

Peroxidase-like activity of nanocrystalline cobalt selenide and its application for uric acid detection

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Abstract: Dendrite-like cobalt selenide nanostructures were synthesized from cobalt and selenium powder precursors by a solvothermal method in anhydrous ethylenediamine. The as-prepared nanocrystalline cobalt selenide was found to possess peroxidase-like activity that could catalyze the reaction of peroxidase substrates in the presence of H₂O₂. A spectrophotometric method for uric acid (UA) determination was developed based on the nanocrystalline cobalt selenide-catalyzed coupling reaction between *N*-ethyl-*N*-(3-sulfopropyl)-3-methylaniline sodium salt and 4-aminoantipyrine (4-AAP) in the presence of H₂O₂. Under optimum conditions, the absorbance was proportional to the concentration of UA over the range of 2.0–40 μM with a detection limit of 0.5 μM. The applicability of the proposed method has been validated by determination of UA in human serum samples with satisfactory results.

Keywords: enzyme mimics, cobalt selenide, peroxidase-like activity, uric acid, human serum

Introduction

As efficient biological catalysts, enzymes are involved in almost all biological reactions. Besides their important roles *in vivo*, enzymes are also widely used in biomedical, chemical, food, environmental, and agricultural sciences. In spite of their high substrate specificities and catalytic efficiencies, most natural enzymes can be digested by proteases as they are globular proteins. Their catalytic activities can be easily suppressed under extreme conditions because the protein structure can be disrupted by environmental changes, such as heating or addition of chemical denaturants. Accordingly, it is highly appealing to search for natural enzyme mimics with good stability and low cost.

In clinical analysis, various enzymes are used for the detection of trace amounts of important components in biological samples. As one of the most commonly used biomedical testing tools, peroxidase can catalyze redox reactions between electron donors and H₂O₂. The use of chromogenic, fluorescent, and chemiluminescent electron donors with horseradish peroxidase (HRP) has proven highly useful for detecting H₂O₂ and tracking reactions that produce H₂O₂. In addition, peroxidase-labeled immunoglobulin is a common probe in immunohistochemistry and in enzyme amplification immunoassay systems. However, as with other enzymes, stability is a major problem that restricts the application of peroxidase. A short-term stability test shows that HRP shows dramatically decreased catalytic ability over a few hours at room temperature and loses most of its activity after 24 h.¹ Since the pioneering study reported by Gao et al² that showed that magnetic Fe₃O₄ nanoparticles exhibit intrinsic peroxidase-like activity, increasing attention has been paid to nanoscale peroxidase mimics such as V₂O₅ nanowires,³ bimetallic Fe–Co alloy nanoparticles,⁴ cupric oxide nanoparticles,⁵

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porous platinum nanoparticles on graphene oxide,⁶ graphene quantum dots,⁷ citrate-capped platinum nanoparticles,⁸ AgX nanoparticles,⁹ platinum nanoparticles in bovine serum albumin scaffold,¹⁰ and gold nanoparticles.^{11,12} Nanoscale peroxidase mimics possess several advantages over naturally occurring peroxidase enzymes, including almost-unchanged catalytic activity over a wide range of pH and temperatures, low cost, tunable catalytic activities, as well as ease of storage and treatment. These features make nanoscale peroxidase mimics suitable candidates for potential applications in the fields of biomedicine and environmental chemistry.^{13–15} Humans lack the enzyme uricase, which results in uric acid (UA; 2,6,8-trihydroxypurine) being the end product of purine metabolism *in vivo*.¹⁶ Abnormal UA levels are a risk factor for diseases such as hyperuricemia, gout, and urate nephropathy. Thus, it is of great importance to establish simple and effective analytical methods for measuring UA in biofluids for physiological investigations, health assessment, and disease diagnosis. To date, an assortment of analytical methods for determining UA levels in biological samples have been proposed, such as fluorescence,¹⁷ chemiluminescence,¹⁸ electrochemistry,¹⁹ high-performance liquid chromatography,²⁰ and capillary electrophoresis.²¹ Colorimetric chemosensors based on nanozymes have also attracted considerable attention for UA detection because of their simplicity and feasibility, as well as the fact that there is no requirement for sophisticated instrumentation.^{22,23}

