

Facile colorimetric detection of As(V) in Rice with immobilized acid phosphatase on hollow metal-organic frameworks hybrid with peroxidase-like activity

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ABSTRACT

Quantitative analysis of As(V) in rice is of great significance for food safety and heavy metal pollution control. Here, a facile colorimetric method for As(V) detection was constructed by using immobilized acid phosphatase (ACP) in hollow metal-organic frameworks hybrid. Metalloporphyrin and gold nanoparticles modified hollow zeolite imidazole framework-8 [Au/HZIF-8@TCPP(Fe)], named AuHT, was chosen here as ACP immobilizing carrier with peroxidase-like activity. Firstly, the morphology, structure, immobilization efficiency and catalytic ability of obtained AuHT@ACP were fully characterized. Then, based on the inhibition of As(V) on immobilized ACP and cascade reaction mediated by ACP and AuHT, a colorimetric biosensor was established with excellent simplicity. After comprehensive validation, this colorimetric method presented the advantages of wide linear range (10.0–1000.0 µg/L), low LOD (4.0 µg/L), nice accuracy (recovery of 93.7–109.6 %) and good selectivity. Finally, this method was applied to visual detection of As(V) in rice samples with different varieties.

1. Introduction

Frequent occurrence of major incidents of heavy metal pollution in grain has become a highly concerned public safety issue (Wang et al., 2023). Among all heavy metal pollutants, arsenic (As) is listed as one of “Ten chemicals of major public health concern” by World Health Organization (WHO). Especially, inorganic arsenic, including arsenate [As(V)] and arsenite [As(III)], is classified as a group 1 carcinogen to humans and one of the main excessive heavy metal elements in food crops (Jomova et al., 2011). Rice, which is the main food for more than half of the global population, has better ability to enrich As than other xerophytic crops due to its special irrigated farming model. Therefore, rice is one of the important sources of human exposure to inorganic As (Mawia et al., 2021). The maximum content of inorganic arsenic in polished rice is strictly limited to 0.2 mg·kg⁻¹ by China food and drug administration (CFDA) (Chen et al., 2022). Hence, quantitative analysis of inorganic As in rice is of great significance for food safety and heavy metal pollution control.

Currently, the detection of As(V) has mainly relied on various instrumental methods and biosensors. Traditional instrumental methods applied to As(V) analysis include ion chromatography-inductively

coupled plasma mass spectrometry (IC-ICP-MS), high performance liquid chromatography with ultraviolet absorbance detection or electrospray ionization mass spectrometry (HPLC-UV/ESI-MS), atomic fluorescence spectroscopy (AFS) and atomic absorption spectrometry (AAS). (Gilmartin & Gingrich, 2018; Huber et al., 2017; Matsumoto-Tanibuchi, Sugimoto, Kawaguchi, Sakakibara, & Yamashita, 2019; Qi et al., 2018; Zhang, Jiang, Chen, Yu, & Wang, 2023). Although these methods can offer highly accurate and sensitive results, the requirement of expensive equipment, professional operators, and complex sample pretreatment limit their extensive development in practical application. Instead, biosensors composed of recognition units and signal units have attracted great attention because of their outstanding performance, ease of operation and nice portability (Sanllorente-Méndez, Domínguez-Renedo, & Arcos-Martínez, 2012; Wang, Yang, Yan, Zhang, & Xu, 2022; Wen, Liang, Zeng, Zhang, & Qiu, 2019; Xu et al., 2021; Zhang, Zhang, & Yu, 2016; Zhong et al., 2019). For recognition unit, acid phosphatase (ACP) has been frequently adopted because of the selective inhibitory effect of As(V) on its catalytic activity (Sanllorente-Méndez et al., 2012; Xu et al., 2021; Zhang et al., 2016). For signal mode, compared to fluorescent and electrochemical methods, colorimetric method possesses significant advantages in terms of cost, time, and operation

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simplicity (Piriya et al., 2017; Verma et al., 2022).

Recently, searching for highly efficient nanomaterials for the construction of colorimetric As(V) biosensors has gained great attention. Typical examples are nanozymes, which refer to nanomaterials with different enzyme-mimicking activity. (Gao et al., 2007; Mohamad, Teo, Keasberry, & Ahmed, 2019). Several kinds of nanozymes, including gold nanoparticles (AuNPs), metal-organic frameworks (MOFs), iron oxide and cobalt oxide, have been reported as peroxidase mimics and widely utilized for the construction of colorimetric biosensors (Wu et al., 2019; Yang et al., 2022). Qiu's group developed colorimetric platforms for As(V) detection based on peroxidase-like activity of cobalt oxyhydroxide ($\text{Co}(\text{OH})_2$) and FeOOH nanorods (Wen et al., 2019; Zhong et al., 2019). However, besides the introduction of novel nanozymes, high susceptibility of free ACP to various factors (thermal, pH, and chemical solvents) can influence the analytical features of biosensors (Van Etten & Waymack, 1991). To overcome this limitation, the strategy of immobilizing natural enzyme on selected nanozyme could be adopted to enhance natural enzyme stability, form cascade reaction and construct facile colorimetric biosensor (Hu et al., 2022; Huang et al., 2022). However, research about this attractive strategy for As(V) detection has been in the initial stage with seldom reports.

In our former study, meso-tetra (4-carboxyphenyl) porphyrin-Fe(III) [TCPP(Fe)] and AuNPs were both modified on the surface of hollow zeolitic imidazolate frameworks 8 (ZIF-8) to form a hybrid nanozyme (Au/HZIF-8@TCPP(Fe), which was abbreviated as AuHT) with excellent peroxidase like activity for quick colorimetric determination of choline in infant formula milk powder (Zhang et al., 2022). The outstanding catalytic activity and inherent hollow skeleton structure of AuHT made it nice candidate for enzyme immobilization and further formation of cascade reactions.

In this study, to construct a facile colorimetric method for As(V) detection, ACP was firstly immobilized through pore infiltration mechanism in AuHT to form cascade natural enzyme/nanozyme reactor (Lian et al., 2017). Then, the morphology, structure and catalytic activity of AuHT@ACP were comprehensively studied. Based on the inhibitory effect of As(V) on immobilized ACP, a facile colorimetric biosensor was constructed, validated and applied to As(V) detection in rice samples.

2. Experimental section

2.1. Materials and reagents

AuHT was home made material in our lab in Wuhan polytechnic university. Iron chloride hexahydrate, ACP from wheat germ, 4-nitrophenol (PNP), ascorbic acid 2-phosphate (AAP), and As(V) standard solution were all provided by Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Methanol and anhydrous sodium acetate were both purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Tris(hydroxymethyl) aminoethane [Tris] was purchased from Beijing Lanjiek Technology Co., Ltd. (Beijing, China). Bradford protein assay kit was supplied by Beyotime Biotechnology Co., Ltd. (Shanghai, China). 4-Nitrophenyl phosphate disodium hexahydrate (PNPP) and 3, 3', 5, 5'-Tetramethyl-benzidine (TMB) were both obtained from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Hydrogen peroxide solution (30 %) and rhodamine B were both bought from Tianjin Tianli Chemical Reagent Co., Ltd. (Tianjin, China). Three different varieties of rice samples, including Northeast rice (a type of japonica rice produced in Heilongjiang Province), Huanghuazhan rice (a type of indica rice produced in Guangxi Province) and Pearl rice (a type of japonica rice produced in Ningxia Province), were all purchased from local supermarket.

