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Development and evaluation of a polydiacetylene based biosensor for the detection of H5 influenza virus

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A B S T R A C T

H5N1 avian influenza has caused serious economic losses as well as posed significant threats to public health, agriculture and wildlife. It is important to develop a rapid, sensitive and specific detection platform suitable for disease surveillance and control. In this study, a highly sensitive, specific and rapid biosensor based on polydiacetylene was developed for detecting H5 influenza virus. The polydiacetylene based biosensor was produced from an optimized ratio of 10,12-pentacosadiynoic acid and 1,2-dimyristoyl-sn-glycero-3-phosphocholine, with the anti-H5 influenza antibody embedded onto the vesicle surface. The optimized polydiacetylene vesicle could detect H5 influenza virus sensitively with a detection limit of 0.53 copies/μL, showing a dramatic blue-to-red color change that can be observed directly by the naked eye and recorded by a UV-vis spectrometer. The sensitivity, specificity and accuracy of the biosensor were also evaluated. The sensor could specifically differentiate H5 influenza virus from H3 influenza virus, Newcastle disease virus and porcine reproductive and respiratory syndrome virus. Detection using tracheal swabs was in accord with virus isolation results, and comparable to the RT-PCR method. These results offer the possibility and potential of simple polydiacetylene based bio-analytical method for influenza surveillance.

1. Introduction

Avian influenza has emerged as a global concern because of serious economic losses and significant impact on agriculture, wildlife and public health in the past decade. Wild birds, particular the ducks, geese, swans, gulls, and shorebirds, are considered as potential reservoirs and carriers for all the 16 hemagglutinin and 9 neuraminidase subtypes of influenza A viruses (Fouchier et al., 2005; Webster et al., 1992). Since the first detection among poultry in China in 1996, the highly pathogenic H5N1 influenza virus has caused an epidemic in poultry (Zhou et al., 2006), and it continues to cross species barriers to infect humans and other mammals, often with a fatal outcome (Peiris et al., 2007). H5N1 virus detection in wildlife and poultry is critical for disease prevention and control programs (Prabakaran et al., 2009).

The H5N1 influenza virus was first isolated from geese in 1996 in China (Xu et al., 1999). In the next year, it infected 18 people in Hong Kong, six of whom died of the infection (Claas, 1998; Subbarao et al., 1998). The recurrence of highly pathogenic avian influenza virus A H5N1 was firstly reported in mid-December 2003 and continued throughout the year of 2005 (Chen et al., 2007). Since the first outbreak of H5N1 in birds, it has spread to wild birds and poultry in several continents and has resulted in 562 confirmed human cases and 329 deaths up to 22 June, 2011 (Kongchanagul et al., 2008; Li et al., 2004; Wolrd Health Organization, 2011). It has not only endangered the poultry industry and wildlife population, but also posed a continuing global human public health risk. Although transmission to humans is still limited, the continuous close contact between man, especially children, and birds in the country raises many concerns for the possibility of human adaptation with its consequent pandemic threats (Ibrahim et al., 2011). Thus, the monitoring and surveillance of H5N1 influenza virus is

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still an important task in protecting agriculture, human health, and natural resources.

Numerous methods for pathogen detection have been developed. There are, however, limited pathogen detection techniques that are as rapid and suitable for field investigations. Specifically, conventional methods take a relatively long time from hours to several days to provide results (He et al., 2010; Ho et al., 2009; Lazcka et al., 2007). Their application often involves complex mechanisms and operation that requires specialized instrumentation and trained personnel, therefore they cannot display their effects in settings other than laboratory environments (Chen et al., 2007). A simple, rapid, robust and reliable test, suitable for field study, is needed urgently (World Health Organization, 2006).

Polydiacetylene (PDA) is a conjugated polymer, formed through 1,4-addition polymerization of diacetylenes in the self-assembly process under UV irradiation, and has switchable chromism and fluorescence (Pindzola et al., 2006; Reppy and Pindzola, 2007). This colorimetric detection system using PDA has been used widely in biosensors, as it has several merits: a simple and rapid detection system, easy recognition through color change, and label-free detection (Kolusheva et al., 2001; Lee et al., 2007b). PDA-based assemblies, regardless of being in the form of vesicles or layered films, exhibit rapid blue to red colorimetric transitions in response to a wide range of stimulin, such as temperature, pH, mechanical perturbations, and solvents (Reppy and Pindzola, 2007). This allows color discrimination not only by eye, but also by a spectrophotometer for qualitative and quantitative analyses. Several attempts were carried out for detecting parasites, bacteria, and some proteins using PDA vesicles embedded with various bioreceptors (Deng et al., 2009; Ma et al., 2003; Orynbayeva et al., 2007; Park et al., 2009; Park et al., 2008; Su et al., 2004; Xia et al., 2010).