Cobalt selenide nanostructures have proven to be active catalysts for oxygen and hydrogen evolution reactions owing to the suitable electronic configuration of their Co ions.^{24,25} In this work, we study the peroxidase-like catalytic activity of nanocrystalline cobalt selenide that was obtained via solvothermal synthesis. The as-synthesized nanocrystalline cobalt selenide can catalyze the oxidation of peroxidase substrates in the presence of H₂O₂, resulting in a color change. This new type of enzyme mimic shows a much higher affinity for H₂O₂ than either HRP or other peroxidase mimics. A spectrophotometric method for UA determination was developed based on the nanocrystalline cobalt selenide-catalyzed coupling reaction between *N*-ethyl-*N*-(3-sulfopropyl)-3-methylaniline sodium salt (TOPS) and 4-aminoantipyrine (4-AAP) in the presence of H₂O₂. The optimum pH for the nanocrystalline cobalt selenide-catalyzed TOPS/4-AAP–H₂O₂ reaction system was 8.5, which is the same as the optimum pH for uricase activity. Thus, our proposed approach avoids the process of pH adjustment after the uricase reaction, which is necessary in previously reported methods using 3,3',5,5'-tetramethylbenzidine (TMB) as a colorimetric substrate.^{22,23} The proposed method

was successfully applied to the determination of UA in human serum samples. Thus, we believe that this colorimetric UA sensor will have potential clinical applications in the future.

Experimental

Chemicals and reagents

TOPS, 4-AAP, TMB, *o*-dianisidine dihydrochloride, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), cobalt powder, selenium powder, UA, triglyceride, cholesterol, creatinine, ascorbic acid, and glucose were all purchased from Aladdin Reagent Company (Shanghai, China). Uricase was purchased from Sigma-Aldrich Co, Ltd (St Louis, MO, USA). H₂O₂ (30%) was purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). All chemicals from commercial sources were of analytical grade and were used without further purification. Water was purified using a Milli-Q® system (EMD Millipore, Billerica, MA, USA).

Apparatus

Ultraviolet/visible (UV/vis) absorption spectra were obtained with a UV-2450 UV/vis spectrophotometer (Shimadzu, Kyoto, Japan). Transmission electron microscopy (TEM) images were collected with a Tecnai G2 F20 microscope (FEI, Hillsboro, OR, USA) at an accelerating voltage of 200 kV at room temperature.

Synthesis of nanocrystalline cobalt selenide

Nanocrystalline cobalt selenide was synthesized via a previously reported solvothermal method.²⁶ Briefly, cobalt powder (2 mmol) and one equivalent of selenium powder were ground together in an agate roller. The precursors and anhydrous ethylenediamine (27 mL) were added to a Teflon-lined autoclave and heated at 140°C for 12 h. A black product was collected, and it was washed with absolute ethanol three times, then dried at 60°C for 4 h before further characterization.

Peroxidase-like activity of nanocrystalline cobalt selenide

Typically, 200 µL of nanocrystalline cobalt selenide (1.3 mg/mL), 250 µL of TOPS (3.2 mM), 300 µL of 4-AAP (21 mM), and 1.0 mL of H₂O₂ (0.5 mM) were added to 2.25 mL of Tris-HCl buffer solution (50 mM, pH = 8.5). The resulting solution was incubated in a water bath at 40°C for 10 min. The absorption of the purple product at 550 nm was measured using a Shimadzu UV-2450 spectrophotometer.

UA assays

UA solutions were prepared in Tris-HCl buffer solution (50 mM, pH =8.5). UA determination was carried out as follows: 10 μ L of uricase (100 U/mL) was added to 200 μ L of UA solution (various concentrations). The resulting solution was incubated in a water bath at 40°C for 10 min. Subsequently, 3.04 mL of Tris-HCl buffer solution (50 mM, pH =8.5), 250 μ L of TOPS (3.2 mM), 300 μ L of 4-AAP (21 mM), and 200 μ L of nanocrystalline cobalt selenide (1.3 mg/mL) were added to the reaction and incubated in a water bath at 40°C for 30 min. The absorption of the resulting solution at 550 nm was measured using a UV-2450 UV/vis spectrophotometer (Shimadzu). The detection limit was estimated from three times the standard deviation of the background signals.

Detection of UA in human serum samples

Clinical human serum samples were donated by the Affiliated Quanzhou First Hospital of Fujian Medical University (Quanzhou, China). The serum samples were diluted 40 times and pretreated by ultrafiltration (Millipore, 3 kDa). The subsequent procedure for UA analysis was the same as that used for standard UA solutions. The recoveries of UA were determined using spiked samples. This study was approved by the Medical Ethics Committee of the Affiliated Quanzhou First Hospital of Fujian Medical University. Participants signed an informed consent before the experiment, and the information of the volunteers was kept confidential.