2.2. Instruments

High-resolution transmission electron microscope (HRTEM, JEOL 2100F), X-ray powder diffraction (XRD, Rigaku SmartLab), Fourier

transform infrared spectrometer (Shimadzu-IRtracer 100, Japan), X-ray photoelectron spectroscopy (Thermo Scientific ESCALAB Xi+, USA) and confocal laser scanning microscope (CLSM, OLYMPUS FV 1200) were all utilized in the characterization of AuHT@ACP. Multifunctional enzyme labeling instrument (Enspire-2300) was used to measure the immobilization capacity of AuHT for ACP. Electron paramagnetic resonance spectra (EPR, EMXPLU-12) was used for the detection of $\cdot\text{OH}$. Thermomixer (Thermostat C, Eppendorf) and UV-vis spectrum (EVOLUTION 220) were both used in the catalytic activity study of synthesized hybrid material and detection of As(V). Smartphone (iPhone 13, Apple) was used to record all color images in this study. As(V) concentration in real samples was also analyzed by AFS method (AFS-3000 Atomic Fluorescence Spectrometer, Beijing Kechuang Haiguang Technology Co., Ltd.).

2.3. Immobilization of ACP in AuHT

ACP was immobilized in the hollow structure of AuHT by pore infiltration strategy (Feng et al., 2015). Before effective immobilization, two key parameters during immobilization process, including enzyme amount (2.0–10.0 mg) and immobilization time (2–10h), were firstly optimized when AuHT was set at 10.0 mg and the whole immobilization volume was set as 18.0 mL. After that, 8.0 mg ACP (0.15 U/mg) was dissolved in 8.0 mL ultra-pure water by slow stirring (200 rpm, 5 min). 10.0 mg AuHT was dispersed in 10.0 mL ultra-pure water by ultrasound (100 W, 1 min). Then, two solutions were mixed, stirred at room temperature for 6 h (200 rpm) and centrifuged for 10 min (8000 rpm). The solid product was collected, washed with ultra-pure water for three times and dried by vacuum at 37 °C for 12 h to obtain immobilized enzyme, named AuHT@ACP. Then, the morphology and structure of AuHT@ACP were fully characterized.

To evaluate immobilization capacity of AuHT towards ACP, bradford protein assay kit was utilized to determine protein concentration before and after immobilization process (Zhang, Sun, Wu, Qin, & Liu, 2023). 5.0 mL protein sample (ACP solution before immobilization or the supernatant obtained after immobilization) and 520.0 μL G250 staining solution were mixed and reacted at room temperature for 5 min. Then, UV adsorption at 595 nm was measured to calculate the amount of immobilized enzyme.

2.4. Catalytic activity of AuHT@ACP

By using PNPP as typical substrate, catalytic activity of immobilized ACP was investigated, including the influence of pH and temperature, and its important apparent kinetic parameters (Srivastava & Anand, 2014). Briefly, 75.0 μL AuHT@ACP (1.0 mg/mL) and 75.0 μL PNPP (1.0 mM) were mixed in 850.0 μL acetate buffer (0.2 M) with different pH value (2.5, 3.0, 3.5, 4.0, 5.0, 6.0). Each mixture was incubated at 37 °C for 5 min to investigate the influence of pH. Similarly, to explore the effect of temperature, the mixture including AuHT@ACP, PNPP and acetate buffer (pH 4.0) was reacted at different temperatures (25, 30, 37, 40, 45, 50, 55, 60 °C) for 5 min. After reaction, the absorbance at 317 nm of various solution was monitored by UV-vis spectrum. Also, the influence of pH and temperature on the catalytic activity of free ACP was investigated with ACP (0.365 mg/mL) instead of AuHT@ACP (1.0 mg/mL) in above process. For data handing, the activity of enzyme with the production of the largest amount of PNP was set as 100 %. Relative activity was defined to be the ratio of PNP amount obtained under different condition to the largest amount of PNP.

Moreover, the steady-state kinetic analysis of AuHT@ACP and free ACP was comparatively carried out under optimal pH and temperature. By changing PNPP concentration in the range of 2.69–53.8 μM (2.69, 5.38, 10.76, 21.52, 32.28, 40.35, 53.8 μM), different reaction velocities were obtained to calculate Michaelis constant (K_m) and the maximum velocity (V_{max}), respectively. For the kinetic study of AuHT@ACP, 75.0 μL AuHT@ACP (1.0 mg/mL), 75.0 μL PNPP (different concentration) and 850.0 μL acetate buffer (0.2 M, pH 4.0) were mixed and incubated at

37 °C for 5 min to measure the UV absorbance at 317 nm. For the kinetic study of free ACP, 75.0 μ L ACP (0.365 mg/mL) replaced AuHT@ACP while the other solution and operational steps were the same as that in the kinetic study of AuHT@ACP. To study the inhibitory effect of As(V), three different As(V) standard solutions (100, 300, 500 μ g/L) were added into the kinetic study system of free ACP and immobilized ACP, respectively (Wang et al., 2018). Other solution and operational steps were the same as above.

To explore the influence of ACP immobilization on catalytic property of AuHT, the peroxidase like activity of AuHT@ACP was studied by using H₂O₂ and TMB as substrates (Yang et al., 2022). The comparative study about catalytic performance of AuHT and AuHT@ACP was conducted under the reaction condition of pH 4.0 and incubation at 37 °C. While, the apparent kinetic parameters of AuHT@ACP towards H₂O₂ and TMB were investigated, respectively. Also, under optimal condition and H₂O₂ concentration set at 1000.0 μ M, UV signals of colorimetric systems mediated by AuHT and AuHT@ACP were compared. By using DMPO as $\cdot$ OH capturing agent and EPR as detection method, production amounts of $\cdot$ OH from AuHT@ACP-H₂O₂ system and AuHT-H₂O₂ system were measured and compared.

2.5. Colorimetric determination of As (V)

The key point of this colorimetric method was inhibitory effect of As (V) on immobilized ACP (Wang et al., 2022; Xu et al., 2021). Here, AAP was chosen as the substrate of AuHT@ACP to obtain AA, which could react with H₂O₂ and then attenuate chromogenic reaction catalyzed by peroxidase-like nanozyme.

100.0 μ L As(V) standard solution with different concentrations (10.0, 25.0, 50.0, 100.0, 200.0, 400.0, 600.0, 750.0, 1000.0, 1500.0 μ g/L) and 100.0 μ L AuHT@ACP solution (1.0 mg/mL) were mixed and reacted at 37 °C for 20 min at first. Then, 100.0 μ L AAP (15.0 mM) was added to the mixture and incubated at 37 °C for another 20 min. After that, 50.0 μ L TMB (20.0 mM), 600.0 μ L acetate buffer (0.2 M, pH 4.0) and 50.0 μ L H₂O₂ (1.0 mM) were all added to the former mixture and incubated at 37 °C for another 10 min. When the reaction was finished, the color of various solutions was recorded by a smartphone while UV-vis signals at 652 nm were measured to investigate the linear relationship and limit of detection (LOD). Moreover, three As(V) samples with different concentrations (50.0, 100.0, 500.0 μ g/L) were added to real sample (Northeast rice) and detected respectively to verify the accuracy of this method. As(V) sample with the concentration of 100.0 μ g/L was measured for six times to test the repeatability of this method. For interference experiment, a large number of interference chemicals (Hg²⁺, Fe³⁺, Cu²⁺, Al³⁺, Mg²⁺, Na⁺, SO₄²⁻, Cl⁻, CO₃²⁻, NO₃⁻, PO₄³⁻) were measured according to previous steps above.

