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A Label-free Aptasensor for Rapid Detection of H1N1 Virus based on Graphene Oxide and Polymerase-aided Signal Amplification

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Abstract

Single-stranded DNA aptamers specific to the hemagglutinin (HA) protein of avian influenza virus (A/Puerto Rico/8/1934) were obtained by SELEX process in 13 cycles. The aptamer with the highest affinity and specificity was applied to an affinity bioassay. An aptamer hairpin (aHP) was prepared that consists of two DNA regions, viz. (a) the aptamer for the HA protein and (b) an oligonucleotide designed to form a stem-loop structure. In the absence of target, the aHP maintains its hairpin structure and was adsorbed to the Graphene Oxide (GO). The fluorescence of SYBR Green I (SGI) is almost quenched. On addition of the target, aHP unfolds and the GO is no longer attached but released to the solution. By applying a polymerase elongation reaction, a long dsDNA product is generated when SGI is added. The proposed method could detect HA and H1N1 virus with a limit of 2.5 μg/mL and 1×10^2 TCID50, respectively. Consequently, this paves the way for influenza virus detection and is employed in basic research and medical diagnosis.

Keywords: Aptamer; Influenza virus; Graphene oxide; Detection

Introduction

Influenza virus belongs to the *Orthomyxoviridae* family and includes three types: A, B and C. Among these types, the influenza A virus leads to seasonal epidemics, sporadic pandemics, which are the major causes of morbidity and mortality. Influenza, an acute infectious disease, is a major source of fever, cough, runny nose and other symptoms of humans and has a great impact on public health. It is estimated that seasonal influenza virus strains infections caused 50,000 deaths each year. These symptoms are actually closely related to the HA. The HA, a glycoprotein, is a spike located on the surface of influenza virus' envelope which is responsible for hemagglutination. It is reported that the HA could bind to sialic acid receptors located on the surface of host cells and mediated the subsequent entry via membrane fusion which is significant for initial viral infection [1-3]. It is proteolytically hydrolyzed to HA1 and HA2 subunits. HA1 is receptor-binding domain while HA2 fusion peptide inserts itself into the endosomal membrane, allowing contact of both the viral and endosomal membranes [4]. Accordingly, HA could be employed as a biomarker for detection and as an important target for depressing its function.

Aptamers, as promising alternatives to antibodies, are ssDNA and RNA molecules obtained from the Systematic Evolution of Ligands by Exponential enrichment (SELEX) process that can bind to a wide range of target molecules with high specificity and affinity, including protein, organic molecules, drugs, various cell surface receptors, and whole cells, to name a few [5-7]. They have been applied to detect pathogenic bacteria, separate the targets, and identify biomarkers [8,9]. Meanwhile, they have also been employed as an efficient therapeutic tool against viruses due to their remarkable properties [10-12]. So far, several high-affinity DNA and RNA aptamers have been successfully applied in suppressing the function of viral proteins, such as human immunodeficiency virus HIV glycoprotein 120 (gp120) [13], human hepatitis B virus polymerase (P protein) [14] and influenza virus NS1 protein [15]. In the past five years, there are already quite some reported works for the selection of HA aptamer by SELEX process. The aptamers selected by Gopinath had a higher affinity and specificity for the HA of H1N1 [16]. However, they were RNA and not stable. The function of DNA aptamer selected by Jeon was to target HA protein of influenza virus and inhibit it, which may be promising candidate for treatment of influenza virus infection [17].

Park paid attention to hemagglutination inhibition test in vitro with selected aptamer [18]. However, few works have been reported on aptamers for H1N1 (A/Puerto Rico/8/1934) influenza virus' detection. Hence, it is necessary to screen a DNA aptamer with highly specificity to the HA protein of the virus H1N1, and use it to establish a sensitive detection system.