Results and discussion

Peroxidase-like activity of nanocrystalline cobalt selenide

Nanocrystalline cobalt selenide was synthesized via a solvothermal method in anhydrous ethylenediamine using cobalt and selenium powders as precursors.²⁶ The TEM images (Figure 1) reveal that the as-prepared cobalt selenide consisted of dendrite-like nanostructures.

H₂O₂-mediated oxidative coupling of TOPS with 4-AAP was used to investigate the peroxidase-like activity of nanocrystalline cobalt selenide. As shown in Figure 2A, nanocrystalline cobalt selenide could catalyze the reaction to produce a purple color with an absorbance maximum at 550 nm. In contrast, a negligible color change occurred in the absence of the catalyst, suggesting the peroxidase-like activity of the nanocrystalline cobalt selenide. Similar experiments were also performed using other peroxidase substrates in place of TOPS/4-AAP. As shown in Figure 2B,

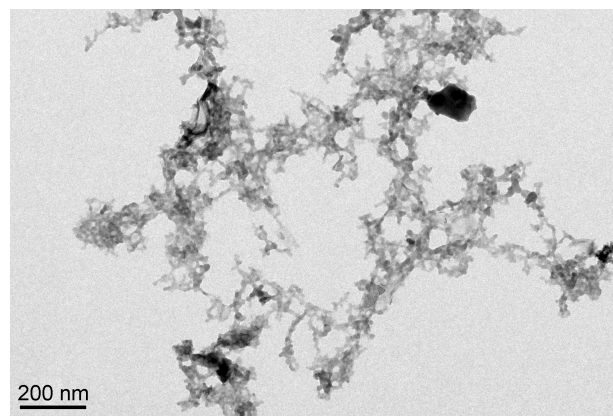


Figure 1 TEM image of the as-prepared cobalt selenide.

Abbreviation: TEM, transmission electron microscopy.

the nanocrystalline cobalt selenide could also catalyze the oxidation reactions of other peroxidase substrates such as TMB and *o*-dianisidine in the presence of H₂O₂.

The catalytic activity of nanocrystalline cobalt selenide was further investigated using enzyme kinetics principles and methods. The apparent steady-state kinetic parameters were obtained by varying the H₂O₂ concentration, while maintaining the other factors as constant. Figure 3 shows the effect of H₂O₂ concentration on reaction rate, with the dots representing the experimental data and the solid curve showing the fitting to the Michaelis–Menten model for enzyme kinetics. The results show that typical Michaelis–Menten curves can be obtained for the reaction over the investigated concentration ranges. The apparent K_m value (the substrate concentration at which the reaction rate is half of the maximum) is 72.2 μ M, which is significantly lower than that of HRP,²⁷ suggesting that this new enzyme mimic has an affinity for H₂O₂ that is at least an order of magnitude higher than that of the natural enzyme.

Optimization of experimental conditions

As with peroxidase, the catalytic activity of nanocrystalline cobalt selenide is dependent on the reaction temperature and pH. The peroxidase-like activity of the nanocrystalline cobalt selenide was investigated by varying the reaction temperature from 25°C to 50°C. As can be seen from Figure 4A, the absorbance of the reaction system gradually increased as the reaction temperature increased to 40°C. However, the catalytic activity of nanocrystalline cobalt selenide plateaued once the reaction temperature crossed 40°C. This makes the catalytic system suitable for combination with various oxidase-related biological reactions at physiological temperature. The pH is another important parameter that affects catalytic efficiency. Figure 4B displays the effect of