In this study, PDA vesicles were produced using 10,12-pentacosadiynoic acid (PCDA) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Fig. 1). Monoclonal antibodies against the HA of H5 influenza virus (H5-mAb) were covalently linked to the PDA surfaces. The PDA vesicles were employed to detecting H5 influenza virus and the immunoassay signals were visible to the naked eye. The foundation of PDA vesicles was optimized and sensitivity, specificity, accuracy and repeatability in the laboratory and detection the field samples were evaluated. The results demonstrated the successful formation of a sensitive PDA vesicle-based biosensor for H5 influenza virus detection. This is the first trial of PDA vesicles conjugated with influenza monoclonal antibodies applied in influenza virus detection, and it is worth noticed that this PDA based biosensor worked well in the field, making it a promising rapid detection platform for the future.

2. Materials and methods

2.1. Materials

10,12-Pentacosadiynoic acid (PCDA) was purchased from Alfa Aesar (Alfa Aesar, USA). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-ethyl-3-(3- dimethylamino-propyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA) and HPLC-grade chloroform were purchased from Sigma–Aldrich (Sigma–Aldrich, USA).

2.2. Viruses

H5N1 influenza virus strain (A/environment/Qinghai/1/2008 (H5N1)), H3N2 influenza virus strain (A/swine/Guangxi/7/2005(H3N2)), Newcastle disease virus (NDV) LaSota strain, and porcine reproductive and respiratory syndrome virus (PRRSV) VR2332 strain, specific monoclonal antibody to influenza H5 hemagglutinin (H5-mAb) as well as tracheal swabs collected from wild birds in the field were stored in National Research Center for Wildlife Born Diseases, Institute of Zoology, Chinese Academy of Sciences. The viruses were heat-inactivated, purified following a previous method (Reimer et al., 1967) and stored at −80 °C for use. Finally, the Bradford method was used to determine the concentration of the purified virus (Walker, 1996).

2.3. Construction of polydiacetylene based biosensor (Fig. 1)

2.3.1. Preparation of polydiacetylene vesicles (Fig. 1, steps (a) and (b))

Chloroform solutions of PCDA and DMPC were prepared separately in amber vials and then mixed at certain molar ratios to give a total lipid concentration of 1.0 mM. After a 10 mL volume of the lipid solution in chloroform was rotoevaporated to dryness, 10 mL of phosphate-buffered saline (PBS) solution (10 mM, pH 7.4) was then added and the solution was sonicated in KQ-100B ultrasonicator (Kunshan Ultrasonic Instrument, China) for about 15 min at 80 °C, then probe sonicated for 5 min. Ultimately, a semitransparent vesicle solution was obtained. After sonication, the solution was cooled to room temperature and then stored overnight at 4 °C to induce crystallization of lipid membranes. Polymerization was then carried out under irradiation at 254 nm for 5 min to obtain a blue-colored PDA solution.

2.3.2. Conjugation of mAb on the PDA vesicles (Fig. 1, steps (c)–(e))

NHS and EDC were dissolved separately in PBS buffer to a concentration of 100 mM. The free carboxylic acid groups of PDA were converted into the corresponding succinimidyl active esters by treatment with NHS and EDC at the molar ratio of 1:1:1. Activation of the carboxylic acids was achieved in the stirred vesicle solution for 20 min at room temperature. Then extra NHS and EDC were separated by dialysis in PBS at 4 °C overnight. The activated vesicles were then resuspended in H5-mAb solution at the final concentration of 100 ng/mL, 10 ng/mL and 1 ng/mL respectively in 2 mL PBS buffer to attach covalently the protein via a peptide bond to the activated linker overnight at 4 °C. The solution of BSA was added (150 μg/mL) to avoid the non-specific absorption. To remove extra materials from the main conjugates, dialysis was used overnight at 4 °C.

2.3.3. Performance of immunoreactions for the virus detection (Fig. 1, step (f))

The purified inactivated H5 influenza viruses were diluted to the final concentration of 100 ng/mL, 10 ng/mL and 1 ng/mL respectively with PBS buffer. Each concentration dilution was then added into polymerized antibody-conjugated PDA vesicles and incubated at 35 °C for 20 min. PBS buffer was added to the antibody-conjugated vesicles as the negative control. The color of the vesicle solution was observed with the naked eye, and the absorption spectrogram of the solution was recorded from 400 nm to 800 nm of the wavelength with a 1 mm optical path length on ND-2000 spectrophotometer (Thermo-Fisher, USA).

