

Smartphone-Based Point-of-Care Microfluidic Platform Fabricated with a ZnO Nanorod Template for Colorimetric Virus Detection

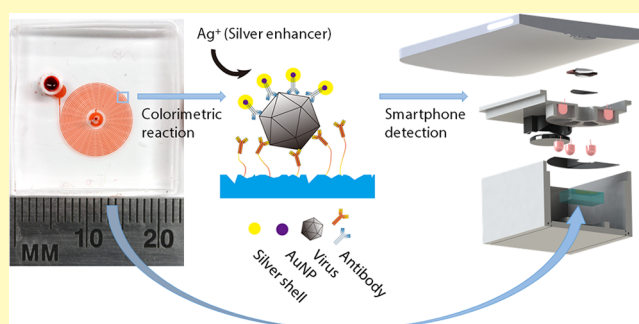
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Supporting Information

ABSTRACT: Viruses pose serious infectious disease threats to humans and animals. To significantly decrease the mortality and morbidity caused by virus infections, there is an urgent need of sensitive and rapid point-of-care platforms for virus detection, especially in low-resource settings. Herein, we developed a smartphone-based point-of-care platform for highly sensitive and selective detection of the avian influenza virus based on nanomaterial-enabled colorimetric detection. The 3D nanostructures, which serve as a scaffold for antibody conjugation to capture the avian influenza virus, are made on PDMS herringbone structures with a ZnO nanorod template. After virus capture, the on-chip gold nanoparticle-based colorimetric reaction allows virus detection by naked eyes with a detection limit of 2.7×10^4 EID₅₀/mL, which is one order of magnitude better than that of conventional fluorescence-based ELISA. Furthermore, a smartphone imaging system with data processing capability further improves the detection limit, reaching down to 8×10^3 EID₅₀/mL. The entire virus capture and detection process can be completed in 1.5 h. We envision that this point-of-care microfluidic system integrated with smartphone imaging and colorimetric detection would provide a fast, cheap, sensitive, and user-friendly platform for virus detection in low-resource settings.

KEYWORDS: point-of-care, smartphone imaging, influenza, ZnO nanowire, colorimetric biosensor, microfluidic device



Viral infectious diseases, such as Ebola, Zika, chikungunya, and influenza, are serious threats for global public health. It is one of the leading causes of morbidity and death. They can also cause significant economic loss, which poses grave burden and stress to the society.^{1–4} According to United States Centers for Disease Control and Prevention, the World Health Organization, and global health partners, up to 650 000 people die from respiratory diseases caused by seasonal influenza each year.⁵ In the latest 2017–2018 influenza season alone (from Oct. 1, 2017 to Mar. 31, 2018), there were 142 pediatric deaths due to influenza in United States alone.⁶ Among influenza viruses, avian influenza virus (AIV) is one of the most important zoonotic origins of human influenza epidemics and the frequent cause of disasters in poultry industry.^{7,8} The H5N1 AIV human infections broke out in Hong Kong in 1997, and then, it spread to Europe and Africa. The continuous transmission resulted in infections affecting hundreds of humans, many human deaths, and millions of birds killed.⁹ In 2013, H7N9 AIV was first reported in China and caused over 1500 human infection cases and many deaths.⁹ From Jan 2015 to Jun 2015, H5N2 and H5N8 AIV caused the largest animal health emergency in both United States and Canada, which decimated poultry flocks in 21 states.¹⁰ Therefore, there

is an urgent need to develop portable and low-cost tools to detect and monitor AIV to significantly mitigate its threats globally. Currently, AIVs are mainly detected by virus isolation in chicken embryos, enzyme-linked immunosorbent assay (ELISA), and reverse transcription polymerase chain reaction.^{11–13} However, these conventional methods cannot meet the urgent needs of rapid and sensitive AIV detection in the low-resource areas because of poor sensitivity, lengthy procedure, and complicated procedures.

We reason that a sensitive and rapid point-of-care (POC) platform with a simple setup to detect viruses at any location would be a great tool for influenza surveillance. Potential virus infection cases can be rapidly detected in the early stage at remoted locations with limited medical sources; therefore, immediate prevention and control procedures can be implemented to prevent virus spread and disease outbreak. POC platforms in healthcare are used to obtain diagnostic results rapidly and accurately at or near the sites of the patient care.^{14–16} In the past decades, many POC biosensors have

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been developed for rapid and sensitive virus detection by integrating microfluidics, sensors, and electronics.^{17–20} Multiple functions can be integrated into a single microfluidic device to make versatile virus detection platforms.^{21,22} Such virus detection platforms are attractive because of their low sample consumption,^{23,24} versatility,²⁵ portability,^{26,27} time-effectiveness, and convenience.^{28,29} Notable platforms utilize signal amplification methods to obtain quantitative and sensitive performance in a single compact device, such as microfluidics-based PCR¹¹ and loop-mediated isothermal amplification.³⁰ However, these strategies either increase the cost by involving specific materials like DNA templates and enzymes or make the fabrication process complicated (Table S1). Besides, detection methods are also concerning for many of these platforms when they are used at remote or resource-limited areas.