Graphene oxide (GO), a two-dimensional nanomaterial, is an efficient bioanalytical platform for the detection of nucleic acids, proteins, metal ions, and small molecules [19-21], drawing wide attention due to its unique and excellent electronic, thermal, and mechanical properties [22-24]. GO makes fluorescence detection promising application in sensing technology [25,26]. Herein, a novel label-free fluorescent approach was constructed for H1N1 detection in conformity with GO and strand displacement reaction by employing SYBR Green I (SGI) for signal amplification. Once target was bound with the aptamer sequence of the hairpin probe (HP), another sequence could form a self-assembled short hairpin structure. Meanwhile, strand displacement reaction would be triggered by generating a large amount of dsDNA in the presence of KF polymerase and dNTPs. Upon addition of GO, the SGI-stained dsDNA would be inefficiently quenched by it, resulting in a significant fluorescence enhancement. In the absence of target, the stained HP would be absorbed onto GO and quenched by it.

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Materials and Methods

Materials

A synthetic ssDNA library containing a 35-base central random sequence (5'-GGGAGCTCAGAATAAACGCTCAA-N35-TTCGAC ATGAGGCCCGGATC-3′) were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China, http://www.sangon.com). Polymerase Klenow Fragment (2 U/μL) and 10×Klenow Fragment buffer (500 mM Tris–HCl, 50 mM $MgCl_2$, 10 mM DTT, pH 8.0) were also obtained from Sangon Biotech Co., Ltd. (Shanghai, China). All materials used in the experiment were dissolved in distilled water purified by a Milli-Q water purification system (electric resistivity 18 MΩ cm^{-}). All the other chemicals were analytical reagent grades, and were used without further purification. SYBR Green I (10^4×concentrate) was purchased from FANBO BIOCHEMICALS Co., Ltd (Beijing, China). Graphite powder was purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China, http://www.xfnano.com). The fluorescent emission spectra were recorded in a quartz cuvette by an LS55 luminescence spectrometer (PerkinElmer, UK) at room temperature. The concentration of oligonucleotides were determined by using the absorbance at 260 nm. The recombinant HA protein of AIV subtype H1N1 (A/Puerto Rico/8/1934) with a concentration of 0.25 mg/mL was offered from Sino Biological Inc. The virus was cultivated in the allantoic cavities of 10-day-old embryonated chicken eggs and maintained at 35°C. After 72 h culturing, the allantoic fluid was centrifuged and the supernatant was harvested, and stored at -70°C prior to use. The titer of virus used for infection was evaluated by the infection of Madin-Darby canine kidney (MDCK) cells [27], and hence, virus titer was expressed as the tissue culture infective doses leading to 50% infected cells (TCID50). The three strains of virus were A/Chicken/Henan/12/2004 (H5N1), A/Chicken/Jiangsu/7/2002 (H9N2) and A/Puerto Rico/8/1934 (H1N1) which were maintained in Molecular Virology Lab of Hunan Normal University.