For real sample pretreatment, 10.0 g rice of each variety was washed in pure water for three times, blotted with kitchen paper and dried at 50 °C in vacuum drying oven for 24 h. Then, 1.0 g dried sample was ground to powder and digested by 20.0 mL HNO₃ (0.15 M) with the operational process the same as that in former report (Xu et al., 2021). The obtained solution was finally detected by our colorimetric method and the concentration of As(V) was subsequently calculated. During the detecting process, 100.0 μ L pretreated rice sample replaced As(V) standard solution while the other solution and operational steps were the same as that in the colorimetric detection of As(V) standard solution. Moreover, to confirm As(V) concentration in three real samples, the obtained solution was analyzed by AFS method. The fluorescence intensities were recorded under following conditions: negative high voltage of 300.0 V, atomizer height of 8.0 mm, lamp current of 80.0 mA, carrier gas flow rate of 300.0 mL·min⁻¹, and shielded airflow rate (800.0 mL·min⁻¹).

3. Results and discussion

3.1. Immobilization of ACP in AuHT

Two important parameters during immobilization process (enzyme amount and immobilization time) were first optimized to identify the most suitable conditions for ACP immobilization. As shown in Fig. 1A, with the increase of ACP amount in the range of 2.0–10.0 mg, the amount of immobilized ACP kept an increasing tendency. However, relative activity of immobilized ACP displayed an increasing tendency in 2.0–8.0 mg and then reached a plateau value in 8.0–10.0 mg. Larger immobilization amount did not always mean higher catalytic activity. This might be explained by an accumulation of enzyme particles and the formation of multilayers that block active sites of the enzymes and cause their deformation (Klapiszewski, Zdarta, & Jesionowski, 2018). Moreover, the influence of immobilization time on the amount and relative activity of immobilized ACP was shown in Fig. 1B. When the immobilization time was extended from 2 h to 10 h, the amount and relative activity of immobilized ACP both displayed increasing tendency in 2–6 h and reached a plateau value in 6–10 h. This phenomenon might be explained by the saturation of ACP immobilization at 6 h (Zhu, Huang, Pigna, & Violante, 2010). Thus, when AuHT was set at 10.0 mg and the whole immobilization volume was set as 18.0 mL, 8.0 mg ACP and immobilization time of 6 h were selected as the optimal condition.

3.2. Characterization of AuHT@ACP

After immobilizing ACP in hollow nanocage of AuHT, the obtained AuHT@ACP was fully characterized by TEM, XRD, FT-IR, XPS and CLSM. From Fig. 2A, hollow structure was still maintained during enzyme immobilization process. Black solid line represented the shell of regular hexahedral ZIF-8 while gray domain represented the hollow core of AuHT with immobilized ACP. This obvious hollow nanostructure was constructed from Au³⁺-Zn²⁺ exchange reaction and Kirkendall effect (Tang et al., 2018). Moreover, XRD spectrum confirmed the stable skeleton of ZIF-8. High-resolution diffraction peaks at 7.43°, 10.55°, 12.88°, 14.88°, 16.62° and 18.16° in Fig. 2B ascribed to ZIF-8 structure (Cheng, Svec, Lv, & Tan, 2019). Also, FT-IR spectrum in Fig. 2C demonstrated successful immobilization of ACP on AuHT carrier. The characteristic peaks at 761, 1000 and 1146 cm⁻¹ (red dotted line in the FT-IR spectrum of AuHT and AuHT@ACP) were ascribed to bending of imidazolate ring out-of-plane and in-plane bending vibrations from ZIF-8 structure (Zhang, Sun, et al., 2023). While, the characteristic peaks at 1239, 1350, 1536 and 3300 cm⁻¹ (black dotted line in the FT-IR spectrum of ACP and AuHT@ACP) were ascribed to amide-III adsorption, amide-II vibration, amide-I stretches and N—H stretching vibration from ACP structure, respectively (Huang et al., 2022). Additionally, the presence of seven elements (Zn, Au, Fe, C, N, O, S) in overall XPS spectrum of AuHT@ACP in Fig. 2D was almost the same as that in AuHT (Zhang et al., 2022). XPS spectrum of C1s (Fig. 2E) could be deconvoluted into three main peaks at 284.8, 286.1 and 288.7 eV, which were attributed to C—C, C—N and C=O, respectively (Zhang et al., 2020; Zhang, Sun, et al., 2023). N1s XPS spectrum (Fig. 2F) has three characteristic bands at 398.12, 399.94 and 401.96 eV, corresponding to pyridinic N, pyrrolic N and -NH₂, respectively (Zhang, Sun, et al., 2023). O1s XPS spectrum (Fig. 2G) presented two characteristic bands at 530.82 and 532.57 eV, ascribing to C=O and C-O/O-H bonds (Zhang, Sun, et al., 2023). Furthermore, as shown in Table S1, the percentages of C and O atoms of AuHT@ACP were 60.57 % and 18.05 %, respectively, which were much higher than that in AuHT, indicating effective immobilization of ACP on AuHT carrier. Additionally, XPS of Au element in Fig. 2H presented two pairs of peaks. Two peaks at 84.51 and 88.37 eV were ascribed to Au nanoparticles while two peaks at 87.41 and 91.44 eV were ascribed to Au³⁺ ions (Tang et al., 2018; Zhang et al., 2022). From the comparison of height of two pairs of peaks, the content of Au nanoparticles was much higher than that of Au³⁺ ion. XPS of Fe

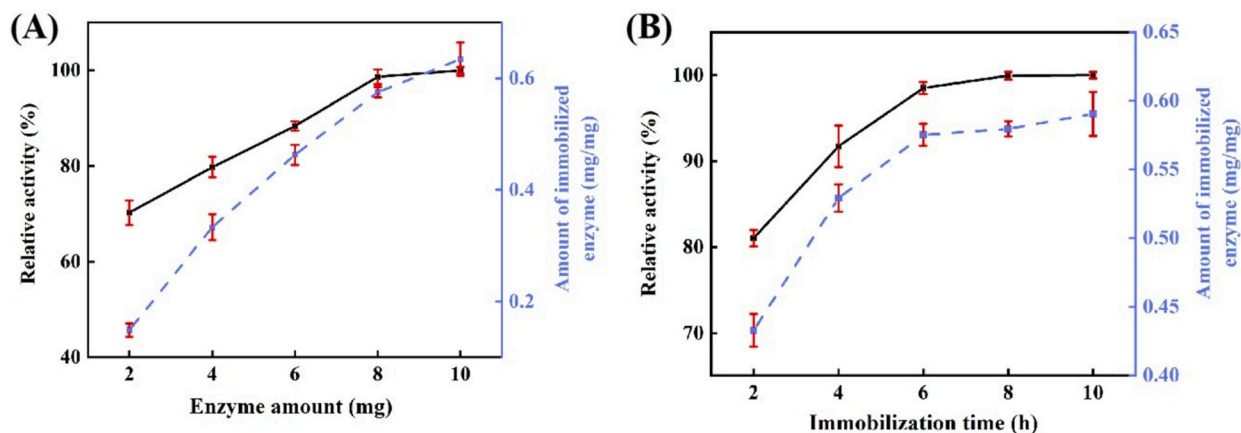


Fig. 1. Optimization of ACP immobilization. The influence of (A) enzyme amount and (B) immobilization time on the amount and relative activity of immobilized ACP.

