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A colorimetric immunoassay of respiratory syncytial virus by using gold nanoparticles/graphene oxide hybrids with mercury-enhanced peroxidase-like activity

Lei Zhan, Chun Mei Li, Wen Bi Wu, and Cheng Zhi Huang

A novel colorimetric immunoassay of respiratory syncytial virus (RSV), one of the leading causes of severe lower respiratory tract infections in all age groups, has been proposed with high sensitivity based on Hg²⁺-stimulated peroxidase-like activity of gold nanoparticles/graphene oxide (AuNPs/GO) hybrids. This metal ion-enhanced immunoassay shows high promise in the field of biomedical sciences.

Natural enzymes with high substrate specificity and high catalytic efficiency have been intensively studied and applied in a variety of fields including biomedical, bioassay and biotechnology. Unfortunately, they bear intrinsic drawbacks such as being easily denatured and digested by protease. Moreover, their preparation, purification and storage are usually time-consuming and expensive, greatly limiting their applications. Hence, searching for natural enzyme mimics and/or artificial enzymes with good stability is urgently required. Since the exciting discovery that magnetite (Fe₃O₄) nanoparticles possess an intrinsic peroxidase-like activity similar to that found in natural peroxidases, nanomaterial-based enzyme mimetics (NMEMs) have attracted great interest and widely applied in biochemical analysis because reported NMEMs take the advantages of low cost, high stability and tunability in comparison with natural enzymes. Subsequently, carbon materials (e.g., graphene oxide (GO), carbon nanotubes, and carbon nanodots), metal oxides (e.g., Co₃O₄, CuO, and MnO₂) and noble metal nanoparticles or nanoclusters (e.g., AuNPs, AuNCs, AgNPs) have been demonstrated to exhibit enzyme mimetic activity. Additionally, hybrid materials (e.g., Fe₃O₄/graphene hybrid, GO/AuNCs hybrid, and Au@Pd nanoparticles-graphene hybrids) have also been reported to have surprisingly high peroxidase-like activity due to the synergistic effects.

Although these unique activities of NMEMs have been widely used for constructing sensitive probes for glucose, glutathione, acetylcholine and DNA detection through their catalytic oxidation of substrates such as 3, 3, 5, 5-tetramethylbenzidine (TMB) and Amplex UltraRed (AUR), their applications in biological systems are relatively rare. A noticeable problem is that the adsorption of biomolecules such as serum albumin and DNA on the surface of nanomaterial reduces the catalytic activity. It has been known that the catalytic reactions usually take place at the surface of nanomaterial, hence the active sites for the substrate may be occupied by the adsorbed molecules, leading to reduced catalytic activity. Furthermore, aggregation of nanoparticles in the biological media causes reduced surface area, accompanying with the significantly decreasing of catalytic activity.

To overcome these limitations, efforts have been made to enhance the catalytic performance of nanomaterials in complex biological fluids. For example, the citrate-capped AuNPs possessed relatively low catalytic activity, our group has presented mercury-stimulated peroxidase mimetic activity of AuNPs in the H₂O₂-mediated oxidation of TMB. In addition, Chang group has found that other metal ions such as Ag⁺, Pb²⁺, Bi³⁺, or Pt⁴⁺ have also participate the reactions of AuNPs with enhanced enzyme-like activity, implying the important role of metal elements for enhanced catalysis.

Inspired by metal ions-enhanced enzymatic activities of nanomaterials, we herein demonstrated the ability of an enhanced colorimetric immunoassay for highly sensitive detection of respiratory syncytial virus (RSV) in complex samples by using Hg²⁺-stimulated enzyme-like activity of AuNPs/GO hybrids, which might meet the challenges of reduced catalytic activity of nanomaterial in biological systems.

Scheme 1 (A) Procedure for the preparation of Ab-AuNPs/GO conjugates; (B) Schematic representation of the Hg²⁺-enhanced peroxidase-like activity ofAuNPs/GO hybrids for colorimetric immunoassay of RSV.

RSV, a single-stranded RNA virus of the paramyxovirus
family, is the leading pathogen causing infant hospitalization and respiratory distress worldwide. Although RSV normally does not cause mortality, it is recognized as a major global public health problem. The cost of RSV-associated illness, both in economic and personal-finance terms, are considerable. Despite decades of research, a safe and successful vaccine remains unavailable for preventing RSV infections. It has been recognized that the treatments for RSV infections are much more effective at the initial stages. Therefore, sensitive and specific diagnosis in the course of early infection is very important.