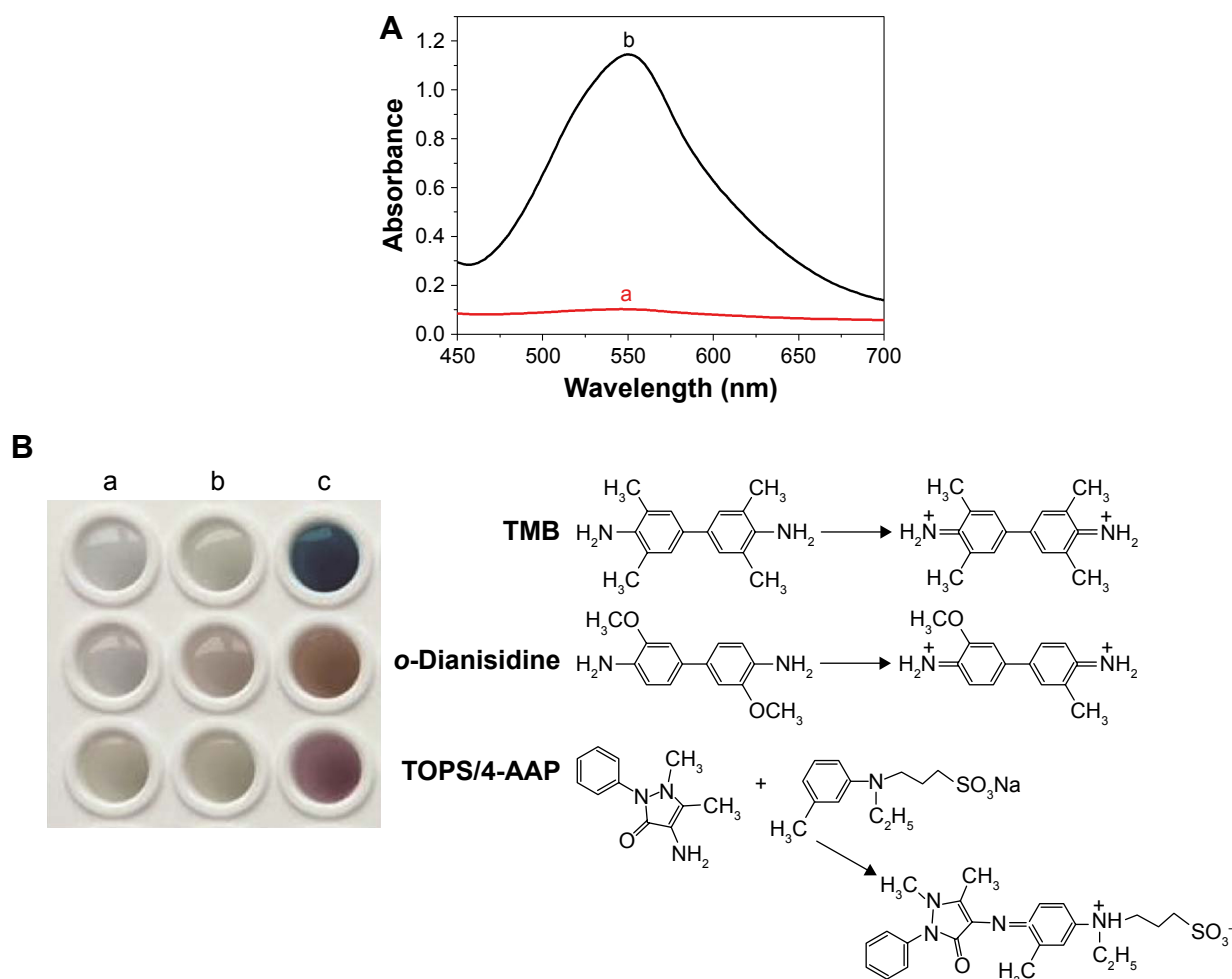


Figure 2 Peroxidase-like activity of nanocrystalline cobalt selenide.

Notes: (A) UV/vis spectra of solutions containing TOPS (0.2 mM), 4-AAP (1.58 mM), and H_2O_2 (125 μ M) (a) in the absence of nanocrystalline cobalt selenide and (b) in the presence of nanocrystalline cobalt selenide (0.065 mg/mL). (B) Reaction and typical photographs of common chromogenic peroxidase substrates being catalytically oxidized by nanocrystalline cobalt selenide in the presence of H_2O_2 , (a) Substrate only, (b) substrate + H_2O_2 , and (c) substrate + H_2O_2 + nanocrystalline cobalt selenide.

Abbreviations: 4-AAP, 4-aminoantipyrine; TMB, 3,3',5,5'-tetramethylbenzidine; TOPS, *N*-ethyl-*N*-(3-sulfopropyl)-3-methylaniline sodium salt; UV/vis, ultraviolet/visible.

