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Visual naked-eye detection of SARS-CoV-2 RNA based on covalent organic framework capsules

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Abstract

The ongoing outbreak of coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has highlighted that new diagnosis technologies are crucial for controlling the spread of the disease. Especially in the resources-limit region, conveniently operated detection methods such as “naked-eye” detection are urgently required that no instrument is needed. Herein, we have designed a novel and facile strategy to fabricate covalent organic framework (COF) capsules, which can be utilized to establish a new colorimetric assay for naked-eye detection of SARS-CoV-2 RNA. Specifically, we employ the digestible ZIF-90 as the sacrificial template to prepare the hollow COF capsules for horseradish peroxidase (HRP) encapsulation. The fabricated COF capsules can provide an appropriate microenvironment for the enzyme molecules, which may improve the conformational freedom of enzymes, enhance the mass transfer, and endow the enzyme with high environmental resistance. With such design, the proposed assay exhibits outstanding analytical performance for the detection of SARS-CoV-2 RNA in the linear range from 5 pM to 50 nM with a detection limit of 0.28 pM which can go parallel to qTR-PCR analysis. Our method also possesses excellent selectivity and reproducibility. Moreover, this method can also be served to analyze the clinical samples, and can successfully differentiate COVID-19 patients from healthy people, suggesting the promising potential in clinical diagnosis.

1. Introduction

The ongoing outbreak of coronavirus disease 2019 (COVID-19), resulted from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread globally and caused substantial infections and deaths [1–4]. As of mid-August 2021, the rapid spread of COVID-19 has affected more than 200 countries, with more than 200 million confirmed cases and nearly 4.3 million deaths. It is of great importance to develop sensitive and accurate SARS-CoV-2 detection methods to promote the epidemic prevention and control of COVID-19. Currently, reverse transcription-polymerase chain reaction (RT-PCR) is the principal method to identify SARS-CoV-2 infection [5–7]. However, RT-PCR requires expensive special equipment, a reverse transcription step and well-trained technicians [8–12]. Considering the high potential threat of the pandemic, it is urgent to establish rapid and simple methods for SARS-CoV-2 detection.

The nanotechnology-based biosensing makes a promising analytical contribution, especially in developing biosensors for viruses detection [13,14]. Owing to the property of large surface-to-volume ratios, nanomaterials can promote highly efficient surface interactions between biosensors and analytes, enabling faster and more accurate detection of viruses [15]. Biosensing technologies include photoelectrochemical (PEC), electrochemiluminescence (ECL), electrochemical and colorimetric methods, etc [16,17]. Although all of these methods can be served for SARS-CoV-2 detection, they differ significantly in their analytical performance. Amongst these, colorimetric assays have attracted widespread attention due to their simplicity, cost-effectiveness, no need for complex instruments, and especially easy to read out with the naked-eye, which may provide new opportunities for SARS-CoV-2 detection [18–20]. Enzyme catalysis is one of the most common colorimetric methods to convert detection events into color changes [21,22]. However, enzymes are easily deactivated under harsh conditions, such as polar organic solvents, extreme pH, high temperatures and protease interactions [23–25]. The fragility of enzymes may
limit their widespread application. Inspired by nature, the design of “smart capsule” is an ingenious strategy for enzyme encapsulation, which can effectively overcome the defects of free enzymes [26,27]. Inorganic nanoparticles and organic polymer nanomaterials are commonly used materials for the preparation of capsules [28,29]. Nevertheless, they may be plagued by shortcomings such as weak functionality or irregular structure [30,31].

