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Rapid Influenza A Antigen Detection using Carbon Nanostrings as Label for Lateral Flow Immunochromatographic Assay

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Abstract— **In this study, we investigated a feasibility of using carbon nanostrings as reporters in developing a lateral flow immunochromatographic system for a rapid influenza A antigen detection as a model. Carbon nanostrings were successfully conjugated to monoclonal antibody specific to the nucleoprotein, one of the most abundant influenza A protein, for a signal visualization. To demonstrate the feasibility of the lateral flow immunochromatographic system using carbon nanostrings as reporters for influenza A antigen detection, the system was applied to detect the antigen in an allantoic fluid infected with pandemic 2009 influenza A H1N1 virus. It was shown that the system was able to detect the influenza A nucleoprotein in a complex biological fluid such as an infected allantoic fluid containing 2.8×103 TCID50/mL (50% tissue culture infectious dose). By using carbon nanostrings as reporters combined with lateral flow immunoassay as the mean of antigen detection, a specific recognition of influenza A antigen could be achieved within 15 min. Compared to the existing methods of influenza A diagnosis such as viral isolation and serological approaches, this simple one-step immunochromatographic system avoids laborious and multiple incubation steps, as well as maintaining a sensitive and specific detection which could be useful for developing a point-of-care diagnostic test in other applications.**

I. Introduction

Rapid and sensitive point-of-care detection of a target protein is of central importance to the clinical diagnostics and other biomedical applications [1]. Many existing methods such as enzyme-linked immunosorbent assay (ELISA) offer sensitive protein detection. However, these techniques are often limited to time-consuming and multiple analytical procedures and requirement of expensive instruments [2]. Recently, an emerging lateral flow immunochromatographic assay (LFIA) has gain considerable attention as a promising tool for rapid protein detection [3].

Within the LFIA system, the target protein is sandwiched between the immobilized capture antibody and the labeled antibody to form a specific immunorecognition complex [4] In order to enable the signal visualization, the antibody must be labeled with labeling molecules. Colored nanoparticles have been used as reporters [5,6]. Among these reporters, gold nanoparticles are commonly used due to their ease in bioconjugation. However, a number of studies have reported the used of carbon nanoparticles as reporters due to their ease in conjugating to a biorecognition unit. In addition, carbon nanoparticles can be synthesized in various sizes and shapes to enable different detection ranges i.e. carbon nanostrings (CNS) [7]. Based upon their properties, CNS could be a good candidate as labeling molecules in LFIA system.

In this study, we investigated the feasibility of using CNS, an elongated form of carbon nanoparticles, as reporters in LFIA system. The functionality of the system is also discussed using influenza A antigen as a model.

II. Materials and Methods

A. Reagents and materials

Carbon nanostrings were purchased from Maiia Diagnostics (Sweden). Monoclonal antibody specific to influenza A nucleoprotein (MAb) was purchased from Innova biotechnology (Thailand). LFIA test strips were supplied from Innova Biotechnology (Thailand). All chemical reagents used in this study were purchased from Sigma-Aldrich (UK), unless stated otherwise.

B. Preparation of allantoic fluid infected with influenza A viruses

Influenza virus A/Thailand/104/2009 (H1N1) was inoculated in 10-day-old embryonated specific pathogen-free chicken eggs as described previously [8]. The allantoic fluid which has been infected with influenza virus A/Thailand/104/2009 (H1N1) was inactivated using binary ethylenimine inactivation method [9]. All viral manipulation was performed under appropriate biosafety level 2 plus conditions.

C. Conjugation of CNS to antibody

CNS stock solution (90 µg) was diluted with 5 mM borate buffer, pH 9. The CNS suspension was then sonicated for 2 min. The diluted CNS was then mixed with 4.5 µg of MAb. A mixture was incubated at 25ºC for 30 min, followed by an addition of 100 µL of 10% BSA (w/v). After incubation of 15 min, the conjugate was centrifuged at 10,000 g at 4ºC for 20 min. The final conjugate was collected and resuspended in a storage buffer containing 1% BSA (w/v) in 5 mM borate buffer, pH 9. The conjugate was stored at 4ºC until used. The surface charge of the CNS and the MAb-conjugated CNS (CNS-MAb) conjugate was determined using Malvern Zetasizer (UK).

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D. *Evaluation of CBNS-MAb conjugate activity using LFIA assay Conjugation of CNS to antibody*

The LFIA test strip was composed of sample pad, conjugate pad, reacting nitrocellulose membrane, and absorbent pad. All 4 components were assembled on a backing card (Fig. 1A). The optimized volume of the conjugate was deposited on the conjugate pad. The antigen-binding activity of the CNS conjugated MAb was firstly evaluated using direct antigen LFIA assay format (Fig. 1B). One microliter of the either allantoic fluid infected with influenza A virus, BSA (0.25 mg/mL), or 5 mM borate buffer (pH 9) only was spotted at the test spot of the reacting nitrocellulose membrane. After dried at 25ºC for 30 min, all components were assembled on the backing card. Sample buffer (100 µL of 5 mM borate buffer, pH 9) was dispensed onto the sample pad to release the conjugate from the conjugate pad. The signal on the test spot was read at 15 min after sample application.

