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ORIGINAL PAPER

Carbon nanotag based visual detection of influenza A virus by a lateral flow immunoassay

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Abstract The authors report on a rapid and direct visual test for the detection of influenza A virus using a carbon nanotag based lateral flow assay. Carbon nanoparticles in the form of nanostrings are acting as reporters. As carbon nanotags accumulate in the test zone due to formation of an antibody-antigen-carbon nanotag antibody complex, and this allows for the direct visualization of the analytical signal. Under optimal conditions, influenza A virus can be determined in allantoic fluid inoculated with the virus with a limit of detection of 350 $TCID_{50}.mL^{-1}$ (i.e., the 50% tissue culture infectious dose). No interference is found for several other tested proteins, and for closely related viruses. Cell lysates containing different seasonal strains of influenza A viruses (including the H1N1 and H3N2 strains) collected from clinical samples were analyzed. It is demonstrated that the method can detect both influenza A viruses without interference by biological matrices. In our perception, this method has a wide potential in that it may be extended to a generally applicable platform for rapid diagnosis influenza A viruses.

Keywords Lateral flow immunochromatographic assay · Carbon nanoparticles · Influenza virus · Antigen detection · Sandwich immunoassay · Direct visualization · Rapid testing

Introduction

Influenza virus is one of the major cause of contagious respiratory illness. Although influenza A virus can cause recurrent epidemics, there have also been occasional severe pandemics. These, for example, include the global outbreak of highly pathogenic avian influenza A(H5N1) virus, the outbreak of influenza A(H1N1)pdm09 virus in 2009 and the spread of influenza A(H7N9) virus in 2013 [1–3]. Serious outcomes of influenza infection and complications can be observed in certain group of patients, especially elderly and young children [4]. Therefore, rapid and accurate diagnosis is important to help planning of treatment regimen i.e. optimization of antiviral therapy, reduction of antibiotic use, and prevention of transmission [5].

Clinical diagnosis of influenza on the basis of clinical signs and symptoms alone is limited. This was due to the similarity of the illness caused by other respiratory pathogens. To help aid clinical judgment and diagnosis of influenza, a number of different techniques has been developed. These techniques include viral culture, molecular assays, and rapid antigen diagnostic tests [6, 7]. Although viral culture and molecular assays allow the information regarding the identification of the outbreak strain and subtypes to be made, they are time-consuming techniques and often require sophisticated and expensive equipment [8]. Unlike other approaches, result interpretation of rapid antigen diagnostic tests can be made within 15–20 min of specimen collection and does not require laboratory expertise.

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Rapid diagnostic tests provide simple and low-cost analysis, making them available as a screening tool in low-resource settings i.e. nursing homes, outpatient settings, and other remote healthcare settings. The majority of these tests are based upon the detection of target analyte on a given platform. These platforms; for example, include paper-based device, membrane-based assay, and lab-on-a-chip device [9, 10]. Such platforms provide advantages of simplicity, portability, inexpensiveness, and rapid analysis for analytical applications. Of these systems offering rapid testing analysis, the capillary-driven platform called "lateral flow immunochromatographic assay" [11]. Within the system, the target analyte is captured between two specific recognition molecules such as antibodies, forming a sandwich complex [12]. In most cases, the test employs two specific antibodies, one of which is conjugated to the reporter molecule, whilst the other is immobilized on the solid support [13]. As the complex flows along the test device, it is separated from sample matrix via capillary force on the porous membrane [14]. Once captured, the target detection can be accomplished via an optical signal readout from the labeled sandwich complex, providing a direct colorimetric visualization.

Of the reporter molecules, gold nanoparticle is commonly used. Previous systems using different sizes of gold nanoparticles to form the dual-layered gold nanoparticles have been reported [15, 16]. Different systems using either other reporter molecules or other signal enhancement techniques [17] such as fluorescent dyes [18, 19], near infrared dyes [20], magnetic nanoparticles [21], silver enhancement [22], surface-enhanced Raman scattering based [23], enzyme amplification [24], and double-antigen targeted strategies have also been reported [16]. However, these systems often require either additional instruments or steps for result interpretation [25]. Alternatively, the use of carbon nanomaterial as a reporter molecule has a great potential for its use in various applications. Due to its unique properties, for example, good chemical stability, biocompatibility, and high contrast, the use of carbon nanoparticles as reporter offers a good prospect for designing a detection platform for biomedical diagnostic application [26].

