Microchim Acta (2017) 184:1233–1237
DOI 10.1007/s00604-017-2147-x

SHORT COMMUNICATION

A sensitive colorimetric assay for cholesterol based on the peroxidase-like activity of MoS_2 nanosheets

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Received: 7 December 2016 / Accepted: 18 February 2017 / Published online: 27 February 2017

Abstract The authors describe a colorimetric method for the determination of cholesterol in human serum. Cholesterol is enzymatically oxidized by oxygen in the presence of cholesterol oxidase (ChOx) to produce 4-cholestene-3-one and hydrogen peroxide (H_2O_2). Due to the peroxidase-like activity of MoS_2 nanosheets, H_2O_2 can oxidize the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) to give a blue product. The increase in the absorbance of the acidic oxTMB solution at 450 nm is used for quantification of cholesterol. Under the optimal conditions, the increase in absorbance is proportional to the concentration of cholesterol in the range from 2 to 200 μmol·L^{-1}, and the limit of detection is 0.76 μmol·L^{-1}. The color change from pale yellow to blue color can also be detected visually for cholesterol concentrations as low as 20 μmol·L^{-1}. Such an enzyme mimetic-based assay possesses advantages over enzyme-based assays in terms of costs, stability against denaturation, and protease digestion. The assay was applied to the determination of free cholesterol and of total cholesterol (after hydrolysis using an esterase) in spiked human serum, and it gave satisfactory recoveries.

Keywords Enzyme mimic · Tetramethylbenzidine · Human serum · Colorimetric method · Hydrogen peroxide · Cholesterol oxidase

Electronic supplementary material The online version of this article (doi:10.1007/s00604-017-2147-x) contains supplementary material, which is available to authorized users.

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The determination of cholesterol level is very important for the diagnosis and prevention of many clinical disorders, such as hypolipoproteinemia, anemia, septicemia, malnutrition hypertension, coronary heart disease, arteriosclerosis, brain thrombosis, lipid metabolism dysfunction and myocardial infarction [1]. Therefore, it is of great significance to develop a reliable and cost effective method to monitor the level of cholesterol in serum. The methods for the detection of cholesterol can be classified into two groups: nonenzymatic and enzymatic methods. Non-enzymatic methods such as colorimetry [2], fluorimetry [3], gas-chromatography-mass spectrometry [4], and high performance liquid chromatography [5] have been applied to detect cholesterol in food and clinical samples. However, these traditional non-enzymatic methods are involved in complicated operational steps and sophisticated equipment, or lacked of sensitivity and selectivity.

Enzymatic methods for the determination of cholesterol have practically replaced the nonenzymatic methods due to the simplicity, rapidity, and specificity [6]. Among the enzyme-based methods for cholesterol, cholesterol oxidase (ChOx)-based electrochemical methods have been developed tremendously due to their rapid response, high sensitivity, and low cost [7]. Cholesterol can be oxidized by oxygen in the presence of ChOx to produce 4-cholestene-3-one and hydrogen peroxide (H_2O_2) [7, 8]. Therefore, the measurement of H_2O_2 by the amperometric method can be used for the indirect quantification of cholesterol. However, the oxidation of H_2O_2 requires a high anodic potential that may oxidize other electrochemical active species in the samples and cause a false positive signal [6–8]. To overcome the weak point, ionic liquid [9], electron transfer mediators (ETM) such as Prussian blue [10], thionine [11], and hydroquinone [12], horseradish peroxidase (HRP) [8], and biocompatible nanomaterials with electrocatalytic effects such as silver nanoparticles, ZnO nanostructures [13], gold nanoparticles [6], carbon nanotubes [6], CuO nanowires [14]

have been introduced to decrease the over potential of the anodic electrode for the effective detection of cholesterol. Besides amperometry, cyclic voltammetry, electrochemical impedance spectroscopy [15], field effect transistor [16], electrogenerated chemiluminescence [17], surface plasmon resonance [18], chemiluminescence [19], photoluminescence [20] have also been applied to assay cholesterol. Although great progresses have been made for the detection of cholesterol by the ChOx-based methods, the development of highly sensitive, convenient, reliable and cost-effective methods for the determination of cholesterol is still a challenge.