E. *LFIA assay of the NP protein*

LFIA test strip component for the NP protein assay was illustrated in Fig. 1C. The optimized volume of the conjugate was deposited on the conjugate pad. One microliter of MAb (1 mg/mL) was spotted on the test spot of the reacting nitrocellulose membrane and used as the capture ligand to capture the NP protein in the sample. Secondary anti-mouse IgG antibody was dispensed at the control line of the membrane and used as a validation of the test system. The reacting membrane was then dried at 25ºC for 30 min. A volume of 100 µL of the sample containing the antigen in 5 mM borate buffer, pH 9 was added to the sample pad. The test spot signal was determined at 15 min after sample application.

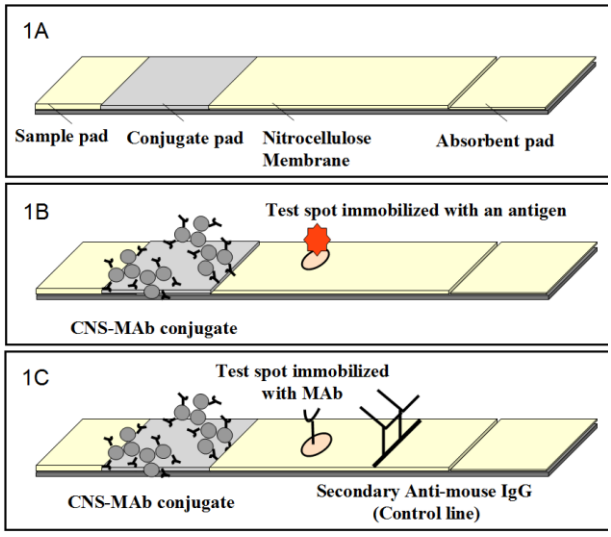


Figure 1. (A) Schematic diagram of LFIA test strip component. (B) Direct antigen detection format of the LFIA system. (C) LFIA assay format of the NP protein.

III. Results and Discussion

In this present study, LFIA system using CNS as reporters was developed for influenza A antigen detection as a model. In the present, diagnosis of influenza A infection has been relied on either viral isolation or a detection of influenza A antigen using ELISA technique. Molecular approaches such as reverse transcriptase polymerase chain reaction (RT-PCR) was also a method of choice. However, these approaches are time-consuming and possess complicated analytical procedures [10, 11]. Clearly, a rapid and sensitive influenza A diagnosis is needed. Rapid test based on LFIA has been used as a tool for rapid influenza A diagnosis due to its speed and simplicity. Most rapid tests for influenza A diagnosis has been relied on the detection of influenza A proteins. Of the influenza A proteins, the nucleoprotein (NP) was found to be one of the most abundant protein present in the virus particle [12]. The NP was therefore used as a target protein in developing rapid test for influenza A diagnosis based on LFIA system using CNS as reporters in this study.

In order to enable the detection signal to be visualized, MAb specific to influenza A NP was conjugated to CNS. Surface charge of the free CNS and the CNS-MAb conjugate was determined using Malvern Zetasizer. It was demonstrated that the zeta potential of the CNS became less negative after the CNS were conjugated to the MAb. The change in surface charge of the CNS could indicate a successful in conjugation of the MAb to CNS. Zeta potential value of the CNS before and after MAb conjugation was determined and summarized in Table 1.

Subsequent to the successful in conjugation, it was also important to evaluate the activity of the MAb after conjugated to the CNS. This was performed using a direct antigen detection format of the LFIA system (Fig. 1B). The allantoic fluid infected with pandemic 2009 influenza A H1N1 virus was used as the antigen and immobilized directly at the test spot on the membrane. After drying, the test strip was assembled as described previously. After the conjugates were released from the conjugate pad, it was shown that the conjugate bound to the immobilized antigen on the test spot of the membrane. This was shown by the presence of the test spot signal (black in color). However, when either BSA or buffer only was spotted at the test spot as the antigen, no test spot signal could be observed. Fig. 2 shows the results of evaluating the activity of the CNS-MAb conjugates. The direct binding between the conjugate and the antigen indicated the successful of the conjugation without cross-reactivity to other protein tested. Furthermore, it was shown that the conjugated MAb retained its activity and could be further used.

TABLE I. Zeta Potentail of the Carbon Nanostrings Before and After Antibody Conjugation

Type	Mean Zeta Potential (mV)
Free CNS	-39.3
CNS-MAb	-27.2

Figure 2. Evaluation of the activity of the CNS-MAb conjugates using direct antigen format of the LFIA system. Positive and negative test spot signals were observed when (A) the allantoic fluid infected with influenza A virus, (B) BSA, and (C) buffer only were used as samples, respectively.

