



Colorimetric determination of the activity of alkaline phosphatase by exploiting the oxidase-like activity of palladium cube@CeO₂ core-shell nanoparticles

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Abstract

Core-shell palladium cube@CeO₂ (Pd cube@CeO₂) nanoparticles are shown to display oxidase-like activity. This is exploited in a method for determination of the activity of alkaline phosphatase (ALP). The Pd cube@CeO₂ nanoparticles were thermally synthesized from Ce(NO₃)₃, L-arginine and preformed Pd cube seeds in water. The Pd cube@CeO₂ nanoparticles catalyze the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by oxygen. This results in the formation of oxidized TMB (oxTMB) with an absorption peak at 652 nm. Ascorbic acid (AA) is generated from the hydrolysis of L-ascorbic acid 2-phosphate (AAP) catalyzed by ALP. It can reduce oxTMB to TMB, and this results in a decrease of the absorbance. The method allows for quantitative determination of the activity of ALP in the range from 0.1 to 4.0 U·L⁻¹ and with a detection limit down to 0.07 U·L⁻¹. Endowed with high sensitivity and selectivity, the assay can quantify ALP activity in biological system with satisfactory results.

Keywords Alkaline phosphatase · Ascorbic acid · Cerium oxide · Pd cube · 3,3',5,5'-tetramethylbenzidine · Oxidase mimic · Colorimetric assay · Ascorbic acid 2-phosphate · Nanozyme

Introduction

Alkaline phosphatase (ALP) plays a key role in catalytic dephosphorylation [1]. ALP promotes the hydrolysis of monoester phosphates, which is capable of producing phosphates and products containing free hydroxy groups [2]. ALP plays an important role in signal transduction and

regulation of intracellular processes [3, 4]. Many methods have been reported for ALP detection, including electrochemistry [5], colorimetry [6], electrochemiluminescence [7], fluorimetry [8] and surface enhanced Raman scattering [9].

Colorimetry has become the preferred method for clinical applications due to its simplicity, readability, low cost, fast response and high throughput [10]. The most widely used colorimetric assay for ALP activity detection is based on the conversion of colorless *p*-nitrophenylphosphate (pNPP) to yellow *p*-nitrophenol. The method is simple and effective, which has been considered as a standard method for ALP activity monitoring. However, pNPP is very sensitive to light and is prone to spontaneous hydrolysis, both of which lead to inaccurate measurement results [11]. Consequently, developing sensitive, selective and accurate method for ALP detection is of great importance. In the past few years, nanozymes, a class of mimic enzymes that show both the unique properties of nanomaterials and catalytic functions, have been reported to replace natural enzymes. So far, many materials have been defined as nanozymes, such as Au@Pd nanoparticles [12], Au@Pt nanostructures [13], Prussian blue nanocubes [14], Fe₃O₄ nanoparticles [15–17] and Pt nanoparticles [18, 19]. Compared with natural enzymes,

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nanozymes are more stable and cheaper, which shows great potential in biosensing [20, 21]. Recent studies have shown that ALP assays can be easily achieved by modulating the catalytic capabilities of nanozymes. For example, Wu et al. have reported a colorimetric assay for ALP activity detection based on the peroxidase-like activity of Prussian blue nanoparticles [22]. Jiang et al. reported a colorimetric assay for the detection of ALP activity by employing copper (II)-based metal-organic frameworks as peroxidase mimic and pyrophosphate as recognition element [23]. Though these methods using peroxidase mimetics show high sensitivity, they are limited by the utilization of unstable H_2O_2 . Therefore, many efforts have been made to develop colorimetric assays for ALP detection using oxidase mimetics since they can directly oxidize 3,3',5,5'-tetramethylbenzidine (TMB) without the addition of H_2O_2 , making it much simpler [10]. However, there are few types of oxidase mimetics that have been reported so far.

