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**A Plasmon-Assisted Fluoro-Immunoassay using Gold Nanoparticle-Decorated
Carbon Nanotubes for Monitoring the Influenza Virus**

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ABSTRACT

A plasmon-assisted fluoro-immunoassay (PAFI) was developed for the detection of the influenza virus by using Au nanoparticle (Au NP)-decorated carbon nanotubes (AuCNTs) that were synthesized using phytochemical composites at room temperature in deionized water. Specific antibodies (Abs) against the influenza virus were conjugated onto the surface of AuCNTs and cadmium telluride quantum dots (QDs), which had a photoluminescence intensity that varied as a function of virus concentration and a detection limit of 0.1 pg/mL for all three types of influenza viruses examined. The clinically isolated influenza viruses (A/Yokohama/110/2009 (H3N2)) were detected in the range of 50–10,000 PFU/mL, with a detection limit of 50 PFU/mL. From a series of proof-of-concept and clinical experiments, the developed PAFI biosensing system provided robust signal production and enhancement, as well as an excellent selectivity and sensitivity for influenza viruses. This nanoparticle-based technique could be potentially developed as an efficient detection platform for the influenza virus.

KEYWORDS: Plasmon-assisted fluoro-immunoassay, Gold nanoparticle-decorated carbon nanotube, CdTe quantum dot, Influenza virus detection platform, Plasmonic resonance energy transfer

1. Introduction

Many kinds of nanomaterials have been recently used in the area of nanobiotechnology research. The unique physicochemical properties of nanomaterials have found a significant number of applications in biosensing, imaging, and drug delivery system (Ahmed et al. 2013; Lee et al. 2014; Leung et al. 2012; Li and Mezzenga, 2013; Wang et al. 2013^b). In particular, nanobiosensing systems have gained popularity owing to its high sensitivity, selectivity, and rapid response time (Liu et al. 2012; Yin et al. 2013). The detection techniques used in various nanobiosensing applications include magnetophoresis, electrochemical analysis, plasmonic coupling immunoassays, and fluoro-immunoassays (Draz et al. 2012; Kim et al. 2013; Li et al. 2013; Viet et al. 2013; Zhou et al. 2012; Zhou et al. 2013). The plasmon-assisted fluoro-immunoassay (PAFI) has been used to analyze specific biomaterials (Ahmed et al. 2014; Li et al. 2012; Nooney et al. 2010; Sharma et al., 2013^b). The PAFI is based on the plasmonic resonance energy transfer (PRET) phenomenon, which causes a photoluminescence (PL) enhancement from the interactions between the plasmonic nanomaterials and the semiconductor nanoparticles (Lee et al. 2004; Lee et al. 2005; Lee et al. 2007). Such hybrid structures can be used to detect the interaction between an antibody (Ab) and its antigen, because of their tuned optical properties. Although numerous plasmonic nanomaterials have been introduced (*e.g.*, gold, silver, platinum, and metal NPs), Au NP-decorated carbon nanotubes (AuCNTs) have received considerable attention, owing to their unique properties. Au NPs are able to exhibit surface plasmon resonance (Jana et al. 2001; Lee et al. 2011^b). Carbon nanotubes exhibit electroconductivity and harbor many π electrons on their surfaces (Jariwala et al.

2013; Sun et al. 2011). Thus, AuCNTs are expected to show a synergistic effect owing to their roles as biosensing platforms, signal enhancers, and signal transducers (McAndrew and Baxendale, 2013; Sharma et al. 2013a; Wang et al. 2013a; Yick et al. 2013).

Combining the above mentioned heterogeneous materials requires sophisticated strategies in order to conserve carbon nanotube (CNT) structures and to bind two materials without the help of organic bridges. One of the well-known processes for preparing AuCNT nanostructures involves reduction with chemical reducing agents such as sodium borohydride or hydrazine (Yu et al. 2014; Zhang et al. 2013). An alternative process involves thiol- or amine-assisted interactions between Au NPs and CNT surfaces (Georgakilas et al. 2007; Li et al. 2011). Attachment of Au NPs onto CNT surfaces has been attempted using the processes of electrodeposition, DNA hybridization, and chemical reaction (Georgakilas et al. 2007; Gobbo et al. 2013; Li et al. 2011; Li and Cooper-White, 2013; Peng et al. 2009). However, these approaches can cause CNT damage and organic/biological electric resistance, which may adversely affect their electrical and mechanical properties (Hirsch, 2002; Holzinger et al. 2001).