Herein, the development of carbon nanotag based lateral flow immunoassay for influenza virus detection was described. Carbon nanoparticles, in particular, carbon nanostrings were used as reporter molecules in lateral flow immunoassay system based upon the previous feasibility study on the use of carbon nanoparticles in rapid antigen detection test [27]. System optimization and evaluation to investigating the potential clinical applicability of the system were performed. The development of the system was described firstly, and followed by system design and optimization steps. Subsequently, to evaluate the performance of the system for influenza A virus detection in complex biological matrices, the system was applied to detect the virus in influenza A-infected samples such as the allantoic fluid and the cell lysate directly.

Experimental

Chemicals and apparatus

Bovine serum albumin (BSA) and polyvinyl pyrrolidone (PVP-10) were obtained from Sigma Aldrich (MO, USA, http://www.sigmaaldrich.com). Nonfat dry milk and Tween 20 were purchased from Bio-Rad (CA, USA, http://www.bio-rad.com). Lateral flow immunochromatographic test strip components including nitrocellulose membrane and absorption pad were obtained from GE Healthcare (Buckinghamshire, UK, http://www.gelifesciences.com). Glass fiber conjugate pad and sample application pad were supplied from Merck Millipore (MA, USA, http://www.merckmillipore.com). Carbon nanoparticles, in the form of nanostrings, were purchased from Maiia Diagnostics (Uppsala, Sweden, http://maiiadiagnostics.com). All other chemicals were of analytical grade, purchased from Sigma Aldrich. Ultrapure water (18.25 MΩ.cm) were obtained using Milli-Q system (Merck Millipore) and used throughout the experiments. Transmission electron microscopy (TEM) and scanning electron microscope (SEM) images were taken on a JEM-2010 (JEOL, Japan), and field-emission scanning electron microscope (FE-SEM, SU5000, Hitachi, Japan). Zetasizer (Malvern Instruments (UK)) was used to measure dynamic light scattering (DLS) of nanoparticles. Biodot dispenser (CA, USA) was used to generate the control line on the nitrocellulose membrane.

Source of antibodies and antigens

Mouse monoclonal anti-influenza A nucleoprotein antibody and secondary goat anti-mouse IgG antibody were supplied from Innova Biotechnology (Bangkok, Thailand, http://www.innovabiotechnology.com) and KPL (MD, USA, https://www.seracare.com), respectively. The allantoic fluid that was inoculated with influenza virus A/Thailand/104/2009 (H1N1) was prepared as described previously [19]. Madin-Darby canine kidney (MDCK) cell cultures infected with influenza A virus using two clinical human isolates (seasonal influenza A H1N1 14/2004 and seasonal influenza A H3N2 04/2004) were produced as described previously [28]. All viral manipulation was performed under appropriate biosafety level 2 plus conditions.

Preparation of carbon nanotag-antibody immunoprobes

Components and preparation of the carbon nanotag-antibody immunoprobes (Fig. 1a, b) can be described briefly in the following procedures: the stock suspension of carbon nanoparticles was homogenized in sonication bath before diluted to different folds with 5 mM borate buffer, pH 9. Monoclonal anti-influenza A nucleoprotein antibody was diluted to the concentration of 0.1 mg·mL^{-1} with 5 mM borate buffer, pH 9. The diluted antibody was added to the diluted suspension of carbon nanoparticles at a molar ratio of 1:10 antibody: carbon nanoparticle. After mixing at 25 °C for 15 min, 100 μL of a blocking solution was added to the mixture with continuous stirring at 25 °C for 20 min. The pellets were then collected by centrifugation at 10,000 g at 4 °C for 20 min. After resuspending the pellets in a resuspending buffer (15 μM BSA, 10 μM PVP-10 in 5 mM borate buffer, pH 9), the final immunoprobes were stored at 4 °C for further use. Characterizations of the particles including the change in size and surface charge were shown in the Electronic Supporting Materials (ESM), Fig. S1).