We describe a colorimetric assay for ALP activity based on the oxidase-like activity of Pd cube@CeO₂ nanoparticles. CeO₂ nanoparticles show low oxidase-like activity [24, 25]. Therefore, we utilize the core-shell composite of Pd cube and CeO₂ to ensure that the material has strong oxidase-like activity and shows high sensitivity for ALP detection. Pd cube@CeO₂ nanoparticles show oxidase-like activity that can directly oxidize TMB to oxidized TMB (oxTMB), resulting in a bluish solution and an intense absorption peak at 652 nm. ALP can catalyze the hydrolysis of L-ascorbic acid 2-phosphate (AAP) to produce ascorbic acid (AA) which can reduce oxTMB to TMB [26]. Consequently, the solution color turns to light blue with a decreased absorption intensity at 652 nm. On the basis of the above facts, a colorimetric assay for ALP detection is developed.

Experimental section

Chemicals and materials

Polyvinylpyrrolidone (PVP, MW \approx 55,000), KBr, 3,3',5,5'-tetramethylbenzidine (TMB), ALP (EC 3.1.3.1), AAP and AA were purchased from Sigma-Aldrich (St. Louis, USA, [www.](http://www.sigmaaldrich.com)

[sigmaaldrich.com](http://www.sigmaaldrich.com)). Na₂PdCl₄ was obtained from Aladdin Reagent company (Shanghai, China, www.aladdin-e.com). L-arginine was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China, www.macklin.cn). Ce(NO₃)₃·6H₂O was supplied by Alfa Aesar (Tianjin, China, www.alfa.com). The water used in all experiments was supplied by a water purifier nanopure water system (18.2 M Ω cm). All chemicals were used as received without further purification.

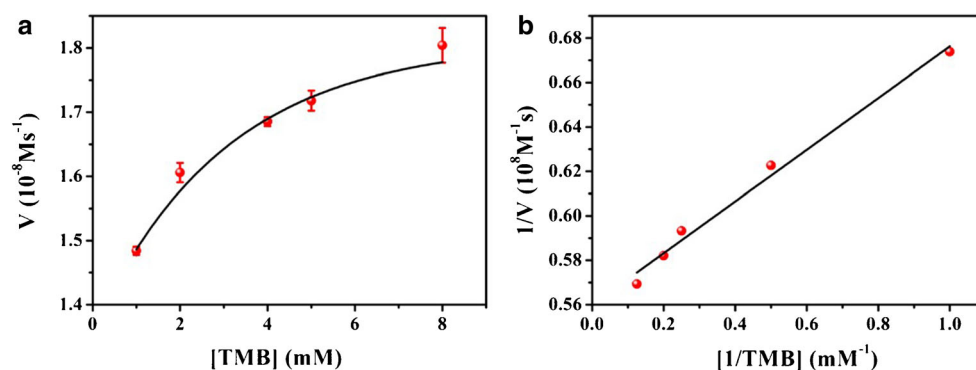
Materials characterization

The UV-vis absorption spectra were recorded on a UV-8000 spectrophotometer (Shanghai Metash Instruments Co., Ltd., China, www.metash.com). Transmission electron microscope (TEM) measurements were performed using a JEM 1400 (JEOL, Ltd., Japan, www.jeol.co.jp) at an acceleration voltage of 80 kV. High resolution transmission electron microscopic (HRTEM) measurements were performed using a JEM-2100 (JEOL Ltd., Japan, www.jeol.co.jp) at an acceleration voltage of 200 kV. The X-ray diffraction patterns of the products were collected on a Rigaku Ultima IV (Japan, www.rigaku.com) with an operation voltage and current maintained at 40 kV and 40 mA. The X-ray photoelectron spectroscopy (XPS) were performed by using a VG Thermo ESCALAB 250 spectrometer (VG Scientific, www.pdf.directindustry.com) operated at 120 W. The binding energy was calibrated against the carbon 1 s line.

