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Enhanced Catalytic Activity of Gold Nanoparticle-Carbon Nanotube Hybrids for Influenza Virus Detection

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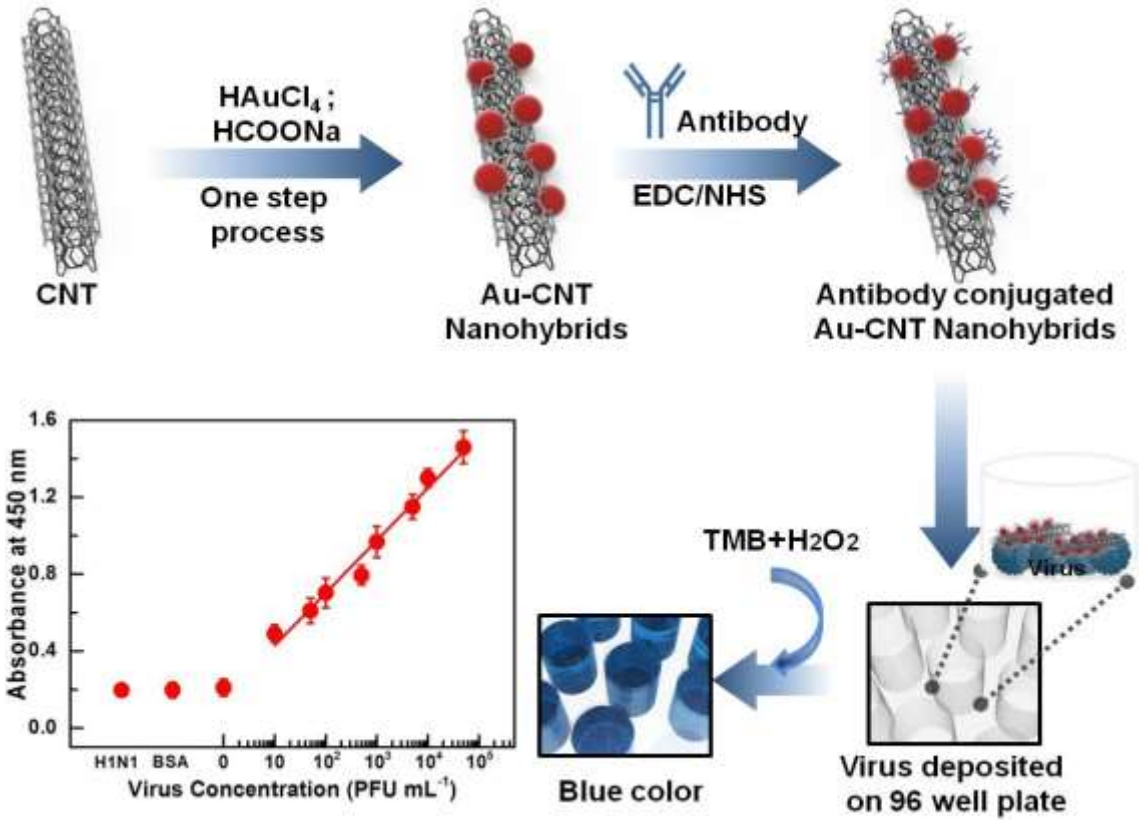
Graphical abstract



ABSTRACT

Multifunctional nanohybrids have created new and valuable opportunities for a wide range of catalysis and biotechnology applications. Here, we present a relatively simple method for producing nanohybrids composed of gold nanoparticles (Au NPs) and carbon nanotubes (CNTs) that does not require an acidic pre-treatment of the CNTs. Transmission electron microscopy (TEM) images and ultraviolet-visible (UV-vis) spectra revealed that Au NPs bonded to the CNT surface. Surface-enhanced Raman scattering (SERS) revealed a stronger signal from Au-CNT nanohybrids than from pristine CNTs. The Au-CNT nanohybrids showed catalytic activity in the oxidation of 3, 3', 5, 5'-tetramethyl-benzidine (TMB) by H_2O_2 and developed a unique blue colour in aqueous solution. Because of the enhanced peroxidase-like activity of these Au-CNT nanohybrids, they were selected for use as part of a highly sensitive colorimetric test for influenza virus A (H3N2). In the presence of influenza A virus (H3N2) in the test system (specific antibody-conjugated Au CNT nanohybrids-TMB-H_2O_2), a deep blue colour developed, the optical density of which was dependent on the virus concentration (10–50,000 PFU/ml). The limit of detection of this proposed method was 3.4 PFU/ml, a limit 385 times lower than that of conventional ELISA (1312 PFU/ml). The sensitivity of this test was also 500 times greater than that of commercial immunochromatography kits. The nanohybrid preparation and colorimetric detection methods reported herein may be easily adapted to other nanohybrid structures with enzyme mimetic properties for broader applications in catalysis and nanobiotechnology.

KEYWORDS: Carbon nanotubes, Gold nanoparticles, Nanohybrids, Surface-enhanced Raman scattering, Enzymatic activity, Colorimetric virus detection

1. Introduction

Recent advances in nanomaterials with enzyme-like activity have opened new avenues for diverse applications in catalysis and nanobiotechnology (Gao et al., 2007; Haider et al., 2015; Hsu et al., 2016; Wei and Wang 2013; Zhang et al., 2013). Over the last several decades, horseradish peroxidase (HRP)-labelled immunoreagents have been widely used in enzyme-linked immunosorbent assays (ELISAs) to detect analytes despite their shortcomings, including their high cost, the large quantity of antibodies needed and poor limits of detection (LOD) (Lin et al., 2013). Substituting HRP with bio-mimetic nanomaterials in ELISA can significantly amplify the detection signal and may reduce the detection costs. Compared to conventional natural enzymes, enzyme-mimetic nanoparticles are more stable, less expensive, and easier to store, and they show enhanced catalytic activity (Zheng and Jiang, 2016). In particular, there has been growing interest in the enhanced enzymatic activity of hybrid nanostructures for potential use in highly sensitive nanobiosensor applications. By combining two or more nanomaterials in a single entity, it is possible to significantly induce catalytic activity and improve the optical, electrical, thermal, and mechanical properties (Zheng et al., 2015). Among the different hybrid nanostructures, the combination of Au NPs and CNTs in a single nanohybrid (Au-CNT) has received considerable attention for numerous applications (Ismaili et al., 2011; Lee et al., 2015; Zhang et al. 2015). CNTs have several exploitable properties, such as large surface area, high mechanical strength, excellent electrical conductivity, and catalytic properties (Eatemadi et al., 2014). Au NPs have a high surface-to-volume ratio and a wide range of size-dependent optical properties and can be easily conjugated to a variety of different proteins without affecting the proteins' biological function, thus making Au NPs excellent biological

carriers (Yeh et al., 2012). Despite their numerous advantages, few efforts have been made to create hybrid structures of CNTs with Au NPs to date. Furthermore, most of the current methods require rigorous and troublesome pre-processing procedures such as boiling in strong nitric acid for five hours (Lee et al., 2015), due to the low dispersibility of CNTs in aqueous media.