In this study, we suggest a novel and easy method for preparing AuCNTs by using phytochemicals. The synthetic reaction was carried out in deionized (DI) water at room temperature *via* sonication and stirring processes, without resorting to external heating or application of high pressure. No electrochemistry equipment was used in the above procedure. AuCNTs were produced as follows. Au ions were first attached onto the surface of CNTs and they were then reduced to Au NPs on the CNT surface. This process was catalyzed using a mixture of gallic acid and isoflavone phytochemicals, two well-known natural antioxidants (Aruoma et al. 1993;

Park et al. 2009) that served as mild reducing agents (Lee et al. 2011a). Furthermore, the generated AuCNTs were applied onto the influenza virus detection platform by using a quantum dot (QDs)-assisted PAFI.

In this study, we developed a PAFI-based detection platform for the influenza virus, using antibody-conjugated AuCNTs and CdTe QDs. In all, we tested three types of influenza viruses, viz. the Influenza virus A/Beijing/262/95 (H1N1), the Influenza virus/New Caledonia/20/99IVR116 (H1N1), and the clinically isolated Influenza virus A/Yokohama/110/2009 (H3N2). The minimum detection limit for the influenza virus was 0.1 pg/mL. The clinically isolated influenza virus was also monitored in the range 50–10,000 PFU/mL, with a detection limit of 50 PFU/mL. Our virus detection platform would be immensely useful not only for detecting the influenza virus, but also for detecting various other viruses and viral diseases.

2. Material and methods

2.1. Materials and instruments

HAuCl₄·3H₂O, multi-walled carbon nanotubes (MWCNTs), gallic acid, EDC, NHS, cadmium perchlorate hydrate, and cysteamine were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Aluminum telluride (Al₂Te₃) was obtained from the Cerac Company (Milwaukee, WI, USA). The isoflavone was isolated from commercial soybeans. 3,3',5,5'-tetramethylbenzidine was purchased from Dojindo (Osaka, Japan). The ECLTM anti-mouse IgG, horseradish peroxidase (HRP)-conjugated whole antibody (Ab) was obtained from GE Healthcare UK, Ltd. (Buckinghamshire, UK). Goat anti-rabbit IgG-HRP was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-Influenza A virus hemagglutinin (HA) Ab Ab66189,

which is a mouse monoclonal antibody [B219M] for the influenza A virus HA H1 and positive against influenza virus A/Beijing/262/95 (H1N1), A/New Caledonia/20/99 (H1N1), and A/Taiwan/1/86 (H1N1), was purchased from Abcam Inc. (Cambridge, UK). Anti-neuraminidase (NA) (New Caledonia/20/1999/(H1N1)), a rabbit polyclonal Ab, was obtained from Immune Technology Corp. (New York, NY, USA). Anti-H3 (H3N2) (Ab82454), a mouse monoclonal Ab [InA227] to H3 (H3N2) that recognizes influenza virus A HA H3, was purchased from Abcam Inc. (Cambridge, UK). Influenza virus New Caledonia/20/99lvR116 (H1N1) and A/Beijing/262/95 (H1N1) were purchased from Sino Biological Inc. (Beijing, China) and HyTest Ltd (Turku, Finland), respectively. Influenza virus A/Yokohama/110/2009 (H3N2) was isolated from a clinically isolated sample, which was kindly provided by Dr. C. Kawakami of Yokohama City Institute of Health, Japan and was used to assess the versatility of this assay system.

The absorbance of AuCNTs and the corresponding PL enhancement were measured using a filter-based multimode microplate reader (Infinite® F500, TECAN, Ltd., Männedorf, Switzerland), and the chemical reactions and surface functional groups were monitored by FT-IR spectroscopy (FT-IR 6300, JASCO, Corp. Tokyo, Japan). The morphologies and sizes of the nanostructures were characterized by TEM (JEM-2100F, JEOL, Ltd., Tokyo, Japan). An X-ray powder diffractometer (RINT ULTIMA, Rigaku, Corp., Tokyo, Japan) was used to characterize AuCNT by using CuK α radiation and a Ni filter. The data were collected from 2 θ = 0–100° at a scan rate of 0.01° per step and 10 s per point. The AuCNTs and MWCNTs were analyzed by Raman spectroscopy (HR-800, LabRAM, HORIBA Ltd., Kyoto, Japan). In order to measure the electroconductivity, AuCNT solution was dropped on the planar interdigitated electrode (planar IDE-Pt/0.25", Synkera, USA) and dried at