element in Fig. 2I presented three pairs of peaks in the range of 710.43–731.91 eV, which were ascribed to satellite peaks, Fe^{3+} and Fe^{2+} . Valence state of Fe element was the same as that of AuHT, indicating main content of Fe^{2+} in AuHT@ACP (Zhang et al., 2020; Zhang et al., 2022). The presence of Fe^{2+} and Au nanoparticles was the key factor corresponding to peroxidase like activity of AuHT and AuHT@ACP (Dong et al., 2022; Navyatha, Singh, & Nara, 2021).

To confirm successful immobilization of ACP in AuHT directly, rhodamine B (RB)-tagged AuHT@ACP was observed by CLSM. RB can specifically bind to ACP and emit red light under excitation wavelength of 543 nm (Mun, Kim, McClements, Kim, & Choi, 2017). As shown in Fig. 2J, black shadows in the microphotograph under a bright field were scattered AuHT@ACP nanoparticles. Under fluorescence field, Fig. 2K showed that large numbers of red dots with different sizes were evenly dispersed, indicating the location of ACP. When these two images overlapped in Fig. 2L, the stained part and black nanoparticles were almost identical, indicating successful immobilization of ACP in hollow MOF hybrids.

Moreover, the immobilization capacity of AuHT was assayed and calculated by using Bradford assay. After immobilization, ACP concentration in the supernatant was 0.125 mg/mL. From calculation, 0.575 mg ACP was immobilized in 1.0 mg AuHT through pore encapsulation strategy. This value was obviously higher than that of other carrier for ACP, such as calcined $\text{Mg}/\text{Al}-\text{CO}_3$ layered double hydroxides (0.292 mg ACP immobilized in 1.0 mg carrier) (Zhu et al., 2010), meaning excellent immobilization capacity of AuHT.

3.3. Catalytic activity of AuHT@ACP

To study the catalytic activity of immobilized ACP, a commonly used phosphatase substrate, PNPP, was chosen here. ACP catalyzes the conversion of PNPP to its product PNP, which has UV absorption signal at 317 nm under acidic condition (Xia, Zhang, Yang, Dai, & Yang, 2021). By using this analysis protocol, the influences of pH and temperature on catalytic activity of free ACP and AuHT@ACP were comprehensively investigated. As shown in Fig. 3A, both free ACP and immobilized ACP showed decreasing activity with rising pH in the range of pH 2.5–6.0. Notably, AuHT@ACP exhibited higher catalytic activity than free ACP in pH 2.5–4.0 and a bit lower activity than free ACP in pH 5.0–6.0. This phenomenon could be explained by the change of surface charge of ACP and subsequent influence on the attachment of ACP to the internal surface of central cavities (Chen et al., 2020; Lian et al., 2017). From Fig. 3B, AuHT@ACP and free ACP showed almost the same catalytic activity in the temperature range of 25–45 °C. While, when the temperature raised over 45 °C, AuHT@ACP showed obviously higher activity than free ACP. The catalytic superiority of AuHT@ACP at higher

temperature might be resulted from protective effect of hollow skeleton structure on ACP (Chen et al., 2020). After the investigation, pH 4.0 and 37 °C were adopted in subsequent experiment to study enzyme kinetic parameters.

By altering PNPP concentration in the range of 2.69–53.8 μM , two important apparent kinetic parameters (K_m and V_{max}) of free ACP and AuHT@ACP were studied, respectively. According to Michaelis-Menten equation, the velocity of ACP-catalyzed reaction could be plotted as a function of PNPP concentration. From the double-reciprocal plot of this equation, named Lineweaver-Burk plot, linear relationship between $1/v$ and $1/[s]$ is presented as follow:

(1)

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[s]} + \frac{1}{V_{max}}$$

Here, $[s]$ is the concentration of PNPP set as independent variable while $[v]$ is the reaction velocity set as dependent variable (Seibert & Tracy, 2021). From the plots of $1/[v]$ vs $1/[s]$ for free ACP and AuHT@ACP shown in Fig. 3C–D, K_m and V_{max} of free ACP were calculated to be 4.961 mg/L and 1.516 mg/L $\cdot\text{min}^{-1}$ while K_m and V_{max} of AuHT@ACP were calculated to be 5.432 mg/L and 1.608 mg/L $\cdot\text{min}^{-1}$, respectively. A slightly higher K_m value of AuHT@ACP elaborated its weaker affinity with substrate compared to free ACP. This minor increase of K_m value during immobilization process is a common occurrence, which is similar to that of immobilized ACP on chitosan beads (Srivastava & Anand, 2014). This might be ascribed to two possible reasons, one was the hindering effect of hollow ZIF-8 cage on the diffusion of substances while the other was the conformational changes of ACP occurred after immobilization (Srivastava & Anand, 2014). Furthermore, storage stability of AuHT@ACP was studied with relative activity of immobilized ACP remaining above 70.0 % after 17 days' storage (Fig. S1). Although the research duration of storage stability of AuHT@ACP was a bit shorter than some other immobilized ACP (Srivastava & Anand, 2014; Zhu et al., 2010), it still satisfied the application of immobilized enzyme in the construction of various types of biosensing devices.

Moreover, the inhibitory mode and inhibitory kinetics of As(V) towards free ACP and immobilized ACP were comparatively studied through the addition of different As(V) standard solutions into the kinetic study system. As shown in Fig. 3E–F, from the plots of $1/[v]$ vs $1/[s]$ obtained under different As(V) concentration, the intercepts ($1/V_{max}$) remained unchanged and the slopes (K_m/V_{max}) increased with increase of As(V) concentration, indicating the fixed value of V_{max} and a