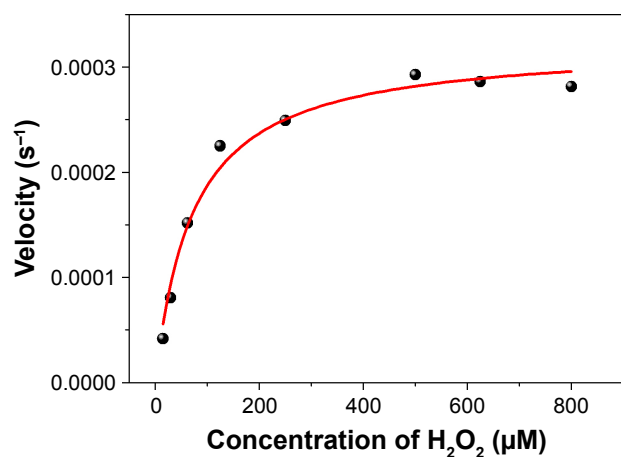


Figure 3 Steady-state kinetic assay for nanocrystalline cobalt selenide.

Notes: Experiments were carried out in Tris-HCl buffer solution (50 mM, pH =8.5) at 40°C. The concentrations of TOPS, 4-AAP, and nanocrystalline cobalt selenide were fixed at 0.2 mM, 1.58 mM, and 65 μ g/mL, respectively. The concentration of H_2O_2 was varied from 15 to 800 μ M.

Abbreviations: 4-AAP, 4-aminoantipyrine; TOPS, *N*-ethyl-*N*-(3-sulfopropyl)-3-methylaniline sodium salt; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

pH on the catalytic activity of nanocrystalline cobalt selenide. Unlike the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) or the TMB system, the nanocrystalline cobalt selenide-catalyzed TOPS/4-AAP system can be successfully performed at pH 8.5, which is the optimum pH for uricase activity. This makes the herein-investigated system interesting for the investigation of multienzyme assays with uricase.

To maximize the activity of nanocrystalline cobalt selenide, the effects of varying concentrations of the substrates were also investigated. The relationship between the color intensity of the product and the concentration of TOPS was investigated between 0.04 and 0.4 mM. It can be seen from Figure 4C that the slope at low TOPS concentrations is much steeper than that at higher concentrations. In the former region, product formation could be limited by the quantity of TOPS available in the system. At higher concentrations of TOPS