To circumvent such pre-processing complexities, we introduce a single-step procedure to prepare Au-CNT nanohybrids using sodium formate (HCOONa) as the reducing agent of chloroauric acid ($HAuCl_4$). This process is environmentally friendly and inexpensive. The Au-CNT hybrid structure produced was further applied to develop a colorimetric detector of influenza virus (used as a model analyte). The influenza virus represents is a growing threat to public health that demands sensitive and accurate diagnosis to prevent virus spread and facilitate early therapy (Ahmed et al., 2014; Chou et al., 2011; Nidzworski et al., 2014).

2. Materials and methods

2.1. Materials

Gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), gold nanoparticles (Au NPs, 10 nm) and human serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium formate (HCOONa), sodium acetate (NaOAc), and hydrogen peroxide (H_2O_2) were purchased from Wako Pure Chemical, Inc. (Osaka, Japan). Multi-walled carbon nanotubes (MWCNTs), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide **(**EDC), N-hydroxysuccinimide (NHS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). The chromogenic substrate 3, 3', 5, 5'-

tetramethyl-benzidine (TMB) was obtained from Dojindo (Osaka, Japan). Anti-influenza A virus HA H1 antibodies [B219M] (ab661189, Lot: GR40088-11) were purchased from Abcam, Inc. (Cambridge, UK). Influenza A (H3N2) hemagglutinin monoclonal antibodies (Anti-H3N2 antibody HA MAb, Lot: HB04N0160) were purchased from Sino Biological, Inc. (Beijing, China). Clinically isolated influenza virus A/Yokohama/110/2009 (H3N2) was kindly provided by Dr. C. Kawakami of the Yokohama City Institute of Health, Japan. HRP-linked anti-mouse IgG antibodies (from sheep) were purchased from GE Healthcare UK, Ltd. (Buckinghamshire, UK). All experiments were carried out using high-purity deionized (DI) water (>18 MΩ·m).

2.2. Preparation of Au-CNT nanohybrid structures

The one-step preparation of *in situ* hybrid Au-CNT nanostructures was performed as follows: 1 ml of 20 mM $HAuCl_4 \cdot 3H_2O$ and 2 mg of MWCNTs were mixed for 5 min. Subsequently, 2 ml of 200 ~500 mM HCOONa was added, and the mixture was maintained at room temperature. $HAuCl_4$ facilitates the dispersion of MWCNTs in aqueous media by surface etching, and HCOONa reduces the gold ions attached to the CNT surface to facilitate nanostructure formation.

2.3. Characterization of antibody specificity

The specificity of the anti-H3N2 HA MAb for influenza virus A/Yokohama/110/2009 (H3N2) was evaluated using ELISA. Briefly, viral stocks were diluted with phosphate-buffered saline (PBS, pH 7.5) to a final titre of 5000 plaque-forming units (PFU)/ml to perform ELISA. Virus solution (100 μl) was then added to

each well of a nonsterile polystyrene 96-well flat-bottom microtiter plate (Becton Dickinson Labware, NJ, USA) and was incubated overnight at 4°C to allow adsorption of the virus onto the plates. The plates were then rinsed with PBS (pH 7.5) and surface pores were blocked with 100 µl of 2% skim milk for 2 h at room temperature. Anti-H3N2 HA Mab (1 µg/ml), anti-H1N1 HA antibody (HA Ab66189; 1 µg/ml), and 1 ng/ml bovine serum albumin (BSA) (100 µl/well) were added to the pre-adsorbed wells, and the plates were incubated for 1 h at room temperature. The secondary antibody, HRP-labelled anti-mouse IgG (GE Healthcare UK Limited, UK), was then added to each well, followed by the chromogenic substrate, TMB. The absorbance of the enzymatic product at 450 nm was measured using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA) to quantify the interaction of the antibodies with the influenza viruses.

2.4. Binding of anti-influenza A (H3N2) antibody to Au-CNT nanohybrids

One millilitre of Au-CNT solution (1 µg/ml), EDC (4 mM) and NHS (10 mM) were mixed and incubated for 10 min, followed by the addition of 1 µl HA MAb (final concentration, 5 µg/ml). The solution was stirred at 4°C for 8 h. Finally, the supernatant and unbound antibodies were removed through centrifugation, and the antibody-conjugated Au-CNT nanohybrids were redispersed in 1 ml Milli-Q water. Amide bonding is the mechanism of antibody/Au-CNT nanohybrid binding.

2.5. Colorimetric detection of influenza A (H3N2) virus in human serum

Influenza virus A (H3N2) stock solution (8×10^6 PFU/ml) was serially diluted with human serum to create sensing subjects for this experiment. Virus solution (100 µl) was

then added to each well of a nonsterile polystyrene 96-well flat-bottom microtiter plate (Becton Dickinson Labware) and incubated overnight at 4°C to allow the adsorption of the virus to the plates. BSA (100 µl, 1 ng/ml) and influenza A (H1N1) virus (100 µl, 1 µg/ml) were used as negative controls for selectivity. The plates were then rinsed with PBS (pH 7.5) and blocked with 100 µl of 2 % skim milk for 2 h at room temperature. Fifty microliters of 1 ng/mL antibody-conjugated Au-CNTs was added to the pre-adsorbed wells, and the plates were incubated for 1 h at room temperature. After washing three times with PBS (pH 7.5), a 100 µl mixture of TMB (5 mM) and H_2O_2 (10 mM) was added into each of the wells. The microplate was then incubated for 10 min at room temperature, which resulted in the development of a blue colour, whose intensity was dependent on the viral titre. Finally, 100 µl of 10% H_2SO_4 was added to each well to stop the reaction. The absorbance at 450 nm was measured using a microplate reader (Model 680, Bio-Rad). Based on the absorbance values at different influenza virus concentrations A/Yokohama/110/2009 (H3N2), a dose-dependent curve was constructed.

2.6. Spectroscopy and structural characterization

Transmission electron microscopy (TEM) images were acquired using a TEM (JEM-2100F, JEOL, Ltd., Tokyo, Japan) operated at 100 kV. The ultraviolet-visible (UV-vis) spectra of the nanostructured films were recorded using a Tecan Infinite M200 spectrophotometer (Infinites F500, TECAN, Ltd., Männedorf, Switzerland). Antibody-conjugated Au-CNTs were monitored by Fourier transform infrared spectroscopy (FT-IR spectroscopy) (FT/IR 6300, JASCO Corp., Tokyo, Japan).

3. Results and discussion

3.1. Design and preparation of Au-CNT nanohybrids, and application to virus sensing

Several methods for preparation of Au-CNT nanohybrids have been reported. However, most of these methods are complicated multi-step processes that require the use of many chemicals as reducing or stabilizing agents (Lee et al., 2015; Zhang et al. 2013). To overcome these difficulties, we used sodium formate as the reducing agent for $HAuCl_4$, with the result that the Au NPs spontaneously bonded to the surface of the CNTs (Scheme 1). In this nanohybrid formation method, there is no need for acidic pre-treatment of CNTs, extra stabilizing agents, or a multitude of procedural steps. The most likely mechanism for our method involves acid etching of the hydrophobic CNT surface by $HAuCl_4$, which introduces structural defects that allows the attachment of Au^{3+} ions.