Fabrication of carbon nanotag based lateral flow assay

Carbon nanotag based lateral flow assay test system consisting of 4 components including sample application pad, conjugate pad, nitrocellulose membrane, and absorption pad was assembled on a backing card as illustrated in Fig. 1c. Within the nitrocellulose membrane, monoclonal anti-nucleoprotein antibody (1 mg·mL^{-1}) was spotted approximately 10 mm from the proximal end of the membrane to generate the test spot. Further, the control line was plotted as a horizontal line above the test spot approximately 5 mm using Biodot dispenser system. With a dispense rate of 1 μL.cm^{-1}, secondary goat anti-mouse IgG antibody (1 mg·mL^{-1}) was dispensed on the membrane to form the control line. For the conjugate pad preparation, a desired volume of the carbon nanotag-antibody immunoprobes was loaded on the conjugate pad and dried at

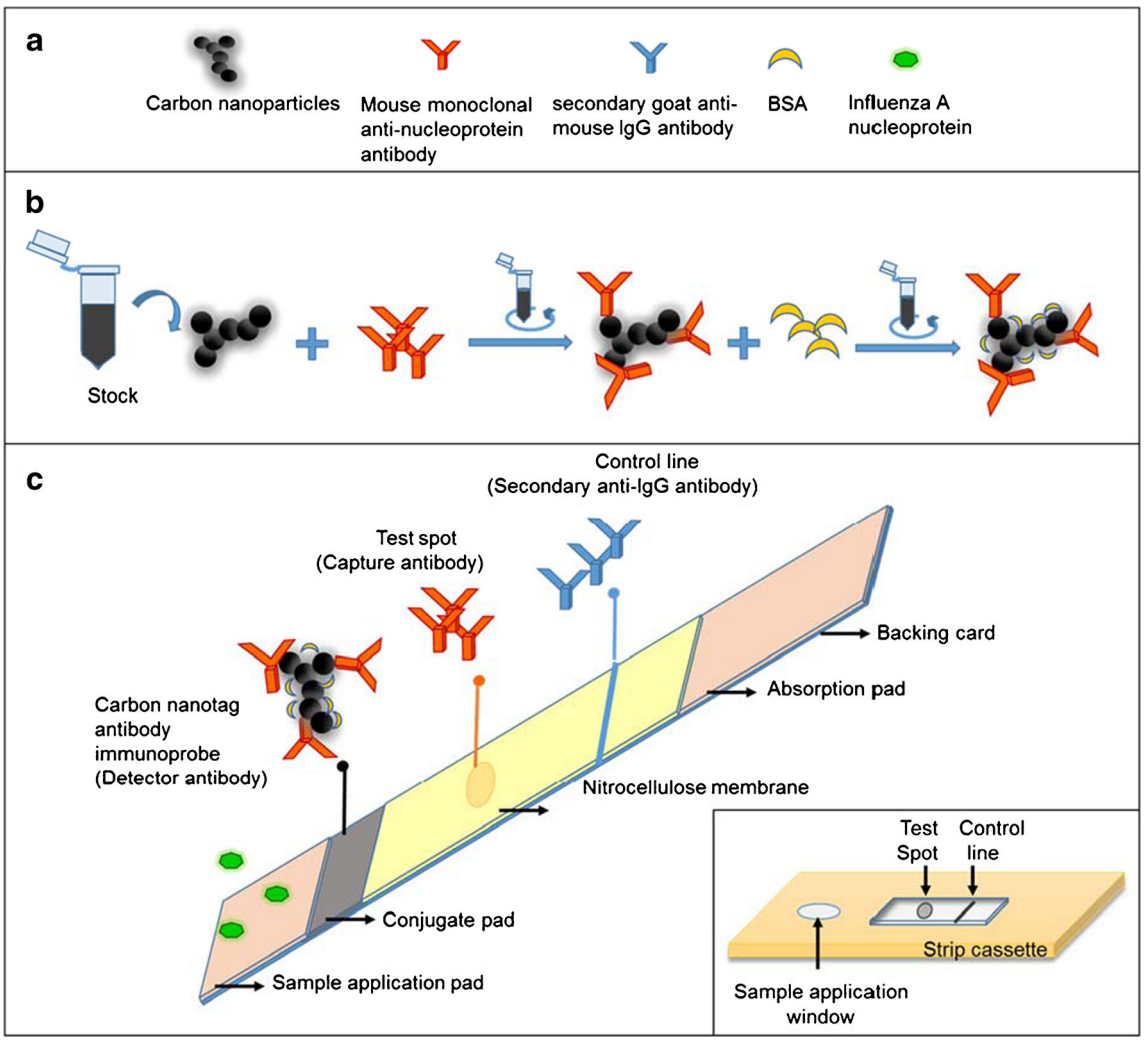

Fig. 1 Schematic illustration for **a** the components and **b** preparation of the carbon nanotag-antibody immunoprobes, and **c** the fabrication of carbon nanotag based lateral flow immunoassay

25 °C. After all the components were assembled, the mounted test strip was inserted in a strip cassette.

