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Fluorometric detection of influenza viral RNA using graphene oxide

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ABSTRACT

Simple and reliable detection of influenza viruses is important for timely prescription of antiviral therapy. Here, we developed a facile fluorometric system for detection of influenza subtype viral genes using graphene oxide (GO). A fluorescent DNA probe complementary to hemagglutinin gene of influenza virus is degraded by the 5′ to 3′ exonuclease activity of *Taq* polymerase during PCR. Upon addition of GO, the released fluorophore retains fluorescence without adsorption onto GO, whereas the intact fluorescent DNA probe is adsorbed onto GO with fluorescence quenching. Our multi well plate system can detect as low as 3.8 pg of influenza viral RNA.

1. Introduction

Seasonal and pandemic influenza, such as swine-origin influenza that erupted in 2009, imposes a considerable health and economic concern [1]. Influenza viruses belonging to the family *Orthomyxoviridae* contain a single-stranded RNA genome, and are classified into four types (A, B, C, and D) according to their core structural proteins. Among these, the influenza A type, which is usually found in humans, is further divided into several other subtypes according to major surface antigens such as hemagglutinin (H) and neuraminidase (N) [2,3].

For rapid detection of influenza viral infections, viral surface antigen detection methods such as a rapid influenza diagnostic test have been widely used. This method can identify the influenza virus based on a viral antigen-specific immunoassay using a paper strip [4] and can be used to detect influenza virus in a short time (~1 h) without the need for virus isolation. Despite its rapidity, it is however difficult to distinguish the subtypes of influenza viruses because the antibodies used in immunoassays show cross-reactivity and low detection sensitivity [5]. Currently, an influenza viral gene detection method by reverse transcription (RT) followed by real-time polymerase chain reaction (real-time PCR) has become a powerful tool to identify influenza virus subtypes. However, intercalating dye-based real-time PCR often shows false-positive results because the fluorescent dye binds to non-specific amplified products during the PCR [6]. Thus, the development of a simple and reliable method for the detection of influenza viral genes is still warranted.

In recent years, graphene oxide (GO) has attracted considerable attention as a DNA sensor due to outstanding electronic, thermal, and mechanical properties with good chemical stability [7,8]. GO is a soluble two-dimensional carbon material that consists of epoxy, hydroxyl, and carboxyl functional groups [9]. The GO surfaces can adsorb single-stranded DNA (ssDNA) owing to the pi-stacking interactions and hydrogen bonding between the aromatic ring structure of the exposed DNA bases and the honeycomb structure of the GO, whereas double-stranded DNA is not adsorbed onto GO due to fewer exposed DNA bases [10]. In addition, GO quenches fluorescence via fluorescence resonance energy transfer between the fluorophore and pi-systems of GO [11]. Previously, we exploited the properties of GO as a DNA sensor for adsorbing nucleic acids with robust fluorescence quenching efficiency, and developed a GO-based fluorometric system for detecting of mutant fusion genes of leukemia [12].

The GO-based RT-PCR system for influenza viral gene detection is based on two reaction: RT-PCR and GO-based fluorescence quenching of a fluorescent DNA probe (Fig. 1a). A FAM-labeled DNA probe is designed, complementary to the hemagglutinin gene sequence of target influenza virus strains (i.e. H3N2). During the RT-PCR for amplification of the hemagglutinin gene, the subtype specific primers are elongated. This leads to the hydrolysis of the hemagglutinin gene-bound FAM-DNA probe due to the 5′ to 3′ exonuclease activity of *Taq* polymerase, resulting in the release of the FAM fluorophore. When the PCR product is

Abbreviations: GO, graphene oxide; qRT-PCR, quantitative reverse transcription polymerase chain reaction; LOD, limit of detection; Ct, cycle threshold; FAM, 6-fluorescein amidite; S/B ratio, signal to background ratio