The Au-CNT nanohybrids catalyze the oxidation of TMB by H_2O_2 to develop a blue colour in aqueous solution. Au-CNTs can initiate reactions that are related to the decomposition of the O-O bond between H_2O_2 and an HO• radical. The HO• radical then oxidizes TMB, which results in the development of a typical blue colour (Scheme 1). Accordingly, the colour change depends on the concentration of Au-CNT nanohybrids. In the virus-sensing system, the antibody-conjugated Au-CNT nanohybrids bind with the target viruses on a 96-well plate, and unbound Au-CNT nanohybrids are washed out. Following the addition of TMB and H_2O_2, a typical blue colour develops, with its intensity depending on the concentration of bound Au-CNT nanohybrids. This phenomenon can be used as a strategy for quantitative influenza virus

detection and is a straightforward approach for spectrophotometric viral quantitation.

[Scheme 1]

3.2. Morphology of Au-CNT nanohybrids

The morphology of Au-CNT nanohybrids is shown in Figure 1B–F. Au NPs (seen as black dots in Fig. 1) are securely attached to the CNT surface and can be clearly distinguished from pristine CNTs (Fig. 1A). Au NPs of different sizes, depending on the concentration of HCOONa present during synthesis, (i.e., ~60 nm (200 mM HCOONa, Fig. 1B), ~40 nm (300 mM HCOONa, Fig. 1C), ~25 nm (400 mM HCOONa, Fig. 1D), or ~10 nm (500 mM HCOONa, Fig. 1E)), as well as some nanoclusters, are distributed on the CNT surface. This allows a wide range of absorbance and is advantageous for energy applications. Moreover, Au NPs and some nanoclusters attach densely over a large surface area of the CNTs (Fig. 1F). This is crucial for the application of nanohybrids in biosensor applications such as plasmon-assisted fluoro-immunoassays (Lee et al., 2015), enhanced colorimetric detection and electrochemical sensors. Usually, nanoparticles of smaller size showed greater peroxidase-like catalytic activity due to the large surface to volume ratio for interaction with substrates (Jv et al., 2010). In this study, Au-CNT nanohybrids with 10 nm Au NPs were further used to observe SERS and catalytic activities.

[Figure 1]

The time course of Au-CNT nanohybrid preparation was also studied. A time sequence of TEM images of Au-CNT (~10 nm Au NPs) preparation is shown in Figure

S1. The initial TEM image (10 min after reaction start, Fig. S1A) showed few Au NPs attached to the CNT surface. More Au NPs and some nanoclusters attached to the CNT surface over time (Fig. S1B after 20 min and Fig. S1C after 40 min of reaction) such that within 1 h, a large number of Au NPs had attached to a large fraction of the CNT surface area at room temperature (Fig. S1D).

3.3. SERS properties of Au-CNT nanohybrids

UV spectroscopic examination revealed a broad absorbance spectrum of Au-CNT nanohybrids due to non-uniform attachment of Au NPs to the CNT surface (Fig. 2A). In contrast, no plasmonic peak was observed for bare CNTs. The SERS spectra for Au-CNT nanohybrids were recorded by Raman spectroscopy (Ramboss 500i, DongWoo Optron Co., Ltd., Gwangju, Korea) using 532-nm excitation. Four peaks (3 strong, 1 weak) were observed in the Raman spectra as seen in Fig. 2B. A closer view of Fig. 2B shows clearly distinguishable peaks (Fig. 2C & D). The spectrum contains a D band at 1340 cm^{-1} that arises due to the presence of local structural defects known as sp^3 defects (Lehman et al., 2011). The other peak at 1590 cm^{-1}, called the G-band, is the spectral product of sp^2-containing graphitic carbon materials. Another low-intensity peak at 1620 cm^{-1}, known as the D′ mode, appeared due to the local structural defects and amorphous carbon (Lehman et al., 2011; Osswald et al., 2005). Additionally, a single strong peak can be seen at 2710 cm^{-1} in Fig. 2D, which corresponds to second-order scattering (2D mode) as a result of two-phonon resonance (Sharma et al., 2013).

The Raman intensity of the Au-CNT nanohybrids was enhanced by approximately 6 times compared to that of the pristine CNTs; the 2D mode was also enhanced for the

nanohybrids. In this study, enhanced SERS intensity from Au-CNT nanohybrids was observed due to the local optical field created by electronic interaction between the Au NPs and CNTs.

[Figure 2]

3.4. Dispersibility and catalytic activity of Au-CNT nanohybrids

Due to the accumulation of Au NPs on the CNT surface, the dispersibility of the CNTs in aqueous media improved significantly (Fig. 3A). The Au-CNT nanohybrids formed a stable and well-dispersed suspension in water within one minute and also formed flocs. In contrast, the CNTs without nanohybrid function were less dispersed in aqueous media. A feasibility study of peroxidase activity was carried out using Au-CNTs and CNTs. As shown in Fig. 3B, the Au-CNT solution (before adding H_2SO_4 to stop the reaction) developed a deep blue colour within one minute of adding the TMB-H_2O_2 solution, whereas the CNT and Au NP solution developed a pale blue colour over a longer period of time. Moreover, the optical density of the Au-CNT solution was approximately six and three times higher than that of the CNT and Au NP solution, respectively. This indicates that adding Au NPs to a hybrid structure can accelerate the catalytic oxidation of TMB with H_2O_2. This finding may be helpful for the development of rapid and sensitive colorimetric biosensors.

[Figure 3]

3.5. Colorimetric detection of influenza A (H3N2) virus using Au-CNT nanohybrids

The strong peroxidase-like activity of Au-CNT nanohybrids with TMB-H_2O_2 was

further applied to detect clinically isolated influenza A (H3N2) virus in human serum. The optimum conditions for catalytic activity of Au-CNT nanohybrids were investigated by varying the pH from 3 to 10, the temperature from 10ºC to 50ºC, reaction time from 0 to 8 min, using 4 different buffer solutions, varying H_2O_2 concentration from 0 to 20 mM and TMB concentration from 1 to 10 mM. The optimal pH and temperature were approximately pH 7.5 and 25ºC respectively. These were adopted as standard conditions for subsequent measurement of nanohybrid catalytic activity (Fig. S2A and B). Nanohybrids consistently showed the highest peroxidase activity within 5 min in all four buffer solutions (Fig. S2C and D). PBS buffer was chosen as the optimal medium for this experiment. It was also observed that the nanohybrid catalytic activity required a H_2O_2 and TMB concentrations at a ratio of 2:1 to maximize peroxidase activity (Fig. S3A and B).

The specificity of the anti-influenza A HA MAb for influenza A (H3N2) virus was confirmed using ELISA. Fig. 4A shows that the optical density of HA MAb was higher than that of either HA Ab 66189 or BSA, implying the specificity of anti-influenza A HA MAb for the influenza A (H3N2) virus. The conjugation of anti-influenza A (H3N2) HA MAb with the Au-CNT nanohybrids was confirmed using FTIR spectroscopy (Fig. 4B). The FTIR bands at 3100–3500 cm^{-1} and 1630–1690 cm^{-1} represented amide N–H stretching and amide C=O stretching vibrational modes respectively, which occurred because of amide bonding between the Au-CNT nanohybrids and anti-H3N2 HA MAb antibodies.