Herein, we report a POC virus detection platform by combining nanostructure-integrated polydimethylsiloxane (PDMS) microfluidics with colorimetric detection. PDMS is environmentally friendly and frequently used in POC devices.^{23,31–33} Laboratory and field measurements demonstrate that PDMS does not bioaccumulate.³⁴ Nanostructures are commonly used to increase surface area.^{35–37} Compared to the flat surface, the three-dimensional (3D) nanostructures can provide more binding sites for probes and offer topological and morphological features to encourage the interaction between surface probes and detecting targets.^{38–40} Moreover, the diffusion distance is efficiently reduced for detecting targets and surface probes within the nanostructures.^{41,42} Normally, the PDMS surface from the replica is flat at the nanoscale. Although nanostructures can potentially be used as the template of PDMS similar to that of microstructures, it is hard to peel off PDMS from these tiny structures and copy the morphology of the template. We developed a new method to create a nanostructured PDMS surface by using inexpensive ZnO nanorods as the template for PDMS molding. Instead of directly peeling off PDMS from the ZnO nanorod template, PDMS is released from the template by dissolving ZnO nanorods in the diluted acid, which keeps the PDMS nanostructures undamaged. The whole fabrication process is of low cost and can be implemented outside cleanroom. Colorimetric reaction is an attractive simple and inexpensive detection strategy, which allows quantification of chemical elements or chemical compounds with different colors or different gray values. Thus, they are frequently used to allow either diagnosis with naked eyes or quantitative detection with minimal instrumentation.^{43–45} However, conventional colorimetric detection suffers from low sensitivity, compared with fluorescence detection. In our study, we use gold nanoparticle (AuNP)-based colorimetric detection with a silver enhancer to boost the detection limit down to 2.7×10^4 EID₅₀/mL by naked eyes, which is one order of magnitude better than that of conventional ELISA. Aided with a grayscale standard chart, quantification can be achieved. Smartphones have been used as the fast and powerful imaging and data processing tool for the POC applications.^{16,46–48} Therefore, we developed a smartphone-based relatively low-cost (Table S1) and portable virus detection system, which shows a better or similar detection limit and good specificity (Table S2). With a pair of polarizers, the sensitivity is further increased to 8×10^3 EID₅₀/mL, which is more than three times higher than that of the conventional fluorescence-based ELISA.

MATERIALS AND METHODS

Gold Nanoparticle Synthesis. AuNPs (13 nm) are synthesized in the sodium citrate solution according to the reported method.⁴⁴ Briefly, 1 mL of gold(III) chloride trihydrate (HAuCl₄·3H₂O, 1%) is mixed with 100 mL deionized water and heated to boiling under stirring. Then, 10 mL sodium hydrate solution (38.8 mM) is injected into the solution. The mixture is kept under heat for 5 min until its color turns from purple to wine red. Finally, another 20 min heating is performed to obtain 13 nm AuNP solution.

Antibody Conjugation to the Gold Nanoparticles. First, carboxymethyl-PEG-thiol (MW = 5000) is used to functionalize AuNP with carboxyl surface groups. Tween 20 solution (1 mL) (20%) is added into 25 mL of AuNP solution and stirred for 25 min at room temperature (RT). After rapidly injecting 0.2% carboxymethyl-PEG-thiol solution, the mixture is kept stirred for 24 h at RT. The mixture is cleaned with centrifugation (10 000 rcf, 15 min) thrice to purify AuNPs with carboxyl groups. Then, the monoclonal antibody (mAb) against the hemagglutinin of H5 AIV is conjugated to the AuNPs via EDC/NHS coupling.⁴⁹ H5 mAb-conjugated AuNPs (mAb-AuNP) are cleaned by centrifugation (10 000 rcf, 15 min) thrice and resuspended in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.05% sodium nitride (Na₃N).

Colorimetric Reaction in a Multiwell Plate. mAb solution (20 μg/mL) is added into the 96-well plate and kept overnight at RT to allow mAb bond to the multiwell surface nonspecifically. After washing thrice with Tris-buffer, 1% BSA solution is added to the wells for 2 h to block nonspecific binding of viruses. Then, virus solutions with different concentrations are added to the wells and incubated for 1 h. After washing, mAb-AuNPs are added to bind to the captured viruses in the wells. Finally, the silver enhancer (Sigma-Aldrich) is added to the wells and incubated for 10 min. After the colorimetric reaction, photos are taken under the same illumination and exposure conditions to measure the gray values of each well. On the other hand, we measure the absorption spectra of the colorimetric reaction solution using a plate reader.

Design and Fabrication of PDMS Replica. The size of the PDMS device is about 2 cm × 2 cm × 0.5 cm. The detailed design of the device is in Figure S1. To fabricate the device, a mold of the spiral microfluidic device containing herringbone structures is fabricated with standard photolithography. The first layer of the mold is designed for the microfluidic channel only (100 μm wide, 25 μm high). The second layer is designed for the herringbone structures (100 μm wide, 25 μm high) within the microfluidic channel. To fabricate the mold, first, a 25 μm-thick SU-8 25 is spin-coated on a 4-inch wafer. After soft bake, first photolithography is performed with the film containing the channel only. After post exposure bake (PEB), a second 25 μm-thick SU-8 25 is spin-coated on the top of the first layer followed by soft bake. Then, second photolithography defines the herringbone structures by accurately aligning the first and second layers on the Su-8 film containing both the channel and herringbone structures. Finally, PEB, Su-8 development and hard bake are carried out to finish the fabrication process.

Microfluidic Device Fabrication Based on the ZnO Nanorod Template. With the SU-8 mold, a negative PDMS replica is obtained first. With this negative PDMS replica, a positive PDMS replica is achieved by pretreating the negative replica's surface with hand soap. Then, ZnO nanorods are synthesized on the surface of the positive replica according to the reported method.³⁵ Briefly, the positive PDMS replica is activated with KMnO₄ for half an hour. Then, the replica is immersed in the ZnO growth media (0.1 M of zinc nitrate, 4% (v/v) of ammonium hydroxide solution, and 10% (v/v) of ethanolamine) under 140 °C for 1 h. After reaction, ZnO nanorods are formed on the surface of the PDMS replica. With this replica containing ZnO nanorods as the template, the PDMS device layer containing channels with the nanostructured surface is obtained. After the surface treatment of the ZnO nanorod forest on PDMS with the hand soap (Softsoap) in 75% ethanol, PDMS can be easily peeled off from the mold.