room temperature. Then, the current change of the deposited area was monitored by linear sweep voltammetry from -1 V to 1 V (SP-150, BioLogic, France). A plate reader (Model 680, Bio-Rad, Hercules, USA) was used to confirm the presence of Ab-conjugated nanomaterials. The PL image of the hybrid nanostructure was observed using a confocal laser-scanning microscope (LSM 700, Carl Zeiss Microimaging, GmbH, Göttingen, Germany).

2.2. Synthesis of AuCNTs and CdTe QDs

AuCNTs were synthesized at room temperature by using commercially available reagents. Forty milligrams of MWCNT was dispersed in 100 mL of nitric acid and boiled for 5 h to prepare the hydrophilic MWCNTs. Subsequently, 0.01 mmol of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ and 2 mg of acid-treated MWCNTs were dispersed in 30 mL of DI water by sonication for 30 min. Subsequently, 600 μL of GI solution was added into the MWCNT/Au ion solution, and then stirred vigorously for 1 h. The GI solution was used as a reducing agent and stabilizer, and prepared as follows: 10 mg of isoflavone was dissolved in 10 mL of the 0.01 M gallic acid solution. The cysteamine-coated CdTe QDs were synthesized as reported in detail elsewhere (Gaponik et al. 2002; Lee et al. 2010).

2.3. Preparation of antibody-conjugated AuCNTs and CdTe QDs

In order to conjugate the Abs to the AuCNTs, amine-functionalized AuCNTs and Ab-conjugated EDC/NHS were prepared. To modify the surface of the Au NPs, 1 mg of AuCNTs was dispersed in 10 mL of DI water. Then, 1 mL of 0.01 M cysteamine was added into the AuCNT solution. After 30 min of stirring, this mixture was centrifuged to separate the amine-functionalized AuCNTs. Additionally, 100 μL of the

4 mM EDC and 10 mM NHS were added in the 96-well plate and incubated and gently shaken for 30 min at 200 rpm with 1 μ L of anti-HA Ab (Ab66189) (final concentration of 5 ng/mL) for the EDC/NHS coupling reaction. Finally, 30 μ L of the amine-functionalized AuCNT (1 μ g/ μ L) and activated anti-HA Ab (Ab66189) were mixed in all wells and shaken for 3 h for effective bioconjugation. Anti-NA (New Caledonia/20/1999/(H1N1)) and anti-HA (Ab82454) Abs were also conjugated to the surfaces of the Au CNTs by using the same procedure. Anti-HA (Ab66189) and anti-HA (Ab82454)-conjugated CdTe QDs were also prepared by using the procedure for Ab-conjugated AuCNTs.

2.4. Plasmon assisted fluoro-immunoassay (PAFI) for influenza virus detection

In order to detect the influenza virus *via* PAFI, 45 μ L of Ab-conjugated AuCNTs and 45 μ L of the CdTe QDs were mixed in each of the 96 wells. Consequently, serially diluted influenza virus was added into each well and shaken for 1 h. During this process, the Ab-conjugated AuCNTs and the CdTe QDs were found to bind each other in the presence of the influenza virus, owing to the affinity between the antigen on the surface of virus and its corresponding antibody. To evaluate the efficacy of PAFI as a detection platform, the following three types of influenza viruses were used: A/Beijing/262/95 (H1N1), New Caledonia/20/99IvR116 (H1N1), and A/Yokohama/110/2009 (H3N2). The PL intensities were measured as a function of the concentration of the influenza virus. The PAFI was carried out at an excitation wavelength of 380 nm, and the excitation and the emission slits were 5 and 10 nm in width, respectively. The PL intensity of this system was monitored at 518 nm during signal detection. In order to evaluate the PAFI system with different influenza virus, all detection experiment was carried out over 3 times.

3. Results and Discussion

3.1. Design and preparation of AuCNTs for PAFI

Fig. 1. (I) Synthetic scheme for the preparation of Au nanoparticle (NP)-decorated CNT nanostructures (AuCNTs) and (II) the process of influenza virus detection by using PAFI, non-scalable.