2.3.4. Colorimetric response

A quantitative value for the extent of blue-red color transition is given by the colorimetric response (CR), which is defined:

$$CR = \left[\frac{(PB0 - PBf)}{PB0}\right] \times 100\%$$

where $PB = A_{blue}/(A_{blue} + A_{red})$, A is the absorbance value at either the blue component (≈650 nm) in the UV–vis spectrum or the red component (≈540 nm), PB_0 is the initial percent blue of the vesicle–antibody conjugate solution before addition of antigen, and

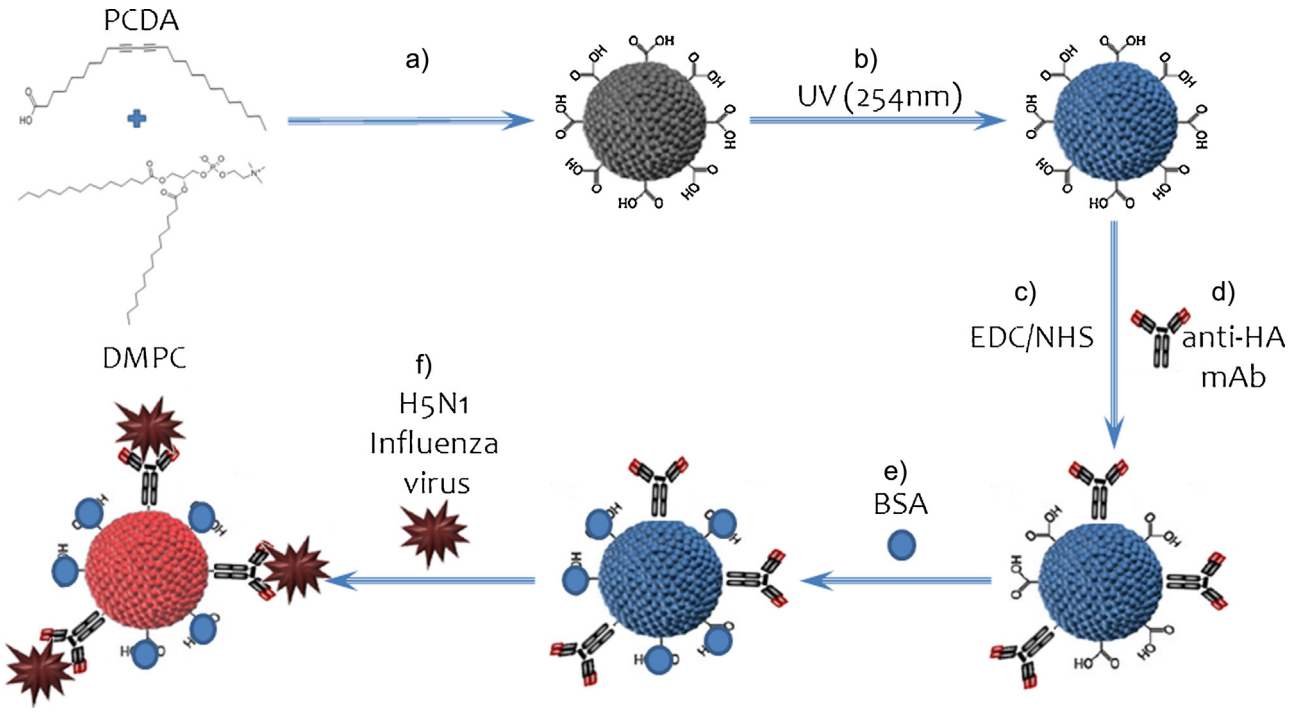

Fig. 1. Schematic diagram illustrating the fabrication steps of the PDA based biosensor for H5 influenza virus detection. (a) Preparation of PDA vesicle by PCDA and DMPC. (b) Polymerization under UV irradiation at 254 nm. (c) Activation by NHS/EDC. (d) Conjugation of anti-HA mAb onto the PDA vesicles. (e) Blocking by BSA to avoid non-specific asorption. (f) Detection of target H5N1 influenza virus.

PB_f is the final percent blue obtained for the vesicle–antibody conjugate solution after incubation with antigen (Deng et al., 2009; Su et al., 2004; Xia et al., 2010).

2.4. Processing of samples

A total of 93 tracheal swabs collected from wild birds in the field were used in this study. The sample process was followed as described previously (Dong et al., 2011; Wang et al., 2011). Tracheal swabs were eluted with PBS containing 2000 U/mL penicillin and 1000 μg/mL streptomycin and vortexed vigorously for 15–20 s and then squeezed to remove as much of the organic material as possible. The squeezed dry-cotton swabs were discarded in a bio-hazard waste bag for autoclaving, the liquid swab samples were kept at 4 °C for 12 h. Samples were centrifuged at 5000 × *g* for 10 min at 4 °C and supernatant was collected into sterile tubes. The processed samples were kept at 4 °C the test was to be done within 48 h, otherwise at −80 °C.

