TMB colorimetric cholesterol biosensor based on reduced graphene oxide-Hemin nanocomposites

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Abstract. Cholesterol level is an important reference index in clinical diagnosis. Herein, a highly sensitive TMB colorimetric cholesterol biosensor was constructed based on the good catalytic activity of reduced graphene oxide-Hemin nanocomposites (HGNs) for H2O2 and the color rendering of TMB. Cholesterol is decomposed into H2O2 catalysed by cholesterol oxidase and cholesterol esterase. Colorless TMB can produce blue cation roots when HGNs catalyzes H2O2 reduction. The content of cholesterol can be obtained by spectrophotometric determination of the absorbance of the solution. In the range of 0-100 µg/mL, the absorbance is linearly related to the cholesterol concentration, the regression equation was Y=0.000629X+0.02876 with a correlation coefficient of 0.99395. The sensor also has a great recovery rate (100.4-101.6%) and RSD (1.78-2.15%) in the detection of human serum samples. This indicated that TMB colorimetric cholesterol biosensor has great potential in clinical diagnosis.

1. Introduction
Cholesterol is the most abundant steroid in the human body, mainly stored in the brain and muscles. Serum cholesterol is closely related to human multiple diseases such as heart disease, diabetes mellitus, atherosclerosis, nephrotic syndrome, liver and gallbladder diseases [1]. Serum cholesterol content has become an important reference index in clinical diagnosis. Therefore, it is of great practical significance to study a sensitive and rapid method for detecting cholesterol [2].

Recently, graphene as a new type of nanomaterials has been widely studied. Reduced graphene oxide (RGO) is a deoxidized product of graphene oxide (GO), which contains a large number of boundary points, structural defects and functional groups. RGO provides a rich site for the detection of adsorption and reaction of substances on it. It can increase the adsorption rate and fixation of enzymes, and has good biocompatibility to retain the activity of enzyme loading. Moreover, the retention of carboxyl groups on RGO improves its hydrophobicity [3].

Hemin is an iron porphyrin complex with low cost and stable properties, which can catalyze the reduction of hydrogen peroxide. However, its catalytic activity and chemical stability are unsatisfactory [4]. Because of its strong conjugated structure, RGO can adsorb porphyrin molecules through pi-pi stacking to form RGO-Hemin nanocomposites (HGNs). The introduction of RGO can enhance the catalytic activity and stability of Hemin. Moreover, the HGNs exhibit a higher intrinsic peroxidase-like activity in H2O2 catalytic reduction experiments [5, 6].

Herein, a simple and sensitive TMB colorimetric cholesterol biosensor based on HGNs was introduced. Cholesterol was decomposed into H2O2 catalyzed by cholesterol oxidase and cholesterol...
esterase (CHOD&CHER). Then, HGNs catalysed the H$_2$O$_2$-mediated oxidation peroxidase substrate TMB, resulting a color change. The colorless TMB was oxidized into blue oxidized TMB in the presence of H$_2$O$_2$ by the assistance of HGNs. The content of cholesterol can be obtained by spectrophotometric determination of the absorbance of the solution [7, 8].

2. Experimental

2.1. Reagents and apparatus
GO was purchased from Xiamen NANO Materials Tech Co., Ltd. (Nanjing, China). All analytical reagents used in the experiment were obtained from Aladdin Reagents Ltd (Shanghai, China) and Sinopharm chemical Reagent Co., Ltd (Beijing, China), and used without further purification. All the solutions were prepared with ultrapure water of 18 Ω·cm purified from a Milli-Q purification system (Milli-Pore, Bedford, MA, USA). The human blood serum was acquired from 181st Hospital of Chinese People's Liberation Army (Guilin, China). Colorimetry and spectrophotometry were performed on an UH5300 UV-Visible spectrophotometer (Hitachi High Technologies Corporation, Japan).

2.2. Synthesis of the HGNs
HGNs were prepared by hydrazine hydrate as reductant. 10 mg hemin was mixed with 10 mL 1.0 g/mL GO solution, then 100 µL 80% hydrazine hydrate was added to react for 4 hours at 60°C in water bath, then HGNs were obtained by centrifugal precipitation and re-dissolving in 10 mL ultrapure water.

2.3. Detection of cholesterol with the TMB colorimetric cholesterol biosensor based on HGNs
500 µL cholesterol was mixed with 2 µL 1.0 mg/mL cholesterol oxidase and 1.0 mg/mL cholesterol esterase (CHOD&CHER), and reacted at 30°C for 60 minutes. Then 1.0 mg/mL TMB solution and HGNs were added in turn and reacted at room temperature for 60°C minutes. UH5300 UV-Visible spectrophotometer was used to measure the solution. The absorption peak was observed at 652 nm. The peak value was positively correlated with cholesterol concentration.

