

Article

Enzyme-Triggered In Situ Assembly of Fe₃O₄ Nanozyme Synthesis Enables Portable Point-of-Care Detection of Acid Phosphatase

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Abstract

Acid phosphatase (ACP) is a clinically important enzyme whose early-stage detection is hindered by its extremely low abundance, nonspecific tissue distribution, and rapid loss of activity under conventional analytical conditions. Herein, we present a target-driven in situ nanozyme synthesis strategy that enables rapid and ultrasensitive point-of-care testing (POCT) of ACP. In this approach, ACP catalyzes the hydrolysis of L-ascorbic acid 2-phosphate sesquimagnesium (AAPS), producing ascorbic acid (AA). The generated AA partially reduces Fe³⁺ ions to Fe²⁺, thereby initiating alkaline co-precipitation and in situ formation of Fe₃O₄ nanoparticles. Polyvinylpyrrolidone (PVP) stabilizes the nanoparticles and preserves catalytic accessibility, while their intrinsic magnetism allows for efficient magnetic separation to eliminate matrix interference. The resulting Fe₃O₄@PVP nanozymes display pronounced peroxidase-like activity, catalyzing hydrogen-peroxide-mediated oxidation of 3,3',5,5'-tetramethylbenzidine (TMB). Quantitative readout can be achieved using either spectrophotometric analysis or smartphone imaging. The sensing platform achieves a detection limit of 0.021 U/L within 40 min and demonstrates excellent sensitivity, selectivity, and operational robustness. Successful validation in human serum confirms its clinical feasibility, while smartphone-based imaging enables portable and low-cost quantification suitable for decentralized diagnostics. Collectively, this work establishes a generalizable paradigm for target-triggered nanozyme generation aimed at detecting low-abundance and labile biomarkers.

Keywords: acid phosphatase; Fe₃O₄ nanozymes; peroxidase; colorimetric assay; smartphone detection



Received: 17 April 2026

Revised: 19 May 2026

Accepted: 5 June 2026

Published: 15 June 2026

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1. Introduction

Acid phosphatase (ACP) is a lysosomal phosphomonoesterase that hydrolyzes phosphate esters under mildly acidic conditions (pH 4–6), maintaining intracellular phosphate homeostasis and regulating metabolic pathways [1]. In mammals, ACP is mainly secreted by the prostate, bone, and liver, contributing to lysosomal turnover and extracellular phosphate metabolism [2–5]. Beyond its physiological functions, ACP has been implicated in tumorigenesis, particularly in prostate cancer, where its dysregulation promotes oncogenic signaling, proliferation, angiogenesis, and apoptosis resistance [6–9]; however, its clinical

utility for early diagnosis is limited due to extremely low basal serum levels, with significant elevation typically occurring only in advanced or metastatic disease. This intrinsic scarcity, combined with inherent enzymatic instability during sample handling, poses a major analytical challenge for early detection [10–14]. Moreover, the lack of rapid point-of-care testing (POCT) platforms leads to prolonged turnaround times, delaying clinical decision making [15,16]. Therefore, developing ultrasensitive, rapid, and portable systems for accurate trace ACP quantification remains an urgent unmet need.

A broad range of analytical technologies has previously been explored for ACP determination, including electrochemical sensing, chromatographic analysis, and magnetic resonance imaging-based methods [17]. Although these techniques provide high analytical sensitivity, their clinical implementation is often constrained by sophisticated instrumentation, time-consuming sample preparation, and extended processing workflows [18,19]. More critically, prolonged *ex vivo* manipulation may partially inactivate ACP prior to measurement, increasing the likelihood of analytical bias or false diagnostic outcomes [20]. By contrast, colorimetric assays represent an attractive alternative owing to their operational simplicity, rapid response, and minimal equipment requirements [21]. Nevertheless, conventional single-signal colorimetric platforms remain susceptible to environmental fluctuation and matrix interference. These limitations are particularly problematic in early cancer diagnostics, where serum ACP elevation is subtle due to low tumor burden and altered metabolic activity [22–24]. Moreover, metabolic reprogramming in cancer cells frequently disrupts systemic amino-acid homeostasis and one-carbon metabolism, further complicating accurate biochemical quantification [25,26]. To overcome these challenges, enzyme-linked cascade amplification strategies have emerged as powerful sensing architectures. By integrating enzymatic recognition with signal amplification, these systems preserve the simplicity of colorimetric detection while substantially improving analytical sensitivity. Such approaches provide a promising framework for next-generation POCT platforms capable of detecting trace disease biomarkers.

Recent progress in nanozyme research has significantly expanded enzyme-mimetic sensing technologies. Compared with natural enzymes, nanozymes exhibit enhanced structural stability, tunable catalytic activity, and scalable synthesis. Metal-based nanomaterials are particularly advantageous because of their high density of catalytic sites, adjustable coordination environments, and efficient electron-transfer properties [27,28]. For example, Song reported a label-free enzyme-linked colorimetric assay for alkaline phosphatase, achieving a remarkably low detection limit of 0.037 U/L. In this system, enzymatic conversion of L-ascorbic acid 2-phosphate sesquimagnesium (AAPS) to ascorbic acid (AA) drives the reduction of metal ions to generate nanoparticles, effectively minimizing false positives commonly encountered in conventional assays [29]. However, systems relying on a single catalytic pathway often suffer from limited robustness. In contrast, iron-based nanozymes offer a distinct advantage by exploiting $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox cycling to drive Fenton-like reactions, thereby generating reactive oxygen species and enabling multiple enzyme-mimetic functions simultaneously [30–34]. This multivalent catalytic behavior enhances both analytical reliability and signal amplification, highlighting the potential of iron-based nanozymes for accurate POCT diagnostics.

Despite these advances, metal nanoparticles frequently undergo aggregation under physiological conditions or fluctuating pH environments, which diminishes catalytic efficiency and sensing performance [35–37]. Polyvinylpyrrolidone (PVP) effectively mitigates this limitation through strong surface adsorption and steric stabilization, maintaining colloidal dispersion without blocking active sites. Parallel developments in smartphone-assisted analytics have further accelerated POCT innovation, enabling rapid digital image analysis using widely accessible mobile devices [38–42].

Building on these concepts, we developed a target-triggered in situ nanozyme synthesis platform in which ACP functions as the specific trigger of nanozyme formation. ACP hydrolysis of AAPS generates AA, which reduces a fraction of Fe^{3+} to Fe^{2+} and drives co-precipitation of Fe_3O_4 nanoparticles under alkaline conditions. Importantly, the amount of AA generated is directly proportional to the ACP activity, which, in turn, determines the quantity of Fe_3O_4 nanozymes formed. To maintain colloidal stability and preserve catalytic performance, PVP is introduced as a stabilizing ligand to prevent nanoparticle aggregation. Furthermore, the intrinsic magnetism of Fe_3O_4 enables facile magnetic separation, removing untransformed substrates and potential interferents, thereby reducing background interference and further improving detection reliability. The generated $\text{Fe}_3\text{O}_4\text{@PVP}$ nanozymes catalyze H_2O_2 -mediated oxidation of TMB, producing a blue chromogenic signal quantitatively correlated with ACP concentration (Figure 1). Compared with conventional assays, this in situ strategy eliminates pre-synthesized catalysts, reduces false positives, and achieves ultrasensitive detection within 40 min. Smartphone integration further enables portable quantitative analysis, establishing a broadly applicable POCT framework.