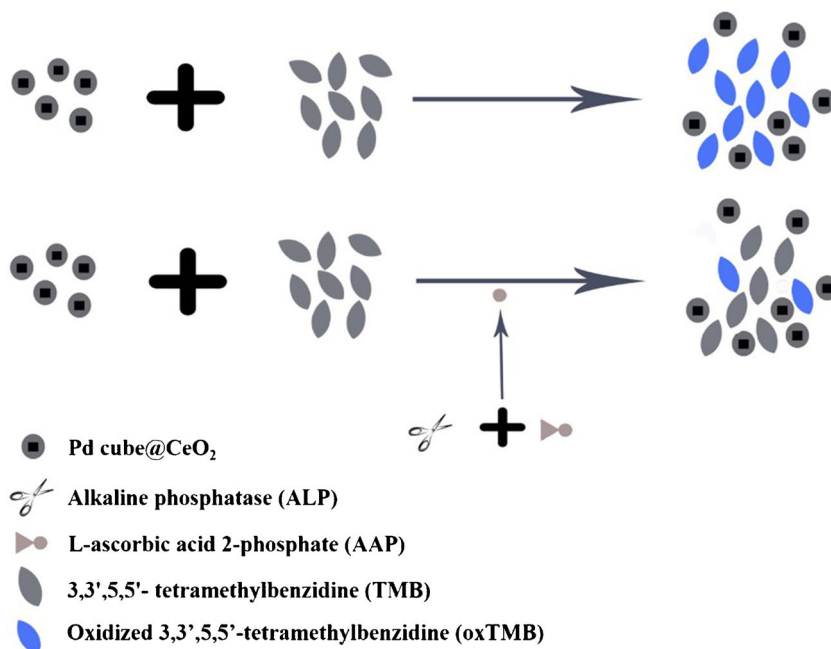
Detection assay procedure

10 μ L of ALP with different activities, 10 μ L of AAP (10 mM), 70 μ L of Tris-HCl buffer (pH = 9.0, 10 mM) and 10 μ L of MgCl₂ solution (50 mM) were sequentially introduced to 1.5 mL centrifuge tube. After thoroughly mixing and incubating at 37 $^{\circ}$ C for 30 min, 100 μ L of Pd cube@CeO₂ nanoparticles (0.25 mg·mL⁻¹), 100 μ L of TMB (5 mM) and 700 μ L of acetic acid buffer (pH = 4.0, 10 mM) were added and incubated at 37 $^{\circ}$ C for another 10 min. Finally, the solution was transferred for the UV-vis absorption spectral measurements.

Fig. 1 Kinetic assays of TMB oxidation by Pd cube@CeO₂ nanoparticles. **a** The fit based on Michaelis-Menten kinetic equation. **b** The corresponding Lineweaver-Burk plot



Scheme 1 Schematic illustration of the detection principle of this method based on the oxidase-like activity of Pd cube@CeO₂ core-shell nanoparticles



Results and discussion

Highly monodisperse Pd cube@CeO₂ composites are prepared by L-arginine-triggered self-assembly of CeO₂ on Pd cube. The material characterization part is shown in Fig. S1–S3. Typically, monodisperse Pd cubes with size of 12 nm are synthesized based on previous work (Fig. S1). And then, the Pd cube@CeO₂ nanoparticles are synthesized by heating the mixture of Ce(NO₃)₃, L-arginine, and pre-synthesized Pd cube at 80 °C for 3 h [27].