Before performing the sensing experiments, we carefully tested antibody selectivity using the defined reaction mixture of influenza A (H3N2) with specific

antibody [anti-H3N2 HA MAb]-conjugated to Au-CNTs and TMB-H_2O_2, and three separate modified mixtures as follows: 1) substitution of the specific antibody [anti-H3N2 HA MAb] with a non-specific antibody [anti-H1N1 Ab 66189] in the defined reaction mixture; 2) removal of TMB from the defined reaction mixture; 3) removal of H_2O_2 from the defined reaction mixture. In the original reaction mixture, a deep blue colour developed upon addition of the TMB-H_2O_2 solution, and a strong characteristic absorption peak at 655 nm was observed (Fig. 4C). However, none of the modified mixtures displayed this characteristic peak (Fig. 4C). These results suggest that the proposed sensing method is highly specific and shows colour development only in the presence of the target virus, specific antibody-conjugated Au-CNTs, TMB and H_2O_2.

Different concentrations of influenza A (H3N2) virus were used to determine the linearity between absorbance and virus concentration. The sensitivity of this system for the influenza A (H3N2) virus was found to be in the range of 10–50,000 PFU/ml. The limits of detection (LOD) of our proposed method and the conventional ELISA method were calculated as 3.4 and 1312 PFU/ml, respectively, based on the standard deviation method (Fig. 4D) (Apostol et al., 2009). However, no significant colour developed for BSA or the H1N1 virus.

[Figure 4]

The effects of interfering substances were also investigated by using IgG (Goat anti-bovine IgG, Jackson Immuno Research Laboratories Inc., PA, USA) and BSA to help confirm the reliability of proposed methods. The results revealed that the proposed colorimetric immunoassay was not interfered with by other substances even in complex

biological matrices (Fig. S4).

The sensitivity of the proposed method was compared with that of a commercially available rapid influenza diagnostic kit, which we have studied previously (ImunoAce Flu, TAUNS Laboratories, Inc., Numazu, Shizuoka, Japan, Ahmed et al., 2014), and the conventional ELISA method (Table 1 & Fig. 4D). A linear response to virus detection with ELISA was seen up to 1000 PFU/ml, whereas in the ImunoAce Flu kit, it was seen up to 5000 PFU/ml. This indicated that our system is 100 times and 500 times more sensitive than conventional ELISA and commercial immunochromatography, respectively. This new approach to the preparation of *in situ* hybrid nanostructures can be used to develop simple, highly sensitive sensing platforms that can detect targets without the need for complex equipment or related training, and it has great potential for use in low-cost colorimetric point-of-care (POC) diagnosis techniques.

[Table 1]

5. Conclusions

In this study, we introduced a straightforward method for the preparation of Au-CNT nanohybrids via the *in situ* accumulation of Au NPs on CNTs under mild conditions. This approach allows us to substantially improve the SERS signal, the solubility of CNTs in aqueous solution, and the peroxidase-like activity of Au–CNT nanohybrids. Based on the enhanced catalytic properties of the nanohybrids, we developed a colorimetric assay to detect clinically isolated influenza virus A (H3N2). A linear response was seen in up to 10 PFU/ml in human serum, which is 100 times and 500 times more sensitive than conventional ELISA and a commercial

immunochromatography kit, respectively. Hence, our findings provide positive proof of concept for the potential of Au–CNT nanohybrids in the development of colorimetric biosensors that are simple, robust, and cost-effective.

Acknowledgements

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Figure Legends

Scheme 1. Schematic illustration of the one-step preparation of Au-CNT nanohybrids using HCOONa and $HAuCl_4$. The antibody was conjugated with the Au-CNT nanohybrids through amide bonding, and proof of peroxidase-like activity based on colorimetric detection of virus deposited on 96-well plates was established. In the absence of Au-CNT nanohybrids, the 3,3',5,5'-tetramethylbenzidine (TMB)-H_2O_2 mixed solution was colourless. In the presence of Au-CNT nanohybrids, the oxidized TMB (oxTMB)-H_2O_2 solution produced a strong blue colour.

Fig. 1. Transmission electron microscope images of Au-CNT nanohybrids: (A) CNTs; (B) Au-CNT nanohybrids with ~60-nm-sized Au NPs; (C) Au-CNT nanohybrids with ~40-nm-sized Au NPs; (D) Au-CNT nanohybrids with ~25-nm-sized Au NPs; (E) Au-CNT nanohybrids with ~10-nm-sized Au NPs; (F) Au-CNT nanohybrids over a large-scale area (~3 μm).

Fig. 2. Spectroscopic analysis of Au-CNT nanohybrids: (A) UV-visible spectra of Au-CNT nanohybrids; (B) SERS profiles of Au-CNTs; (C & D) A closer view of the SERS profile of (B).

Fig. 3. Dispersibility and catalytic activity of Au-CNTs: (A) Photograph showing the dispersibility of CNTs and Au-CNTs in aqueous media; (B) Comparison of the peroxidase activities of Au NPs, CNTs and Au-CNTs on TMB in the presence of H_2O_2 (inset: note the difference in the solution colours before reaction-stopping agent was added). Error bars in (B) denote standard deviation (n=3).

Fig. 4. Detection of influenza virus using Au-CNT hybrid nanostructures: (A) ELISA

results showing antibody specificities for the target virus; (B) FTIR spectra of antibody binding with Au-CNTs; (C) Selectivity test of the proposed sensing method using the defined reaction mixture (a) of influenza A (H3N2) with specific antibody [anti-H3N2 HA MAb]-conjugated to Au-CNTs and TMB-H_2O_2, and three separate modified mixtures as follows: b) substitution of the specific antibody [anti-H3N2 HA MAb] with a non-specific antibody [anti-H1N1 Ab 66189] in the defined reaction mixture; c) removal of TMB from the defined reaction mixture; d) removal of H_2O_2 from the defined reaction mixture. (D) Absorbance versus concentration of the influenza virus A/Yokohama/110/2009 (H3N2). BSA was used as a negative control; H1N1 denotes influenza virus A/New Caledonia/20/1999 (H1N1) that was used a control to test specificity for H3N2 virus detection. Closed and open circles denote proposed and conventional ELISA methods, respectively. Error bars in (A) and (D) denote standard deviation (n=3).

Scheme 1, Ahmed et al.