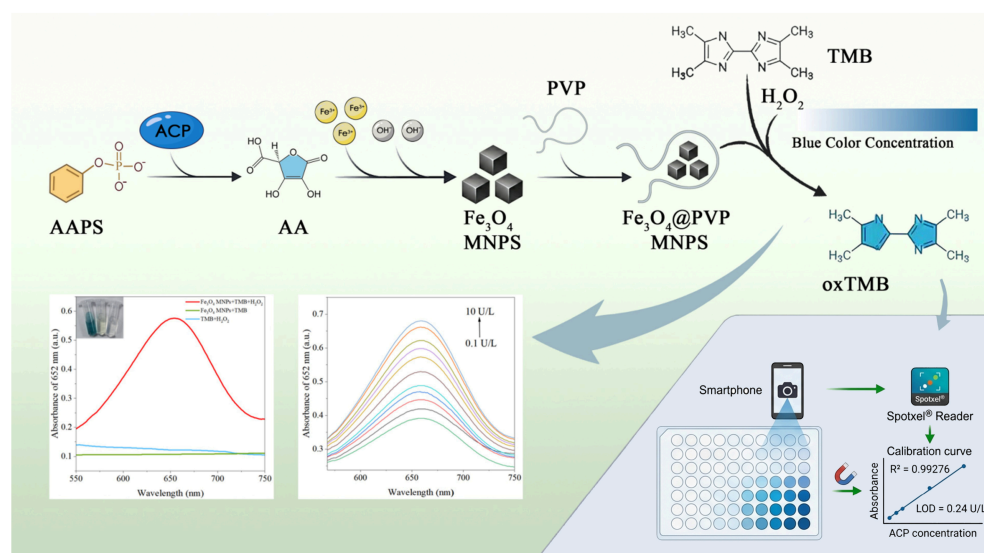


Figure 1. Mechanism of the ACP colorimetric assay.

2. Materials and Methods

2.1. Materials and Reagents

Reagents were employed directly upon receipt, with no additional purification steps performed. For all analytical experiments conducted, deionized water served as the exclusive water source. L-ascorbic acid 2-phosphate sesquimagnesium (AAPS), L-ascorbic acid (AA), polyvinylpyrrolidone (PVP), sodium hydroxide (NaOH), and ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) were procured from Sinopharm Chemical Reagent Shanghai Co., Ltd. (Shanghai, China). 3,3',5,5'-Tetra methylbenzidine (TMB), acid phosphatase (ACP), terephthalic acid (TPA), H_2O_2 , L-histidine (L-His), leucine, cysteine, phenylalanine, cystine, lysine, and arginine were obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). Human serums were procured from Hengyuan Biological Technology Co., Ltd. (Shanghai, China), Gemini Bioproducts, LLC. (West Sacramento, CA, USA), Thermo Fisher Scientific (Shanghai, China) Co., Ltd., and Beijing Boleide Biological Technology Co., Ltd. (Beijing, China).

2.2. Measurements and Characterizations

X-ray diffraction (XRD) patterns were obtained using a D8 ADVANCE X-ray diffractometer (Bruker, Karlsruhe, Germany). Scanning electron microscopy (SEM) was first used for morphological characterization, with images acquired via a Hitachi S-4800 microscope from Hitachi Ltd. (Tokyo, Japan). For optical property analysis, a UV-2600 spectrophotometer (Unico, Shanghai, China) was applied to determine the UV–visible absorption spectra. To gain deeper insights into the surface chemical composition, X-ray photoelectron spectroscopy (XPS) analysis was performed using a Thermo Scientific K-Alpha spectrometer, which was provided by Thermo Fisher Scientific Inc. (Waltham, MA, USA). Electron spin resonance (ESR) spectra were characterized by a Bruker EMXPLUS spectrometer (Bruker, Karlsruhe, Germany).

2.3. Synthesis of Fe_3O_4 @PVP MNPs

To realize a target-driven in situ nanozyme synthesis strategy, a control experiment was performed using various FeCl_3 solutions, and the resulting outcomes were examined by UV–vis absorption spectroscopy. ACP bioassays were performed in HAc–NaAc buffer (pH 4.0). AAPS solution (50 μL , 2.5 mM) was introduced into a 96-well plate, followed by the addition of ACP solutions of varying concentrations (50 μL). After incubation for 30 min to ensure complete enzymatic hydrolysis, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (50 μL , 5 mM) was added, gently pipetted to mix, and incubated for one minute. Subsequently, 50 μL of 0.5 M NaOH solution was added and gently pipetted again. The enzymatically generated AA reduced a fraction of Fe^{3+} ions, initiating the formation of Fe_3O_4 nanoparticles under alkaline conditions. UV–vis absorption spectroscopy was used to monitor nanozyme generation.

Additionally, to exclude interference from the mixed system, Fe_3O_4 nanoparticles were synthesized using magnetic separation technology for subsequent experiments: Fresh stock solutions of ACP (10 U/L) and AAPS (2.5 mM) were prepared in 0.01 M HAc–NaAc (pH 4.0), and 2.5 mL of each was mixed in a 50 mL round-bottom flask, followed by incubation at room temperature with gentle stirring (300–400 rpm) for 30 min to ensure homogeneity. Under continuous stirring, 2.5 mL of 5 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution was added first, then 2.5 mL of PVP solution (0.1% *w/w*) dropwise, and after 2 min of stirring, 3.5 mL of 0.5 M NaOH solution was immediately added dropwise, with the reaction vigorously stirred at room temperature for 10 min. The reaction yielded 13.5 mL of a black suspension containing the synthesized Fe_3O_4 nanoparticles. The nanoparticles were then collected via magnetic separation, washed twice with deionized water, and redispersed in 10 mL of deionized water with uniform stirring. This step effectively removes residual free $\text{Fe}^{2+}/\text{Fe}^{3+}$ ions, unreacted AAPS, AA, and other soluble species, ensuring that subsequent catalytic measurements reflect solely the activity of the purified Fe_3O_4 @PVP nanozyme rather than any homogeneous Fenton reactions of dissolved iron ions. The washed nanoparticle dispersion was then set aside for subsequent use.

2.4. Peroxidase-like Activity of Fe_3O_4 @PVP MNPs

Colorimetric assays were employed to evaluate the peroxidase-mimetic activity of Fe_3O_4 @PVP MNPs, with TMB used as the substrate; detailed experimental protocols for this process are provided in Table S1. Once the three mixed solutions were prepared, they were incubated at ambient temperature for a duration of 10 min. After completing the incubation step, a spectrophotometer (Section 2.2) was utilized to acquire UV–vis absorption spectra for each solution group across the wavelength range of 550–750 nm, and the corresponding absorbance values were documented simultaneously.

Additionally, $\cdot\text{OH}$ production was evaluated using TPA as a fluorescent probe. TPA (2 mM) was dissolved in dimethylformamide and mixed with reaction components according to predefined compositions. Samples were incubated in the dark for 12 h before fluorescence spectra were recorded at an excitation wavelength of 315 nm.

2.5. Evaluation of Steady-State Kinetics of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs

To evaluate the steady-state kinetics of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs, 100 μL of $\text{Fe}_3\text{O}_4@\text{PVP}$ magnetic nanoparticles (with 10 U/L of in situ-generated ACP) was mixed with 500 μL of HAc-NaAc buffer (pH 4.0), 200 μL of TMB solution at varying concentrations (0.1–2.5 mM), and 200 μL of H_2O_2 solution (2.5 mM). After preparing the reaction mixture, the absorbance of oxidized TMB (oxTMB) was measured at a wavelength of 652 nm using a UV-vis spectrophotometer.

