of Fe_3O_4 GO nanoparticle based on the principle of π-π static interaction between free aptamer and Fe_3O_4 GO nanoparticles [13, 14]. The role of GO is catching free aptamers, whereas that of magnetic nanoparticle is removing free aptamer bound with GO. Using the magnetic separator, free DNA aptamers immobilized on Fe_3O_4 GO nanoparticles were removed from the sample containing aptamer-bound norovirus GII capsid recombinant.

2.3 Quantification of norovirus GII capsid recombinant in tap water or artificial urine

Several standards containing different concentration of norovirus GII capsid recombinant and samples containing a certain concentration of norovirus GII capsid recombinant spiked in tap water or artificial urine were prepared. The modified DNA aptamer (125 nM, 100 μl) in Tris-HCl (pH 7.5) was mixed with a standard or sample (100 μl). The mixture was incubated for 30 minutes at room temperature. After the incubation, the mixture containing DNA aptamer-bound norovirus GII capsid recombinant and free DNA aptamer (10 μl) was mixed with 20 mM TPA (10 μl) in a borosilicate test tube. Then, the test tube was inserted into the luminometer (Lumat LB 9507, Berthold, Inc, Germany). Finally, CL that was immediately emitted from the test tube after the addition of 4 mM TMPG (100 μl) through a syringe pump of luminomeer was measured for 20 seconds.

3. Results and discussion

3.1 Role of conventional and modified DNA aptamers in guanine chemiluminescence reaction

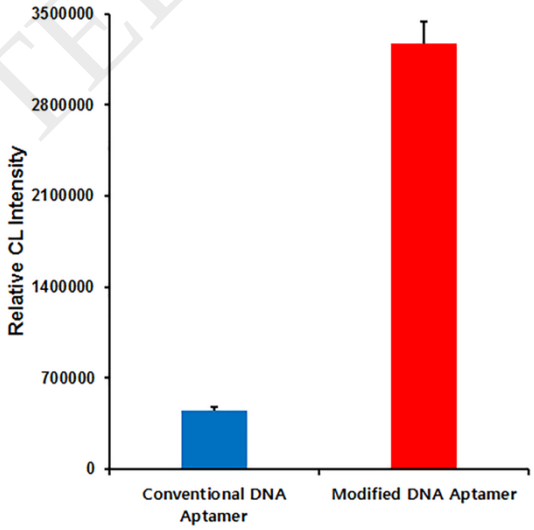


Fig. 1 Relative CL intensities of conventional and modified DNA aptamers in guanine chemiluminescence reaction. Condition: [DNA aptamer] = 200 nM, [TMPG] = 2 mM, [TPA] = 20 mM.

As shown in Fig. 1, the relative CL intensity of the modified DNA aptamer was

approximately 7.2-fold brighter than that of the conventional DNA aptamer because additional guanines of the modified DNA aptamer react with TMPG to generate more high-energy intermediates. In addition, the high-energy intermediates formed from the additional guanines can directly transfer energy to 6-FAM based on the principle of Intra-CRET. The results shown in Fig 1 indicate that a biosensor with the modified DNA aptamer can be more sensitive than that with the conventional DNA aptamer if the binding between the modified DNA aptamer and norovirus GII is as good as that between the conventional DNA aptamer and norovirus GII.