Covalent organic frameworks (COFs) are an emerging kind of porous crystalline materials with excellent stability, regular porosity, pre-designable structures and customizable functions, making them a perfect candidate for enzyme encapsulation [32–35]. COFs not only triumph over the defects of poor crystallinity and uneven pore distribution of most inorganic porous materials, but also conquer the deficiency of metal–organic frameworks (MOFs) that easy collapse in water [36–38]. These remarkable physicochemical properties of COFs make them exhibit great potential for biomolecules encapsulation applications. However, the presence of biomolecules will restrict the synthetic conditions, it is unrealistic to use the traditional “one-pot” method to directly prepare COF capsules for biomolecules encapsulation [39,40]. Herein, we have firstly proposed a novel and facile strategy to fabricate COF capsules for the encapsulation of enzymes, and then applied them to establish a new colorimetric assay for the naked-eye detection of SARS-CoV-2 RNA. In order to solve the conundrum of enzyme-encapsulation, we have employed the digestible zeolitic imidazolate framework-90 (ZIF-90) as a template to prepare an enzyme@ZIF-90/COF core–shell structures. Enzyme molecules are enveloped in ZIF-90 through in situ encapsulation, which can protect them from the harsh synthesis conditions of COFs in the next step. Then, the COF shell is formed by a facile reaction between 2,5-divinylterephthalaldehyde (DVA) and 1,3,5-Tris (4-aminophenyl) benzene (TPB) within 10 min at room temperature. After that, the ZIF-90 core is etched away and masses of enzymes are released into the hollow COF capsule. The relatively capacious microenvironment in the formed enzyme@COF capsules can provide excellent protective effects, while keeping the conformational freedom of enzymes and promoting the mass transfer between the substrates and products, which is crucial for the highly catalytic efficiency of the enzyme. The proposed method has combined the advantages of COFs and MOFs, paving a new way to improve the performance of enzymes. More critically, the outstanding performance of COFs capsules as well as their combination with colorimetric bioassay may provide a new alternative for SARS-CoV-2 detection.

2. Experimental section

2.1. Materials and apparatus

1,3,5-Tris (4-aminophenyl) benzene (TPB) and 2-imidazolecarbaldehyde (HyCA) were obtained from Alfa Aesar (UK). S9.6 antibody (Anti-DNA:RNA antibody) was purchased from KeraFAST (USA). Horseradish peroxidase (HRP), goat anti-mouse IgG and FITC labeled goat anti-mouse IgG (FITC-IgG) were supplied by Sangon Biotech Co., Ltd. (Shanghai, China). 2,5-divinylterephthalaldehyde (DVA) was purchased from Jilin Chinese Academy of Sciences-Yanshen Technology Co., Ltd (Changchun, China). Zinc nitrate hexahydrate (Zn(NO3)2·6H2O), tetrahydrofuran (THF), polyvinylpyrrolidone (PVP), scandium trifluoromethanesulfonate (Sc(OTf)3) and acetonitrile (ACN) were supplied by Aladdin (shanghai, China). The 3',5'-5'-tetramethylbenzidine (TMB) substrate (H2O2 included) was obtained from Beyotime (Shanghai, China). DNA-BIND surface 96-well plate was supplied by Corning (USA). The synthetic DNA and HPLC-purified RNA were supplied by Sangon Biotech Co., Ltd. (Shanghai, China) and Takara Biotechnology Co. Ltd. (Dalian, China), respectively. The oligonucleotide sequences were provided in Table S1. All reagents were analytically pure grade and all solutions were prepared by DEPC treated redistilled deionized water in an RNase-free environment. The buffers and apparatus used in this work are given in the Supporting Information.

2.2. Synthesis of HRP@ZIF-90

The synthesis of HRP@ZIF-90 was carried out according to previous report with some modifications [39]. Briefly, 371.3 mg of zinc nitrate was dissolved in 12.5 mL of tertiary butanol and sonicated for 5 min. Then, 12.5 mL of deionized water containing PVP (50.0 mg), ICA (480 mg), and HRP (25.0 mg) was added into the solution, and stirred for 10 min. The product was collected by centrifugation (5500 rpm, 10 min) and washed with deionized water, and then dried under vacuum at room temperature.

2.3. Synthesis of HRP@COF capsules

HRP@COF capsules were prepared as follows: Firstly, HRP@ZIF-90 (20 mg), TPB (14.4 mg) and DVA (10.8 mg) were dissolved in ACN (2 mL) and sonicated for 5 min. Thereafter, 1 mL of Sc(OTf)3 was added dropwise to the solution and stirred for 10 min. Sc(OTf)3, as a MOF compatible catalyst to replace acetic acid, because proton acid can decompose ZIF-90. The product (HRP@ZIF-90@COF) was collected by centrifugation (5500 rpm, 10 min) and washed with acetone, and then dried under vacuum. Afterwards, the obtained HRP@ZIF-90@COF was used to prepare HRP@COF capsules. 30 mg of HRP@ZIF-90@COF was etched in 15 mL of PB buffer (50 mM, pH = 5) for 4 h. The precipitate was collected by centrifugation and washed with deionized water. Finally, the obtained HRP@COF capsules were dried under vacuum at room temperature.