Subsequently, the Lineweaver–Burk plot was employed to calculate the key Michaelis–Menten parameters, namely K_m (Michaelis constant) and V_{max} (maximum initial reaction rate). For clarity, the definitions of the relevant parameters are provided below: V corresponds to the initial reaction rate, V_{max} represents the maximum initial rate of the reaction, $[S]$ denotes the substrate concentration, and K_m stands for the Michaelis constant.

$$\frac{1}{V} = \frac{K_m}{V_{\text{max}}} \cdot \frac{1}{[S]} + \frac{1}{V_{\text{max}}}$$

2.6. Optimization of Assay Conditions

To investigate how the microenvironment modulates nanozyme performance, we performed a comprehensive optimization of key parameters, namely pH, temperature, reaction duration, and H_2O_2 level. In the pH study, the $\text{Fe}_3\text{O}_4@\text{PVP}$ MNP-TMB mixture was adjusted to a series of pH values spanning 2.0–7.0, held for 10 min, and then the absorbance at 652 nm was recorded with a UV-vis spectrophotometer (Section 2.2). Temperature effects were examined by holding the reaction mixtures at temperatures from 10 to 50 $^{\circ}\text{C}$ over a 10 min incubation; color development and the absorbance at 652 nm were both monitored spectrophotometrically at predetermined time points during this period. The influence of reaction time was evaluated by allowing the mixture to stand at room temperature and measuring the absorbance at 652 nm at regular intervals across a 0.5–30 min window. For H_2O_2 concentration, 200 μL portions of H_2O_2 solutions with concentrations ranging from 5 to 25 mM were introduced under ambient conditions, and the 652 nm absorbance was immediately acquired.

2.7. Selectivity of the Colorimetric Assay

The selectivity of the ACP-based assay was evaluated by examining its response toward a panel of amino acids, namely leucine, cysteine, phenylalanine, cystine, lysine, arginine, and histidine, along with other common interfering compounds like urea and Na^+ . Stock solutions of each potential interferent were prepared at approximately one-fold, two-fold, and five-fold of their physiological concentrations and subsequently mixed with the optimized $\text{Fe}_3\text{O}_4@\text{PVP}$ MNP-TMB probe. To further probe the influence of ALP on the reaction, a 10 U/L ALP solution was first prepared. Two separate sets of experiments were then conducted following the previously described procedure: one employing the $[\text{ALP} + \text{AAPS} + \text{Fe}^{3+} + \text{H}_2\text{O}_2 + \text{OH}^- + \text{TMB}]$ system and the other the $[\text{ACP} + \text{AAPS} + \text{Fe}^{3+} + \text{H}_2\text{O}_2 + \text{OH}^- + \text{TMB}]$ system. The absorbance of the resulting mixtures was recorded at 652 nm. All selectivity tests were carried out in triplicate ($n = 3$) to ensure reliability.

2.8. Colorimetric Assay of ACP and Smartphone-Based Color Readout

For dose-response measurements, $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs were generated in situ by introducing 100 μL of ACP solutions covering the concentration range 0.1–10 U/L. After magnetic separation, the reaction mixture was then supplemented sequentially with 500 μL of HAc-NaAc buffer (pH 4.0), 200 μL of H_2O_2 (2.5 mM), and 200 μL of TMB (0.5 mM). After incubation at 35 °C for 10 min, the absorbance spectrum from 550 to 750 nm was collected using a UV-vis spectrophotometer (Section 2.2). Each concentration was tested in triplicate ($n = 3$).

For the smartphone-assisted readout, a 200 μL aliquot of each reacted solution was transferred into the wells of a 96-well plate. Colorimetric signals were captured using a smartphone installed with the Spotxel[®] Reader application, a freely available tool that quantifies color intensity in multi-well formats. The app was operated in blue filter mode, and the color intensity of the blue channel was extracted from the well images. By correlating this intensity with the known acid phosphatase concentrations, a quantitative calibration relationship was established.

2.9. Human Serum Sample Analysis

To demonstrate the practical applicability of the proposed sensing platform in ACP detection, human serum samples obtained from four independent commercial suppliers (Section 2.1) were employed as representative model matrices. Each sample was centrifuged at 10,000 rpm for 20 min, and the supernatant was diluted two-fold with ultrapure water. Subsequently, 50 μL of the diluted serum was mixed with 50 μL of AAPS solution (2.5 mM) and incubated for 30 min at room temperature. Then, 50 μL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (5 mM) and 50 μL of PVP solution (0.1% w/w) were added, followed by 50 μL of NaOH (0.5 M). After the in situ synthesis was complete, the generated $\text{Fe}_3\text{O}_4@\text{PVP}$ nanoparticles were magnetically collected, washed twice with deionized water to remove the serum matrix, and redispersed in 500 μL of HAc-NaAc buffer (pH 4.0). The washed nanoparticle dispersion was then used in the optimized reaction system with H_2O_2 and TMB, and the ACP concentration was determined using the colorimetric method described above.

3. Results and Discussion

3.1. Detection of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNP Production

To check the experimental design, we carried out a control experiment with different $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solutions. We looked at the results with UV-vis absorption spectroscopy. Figure S1a shows that the mixture of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, ACP, and AAPS gave a typical plasmon peak of Fe_3O_4 MNPs at about 309 nm after 10 min at room temperature. The Fe_3O_4 MNPs formed because Fe^{3+} was reduced by AA. AA was made from the enzyme reaction of ACP and AAPS. But other mixtures (Fe_3O_4 MNPs + ACP and Fe_3O_4 MNPs + AAPS) did not show the peak. These first results showed that the AA from the breakdown of AAPS could help form Fe_3O_4 MNPs in the reaction solution. Then we made ACP solutions with different concentrations and let them react with AAPS for 10 min. In this step, the amount of AA made depended on the ACP concentration. After we added the FeCl_3 solution, a typical plasmonic peak appeared within 10 min. This confirmed that Fe_3O_4 MNPs formed. The peak positions changed with the ACP concentration (Figure S1b). When the ACP concentration was high (10 U/L), the plasmonic peak of the Fe_3O_4 MNPs was exactly at 309 nm [43]. When the ACP concentration was low (0.01 U/L), the peak moved to shorter wavelengths. The peak position changed because the sizes and shapes of the nanoparticles were different under different AA concentrations.

3.2. Characterization of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs

The morphological and structural properties of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs were characterized via SEM (Figure 2a). Correspondingly, the detailed morphological features of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs are presented in Figure 2b. It was observed that the particle size distribution of the $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs ranges from 10 to 55 nm, with a higher proportion of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs falling within the 30–35 nm range.