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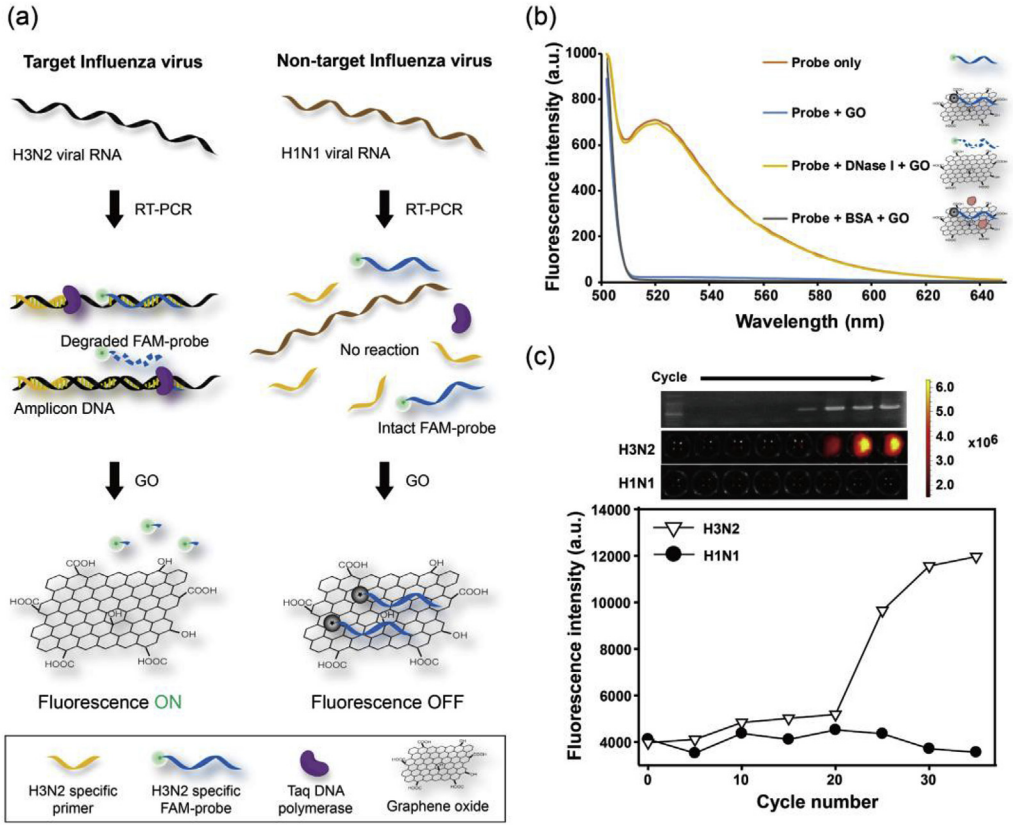

Fig. 1. (a) Schematic illustration of the fluorometric detection of influenza viral RNA based on GO and the 5′ to 3′ exonuclease activity of *Taq* polymerase during RT-PCR. (b) DNase I treatment and fluorescence difference after GO incubation. Shown are the fluorescence emission spectra (λ_{ex} = 485 nm) of the FAM-DNA probe and the DNase I digested FAM-DNA probe after GO incubation. (c) The fluorescence of the FAM-DNA probe was enhanced with PCR cycle progression. The H3N2 (target) and H1N1 (non-target) viral RNAs were subjected to RT-PCR with H3N2 hemagglutinin gene-specific primers and the FAM-DNA probe. After incubation with GO, the fluorescence intensity was measured at various PCR cycle intervals (λ_{ex} = 485 nm and λ_{em} = 535 nm). Upper panels: agarose gel electrophoresis and fluorescence image showing the accumulation of PCR amplicons and increase of fluorescence intensity with PCR cycle progression.

incubated with GO, the released FAM is not adsorbed onto GO and a fluorescence signal can be observed. However, in the absence of target influenza viral RNA, the PCR would be aborted and the fluorescent DNA probe would remain intact. As a result, the intact fluorescent DNA probe would be easily adsorbed onto GO, resulting in the quenching of FAM fluorescence.

