Amplified visual immunosensor integrated with nanozyme for ultrasensitive detection of avian influenza virus

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Optimization of Au nanostructures catalytic activity

To get the best enzymatic activity from the synthesized Au nanostructures, several parameters were optimized. As shown in figure S1, Au nanostructure showed its best catalytic activity within 10 min after adding a mixture of TMBZ (10 nM) and H_2O_2 (5 nM) on it.

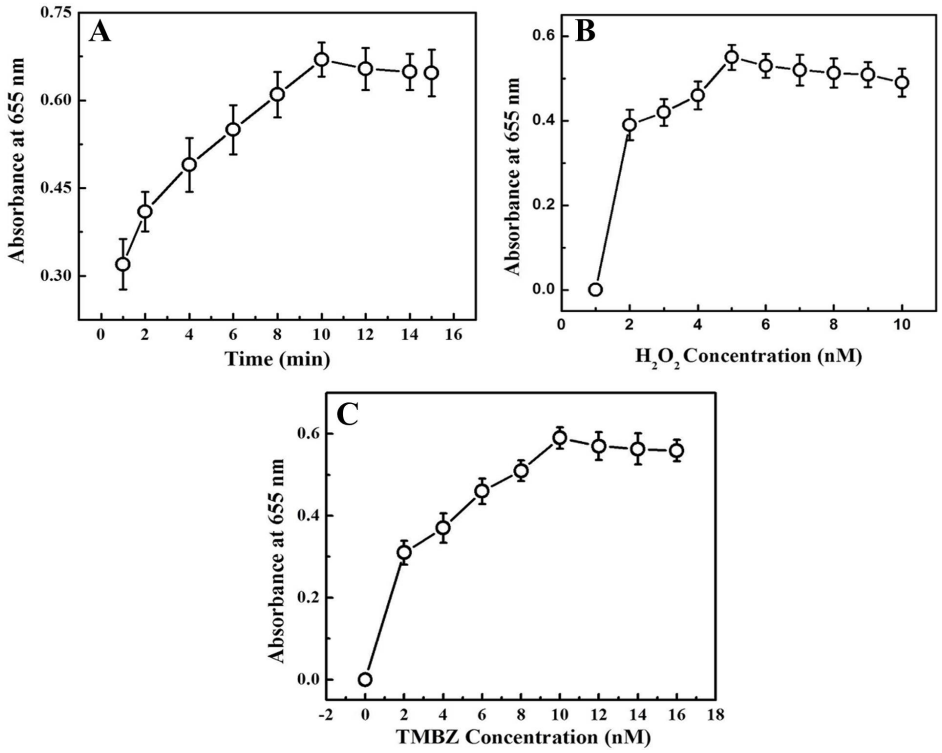

Figure S1. Optimizations of Au nanostructures catalytic activity: (A) Time dependent change of optical density; (B) H_2O_2 concentration Vs optical density when TMBZ concentration was fixed (10 nM) and (C) TMBZ concentration Vs optical density when H_2O_2 concentration was fixed (5 nM).

Binding confirmation of anti-H5N1 Ab 135382 with gold ions

The stability of antibodies in different pH was examined using ELISA method and showed in figure S2. At low pH ≤ 2, irreversible precipitation of antibodies occurred and may lose their bio function. The activities of antibodies also reduced with increasing pH values at alkaline media. Hence, a pH value of ~4 was kept during conjugation of antibodies with gold ions (Fig. S3A). The binding of antibodies and gold ions (Ab-Au^{3+}) complex was confirmed by FTIR analysis. In Figure S3B, two new peaks arose compared to bare antibodies and $HAuCl_4$ in the range of 3200-3000 cm^{-1}, indicating new bonding between antibodies and $HAuCl_4$.

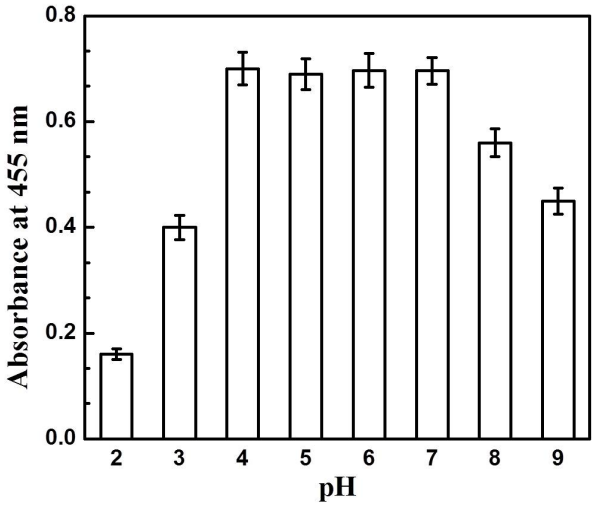

Figure S2. Antibodies stability at different pH.