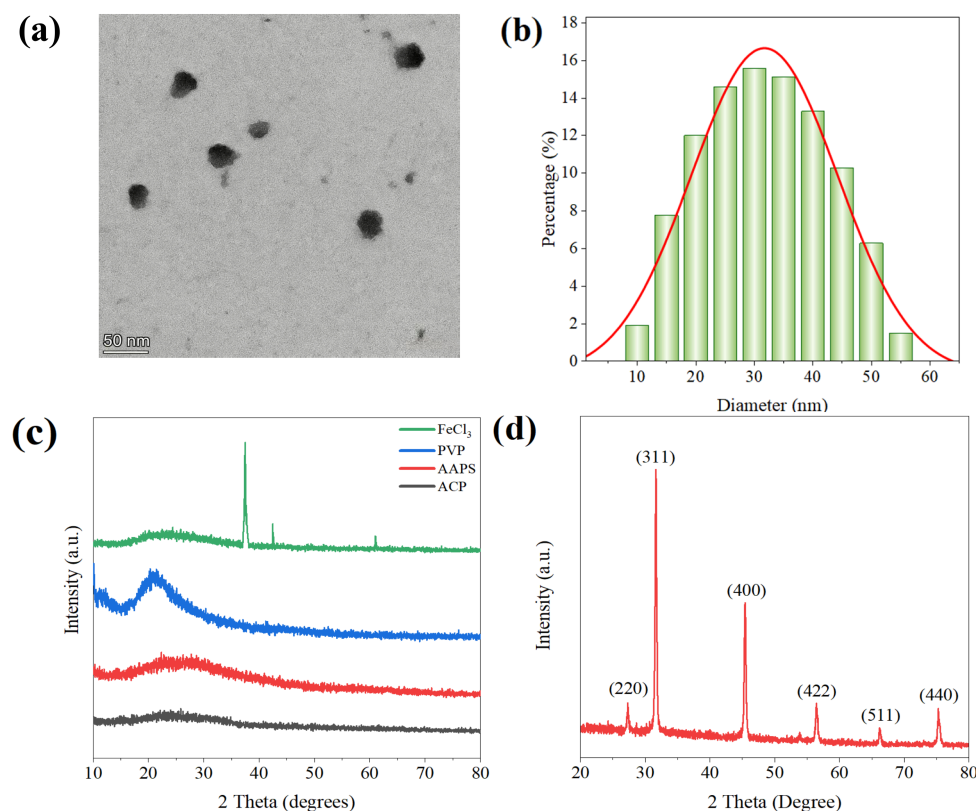


Figure 2. (a) SEM observation of the surface morphology and architecture of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs; (b) histogram displaying the size distribution of $\text{Fe}_3\text{O}_4@\text{PVP}$ nanoparticles; (c) X-ray diffraction of the precursors (AAPS, PVP, FeCl_3 , and ACP); (d) X-ray diffractogram of the as-synthesized $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs.

To further confirm the in situ formation of $\text{Fe}_3\text{O}_4@\text{PVP}$ nanoparticles, the reaction mixture (ACP + AAPS + FeCl_3 + NaOH + PVP) was incubated for different time periods (2, 5, 10, 15, 20, 25, and 30 min). At each time point, the resulting nanoparticles were collected by magnetic separation, washed twice with deionized water, and immediately subjected to particle size analysis. As shown in Figure S2, the average particle diameter gradually increases from approximately 20 nm at 2 min to about 30–35 nm at 10 min, after which it remains essentially stable. This time-dependent growth directly demonstrates that nanoparticles are being formed and maturing within the reaction mixture. Furthermore, the successful magnetic collection of the nanoparticles at each time point confirms their magnetic nature, which is a characteristic property of Fe_3O_4 . In contrast, in the control experiment without AAPS, magnetic separation yielded no detectable nanoparticulate material, and SEM imaging confirmed the absence of nanoparticle formation. These control results demonstrate that nanoparticle formation is strictly dependent on both ACP and its substrate AAPS, consistent with the proposed mechanism that ACP-catalyzed hydrolysis of AAPS generates AA, which, in turn, triggers the formation of $\text{Fe}_3\text{O}_4@\text{PVP}$ nanoparticles.

The crystal structure of AAPS, PVP, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, ACP, and $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs prepared via the in situ ACP method was characterized by XRD analysis. As shown in

Figure 2c, no characteristic diffraction peaks of metallic Fe_3O_4 are observed; only features corresponding to amorphous $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and excipients appear, confirming that $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ exists as Fe^{3+} with no metallic impurities. In contrast, as shown in Figure 2d, distinct diffraction peaks at 30.1° , 35.5° , 43.1° , 53.4° , 57.0° , and 62.6° can be clearly observed, which correspond to the (220), (311), (400), (422), (511), and (440) crystal planes of Fe_3O_4 (JCPDS No. 19-0629), respectively. The sharp peak shapes and low baseline noise indicate good crystallinity of the prepared $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs.

It is worth noting that the ACP-catalyzed hydrolysis of AAPS releases phosphate anions at mM concentration alongside AA. Theoretically, these phosphate ions could co-precipitate with $\text{Fe}^{2+}/\text{Fe}^{3+}$ to form iron (II) phosphate, iron (III) phosphate, or related hydrogen phosphate salts. However, under our strongly alkaline synthesis conditions (0.5 M NaOH), the formation of $\text{Fe}(\text{OH})_2/\text{Fe}(\text{OH})_3$ and their subsequent rapid co-precipitation into Fe_3O_4 is kinetically dominant over the nucleation and growth of iron phosphate phases. Importantly, the XRD pattern shows no characteristic diffraction peaks of crystalline FePO_4 or $\text{Fe}_3(\text{PO}_4)_2$, confirming that Fe_3O_4 is the primary crystalline product. Furthermore, even if minor amounts of amorphous iron phosphate do form, they are non-magnetic and are effectively removed during the magnetic separation step (Section 2.3) before the catalytic assay, thus posing no interference to the subsequent colorimetric detection. This is further supported by the excellent analytical performance observed in selectivity and real sample studies.

We further employed XPS to determine the valence state of the Fe_3O_4 species. The full survey spectrum (Figure S3) confirms the presence of Fe, C, N, and O elements. Previous reports indicate that the $\text{Fe } 2p_{3/2}$ peak for Fe_3O_4 lacks a satellite peak [44]. Our results (Figure S4a) also show no such satellite peak. The binding energies of $\text{Fe } 2p_{3/2}$ and $\text{Fe } 2p_{1/2}$ are 710.6 eV and 724.1 eV, respectively. Figure S4b displays the XPS spectrum of $\text{Fe } 3p$ for Fe_3O_4 . The expected $\text{Fe}^{2+}:\text{Fe}^{3+}$ ratio in Fe_3O_4 is approximately 1:2. Using the parameters defined above, deconvolution of the peaks yields an $\text{Fe}^{2+}:\text{Fe}^{3+}$ ratio of 0.29:0.61.

To evaluate the stability of the reaction system, we compared the particle size and peroxidase activity of a 10 U/L ACP in situ-synthesized $\text{Fe}_3\text{O}_4@\text{PVP}$ MNP solution before and after two months of storage at room temperature. As shown in Figure S5a,b, neither the particle size nor the peroxidase activity changed significantly after two months, indicating that the $\text{Fe}_3\text{O}_4@\text{PVP}$ MNP system prepared via ACP in situ synthesis exhibits excellent physicochemical stability.

3.3. Peroxidase-Like Activity

Although the intrinsic peroxidase-like activity of Fe_3O_4 nanoparticles has been well documented since the pioneering work of Gao et al. [45] in 2007, it was necessary to functionally verify that the $\text{Fe}_3\text{O}_4@\text{PVP}$ nanoparticles generated through our specific ACP-triggered in situ synthesis route retain sufficient catalytic activity under the assay conditions.

All catalytic activity assays described below were performed on the $\text{Fe}_3\text{O}_4@\text{PVP}$ nanoparticles after magnetic separation and washing, thus eliminating any potential interference from residual free iron ions. To characterize the peroxidase-related activity of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs, we first assessed their peroxidase-mimicking properties using the color change of TMB (common chromogenic enzyme substrate) and confirmed the results through synthesis experiments (Figure 3). The detection principle is based on the hydrolysis of AAPS catalyzed by ACP to generate AA, which subsequently reduces Fe^{3+} to form $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs. To further elucidate the catalytic mechanism, a series of control experiments were performed: In the $[\text{H}_2\text{O}_2 + \text{TMB}]$ system without $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs, the absorption in the visible region was minimal, indicating no oxidation reaction occurred.