Methods

SELEX procedure: The oligonucleotide library consisted of a 35-base random region flanked by two primer regions: 5′-GGGAGCTCAGAATAAACGCT CAA-N35-TTCGACATGAGGCCCGGATC-3′. The detailed procedure for selecting HA protein aptamers was implemented as follows. The HA protein with 0.05 mol/L$NaHCO_3$ (pH 9.6) was coated on a 96-well ELISA Plate at 4°C overnight. 3% bovine serum albumin (BSA) was added at 37°C for 2 h to block the wells coated with HA protein and blank wells. The ssDNA library was denatured at 95°C for 5 min in binding buffer (20 mmol/L HEPES buffer pH 7.35, 120 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 5 mmol/L KCl) and then immediately placed into iced water for 10 min. To decrease background binding, yeast tRNA was added to the binding buffer from the second round to the end round. For negative selection, ssDNA libraries added to BSA-blocked blank wells were maintained at 37°C for 40 min. Uncombined ssDNAs were then transferred to the wells coated with HA protein at 37°C for 40 min. The wells were washed five times with washing buffer (binding buffer +0.05% Tween 20) and filled with eluting buffer (20 mmol/L Tris-HCl, 4 mol/L guanidiniumisothiocyanate, 1 mmol/L DTT, pH 8.3) at 80°C for 10 min. Subsequently, 3 mol/L sodium acetate (pH5.2) and dehydrated ethanol were added and kept at -80°C for 40 min, and centrifuged at 30,857×*g* for 10 min at 4°C. The sediment was washed twice by absolute ethyl alcohol and dissolved in 20 μL double-distilled water after drying. The ssDNA was amplified by PCR (5 min at 95°C, then 30 s at 95°C, 30 s at 65°C, and 30 s at 72°C, 20 cycles, followed by 5 min at 72°C). The asymmertric PCR (5 min at 95°C, then 30 s at 95°C, 30 s at 65°C, and 30 s at 72°C, 40 cycles, followed by 5 min at 72°C) was used to acquire the ssDNA as the enriched library for the next selection round. The amount of ssDNAs at each step was measured by UV-Vis spectroscopy. The aptamer binding ratio was obtained by analyzing the fluorescence of eluted ssDNA every two rounds. After 13 rounds' enrichment, the PCR products selected from the 13th round were subcloned and sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). Their secondary structures were performed using the DNAMAN software.

Performance of target protein sensing and selective assay: The sample was mixed with hairpin probe (5 μL, 50 nM 5'-GGGAGCTCAGAATAAACGCTCAAGGCACGGCATGTGTG GTATGTGGTGCCTGTACTCGTTCGACATGAGGCCCGGATC CATGCTAACAAGCATG-3') in PBS (phosphate buffered saline) buffer at 95°C for 5 min and kept at room temperature for 20 min. After that, the HA proteins ranging from 0 μg/mL to10 μg/mL were added to the solution. After incubation for 40 min, 4 μL SGI (50×concentrate) and 1.5 μL GO (1 mg/mL) were successively injected into the reactions and incubated at 37°C for 10 min. The polymerization reaction was then performed by mixing with 10 mM dNTPs and 2 U/mL KF polymerase for 10 min, and then terminated by heating at 80°C for 5 min. Subsequently, the fluorescence intensity was measured in the quartz cuvette. Fluorescence intensity was recorded at 520 nm with an excitation wavelength of 496 nm. Bovine serum albumin (BSA), lysozyme and the control were chosen to verify the selectivity of this approach. Their concentrations were 5 μg/mL. All experiments were repeated three times.

Analysis of virus sample: After various titers of virus were mixed with hairpin in working solution (PBS) at 37°C for 40 min, SGI and GO were added into the systems for 10 min. dNTP and KF polymerase were mixed into the reaction buffer. Continually, the reaction was performed at 37°C for 10 min, and the fluorescence signals were measured in a quartz cuvette at an excitation wavelength of 496 nm and an emission wavelength of 520 nm, respectively. Its specificity was also detected using two different strains:H9N2, H5N1 under the same conditions. All experiments were repeated in triplicates.

Results and Discussion

In vitro selection of ssDNA aptamers for recognition of the HA protein

In order to obtain DNA aptamers with high affinity and specificity, thirteen repeated separation-amplification cycles were completed, and the ssDNA and HA protein concentrations decreased with each subsequent selection round. After 13 selection rounds, the aptamers showed higher affinities for HA protein. The amounts of HA protein and ssDNA pools added in each round are shown in Table 1. In addition, the quantity of BSA and yeast tRNA increased with the selection round to ensure the competitive binding. The binding ratios of aptamers against HA increased with the selection process and the percentage of bound ssDNA did not increase considerably after round 11 as shown in Figure S1. These results displayed that the affinity of the ssDNA pool to HA protein appeared to be nearly constant after round 11. After being cloned and sequenced, two independent candidates were acquired and named aptamers 1, 2. The predicted secondary structures of the two aptamers were also determined by DNAMAN software, and their typical stem-loop motifs are shown in Figure 1. As aptamer 1 showed