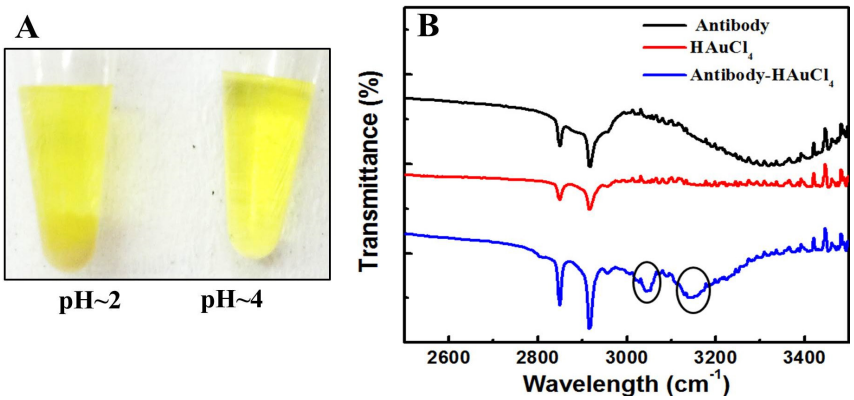

Figure S3. Antibody stability and its binding confirmation. (A) Naked eye image of antibody in different pH; (B) FTIR spectra of antibodies binding with gold ions.

ELISA for Antibodies-Gold Ion Binding Test

Avian influenza virus A (H5N21) solution (100 µL, 1µg/mL) was added to each 96-well plate and incubated overnight at 4°C. After washed three times with PBS buffer solution (pH 7.5), blocked the wells with 100 µL of 2% skim milk for 2 h at room temperature, then 50 µL of antibody (Ab135382)-gold ion bioconjugates was added to each wells. Gold ions (50 µL, 5 mM), antibodies (50 µL, 1 ng/mL) and BSA (50 µL,1ng/mL) was used as a negative control to check the binding confirmation of the system. After 1h, wells were washed three times with PBS (pH 7.5) and 50 µL

of TMBZ (10 nM) solution was added into each of the wells and spontaneously characteristic color was developed. Clearly, strong optical density was observed from antibody-gold ion bioconjugates in compare to bare gold ions, antibodies and BSA indicating the binding confirmation between antibodies and gold ions (Fig. S4).

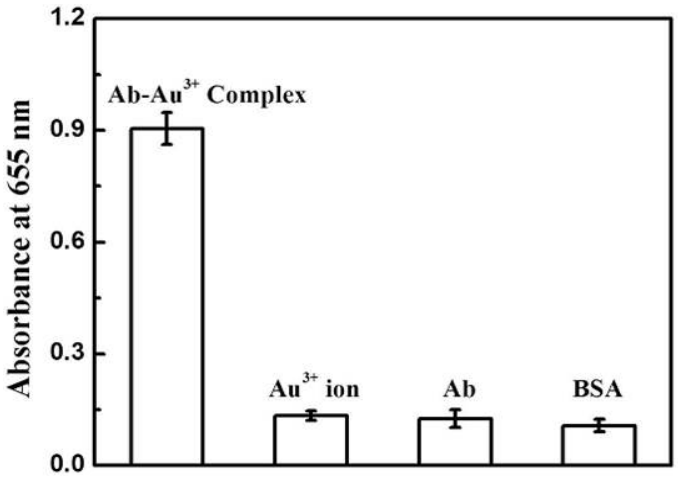

Figure S4. ELISA results for antibodies-gold ion binding.

Specificity of proposed bioassay

The catalytic activity of the proposed bioassay was investigated using four different reaction mixtures: a) recombinant influenza virus A (Avian/Vietnam/1203/04) (H5N1) /Ab135382–gold ion conjugates/TMBZ; b) replacing the specific Ab135382–gold ion conjugates with another anti-H5N2 HA–gold ion conjugates in reaction mixture a); c) removing TMBZ from the reaction mixture (a); and d) removing gold ion (only Ab 135382) from the reaction mixture (a). A deep bluish-green color developed in sample mixture (a), and a strong characteristic absorption peak at 655 nm was also observed (Fig. S5). However, no such characteristic peak was observed for other mixtures (b, c and d), suggesting that the proposed sensing method is highly specific and selective; and color development occurs only with the target virus, its specific Ab-conjugated gold ion and TMBZ.