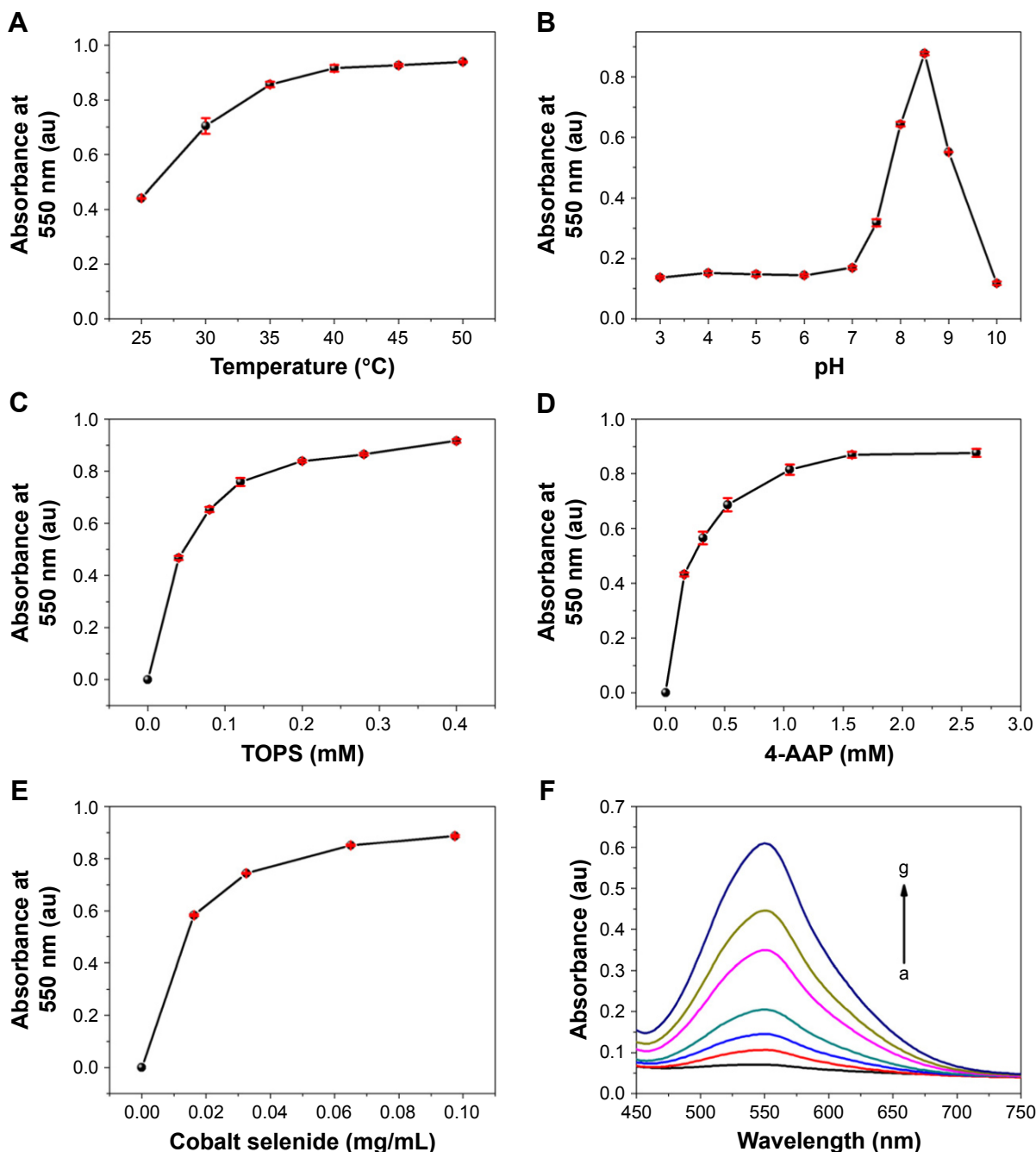


Figure 4 Catalytic activity of nanocrystalline cobalt selenide.

Notes: (A) Temperature dependence of the absorption of a system containing 0.2 mM T OPS, 1.58 mM 4-AAP, 0.125 mM H₂O₂, and 65 μg/mL nanocrystalline cobalt selenide at 550 nm. (B) pH dependence of the absorption of a system containing 0.2 mM T OPS, 1.58 mM 4-AAP, 0.125 mM H₂O₂, and 65 μg/mL nanocrystalline cobalt selenide at 550 nm. (C) Dependence of the absorption of a system containing 1.58 mM 4-AAP, 0.125 mM H₂O₂, and 65 μg/mL nanocrystalline cobalt selenide at 550 nm on T OPS concentration. (D) Dependence of the absorption of a system containing 0.2 mM T OPS, 0.125 mM H₂O₂, and 65 μg/mL nanocrystalline cobalt selenide at 550 nm on 4-AAP concentration. (E) Dependence of the absorption of a system containing 0.2 mM T OPS, 1.58 mM 4-AAP, and 0.125 mM H₂O₂ at 550 nm on nanocrystalline cobalt selenide concentration. (F) Response to H₂O₂ based on peroxidase-like activity of nanocrystalline cobalt selenide. H₂O₂ concentrations (a–g): 0, 8, 10, 20, 40, 50, and 70 μM. The reaction system contained 0.2 mM T OPS, 1.58 mM 4-AAP, 0.125 mM H₂O₂, and 65 μg/mL nanocrystalline cobalt selenide.

Abbreviations: 4-AAP, 4-aminoantipyrine; T OPS, N-ethyl-N-(3-sulfopropyl)-3-methylaniline sodium salt.

(>0.2 mM), the absorbance of the system reaches a plateau. A similar investigation was also conducted on the effect of 4-AAP concentration. As shown in Figure 4D, the absorbance of the system was enhanced as the concentration of 4-AAP increased to 1.58 mM, after which it was almost constant.

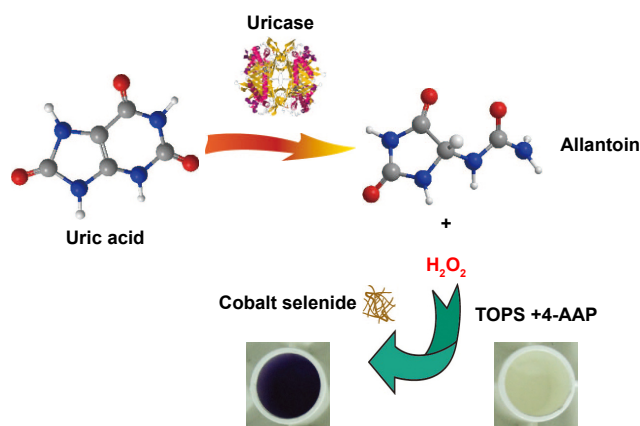
The amount of nanocrystalline cobalt selenide also affected the chromogenic reaction. Because a higher concentration of nanocrystalline cobalt selenide catalyzed the oxidation of the substrates more rapidly, a higher proportion of the product was present in the tested solution.