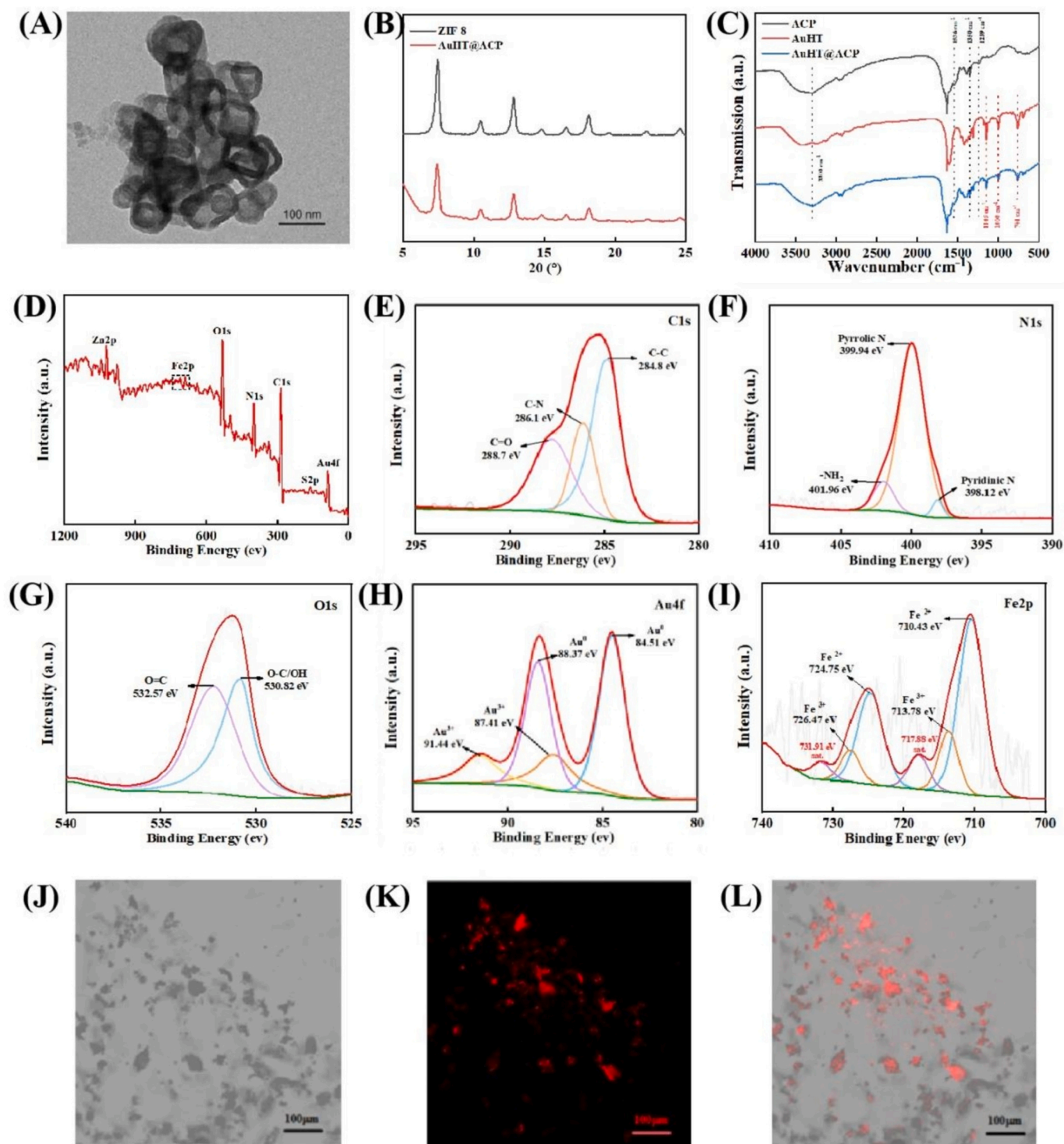


Fig. 2. Morphology and structure characterization. (A) TEM image of AuHT@ACP; (B) XRD spectra of ZIF-8 and AuHT@ACP; (C) FT-IR spectrum of ACP, AuHT and AuHT@ACP; (D) overall XPS spectrum of AuHT@ACP; (E-I) XPS spectra of C, N, O, Au, and Fe element; CLSM images of AuHT@ACP under (J) the bright field and (K) the fluorescence field; (L) overlapped image from (F) and (G).

competitive inhibition mechanism of As(V) towards ACP (Wang et al., 2018; Wen, Liang, et al., 2019). Moreover, the inhibition constants (K_i) of As(V) towards free ACP and AuHT@ACP were calculated according to the following formula:

(2)

$$\frac{1}{v} = \frac{K_m}{V_{max}} \left\{ 1 + \frac{[I]}{K_i} \right\} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

K_i of As(V) towards free ACP and immobilized ACP were calculated to be 322.58 and 344.83 $\mu\text{g/L}$, respectively. This minor difference

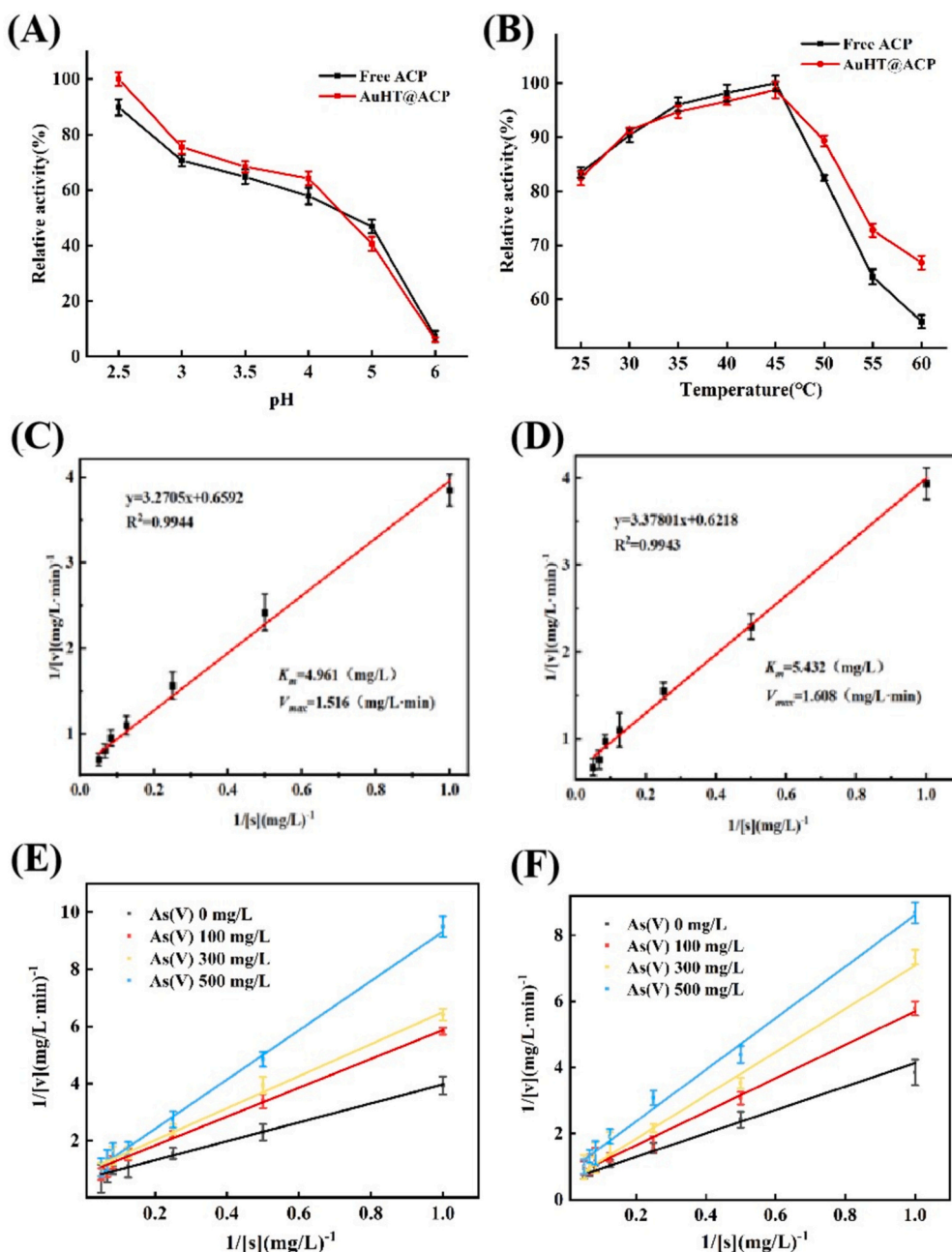


Fig. 3. Catalytic activity of immobilized ACP. Effect of (A) pH and (B) temperature on free ACP and AuHT@ACP; Lineweaver-Burk plots of (C) free ACP and (D) AuHT@ACP; Lineweaver-Burk plots obtained under different As(V) concentrations for (E) free ACP and (F) AuHT@ACP.

between K_i values might be explained by the conformational changes of ACP occurred after immobilization (Srivastava & Anand, 2014;).