2.5. Virus Isolation

Conventional virus isolation was carried out as described previously (Dong et al., 2011; OIE, 2005). The processed samples were inoculated into the allantoic cavity of 9-day-old SPF embryonated chicken eggs. Inoculated embryos were incubated at 37 °C and allantoic fluid was harvested 48–72 h p.i. (Li et al., 2004; Li et al., 2003). Subtypes of influenza viruses were determined by hemagglutination-inhibition (HI) and neuraminidase inhibition (NI) tests as described previously (Alexander and Spackman, 1981; Dong et al., 2011).

2.6. Quantitative RT-PCR

Viral RNA was extracted from infected allantoic fluid with Trizol LS Reagent (Invitrogen, USA) and was reverse-transcribed into cDNA by using universal influenza primers with the M-MLV reverse transcriptase (Promega, USA) (Dong et al., 2011; Li et al., 2011; Liang et al., 2010; Zhou et al., 2008). Quantitative RT-PCR (qRT-PCR) was performed using primers designed based on published NP sequences and previously reported (Liang et al., 2010). The levels of PCR products were monitored with the Stratagene Mx3005 using SYBR Green PCR Master Mix (TaKaRa, Japan). The PCR conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 20 s. Cycling was terminated after 40 cycles with 95 °C for 1 min, 60 °C for 1 min, and 95 °C for 30 s. Dissociation curves of the products were generated by increasing the temperature of samples incrementally from 55 to 100 °C as the final step of the real-time PCR. Amplified products were run on a gel and extracted using a PCR and Gel Purification kit (TianGen, China). For the purpose of assay validation, purified products were cloned into pMD18-T with a TA Cloning Kit (TaKaRa, Japan) and sequenced to verify proper target amplification using M13 forward and reverse primers.

2.7. Statistical analysis

The means and standard deviations were calculated based on three separate replicates. Statistical analysis including simple linear regression model by method of least square, *t*-test and ANOVA method was performed by Excel Software and R Software (version 2.13.1). Difference was considered to be significant when $P < 0.05$.

3. Results

3.1. Optimization of vesicle formation

In this study, the PDA vesicles were made using different molar ratios of PCDA (10:0, 8:2, 6:4, 4:6 and 2:8). The carboxyl group fraction of the PCDA is an important factor for the conjugation of antibodies because it can react chemically with amine groups of the antibodies. The major role of DMPC is to increase the fluidity of the membrane of the vesicle. A higher fluidity of the vesicle

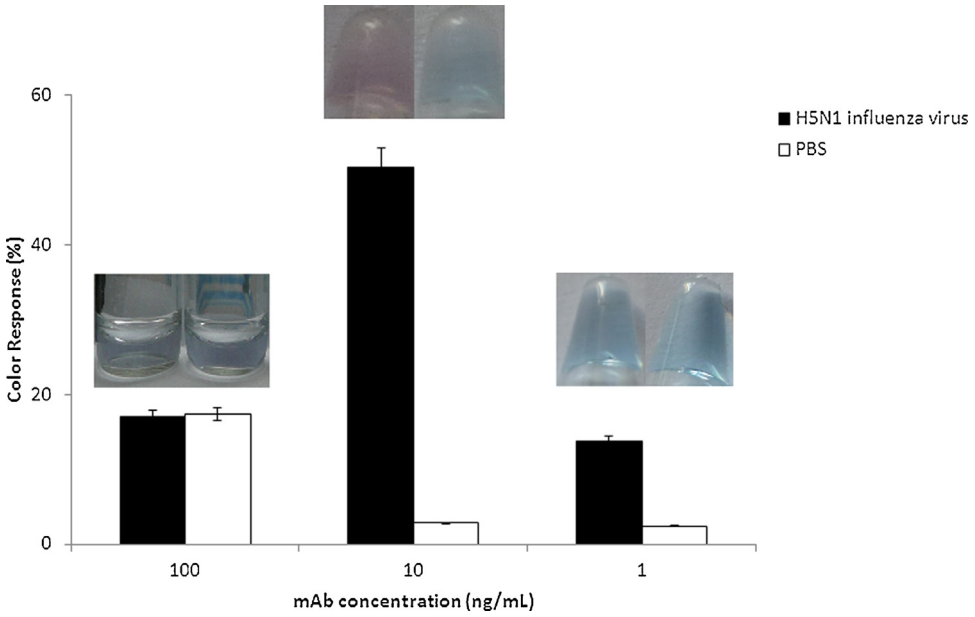

Fig. 2. Diagram representing the CR values derived from different amount of conjugated antibodies (100 ng/mL, 10 ng/mL, and 1 ng/mL). Color changes were induced by adding influenza virus of 13.5 copies/μL (black column) or PBS (white column) and incubated at 35 °C for 20 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

membrane implies that the vesicle can react more easily to stimuli. The ratio was optimized by controlling the PCDA and DMPC molar ratio during the formation of vesicles considering both fluidity and conjugation efficiency of the antibodies onto the PDA vesicles.