Signal detection and performance of the system

The analytical procedure of the test system was performed by loading a sample liquid (100 μL) onto a sample application window of the strip cassette. After the reaction was allowed to process for 15 min, the signal at the test spot and the control line were visualized and recorded. The intensity value of the test spot was obtained using Image J software (http://imagej.nih.gov/ij/). The normalized signal intensity of the sample was obtained by subtracted the signal intensity obtained from the negative control without the target from that obtained from the sample. In all experiments, the averaged data and standard deviation were calculated from three individual experiments. In case of negative and positive control, a sample running buffer (5 mM borate buffer, pH 9) and influenza A-infected allantoic fluid was used as negative and positive controls, respectively. For specificity evaluation, other molecules including human serum albumin, BSA, lysozyme, dopamine, and glucose, tryptone the concentration of 5 $\mu g \cdot mL^{-1}$, and other closely related viruses including influenza B and canine distemper virus were used for system evaluation. After applying the volume of 100 μL of the either protein or virus sample, the test was processed in the same manner as described previously.

Detection of the influenza A virus in biological matrices

To evaluate the performance of the test system in detecting the influenza A virus in biological matrices, the allantoic fluid inoculated with influenza virus A/Thailand/104/2009 (H1N1) and MDCK cell cultures infected with either seasonal influenza A H1N1 14/2004 strain or seasonal influenza A H3N2 04/2004 strain were used as a sample. Prior to the sample application, the sample was mixed with a sample running buffer in a ratio of 1:1. Subsequently, a volume of 100 μL of the mixed sample was applied onto a sample application window of the strip cassette and allowed to process as described previously.

Results and discussions

Principle of the carbon nanotag based lateral flow assay

Schematic diagram in Fig. 2 illustrated the principle of the system, which is relied upon a specific recognition between the antigen and the antibody molecules. Carbon nanoparticles, in the form of nanostrings, was used as reporter molecules. When the sample containing the target antigen was used as a sample, the antigen was captured by carbon nanotag antibody immunoprobes loaded on the conjugate pad of the test strip. As the sample migrated through a porous membrane by capillary action, the antigen-carbon nanotag antibody complex was separated gradually from sample matrices by the mean of chromatography and continued to move along the test strip. Once the complex reached the nitrocellulose membrane, it was bound with the capture antibody immobilized on the membrane, forming a sandwich antibody-antigen-carbon nanotag antibody complex (Fig. 2a). The accumulation of the carbon nanotag sandwich immunocomplex in the fiber pores of the membrane at the test spot area resulted in the color change in signal detection from no color to black color (SEM image, Fig. 2a). The excess immunoprobes continued to move along the test and was bound to secondary anti-mouse IgG antibody at the control line. Fig. 2b shows the diagram and image when there was no target antigen present in the sample.

System optimization

In order to obtain the optimal condition of the system, the major factors that contribute directly to the performance of the system were systematically investigated. The following parameters were optimized: (a) ratio of the amount of the particles to the amount of the antibody for the immunoprobe preparation; (b) the amount of the immunoprobe loaded in the system; and (c) the amount of the antibody immobilized in the test area. Respective data and Figures are given in the ESM (Fig. S2-S4). We found the following experimental conditions to give best results: (a) the carbon nanotag immunoprobe at the ratio of 10; (b) the volume of 2 μL of the immunoprobes; and (c) the concentration of 1 $mg.mL^{-1}$ of the antibody immobilized at the test area. These optimized conditions were therefore adopted in all further experiments.

Performance of the carbon nanotag based lateral flow assay

To investigate the ability of the optimized system for the detection of influenza A virus, the allantoic fluid inoculated with influenza virus A/Thailand/104/2009 (H1N1) was used as a sample. Under optimized experimental conditions, the performance of the system was evaluated with different concentrations of the virus ranged from 0 to 2.8×10^3 $TCID_{50}.mL^{-1}$ (50% tissue culture infectious dose). As depicted in Fig. 3a, as the concentration of the target virus antigen was increased, the detection signal strength was shown to be higher. This suggests that the detection signal was increased gradually proportional to the number of the target influenza A virus present in the sample. The higher signal strength can be attributed to the increased number in the clusters of carbon nanotag labeled sandwich immunocomplex on the test spot area. With a direct signal visualization, a black spot detection signal can be