2.4. Preparation of IgG-HRP@COF

The IgG-HRP@COF was prepared by adding 50 μL of IgG (1 mg mL−1) to 1 mL of HRP@COF dispersion (0.3 mg mL−1) with stirring for 2 h. After that, the product was collected by centrifugation, washed with deionized water and dispersed in PBS for further use.

2.5. Fabrication of visual platform for SARS-CoV-2 RNA detection

Firstly, 100 μL of DNA binding buffer containing 0.1 μM probe DNA (p-DNA) was dropped in each well of the DNA-BIND 96-well plate and incubated at 37 °C for 1 h. The plate was rinsed three times with 1 × PBS containing 0.05 % Tween-20 (PBST). Then 100 μL of blocking buffer was dropped in each well for 1 h, and followed by rinsing three times with PBST. After that, 100 μL of hybridization buffer containing 1 nM SARS-CoV-2 RNA were added to each well and incubated for 2 h. After rinsing three times with PBST, 100 μL of S9.6 antibody (20 μg/mL) was added and incubated for 1 h. Subsequently, 100 μL of IgG-HRP@COF was added to each well. After incubation for 1 h, the plate was washed with PBST. Next, 100 μL of TMB solution was dropped in each well and incubated for 15 min. Finally, 100 μL of H2SO4 (2 M) was added to terminate the reaction and the absorbance was determined by microplate reader at 450 nm.

2.6. Clinical samples preparation

The RNA extraction was performed from each clinical sample using a Nucleic Acid Isolation Kit from Bioperfectus Technologies (Taizhou, China) according to the manufacturer’s instructions. The concentration of the extracted RNA was analyzed by UV–vis spectrometer. For SARS-CoV-2 RNA detection, the extracted RNA was diluted to the same concentration.

The total RNA clinical samples of healthy and COVID-19 patients were provided by the Second Affiliated Hospital of Southeast University. The research was ratified by the scientific ethical committee of the Second Hospital of Nanjing and Nanjing University (the project number is 2020-LS-ky003), and informed consent was procured in all cases.
3. Results and discussion

3.1. Principle of the method

Scheme 1 depicts the fabrication process of COF capsules and the mechanism of the colorimetric assay for naked-eye detection of SARS-CoV-2 RNA. Firstly, the enzyme molecules are enclosed in ZIF-90 through in situ encapsulation to gain adequate protection. Then, the COF shell is formed through a Schiff-base reaction between 2,5-divinylterephthalaldehyde (DVA) and 1,3,5-Tris (4-aminophenyl) benzene (TPB) within 10 min at room temperature [32]. Afterwards, the ZIF-90 core is etched away and masses of enzymes are released into the hollow COF capsule, forming the HRP@COF capsules. The excellent biocompatibility of COFs can provide an appropriate microenvironment for enzymes, and the dependable exoskeleton can endow the enzyme with high environmental resistance. Subsequently, the goat anti-mouse IgG is immobilized on the HRP@COF capsules via host–guest interaction to produce IgG-HRP@COF capsules [41]. The high porosity of COFs allows them to load large amounts of IgG, and the exoskeleton of COFs endues IgG with significantly enhanced stability. For the detection of SARS-CoV-2 RNA (RdRP SARSSr-P2 is selected as the target sequence for this work), probe DNA is firstly bound to the DNA-BIND 96-well plate. In the presence of target RNA, a rigid DNA: RNA hybrid is formed, which can be firmly combined by S9.6 antibody due to the high affinity of the S9.6 antibody for DNA-RNA hybrids. After that, the IgG-HRP@COF capsules can conjugate with the S9.6 antibody via the recognition between the primary antibody and the secondary antibody. With the help of TMB and H₂O₂, significant color changes can be observed. In this way, SARS-CoV-2 RNA can be detected by the simple naked-eye and analyzed quantitatively by UV–vis spectra.