3. Results and discussion

3.1. Principle of the TMB colorimetric cholesterol biosensor based on HGNs
The principle of the TMB colorimetric cholesterol biosensor based on HGNs was schematically shown in Figure 1. Cholesterol is decomposed into H$_2$O$_2$ catalyzed by CHOD&CHER. After adding TMB solution and HGNs, H$_2$O$_2$ is decomposed under the catalysis of HGNs, and colorless TMB is converted into blue cations. The reactions can be explained by the following equations:

\[
\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD&CHER}} \Delta - \text{Cholestenone} + H_2O_2
\]  

\[
H_2O_2 + TMB \xrightarrow{\text{HGNs}} H_2O + TMB_{OX}
\]
3.2. Characterization of the HGNs nanocomposites
The image for HGNs nanocomposite solution was shown in Figure 2A. TEM image for HGNs nanocomposite can also be observed in Figure 2B, the morphology of the flake structure was evident, and the HGNs were well dispersed with the uniform diameter in the range of 200-400 nm in size. Figure 2C also described the particle size of HGNs, the HGNs particle size was about 340 nm. The Zeta potential of HGNs were shown in Figure 2D, the Zeta potential of HGNs was about 3 mV.

3.3. The feasibility of TMB colorimetric cholesterol biosensor based on HGNs
The feasibility of TMB colorimetric cholesterol biosensor based on HGNs was evaluated at different system, Figure 3A and Figure 3B were respectively experimental pictures and absorbance image of feasibility of TMB colorimetric cholesterol biosensor based on HGNs, curve a contained TMB and

Figure 1. Principle of the TMB colorimetric cholesterol biosensor based on HGNs.

Figure 2. (A) Image for HGNs nanocomposite solution; (B) TEM image for HGNs nanocomposite; (C) The particle size of HGNs; (D) The Zeta potential of HGNs.

Figure 3. (A) Experimental picture; (B) Absorbance image of feasibility of TMB colorimetric cholesterol biosensor based on HGNs.
HGNs, curve b contained CHOD&CHER, TMB and HGNs, curve c contained cholesterol, CHOD&CHER, TMB and HGNs, curve d contained $H_2O_2$, TMB and HGNs, there was no absorption peak in curve a and curve b, while there was obvious absorption peak in curve c and curve d. This indicated that the TMB colorimetric cholesterol biosensor based on HGNs can detect cholesterol effectively. Experimental pictures and the linear relationship between absorbance and the concentration of $H_2O_2$ within the range of 5-30 µM were shown in Figure 3C and Figure 3D, the color of the system deepened as the concentration of $H_2O_2$ increased, the regression equation was $Y=0.00314X+0.026$ with a correlation coefficient of 0.99406 within the range of 5-30 µM.

3.4. The performance of the TMB colorimetric cholesterol biosensor based on HGNs
Under the optimized experimental conditions, the cholesterol detection was performed by the TMB colorimetric cholesterol biosensor based on HGNs. The color of the system was a cholesterol concentration-dependent manner, as as indicated in Figure 4A. Figure 4B was absorbance of different concentrations of cholesterol, the linear relationship between absorbance and the concentration of cholesterol within the range of 0-100 µg/mL was shown in Figure 4C, the regression equation was $Y=0.000629X+0.02876$ with a correlation coefficient of 0.99395. Figure 4D was an analysis of the specificity of RGO-CS-Fc/Pt NPs electrochemical biosensor, the responses caused by Gal, AA, UA, and Dop could be negligible. Which indicated that the TMB colorimetric cholesterol biosensor based on HGNs has a good selectivity and high specificity and can be used for the detection of cholesterol without any effect caused by possible interferents.
Figure 4. (A) Experimental picture of different concentrations of cholesterol; (B) Absorbance of different concentrations of cholesterol; (C) The linear relationship between absorbance and the concentration of cholesterol within the range of 0-100 µg/mL; (D) Absorbance of Gal, AA, UA, Dop, cholesterol and its mixture solution.

3.5. Practicability for the detection of cholesterol in human serum sample.

The TMB colorimetric cholesterol biosensor based on HGNs was used to detect human serum samples under the optimal conditions by direct detection method. The human serum was come from the 181 Hospital of the Chinese People's Liberation Army (Guilin, China). Results indicated in the Table 1 showed good recovery of the known samples in the range of 100.4-101.6% with the RSD values of 1.78-2.15%. This results showed that the developed TMB colorimetric cholesterol biosensor based on HGNs has the ability to overcome the potential interference in human serum samples, indicating that the sensor has a good application prospect in medical diagnosis.

4. Conclusion

In summary, a highly sensitive TMB colorimetric cholesterol biosensor was constructed based on the good catalytic activity of HGNs for H₂O₂ and the color rendering of TMB. In the range of 0-100 µg/mL, the absorbance is linearly related to the cholesterol concentration, the regression equation was Y=0.000629X+0.02876 with a correlation coefficient of 0.99395. The sensor also has a great recovery rate in the detection of human serum samples. This indicates that TMB colorimetric cholesterol biosensor has great potential in clinical diagnosis.
Table 1. Detection of cholesterol in human serum samples

| Cholesterol Concentration Added (mg/mL) | Measured Absorbance | Concentration Found (mg/mL) | Recovery (%) | RSD (%) |
|----------------------------------------|---------------------|-----------------------------|--------------|---------|
| 0.25                                    | 0.184               | 0.254±0.006                 | 101.6        | 2.15    |
|                                        | 0.187               |                             |              |         |
|                                        | 0.192               |                             |              |         |
|                                        | 0.338               |                             |              |         |
| 0.5                                     | 0.345               | 0.502±0.009                 | 100.4        | 1.78    |
|                                        | 0.350               |                             |              |         |
|                                        | 0.646               |                             |              |         |
| 1.0                                     | 0.666               | 1.006±0.022                 | 100.6        | 2.12    |
|                                        | 0.673               |                             |              |         |

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