The synthesis of AuCNTs comprised two steps (Fig. 1), viz. the preparation of plasmonic nanomaterials (step I), and the processing of PAFI for influenza virus detection by using AuCNTs and QDs (step II). Firstly, the acid-treated multi-walled CNTs (MWCNTs) were dispersed in DI water containing a gold precursor (Au^{3+}) by using sonication. Owing to the π -electrons and oxygen moieties on the surface of the acid-treated MWCNTs, Au^{3+} is able to attach onto the surface of MWCNTs through

electrostatic attraction. In order to decorate Au NPs onto the MWCNTs, the Au ions were reduced to Au NPs, using a mixture of gallic acid and isoflavone (GI solution). AuCNTs were obtained after 1 h of stirring and washing with DI water. To perform the PAFI for influenza virus detection by using AuCNTs, anti-hemagglutinin (HA) Ab or anti-neuraminidase (NA) Ab specific for the influenza virus were conjugated onto the surfaces of AuCNTs and QDs by using a *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS)-coupling reaction (step II in Fig. 1). In the presence of the influenza virus, the distance between the Ab-conjugated AuCNTs and the QDs diminishes by an affinity between an antigen and its Ab. Depending on the concentration of the influenza virus, the formation of the AuCNT and QD hybrid structures caused a variation in the PL intensities. In the PAFI system, plasmonic material is not single Au NP but AuCNT assembly structure. Thus the energy transfer to enhance the PL property would be taken a place at the sandwich structure between plasmonic AuCNT structure and fluorescent CdTe QDs with virus. Moreover, this sandwich structure could lead the PL enhancement through the collective effect between plasmonic materials and fluorescent nanoparticles (Lee et al. 2004).

3.2. Morphology of AuCNTs

Fig. 2. TEM images of (A) MWCNTs and (B) AuCNTs.

In order to confirm the decoration of Au NPs onto MWCNTs, the morphology of MWCNTs and AuCNTs were examined using transmission electron microscopy (TEM). The MWCNTs showed the presence of a slick surface that was over 3 μm in length (Fig. 2A and Fig. S1A, respectively). However, after two steps of the decoration reaction, a large number of Au NPs were detected on the surface of MWCNTs (Fig. 2B and Fig. S1B). The carbon structure of MWCNT is transparent, but as heavy metals show high electron absorption, Au NPs are easily distinguishable in the TEM images. The average particle size of the Au NPs was 20 nm, and they were well dispersed with a high density over the large surface area of the MWCNTs (Fig. S1B).

3.3. Optical, surface-enhanced Raman scattering, physicochemical, and electrical properties of AuCNTs

Fig. 3. Physicochemical properties of the AuCNTs. (A) UV/Vis spectra, (B) XRD data, (C) Raman spectra of MWCNT with Au ions (red dash line) and AuCNTs (black line), and (D) FT-IR spectra of the amine-functionalized AuCNTs.

The optical absorbance of AuCNTs was measured using a filter-based absorbance-mode microplate reader. Typically, the plasmon peak of Au NPs that are 20 nm in radius occurs at 525 nm. However, the surface plasmonic absorbance of Au NPs was observed as a black spectrum at 550 nm (Fig. 3A), and its bands were broadened and non-symmetric; however, large-sized (>50 nm) particles were not observed in the corresponding TEM image. Plasmon-coupling between adjacent Au NPs might have occurred, owing to the delocalized π -electron cloud on the surface of MWCNTs. This interaction caused a broadened and non-symmetric plasmonic band of Au NPs. Thus MWCNT can play a role as mediator for plasmonic coupling interaction, thus optical property of this hybrid structure could be enhanced (Lee et al. 2012). However, in the MWCNT case, the specific absorbance as plasmonic peak was not observed by UV/Vis spectroscopy. Thus, only MWCNT was not suitable for

PAFI detection platform.