3.2. Characterization of materials

The core–shell structure of the HRP@COF capsule has been verified by transmission electron microscopy (TEM) and high resolution transmission electron microscopy (HR-TEM). Fig. 1 A shows that HRP@ZIF-90 has a regular cubic morphology with an average diameter of 400 nm. Fig. 1 B reveals that the COF shell retains the well-defined shape of HRP@ZIF-90. The HR-TEM images (Fig. 2) further confirm the structural transformation of the materials. In addition, the elemental mapping images also prove the successful construction of the HRP@COF capsules. As revealed in Fig. 1D, Fe (the characteristic element of HRP) is evenly dispersed in the COF capsule, demonstrating the successful encapsulation of HRP. Additionally, the signal of Zn is extremely weak, indicating that the etching is successful and ZIF-90 has been digested. Subsequently, powder X-ray diffraction (PXRD) has been conducted to verify the crystal structure of the synthesized materials (Fig. 3A). The PXRD patterns of ZIF-90 and COF are both observed in HRP@ZIF-90@COF, demonstrating the successful preparation of HRP@ZIF-90@COF. However, in the PXRD pattern of HRP@COF, the characteristic peaks of ZIF-90 disappear, confirming that ZIF-90 has been etched. To further validate the loading of IgG, fluorescein isothiocyanate (FITC) labeled IgG (FITC-IgG) has been introduced. The confocal laser scanning microscopy image of FITC-IgG-HRP@COF confirms the presence of a large amount of FITC-IgG on the COF capsules (Fig. 3B).

3.3. Protective effect and reusability of the COF capsules

The protective effect of the COF capsules has been investigated under different harsh conditions (Fig. 3C), such as high temperature, acid, organic solvent (THF) and proteases (trypsin). Firstly, the influence of high temperature is assessed. After thermal treatment at 60 °C for 1 h, free HRP and HRP@ZIF-90 retain only 12 % and 65 % of their original activity, respectively, while HRP@COF remains 89 % of its original activity. The tolerance of COF capsules to acid is evaluated in a phosphate buffer (pH = 4). It can be known that HRP@COF keeps 86 % of its original activity, while free HRP retains 25 %, and HRP@ZIF-90 retains only 30 % of its original activity. After exposure to THF for 1 h, HRP@COF keeps 94 % of its original activity, while free HRP retains 25 %, and HRP@ZIF-90 retains only 30 % of its original activity. After exposure to THF for 1 h, HRP@COF keeps 94 % of its original activity, while free HRP keeps only 18 %. Similar results are observed after treatment with trypsin (2 mg mL⁻¹) for 1 h. HRP@COF remains nearly 100 % of the activity, while free HRP remains only 14 %. These results suggest that HRP@COF capsules can have a superior protective effect. The stability of the
HRP@COF capsules stored at room temperature has also been evaluated. As shown in Fig. S1, HRP@COF capsules retain about 80% of its original activity after one week, showing excellent storage stability. In addition, the reusability of HRP@COF capsules has been assessed (Fig. 3D). After one catalytic reaction is completed, the HRP@COF can be recovered by centrifugation, and then re-dispersed in water for the next catalytic reaction. As revealed in Fig. 3D, HRP@COF still retains 90% of its original activity after 5 cycles, indicating excellent reusability.

Fig. 1. TEM images of (A) HRP@ZIF-90, (B) HRP@ZIF-90@COF and (C) HRP@COF capsules. (D) Elemental mapping images of HRP@COF capsules.

Fig. 2. HR-TEM images of HRP@ZIF-90 (A,D), HRP@ZIF-90@COF (B,E) and HRP@COF capsules (C,F). The indicated scale bar is 10 nm.
3.4. Feasibility verification of the sensing method

To verify the feasibility of this sensing method, the UV–vis spectra under different conditions has been recorded. As shown in Fig. 4, the control group added with TMB solution (curve a) reveals no absorbance at 450 nm. Curve b and curve c represent the absorbance without and with SARS-CoV-2 RNA, respectively. In the absence of SARS-CoV-2 RNA (curve b), the solution only exhibits a low absorbance at 450 nm, since the HRP@COF capsules cannot be bound to the 96-well plate. However, in the presence of SARS-CoV-2 RNA (curve c), the absorbance increases significantly, which demonstrates the feasibility of this sensing method for SARS-CoV-2 RNA detection.

3.5. Analytical performances

To obtain the best performance of the assay, experimental parameters including HRP@COF concentration, probe DNA concentration, S9.6 incubation time, IgG-HRP@COF immobilization time and the size of probe DNA have been optimized (data for the optimization experiments is provided in Fig. S2 and Fig. S3). The optimal condition is as follows: HRP@COF concentration, 0.3 mg mL\(^{-1}\); probe DNA concentration, 0.1 μM; S9.6 incubation time, 60 min; IgG-HRP@COF immobilization time, 60 min; the size of probe DNA, 25 nt.