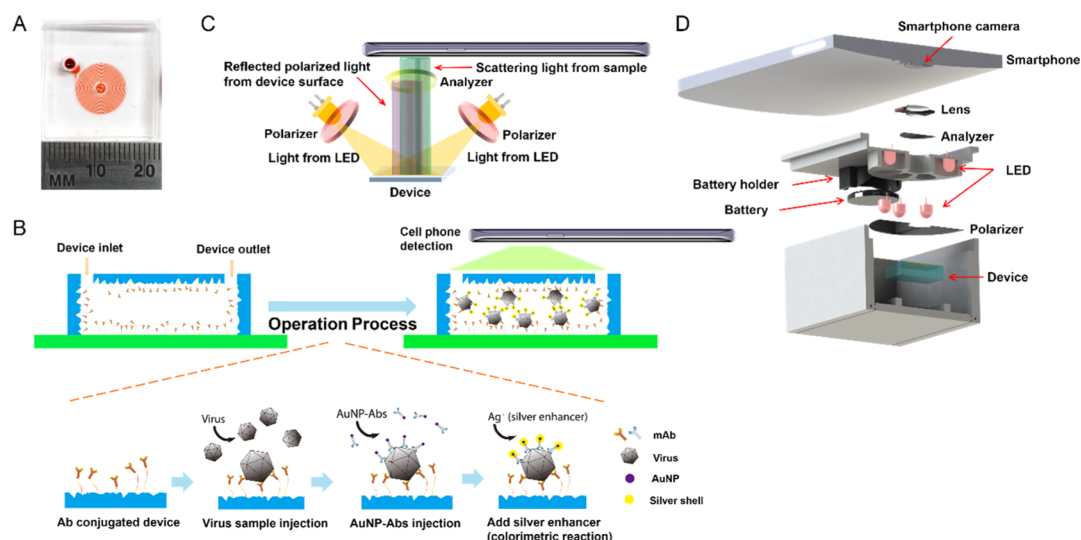


Figure 1. Design of the nanostructured microdevice and the portable smartphone-enabled virus detection system. (A) Photo of the microfluidic device. (B) Schematic of the sandwich virus detection assay in the device. (C) Setup of the optical system. (D) Design of the smartphone imaging system.

Antibodies Conjugation inside the Microfluidic Channels.

The surfaces of the PDMS channel layer as well as glass slides are functionalized with 4% v/v (3-glycidyloxypropyl)trimethoxysilane (GPTMS, Sigma) in ethanol. A thin layer of uncross-linked PDMS is used as the adhesive layer between the PDMS channel layer and glass slide. The thin PDMS adhesive layer is prepared by spin-coating 20% v/v PDMS in toluene on a glass slide with 1500 rpm. After device bonding, all of the inner surfaces of the channels are modified with GPTMS. The H5 mAb was produced in mouse by the Wiley Lab/Avian Virology of the Pennsylvania State University. H5 mAb (20 $\mu\text{g}/\text{mL}$) is introduced into the channels and allowed for the epoxy ring-opening reaction. After overnight incubation, the channels are washed with 50 mM Tris-buffer and incubated with 0.2 mM glycine (in Tris-buffer) for 1 h to block the unreacted epoxy group. Finally, the channel surface is blocked with 5% w/w BSA solution for 1 h to minimize nonspecific adsorption.

Finite Element Method Simulation of the Herringbone Structure. A finite element method (FEM) model was established to simulate the fluidic field inside the device by the software COMSOL Multiphysics. The laminar flow stationary study was chosen. A straight channel with the herringbone structure was constructed to study the flow condition. In the inlet of the channel, the inflow condition was a normal inflow with a flow rate of 5 $\mu\text{L}/\text{min}$. In the outlet, the outflow conditions of normal flow and suppress backflow were set up. No slip condition was built on the walls. A physics-controlled finer mesh was chosen to produce meshes in the model. The results of velocities in the x , y , z directions were outputted to gain the streamlines in the cross-sectional area of the channel.

Virus Detection with Colorimetric ELISA or Fluorescence ELISA inside the Plate. The virus sample (50 μL) is added into each well of the 96-well plate and incubated overnight under 4 $^{\circ}\text{C}$ for virus coating. After washing wells thrice with Tris-buffer, the wells are blocked with 5% w/w BSA solution under RT for 1 h. Then, 50 μL detection mAbs solution (colorimetric ELISA: mAb-AuNPs; fluorescence ELISA: mAb-biotin) is added into each well and incubated for 1 h. After washing wells thrice with Tris-buffer, the detection reagent (colorimetric ELISA: silver enhancer; fluorescence ELISA: Cy3-straptavidin) is added into each well and incubated (colorimetric ELISA, 20 min; fluorescence ELISA: 30 min). Finally, after washing thrice with Tris-buffer, the results are measured by the plate reader (colorimetric ELISA, absorbance at 500 nm; fluorescence ELISA: fluorescence).

Virus Detection with Colorimetric Reaction Inside the Device. Virus samples (300 μL) with different concentrations were injected into the devices by a syringe pump with 5 $\mu\text{L}/\text{min}$ for 1 h.

After washing the channel with Tris-buffer, mAb-AuNPs were introduced into the channel and incubated for 45 min, allowing for binding to the captured viruses. Finally, the PDMS layer containing the microfluidic channel was peeled off from the glass slide. The silver enhancer kit was added to cover the entire channel surface. After 15 min colorimetric reaction, the PDMS surface was washed with Tris-buffer and then covered with a cover slip.

Quantification of Colorimetric Reaction with the Smartphone Imaging System. A smartphone-integrated imaging system was designed to take photos of the channel after the colorimetric reaction. The components of the imaging system from top to bottom are the smartphone camera, lens, polarizer 1 (analyzer), light-emitting diode (LED), polarizer 2 (polarizer), and the device, respectively. When processing the image with the smartphone application, the signal is calculated by subtracting the mean grayscale values of background on the four corners from the mean grayscale value of the entire microfluidic channel area (not including the center outlet area).