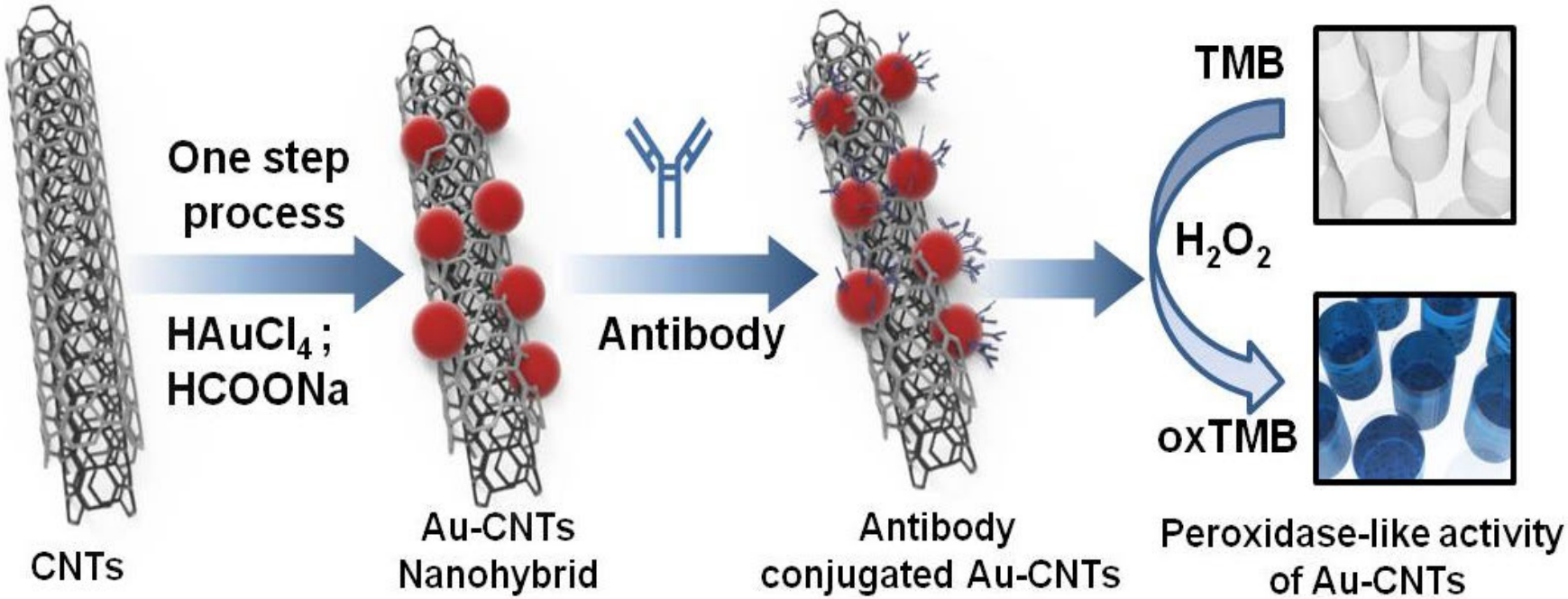


Fig. 1, Ahmed et al.

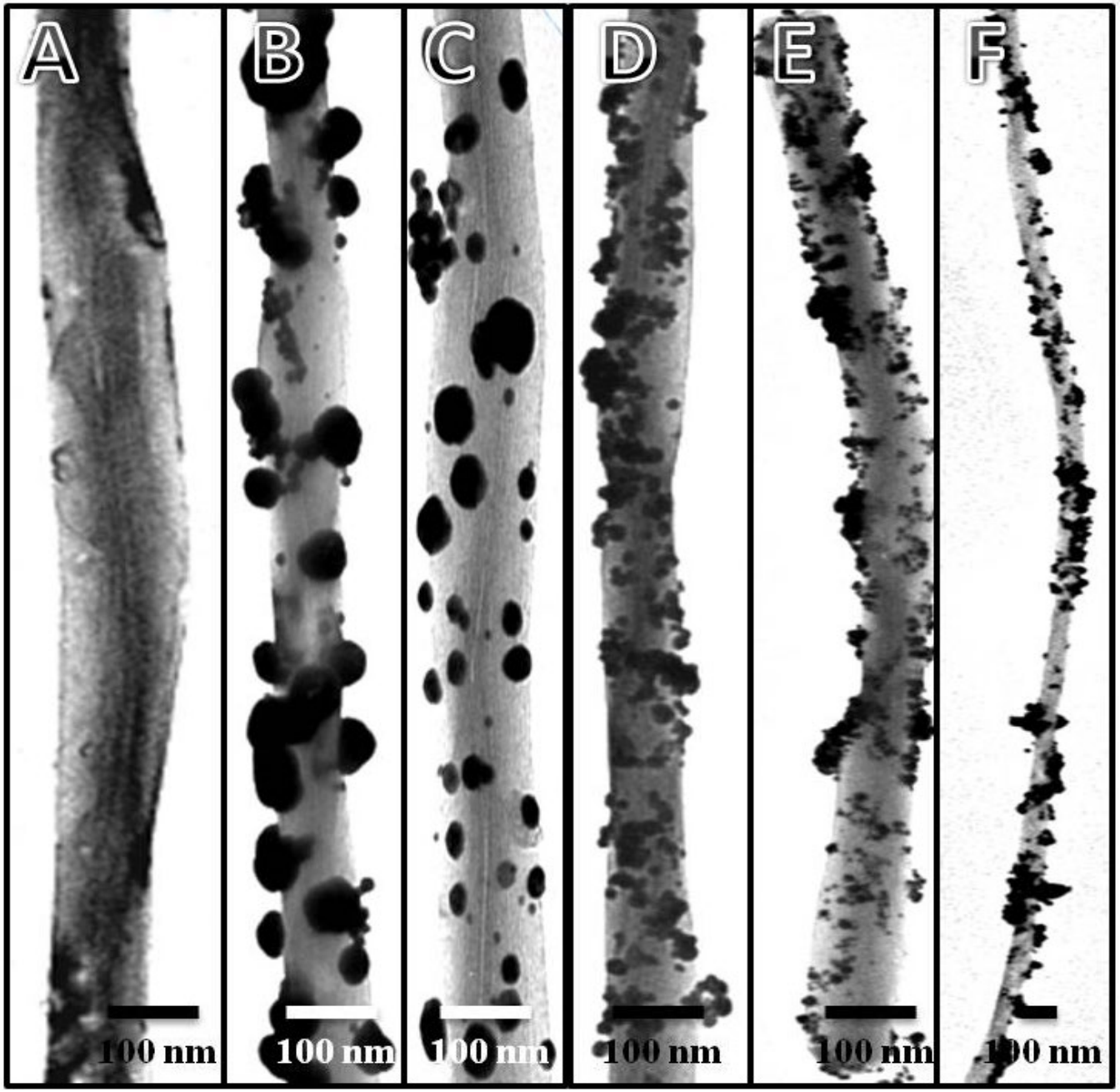
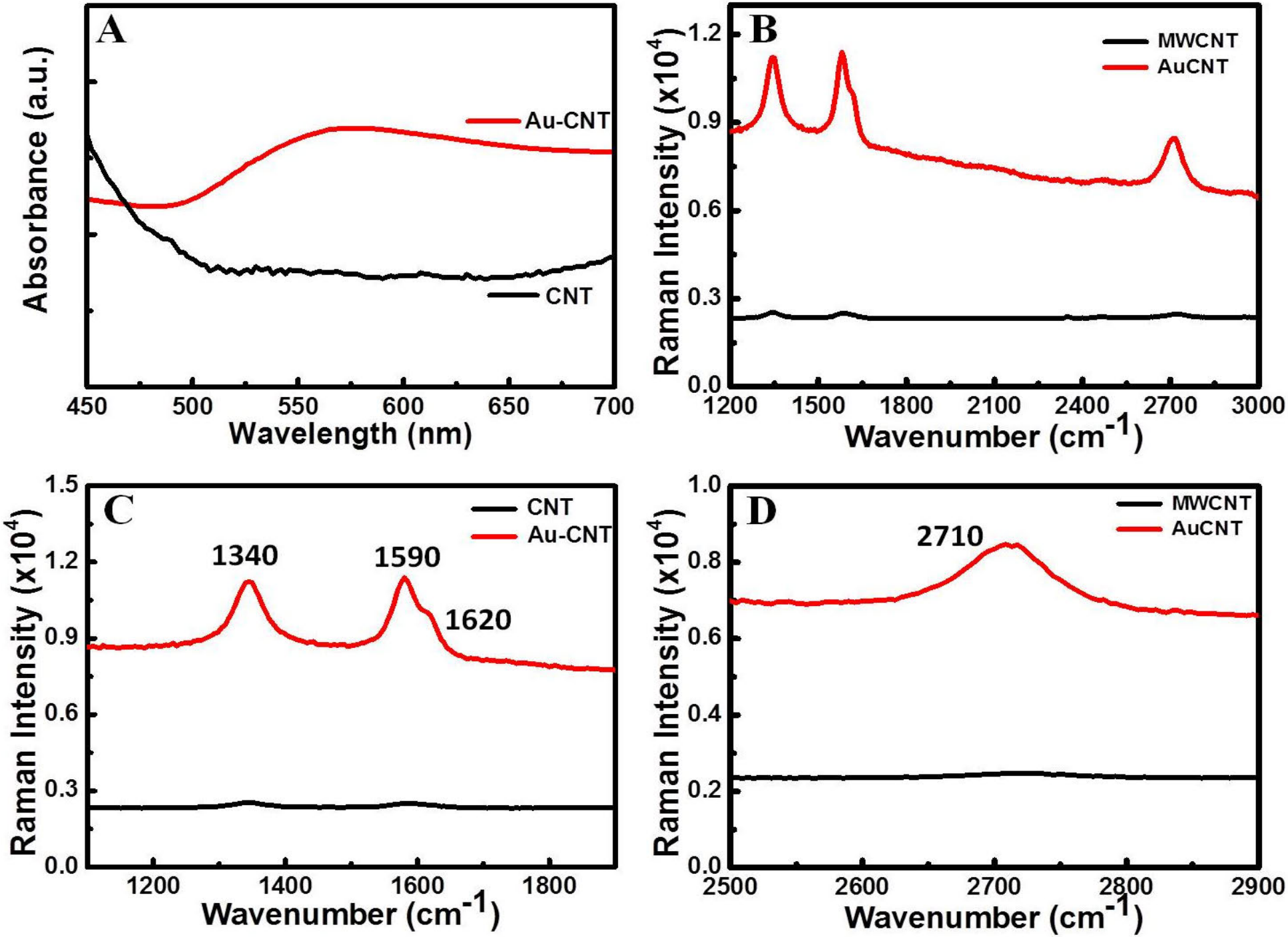