The color response (CR) value of the vesicles incubated at 80 °C for 10 min was introduced to measure the sensitivity of the vesicles under the stimuli. The CR value was the highest of the vesicles with a molar ratio of 6:4 (PCDA:DMPC) (data not shown). With the decrease of PCDA molar composition in the vesicle from 100% to 60%, the sensitivity of the vesicles increased due to the insertion of DMPC in the vesicle membrane. However, when the molar ratio of PCDA to DMPC decreased to 4:6 and 2:8, the sensitivity of the vesicles maintained at the same level as the molar ratio of 6:4. Therefore, the optimum ratio of PCDA and DMPC was determined as 6:4 for stable formation of the PDA vesicle.

3.2. Optimization of antibody conjugation

In order to improve the detection sensitivity, the mAb concentration had to be optimized on the PDA vesicle surface because the appropriate distance between receptors improves the accessibility of antibodies and minimizes the effect of steric hindrance (Choi et al., 2004; Kim et al., 2009a,b,c; Lee et al., 2005; Zhang et al., 2006). PDA vesicles have to maintain a proper surface flexibility (Lee et al., 2007a,b). In this study, the concentration of H5-mAb was optimised because the flexibility of the PDA vesicle would decrease with increased density of receptors. The PDA vesicle was modified with NHS and EDC, with the carboxyl group being replaced by an amine-reactive NHS-ester moiety (Kwon et al., 2010). Then various concentrations of H5-mAb (100, 10, and 1 ng/mL) were conjugated to the NHS-activated PDA vesicle solutions to optimize the concentration of H5-mAb. To induce colorimetric changes by immunoreaction, H5 influenza virus (13.5 copies/μL) was added and the vesicles were incubated at 35 °C for 20 min.

The vesicle showed the strongest color response when the antibody concentration was 10 ng/mL. The sensitivity of the vesicle reduced in the concentration of 1 ng/mL because the density of antibodies conjugated on the vesicles surface was too low (Fig. 2). In the case of 100 ng/mL antibody concentration, the sensitivity also decreased, caused by the decreased flexibility of vesicle surface as well as the increased steric hinderance due to the high density of receptors. From these results, we determined that the optimal concentration of the H5-mAb on the vesicle was 10 ng/mL, as the colorimetric changes indicated the highest and most efficient responses at the H5-mAb concentration of 10 ng/mL.

3.3. Time influence on CR value

The response time of this PDA based biosensor was also determined by recording the CR value at different time. A total of 5 time points, including 5 min, 10 min, 15 min, 20 min, and 40 min after samples added, were recorded. The result demonstrated that the bio-recognition events occurred within 20 min (Fig. 3). It also indicated that samples without H5 influenza viruses or anti-H5 antibodies did not induce color changes in the PDA based biosensor within 20 min. Thus, the detection time was determined as 20 min considering the sensitivity and specificity at the same time.

3.4. Sensitivity and specificity of H5 influenza virus using the optimized PDA vesicles

The performance of the PDA based biosensor manufactured under the above optimum conditions was investigated using H5 influenza virus. H5 influenza virus (13.5 copies/μL) and PBS (pH 7.4) were added into the PDA vesicles with and without H5-mAb. The vesicles changed from blue to pink in the case of H5 influenza virus (13.5 copies/μL) being added into the H5-mAb conjugated PDA (Fig. 4). The color of the vesicles remained blue in other cases. The result showed that the PDA based biosensor can recognize successfully the H5 influenza virus. The detection limit of the biosensor was determined by testing for the lowest detectable H5 influenza virus concentration (expressed as copies/μL). The H5 influenza virus was diluted into different concentrations (13.5, 5.4, 2.7, 1.35, 0.54, 5.4×10^{-2}, 5.4×10^{-3}, and 5.4×10^{-4} copies/μL) in PBS buffer (pH 7.4) and added into the biosensor. The color response was monitored and recorded after the mixture incubated at 35 °C for 20 min. All experiments at each concentration point were separately repeated three times. Among them, one of the best resolution