3.2 Interaction between norovirus GII and modified DNA aptamer

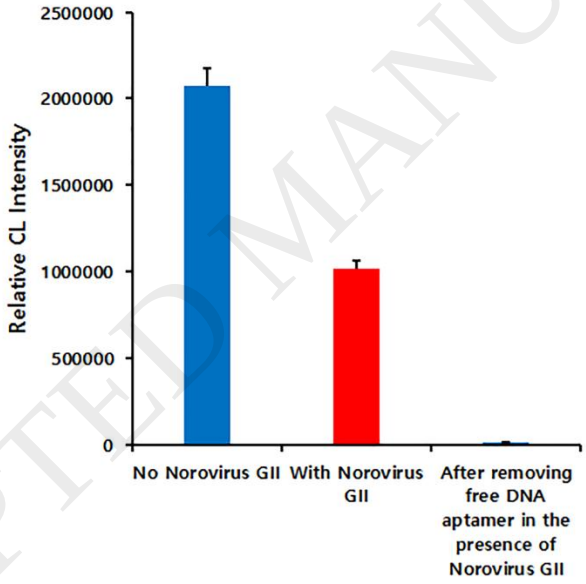


Fig. 2 Interaction of norovirus GII and modified DNA aptamer. [Modified DNA aptamer] = 250 nM, [Norovirus GII capsid] = 5 μg/ml, [TMPG] = 2 mM, [TPA] = 20 mM, Incubation time: 30 min at room temperature.

Fig. 2 shows that the modified DNA aptamer can rapidly bind with norovirus GII. The results of Fig.2 indicate that guanines of DNA aptamer bound with norovirus GII cannot react with TMPG to emit light because the relative CL intensity in the presence of norovirus GII is smaller than that in the absence of norovirus GII. As a proof, we confirmed that the modified DNA aptamer bound with norovirus GII, remaining after removing the free modified DNA

aptamer using GO/Fe_3O_4 nanocomposites, cannot emit light as shown in the right bar graph of Fig. 2.

Fig. 3 is the plausible reaction pathways in guanine chemiluminescence reaction derived based on the results of Fig. 2. The complex of the modified DNA aptamer-bound norovius GII cannot emit light, whereas the modified DNA aptamer remaining after the formation of the complex can emit light.

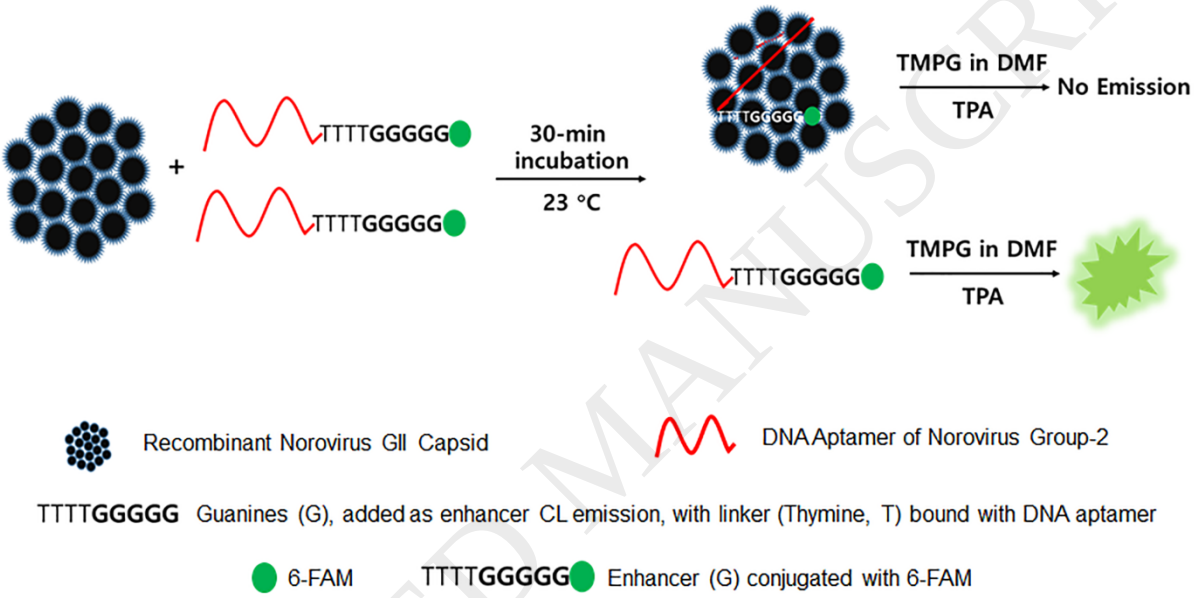


Fig. 3 Complex of norovirus GII-bound the modified DNA aptamer and guanine chemiluminescence