Fig. 2, Ahmed et al.



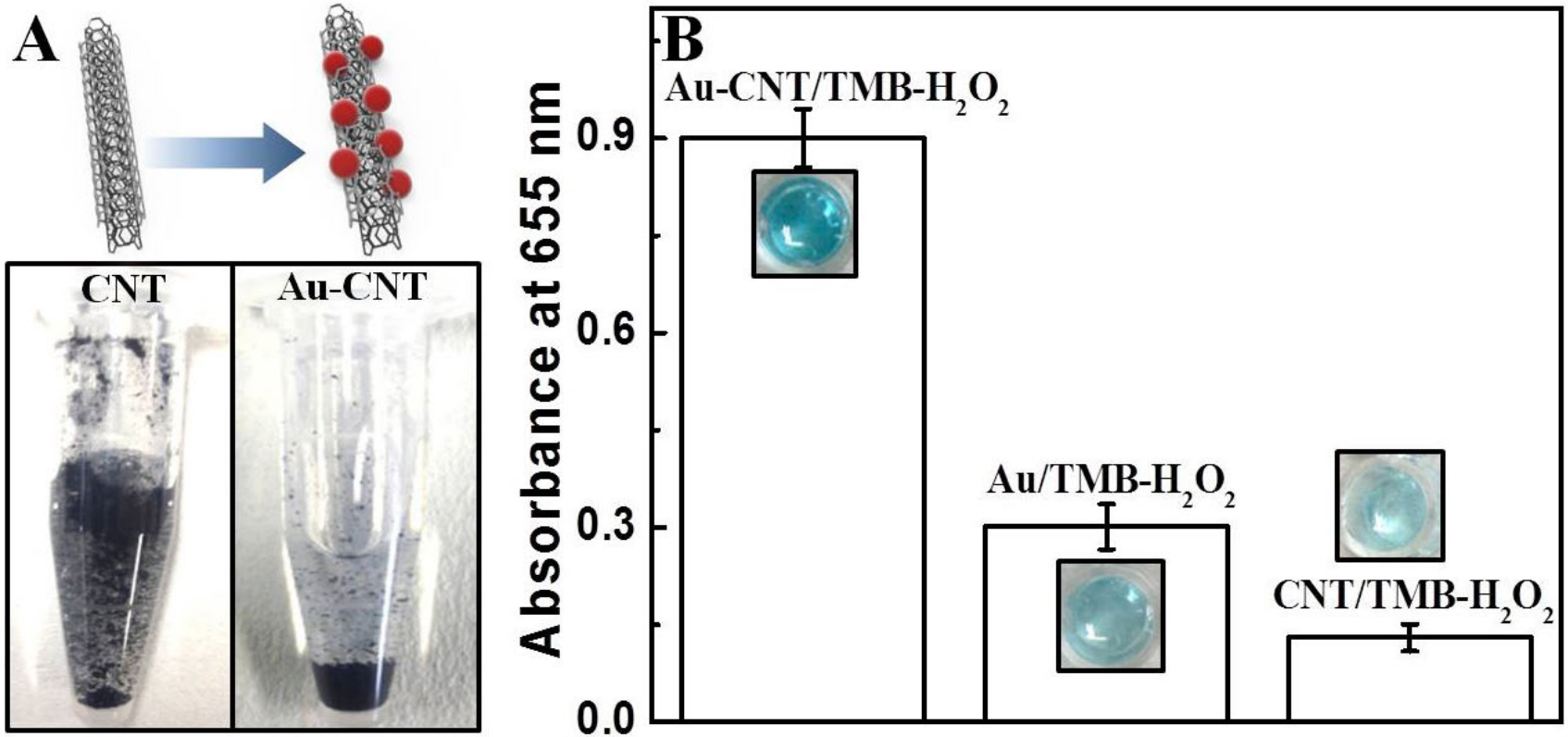

A
CNT
Au-CNT
B
Au-CNT/TMB-H2O2
Au/TMB-H2O2
CNT/TMB-H2O2
Absorbance at 655 nm
0.0
0.3
0.6
0.9

Fig. 4, Ahmed et al.