ACP immobilization process has almost no impact on peroxidase like activity of AuHT as shown in Fig. 4. Under optimal condition with the concentration of H_2O_2 set at 1000.0 μM , UV signals of reaction solution mediated by AuHT and AuHT@ACP were almost equal (Fig. 4A). From EPR results in Fig. 4B, $\cdot OH$ content produced by AuHT@ACP- H_2O_2 system and AuHT- H_2O_2 system were nearly equal too. From enzyme kinetic parameters study shown in Fig. S2, K_m and V_{max} of AuHT@ACP towards H_2O_2 were 1.66 mM and $4.20 \times 10^{-8} M \cdot s^{-1}$ while K_m and V_{max} of AuHT@ACP towards TMB were 0.876 mM and $8.68 \times 10^{-8} M \cdot s^{-1}$, respectively. These values were all close to that of AuHT in our former study (Zhang et al., 2022), indicating good peroxidase like activity of AuHT@ACP.

3.4. Colorimetric determination of As(V)

Based on nice catalytic activity of immobilized ACP and outstanding peroxidase-like property of AuHT carrier, a visual biosensor for colorimetric detection of As(V) was established. As shown in Scheme 1 (graphical abstract) and Fig. 5A, immobilized ACP could catalyze the conversion of ascorbic acid 2-phosphate (AAP) to its product ascorbic acid (AA), which possesses antioxidant capacity and inhibits AuHT mediated color reaction between H_2O_2 and TMB. As(V), an typical competitive inhibitor of ACP, could bind to ACP specifically and occupy its active site, leading to its decreased activity. Thus, with the addition of As(V), catalytic activity of immobilized ACP was inhibited, resulting in less production of AA, high efficiency of color reaction and darker blue color of reacted solution. Finding the relationship between As(V) content and color change could provide an efficient means for As(V)

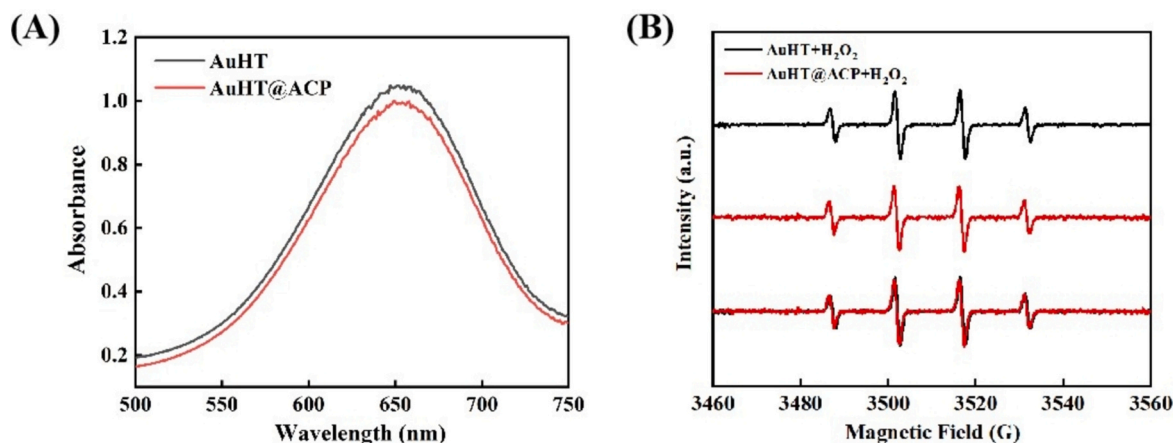
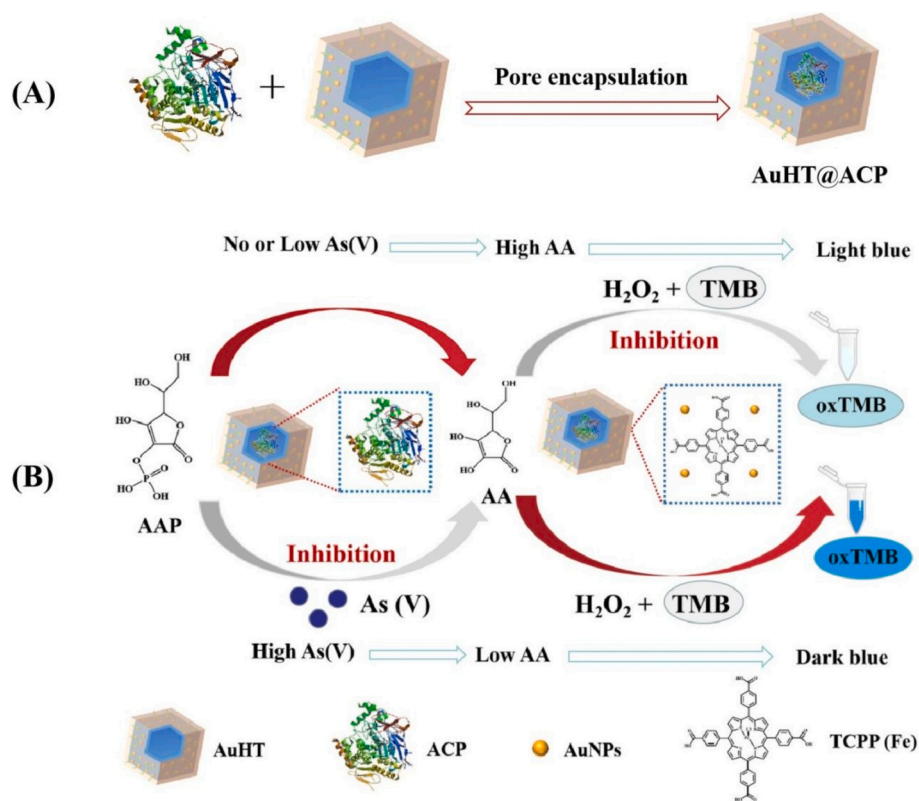


Fig. 4. Peroxidase-like activity of AuHT@ACP. (A) Comparison chart of peroxidase like activity of AuHT and AuHT@ACP (the concentration of H_2O_2 was 1000.0 mM); (B) EPR spectrum of AuHT and AuHT@ACP.



Scheme 1. (Graphical abstract) (A) Preparation of AuHT@ACP; Colorimetric biosensor for As(V) detection based on AuHT@ACP.

detection.