Transition metal dichalcogenides (TMDC) with lamellar structure have been attracted growing attention because some of them are semiconductors with sizable bandgaps and are naturally abundant [21]. MoS_2 nanosheets, as a prototypical TMDC material, have showed potential applications in the biomedical region such as NIR photothermal therapy [22], drug delivery [23], antibacterial activity [24], etc. Recently, MoS_2 nanosheets were discovered to possess an intrinsic peroxidase-like activity by our group [25]. Enzyme mimetics possess many advantages over nature enzymes such as low cost, more stable against denature or protease digestion [26]. Colorimetric methods are particularly attractive for point-of-use applications because they can be simply read out by the unaided eyes [27–30]. Herein we propose a sensitive colorimetric assay for cholesterol in human serum by taking advantage of commercial MoS_2 nanosheets as the peroxidase mimic (Scheme 1). Cholesterol is oxidized by oxygen (O_2) in the presence of ChOx to produce 4-cholestene-3-one and hydrogen peroxide (H_2O_2). Then, MoS_2 nanosheets catalyze the oxidation of peroxidase substrate TMB by H_2O_2 to produce a blue color product. The absorbance of solution can be used for the direct quantification of cholesterol.

Materials and methods

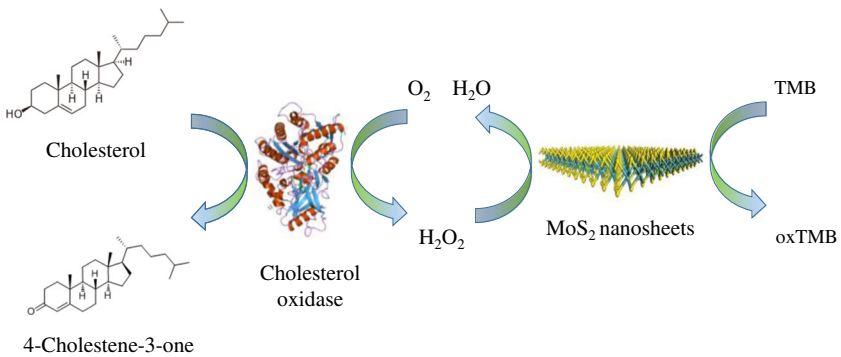
Reagents

Cholesterol (powder, ≥99%) and ChOx from Streptomyces sp. (lyophilized powder, 37 units·mg^{-1} protein) were bought from Sigma (http://www.sigmaaldrich.com/china-mainland.html). MoS_2 nanosheets solution (18 mg·L^{-1}) was purchased from Nanjing XFNANO Materials Tech Co., Ltd. (China, http://www.xfnano.com/). Tris(hydroxymethyl)methyl aminomethane (Tris, BR) and H_2O_2 (AR) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China, http://en.reagent.com.cn/). TMB was bought from Bio Basic Inc. (Canada, http://store.biobasic.com/). Other chemicals used were analytical grade. Ultrapure water (18.2 MΩ·cm) was used throughout the experiment.

Detection of cholesterol in human serum

For the detection of free cholesterol in human serum, the serum sample was diluted 100-fold with water before detection. For the detection of total cholesterol in human serum, the cholesterol ester was hydrolyzed to free cholesterol in advance. The protocol for the hydrolysis of cholesterol ester was as following. 0.2 mL serum sample was mixed with 1.8 mL potassium hydroxide ethanol solution (8.9 mmol·L^{-1}) for 1 h at 37 °C to hydrolyze cholesterol ester. Then the sample was mixed with 2.0 mL water and 4.0 mL n-hexane (as extraction solvent). After centrifugation at 5000 rpm for 5 min, 2 mL supernatant was extracted and the solvent was evaporated to dryness under a stream of nitrogen. The residue was dissolved with 1 mL isopropanol-Triton X-100 aqueous solutions (isopropanol: 7.2%, Triton X-100:2.8%, v·v^{-1}).

120 μL pretreated serum sample was mixed with 30 μL ChOx (2 U·mL^{-1}) and 50 μL Tris-HCl buffer (10 mmol·L^{-1}, pH 6.9). After incubation for 2 min, 200 μL Tris-HCl buffer (10 mmol·L^{-1}, pH 6.9), 50 μL MoS_2 nanosheets (18 μg·mL^{-1}), and 50 μL TMB (12 mmol·L^{-1}) were added and the mixture was incubated at 25 °C for 30 min. Finally, 50 μL H_2SO_4 solution (20%, v·v^{-1}) was added to quench the color reaction and the absorption spectrum was recorded by a Lambda 750 UV-Vis-NIR spectrophotometer (PE, USA) over the range of 350 to 550 nm. The absorbance of acidic oxTMB at 450 nm was used to quantitatively measure cholesterol in human serum.



