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Rapid detection of avian influenza A virus by immunochromatographic test using a novel fluorescent dye

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ABSTRACT

Sensitive and rapid diagnostic systems for avian influenza (AI) virus are required to screen large numbers of samples during a disease outbreak and to prevent the spread of infection. In this study, we employed a novel fluorescent dye for the rapid and sensitive recognition of AI virus. The styrylpyridine phosphor derivative was synthesized by adding allyl bromide as a stable linker and covalently immobilizing it on latex beads with antibodies generating the unique Red dye 53-based fluorescent probe. The performance of the innovative rapid fluorescent immnunochromatographic test (FICT) employing Red dye 53 in detecting the AI virus (A/H5N3) was 4-fold and 16-fold higher than that of Europium-based FICT and the rapid diagnostic test (RDT), respectively. In clinical studies, the presence of human nasopharyngeal specimens did not alter the performance of Red dye 53-linked FICT for the detection of H7N1 virus. Furthermore, in influenza A virus-infected human nasopharyngeal specimens, the sensitivity of the Red dye 53-based assay and RDT was 88.89% (8/9) and 55.56% (5/9) relative to rRT-PCR, respectively. The photostability of Red dye 53 was higher than that of fluorescein isothiocyanate (FITC), showing a stronger fluorescent signal persisting up to 8 min under UV. The Red dye 53 could therefore be a potential probe for rapid fluorescent diagnostic systems that can recognize AI virus in clinical specimens.

1. Introduction

The rise in the number of influenza outbreaks and outpatient visits for influenza-like-illness suggests an increased risk for global human public health (CDC, 2016b). Recently, incidents of human infection by avian and other zoonotic influenza viruses, such as avian influenza (AI) virus subtypes A/H5N1, A/H7N9, and A/H9N2, and swine influenza virus subtypes A/H1N1 and A/H3N2, have been reported (WHO, 2016).

The H7 subtypes of the AI virus are classified into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses based primarily on mortality rates following chicken pathogenicity testing (Belser et al., 2011). Since 2002, H7 subtype viruses have caused more than 100 cases of human infection in Europe and North America, resulting in both ocular and respiratory illnesses (Fouchier et al., 2004; Skowronski et al., 2006; Tweed et al., 2004).

World Health Organization (WHO) recommends rapid influenza testing only in cases of patients with lower respiratory tract illnesses, especially in children and adults with medical conditions that increase their risk for influenza complications (WHO, 2005). Therefore, highly sensitive, rapid, quantitative, low-cost, and reliable tests are urgently needed for diagnosing infectious diseases (Caliendo et al., 2013).

Currently, a major challenge is to develop a highly sensitive point-of-care test (POCT) for the detection of avian influenza virus (Fedorko et al., 2006). In this respect, considerable efforts have been directed toward developing fluorescent probes for immunochromatographic tests in field-deployable biosensors. Fluorescent dyes are increasingly being used for the identification, detection, quantification, and characterization of biological molecules (Mahmoudian et al., 2011). However, few studies targeted the development of fluorescent dye for

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immunochromatographic tests and most research has been limited to Europium (Juntunen et al., 2012) and quantum dots (Cheng et al., 2014; Di Nardo et al., 2016; Le et al., 2016).

The labeling of antibodies with fluorescent moieties is a key for biological application (Coons and Kaplan, 1950). The presence of multiple primary amines in the active site of an antibody can result in fluorophore conjugation but changes the characteristics of antigen binding and, in extreme cases, completely inactivates the antibody (Werthen and Nygren, 1988). Steric hindrance and the absence of additional reactive sites on the fluorophore are presumed to limit the degree of antibody modification by the conjugation reaction (Vira et al., 2010). Finally, there is a limit to the number of fluorescent molecules that can be attached to an antibody limiting their application as diagnostic agents, as higher photoluminescence is required for better performance. To improve the performance, fluorescent dyes have been examined in terms of photostability and brightness. However, it is largely unknown how changes in the molecular structure of a dye give rise to profound differences in its fluorescence properties (Zhu et al., 2002).

A fluorescent dye is a chemical compound that absorbs light and then remits it at a longer wavelength. There were two main considerations in the selection of the fluorescent dye: a sufficiently large difference between its absorption and emission wavelengths to minimize interference and the ability to easily introduce a linker to the dye which can react with lysine or arginine. We selected a Red dye with a pyridine-styrylpyridine structure, which satisfied both the conditions. Styrylpyridine is one of the most common fluorescence dyes (Ams et al., 2009) often used as a fluorophore in imaging (Campos et al., 2016). However, there are limitations due to its weak fluorescence. In this study, we sought to increase the sensitivity of styrylpyridine probes by connecting large numbers of fluorescent phosphor groups to the latex surface.

To evaluate the potential of pyridine-styrylpyridine Red dye 53 as a novel fluorescent probe, we synthesized and assessed the fluorescent dye-linked rapid diagnostic system in immunoassays.