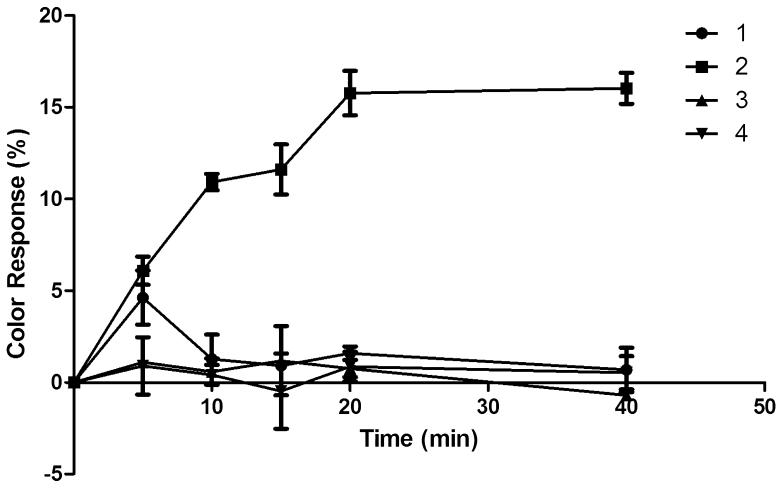

Fig. 3. Diagram representing the CR values derived from (1) adding PBS into PDA vesicles with anti-H5 mAb, (2) adding H5 influenza virus into PDA vesicles with anti-H5 mAb, (3) a adding H3 influenza virus into PDA vesicles with anti-H5 mAb, and (4) adding H5 influenza virus into PDA vesicles with anti-H9 mAb. The vesicles were incubated at 35 °C, and recorded the spectrum for 5 min, 10 min, 15 min, 20 min, and 40 min, respectively.

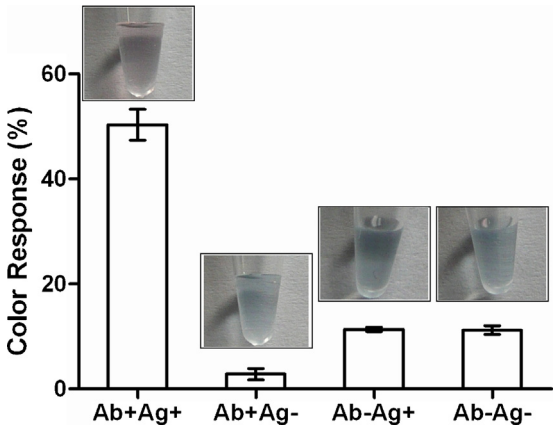

Fig. 4. Diagram representing the CR values derived from (1) adding H5 influenza virus (13.5 copies/μL) into PDA vesicles, (2) adding PBS into anti-HA mAb conjugated PDA vesicles, (3) adding H5 influenza virus (13.5 copies/μL) into PDA vesicles without anti-HA mAb, and (4) adding PBS into PDA vesicles without anti-HA mAb. The vesicles were incubated at 35 °C for 20 min.

images at each point was selected and inserted in figures. The result showed that the biosensor was able to detect H5 influenza virus down to 1.35 copies/μL with CR value >34 (Fig. 5).

A linear regression equation $y = 22.34x + 32.29$ ($R^2 = 0.996$) was calculated to analyze the relationship between the color responses of the PDA based biosensor and the concentration of H5 influenza virus using the simple linear regression model (Fig. 6). The t tests on the regression coefficients ($p = 43.10 < 2e{-}16$, $p < 0.0001$), the intercept ($p = 2.01e{-}15$, $p < 0.0001$) and analysis of variance (ANOVA) ($p = 2.006e{-}15$, $p < 0.01$) were all significant. According to the correlation curve, the linear relationship between color response and the log of virus concentration spans 4 orders of magnitude, which made the quantitative analysis for measuring the concentration of H5 influenza virus possible. Combining with the result of detect limit above, the biosensor successfully recognized the H5 influenza virus at concentration of 1.35 copies/μL with CR value >34. Consequently, the CR value of 34 was accepted as the cutoof in these PDA vesicles. A control experiment with an antibody against H9 subtype influenza viruses was conducted. The result showed that vesicles with the anti-H9 mAb did not respond to H5 influenza viruses (Fig. 3). The specificity of the biosensor was

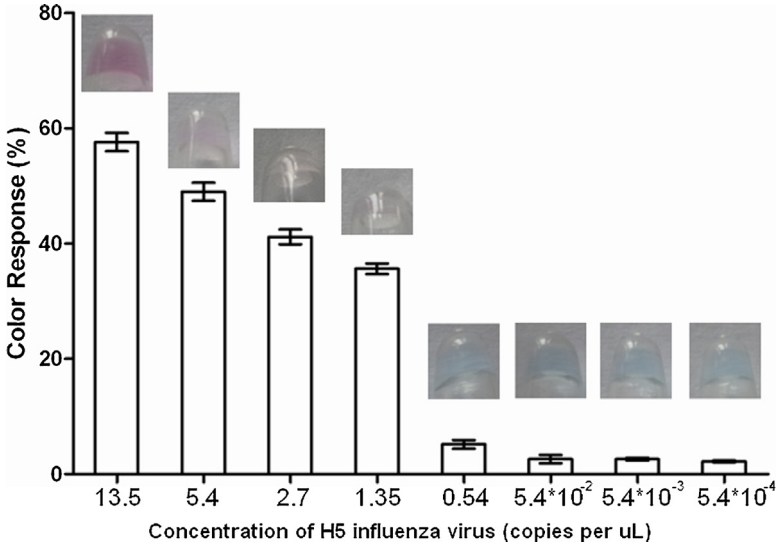

Fig. 5. Different color responses induced by different concentration of H5 influenza virus (13.5, 5.4, 2.7, 1.35, 0.54, 5.4×10^{-2}, 5.4×10^{-3}, and 5.4×10^{-4} copies/μL) after incubation at 35 °C for 20 min.