RESULTS AND DISCUSSION

Design the Virus Detection Platform and Smartphone Imaging System. The microfluidic device contains a spiral channel and herringbone structures (Figure 1A), both of which can increase the interaction of viruses in the flow with mAbs functionalized on the channel wall, thus improving the virus capture efficiency. The inner walls of the flow channel have a nanostructured surface, mirroring the morphology of the ZnO nanorods, which significantly increases the surface area to improve the virus capture efficiency. The device captures viruses immunologically and detects captured viruses with on-chip immunoassay coupled with a colorimetric reaction (Figure 1B). The more captured viruses, the more AuNPs with silver shells (Ag@AuNPs) will form on the device surfaces, which makes the device surface look darker. The gray value of colorimetric reaction is used to quantify the virus concentration either by naked eyes or a simple smartphone-based imaging system.

To boost the virus detection sensitivity especially at low resource areas, a low-cost and portable smartphone imaging system is developed to quantify the darkness of the channel area of the device after the colorimetric reaction (Figure 1D,E). The smartphone imaging system provides a stable and reproducible imaging environment for the microfluidic device.

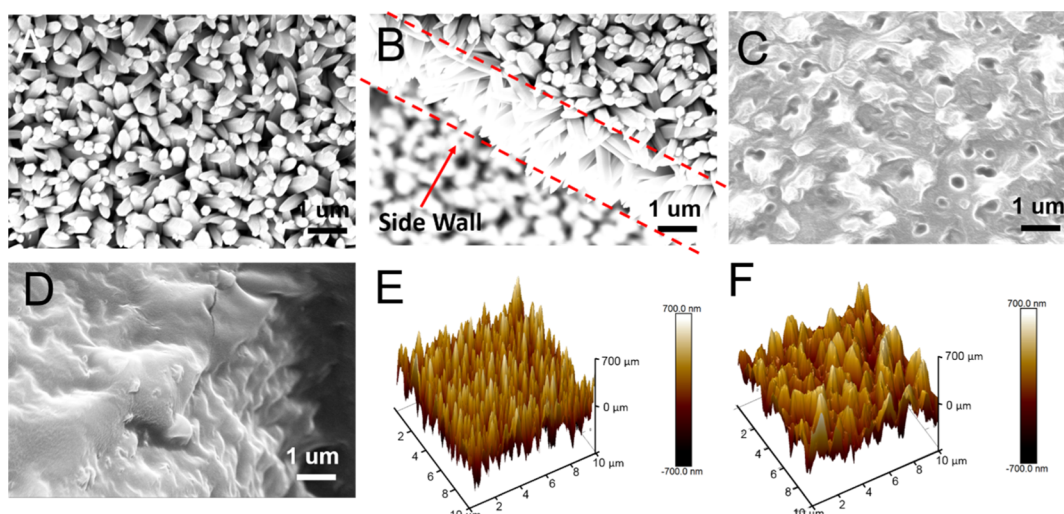


Figure 2. Nanoscale surface morphology characterization of the ZnO–PDMS mold and the PDMS device. (A) SEM of ZnO nanorods on the ZnO–PDMS mold. (B) SEM image of ZnO nanorods on the sidewalls of the ZnO–PDMS mold. (C) SEM image of the nanostructured surface on the bottom of the PDMS device. (D) SEM image of the nanostructured surface on the sidewalls of the PDMS device. (E) AFM image of the ZnO nanorods on the ZnO–PDMS mold. (F) AFM image of the nanostructured surface of the PDMS device.

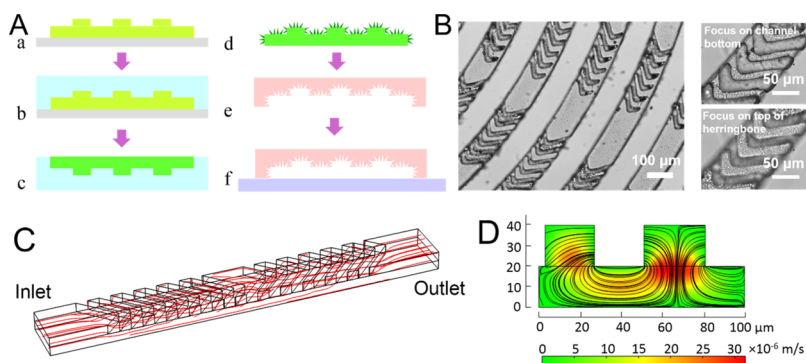


Figure 3. Device fabrication and flow simulation. (A) Fabrication process of the device: (a) fabrication of the SU 8 channel mold on the silicon substrate; (b) negative PDMS mold replication from the SU 8 mold; (c) positive PDMS mold replication with the negative PDMS mold; (d) ZnO nanorod synthesis on the positive PDMS mold; (e) fabrication of PDMS channels with nanostructured inner surfaces using the ZnO–PDMS mold; (f) PDMS layer bonding to the glass slide. (B) Optical microscopic images of herringbone structures of the microfluidic device with the nanostructured surface. (C) FEM simulation of streamlines along the microfluidic channel with the herringbone structure. (D) FEM simulation of the velocity (color-coded) and streamlines in the cross-sectional plane of the microchannel.

From top to bottom, the system includes a smartphone with a camera, lens, analyzer, LEDs, polarizer, and microfluidic device. The portable lens is used to shorten the focus distance and obtain a better view of the channel area. The pair of polarizers (one analyzer and one polarizer) eliminates specular reflection to reach camera. The camera of the smartphone takes image from the light scattered from the sensing area of the device, and the mobile application evaluates the gray value of images and finally shows the concentration of viruses in the solution based on a pre-established standard curve that correlates gray value with virus concentration.