The preparation of AuNPs/GO hybrids was made through a facile and efficient strategy with the use of Tannic acid (TA) as a reducing and stabilizing agent (Scheme 1A). It should be noted here that the use of TA is mainly based on the considerations that TA is a water-soluble, polyphenolic compound (Fig. S1, ESI†) and has been used to disperse GO sheets in aqueous medium owing to the π–π interactions between the aromatic rings of TA and GO sheets, and also acts as a reducing agent to synthesize AuNPs and PtNPs at room temperature. The as-prepared AuNPs/GO hybrids were examined by the UV-vis spectroscopy and transmission electron microscopic (TEM) imaging (Fig. 1). GO/TA solution has the superimposed absorption bands of TA and GO at 220 nm, while a strong surface plasmon resonance (SPR) absorption appears at 550 nm with the addition of HAuCl₄, indicating the formation of AuNPs. TEM measurements reveals that AuNPs are well-dispersed decorated on graphene with the average size of 41.9 nm. For comparison, TA-reduced AuNPs in the absence of GO was also obtained through the same procedures.

![Fig. 1 Characterization of the as-prepared AuNPs/GO hybrids. (A) UV-vis absorption spectra of aqueous dispersion of GO, TA, GO/TA, AuNPs and AuNPs/GO hybrids. (B) TEM image of AuNPs/GO hybrids. The initial concentration of HAuCl₄ in the sample was 0.5 mM.](image1)

The enzyme mimic catalytic activity of the AuNPs/GO hybrids was firstly evaluated by the oxidation of enzyme substrate TMB in the presence of H₂O₂. A typical colour change was observed upon the addition of AuNPs/GO hybrids, while no obvious reaction occurred in the absence of catalyst, suggesting that the as-prepared AuNPs/GO hybrids have peroxidase-like activity (Fig. 2A a&b). However, the activity of the AuNPs/GO hybrids got lost after their binding with antibody (Ab) (Fig. 2A c). This observation showed that protein, which would effectively prevent the substrates binding to the surface of AuNPs/GO hybrid, caused a significant decrease in the catalytic activity of AuNPs/GO catalyst. Interestingly, the catalytic effect of AuNPs/GO got stimulated upon further addition of Hg²⁺ even though biomolecules adsorbed on the surface (Fig. 2A d), and with increasing Hg²⁺ concentration, the catalytic effect of AuNPs/GO, as reflected by the absorption, got increased (Fig. 2B). As a control, Hg²⁺ was used to catalyze the reaction, which showed negligible catalytic effect even if the concentration up to 1 mM. Considering both the assay sensitivity and heavy metal ions’ toxicity, 10 μM Hg²⁺ was chosen for further experiments.

As compared to other metal ions including Li⁺, Na⁺, K⁺, Ag⁺, Mg²⁺, Ca²⁺, Fe²⁺, Ba²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Mn²⁺, Pd²⁺, Cd²⁺, Pb²⁺, Al³⁺, Cr³⁺, Fe³⁺, the Hg²⁺-stimulated catalytic ability of Ab-AuNPs/GO conjugates was found to be much stronger, mainly because of the high-specificity metalophilic Hg²⁺-Au interactions (Fig. S2, ESI†), which induced the deposition of Hg atoms onto the surfaces of the AuNPs. In other words, the catalytic activity of Ab-AuNPs/GO conjugates was cured by the formation of metal-Au alloys.

![Fig. 2 Peroxidase-like activity of Ab-AuNPs/GO conjugates stimulated by Hg²⁺. (A) UV-vis absorbance spectra of solutions containing TMB/H₂O₂ (a), Ab-AuNPs/GO + TMB/H₂O₂ (b), Ab-AuNPs/GO + TMB/H₂O₂ + Ab (c) and Ab-AuNPs/GO treated with Hg²⁺ in the substrate (TMB/H₂O₂) solution (d). Inset was the corresponding photograph. (B) Absorbance spectra of Ab-AuNPs/GO conjugates solution treated with various concentrations of Hg²⁺ ranging from 0 to 1 mM.](image2)

The surprisingly high activity of the conjugates compared to the low catalytic activity of both Ab-GO and Ab-AuNPs in terms of the metal ions-stimulated suggested that the synergetic coupling effects between AuNPs and GO existed (Fig. S3, ESI†), which might result from the large deposition of AuNPs on the GO sheet, on which the AuNPs were fully dispersed, making the Hg²⁺-stimulated peroxidase-like activity efficiently to initiate the high-performance catalysis.