SELEX Rounds	HA protein (ugwell)	ssDNA poll (pmol/well)	tRNA (ug/ml)
1	4	400	0
2	2	200	0.125
3	1	100	0.125
4	0.5	100	0.25
5	0.5	80	0.25
6	0.25	60	0.5
7	0.25	50	0.5
8	0.1	40	1
9	0.1	30	1
10	0.05	20	1.5
11	0.05	10	1.5
12	0.05	10	2
13	0.025	5	2

Table 1: Hemagglutinin (HA) protein, ssDNA pool and tRNA input in 13 rounds selection.

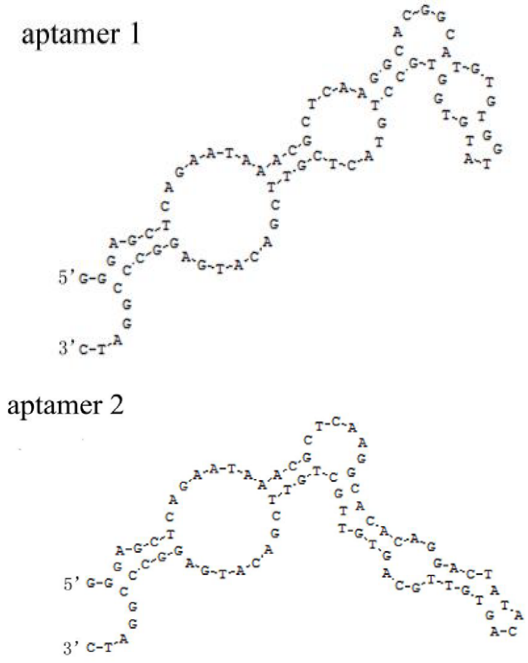
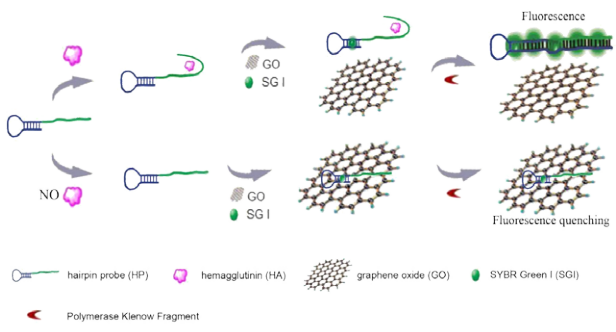


Figure 1: Predicted secondary structures of the selected ssDNA aptamer candidates using DNAMAN software. (a) aptamer sequence 1; (b) aptamer sequence 2.



Scheme 1: The detection principle for the target.

higher binding affinity for HA protein (Kd=78 ± 1 nmol/L) compared with aptamer 2, aptamer1 was selected for further experiments.

Design of rapid detection for target

Although several similar methods have been reported, however, they have their some shortcomings. Therefore, it is necessary to develop a highly sensitive and selective fluorescent means for HA and H1N1 detection. As shown in Scheme 1, the aptamer hairpin (HP) was composed of two DNA regions including the HA protein aptamer sequence and the oligonucleotide sequence specially designed which could form stem-loop structure. In the absence of HA protein, HP maintained its hairpin structure and its fluorescence signals could not be detected after adding SGI and GO. Because the aptamer was strongly absorbed onto the GO due to π-π stacking interactions between the nucleotide bases and GO [28], it was resulted in that KF polymerase and dNTPs could not work and the fluorescence was quenched. When the target was added, HP would not be absorbed onto the GO, and polymerase elongation reaction generated a long dsDNA product, triggering a strong increase in the fluorescence intensity upon introducing the SGI. In addition, it was first necessary to examine the ratio of GO and HP to improve the detection efficiency. As shown in Figure 2, the fluorescence decreased obviously with GO increase, and remained stable until its concentration reached 15 μg/mL, which suggested the fluorescence of 50 nM HP could be effectively quenched by 15 μg/mL GO via fluorescence resonance energy transfer (FRET) [29]. Thus, 15 μg/mL was chosen as the optimal concentration for next experiments. As for the feasibility assay, Figure 3 showed that the fluorescence of aptamer spiked with GO and SGI was weak (curve b)