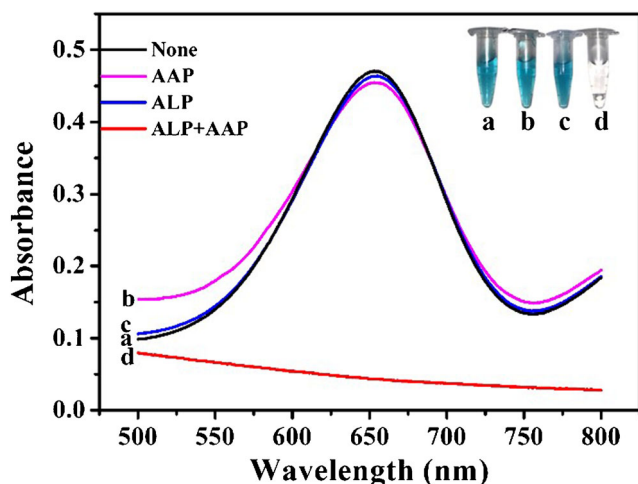


Fig. 2 UV-vis absorption spectra of the detection system in the absence (a) and presence of AAP (b), ALP (c), ALP and AAP (d). Inset shows the corresponding photos

Kinetics of 3,3',5,5'-tetramethylbenzidine (TMB) oxidation

In order to quantitatively evaluate the intrinsic oxidase-like activity of Pd cube@CeO₂ core-shell nanoparticles, we study the steady-state kinetic by varying the concentrations of TMB. The kinetic parameter for Pd cube@CeO₂ nanoparticles is determined by fitting the data of Fig. 1a into Michaelis-Menten equation. The initial velocity can be calculated by using the molar absorption coefficient of 3900 M⁻¹ cm⁻¹ for oxTMB. Michaelis-Menten constant (K_m) is the substrate concentration at which the reaction rate is half-maximum. A smaller K_m indicates a stronger binding affinity of enzyme to substrate and hence often leads to a higher enzymatic activity [28]. The K_m value can be obtained from the Lineweaver-Burk plot. From Fig. 1b, K_m value is calculated to be 0.21 mM, which is lower than the K_m values mentioned in previous report [25]. It indicates that Pd cube@CeO₂ nanoparticles show good affinity to TMB.

Detection mechanism

The Pd cube alone is not sensitive to the detection of ALP (Fig. S4), so the Pd cube@CeO₂ core-shell nanoparticles are adopted. The detection principle of Pd cube@CeO₂ nanoparticles for ALP is shown in Scheme 1. Pd cube@CeO₂ nanoparticles show strong oxidase-like activity, which can oxidize TMB to oxTMB without the need for H₂O₂ and result in a strong absorption peak centered

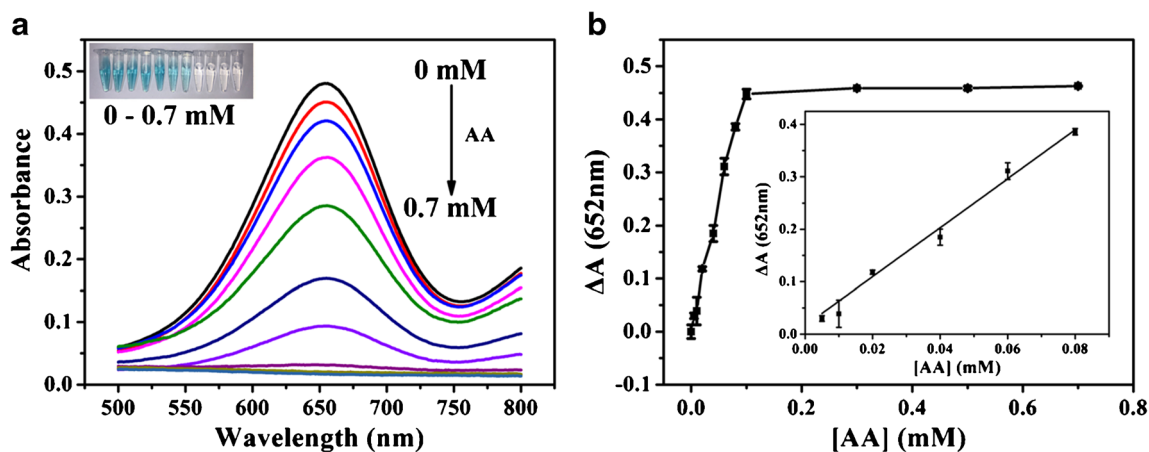


Fig. 3 **a** UV-vis absorption spectra of Pd cube@CeO₂nanoparticles-TMB in the presence of various concentrations of AA (from top to down: 0, 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.3, 0.5 and 0.7 mM). Inset