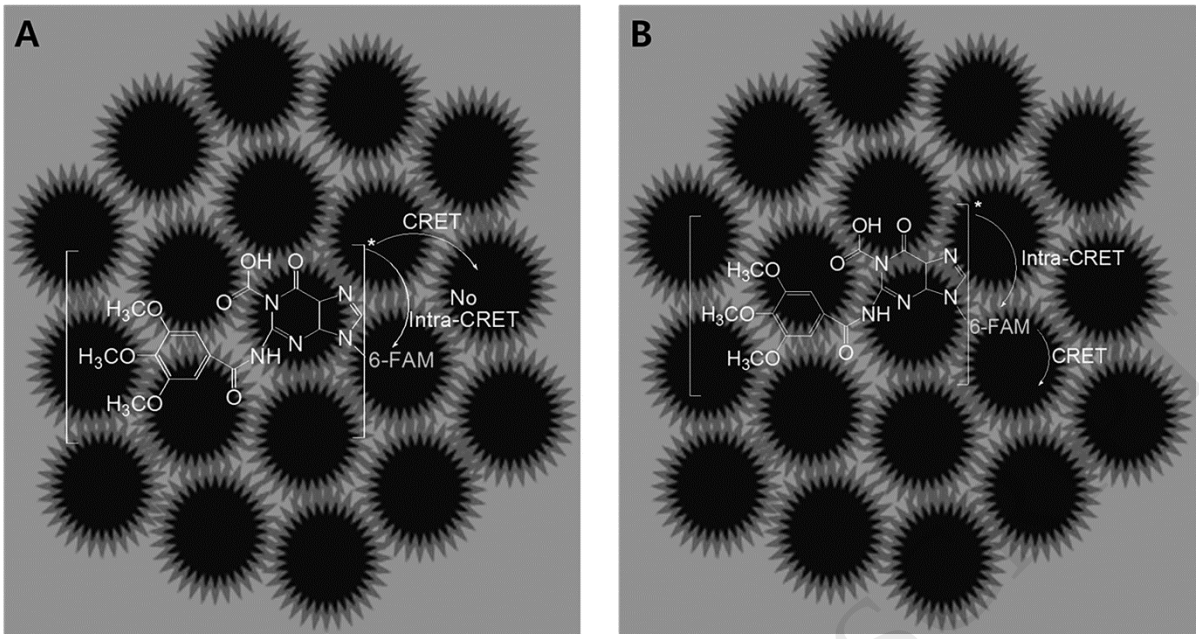


Fig. 4 Possible CRET mechanisms of additional guanines bound with norovirus GII capsid in Guanine chemiluminescence reaction.

As shown in Figs. 2 and 3, 5 additional guanines of the modified DNA aptamer cannot bind with norovirus GII. Thus, we expected that the extra guanines of the modified DNA aptamer can emit light in guanine chemiluminescence reaction. However, no emission from the complex of norovirus GII and the modified DNA aptamer was observed as shown Figs. 2. The plausible mechanisms to interpret the results of Fig 2 are shown in Fig. 4. As shown in Fig. 4A, the high-energy intermediate formed from the reaction of TMPG and 5 additional guanines cannot transfer energy to 6-FAM because the energy transfer between the high-energy intermediate and the surface of norovirus GII capsid is faster than that between the high-energy intermediate and 6-FAM. As another plausible reaction mechanism (Fig 4B), 6-FAM excited with the intra-CRET between high-energy intermediate and 6-FAM cannot emit light if norovirus GII capsid bound with the modified DNA aptamer acts as a strong quencher in guanine chemiluminescence. The role of norovirus GII capsid may be similar to that of GO.