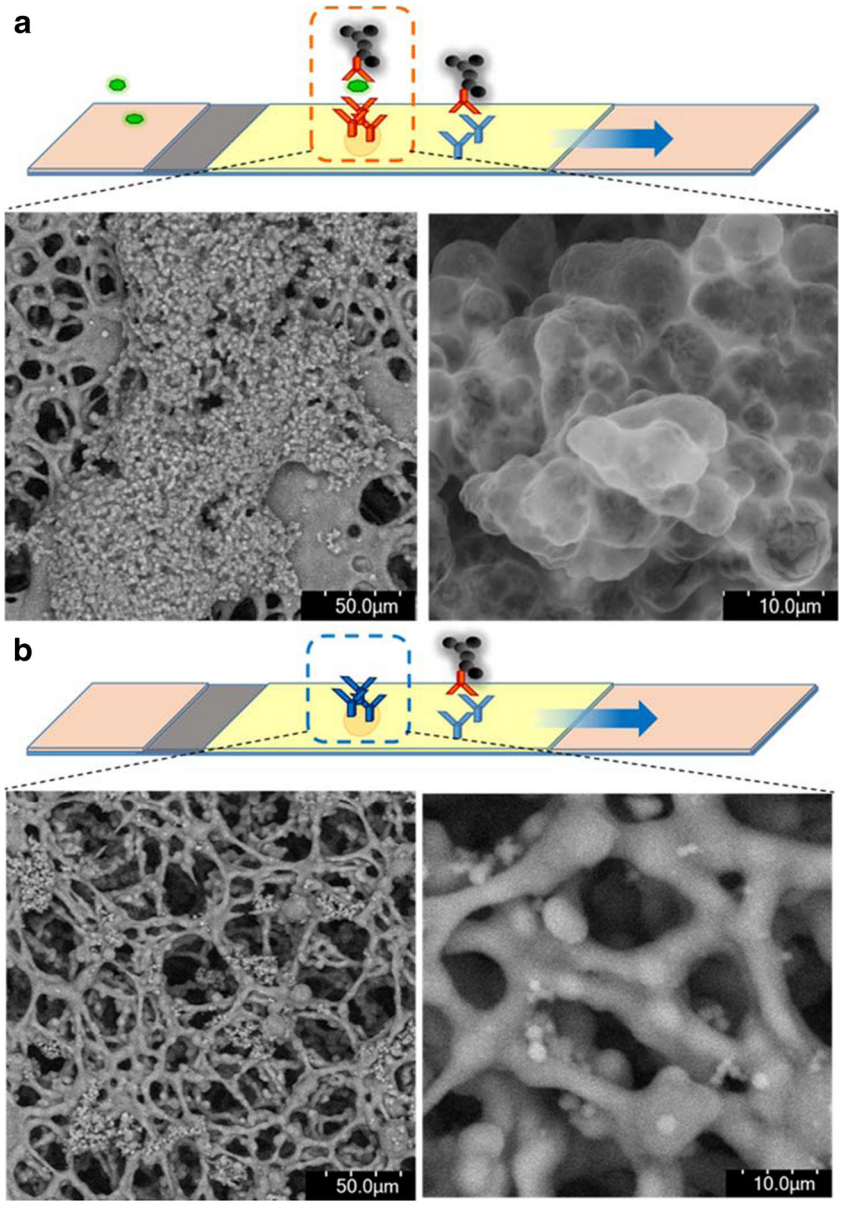

Fig. 2 Schematic illustration and SEM images of the carbon nanotag based lateral flow immunoassay in **a** a presence and **b** absence of the target analyte. *Arrow* indicates the flow direction of the sample liquid on the test system

observed visually at the concentration at 3.5×10^2 $TCID_{50}.mL^{-1}$. The image of the carbon nanotag based lateral flow assay test system in the presence of various influenza A virus concentrations is shown in the inset panel. Semi-quantitative analysis was also performed by recording the signal intensity at the test spot area as described earlier. The averaged normalized signal intensity of the sample was plotted against the concentration of the target virus. The limit of detection of the system was demonstrated to be 3.5×10^2 $TCID_{50}.mL^{-1}$, with the averaged normalized signal intensity value of 3165 ± 942 a.u. obtained from three individual experiments.