The structural characteristics of the AuCNTs were elucidated based on the powder X-ray diffraction (XRD) pattern (Fig. 3B). A strong diffraction peak corresponding to the 002 plane of the MWCNTs was presented at $2\theta = 26.2^\circ$ in the XRD pattern (ICSD card no: 01-075-1621). Meanwhile, several new diffraction patterns and weak carbon peaks were measured in the AuCNTs (Fig. 3B, red pattern). The presence of Au NPs in the nanostructure was confirmed by the characteristic diffraction peaks from the face-centered cubic packing arrangement of bulk Au *i.e.*, the (111), (200), (220), (311), (222), and (400) planes at 2θ values of 38.2° , 44.4° , 64.6° , 77.5° , 81.7° , and 98.1° (ICSD card no: 00-004-0784), respectively. Au NPs possess a higher crystallinity than the carbon structures, because of the metal NPs. Thus, the diffraction patterns of Au NPs were stronger than those of the carbon face. The surface-enhanced Raman scattering (SERS) was measured by Raman spectroscopy at the excitation wavelength of 514 nm (Fig. 3C). The peak at 1340 cm^{-1} indicates that the D band occurred due to the local disorder present in the AuCNT structure (Sharma et al. 2013a). In addition, a G peak appeared near 1580 cm^{-1} , which is related to the characteristic feature (especially to the sp^2 -hybridized carbon allotropes) of the graphitic layers (Baro et al. 2013). As expected, the Raman spectrum intensity was more enhanced in the AuCNTs than in the MWCNTs (Sharma et al. 2013a). The spectrum intensity of AuCNTs was 5 times higher than that of MWCNTs. Therefore, the SERS effect was induced by decoration of Au NPs onto the MWCNT surface. The functional group of the amine-functionalized AuCNT was analyzed using Fourier Transform Infrared (FT-IR) spectroscopy. For the EDC/NHS coupling reaction with the carboxylic group of the influenza virus antibody, the N-H vibration of AuCNT was detected around 1250 cm^{-1} and $3400\text{--}3500\text{ cm}^{-1}$ (Fig. 3D), respectively. These bands around $1450\text{--}1580\text{ cm}^{-1}$

are characteristic of the aromatic bonds of MWCNT. The C–O single-bond vibration was observed at 1010 cm⁻¹. The electroconductivities of the AuCNTs and MWCNTs were measured using a finger type Pt electrode *via* the linear sweep method. The average linear resistance of AuCNT was approximately 0.007 mΩ, whereas that of MWCNT was much higher (Fig. S2). From these physicochemical results, it was evident that the AuCNT composite exhibits enhanced properties such as SERS and electrical conductivity. Furthermore, we could experimentally establish the fact that critical damage to the AuCNT structure indeed did not occur, and that AuNPs were directly attached onto the surface of the CNTs, owing to a mild reduction reaction.

3.4. PAFI performance for influenza virus detection by using AuCNT

In order to assess the PAFI performance for the influenza virus detection, three types of antibodies, *viz.* anti-HA (H1) (Ab66189), anti-neuraminidase (NA) (New Caledonia/20/1999/(H1N1)), and anti-HA (H3N2) (Ab82454) were conjugated onto the surface of AuCNTs (step II in Fig. 1). In addition, the surfaces of CdTe QDs were also conjugated with anti-HA (H1) (Ab66189) and anti-HA (H3N2) (Ab82454) antibodies by using the same method. In this case, the Ab-conjugated AuCNTs play the role of plasmon-supplying substrates, whereas the Ab-conjugated CdTe QDs act as PL-monitoring materials. Prior to virus detection, conjugation of the Abs to the AuCNTs or CdTe QDs was verified using an ELISA (Figure S3). Anti-HA (Ab66189) and anti-NA (New Caledonia/20/1999/ (H1N1))-conjugated AuCNTs, and anti-HA (Ab66189)-conjugated CdTe QDs displayed strong signals (Fig. S3A, B, and C, respectively). In the case of the anti-HA (Ab82454)-conjugated AuCNTs and the CdTe QDs, the recorded absorbance was lower than that of the other Ab-conjugated NPs (Fig. S4). These results indicate that all antibodies were successfully

conjugated onto the surface of the AuCNTs and the CdTe NPs.

Fig. 4. Confocal laser-scanning microscopy images of the AuCNT-QD hybrid structure obtained via the anti-HA Ab conjugation reaction with influenza virus A/Beijing/262/95 (H1N1). (A)–(C) Before and (D)–(F) after the conjugation reaction with influenza virus. (A) and (D) are fluorescent images; (B) and (E) are DIC images; (C) and (F) are merged images.