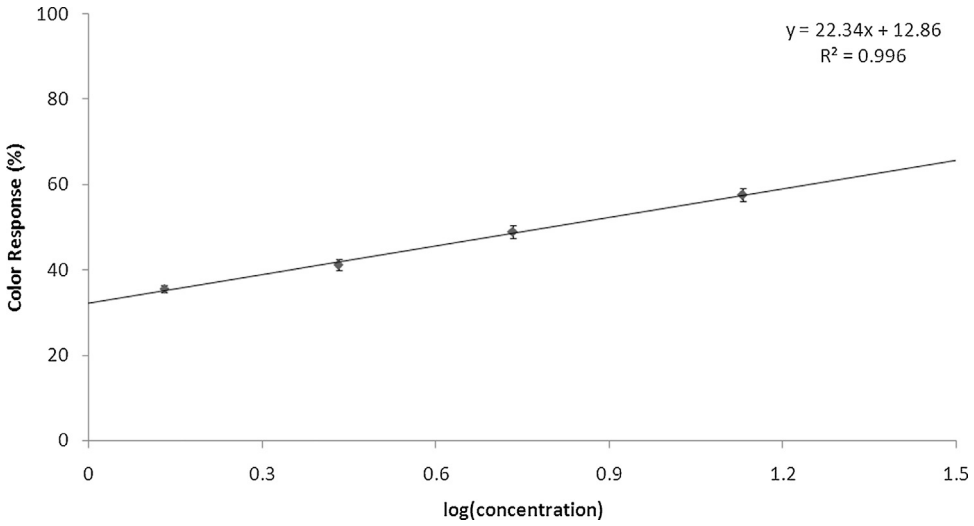

Fig. 6. The linear relationship between log(consentration$_{H5\ influenza\ virus}$) and CR values.

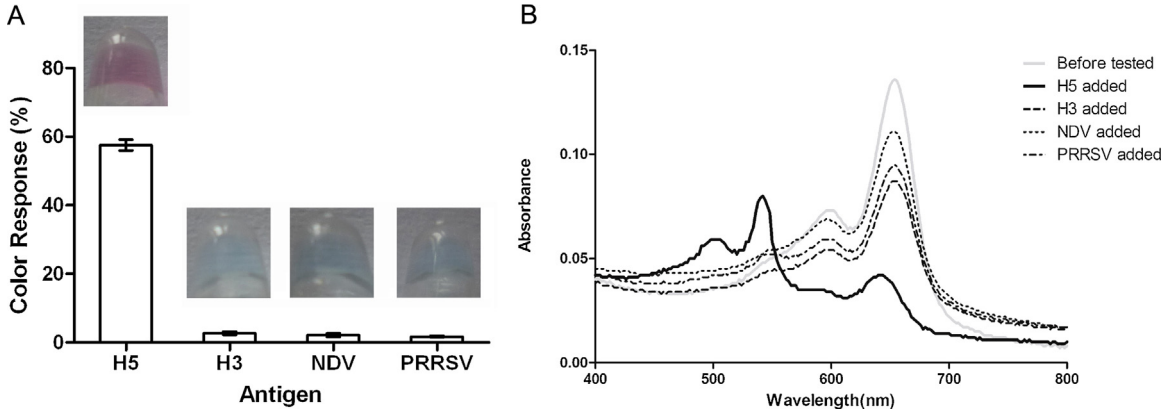

Fig. 7. Diagram representing the CR values (A) and UV–vis spectra (B) after incubation at 35 °C for 20 min with different pathogens (1) H5: H5N1 influenza virus A/environment/Qinghai/1/2008 (H5N1), (2) H3: H3N2 influenza virus strain (A/swine/Guangxi/7/2005 (H3N2)), (3) NDV: Newcastle disease virus LaSota strain, and (4) PRRSV: porcine reproductive and respiratory syndrome virus VR2332 strain.

determined by testing an H5 influenza virus strain (H5), an H3 influenza virus strain (H3), a Newcastle disease virus strain (NDV) and a porcine reproductive and respiratory syndrome virus strain (PRRSV) at the concentration of 13.5 copies/μL. The biosensor can recognize H5 influenza virus by observing a blue to red color transition. No colorimetric response occurred in the presence of H3 influenza virus, Newcastle disease virus and porcine reproductive and respiratory syndrome virus (Fig. 7). The results showed that the PDA based biosensor can recognize H5 influenza virus specifically.