As indicated in Figure 4E, the absorbance was rapidly enhanced with increasing nanocrystalline cobalt selenide concentration, reaching a plateau at levels >0.065 mg/mL. Therefore, a nanocrystalline cobalt selenide concentration of 0.065 mg/mL was chosen as the standard condition for subsequent analysis. Under the optimum reaction conditions (0.065 mg/mL nanocrystalline cobalt selenide, 1.58 mM 4-AAP, 0.2 mM TOPS, pH 8.5 , and 40°C), the absorbance was found to be proportional to the concentration of H_2O_2 (Figure 4F), which enables the use of the system as a colorimetric sensor for H_2O_2 determination.

Detection of UA

As H_2O_2 is the main product of the uricase-catalyzed reaction, the nanocrystalline cobalt selenide-catalyzed oxidative coupling of TOPS/4-AAP can be combined with the uricase catalysis of UA to provide a colorimetric method for the detection of UA (Scheme 1). As shown in Figure 5, a purple product with a maximum absorbance at 550 nm was formed when the product of the reaction between uricase and UA was introduced into the nanocrystalline cobalt selenide-catalyzed system. The results show that both uricase and UA were essential for the production of the colorimetric product, because the exclusion of either component would yield no H_2O_2 .

The absorbance increased markedly as the concentration of UA was increased. A linear calibration graph was obtained between 2.0 and 40 μM (Figure 6A). The correlation coefficient was 0.998 , and the limit of detection obtained for the experiment was 0.5 μM (signal/noise ratio=3). The relative standard deviation for six repeated measurements using 30 μM UA was 3.2% . The analytical performance of the proposed



Scheme 1 Schematic illustration of the colorimetric assay for the detection of uric acid.

Abbreviations: 4-AAP, 4-aminoantipyrine; TOPS, *N*-ethyl-*N*-(3-sulfopropyl)-3-methylaniline sodium salt.

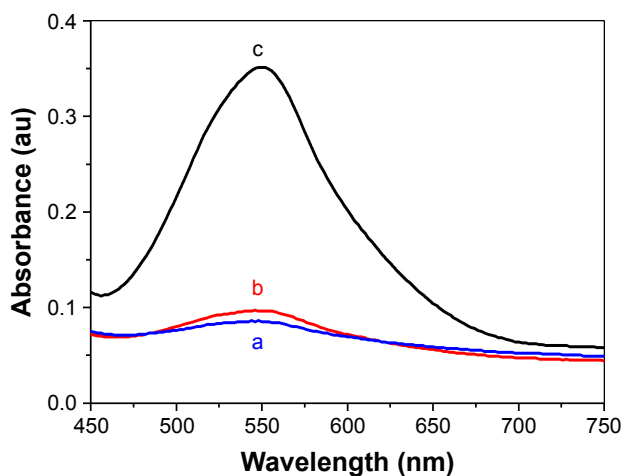


Figure 5 UV/visible spectra of the colorimetric sensor in the presence of (a) uricase, (b) uric acid, and (c) uricase + uric acid.

Abbreviation: UV, ultraviolet.

UA sensing approach was compared to those of other methods based on peroxidase mimics reported in recent years.^{23,28} The developed sensor showed a wide linear range, good sensitivity, and relative low limit of detection.^{23,28}

To identify the selectivity of the proposed UA sensor, responses in the presence of creatinine, ascorbic acid, glucose, cholesterol, and triglyceride were studied. As can be seen from Figure 6B, the absorbance at 550 nm was negligible even when the concentrations of the alternative substrates were 10 times higher than that of UA. The color difference can be easily distinguished with the naked eye, indicating that the developed colorimetric method exhibited high selectivity toward UA.