2. Experiments

2.1. Reagents

Two different aliphatic amine latex beads (100 and 200 nm) were purchased from Life technology (Carlsbad, USA). Influenza A nucleoprotein (NP) protein was obtained from Novus Biologicals (Littleton, USA). Monoclonal antibodies (mAb), including anti-influenza A NP (7307 and 7304), were purchased from Medix Biochemica (Espoo, Finland). Europium beads (200 nm) were acquired from Bangs Laboratories Inc. (Fishers, USA). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS) were purchased from Thermo Scientific (Waltham, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Virus stock and titration

Avian influenza viruses were obtained from the Centers for Disease Control & Prevention. All viruses were inoculated in amniotic fluid of 10-day-old specific pathogen-free embryonated chicken eggs for 48 h at 37 °C. The virus titer (hemagglutination unit (HAU)/mL) was determined by hemagglutination assay (HA) as previously described (Yeo et al., 2014b).

2.3. Synthesis of Red dyes

General procedure of synthesis of Red dye and the conjugation of fluorescent compounds to latex beads (Red dye 47, 49, 53, and 58) was described in the Method section of Supplementary information.

2.4. Conjugation of fluorescent dyes with antibody

The latex-Red dyes were further conjugated to an anti-influenza monoclonal antibody via nucleophilic substitution reaction. A bond between allyl bromide and a primary amine was formed by mixing 20 μL of latex-Red fluorescence dye with 60 μL of antibody solution (1 mg/mL) in a final volume of 500 μL of 0.1 M sodium phosphate, pH 8.0, and allowing it to react for 24 h at 30 °C. The nonpolar moiety in the terminal part of the Red dye was deactivated by adding 188 μL of 20 mM glycine. The solution was incubated for 24 h at 30 °C, which resulted in the decoration of the surface of the conjugate. Subsequently, the conjugate was washed and separated from the free antibody by centrifugation at 27,237×*g* for three times. Finally, the resultant latex-Red dye-mAb was stored in 200 μL of 2 mM Borax buffer, pH 9.0 containing 1% bovine serum albumin (BSA) at 4 °C before use.

In the case of Europium, the covalent conjugation of the antibody to Europium beads was performed by a well-established procedure based on the EDC/NHS chemical reaction whereby NHS esters react with primary amines on the antibody to yield stable amide bonds (Fischer, 2010). Briefly, 10 μL Europium (200 nm, 1% w/t) was added to 500 μL 0.1 M Tris-HCl (pH 7.0) and incubated for 1 h at 25 °C in the presence of 0.13 mM EDC and 10 mM sulfo-NHS. The EDC and sulfo-NHS surplus was removed by centrifugation at 27,237×*g* for 5 min. The activated Europium was then mixed with 60 μL antibody (1 mg/mL) in 500 μL 0.1 M sodium phosphate (pH 8.0) and allowed to react for 2 h at 30 °C. After centrifugation at 27,237×*g* for 5 min, the Europium-conjugated antibody was collected, washed with 2 mM phosphate-buffered saline (PBS) (pH 8.0), resuspended in 100 μL storage buffer (1% BSA in PBS) and stored at 4 °C. For FICT assay, all conjugates were 2-fold diluted in storage buffer.

2.5. Measurement of the spectroscopic properties of Red dyes

As previously described, the absorption and emission spectra of the original fluorescent phosphor and the conjugate with antibody on the latex were measured by ultraviolet/visible spectrometer (Optizen Pop, Mecasys, Korea) and a spectral scanning multimode reader (Varioskan Flash, Thermo Electron Corporation, USA), respectively (Yeo et al., 2014b).

2.6. Preparation of test strips

Immunochromatographic strips were prepared as previously described (Yeo et al., 2016a, 2016b). The membrane was coated with 0.5 mg/mL of goat anti-mouse IgG on the control line (CL) and 2.5 mg/mL of anti-influenza A NP antibody on the test line (TL). After drying the membrane at 30 °C for 2 h, the diagnostic strip was used in the fluorescent immnunochromatographic test (FICT).

2.7. Rapid FICT

Each strip was tested by dropping 6 μL of fluorescent conjugates on conjugate pad and then, a mixture of 75 μL sample and 75 μL lysis buffer (100 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 0.2% SDS, 0.1% sodium azide, and 1% Triton 100) was added to the sample pad. After 15 min incubation, the fluorescence intensity was measured with a portable fluorescent strip reader (excitation at 355 nm and emission at 612 nm) (Medisensor, Daegu, South Korea) (Ham et al., 2015). Both TL and CL signals were measured and the TL/CL ratio was calculated automatically. To evaluate the performance of Red dye 53-linked FICT assay, tests were conducted with virus or antigen in distilled water (DW). A commercial influenza virus A/B rapid detection test (RDT) (Standard Diagnostics, Yongin, South Korea) was used for comparisons, following the manufacturer's instructions.

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