As shown in Fig. 5B, with As(V) concentration increased in the range of 10.0–1500.0 $\mu\text{g/L}$, the color of AuHT@ACP mediated reaction solution turned blue gradually while UV signal elevated obviously. For quantitative detection of As(V), the calibration curve between UV signal and As(V) concentration was investigated. Here, with As(V) concentration [C, $\mu\text{g/L}$] as independent variable and UV signal as dependent quantitative variable [A, the absorbance at 652 nm], the regression equation was $A = 7.25 \times 10^{-4}C + 0.1579$ ($R^2 = 0.997$) shown in Fig. 5C. The linear range was 10.0–1000.0 $\mu\text{g/L}$ and the LOD was 4.0 $\mu\text{g/L}$. when As(V) concentration was higher than the upper limit of this linear range (1000.0 $\mu\text{g/L}$), the measured UV signal became lower than the data obtained from linear equation. While, when As(V) concentration was

lower than the LOD, the measured UV signal was approximate to that of blank sample and showed no relation to the change of As(V) concentration. Thus, when As(V) concentration was outside of this linear range, proper sample pretreatment was needed to conduct As(V) detection. From Table 1, compared to some other optical biosensors for As(V) detection (Wen et al., 2019; Zhang et al., 2016; Pal, Akhtar, & Ghosh, 2016), our colorimetric biosensor presented wider linear range or lower LOD, indicating satisfied analytical property.

Furthermore, the reproducibility, accuracy and selectivity of this colorimetric biosensor were fully validated. From six measurements of 100.0 $\mu\text{g/L}$ As(V) standard solution, the relative standard deviation (RSD) of detected signals was calculated to be 6.13 %, meaning qualified reproducibility. By using a pretreated real sample (Northeast rice)

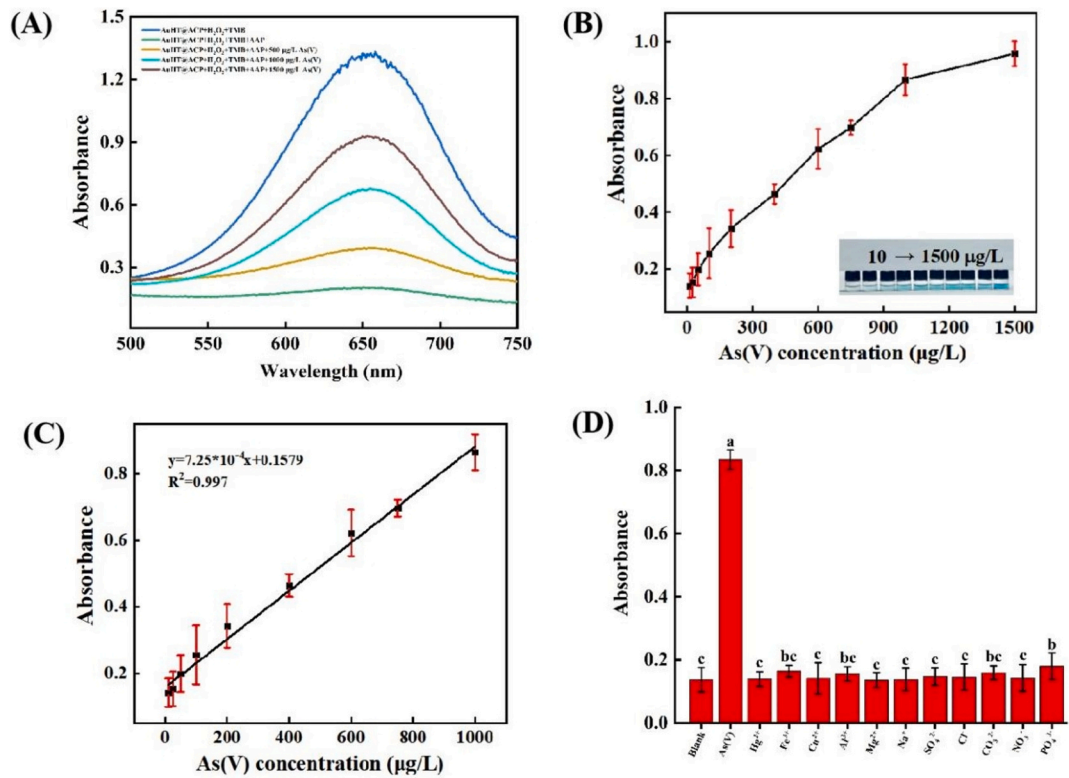


Fig. 5. Colorimetric method for As(V) detection. (A) UV signals of various reacted solution, including AuHT@ACP + H₂O₂ + TMB; AuHT@ACP + H₂O₂ + TMB + AAP; AuHT@ACP + H₂O₂ + TMB + 500.0 µg/L As(V); AuHT@ACP + H₂O₂ + TMB + 1000.0 µg/L As(V); AuHT@ACP + H₂O₂ + TMB + 1500.0 µg/L As(V); (B) UV signals of reaction system containing AuHT@ACP, AAP, H₂O₂, TMB and As(V) standard solution with different concentrations (10.0–1500.0 µg/L); (Insert image showed the color of different reaction systems under various As(V) concentration); (C) linear relationship between As(V) concentration and obtained UV signal; (D) selectivity of the colorimetric method constructed by AuHT@ACP, different letters (a, b, c) denote significant differences at $p < 0.001$ (Tukey's test).

Table 1
Comparison of various analytical methods for As(V) detection.

Detection methods	Linear range (µg/L)	LOD (µg/L)	Consuming times	Advantages and Disadvantages	Reference
Colorimetric biosensor	10.0–1000.0	4.0	50 min	Rapid, inexpensive, easy to use, limited sensitivity	This work
Colorimetric biosensor	0.67–66.67, 66.67–2666.67	0.44	62 min	Rapid, convenient, wider detection range, lower LOD	(Wang et al., 2022)
Colorimetric biosensor	0–8, 8–200	0.1	60 min	Higher sensitivity, effective, low cost and good selectivity	(Zhong et al., 2019)
Electrochemical biosensor	0.04–200	0.012	30 min	Limited sensitivity, good selectivity	(Zhang et al., 2016)
Colorimetric biosensor	22.5–7500	7.50	86 min	Label-free step, convenient operation, on-site assay, low cost and high sensitivity, time consuming	(Wen, et al., 2019)
Electrochemical biosensor	4–500	3.70	150 min	Nice sensitivity, specificity, visual analysis, limited linear range	(Wen, Liang, et al., 2019)
Fluorescent biosensor	0.5–200	0.39	130 min	High sensitivity, excellent selectivity, time consuming	(Xu et al., 2021)
Fluorescent biosensor	3.33–300	1.05	105 min	Easy to use, cost-effective, environmental friendly, limited sensitivity	(Pal et al., 2016)
Fluorescent biosensor	10–100	7	60 min	Simple, rapid, immobilization-free	(Yang, An, Yin, & Li, 2020)
Electrochemical biosensor	3.75–150	1.54	20 min	Low LOD, wide linear range, high selectivity, time consuming	(Fu et al., 2022)
Electrochemical biosensor	0.75–15,000	0.0075	5.5 h	High sensitivity, rapid, expensive equipment	(Zhang, Jiang, et al., 2023)
IC-ICP-MS	0.2–6	0.13	25 min	Rapid, expensive equipment	Matsumoto-Tanibuchi et al., 2019)
HPLC-ICP-MS	0.5–20	2.3	15 min	Rapid, expensive equipment	(Gilmartin & Gingrich, 2018)
HPLC-UV	562–1.21 × 10 ⁵	562	10 min	High efficiency, cost, energy consumption, ambient and sensitivity advantages	(Qi et al., 2018)
HPLC-ESI-MS	5.62–606	5.62	30 min	High sensitivity, accurate, expensive equipment	(Huber et al., 2017)
AFS	0.1–8	0.0028	30 min		
AAS	0.5–5	0.022	30 min		

Table 2

As(V) content in different rice samples detected by our proposed method and AFS method.