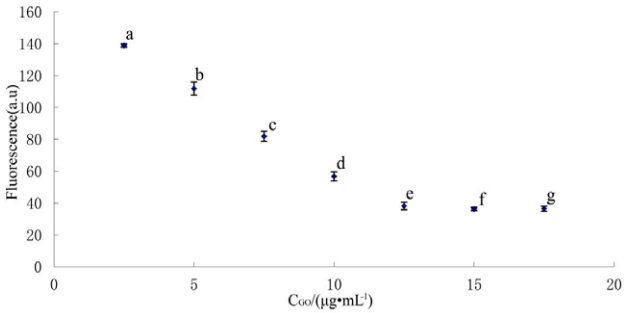


Figure 2: Optimization of concentration ratio of graphene oxide toaptamer (50 nM) with SGI. (a to g: 2.5,5,7.5,10,12.5,15,17.5 μg/mL) Excitation: 496 nm. Error bars indicate standard deviation (n=3).

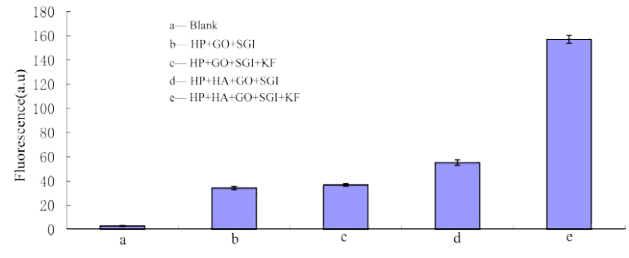


Figure 3: The feasibility of KF polymerase-aided amplification assay. The fluorescence spectras of the proposed method were under different conditions. (a) Blank: buffer; (b) 50 nM HP + GO + SGI; (c) 50 nM HP + GO + SGI + KF; (d) 50 nM HP + HA + GO + SGI; (e) 50 nM HP + HA + GO + SGI + KF. The concentrations of HA protein and GO were respectively 5 μg/mL and 15 μg /mL.Excition: 496 nm, and emission: 520 nm. Error bars indicate standard deviation (n=3).

and nearly the same as the background signals. In the absence of HA, the polymerase elongation reaction would not be triggered, resulting in weak fluorescence signals even after KF polymerase and dNTPs were added (curve c). In the presence of target, fluorescence enhanced slightly (curve d) as the reaction was initiated, and an obvious fluorescence peak appeared upon mixing KF polymerase and dNTPs subsequently (curve e). Therefore, the result revealed clearly that the KF polymerase-aided reaction would increase greatly the fluorescence intensity, which displayed that the assay is feasible for detection HA.

Analysis of sensitivity and selectivity of the assay

As for the sensitivity assay, Figure 4A displayed that the fluorescence intensity considerably increased with HA increase ranging from 0 μg/mL to 10 μg/mL. The fluorescence intensity showed a linear correlation in 2.5 μg/mL to 7.5 μg/mL (inset of Figure 4A), with a correlation coefficient (R^2=0.9978), where Y and X are the changes of fluorescence intensity and target concentration, respectively. More importantly, the detection limit was 2.5 μg/mL based on theoretical calculation (3σ rule). Additionally, BSA and lysozyme were employed to evaluate the selectivity of this approach. As shown in Figure 4B, the fluorescence signals did not obviously increase after the addition of them, and had substantial enhancement when HA was added. Due to the inherent specificity of the aptamer toward its target, the fluorescence increased remarkably after adding only HA. The result indicated that this method could be applied to detect HA with high specificity. Meanwhile, influenza viruses were also assessed to test the applicability of this method for real sample detection. It was detected sample of the influenza virus with lower detection limit of 1×10² TCID50 (Figure 5A). Thus, the method was a highly sensitive way for detecting H1N1.