The Ab-conjugated nanostructures were used for constructing the PAFL system. The detection of the influenza virus was made possible by using a confocal laser-scanning microscope (Fig. 4). Influenza virus A/Beijing/262/95 (H1N1) was dosed into the anti-HA Ab-conjugated AuCNTs and onto the CdTe system. In the presence of the virus, the distance between the HA Ab-conjugated AuCNTs and the HA Ab-conjugated CdTe QDs was reduced, owing to the affinity between the antigen on the surface of the virus and conjugated antibody. Subsequently, the hybrid nanowire structure showed green PL (Fig. 4D–F). However, no PL was observed at all in the absence of the virus (Fig. 4A–C).

Fig. 5. The calibration curve of the PL intensity corresponding to the concentration of (A) Influenza virus A/Beijing/262/95 (H1N1) and (B) Influenza virus/New Caledonia/20/99lvR116 (H1N1).

In addition, the sensitivity of the PAFI was demonstrated with two different influenza viruses; A/Beijing/262/95 (H1N1) and New Caledonia/20/99lvR116 (H1N1), respectively. All procedures were carried out in a 96-well plate. As the concentration of the influenza virus was increased, the PL intensity corresponding to both of the viruses also increased (Fig. S5). This implies that the binding affinity between the Ab-conjugated AuCNTs and the CdTe QDs was dependent on the virus concentration. Furthermore, using anti-HA Ab (Ab66189)-conjugated AuCNTs and CdTe QDs, we carried out the PAFI for the influenza virus A/Beijing/262/95 (H1N1). The detection was carried out at 518 nm, and a calibration curve corresponding to the virus concentrations was obtained (Fig. 5A). In this case, the detection limit was 1 ng/mL. As the same antibody was conjugated onto the surface of both the nanomaterials, a binding competition took place between the Ab-conjugated AuCNTs and the CdTe QDs against virus. To improve the detection limit of PAFI, various different antibodies were conjugated onto the surface of each of the nanomaterials: anti-NA Ab (New

Caledonia/20/1999/(H1N1))-conjugated AuCNTs and anti-HA Ab (Ab66189)-conjugated CdTe QDs (Fig. 5B, anti-NA Ab; red color Ab and anti-HA Ab; blue color Ab). Using these two different nanostructures the influenza virus/New Caledonia/20/99lvR116 (H1N1) was monitored at the same PL emission wavelength. The calibration curve shows a more reliable linearity than that with the same Ab-conjugated system (Fig. 5B). The sensitivity was more improved, and the detection limitation was 0.1 pg/mL.

3.5. Detection of the clinically isolated Influenza virus

Fig. 6. The calibration curve of the clinically isolated influenza virus A/Yokohama/110/2009 (H3N2).

The clinically isolated sample (influenza virus A/Yokohama/110/2009 (H3N2)) was also detected by PAFI using the anti-HA Ab (Ab82454)-conjugated AuCNTs and CdTe QDs at 518 nm in the 96-well plate. The PL intensity was dramatically changed as a function of the virus concentration. In the presence of the influenza virus, the PL intensity showed an 8-fold increase, as compared to the corresponding intensity in

the absence of the same virus (Fig. S6). The calibration curve was obtained in the range 50–10,000 PFU/mL (Fig. 6.) and the detection limit was 50 PFU/mL. This implies that the clinically isolated virus was successfully detected by PAFI using the Ab-conjugated AuCNTs and the CdTe QDs. The selectivity of PAFI was confirmed using the anti-HA Ab (Ab66189)-conjugated AuCNTs and the CdTe QDs. The anti-HA Ab (Ab66189) could only recognize the HA present in the influenza virus (H1N1). The PL for influenza virus A/Yokohama/110/2009 (H3N2) was low, similar to that of the negative control (BSA) (Fig. S7).