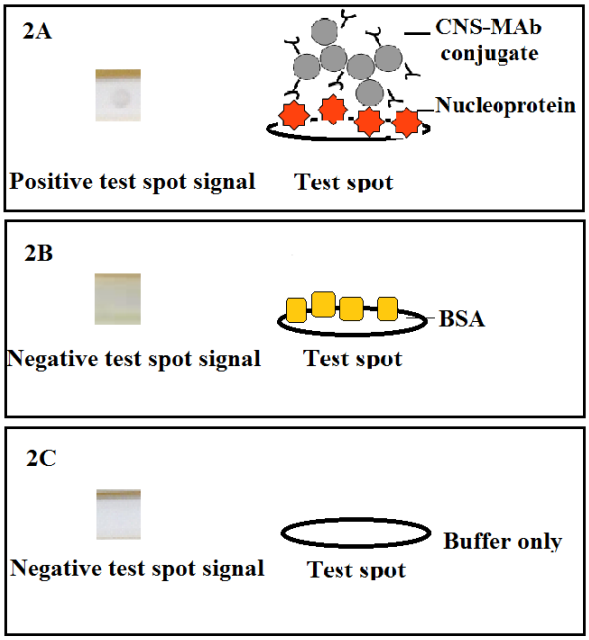
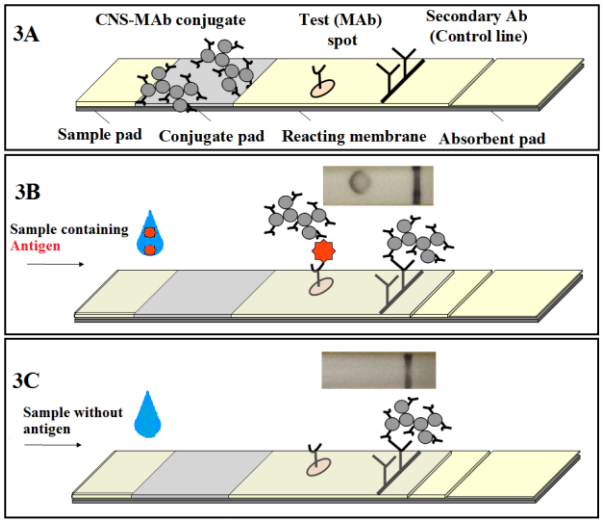


Figure 3. (A) Schematic diagram of sandwich LFIA system using CNS as reporters. Positive and negative results were shown when (B) the allantoic fluid infected with influenza A virus, and (C) buffer only were used as samples, respectively.



To demonstrate the feasibility of using CNS-based LFIA test system for rapid influenza A antigen detection, sandwich LFIA system was developed. A schematic diagram of the sandwich LFIA system was illustrated in Fig. 3A. Within the test system, the free MAb and the CNS-MAb conjugate were used as the capture and the detector antibody, respectively to detect the NP in the sample.

When the allantoic fluid containing pandemic 2009 influenza A H1N1 virus at 2.8×103 TCID50/mL (50% tissue culture infectious dose) was used as a sample, the test exhibited a clear positive signal. This was demonstrated by a visualization of the detection signal as a black color spot on the membrane, resulting from a formation of sandwich immunocomplex of free MAb/influenza A NP/CNS-MAb conjugates (Fig. 3B). The excess unbound conjugate further migrated along the device was then captured by the secondary anti-mouse IgG antibody immobilized at the control line of the membrane as demonstrated by the presence of the signal at the control line. The presence of the signal at both the test spot and the control line indicated that the test system was functioning correctly and that the system was validated.

In contrast, when chasing buffer only was used as a sample, only a clear control line signal was observed (Fig. 3C). A clean negative test spot signal indicated that the system specifically detected only the target analyte.

IV. Conclusion

In this study, we demonstrated that carbon nanoparticles such as CNS could be used as reporters in the LFIA system for the detection of influenza A antigen detection as a model. Due to its intense color and its ease in conjugation to biorecognition units, CNS could be used as alternative labeling molecules to targeting molecules such as antibody to enable a detection signal to be visualized. Here, we have demonstrated that the conjugate retained its antigen-binding activity. The valid positive and negative test spot signal suggested that the conjugate was optimal and specific to the target analyte, By integrating with the LFIA system, rapid influenza A antigen detection based on LFIA principle was successfully developed. The system was shown to be able to detect the influenza A nucleoprotein in a complex biological fluid such as an allantoic fluid. Further extension to apply the system to different influenza A viral isolates may help evaluate the robustness of the system in clinical application. In summary, CNS shows a great potential for future development of rapid point-of-care diagnostic test for biomedical and other applications.

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