This also confirms that no Fenton-active free iron ions were present in the system after magnetic purification, as they would otherwise have catalyzed H_2O_2 decomposition and TMB oxidation. Similarly, the $[\text{Fe}_3\text{O}_4@\text{PVP MNPs} + \text{TMB}]$ system also showed only weak absorption, suggesting that dissolved oxygen had negligible impact and that $\text{Fe}_3\text{O}_4@\text{PVP MNPs}$ themselves do not possess intrinsic peroxidase-like activity. However, when H_2O_2 was introduced into the $[\text{Fe}_3\text{O}_4@\text{PVP MNPs} + \text{TMB}]$ system, the solution color changed markedly from colorless to deep blue, confirming that TMB oxidation took place in the $[\text{Fe}_3\text{O}_4@\text{PVP MNPs} + \text{H}_2\text{O}_2 + \text{TMB}]$ system. These findings confirm that $\text{Fe}_3\text{O}_4@\text{PVP MNPs}$ can serve as a highly sensitive colorimetric assay for the quantitative detection of ACP.

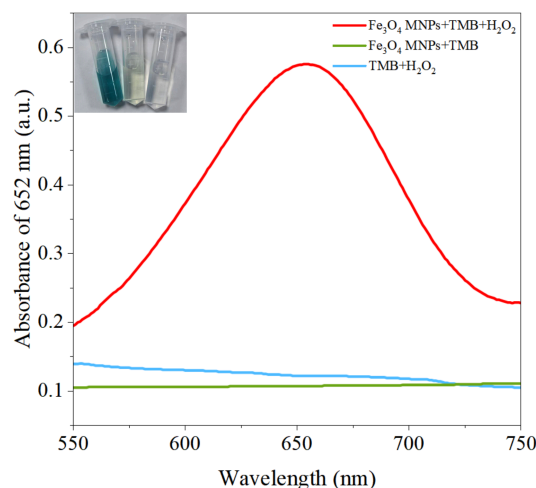


Figure 3. Comparison of peroxidase-mimicking activity of $\text{Fe}_3\text{O}_4@\text{PVP MNPs}$ under different conditions.

3.4. Steady-State Kinetics

To clarify the mechanism underlying $\text{Fe}_3\text{O}_4@\text{PVP MNPs}$ ' peroxidase-mimetic activity, we further investigated the steady-state kinetics of TMB—its substrate—in the $[\text{TMB}-\text{Fe}_3\text{O}_4@\text{PVP MNPs}]$ complex. By utilizing experimental kinetic data, we constructed a plot of initial reaction rate against substrate concentration, which yielded a typical Michaelis–Menten curve (Figure 4a). Subsequently, the Lineweaver–Burk plot (double reciprocal plot) was derived from the data presented in Figure 4b.

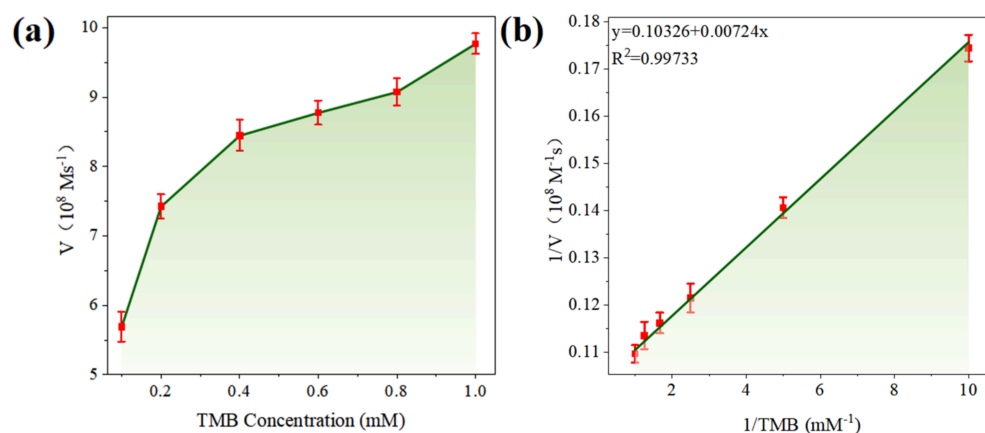


Figure 4. Steady-state kinetic analysis and investigation of the catalytic behavior of $\text{Fe}_3\text{O}_4@\text{PVP MNPs}$: (a) Michaelis–Menten saturation curve obtained with TMB as the substrate catalyzed by $\text{Fe}_3\text{O}_4@\text{PVP MNPs}$; (b) double-reciprocal Lineweaver–Burk plot derived for TMB oxidation mediated by the nanozymes.

The calculated $V_{\max}$ and K_m values of $\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs were $9.708 \times 10^{-8} \text{ M/s}$ and 0.07 mM , respectively. Notably, its K_m value was lower than those of other catalysts listed in Table S3. This finding confirms that $\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs exhibit strong affinity for TMB.

3.5. Optimization of Reaction Conditions

To detect ACP with high sensitivity and selectivity, we need to optimize key experimental conditions. These conditions include pH, incubation time, temperature, and H_2O_2 concentration. It should be noted that the pH-dependent oxidation of TMB is fundamentally governed by the protonation state of TMB itself, with the monoprotonated form being the reactive species susceptible to oxidation [46], as previously established. Therefore, the pH optimization in this study was performed not to reinvestigate this well-known property of TMB. Instead, we wanted to find the best pH that balances two factors. The first factor is the catalytic efficiency of the $\text{Fe}_3\text{O}_4\text{@PVP}$ nanozyme, which can be influenced by its surface charge and colloidal stability at different pHs. The second factor is the chromogenic performance of TMB in our specific reaction system.

In this study, single-factor experiments and response surface methodology were further used to explore how different conditions affect the peroxidase-like activity of $\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs. This work had two core goals: first, to carry out enzyme kinetics research on $\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs, and second, to identify the optimal conditions for ACP detection. With the aim of maximizing colorimetric assay performance, each parameter was quantitatively evaluated through systematic assessment using Equation (1).

$$\text{Relative Activity} = \frac{A}{A_0} \cdot 100\% \quad (1)$$

Data from Figure 5a demonstrate that the relative peroxidase-like activity of the $\text{Fe}_3\text{O}_4\text{@PVP}$ MNP system increased significantly as the buffer pH rose from 2.0 to 4.0, peaking at pH 4.0, and declined progressively at higher pH values. This optimal pH of 4.0 is consistent with the well-established property of TMB, whose monoprotonated form is the species prone to oxidation and predominates under mildly acidic conditions. At the same time, this pH also provides favorable conditions for the colloidal stability and surface catalytic activity of the $\text{Fe}_3\text{O}_4\text{@PVP}$ nanozyme. Therefore, pH 4.0 represents the balanced optimum for the overall assay performance and was selected for all subsequent experiments.

For reaction time optimization, results from Figure 5c show that the reaction in the $\text{Fe}_3\text{O}_4\text{@PVP}$ MNP-TMB system rose sharply within the first 5 min, then stabilized at the 10 min mark, and finally began to decrease slowly between 10 and 30 min. This trend suggests the reaction was nearly complete by 10 min. To further investigate the influence of H_2O_2 dosage on catalytic performance, we systematically examined the effects of varying concentrations of $\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs on the reaction system (Figure 5d).