Scheme 1 Schematic illustration of colorimetric detection of cholesterol by using ChOx- and MoS_2 nanosheets- catalyzed reactions

Results and discussion

Optimization of method

The colorimetric assay for cholesterol is based on the enzyme catalytic action of ChOx and the pseudo-enzymatic action of MoS_2 nanosheets. The structure information of the MoS_2 nanosheets such as UV spectrum, TEM image is shown in Fig. S1 in the Electronic Supporting Material (ESM). ChOx catalyzes the oxidation reaction of cholesterol by O_2 to produce H_2O_2, which subsequently oxidize TMB under the catalysis of MoS_2 nanosheets to generate a blue production, the charge-transfer complexes derived from the one-electron oxidation of TMB (oxTMB) with two characteristic absorption peaks at 369 and 652 nm [25]; After the color reaction was quenched by H_2SO_4, the absorption peak of acidic oxTMB was shifted to 450 nm (Fig. S2 in ESM). The comparison of absorption spectra of cholesterol, ChOx, TMB, and oxTMB is shown in Fig. S3 in ESM. The optimal conditions for the catalytical oxidation reaction of TMB by H_2O_2, such as concentrations of MoS_2 nanosheets, TMB, pH were chosen according to our previous experiments [25]. The following parameters were optimized: (a) Concentration of ChOx; (b) Temperature; (c) Incubation time. Respective data and figures are given in Fig. S4 in the ESM. We found the following experimental conditions to give best results: (a) 30 μL ChOx (2 $U \cdot mL^{-1}$); (b) 50 μL TMB (12 $mmol \cdot L^{-1}$); (c) 50 μL MoS_2 nanosheets (18 $\mu g \cdot mL^{-1}$); (d) 200 μL Tris-HCl buffer (10 $mmol \cdot L^{-1}$, pH 6.9); (e) incubation temperature 25 °C; (f) incubation time 30 min.

Calibration curve

The absorbance of mixture solution toward different concentration of cholesterol was measured under the optimal conditions. As shown in Fig. 1a, the absorbance of mixture solution increases with the concentration of cholesterol. The calibration curve (Fig. 1b) indicates that there is a good linear relationship between the absorbance at 450 nm and the concentration of cholesterol over the range from 2 to 200 $\mu mol \cdot L^{-1}$ (R^2 = 0.999). The detection limit is about 0.76 $\mu mol \cdot L^{-1}$ (3σ/K, in which σ is the standard deviation for the blank solution, and K is the slope of the calibration curve). The comparison of assay performance of this colorimetric method with previously reported methods is shown in Table 1. This method exhibits comparable assay performance, such as high sensitivity and board linear range. The assay sensitivity is much higher than the colorimetric methods by using graphene quantum dots [27], gold nanoparticles supported on MoS_2 nanoribbons [28], Nanoclay [29], $Cu_2(OH)_3Cl$-CeO_2 nanocomposite [30] as peroxidase mimetics. In addition, the color variation of mixture solution from pale yellow to blue color or from pale yellow to deep yellow color after addition of sulfuric acid is visually observed at the presence of different concentrations of cholesterol (Inset in Fig. 1b), offering a convenient approach to detect cholesterol by the unaided eyes. Observable color change can be distinguished at the concentration of cholesterol as low as 20 $\mu mol \cdot L^{-1}$.

CS: chitosan, PB: Prussian blue, IL: ionic liquid, ChE: cholesterol esterase, HRP: horseradish peroxidase, LDHs: layered double hydroxides, NPs: nanoparticles, NCs: nanoclusters, NWs: nanowires, GQDs: graphene quantum dots, NRs: nanoribbons, MCWNTs: multi-walled carbon nanotubes, PTH: poly-thionine, FNAB: 1-fluoro-2-nitro-4-azidobenzene, H-GNs: hemin-graphene nanosheets, TMB: 3,3′,5,5′-tetramethylbenzidine, GCE: glassy carbon electrode, GRE: graphite electrode, ITO: indiumtin oxide coated glass, GE: gold electrode, GCG: gold coded glass,