To assess the analytical selectivity of the system, other proteins were included in the evaluation as a sample. These molecules were human serum albumin, BSA, lysozyme, dopamine, glucose, and tryptone, at the concentration of 5 $\mu g.mL^{-1}$. In addition, other viruses including influenza B and canine distemper viruses were also used as a sample in order to assess the specificity of the system against other closely related viruses. Fig. 3b shows a photo image of the test system in the presence of allantoic fluid containing influenza A virus at the concentration of 2.8×10^3 $TCID_{50}.mL^{-1}$, compared to those of the strips in presence of other proteins and viruses. The averaged normalized signal intensity plotted against other proteins and viruses was also displayed ($n = 3$). It was shown that the system only exhibited a distinct black color test spot signal with a high normalized signal intensity value (averaged value of 35,871 ± 4052 a.u., $n = 3$) in the

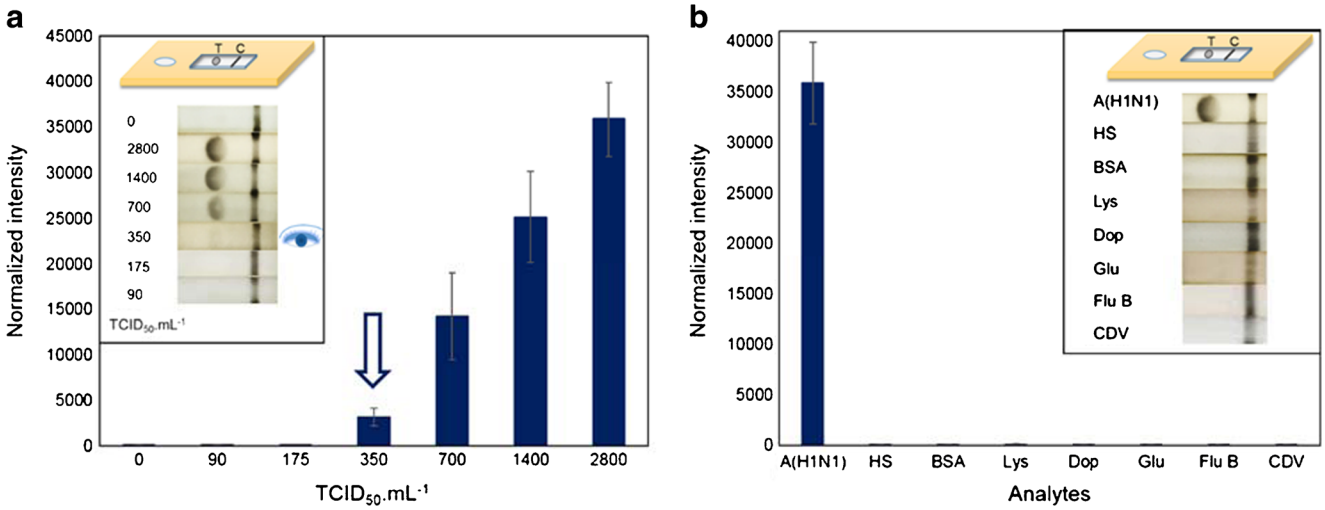

Fig. 3 **a** Performance evaluation and **b** analytical selectivity of the carbon nanotag based lateral flow immunoassay. The averaged normalized signal intensity of the test spot area was plotted against either different concentrations of influenza A virus or other tested molecules and closely related viruses. *Error bars* indicate standard deviation obtained from three individual experiments. *Inset panel* shows a photo image of a direct signal visualization. *Arrow* indicates the limit of detection

presence of allantoic fluid containing influenza A virus. On the other hand, no test spot signal was observed when other proteins and viruses were used as a sample. This result suggests that the system demonstrated the specificity against other tested proteins and viruses.

To determine how the system differs from other systems, a number of studies using different techniques were reviewed. Firstly, it is worth mentioning the diagnosis using traditional viral culture method. As described previously, traditional culture method is not only time-consuming technique, but it also represents only a fraction of bacteria and virus in the community relying on the culture condition selected [29]. Therefore, point-of-care test is of importance for rapid screening and diagnosis of influenza virus infection. With the advancement in the field of nanotechnology, this has led to the development of several nanomaterial-based methods for the identification of influenza virus. Table 1 summarizes an overview on recently reported techniques including the system reported in this study. Performances of each methods including sensitivity and specificity of the system were also reported.