Characterization of the ZnO Template and the Channel Inner Surface. When PDMS is peeling off from the ZnO nanorod template grown on the hard surface (e.g., silicon and glass), the detachment occurs between the nanorod roots and the thin and smooth ZnO layer below on the substrate, instead of the interface between nanorod tips and PDMS (Figure S2A). Therefore, the ZnO nanorods tend to break and stay within the PDMS, becoming the defects of the PDMS channel layer. To solve this challenge, we grow ZnO

nanorods on a soft material such as PDMS instead of the rigid material. The ZnO nanorods are synthesized on the KMnO_4 -activated PDMS in a batch process using a hydrothermal deposit method.³⁵ The method is of low cost and can be easily scaled up. The synthesized ZnO nanorods are uniform on the surface of PDMS, including channel bottoms and sidewalls (Figure 2A,B). For all of the nanorods, their growth directions are perpendicular to the respective surface, which are different from the seed-induced methods of randomly oriented ZnO growth. The diameter and height of the nanorod are ~ 300 nm and ~ 2 μm (Figure S2A), respectively, which offers a nanostructure with a high aspect ratio on the surface.

The ZnO nanorod forest on the positive ZnO–PDMS mold is used as the template to obtain PDMS with nanostructures. From our experience, ZnO nanorods cannot be used directly. They induce the formation of silver nanoparticles even without the existence of Au nanoparticles. This nonspecific colorimetric reaction caused by ZnO nanorods increases the background significantly, thus decreasing the sensing ability. After peeling off PDMS from the ZnO nanorod template, in

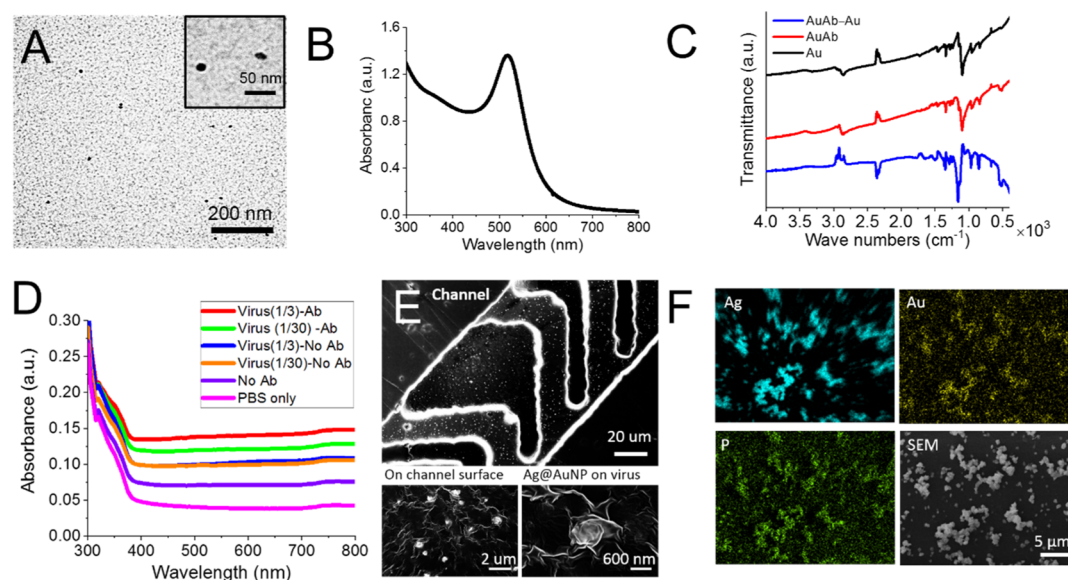


Figure 4. Development of colorimetric virus detection. (A) TEM of AuNPs. (B) UV-vis absorption spectra of AuNPs. (C) FT-IR spectra of AuNPs and mAb-AuNPs. (D) Absorption spectra of colorimetric reactions in the multiwell plate, including 1:3 virus dilution with mAb-AuNPs [virus (1/3)-Ab], 1:3 virus dilution without mAb-AuNPs [virus (1/3)-No Ab], 1:30 virus dilution with mAb-AuNPs [virus (1/30)-Ab], 1:30 virus dilution without mAb-AuNPs [virus (1/30)-No Ab], and negative controls of no mAb-AuNPs (No virus-Ab) and PBS control (control). SEM images at different magnifications (E) and EDS mapping (F) of colorimetric reaction products in the microfluidic device. Ag: silver from Ag@AuNPs; Au: gold from Ag@AuNPs; and P: phosphorus on the virus membrane.

most of the areas, ZnO nanorods completely detach from the mold with surface-grown ZnO nanorods. If there are any remains, they can be easily removed in diluted acid (Figure S2B), leaving the positive PDMS surface of the mold undamaged. Thus, the positive PDMS mold is reusable. In this way, as shown in the scanning electron microscope (SEM) images, both the ceiling and the sidewall of PDMS channel inner surfaces have nanostructured surfaces with a high aspect ratio transferred from the ZnO nanorod forest, making nanoscale surface topology on the PDMS (Figures 2C,D and S2C). The z range and the root mean square of the PDMS device surface measured by atomic force microscopy (AFM) are 1082 and 164 nm (Figure 2F), respectively, which are comparable to those of the ZnO nanorod forest on the mold, 1347 and 169 nm (Figure 2E), respectively. It indicates that transfer of the nanoscale topology from the ZnO-PDMS mold to the PDMS device layer is faithful and accurate. Moreover, measured by AFM, the surface area of rough PDMS increases by more than 43%, offering larger binding area for mAbs and viruses.