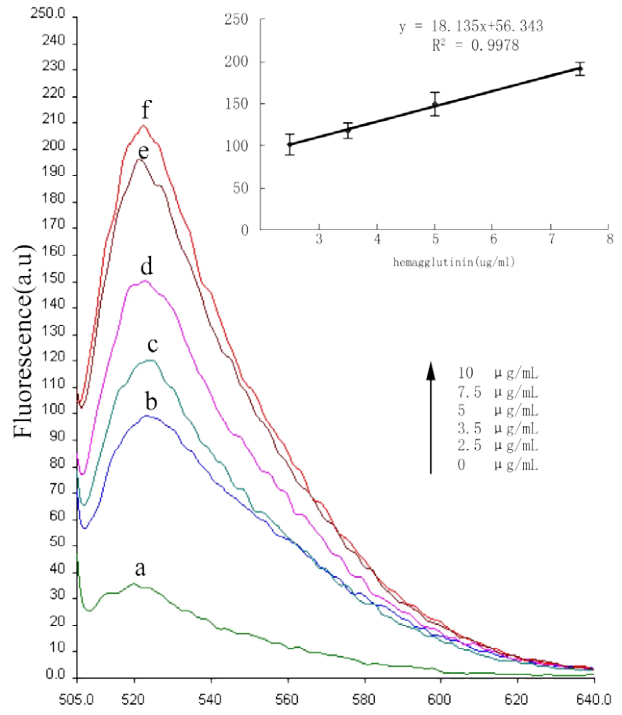


Figure 4a: Fluorescence spectral responses of KF polymerase-aidedamplification assay for HA protein of varying concentrations (a to f: 0, 2.5, 3.5, 5, 7.5, 10 μg/mL). Insert: The linear relationship of the fluorescence intensity on HA protein concentrations from 2.5 μg/mL to 7.5 μg/mL. The error bars embody the standard deviations of repeated results.

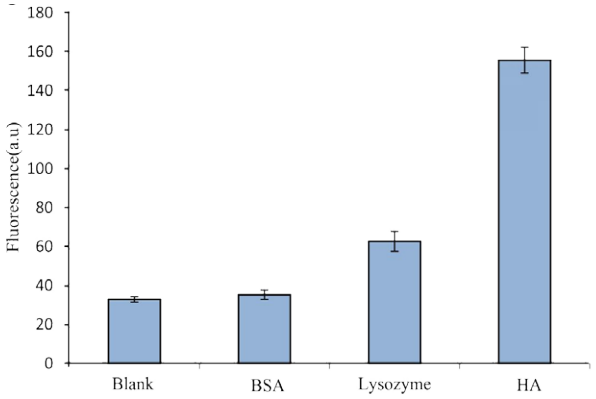


Figure 4b: The specificity of this assay with different targets. (a) Blank,(b) BSA, (c) Lysozyme, (d) A. (all at a concentration of 5 μg/mL) Each data indicates the average of at least three experiments.