As a preliminary step, concentrations of FAM-DNA probe and GO to maximize the difference in fluorescence signal between target and non-target influenza viral RNA was optimized to be 600 nM and 10 μg mL^{-1}, respectively (Fig. S1). To verify that the fluorescence signal difference indeed resulted from the release of the fluorophore from the FAM-DNA probe, the FAM-DNA probe was mixed with a DNase I, which is a DNA-degrading enzyme. Degradation of the fluorescent DNA probe by DNase I is analogous to the 5′ to 3′ exonuclease activity of *Taq* polymerase. After addition of GO to a solution containing the FAM-DNA probe, fluorescence was completely quenched due to adsorption of DNA probe onto the GO sheet (Fig. 1b). In contrast, when the FAM-DNA probe was degraded with a DNase I, subsequent addition of GO (10 μg mL^{-1}) did not diminish the fluorescence of the sample (0.2 U μL^{-1}). However, fluorescence of the FAM-DNA probe was quenched upon the addition of GO to the mixture when bovine serum albumin was used instead of DNase I. Thus, degradation of the FAM-DNA probe during the PCR would result in steady fluorescence even after the addition of GO, due to the release of FAM from the FAM-DNA probe.

Next, to demonstrate that the fluorescence intensity in the GO-based assay reflected the accumulation of the PCR product from the target viral gene amplicon DNA during the PCR, we measured the fluorescence of the PCR mixture as the PCR cycle progressed. A 5-μl aliquot of the PCR mixture containing 600 nM of fluorescent DNA probe was removed from thermal cycler at specified cycles. The aliquot of the PCR mixture was diluted to 50 μL with a reaction solution containing GO (10 μg mL^{-1}) in a 96-well plate, and the fluorescence was measured using a multilabel plate reader. The fluorescence signal from the FAM-DNA probe after GO quenching with RT-PCR of H3N2 influenza RNA (*i.e.* target influenza virus) progressively increased as the PCR cycle number increased, whereas the fluorescence signal was not increased with H1N1 influenza RNA (*i.e.* non-target influenza virus) (Fig. 1c). In the presence of H3N2 influenza RNA, amplicon bands of the expected size (578 bp) were observed with increased intensity as the PCR cycle progressed (top panel in Fig. 1c). Thus, the increase of fluorescence in the GO-based assay system indeed corresponds to the accumulation of the viral gene amplicon in the PCR.

We next examined the sensitivity of the GO-based RT-PCR system compared with the sensitivity of SYBR green-based real-time qRT-PCR. Two-fold serially diluted H3N2 (*i.e.* target influenza) or H1N1 (*i.e.* non-target influenza) RNAs were subjected to RT-PCR amplification with H3N2 hemagglutinin gene-specific FAM-DNA probe. After GO incubation (10 μg mL^{-1}) for 15 min in a 96-well plate, the fluorescence emission spectra (Fig.S2) and fluorescence intensity were measured (Fig. 2a). The fluorescence intensity was gradually increased with increasing amounts of H3N2 RNA, while the fluorescence intensity with H1N1 RNA was not increased with increasing amounts of RNA. A gradual increase of fluorescence intensity with increasing amounts of target viral RNA (H3N2) was reflected by the accumulation of the H3N2 amplicon (top panel in Fig. 2a). Visualized fluorescence signals in the black 96-well plate also increased as the amount of H3N2 RNA increased (middle panel in Fig. 2a), which was consistent with a linear relationship between the fluorescence intensity and the amount of H3N2 RNA on a log scale. A statistically significant ($P < 0.05$) fluorescence signal was measured with influenza RNA as low as 37 pg, and the limit of detection (LOD) was calculated to be 3.8 pg (see Supplementary Information for equation). The same serially diluted H3N2 influenza viral RNA from 9400 to 37 pg was analyzed by conventional real-time qRT-PCR with the same primers used in the GO-based RT-PCR system (Fig. S3, fluorescence curves). As shown in Fig. 2b, the analyses of the cycle threshold (Ct) values obtained from the real-time qRT-PCR were significantly ($P < 0.05$) different, which were as low as 73 pg and