For example, Zhao et al. described the use of gold nanoparticle-based microarray assay to detect avian influenza A(H5N1) viral RNA with the limit of detection of 10^3 $TCID_{50}$ per assay [30]. Different approaches using nanoparticles to either concentrate the sample, enable the signal detection, or amplify the signal were also reported with various detectable level of target influenza protein and virus particle. These platforms; for example, include electrochemical sensor, integrated microfluidic system, and field-effect transistor system [31–33]. Other systems using similar immunoassay principle were also investigated. Whilst Zhang et al. reported europium nanoparticle-based immunoasssay for a detection of influenza virus with the limit of detection of 10^1 to 10^3 egg infective dose $(EID)_{50}.mL^{-1}$, a paper-based immunoassay using gold nanoparticle to enable a detection of influenza virus with a range of 2.7×10^3 to 2.7×10^4 plaque-forming unit per assay was described [34, 35]. It should also be noted that the antibody specific to influenza virus used in this system is different to those of other systems. In this system, the antibody chosen as a targeting ligand is specific to the nucleoprotein, rather than other proteins such as hemagglutinin and neuraminidase, which are more susceptible to antigenic change. Nucleoprotein is recognized as one of the most abundant proteins in the virion and is less sensitive to antigenic drift. This, therefore, provides an advantage to the system by enabling the detection of all subtypes of influenza A virus to be possible, compared to other systems.

In comparison to the lateral flow immunoassay system, although these approaches provided similar or even superior detection sensitivity, these techniques are complicated techniques requiring expensive equipment, highly-trained personnel, and extensive validation for a routine use. Therefore, with respect to these approaches, lateral flow immunoassay platform, designed as rapid point-of-care diagnostic test, can provide significant advantages by allowing a direct, simple, but sensitive determination of influenza virus. With regards to the previous lateral flow immunoassay systems reported for the detection of influenza virus, dual-layered and double-targeted gold nanoparticles and fluorescent dye-doped silica nanoparticles were used as reporters [16, 19]. However, it should be noted that the strategies of using either different size of gold nanoparticles in combination with combined antigen detection method or fluorescence based method are more complicated strategies, compared to the single-label direct visualization system. For example, the system not only requires

Table 1 An overview on recently reported nanomaterial-based methods for determination of influenza A virus

Nanomaterial	Method	Virus subtype	Limit of detection	Specificity	Ref.
Gold nanoparticle with silver staining method	DNA–based Microarray assay (scanomatric detection)	A(H5N1)	10^2 fM per assay (PCR fragments) 10^3 $TCID_{50}$ per assay (viral RNA)	No cross reactivity to A(H1N1) and A(H3N2)	[30]
Graphene oxide, Multi-walled carbon nanotube	Electrochemical sensor (differential pulse voltammetry) with nanoparticle-based approaches	A(H7N9)	0.81 $pg.mL^{-1}$	No cross reactivity to tested proteins (BSA, CEA, human IgG), and A(H5N1) subtypes	[31]
Magnetic nanoparticle	Magnetic nanoparticle-integrated microfluidic separation with fluorescence immunoassay	A(H1N1)	0.007 HAU	-	[32]
Silicon nanowire, magnetic nanoparticle	Field-effect transistor biosensor	A(H3N2)	29 viruses per μL	Further improvement of specific binding is being addressed	[33]
Europium nanoparticle	Immunoassay (europium -based immunoassay	A and B	10^1 to 10^3 $EID_{50}.mL^{-1}$	No cross reactivity to the tested viruses (respiratory syncytial virus, herpes simplex virus, adenovirus, coronavirus, rhinovirus	[34]
Gold nanoparticle	Paper-based immunoassay (gold enhancement method)	A(H1N1) A(H3N2)	2.7×10^3 to 2.7×10^4 plaque-forming unit per assay	No cross reactivity with one negative sample	[35]
Carbon nanoparticle	Lateral flow immunoassay	A	3.5×10^2 $TCID_{50}.mL^{-1}$	No cross reactivity to tested proteins (i.e. human serum albumin, BSA, lysozyme, dopamine, glucose, and tryptone), and viruses (influenza B, and canine distemper virus)	This study

sophisticated protocol to generate the optimal probe, it also needs both extensive optimization procedures to prevent the non-specific signal that can be generated and specialized equipment for signal interpretation.