4. Conclusion

In this study, the influenza virus monitoring was successfully demonstrated by interaction between plasmonic nanomaterials and fluorescent particles. In particular, novel PAFI system was developed using AuCNT and CdTe nanomaterials. To perform the PAFI, the AuCNTs were prepared by a simple 2 step-process, using a mild reducing agent without thermal assistance or harsh reducing agents. A large number of Au NPs were decorated onto the surfaces of the CNTs, and they showed a surface plasmon resonance effect at the AuCNT surface. Thus, the AuCNT surface played an important role as the plasmonic substrate for the PAFI. Various influenza viruses were monitored and the detection limits of PAFI against influenza viruses A/Beijing/262/95 (H1N1) and New Caledonia/20/99lvR116 (H1N1) were 1 ng/mL and 0.1 pg/mL, respectively. For the detection of the New Caledonia virus, two different types of antibodies were attached onto the surface of each of the nanomaterials, and then used for monitoring the virus. In this case, the detection limitation was higher than that of the single antibody system (Beijing virus detection), owing to less competitive binding affinity. In previous reports, the detection limitation for proteins

and viruses was reported to be approximately in the ng range (Ahmed et al. 2014; Li et al. 2012; Nooney et al. 2010). As compared to the other PAFI detection systems, the detection limitation for this particular system was improved by about 10-fold. The clinically isolated influenza virus A/Yokohama/110/2009 (H3N2) was also tested, and the corresponding sensitivity was 50 PFU/mL. The PAFI system also showed excellent selectivity, which was 100-fold higher than that of the commercial diagnostic kit. This system may be applied not only to the influenza virus, but also to various other infectious viruses.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version.

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[Supplementary Information]

Plasmon-Assisted Fluoro-Immunoassay of Au Decorated CNT for Influenza Virus Monitoring

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ELISA for Confirmation of Ab Conjugation on the Surface of AuCNTs and CdTe QDs

After preparation of Ab-conjugated AuCNTs via EDC/NHS coupling reaction, 100 μ l of 1000 times diluted anti-mouse IgG-HRP (Santa Cruz Biotechnol., CA) was added into 1 ml of Ab-conjugated nanomaterials. When anti-neuraminidase (NA) Ab (New Caledonia/20/1999/(H1N1)) was used goat anti-rabbit IgG-HRP (Immune Technology Corp., New York, NY, USA) was used for this ELISA. Subsequently, the samples were incubated at room temperature for 1 h, and then purified by centrifuge at 9170 g for 10 min, three times. HRP was developed with 50 μ l TMBZ substrate solution (10 μ g/ml TMB) and 10% H₂O₂ in 100 mM NaOAc (pH 6.0) for 5–30 min at 25°C. The reaction was quitted by adding 25 μ l of 10% H₂SO₄, and then measured the absorbance at 450 nm with a reference at 655 nm using a micro plate reader

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30 (Model 680, Bio-Rad, Hercules, CA, USA). Anti-HA Ab (Ab66189)-, anti-NA Ab (New
31 Caledonia/20/1999/(H1N1))- and anti-HA Ab (Ab82454)-conjugated AuCNTs, and
32 anti-HA Ab (Ab66189)- and anti-HA Ab (Ab82454)-conjugated CdTe QDs were
33 measured by the same protocol as above procedure.

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Supplementary Figures

Figure S1. Low magnification TEM images of (A) MWCNT and (B) AuCNT

Figure S2. Line conductivity measurement: I/V curve of AuCNT and MWCNT

Figure S3. ELISA for confirmation of Ab conjugation on the surface of anti-HA (Ab66189) conjugated AuCNTs (A), anti-NA conjugated AuCNTs (B), and anti-HA (Ab66189) conjugated CdTe QDs (C). Error bars denote standard deviation (SD) (n=3).

Figure S4. ELISA for confirmation of Ab conjugation on the surface of anti-HA (Ab82454) conjugated AuCNTs (A) and anti-HA (Ab82454)-conjugated CdTe QDs (B). Error bars denote standard deviation (SD) (n=3) at 450 nm.

Figure S5. PL spectra monitoring for influenza virus A/Beijing/262/95 (H1N1) (A) and influenza virus/New Caledonia/20/99lvR116 (H1N1) (B).

Figure S6. PL spectra of PAFI system for clinical isolated influenza virus A/Yokohama/110/2009 (H3N2) detection.

Figure S7. PL intensity monitoring for selectivity test of PAFI. When anti-HA Ab (Ab66189)-conjugated AuCNTs and CdTe QDs were used, PL intensity of influenza virus A/Beijing/262/95 (H1N1) was specifically detected, but that of influenza virus A/Yokohama/110/2009 (H3N2) was very low, similar to that of negative control (BSA).