shows the photos of the corresponding solutions. **b** AA concentration dependent changes of the absorption intensity at 652 nm. Inset shows the liner relationship between the ΔA and AA concentration

at 652 nm (a, Fig. 2). When either AAP or ALP is introduced into detection assay, the changes of solution color and absorbance is negligible compared to blank solution (b and c, Fig. 2). After both AAP and ALP are added to the detection system, the solution turns to colorless and the absorption intensity at 652 nm decreases obviously (d, Fig. 2), which is attributed to the fact that AA generating form ALP-catalyzed hydrolysis of AAP can inhibit the oxidation of TMB [22, 29]. Based on the changes of solution colors and absorbance, this method can be used for ALP detection. The method does not involve H₂O₂ in the reaction, making it much simpler.

Optimization of assay conditions

Before the application of this assay for ALP activity detection, several parameters such as pH value of buffer,

incubation time, the concentrations of Pd cube@CeO₂ nanoparticles, TMB and AAP should be optimized. We utilize ΔA ($\Delta A = A_0 - A$) as the criterion to optimize the detection conditions, where A_0 and A are the absorbance at 652 nm of the detection system in the absence and presence of ALP, respectively.

Optimization of pH

In order to determine the effect of pH values on ALP detection, we performed the assay in acetic acid buffer with different pH values (3.6–5.6). As shown in Fig. S5, with the increase of pH values, A_0 increases firstly and then decreases, while A keeps almost constant in the whole pH range. When the pH is 4.0, ΔA reaches a maximum value.

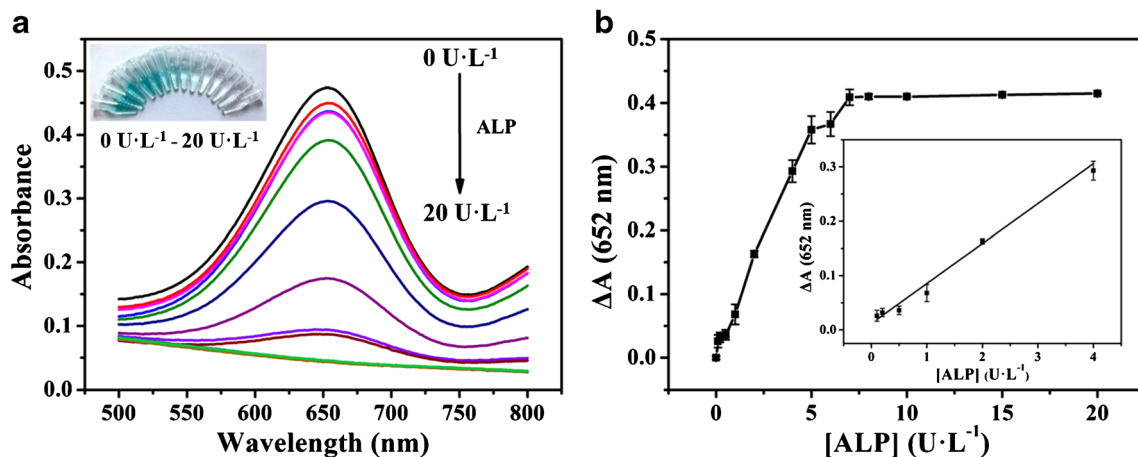


Fig. 4 **a** UV-vis absorbance spectra of Pd cube@CeO₂nanoparticles-TMB in the absence and presence of various concentrations of ALP (from up to down: 0, 0.1, 0.2, 0.5, 1, 2, 4, 5, 6, 7, 8, 10, 15, and 20 U·L⁻¹), the

inset show the photos of corresponding solutions. **b** ALP concentration dependent changes of the absorption intensity at 652 nm. Inset shows the liner relationship between the ΔA and ALP concentration