In order to improve these difficulties, different approach using carbon nanoparticles, in the form of nanostrings, as reporter molecules, for the detection of influenza A virus are therefore explored and investigated in this work. Carbon

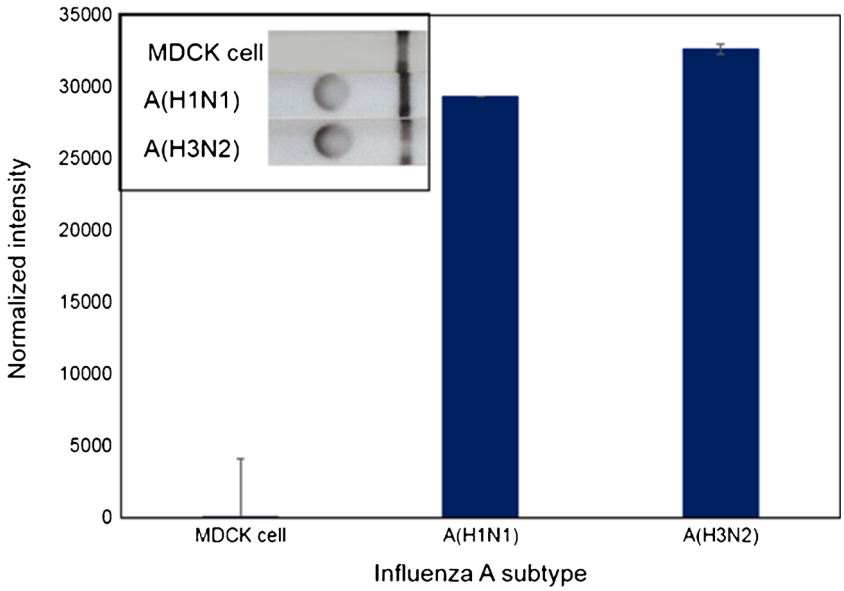

Fig. 4 Evaluation of the carbon nanotag based lateral flow immunoassay performance in a relevant clinical application. *Inset panel* shows a photo image of the system when applied to the cell cultures infected with either seasonal influenza A H1N1 14/2004 strain or seasonal influenza A H3N2 04/2004 strain. Analytical performance was demonstrated by the averaged normalized signal intensity of the test spot area plotted against different virus subtypes and uninfected cells. *Error bars* indicates standard deviation

nanoparticle have been recognized as a versatile label for a rapid diagnostic assay. The use of carbon nanoparticles as reporters in lateral flow assay system can provide a better sensitivity, compared to the use of either gold nanoparticles or latex beads. Among different nanomaterials used as reporters in lateral flow assay system, it was reported that carbon nanoparticles are the cheapest one and their suspensions are very stable and easy to prepare. [26]. With these unique properties of carbon nanomaterials such as good stability, high contrast, and inexpensive, we have demonstrated that the carbon nanotag based lateral flow assay can be successfully fabricated for a rapid and direct visual determination of influenza virus with a limit of detection comparable to other existed techniques and systems.

A final point that merits a further discussion is how the system can potentially applied to the relevant clinical application. In order to evaluate the potential of the system, it is important to assess the performance of the system for the detection of influenza A virus in related biological sample matrix i.e. in the cell lysate format. To this regard, MDCK cell cultures infected with either seasonal influenza A(H1N1) 14/2004 strain or seasonal influenza A(H3N2) 04/2004 strain were used as a sample, with uninfected cells were used a negative control. It was shown that when influenza A infected cell cultures were used as a sample, a distinct black color test spot was observed with a high averaged normalized signal intensity value (29,338 ± 353 and 32,671 ± 304 a.u., respectively) (Fig. 4). Oppositely, when uninfected cells were applied to the system, no test spot signal was detected, with a relatively low averaged normalized signal intensity value. These results demonstrate that the system have the potential to detect the virus in biological matrices, with no cross reactivity to other tested proteins.

Conclusions

We have demonstrated the development of a rapid detection platform based on lateral flow chromatographic assay. With the combination of carbon nanoparticles, in the form of nanostrings, as reporters, visual detection of influenza A virus was demonstrated. Rapid detection of the target virus was achieved through a direct color visualization of the signal at the test area. Under optimal conditions, the system is capable of detecting influenza A virus in the influenza A-inoculated allantoic fluid, with no cross reactivity to other tested proteins. In addition, successful detection of the clinical strains of the influenza A virus in the cell lysate format without interference from biological matrices was also demonstrated. Compared to the traditional culture method, molecular approaches, and other nanomaterial-based methods, carbon nanotag based lateral flow assay provides a rapid and direct approach for the detection of influenza A virus. In conclusion, this work has demonstrated the potential application of such detection platform to detect influenza A virus in complex biological matrices, including allantoic fluid and the cell-associated format, which can be extended for the future development of rapid tests in clinical application.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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