3.3 Effect of buffer

As shown in Fig. S2, the interaction between norovirus GII capsid and the modified DNA

aptamer is dependent on the property of the buffer solution. The complex of norovirus GII-bound the modified DNA aptamer was formed in Tris-HCl, whereas the reaction between norovirus GII and DNA aptamer is too slow to observe any change in PBS and TBS while incubating for 30 minutes at room temperature. Also, the formation of complex in Tris-HCl (pH 7.5) was faster than that in Tris-HCl (pH 7). Based on the results shown in Fig. S2, we selected Tris-HCl (pH 7.5) for the development of a biosensor capable of quantifying norovirus GII in a sample.

3.4 Effect of TMPG concentration

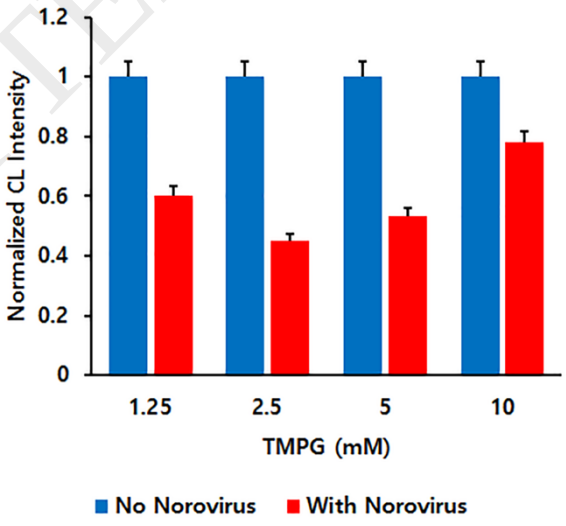


Fig. 5 TMPG Effect in guanine chemiluminescence reaction.

Relative CL intensity in guanine chemiluminescence reaction was dependent on the

concentration of TMPG. Relative CL intensity in the absence of norovirus GII capsid was enhanced with the increase of TMPG concentration However, relative CL intensity of 10 mM TMPG was lower than that of 5 mM TMPG due to the self-quenching of light emitted under 10 mM TMPG.

Fig 5 shows that the discrepancy of relative CL intensities in the absence and presence of norovirus GII capsid is dependent on the concentration of TMPG. As shown in Fig. 5B, the difference of relative CL intensities of 2.5 mM TMPG in the absence and presence of norovirus GII capsid was the largest. Based on the results shown in Fig. 8, 2.5 mM TMPG was selected for the development of biosensor capable of sensing norovirus GII in a sample.

3.5 Effect of incubation time

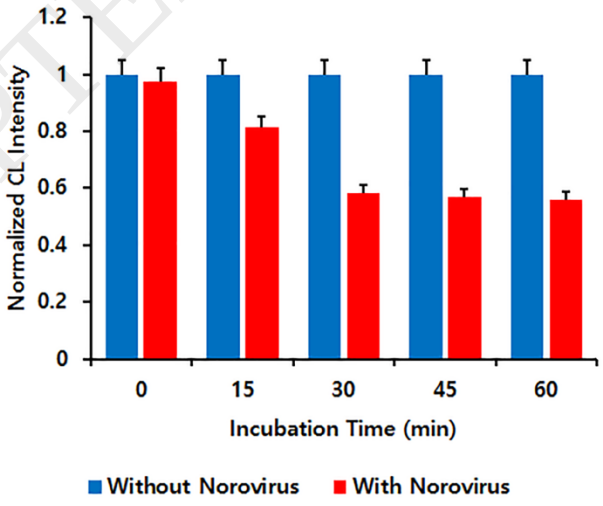


Fig. 6 Effect of incubation time to form the complex of norovirus GII-bound the modified DNA aptamer.

As shown in Fig. 6, the complex of norovirus GII-bound the modified DNA aptamer was

completely formed after the 30-min incubation at room temperature within the acceptable error range (± 5.0 %) when norovirus GII capsid (3μg/ml) was mixed with the modified DNA aptamer (250 nM). Thus, 30-min incubation at room temperature was selected for the development of biosensor for the quantification of norovirus GII in a sample.