Rice Samples	Spiked concentration ($\mu\text{g}\cdot\text{kg}^{-1}$)	Detected concentration by our method ($\mu\text{g}\cdot\text{kg}^{-1}$)	Recovery (%)	Detected concentration by AFS method ($\mu\text{g}\cdot\text{kg}^{-1}$)
Northeast rice	—	132.6 ± 13.1	—	129.1 ± 1.5
	50.0	187.4 ± 17.6	109.6	—
	100.0	226.3 ± 8.6	93.7	—
	500.0	648.4 ± 17.4	103.1	—
Huanghuazhan rice	—	104.5 ± 14.8	—	93.5 ± 0.9
Pearl rice	—	132.4 ± 11.7	—	137.7 ± 1.0

solution as background, three As(V) samples with different concentrations (50.0, 100.0, 500.0 $\mu\text{g/L}$) were added and detected to verify the accuracy of this method. From Table 2, the obtained recovery rates were all in the range of 93.7–109.6 %, which was in the allowable range of 100.0 ± 10.0 % and demonstrated nice accuracy of this method. Moreover, to explore the selectivity of this method, some possible co-existing ions including Hg^{2+} , Fe^{3+} , Cu^{2+} , Al^{3+} , Mg^{2+} , Na^+ , SO_4^{2-} , Cl^- , CO_3^{2-} , NO_3^- and PO_4^{3-} were all detected by this colorimetric biosensor. The concentration of tested interfering substance were set to be 1000.0 $\mu\text{g/L}$, which was the upper limit of As(V) linear range. As depicted in Fig. 5D, the detected signal of As(V) was 0.834 while the signals of other ions were close to that of blank sample. However, it should be noted that blank sample also presented UV signal about 0.1. This phenomenon might be explained by oxidase-like activity of AuNPs in AuHT (Lou-Franco, Das, Elliott, & Cao, 2020), which could oxidize TMB to oTMB product without the participation of H_2O_2 . The slight interference from AuNPs could be treated as background signal in As(V) detection and could not become a major issue in real-world samples analysis. Thus, from comparison of the signals of As(V) and possible co-existing ions, satisfied selectivity of this colorimetric method was confirmed, which was mainly resulted from highly selective inhibitory of As(V) towards immobilized ACP.

To gain comprehensive evaluation of this colorimetric method, comparison of various analytical methods for As(V) detection was showed in Table 1. The excellent advantages of this designed method were rapidity, low cost and simplicity. In terms of time, the detection of As(V) by using our colorimetric method could be finished in just 50 min, which was shorter than that of most of electrochemical biosensors or fluorescent biosensors. In terms of cost and simplicity, the signal of this colorimetric method could be identified by naked eyes, without expensive instruments or complicated operational steps. However, the unavoidable disadvantage of this method was the limited sensitivity. From Table 1, the LOD of this method was 4.0 $\mu\text{g/L}$, which was a bit higher than that of the other biosensors or instrumental methods. Nevertheless, this value was much lower than the limited maximum content of inorganic arsenic in polished rice (0.2 mg/kg). Thus, this method could be utilized in the analysis of As(V) content in rice samples.

3.5. Detection of As(V) in rice samples

After effective verification, this colorimetric method was utilized in the determination of As(V) in rice samples with different varieties. In sample pretreatment, strong acid digestion was adopted to destroy organic compounds and recover inorganic ions. Then, the obtained real samples were analyzed by this method. Like detecting strategy of standard As(V) samples, pretreated rice samples containing different amount of As(V) could react with AuHT@ACP system, inhibit the catalytic activity of immobilized ACP, decrease producing quantity of AA and depress its influence on AuHT mediated color reaction. Thus, pretreated samples with higher As(V) content resulted in dark blue color and higher

UV absorbance while pretreated samples with lower As(V) content resulted in light blue color and lower UV absorbance. After determination and calculation, as shown in Table 2, As(V) concentrations in three different rice samples were in the range of 104.5–132.6 $\mu\text{g/L}$, which was within the scope of data reported in former literature (Chen et al., 2022; Dai et al., 2014; Wang et al., 2022). Actually, As(V) content in three different rice samples were all lower than 200.0 $\mu\text{g/kg}$, indicating low pollution of As(V) and nice quality of these rices. Huanghuazhan rice possessed the lowest As(V) content while Northeast rice and Pearl rice possessed almost equal As(V) content. This result was also confirmed by AFS method as shown in Table 2. Similar data obtained by two methods validated high accuracy and good selectivity of this colorimetric method indirectly. The difference in As(V) content could be ascribed to their different enrichment abilities to As(V) and different places of production.

Thus, As(V) content in three different rice samples were accurately determined by this colorimetric method combined with sample pretreatment of strong acid digestion. In future, the application of this method could extend to the detection of As(V) content in soil, tap water, river water, seaweed, seafood and some other types of food based on its excellent analytical performance.

4. Conclusion

In summary, by using AuHT as an effective enzyme carrier, ACP was immobilized through pore encapsulation strategy. The obtained AuHT@ACP was characterized by TEM, XRD, FT-IR, XPS and CLSM. The catalytic activity of immobilized ACP and peroxidase like activity of AuHT were fully studied. Based on competitive inhibitory effect of As(V) on ACP and cascade reaction mediated by ACP and AuHT, a facile colorimetric method was constructed and effectively verified. This colorimetric method was then utilized in the determination of As(V) content in three different rice samples. Our study not only offered a facile colorimetric means for As(V) detection, but also provided a versatile nanomaterial for enzyme immobilization and biosensor construction. AuHT with hollow skeleton could be utilized as excellent carrier for various enzyme (not only ACP) immobilization through pore adsorption strategy. Then, the formed natural enzyme-nanozyme system can be adapted to detect other contaminants. For example, acetylcholinesterase (AChE) and choline oxidase (ChOx) can be co-immobilized in AuHT to form ACHE-CHOx-AuHT cascade reaction system. Based on the inhibitory effect of organophosphorus pesticides (OPs) on immobilized AChE, a facile colorimetric method might be established for OPs detection.

CRedit authorship contribution statement

Zhiyang Qin: Validation, Data curation. **Zhuolan Xu:** Writing – original draft, Investigation. **Yixin Liu:** Visualization. **Xinguang Qin:** Supervision. **Gang Liu:** Project administration, Funding acquisition. **Xinlin Wei:** Resources. **Haizhi Zhang:** Writing – review & editing, Methodology, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2024.102051>.

Data availability

Data will be made available on request.

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