3.5. Evaluation of the PDA based biosensor using tracheal samples

A total of 93 tracheal swabs were collected and used to evaluate the above PDA based biosensor, the results were compared with virus isolation methods in 9-day-old SPF embryonated chicken eggs and qRT-PCR (Liang et al., 2010; Office International des Epizooties in World Organisation for Animal Health, 2005). Each sample was tested on three separate experiments. The PDA system employed will show significant color responses against positive samples within 20 min. For samples without a color change, the vesicles were observed and measured for at least 40 min to ensure negative results. The results showed that 14 samples were identified as H5 positive by virus isolation and RT-PCR, and 16 samples showed positive results by PDA based biosensor, including 2 false positive results (Table 1). The results showed that the PDA based biosensor has potential as a fast screening method for surveillance of influenza virus.

Table 1
Detection results of tracheal swabs by traditional virus isolation, PDA-based biosensor and qRT-PCR method.

Detection result	No. of samples identified by		
	Traditional isolation method [a]	PDA	qRT-PCR
Positive	14	16	14
Negative	79	77	79

[a] Samples were determined to H5 influenza virus positive by virus isolation in 9-day-old SPF embryonated chicken eggs according to Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by OIE (Office International des Epizooties in World Organisation for Animal Health, 2005).

4. Discussion

Conventional detection methods are usually time consuming and demanding, and are not practical for field investigations (Ho et al., 2009). Virus isolation in embroynated chicken eggs or cells, followed by haemagglutinin (HA) and neuraminidase (NA) subtyping by serological testing require 1–2 weeks for results. Thus

they are less useful for making infection-control decisions (Office International des Epizooties in World Organisation for Animal Health, 2005). Several rapid tests for detection of H5N1 influenza virus have been developed and evaluated these years (Chen et al., 2008; Cui and Tong, 2008; Wang et al., 2011). The PDA based biosensor was rapid and convenient to use. It was concluded that it had the potential for field use in the investigation of H5N1 influenza outbreaks and surveillance in poultry. Further experiments and studies using this method should be done before its practical application in field investigations. The technique was more sensitive than a duplex real-time PCR assay reported by Zhang et al. (2012) which detected influenza virus at 10^2 copies/μL, and was at least as sensitive as most other existing techniques.

Natural or synthesized glycolipids and other molecules were employed to conjugated on PDA vesicles in previous studies for detection of influenza virus and other pathogens (Baek et al., 2000; Charych et al., 1993; Deng et al., 2009; Reichert et al., 1995). One major limitation to the PDA-based approach is the difficulty in synthesizing complicated probes, especially when the probes contain more than one saccharide group in a molecule as effective binding sites. However, the application of antibodies conjugated into the vesicles could overcome this limitation, and may provide a possible approach for biosensor design and construction to develop the multiplex method for pathogen detection.

In this study, the PDA vesicle was optimized by controlling the composition of PCDA and DMPC, considering both the fluidity and conjugation efficiency of the antibodies onto the vesicles. Several studies have investigated this issue but the results differed. The molar ratio of PCDA and DMPC was optimized to 8:2 in one study (Park et al., 2008), while in the current study, the most sensitive vesicle was achieved when the molar ratio of PCDA and DMPC was 6:4, which was in accord with previous studies (Su et al., 2004).

Besides the molar fraction of the component, the density of antibody could also affect the sensitivity of the vesicles. To some extent, the more antibodies conjugated on the vesicle, the more antigens will be captured by the antibodies. However, too many antibodies may have the effect of steric hindrance that may reduce the sensitivity instead (Kwon et al., 2010). An appropriate distance between receptors improves the accessibility of the target proteins and minimizes the effect of steric hindrance (Choi et al., 2004; Kim et al., 2009a,b,c; Lee et al., 2005; Zhang et al., 2006). The diameter of the vesicles is about 100 nm. It was reported that the apparent mean hydrodynamic diameter of antibodies, measured at an antibody concentration of 0.5 mg/mL, was 10.1 ± 0.6 nm (Sukumar et al., 2004). Thus a significant coverage of antibodies could be achieved, which will have an impact on the sensitivity of the assay according to previous studies. On the other hand, the amount of antibodies that a vesicle could hold is limited, and antibodies exceeded this limitation did not contribute to an improvement in sensitivity. The antibody concentration was optimized in this study. The results showed that the color response improved when the antibody concentration increased to 10 ng/mL. When the antibody concentration went up to 100 ng/mL, the CR value decreased. It could be explained by steric hindrance, which means that when too much antibody was conjugated on the vesicle, they mutually hindered the capture of antigen thereby reducing the color response.

The biosensor developed and evaluated in this study is highly specific and rapid for the field diagnosis of H5 influenza infections. However, the specificity and sensitivity of the assay must be assessed and improved continuously to adapt to the complex sample condition in the field. Because this assay does not require the use of sophisticated equipment or highly skilled personnel and can provide accurate results in a short time frame, we believe it would be useful for diagnosis and management of influenza outbreaks.

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