Under the optimal conditions, the method has been used to analyze different concentrations of SARS-CoV-2 RNA to validate the sensitivity of the assay. As revealed in Fig. 5A, the absorbance increases with SARS-CoV-2 RNA concentration in the range from 5 pM to 5 × 10\(^4\) pM. In addition, it can be observed with the naked-eye that the color of the corresponding solution gradually deepens with the increase of SARS-CoV-2 RNA concentration. The regression equation is \(A = 0.2369 \log c + 0.1061\), with a correlation coefficient of 0.9948. The limit of detection (LOD) and limit of quantification (LOQ) are calculated to be 0.28 pM and 0.93 pM according to the 3Sb/M and 10Sb/M (S = blank standard deviation and M = slope) equations [42–44]. Compared with other methods for SARS-CoV-2 detection (Table 1), this method possesses a wider linear range and a relatively low detection limit, suggesting its excellent performance.

To assess the selectivity of the method, single-base mismatched RNA, three-base mismatched RNA and non-complementary RNA have been chosen as possible interferants. As depicted in Fig. 5C, the absorbance
for SARS-CoV-2 RNA is significantly higher than the other interferents. The results demonstrate that the proposed method has good selectivity for SARS-CoV-2 RNA analysis. Additionally, the reproducibility of the method has been evaluated by the relative standard deviation (RSD) (Fig. S4). Six experiments are performed independently with SARS-CoV-2 RNA concentration of 1 nM. The RSD value for the six experiments is 2.07 %, suggesting that the proposed method has excellent reproducibility. Furthermore, the stability of DNA/RNA hybrid has been investigated, and the result is presented in Fig. S5, which shows satisfactory stability. Moreover, the detection performance in different environments is also investigated. As shown in Fig. 5D, the results obtained in saliva are almost the same as those in PBS, demonstrating that the proposed method can work well in complex biosamples.

3.6. Clinical sample analysis

To further assess the clinical applicability, the proposed method has been employed to analyze clinical samples. The clinical samples are collected from oropharyngeal swabs of five healthy individuals and five COVID-19 patients. As revealed in Fig. 6A, the obtained absorbance of the test samples for COVID-19 patients is significantly higher than that for healthy samples. The scatterplot may visualize a significant difference between the COVID patients and healthy individuals. Additionally, the color of the test sample for the patient and normal are obviously diverse. To testing the accuracy of this method, the expression level of SARS-CoV-2 RNA has also been investigated by qRT-PCR. As presented

![Fig. 5.](image)

![Fig. 6.](image)
in Fig. 6B, the results procured using qTR-PCR and our method are concordant, validating the accuracy of the proposed method. The above results demonstrate that our method can realize visual naked-eye detection of SARS-CoV-2 RNA, which may facilitate the highly urgent results of COVID-19 in resources-limit regions.

4. Conclusions

In summary, we have proposed a novel and facile approach to fabricate COF capsules for the encapsulation of enzymes. Furthermore, the approach has been developed as a new colorimetric assay for the naked-eye detection of SARS-CoV-2 RNA. The excellent biocompatibility of COFs can provide a proper microenvironment for the enzyme molecules, and the dependable exoskeleton can endow enzymes with high environmental resistance. The results show that the enzymes encapsulated in COF capsules possess remarkable stability and reusability, exceeding the traditional porous materials. This method combines the advantages of COFs and MOFs to form capsules, pioneering a new way to improve the performance of enzymes. With such design, COF capsules can exhibit high sensitivity for the SARS-CoV-2 RNA detection with a low detection limit of 0.28 pM. Additionally, the method possesses excellent selectivity, reproducibility and has good applicability in complex biological environments. The proposed method has also been employed to analyze the clinical samples and successfully distinguish COVID-19 patients from healthy people, confirming the feasibility for the diagnosis of COVID-19. Significantly, the results obtained using qTR-PCR and our method are in excellent accord, indicating the accuracy of the method. So the work may provide an alternative for the assay of SARS-CoV-2 RNA, which may possess a promising potential for the application to control the ongoing disease.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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