Fabrication and Surface Modification of the Microfluidic Device. The fabrication of the microfluidic device with nanostructured inner channel surface employs both standard soft lithography and ZnO nanorod synthesis (Figure 3A). Usually, the PDMS device layer is bonded to the glass slide aided by oxygen plasma treatment. However, because all surfaces of the PDMS device layer are nanostructured as the mirror image of the nanostructured ZnO-PDMS mold, they cannot get close enough to the glass slide surface to allow the formation of Si-O-Si covalent bonds. Besides, the oxygen plasma treatment can damage the exposed epoxy groups on both the PDMS and the glass slide surfaces, which is a problem for subsequent surface conjugation of mAbs. To solve this issue, a thin uncured PDMS layer is spin-coated on the glass slide and serves as the adhesion layer to the PDMS device layer to form the microfluidic channel. The thickness of uncured

PDMS needs to be controlled. PDMS adhesive layers with different thickness controlled by the ration of PDMS to toluene were tested (Figure S3B,D). If the PDMS adhesive layer is too thick, uncured PDMS will flow into the channels and block them (Figure S3B); while too thin, the adhesion layer will not be strong enough to tolerate high hydraulic pressure (Figure S3D). To get the uncured PDMS layer with different thicknesses, PDMS is diluted in toluene with different ratios before spin-coating on glass slides. In the ratio range from 2:1 to 6:1, about half thickness of uncured PDMS will be transferred to the PDMS device layer containing channels as the adhesion layer (Figure S3A). A ratio of 4:1 can cover most of the nanostructures on the PDMS surface and let it attached firmly to the glass slide (Figure S3C). It has a transferred thickness of ~ 670 nm, which is in accordance with the ~ 1 μm z range of the nanostructured PDMS surface. After bonding, the surfaces between the channels are smooth, indicating that the rough surface is smoothened by the uncured PDMS, and the PDMS device layer is adhered firmly to the glass slide (Figure S4). While inside channel, nanostructured surfaces of the channel inner walls and herringbone structures are evident, keeping a large area for mAbs bonding. With the uncured PDMS as the adhesion layer, the morphology of the herringbone structures keeps well (Figure 3B). The herringbone structures introduce local mixing inside the microfluidic channels;⁵⁰ therefore, viruses along the trajectories have a better chance to interact with the mAbs on the channel wall to be captured (Figure 3C,D). The higher virus capture efficiency helps to boost the overall detection limit.

Before we developed the on-chip colorimetric virus detection, fluorescence-based ELISA assay was designed to validate the antibody against H5N2 AIV and on-chip virus capture (Figure S5). Excessive mAbs were conjugated on the microchannel surface to capture viruses at different titers. After virus capture and washing, mAb-AuNPs modified with biotins were introduced to bind to virus surface. Then, Cy3-

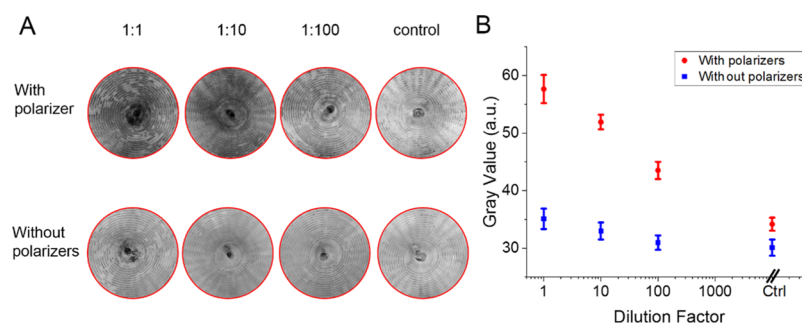


Figure 5. AIV detection using the smartphone imaging system with or without the polarizer pair. (A) Photos of colorimetric reaction with and without polarizers. (B) Comparison of colorimetric reaction with and without polarizers.

streptavidin was applied to quantify captured viruses under the fluorescence microscope. The results showed that the fluorescence intensity had a positive correlation with the virus titer.

Characterization of the Colorimetric Reaction with AuNPs. AuNPs (13 nm) are typical probes in colorimetric reaction. Silver ions can amplify the signal by depositing on the surface of AuNPs as silver shells forming Ag@AuNPs, where the AuNPs serve as the catalysts. TEM confirms that the diameter of synthesized AuNPs is around 13 nm and UV–vis absorption spectrum has a peak at ~ 517 nm (Figure 4A,B), matching with previous reports of AuNPs of 13 nm diameter.⁵¹ Then, mAbs are conjugated to these AuNPs. First, the Fourier transform infrared (FT-IR) spectrum was used to validate the mAbs conjugation on the AuNPs. As shown in Figure 4C, the strong absorption peak at around 1100 cm^{-1} in FT-IR of the PEG-functionalized Au nanoparticles can be assigned to the stretching vibration of C–O from the ethylene glycol monomers.⁵² After the grafting of the antibodies, besides the characteristic adsorption peaks of the AuNPs and PEG molecules, several characteristic absorption peaks of antibodies with weak intensity were observed in the range of $1000\text{--}1800\text{ cm}^{-1}$. Absorbance peaks at around 1720 cm^{-1} are typical from the stretching vibration of C=O in protein backbones. In addition, absorbance peaks at ~ 1646 and 1515 cm^{-1} could be ascribed to the amide I vibration and amide II vibration of proteins from the antibodies, respectively.^{53,54} Therefore, these results demonstrate the successful functionalization of mAbs on the AuNPs.

Then, colorimetric reactions both in the multiwell plate and on-chip were developed. Adding the detection reagent mAb-AuNPs increases absorbance in all groups. Moreover, a higher virus titer results in higher absorbance (Figure 4D). Thus, AuNPs remained in the wells when the viruses were present and the quantity of AuNPs had a positive correlation with the virus titer. The multiwell plate assay validates that the AuNPs were conjugated to the mAb and the overall protocol worked. We then implemented this assay inside the microfluidic device. SEM and Energy Dispersive Spectroscopy (EDS) mapping were used to check the results of on-chip colorimetric reaction directly (Figure 4E,F). According to SEM, large particles around 500 nm were formed on the channel walls, which were much larger than that of AIVs (~ 100 nm), indicating that thick silver shells were deposited on the surface of viruses. Moreover, the EDS mapping showed that the distributions of silver and gold were colocalized to that of phosphorus, which represents virus location. It suggests that the on-chip colorimetric virus detection is specific.