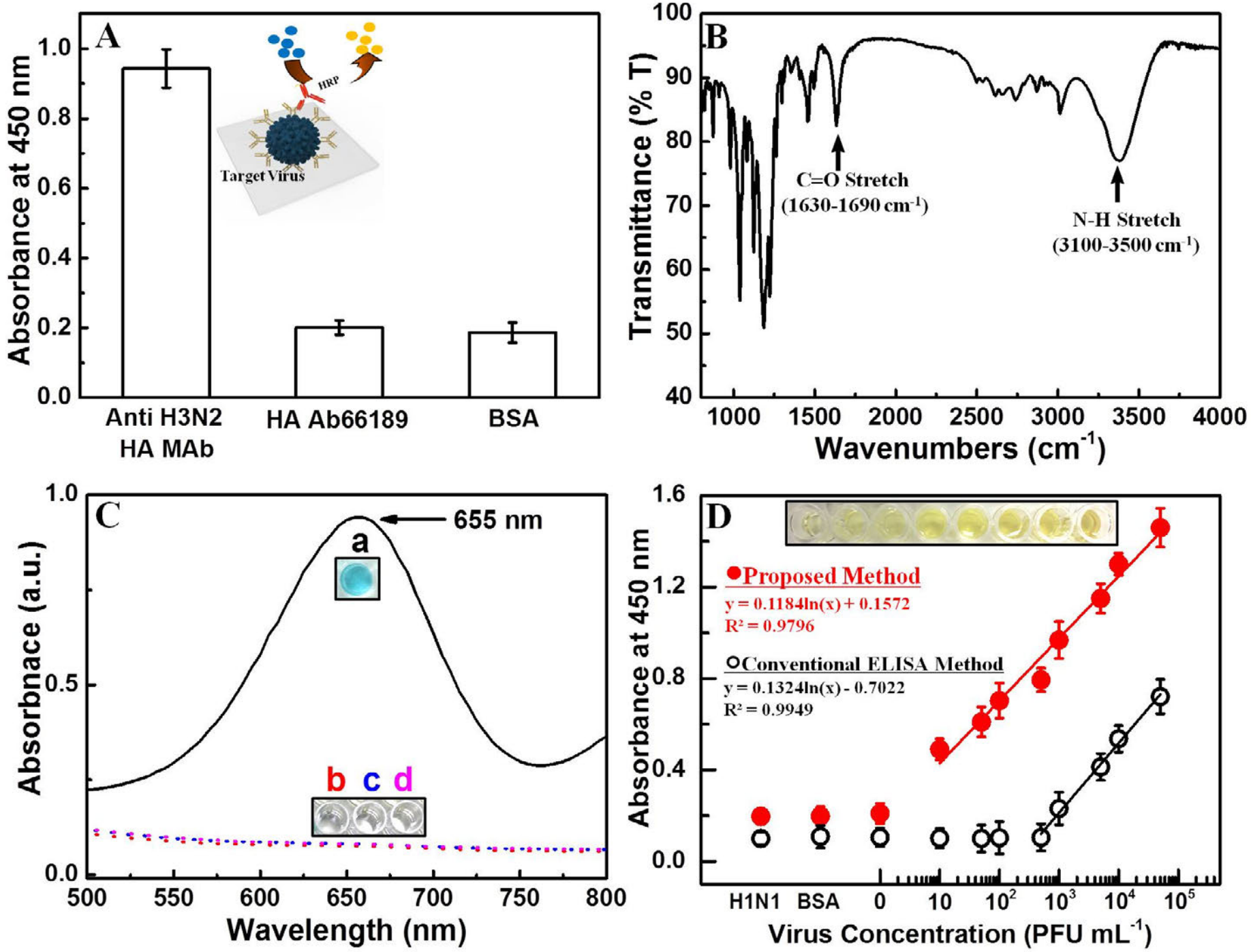


Table 1: Comparison of influenza virus A/Yokohama/110/2009 (H3N2) detection using different methods

Detection method	Virus concentration (PFU/mL)								
	10000	5000	1000	500	100	50	10	1	0
Au-CNT	+	+	+	+	+	+	+	–	–
Conventional ELISA	+	+	+	–	–	–	–	–	–
Commercial immunochromatography kit (Ahmed et al. 2014)	+	+	–	–	–	–	–	–	–

*Note: + and - denote the positive and negative diagnoses, respectively.

Supplementary File

Enhanced catalytic activity of gold nanoparticle-carbon nanotube hybrids for influenza virus detection

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Time dependent morphology of Au-CNT nanohybrids

To study time dependent nanohybrids morphology, 1 ml of 20 mM $HAuCl_4 \cdot 3H_2O$ and 2 mg of MWCNTs were mixed for 5 min. Subsequently, 2 ml of 500 mM HCOONa was added, and at the mixture was maintained at room temperature. TEM images of Au-CNT nanohybrids (~10 nm Au NPs) over time are shown in Figure S1. The initial TEM image (10 min after reaction start, Fig. S1A) showed few Au NPs attached on the CNT surface. With time, more Au NPs and some nanoclusters attached on the CNT surface (Fig. S1B after 20 min and Fig. S1C after 40 min of reaction). Within 1 hour, many Au NPs attached over a large surface area of the CNTs at room temperature (Fig. S1D).

Fig. S1. Transmission electron microscope images of Au-CNT nanohybrids after (A) 10 min; (B) 20 min; (C) 40 min; (D) 60 min of reaction at room temperature.

pH, Temperature, reaction time and buffer solution dependence

The optimum conditions for catalytic activity of Au-CNT nanohybrids were studied on pH, temperature, reaction time and buffer solution before sensing experiments. The peroxidase-like activity of nanohybrids was varying the pH from 3 to 10, the temperature from 10ºC to 50ºC, reaction time from 0 to 8 min, 4 different buffer solutions, the H_2O_2 concentration from 0 to 20 mM and TMB concentration from 1 to 10 mM (Fig. S2 and 3). The optimal pH and temperature were approximately pH 7.5 and 25ºC, respectively and adopted as standard conditions for subsequent analysis of nanohybrids catalytic activity (Fig. S2A and B). Nanohybrids showed highest peroxidase activity within 5 min which not varied too much in different buffer solution (Fig. S2C and D). So, PBS buffer was chosen as optimal media in this experiment.