To achieve excellent analytical performance, several kinds of AuNPs/GO hybrids could be synthesized by changing the concentration of Au precursor (Fig. S4, ESI†), showing that the as-prepared Ab-AuNPs/GO conjugates exhibited the highest catalytic activities in the presence of Hg²⁺ when the Au content was 0.5 mM (Fig. S5, ESI†), which was mainly determined by the loading amount of AuNPs on GO surface. Other experimental parameters, including pH, temperature, the concentration of H₂O₂ and TMB, reaction time and Ab concentration on the catalytic activity of Ab-AuNPs/GO in the absence and presence of Hg²⁺ were individually investigated (Fig. S6, Fig. S7 and Fig. S8A, ESI†).

Under the optimal conditions, we evaluated the application of this Hg²⁺-regulated peroxidase-like activity to detect RSV using a sandwich-based immunoassay, where AuNPs/GO hybrids were used as the enzyme for the oxidation of TMB by H₂O₂ instead of HRP in conventional ELISA (Scheme 1B). Benefiting from the cysteine or NH₃¹⁺-lysine residues on protein, Ab can be easily adsorbed onto the surfaces of AuNPs/GO hybrids. The signals were collected when different dilutions of the Ab-AuNPs/GO conjugate were used in the immunoassay (Fig. S8B, ESI†). Using undiluted conjugate resulted in a relative high nonspecific signal. The 1:2 diluted conjugate was adopted with a good balance between low nonspecific signal and high sensitivity.

Later, we compared the detection performance of Ab-
AuNPs/GO conjugates with or without Hg^{2+} stimulation. As indicated clearly by Fig. 3, the detection of the virus performed in the absence of Hg^{2+} was impossible since the absorption at 652 nm was too weak and no appreciable colour change observed. In contrast, the presence of Hg^{2+} can give remarkable absorption in the detection performance of the virus. Specifically, semi-quantization of RSV could be done by the naked eye, which is identical to the spectrophotometric detection of RSV in the range between 0.1 and 10 pg/mL. This immunoassay based on the Hg^{2+}-stimulated peroxidase-like activity of AuNPs/GO hybrids could provide a limit of detection (LOD) of 0.04 pg/mL, which was 50-fold lower than the commercial ELISA kits (2 pg/mL). The high sensitivity of the newly proposed assay was extremely attractive because the concentration of pathogen would be very low at an early infection stage.

![Fig. 3](image)

**Fig. 3** Validation of the use of Ab-AuNPs/GO probe for RSV detection. (A) Comparison of the detection performance of Ab-AuNPs/GO conjugates with (red) or without Hg^{2+}-stimulated (blue) versus various concentrations of RSV. The inset showed the corresponding colour change. (B) The linear range for RSV immunoassay. The error bars represented the standard deviations of the measurements (n=3).

In conclusion, we have demonstrated that the peroxidase-like activity of AuNPs/GO hybrids which have been reduced by protein adsorption could be stimulated by metal ion, facilitating the design of high-sensitivity nanoprobes in biological fluids. To the best of our knowledge, this is the first attempt to explore Hg^{2+}-enhanced catalytic activity of nanomaterial on signal amplification for immunoassay. This approach, by taking use of the synergistic effect between GO and AuNPs, offers the advantages of low cost, sensitivity, simplicity and stability. In addition, the use of H_{2}O_{2} in this strategy further supplies the possibility to the analysis of various substrates or enzymes if coupled with enzyme-mediated reactions by using nanomaterials. Therefore, the proposed metal ion-tuned enzyme-like activity of AuNPs/GO has a broad prospect in biochemistry and biomedical science.

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**Notes and references**

a Key Laboratory on Luminescence and Real-Time Analytical Chemistry (Southwest University), Ministry of Education, College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, China. E-mail: chengzhi@swu.edu.cn; Fax: +86 23 68367257; Tel: +86 23 68254659

b College of Pharmaceutical Science, Southwest University, Chongqing 400716, China.

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