Table 1 Comparison of the analytical performance of different methods for ALP detection

Biosensing materials	Analytical method	Detection limit (U·L ⁻¹)	Linear range (U·L ⁻¹)	Reference
Ce ₃ (PO ₄) ₄	Fluorimetry	2.3	0–50	[10]
AuNCs	Fluorimetry	0.002	0.1–100	[30]
NGQDs ^a	Fluorimetry	0.07	0.1–5	[1]
coumarin@Tb-GMP	Fluorimetry	10	25–200	[31]
Ce ³⁺ -ATP-Tris	Fluorimetry	0.1	0.1–10	[32]
Au@Ag NPs-GQDs	Fluorimetry	0.005	0.01–2	[33]
Au@Ag NPs-GQDs	Colorimetry	0.009	0.01–6	[33]
AuNRs	Colorimetry	0.01	0.01–0.4	[6]
PB NCs	Colorimetry	0.23	0.6–6	[22]
CoOOH nanoflakes	Colorimetry	0.026	0.04–160	[34]
Pd cube@CeO ₂	Colorimetry	0.07	0.1–4	This work

^a nitrogen-doped graphene quantum dots

Optimization of incubation time

Different reaction time has a great influence on the experimental results, and the effects of time (5, 10, 15, 20, 25 min) are investigated (Fig. S6A). When the reaction time is 10 min, the largest ΔA is obtained (Fig. S6B).

Optimization of Pd cube@CeO₂ concentration

Different concentrations of Pd cube@CeO₂ nanoparticles represent different oxidizing ability of the detection system. We select samples with concentrations of 0.01, 0.025, 0.05, and 0.075 mg·mL⁻¹ (Fig. S7A). When the concentration of Pd cube@CeO₂ nanoparticles is 0.025 mg·mL⁻¹, ΔA gets the maximum value (Fig. S7B).

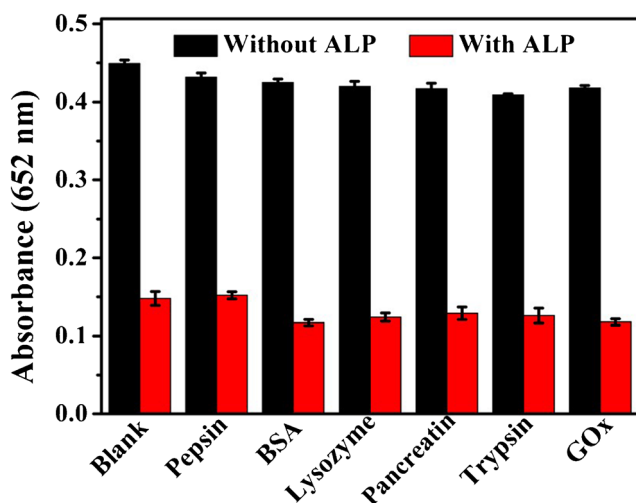


Fig. 5 The absorbance of this detection assay in the presence of possible interfering substances only or coexistence with ALP. The final concentrations of these substances are 5 U·L⁻¹, BSA are 100 μ g·mL⁻¹. Error bars indicate the standard deviation of three independent measurements

Optimization of TMB concentration

To determine the effect of TMB concentration on the sensitivity of the detection system, we chosen different concentrations of TMB from 0.1 to 0.9 mM. As shown in Fig. S8, too high or too low concentration of TMB is not conducive to improving ΔA . Both A_0 and A increase with increasing TMB concentration (Fig. S8A). When the TMB concentration is 0.5 mM, ΔA reaches a maximum value (Fig. S8B).

Optimization of AAP concentration

To test the effect of AAP concentration on the sensitivity of the detection system, we select different concentrations of AAP from 0 to 0.25 mM (Fig. S9A). When the AAP concentration is 0.1 mM, the ΔA value is the largest (Fig. S9B).