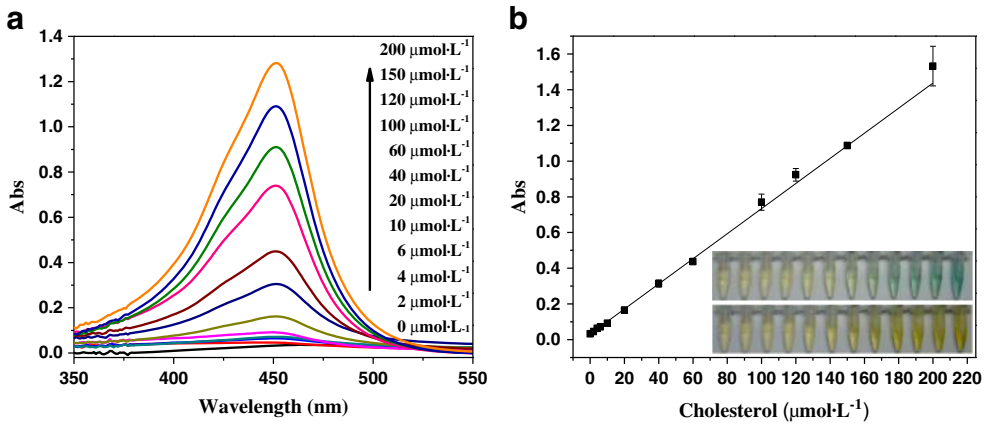


Fig. 1 **a** Absorption spectra of solution in the presence of various concentrations of cholesterol. **b** Calibration curve (absorbance at 450 nm vs concentration of cholesterol). The error bars indicated the standard deviation of three experiments. Inset of B is photos of colored solutions in the presence of different concentrations of cholesterol before (*top*) and after (*down*) adding of 50 μL sulfuric acid (20%, $V \cdot V^{-1}$). Cholesterol concentration ($\mu mol \cdot L^{-1}$, from left to right): 0, 2, 4, 6, 10, 20, 40, 60, 100, 120, 150, 200

Table 1 Comparison of assay performance of different methods for cholesterol

Materials	Detection method	Linear range ($\mu mol \cdot L^{-1}$)	Detection limit ($\mu mol \cdot L^{-1}$)	Ref.
Nafion/ChOx/Au NPs-MWCNTs/GCE	DPV	10–5000	4.3	6
CS/ChOx/Au NPs/ITO	amperometric	1–45	0.5	7
CS/ChOx/LDHs/GCE	amperometric	0.5–800	0.1	8
CS/ChOx-HRP/LDHs/GCE	amperometric	0.08–600	0.04	8
IL/ChOx/PB/GCE	CV	10–400	4.4	9
PB/ChOx/GRE	amperometric	50–800	3.7	10
PTH/ChOx-ChE/Au NPs/GCE	amperometric	2–1000	0.6	11
PTH/ChOx-HRP/GCE	DPV	25–125	6.3	12
Nafion/ChOx/ZnO NPs/GE	amperometric	0.001–0.5	0.00037	13
ChOx/CuO NWs/GCG	potentiometric	5–5000	1	14
ChOx/NanoFe_3O_4/ITO	EIS	6.5–10,345	6.5	15
ChOx-ZnO Nanorods	FET	1–45,000	0.05	16
H-GNs/ChOx/GCE	ECL	0.17–1120	0.06	17
ChOx/FNAB/P3HT/GE	SPR	1300–13,000	1300	18
Cu NCs- Luminol-H_2O_2	CL	50–10,000	1.5	19
PVP-Au NPs/ChOx/BSA-Au NCs	IEF-Fluorescence	1–100	1.4	20
ChOx/GQDs-TMB-H_2O_2	colorimetric	20–600	6	27
ChOx/MoS_2 NRs-Au NPs-TMB-H_2O_2	colorimetric	40–1000	15	28
ChOx/Nanoclay-TMB-H_2O_2	colorimetric	50–244	-	29
ChOx/$Cu_2(OH)_3Cl$-CeO_2	colorimetric	100–2000	-	30
ChOx/MoS_2 nanosheets-TMB-H_2O_2	colorimetric	2–200	0.76	This work

DPV: differential pulse voltammetry, CV: cyclic voltammogram, ECL: electrogenerated chemiluminescence, FET: field-effect-transistor, SPR: surface plasmon resonance, CL: chemiluminescence, IEF: inner filter effect, EIS: electrochemical impedance spectroscopy.

Interference study

The selectivity of this method was estimated by comparing the absorbance of mixture solution in the presence of cholesterol and other interferences such as urea, lactose, glycine, KCl, $MgCl_2$, NaCl, glucose. As shown in Fig. S5 in the ESM, the other interferences tested did not produce a large absorbance at the concentration of even 10 fold of cholesterol, which demonstrates high selectivity for the detection of cholesterol.

Analytical application

In order to explore the applicability and feasibility of this method, cholesterol in human serum samples was detected according to the experimental procedure. The recovery experiment was carried out by standard addition method. As shown in Table S1, the recoveries are in the range of 94.0–100.8%, which demonstrates that this colorimetric method shows great potential for practical application.

Conclusions

In summary, a sensitive colorimetric assay for cholesterol in human serum was developed by taking advantage of MoS_2 nanosheets as the peroxidase mimetic. MoS_2 nanosheets can catalyze the oxidation of peroxidase substrate TMB to produce a color reaction by H_2O_2, which is generated by the oxidation of cholesterol at the presence of ChOx. The color change of mixture solution can be used to quantitative measurement of cholesterol by the colorimetric method and semi-quantitative detection by the visual method. Tedious syntheses, immobilization of ChOx and sophisticated equipment are not needed. Furthermore, this method can also be expanded to detect other biologically important molecules with different H_2O_2-producing oxidases.

Acknowledgements This work was financially supported by NSFC (21577017), Program for New Century Excellent Talents in Fujian Province University, and the Science and Technology Project of Fujian Province, China (2015Y0045).

Compliance with ethical standards The author(s) declare that they have no competing interests.

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