3.6 Effect of the modified DNA aptamer concentration

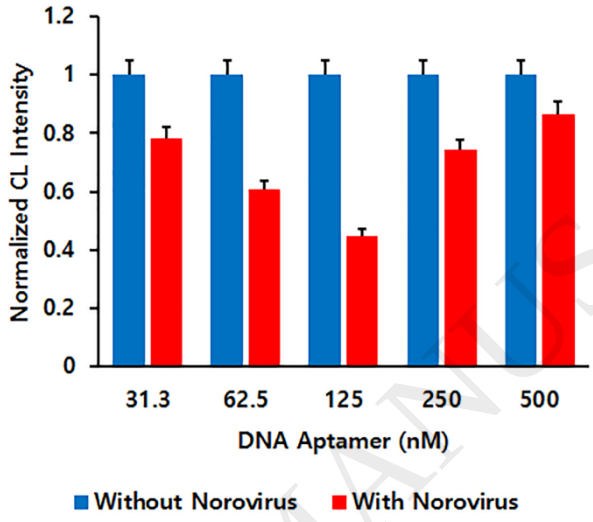


Fig. 7 Concentration effect of the modified DNA aptamer

Relative CL intensity depends on the modified DNA aptamer concentration. In addition, the difference of relative CL intensities in the absence and presence of norovirus GII was dependent on the concentration of the modified DNA aptamer as shown in Fig 7. Based on the results shown in Fig. 7, the best concentration of the modified DNA aptamer was 125 nM in Tris-HCl (pH 7.5) to devise a biosensor for the monitoring of norovirus GII in a sample. This is because (1) the modified DNA aptamer lower than 125 nM wasn't enough to bind with norovirus GII under the limited incubation time of 30 minutes (2) relative CL intensities of the modified DNA aptamer (250 and 500 nM) were self-quenched in the absence of norovirus GII.

3.7 Effect of TPA concentration

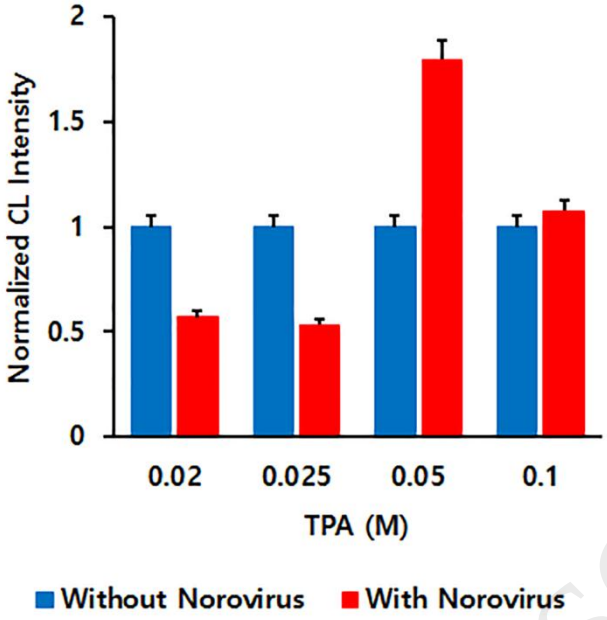


Fig. 8 Concentration effect of TPA

Relative CL intensity was enhanced with the increase of TPA concentration. Fig. 8 indicates that TPA between 20 and 25 mM is the best to develop a biosensor capable of monitoring trace levels of norovirus GII in a sample. This is because relative CL intensity with the addition of TPA higher than 25 mM is self-quenched in the absence of norovirus GII. Thus, relative CL intensity of 50 mM TPA in the absence of norovirus GII was lower than that in the presence of norovirus GII (5 μg/ml). Thus, the relative CL intensity of 50 mM TPA in the presence of norovirus GII was about 1.78-fold higher than that in the absence of norovirus GII. Additionally, the relative CL intensites of 100 mM TPA were self-quenched in the absence and presence of norovirus GII. Thus, the relative CL intensity of 100 mM TPA in the presence of norovirus GII was about 1.07-fold higher than that in the absence of norovirus GII.