Overall, the optimal conditions of pH 4.0, 35°C , 10 min incubation, and 2.5 mM H_2O_2 were identified, with the understanding that the optimal pH primarily reflects the chromogenic properties of TMB, while the other parameters govern the catalytic performance of the $\text{Fe}_3\text{O}_4\text{@PVP}$ nanozyme.

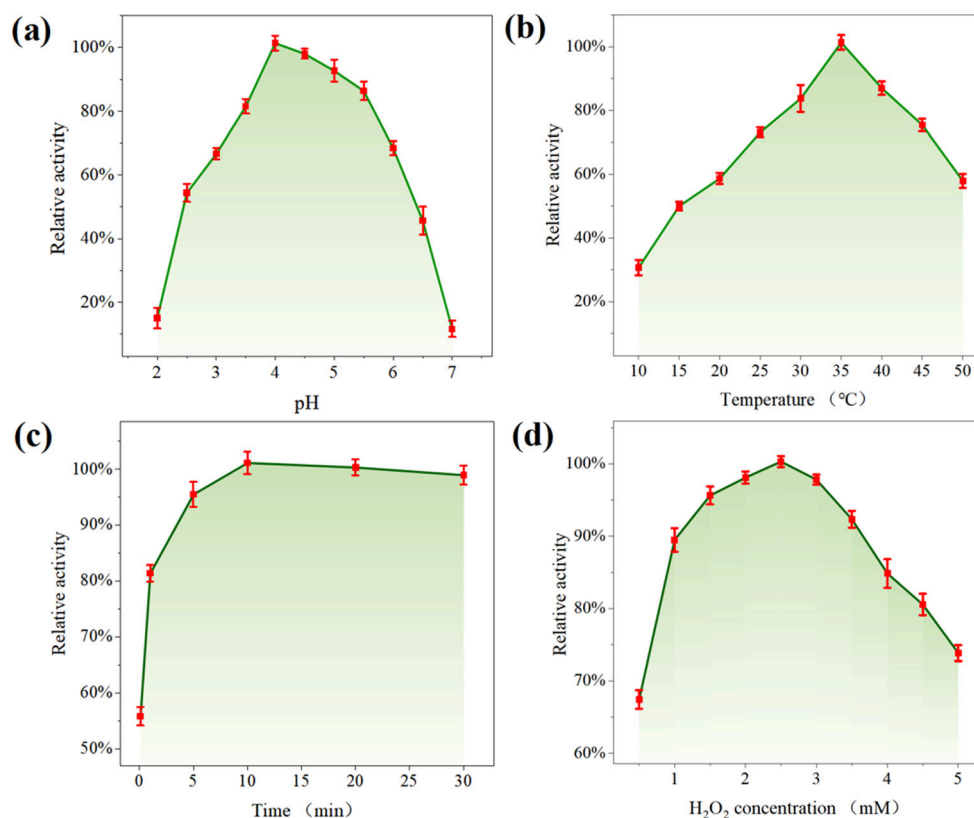


Figure 5. Systematic tuning of assay variables: (a) peroxidase-like activity profile of $\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs across different pH conditions; (b) effect of incubation temperature on the catalytic performance of the nanozymes; (c) time-dependent variation in the enzyme-mimetic activity; (d) dependence of the activity on H_2O_2 concentration.

3.6. Mechanism Study

The catalytic pathway was further clarified by probing the formation of hydroxyl radicals ($\cdot\text{OH}$) within the $[\text{Fe}_3\text{O}_4\text{@PVP MNPs} + \text{H}_2\text{O}_2]$ system. The enzyme-mimetic activity of the $\text{Fe}_3\text{O}_4\text{@PVP}$ nanozymes originates largely from a surface-driven Fenton-type reaction, mediated by the $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox cycle, which accelerates the breakdown of H_2O_2 into highly oxidative $\cdot\text{OH}$. In this process, the continuous interconversion between Fe^{2+} and Fe^{3+} at the nanoparticle surface donates electrons to H_2O_2 , facilitating the rupture of the O-O bond and the subsequent release of $\cdot\text{OH}$. These radicals serve as potent oxidants that convert the chromogenic substrate TMB into its blue oxTMB.

Two complementary approaches were employed to verify $\cdot\text{OH}$ production. First, fluorescence measurements revealed a distinct emission peak at 440 nm upon combining $\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs with H_2O_2 and TPA. This enhancement arises because TPA specifically captures $\cdot\text{OH}$ to yield the strongly fluorescent 2-hydroxyterephthalic acid. Secondly, ESR spectroscopy with DMPO spin trapping provided direct confirmation: a characteristic 1:2:2:1 quartet pattern, typical of the DMPO- $\cdot\text{OH}$ adduct, was recorded in the $[\text{Fe}_3\text{O}_4\text{@PVP MNPs} + \text{H}_2\text{O}_2]$ mixture (Figure 6b), leaving no ambiguity regarding the radical's presence. Moreover, the fluorescence intensity scaled with the concentration of ACP used during in situ nanoparticle synthesis, with weaker signals observed at lower ACP levels. This concentration-dependent response confirms that the quantity of in situ-generated $\text{Fe}_3\text{O}_4\text{@PVP}$ directly determines the yield of $\cdot\text{OH}$, thereby linking the target ACP level to the final colorimetric output. Collectively, these results demonstrate that the in situ-synthesized $\text{Fe}_3\text{O}_4\text{@PVP}$ nanozymes exhibit intrinsic peroxidase-like activity by catalyzing H_2O_2 de-

composition into $\cdot\text{OH}$, a mechanism that can be exploited for the rapid and sensitive detection of ACP.

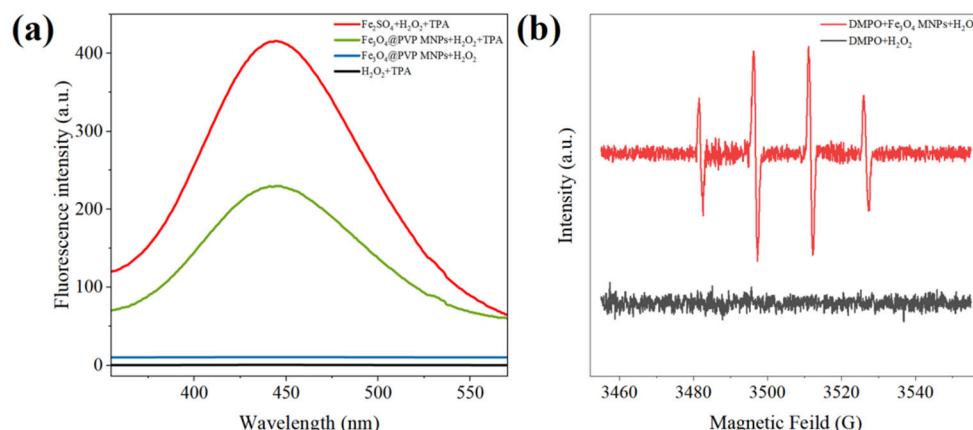


Figure 6. (a) $\cdot\text{OH}$ detection via fluorescence measurement; (b) spin-trapping ESR analysis of $\cdot\text{OH}$.

It should be noted that because the $\text{Fe}_3\text{O}_4@\text{PVP}$ nanoparticles were thoroughly washed via magnetic separation prior to these measurements, the observed $\cdot\text{OH}$ generation and subsequent TMB oxidation can be exclusively attributed to the heterogeneous peroxidase-like catalysis on the nanoparticle surface, rather than to homogeneous Fenton-type reactions of any residual free iron ions.