We studied the effects of AuNP concentrations and reaction time on the colorimetric reaction in the multiwell plate. With the same reaction time of the silver enhancer, the absorbance in the range of visible lights decreases with the decreasing concentration of the mAb-AuNP solution (Figure S6). It indicates that the quantity of AuNPs can be estimated by the gray value of colorimetric reaction, which suggests that it can be used to determine the virus concentration in the microfluidic device after conjugating to the specific mAb. On the other hand, the incubation time of the colorimetric reaction can also significantly affect the gray value. Our results show that with the same dilution factor of the AuNP solution, the absorbance increases with increase of reaction time, which produces a higher gray value (Figure S6). Thus, it is important to make the reaction time consistent during the detection.

We characterized the device using aqueous samples and reagents. To store and ship the device as well as chemicals to remote areas under RT with minimum storage and shipping cost, the device coated with capture antibodies and blocked with BSA can be dried,⁵⁵ sterilized, and sealed in the airtight plastic bags, and the detection antibodies will also be lyophilized and stored in a small glass bottle as a kit.

On-Chip Virus Detection with Colorimetric Reaction.

On-chip virus detection with colorimetric reaction was tested and compared to conventional immunoassay (ELISA) to evaluate the overall performance of the system. H5N2 AIVs cultured in embryonated chicken eggs with the original titer of 8×10^6 EID₅₀/mL were used as the virus target. Serial dilutions of H5N2 AIVs with final titers of 8×10^5 , 2.7×10^5 , 8×10^4 , 2.7×10^4 , 8×10^3 , and 2.7×10^3 EID₅₀/mL were prepared. After flowing in viruses for 1 h and followed by 15 min colorimetric reaction, the devices were washed by PBS and covered with a cover slip. Then, photos of the device were taken and analyzed (Figure 6A). The photos of the channel area become darker with the increasing virus titer, and the detection limit by naked eyes is around 2.7×10^4 EID₅₀/mL.

Imaging System Optimization. First, an enclosed imaging system is designed built on optical bench with a digital microscope (Dino-Lite AM 2111) to measure the grayscale value of the device after the colorimetric reaction (Figure S8). In order to eliminate the grayscale variance introduced by different device thicknesses, the proposed design, as shown in Figure 1C, utilizes the reflective detection mode to measure the scattering light from the microchannels. In specific, the light source, an LED array consisting of four LEDs arranged in a circle, is placed on top of the device to evenly illuminate the whole channel area and minimize the spatial dependence of the light source. After traveling through the central hole of the LED array, the scattering light from the

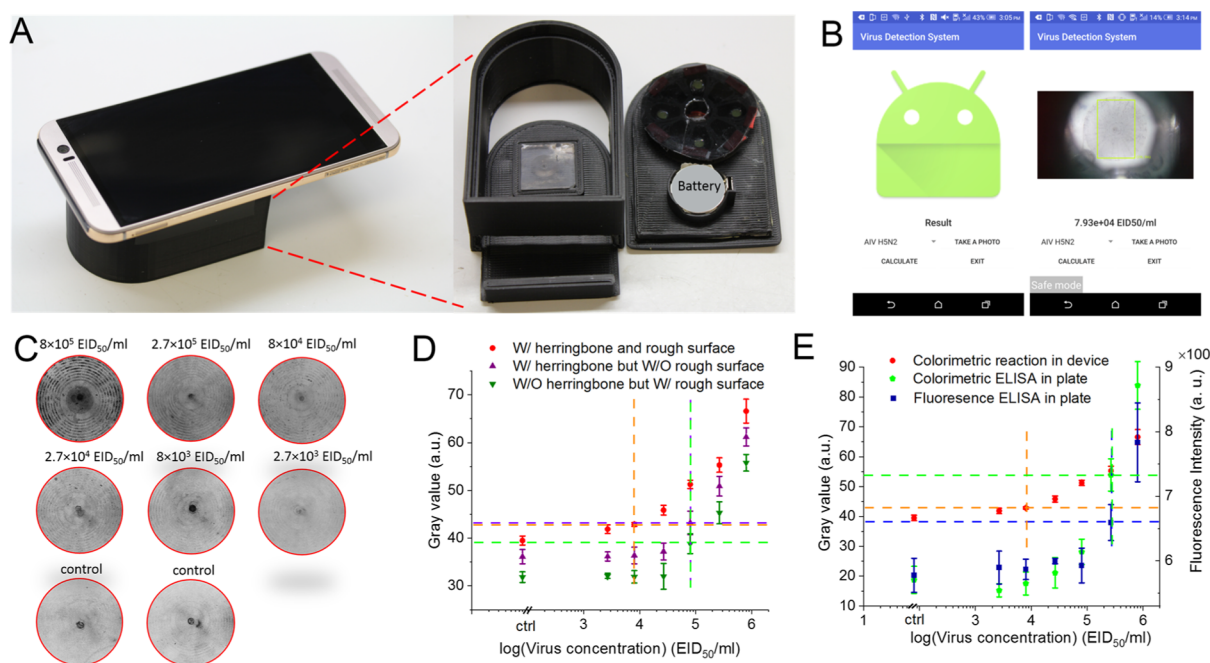


Figure 6. Development of the smartphone-based on-chip colorimetric virus detection system. (A) Photo of the implemented platform. (B) Smartphone application user interface and the result of a virus sample of 8×10^4 EID₅₀/mL. (C) Photos of AIV virus detection with the smartphone-based on-chip colorimetric virus detection system. (D) Comparison of virus detection with/without the herringbone structure and ZnO nanorod replication (roughness surface). (E) Comparison of virus detection between the colorimetric detection system and conventional fluorescence-based ELISA. The vertical lines are the location of detection limits. The horizontal lines are the location of signal intensity at the detection limit.