3.8 Quantification of norovirus GII capsid using the biosensor optimized based on preliminary experimental data

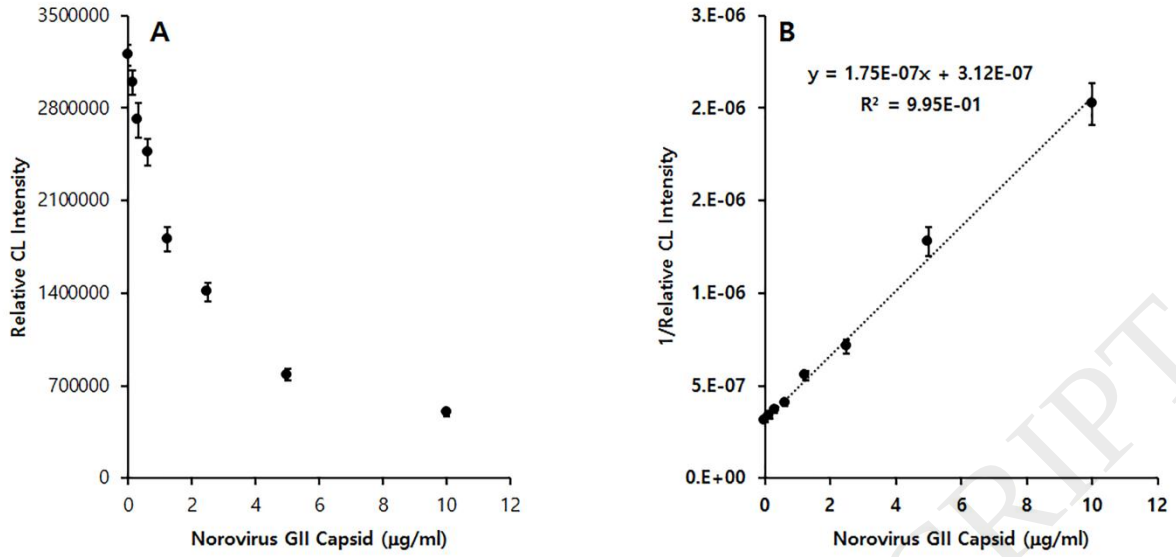


Fig. 9 Calibration curves of the biosensor for the quantification of norovirus GII capsid in water.

As shown in the calibration curve of Fig. 9A, relative CL intensity was decreased with the increase of norovirus GII capsid. In addition, Fig. 9B shows the linear calibration curve with the reverse values of relative CL intensities. The calibration curves of biosensor developed in this research was obtained under the optimized conditions of several variables determined with the preliminary experimental results described above (See sections 3.3 to 3.7). The dynamic range of the linear calibration curve was 0.16 to 10 μg/ml) with the good R-squared value (R^2 = 0.995). The limit of detection (LOD = 3σ) of the biosensor was as low as 80 ng/ml. σ is the standard deviation of average (n = 10) of relative CL intensity measured in the absence of norovirus GII capsid. The results shown in Fig. 9 indicate that the cost-effective biosensor devised with the modified DNA aptamer can be applied as a new and simple method, capable of rapidly quantifying and monitoring norovirus GII, instead of time complicated, expensive, and time consuming methods such as electron microscopy, enzyme immunoassay, and RT-PCR [7-9]. The new biosensor for the quantification of norovirus GII was compared with other biosensors [10, 23-25] reported recently as shown in Table S1. For example, we developed a biosensor with chemiluminescence detection capable of rapidly

sensing norovirus GII in a sample, whereas Chand and co-researchers fabricated a graphene-gold nano-composite aptasensor with electrochemical detection [25].