3.7. Colorimetric Detection of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs

Leveraging the peroxidase-mimetic activity of $\text{Fe}_3\text{O}_4@\text{PVP}$ MNP nanomaterials, an innovative detection method for ACP was developed. To achieve quantitative analysis of ACP, a detection strategy was established by monitoring the absorbance change at 652 nm (defined as $\Delta A = A_0 - A$, where A_0 represents the absorbance without ACP addition and A denotes the absorbance in the presence of ACP).

Under optimized experimental conditions, the introduction of ACP solutions with varying concentrations (0.1–10 U/L) into the $\text{Fe}_3\text{O}_4@\text{PVP}$ MNP-TMB reaction system led to a continuous reduction in the characteristic absorption peak at 652 nm. UV-vis spectroscopic results further confirmed this phenomenon: in the absence of ACP, oxTMB exhibits a distinct absorption peak at 652 nm, while this peak gradually diminishes as ACP concentration increases (Figure 7a). This observation validates the reductive effect of ACP on oxTMB.

Regarding the linear correlation between absorbance change and ACP concentration, Figure 7b illustrates a nearly linear relationship within the 0–6 U/L range, with a more pronounced linearity in the low-concentration interval. Specifically, for ACP concentrations ranging from 0.1 to 10 U/L (Figure 7c,d), the linear fitting equation is $\Delta A_{652} = 0.65014 + 0.01284x$ ($R^2 = 0.99418$). Additionally, parameters for calculating the LOD are defined as follows: LOD refers to the detection limit, S_b represents the standard error of the blank signal, and k denotes the slope of the fitted linear line.

$$\text{LOD} = \frac{3S_b}{k}$$

Based on the aforementioned formula, the LOD is calculated to be 0.021 U/L. As presented in Table S4, when compared with other materials used for ACP detection, $\text{Fe}_3\text{O}_4@\text{PVP}$ MNPs demonstrate distinct advantages, including excellent selectivity, high sensitivity, rapid response, and simple operation procedures. These prominent merits

highlight that the $\text{Fe}_3\text{O}_4\text{@PVP}$ MNP-based detection method serves as a novel and efficient approach for rapid ACP detection.

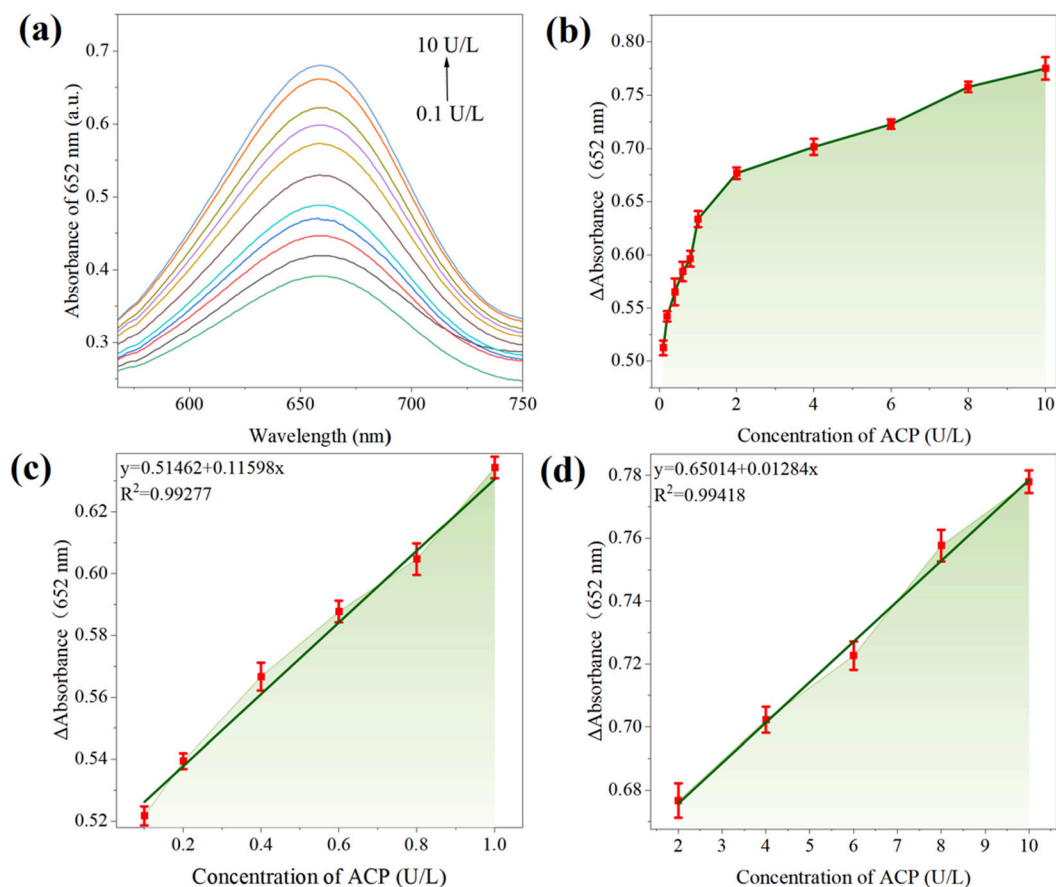


Figure 7. (a) UV–vis absorption spectra of the $\text{Fe}_3\text{O}_4\text{@PVP}$ MNP-TMB system recorded at ACP concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, and 10 U/L; (b) the corresponding variation in absorbance (ΔA) plotted against ACP level across 0.1–10 U/L; (c,d) linear calibration curves established for two distinct ACP intervals, namely 0.1–1 U/L and 2–10 U/L, each demonstrating a strong linear correlation.

3.8. Colorimetric Assay Selectivity

For evaluating the selectivity of ACP-induced inhibition in the $\text{Fe}_3\text{O}_4\text{@PVP}$ MNP sensing system, a systematic UV–vis spectroscopic assay was performed to analyze various common amino acids and common metal ions at normal, $2\times$, and $5\times$ physiological concentrations (Figure S6a,b). The results show that when interfering substances were introduced into the system, the absorbance change at 652 nm showed no significant variation compared to the blank group. This indicates that such interfering substances neither regulated the peroxidase mimicking activity of $\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs nor interfered with the chromogenic reaction between TMB and H_2O_2 . In addition, we considered the selectivity of ALP for the experiment. Under the acidic conditions employed in this experiment (Figure S7), ALP activity is almost completely suppressed. Consequently, replacing ACP with ALP in the sensing platform yielded no detectable reaction, as indicated by an absorbance value close to zero. Conversely, adding ALP to the ACP-based platform did not appreciably alter the absorbance, demonstrating that ALP does not interfere with our detection system. In summary, the [$\text{Fe}_3\text{O}_4\text{@PVP}$ MNPs + H_2O_2 + TMB] colorimetric system constructed in this study exhibits excellent selectivity and effectively avoids interference from the aforementioned potential interferents, providing reliable interference resistance for the accurate detection of ACP in subsequent practical samples.

3.9. Smartphone-Based Detection of ACP

A portable smartphone-based analytical platform was established by integrating the $\text{Fe}_3\text{O}_4\text{@PVP MNP-TMB}$ colorimetric reaction for the rapid quantification of ACP. Within this system, a progressive color transition from colorless through light blue to deep blue was observed as the ACP concentration was elevated from 0.1 to 1 U/L (Figure 8a). High-resolution images of the resulting solutions at varying ACP levels were captured using a smartphone, and the corresponding color intensity at 652 nm was extracted using Spotxel[®] software (version 2.1.6, Figure 8b). Based on these data, a calibration plot of absorbance against ACP concentration was constructed (Figure 8c), yielding a linear regression equation of $y = 0.93883 + 5.88653x$ with an R^2 of 0.99276 over the range 0.1–1 U/L (Figure 8d). The method exhibited a favorable linear response in this range and achieved a detection limit of 0.24 U/L. The designed smartphone-based system is operationally straightforward and cost effective, offering a dependable and practical approach for on-site ACP determination, and holds considerable promise for POCT applications.