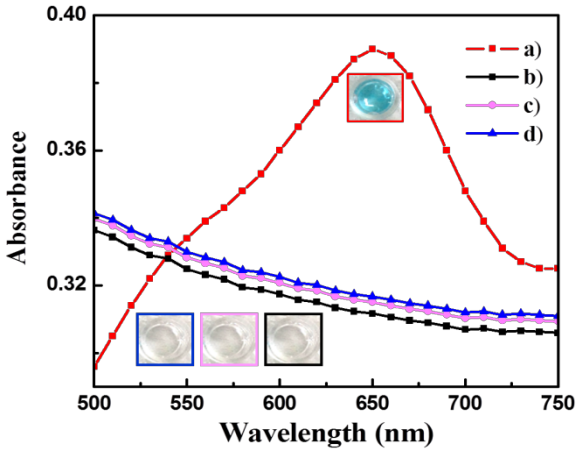

Figure S5. Specificity of proposed bioassay in different mixtures

Detection of avian influenza virus A (H5N1)

A wide range of quantitative analysis for recombinant influenza virus A (H5N1) detection was performed after confirming the specificity and binding of Ab 135382 towards the target virus. The sensitivity of this proposed system for recombinant influenza virus A (H5N1) detection was found to be in the range from 10 pg/mL to 10 µg/mL with an LOD value of 1.11 pg/mL. Visual image of detection system was shown in Figure S6.

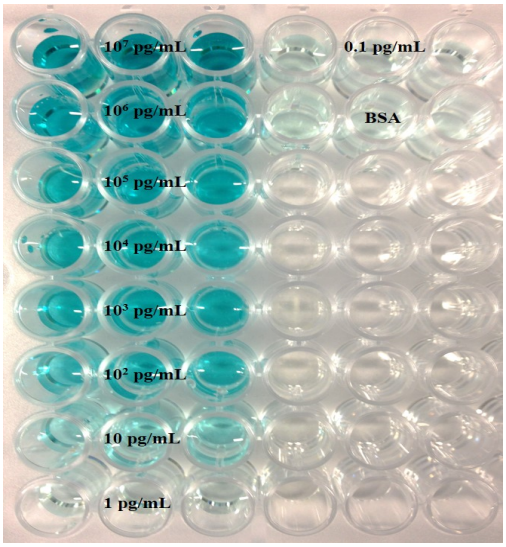

Figure S6. Naked eye image of virus detection

A comparison study with plasmonic bioassay

It is instructive to do a comparative study with other nanotechnology-based analytical techniques. To do that, we have compared the present technique with the plasmonic resonance peak response of synthesized Au NPs (at intermediate stage) with different concentrations of target analytes. As shown in Figure S7, the change of plasmonic peak located at 550nm was not consistent compared to the peak at 655nm (present study) and showed 10 times less sensitivity (100 pg/mL) than the current study.

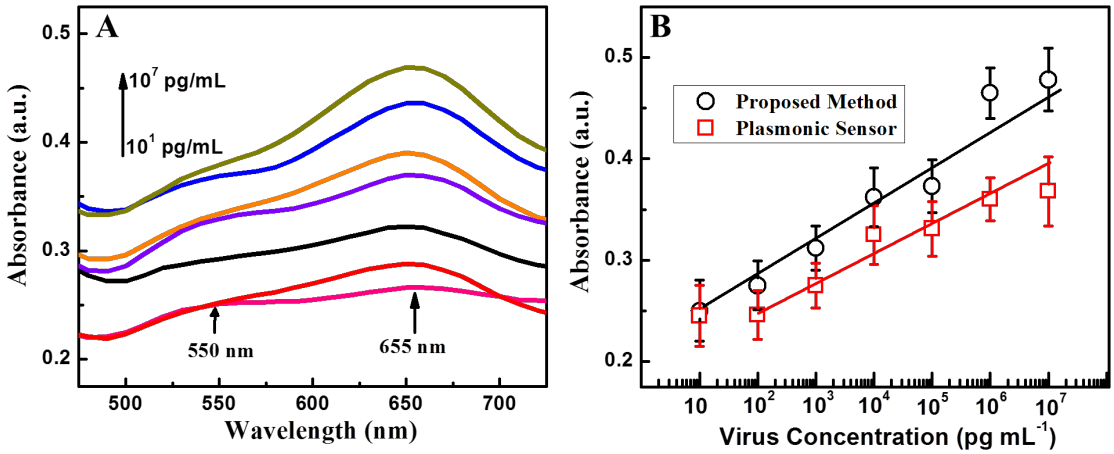

Figure S7. A comparison study with plasmonic bioassay: (A) Changes of plasmonic peaks with different concentrated virus; (B) Virus concentration Vs plasmonic peak, located at 550 nm.