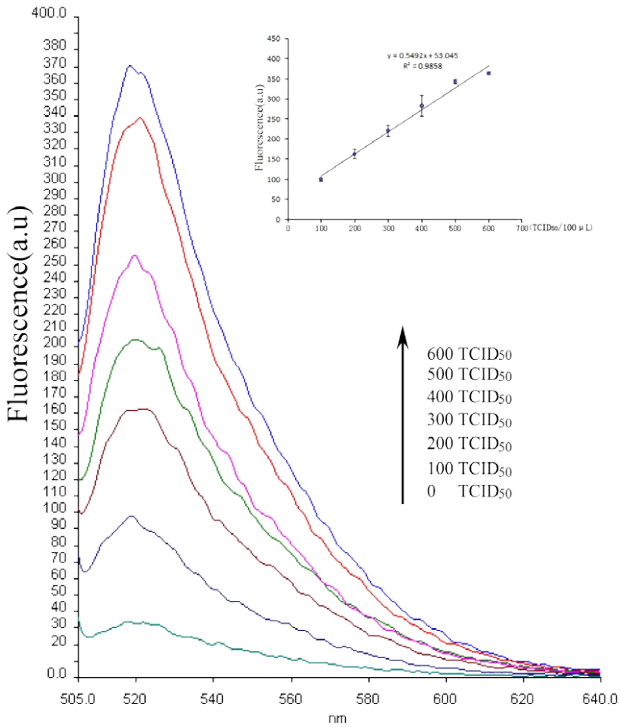


Figure 5a: The detection of the sample virus with different titers.Fluorescence intensity detection. The virus titers are varied from 0 to 6×10² TCID50 (100 μL) from the bottom up. The inserted figure shows that the increases in fluorescence intensity and absorbance value are proportional to the concentration of target virus. Thecorrelation equation is Y=0.5492X+53.045 while with a regression coefficient of 0.9858.

As for selectivity, it was found that fluorescent intensity of the target virus (H1N1) is greater than that of non-target ones (Figure 5B). It was further confirmed that our method was highly selective and specific not only for HA protein but also for the whole influenza virus.

Conclusion

In summary, a ssDNA aptamer against HA protein of the subtype H1N1(A/Puerto Rico/8/1934) was successfully obtained by employing

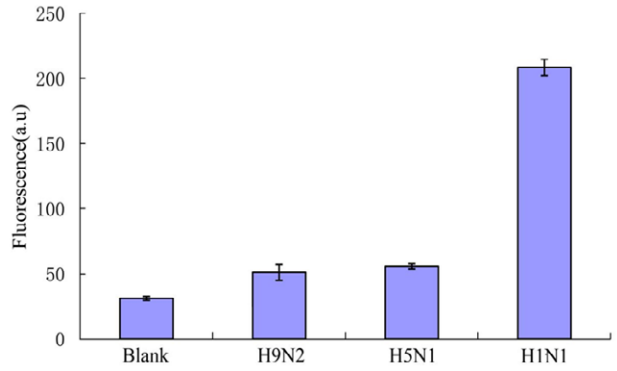


Figure 5b: The detection specificity of the proposed KF polymerase-based signal amplification for virus assay. The titer of H1N1 was 3×10^2 TCID50 (100 μL) compared with other virus, H9N2 and H5N1. Each titer was measured three times and averaged.

asymmertric PCR-based SELEX. Based on this, the assay using KF polymerase-aided signal amplification and GO-based fluorescence quenching showed sensitivity and good selectivity with a detection limit of 2.5 μg/mL for HA protein and had been successfully applied in real samples with a low limit of 10^2 TCID50. As a highly sensitive quenching platform GO has been widely applied in many biomolecule detections. The detection method constructed by Chen was based on the quenching action of GO, and its aptamer need to be labeled with carboxy fluorescein (FAM) [19].

Sheng also used GO for detection without enzyme [23]. Comparatively, two advantages are displayed in our work. Firstly, the labeled aptamer is not required. Secondly, a large amount of dsDNA were generated and the signals were significantly amplified when the strand displacement reaction would be triggered by KF polymerase. Based on the above characters, the aptasensor can effectively detect H1N1 with a lower limit of 1×10^2 TCID50 in 80 min, which is shorter than 2.5 h [30]. Moreover, the assay is easy to operate and simpler than the commercial real-time NASBA assay [31]. More important, it is easily applied in developing countries and other remote areas. Our further study will be focused on that the selected aptamer might be coupled with DNA nanotechnology to achieve more versatile functions as the strategy and to be widely applied in biomedicine [32,33].

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