Analysis of human serum samples

To evaluate the possible application of the sensing system to clinical analysis, the developed method was applied to the determination of UA in human serum samples. Table 1 lists the UA content in three human serum samples determined using both our proposed method and the routine clinical method. In the routine clinical method, the UA assay was performed using a Beckman Coulter (Beckman Coulter, Inc., Brea, CA, USA) automatic analyzer using its instrument-adapting reagents, calibrators, and control solution. The results of the two-sample *t*-test and *F*-test performed at the 95% confidence level demonstrated that the two approaches did not provide significantly different results. The practical applicability of the proposed method was further verified through standard addition experiments. The results showed that the data determined using the UA sensor were in good agreement with the amount of UA added

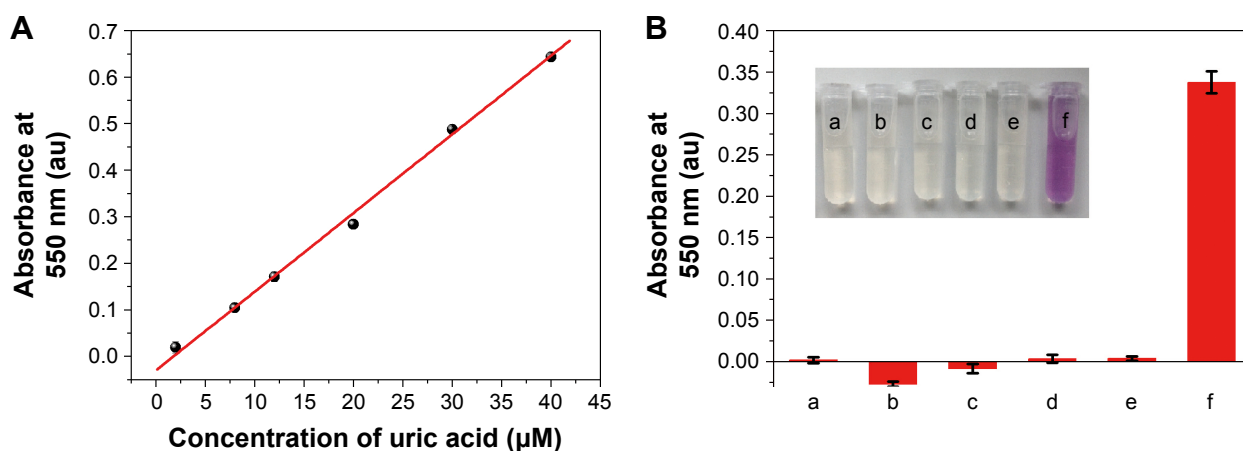


Figure 6 (A) Linear calibration plot for uric acid and (B) selectivity of the assay toward uric acid.

Notes: (a–f) Represent glucose, ascorbic acid, creatinine, cholesterol, triglyceride, and uric acid, respectively. The concentration of uric acid was 25 μM , while those of other solutes were 250 μM . Inset shows the photographs of the corresponding solutions.

to the human serum samples. The recoveries of UA from the spiked samples ranged from 100.5% to 103.2%. This proves that this new method is suitable and satisfactory for UA analysis in clinical applications.

Conclusion

We found that nanocrystalline cobalt selenide fabricated via a solvothermal method possesses intrinsic peroxidase-like activity. The catalysis is dependent on temperature, pH, and concentration of H_2O_2 , similar to HRP catalysis. Kinetic analysis reveals that the catalysis followed a typical Michaelis–Menten model. Based on the intrinsic peroxidase-like activity of nanocrystalline cobalt selenide, a colorimetric UA sensor was constructed. H_2O_2 could be generated by the reaction between UA and oxygen in the presence of uricase. TOPS/4-AAP then reacted with H_2O_2 in the presence of nanocrystalline cobalt selenide as a catalyst to produce a purple product. Under optimum conditions, the assay had a linear range from 2.0 to 40 μM and a detection limit of 0.5 μM . Satisfactory results were obtained when it was applied in the detection of UA in human serum. Therefore, we believe that this approach could be a promising candidate for bioanalysis and clinical diagnosis.

Table I Determination of uric acid in human serum samples

Sample	Uric acid level, μM , mean \pm SD		F-test	t-test
	Proposed method (n=3)	Routine clinical method (n=3)		
1	402.0 \pm 9.0	408.3 \pm 15.3	1.6	0.62
2	179.7 \pm 15.9	173.3 \pm 3.2	5.5	0.68
3	241.7 \pm 4.5	236.0 \pm 15.4	4.1	0.62

Note: $F_{0.05, 2, 2}=19.00$, $t_{0.05, 4}=2.776$.

Abbreviation: SD, standard deviation.

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Disclosure

The authors report no conflicts of interest in this work.

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