Comparison study of detection with commercial kit

The sensitivity of the proposed method was compared to that of a commercially available avian influenza A (H5N1) diagnostic kit (Table 1, Fig. S8). The naked-eye color response to the detection of avian influenza A (H5N1) in the commercial kit was as high as 1 ng/mL, indicating that our system is more sensitive than the commercial kit. Thus, the dual mode enhanced colorimetric technique presented here for the visual detection of avian influenza viruses could be applicable for low-cost, visible, point-of-care diagnosis and also extendable to develop other nanozyme-based biomarkers.

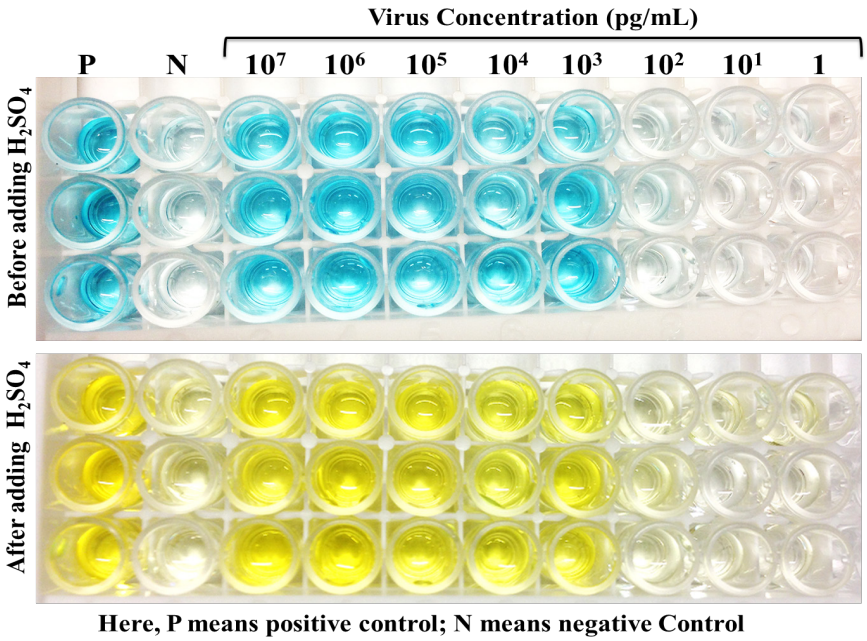

Figure S8. Naked-eye image of avian influenza virus detection using a commercial ELISA kit.

ELISA for antibodies specificity towards viruses

The specificity of anti-H9N2 (ab31674) antibody and Anti-H4 (A/environment/Maryland/1101/06)(H4N6) polyclonal antibody towards avian influenza A (H9N2) and A (H4N6) virus was determined through ELISA method. Viral titer solution (50 µL, 100 HAU/50 µL) was added to each 96-well plate and incubated overnight at 4°C. After washed three times with PBS buffer solution (pH 7.5), blocked the wells with 100 µL of 2% skim milk for 2 h at room temperature, then 50 µL of antibody was added to each wells. BSA (50 µL,1ng/ml) was used as a negative control to check the binding confirmation of the system. Then 50 µL of secondary antibody conjugated with HRP enzyme (1 ng/mL) was added in each wells for 1h at room temperature. After 1h, wells were washed three times with PBS (pH 7.5) and 50 µL of TMBZ (10 nM)/H_2O_2 (5 nm) solution was added into each of the wells and spontaneously characteristic color was developed. Enzymatic reaction was stopped by adding 10% H_2SO_4 solution. Clearly, strong optical density was observed from antibody-antigen in compare to BSA indicating the binding confirmation the specificity of antibodies towards viruses (Fig. S9).

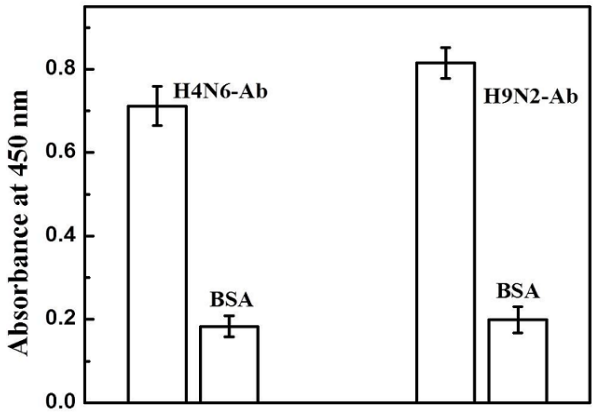

Figure S9. ELISA results for antibodies specificity towards avian influenza viruses.