device is collected by the smartphone camera. Because only the grayscale of the scattering light encodes the virus concentration, an average illumination angle of 35° is utilized to reduce the amount of reflected light entering the camera. Meanwhile, a polarizer film pair (one analyzer and one polarizer) is employed to filter away the residue reflected light. After passing through the polarizer, the incident light reflects off the device surface and maintains its polarization in the *s* direction.⁵⁶ The analyzer in front of the camera filters out the *s*-polarized component of the incident and the reflected light, respectively, therefore producing a high rejection ratio for the reflected light. The grayscale value of the captured image is then quantitatively analyzed and converted into virus concentration by a smartphone application which we developed. The results of different concentrations of AuNPs absorbed in the device are shown in Figures 5A and S9. The grayscale value of the colorimetric reaction without the polarizer pair shows only a slight change once the dilution factor goes higher than 1:10. This limits the signal-to-noise ratio (SNR) due to the fact that the signal captured by the camera is dominated by the reflected light. By placing the polarizer pair close to the LED and the camera, respectively, the SNR can be greatly improved. In our testing system, the change of gray value due to the colorimetric reaction can be resolved with the 1:100 dilution and the detection limit is increased by more than one order of magnitude (Figures 5A,B and S7).

Integration of the Smartphone Imaging System and On-Chip Virus Detection with the Colorimetric Reaction. A smartphone imaging system and a smartphone application are developed for rapid virus analysis using the on-chip colorimetric reaction. The smartphone imaging system contains the same components as the imaging system built on

an optical bench mentioned above but designed and packaged in a portable adaptor with eight LEDs (Figures 1A,B and S10). A standard curve that correlates the gray value of the device and virus concentration is pre-programmed in the code of the application. After on-chip colorimetric reaction, a user needs to insert the device into the device holder of the adaptor and take a photo with the smartphone application. After choosing a square area that is exclusively within the virus detection area (i.e., the microchannel) and tabbing the calculate icon, the concentration of virus will be displayed immediately on the screen of the application (Figure 6A). Moreover, by programming standard curves of different viruses in the code, the platform can potentially be used for detecting different viruses. With this imaging system, the detection limit can be further improved to around 8×10^3 EID₅₀/mL for AIV (Figure 6C–E), which is over 30 times higher than that of the conventional ELISA of 2.7×10^5 EID₅₀/mL (both colorimetric ELISA and fluorescence ELISA). The introducing of herringbone structure or the ZnO nanorod surface increases the control grayscale value as the mixing or large surface area might increase the nonspecific adhesion of silver nanoparticles in the silver enhancer solution. However, although the control grayscale value is increased by introducing these structures, the increase of signal is much higher, leading to a higher signal to noise ratio. The detection limit is about 10 times better than the design with the herringbone structures but without ZnO nanorod replication (smooth surface) and the design with ZnO nanorod replication but without the herringbone structures (Figure 6D), indicating the use of herringbone structures and ZnO nanorod replication (roughness surface) both significantly improving the performance of the device. All devices used in this work are from the same PDMS template. The standard deviation of the gray value in Figures 5B and 6D,E

indicates that there is minimal difference when the template is reused.

CONCLUSIONS

In summary, we developed a POC microfluidic platform for virus detection by plasmonic colorimetric reaction. The PDMS microfluidic device is portable, cheap, and disposable. We invented a new technology to fabricate PDMS device with a nanostructured surface by using the ZnO nanorod forest as the reusable PDMS mold. The surface of PDMS contains 3D nanostructures that mirror from ZnO nanorods with a high aspect ratio in the sub-micrometer scale. The new fabrication process utilizes ZnO nanorods directly synthesized on soft PDMS structures as the molding template, which guarantees truthful replication of the nanostructures without damaging the mold. The use of diluted uncured PDMS as an adhesion layer reliably bonds the nanostructured PDMS surface to a flat substrate. By integrating those nanostructures and micrometer-scale herringbone structures into a single microfluidic device, the virus capture efficiency and detection sensitivity are greatly increased. Moreover, a colorimetric reaction is employed to measure the concentration of the captured virus in the sample. It allows virus detection by naked eyes with a detection limit of 2.7×10^4 EID₅₀/mL, which is one order of magnitude better than conventional ELISA. Furthermore, a smartphone-based imaging system and a smartphone app were developed to rapidly analyze the colorimetric reaction and make the whole system fully portable. Aided by the reflective detection mode design and a pair of polarizers, the sensitivity of this platform can be further improved to 8×10^3 EID₅₀/mL, which is over three times higher than that of conventional fluorescence-based ELISA. The entire virus capture and detection process can be completed in 1.5 h. With simple peristaltic pumps and containers connected to different reaction solutions, the whole protocol can potentially be automated with further instrumentation. Overall, this nanostructured microfluidic device with its smartphone-enabled plasmonic colorimetric virus detection system offers a fast, cheap, portable and user-friendly platform with higher sensitivity for virus detection, which can potentially serve the critical needs in low-resource areas for the surveillance of influenza and other emerging viruses. In the future, to work with more complicated real swab samples, a microfluidic filter design should be added in front of the detection device to avoid device clogging by large aggregates in the samples. Besides, as phones have different cameras with different sensitivity and focusing length, the grayscale value of the images from the same device might be different. To avoid this inconsistency, a low-cost mini camera can be integrated in the imaging system and the images will be transferred to the phone via USB or Bluetooth and processed by the application.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.9b01927>.

Comparison to other POC technologies, detailed device design, PDMS and glass bonding test and result, colorimetric reaction test in the well plate, optical detection system setup, and platform images (PDF)

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Notes

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