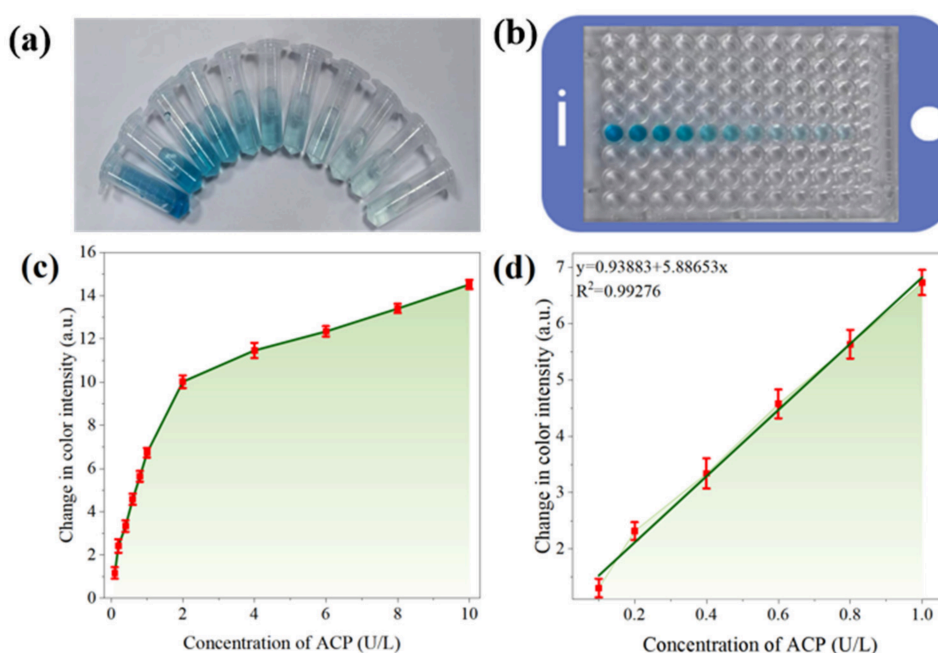


Figure 8. (a) and (b) Progressive color transition of the solution upon elevating the ACP concentration from 0.1 to 10 U/L; (c) absorbance plotted as a function of ACP level; (d) linear regression equation obtained over the 0.1–1 U/L concentration range.

3.10. Real Sample Analysis

To evaluate the applicability and precision of this method, human serum samples were analyzed, and recovery rates were determined via the standard addition approach. Table S5 presents recovery values ranging from 96.5% to 105.0%, with a relative standard deviation (RSD) below 3%. These findings suggest that the proposed approach is highly feasible for ACP quantification in diverse sample matrices. Accordingly, our results confirm that the colorimetric detection system based on the in situ $\text{Fe}_3\text{O}_4\text{@PVP MNP} + \text{TMB}$ platform offers consistent and reliable performance for ACP analysis.

Furthermore, as shown in Table S6, we conducted comparative experiments between HPLC and colorimetry using human serum samples. In practical operation, HPLC involves cumbersome procedures, while colorimetry features rapid and convenient detection advantages. Additionally, there is only a minor difference in the detection results of ACP between the two methods with no statistically significant difference ($p > 0.05$). Therefore, this confirms that our detection platform can achieve rapid detection of ACP.

4. Conclusions

This study presents a target-driven in situ nanozyme synthesis platform for sensitive and portable detection of ACP. ACP hydrolyzes AAPS to generate ascorbic acid, which triggers the formation of Fe₃O₄@PVP nanozymes with intrinsic peroxidase-like activity. The system achieves a detection limit of 0.021 U/L within 40 min and integrates smartphone imaging for on-site analysis. Compared with the standard p-nitrophenyl phosphate (pNPP) colorimetric method, our approach offers three key advantages. Firstly, it provides higher sensitivity through dual signal amplification (enzymatic hydrolysis coupled with nanozyme-catalyzed TMB oxidation). Moreover, magnetic separation physically removes matrix interference, unlike pNPP where endogenous chromophores and turbidity affect readings. Lastly, the acidic working pH (4.0) suppresses alkaline phosphatase activity, ensuring enhanced selectivity. Although our multi-step design is more complex than the single-step pNPP assay, these gains in sensitivity, anti-interference capability, and selectivity justify its use for POCT in complex biological samples. This work establishes a broadly applicable framework for detecting low-abundance and labile biomarkers in decentralized diagnostics.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/bios16060337/s1>, Figure S1: UV-vis spectroscopic study of the formation of Fe₃O₄ MNPs: (a) synergistic role of ACP and AAPS and (b) effect of the concentration of ACP (0.1 U/L, 0.2 U/L, 0.4 U/L, 0.6 U/L, 0.8 U/L, 1.0 U/L); Figure S2: The particle size distribution of the [ACP + AAPS + FeCl₃ + NaOH + PVP] system and the [ACP + FeCl₃ + NaOH + PVP] system at different incubation times (2–30 min); Figure S3: Full XPS spectral surveys of the Fe₃O₄@PVP MNPs; Figure S4: (a) The XPS spectrum of Fe 2p from the fractured surface of the Fe₃O₄ standard sample; (b) The XPS spectrum of Fe 3p from the fractured surface of the Fe₃O₄ standard sample; Figure S5: (a) Comparison of particle size distribution of Fe₃O₄@PVP MNPs two months ago and two months of storage; (b) Comparison of peroxidase mimicking activity of Fe₃O₄@PVP MNPs two months ago and two months of storage; Figure S6: Comparison of inhibition of various amino acids (a) and other ion pairs (b) in the sensing system with ACP; Figure S7: The absorbance at 652 nm of [ACP + AAPS + FeCl₃ + H₂O₂ + TMB] system in the presence of albumin, globulin and ALP (the four one is the [ALP + AAPS + FeCl₃ + H₂O₂ + TMB]); Table S1: Solution addition method for Peroxidase-like Activity of Fe₃O₄@PVP MNPs; Table S2: Solution addition method for fluorescent probe TPA; Table S3: Comparison of apparent steady-state kinetic of nanophase materials; Table S4: Comparison of detection limits of ACP with those reported methods; Table S5: Detection of ACP in human serum samples (*n* = 3); Table S6: ACP analysis in real samples (*n* = 3). For references in the supplementary materials, please refer to [47–56].

Author Contributions: J.K.: Investigation, Writing—original draft, Formal analysis, and Data curation. Y.C.: Investigation, Writing—original draft, and Formal analysis. Z.S.: Investigation, Formal analysis, and Data curation. C.W.: Formal analysis and Data curation. F.K.: Conceptualization, Methodology, Writing—original draft, Validation, and Supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Natural Science Foundation of Fujian Province (Nos. 2025J01702 and 2025J01708) and the fund of the Fujian Provincial Key Laboratory of Hepatic Drug Research (No. KFLX2024002).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are provided within the manuscript or Supplementary Information Files.

Acknowledgments: We are grateful for the support of the Public Technology Service Center of Fujian Medical University. During the preparation of this work, we used AI tools to assist in refining the language, improving readability, and enhancing the academic expression of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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