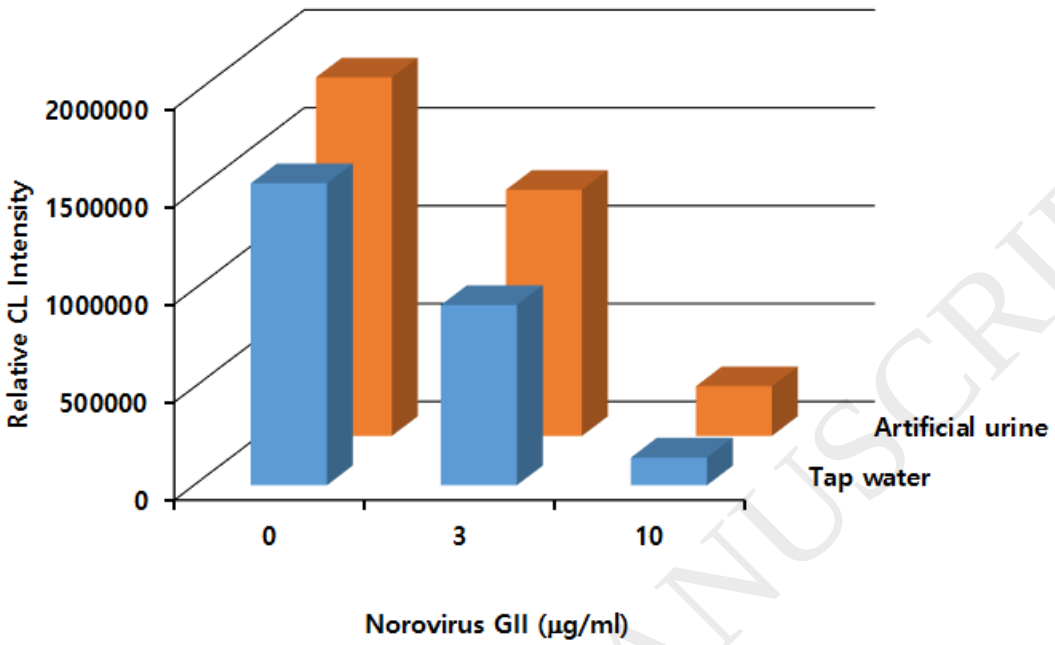


Fig. 10 Quantification of norovirus GII capsid in tap water and artificial urine. The error range of relative CL intensity measured under each condition was 3 ~ 7 %.

In general, viruses are excreted through urine. Also, drinking water can be contaminated with noroviruses. As shown in Fig. 10, relative CL intensity in tap water and artificial urine was decreased with the increase of norovirus GII capsid. However, relative CL intensity was constant in the absence and presence of norovirus GII capsid within the statistically acceptable error range. In addition, the relative CL intensity in artificial urine in the absence of norovirus GII capsid was slightly higher than that in tap water. These results indicate that the modified DNA aptamer can specifically capture norovirus GII in tap water and artificial urine. However, Fig 10 shows that the binding between norovirus GII capsid and the modified DNA aptamer in tap water is faster than that in artificial urine containing more complicated compositions such as various ingredients such as urea, chloride, sodium, creatine, proteins, hormones and metabolites. In addition, it was confirmed that the recovery range

(95.6 ~ 106.6 %) of the new biosensor, capable of specifically quantifying norovirus GII capsid, in tap water and artificial urine was statistically acceptable as shown in Table S2. We confirmed that the specificity of the modified aptamer is good as shown in Fig. S3. The modified aptamer doesn't interact with several bacteria positive controls purchased from KPL.

4. Conclusions

With the development of biosensor in this research, we confirmed that the modified DNA aptamer with 5 extra guanines was able to capture norovirus GII as well as to enhance the brightness of guanine chemiluminescence. The biosensor with excellent specificity and reproducibility was able to rapidly and quantify trace levels of norovirus GII capsid in tap water and artificial urine. Thus, it is possible that the cost-effective and simple biosensor can be applied as an alternative analytical method, instead of complicated, expensive, and time-consuming methods (e.g., electron microscopy, enzyme immunoassay, RT-PCR) that need to be operated by trained inspectors, for the quantification of norovirus GII in a sample.

Additionally, it is expected that the technology applied in the development of the new biosensor can be widely applied to devise alternative biosensors for the analyses of food-borne pathogens such as *Listeria*, *Salmonella*, and *Vibrio*.

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