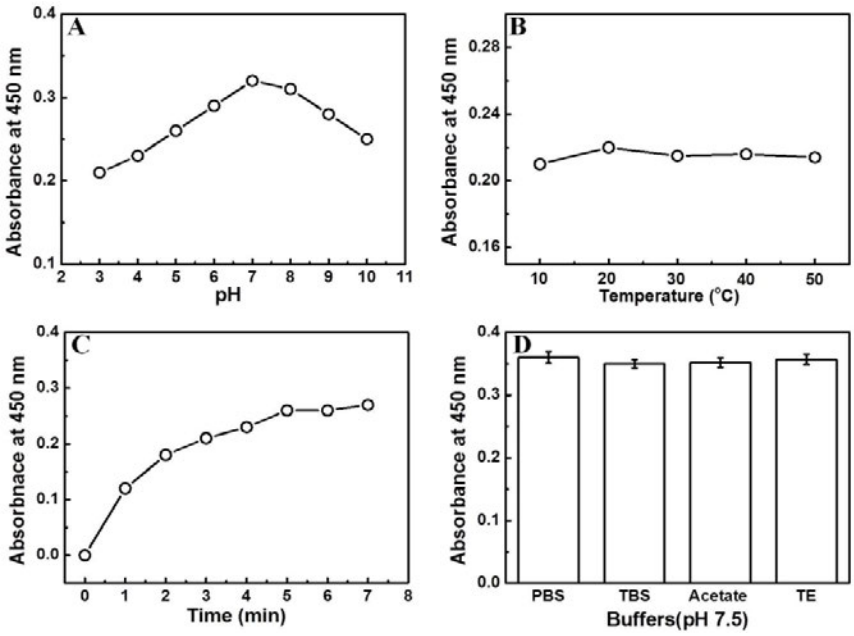

Fig. S2. The peroxidase-like activity of Au-CNTs nanohybrids (1μg/ml) in different: (A) pH (Reaction conditions: 5 mM TMB, 10 mM HRP); (B) Temperature (Reaction conditions: 5 mM TMB, 10 mM HRP at pH 7.5); (C) Reaction time (Reaction conditions: 5 mM TMB, 10 mM HRP and pH 7.5 at 25ºC); (D) Buffer solution (Reaction conditions: 5 mM TMB, 10 mM HRP and pH7.5 at 25ºC). Error bars denote standard deviation (n=3).

Dependence of Au-CNT nanohybrids catalytic activity on H_2O_2 and TMB concentration

It was observed that the catalytic activity of nanohybrids required a H_2O_2 concentration (10 mM) two times higher than TMB (5 mM) to reach the maximal level of peroxidase activity (S3A and B).H_2O_2 and TMB concentration pH 7.5 at 25ºC

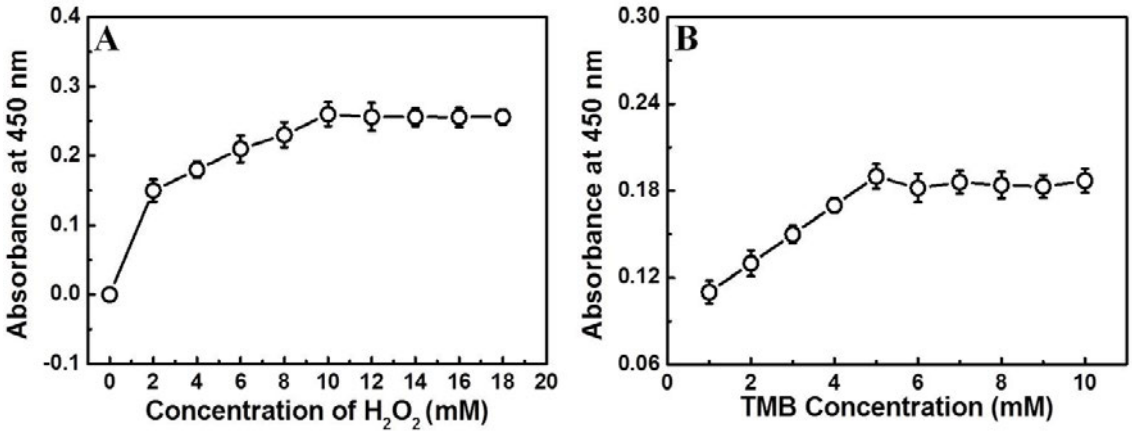

Fig. S3. The peroxidase-like activity of Au-CNTs nanohybrids (1μg/ml) with varying: (A) TMB concentrations (10 mM) are fixed and H_2O_2 concentrations are varied; (B) H_2O_2 concentrations (10 mM) are fixed and TMB concentrations are varied. Reaction conditions were fixed at pH 7.5 and 25ºC. Error bars denote standard deviation (n=3).

Effect of interfering substance on virus detection

The interfering effect of interfering substance (BSA and IgG) in detection media on detection reliability was also investigated. Experiments were performed based on previously mentioned (section 2.5) with IgG (1 ng/ml) and BSA (1 ng/ml). The target influenza A (H3N2) virus (50,000 PFU/ml) was prepared in human serum, then each IgG and BSA was added on it. Virus solution (100 µl) was then added to each well of a nonsterile polystyrene 96-well flat-bottom microtiter plate and incubated overnight at 4°C to allow adsorption of the virus to the plates. The plates were then rinsed with PBS (pH 7.5) and blocked with 100 µl of 2 % skim milk for 2 h at room temperature. One nanogram per milliliter of antibody-conjugated Au-CNTs (50µl) was added to the pre-adsorbed wells, and the plates were incubated for 1 h at room temperature. After washing three times with PBS (pH 7.5), a 100 µl mixture of TMB (5 mM) and H_2O_2 (10 mM) was added into each of the wells. The microplate was then incubated for 5 min at room temperature, which resulted in the development of a blue color. Finally, 100 µl of 10% H_2SO_4 was added to each well to stop the reaction. The absorbance at 450 nm was measured using a microplate reader (Model 680, Bio-Rad). Results revealed that proposed colorimetric immunoassay was not much interfered by interfering substance, BSA or IgG in complex biological matrices (Fig. S4).

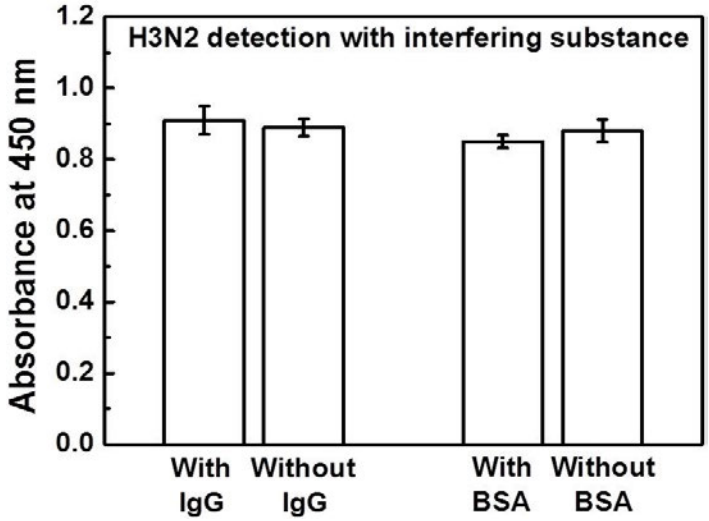

Fig. S4. Interfering effect of IgG and BSA on the reliability of virus detection performance. IgG (Goat anti-bovine IgG) and BSA were used as potential interfering substances. Error bars denote standard deviation (n=3).