Application of this method to the determination of AA and ALP activity

Under the optimum conditions, the Pd cube@CeO₂ core-shell nanoparticles-TMB system is used to detect AA based on its oxidase-like activity. An obvious color change from dark blue to light blue is found with the increasing concentrations of AA (inset of Fig. 3a). In addition, as the concentration of AA increases, the UV-vis absorption intensity at 652 nm decreases gradually (Fig. 3a). As shown in Fig. 3b, a good linear relationship between ΔA and AA concentration can be obtained in the range from 0.005 to 0.08 mM. The linear regression equation is $\Delta A = 4.668 [AA] + 0.016$ ($R^2 = 0.994$) with a detection limit down to 1.3 μ M.

In order to evaluate the performance of this method for ALP detection, the color changes of the detection assay before and after the introduction of various concentrations of ALP are recorded by a digital camera. Obvious color change from dark blue to light blue is found with the increasing

Table 2 Determination of ALP in 100-fold diluted human serum samples

Samples	Added (U·L ⁻¹)	Found (U·L ⁻¹)	pNPP assay (U·L ⁻¹)	Recovery (%)	RSD (n = 4, %)
1	2	2.07 ± 0.14	1.96 ± 0.18	103.5	6.8
2	4	3.88 ± 0.12	4.02 ± 0.13	97.0	3.1

concentrations of ALP (inset of Fig. 4). The UV-vis absorption spectra of the system after the addition of different concentrations of ALP are also recorded. As the concentration of ALP increases, the absorbance at 652 nm decreases gradually (Fig. 4a). As shown in Fig. 4b, a good linear relationship between ΔA and the ALP concentration in the range from 0.1 to 4 U·L⁻¹ is obtained. The linear regression equation is $\Delta A = 0.074 [\text{ALP}] + 0.012$ ($R^2 = 0.984$) with a detection limit down to 0.07 U·L⁻¹. Then, we compare the linear range and detection limit of this method with previously reported methods. The corresponding results are shown in Table 1. The analytical performances of our method are comparable or even better than reported methods.

Selectivity

To demonstrate the selectivity of this assay for ALP, we firstly investigate the effects of possible interferences (pepsin, BSA, lysozyme, panceratin, trypsin and GOx). As shown in Fig. 5, when other enzymes and proteins are individually added to the sensing system, no significant change in the absorbance is observed. While the absorbance at 652 nm decreases significantly after ALP is added individually or simultaneously with other interfering substances. Then, we study other interfering substances that may be present in human serum, including AA, L-cysteine (Cys), uric acid (UA), glutathione (GSH) and L-homocysteine (HCy). As shown in Fig. S10, the absorbance of the detection system keep almost unchanged after the addition of interfering substances. These results indicate that the detection assay has high selectivity for ALP detection.

Real sample analysis

In order to evaluate the practical application of our proposed method, the colorimetric assay is used to analyze ALP in human serum samples. Serum samples are diluted 100-fold and detected by using standard addition method. The solution is awakened to determine the UV-vis absorption peak after adding different concentrations of ALP (2 U·L⁻¹ and 4 U·L⁻¹). As shown in Table 2, the recoveries are in the range from 97.0% to 103.5% with the RSD ranging from 3.1% to 6.8%. The experimental results are quite satisfactory, indicating that the method has great potential in real sample detection.

Conclusion

We have established a colorimetric method for ALP activity detection based on the high oxidase-like activity of Pd cube@CeO₂ nanoparticles. This method shows high sensitivity, good selectivity and great potential to detect ALP in real samples, which provides good practicability and reliability for clinical detection of ALP activity. This assay may not only provide a new idea for construction of nanozymes with high oxidase-like activity, but also broaden the applications of oxidase mimetics. The limitation of the detection system is that it cannot distinguish the iso-enzymes of ALP in human plasma.

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