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A Novel Electrochemical Biosensor for Detection of Cholesterol[1]

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Abstract—We report on a highly sensitive electrochemical biosensor for determination of cholesterol. The biosensor was fabricated by co-immobilizing bi-enzymes, cholesterol oxidase (ChOx), and horseradish peroxidase (HRP). Voltammetric technique such as cyclic voltammetry and impedance experiment were used to study the characterization of modified electrode step by step. The developed sensor is cheap, disposable, portable and exhibits higher sensitivity. The biosensor expressed a wide linear range up to 300 mg dL^{-1} in a physiological condition (pH 7.0), with a correlation coefficient of 0.9969. A sensitivity of 13.28 μA mg^{-1} dL cm^{-2} which makes it very promising for the clinical determination of cholesterol.

Keywords: co-immobilizing, bi-enzymes, HRP, ChOx, cholesterol

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1. Introduction

Cholesterol is a fat-like substance with important natural functions that's found in all mammalian cells. According to the lipid hypothesis, abnormal cholesterol levels (hypercholesterolemia) are strongly associated with cardiovascular disease because these promote atheroma development in arteries (atherosclerosis) [1–3]. This disease process leads to serious life threatening diseases like myocardial infarction (heart attack), stroke, and peripheral vascular disease. The Adult Treatment Panels suggests the normal blood cholesterol level should be lower than 5.2 mM (200 mg dL^{-1}) in total, with over 6.2 mM (240 mg dL^{-1}) as a high level [2–5]. Thus, the analysis of cholesterol in serum is a very important parameter for clinical diagnosis and treatment [6].

Various methods have been reported for monitoring cholesterol in biological fluids [4, 7–18] including colorimetric [6], fluorometric [19, 20], electrochemical methods [7, 21–29] and N-geneous methods [30]. Among various methods available for cholesterol determination, biosensors are comparatively simpler, rapid, sensitive and specific [2–6, 31]. A biosensor is an analytical device which is made up of a transducer and a biological element. The bioelement, such as enzymes, antibodies, nucleic acids, receptors, organelles or microorganisms, interact with the analyte being tested and the concentration of substances or other parameters of biological response are converted into an electrical signal [32]. Immobilization of enzymes on electrodes are of great interest in the fabrication of a biosensor. The great performance of an amperometric biosensor is not only immobilizing enzyme on the electrode, but also enhance the electron transfer in sensor design by using mediators, promoters or other special materials.

Most cholesterol biosensors are developed based on electrochemical reduction of hydrogen peroxide (H_2O_2). For example, cholesterol oxidase (ChOx) and horseradish peroxidase (HRP) are modified on the biosensor, which yields H_2O_2 and the redox production of cholesterol (cholest-4-en-3-one) by an enzymatic reaction [33].

As a well-known carboxymethyl cellulose (CMC) is one type of cellulose derivative that has good film forming properties, can form a transparent film and possesses high mechanical strength. It can be used for the fabrication of biosensing devices due to biocompatibility, high viscosity, nontoxic, as well as its solubility in acidic aqueous medium.

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Compared with those reported for glucose, few biosensors have been reported for cholesterol determination. Recently, researchers developed cholesterol biosensors based on the reduction of H_2O_2 due to their simplicity and specificity. Redox-active enzymes are used to test cholesterol because of their electron activity and catalytic ability. This behavior produces or consumes electrons and can enhance signal transduction on the working electrode. Gholivand and Khodadadian immobilized ChOx and catalase (CAT) on a graphene/ionic liquid-modified glassy carbon electrode (GR-IL/GCE) [34]. Souza and co-worker immobilized hemoglobin (Hb) and ChOx on polyelectrolytes poly(allylamine hydrochloride) (PAH) and poly(ethylene imine) (PEI) through layer-by-layer(LBL) technique [35].

In the present work, we co-immobilized bienzymes, cholesterol oxidase (ChOx) and horseradish peroxidase (HRP), on the surface of glassy-carbon electrode(GCE) with the aid of CMC for the detection of the cholesterol. Due to the excellent biocompatibility, low-cost, and biodegradability of CMC, the fabricated biosensor was free of any special materials toxic to the environment and human. The ChOx/HRP/$K_3[Fe(CN)_6]$/GCE biosensor will promise for the detection of cholesterol in both clinical diagnostics and the food industry.

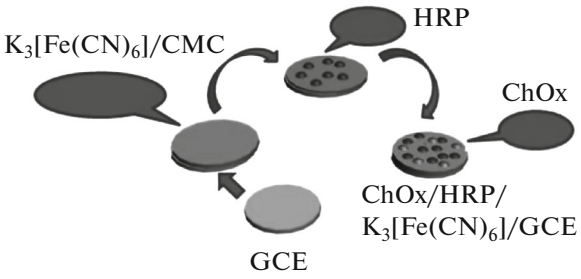
2. EXPERIMENTAL

2.1. Reagents and Materials

Cholesterol oxidase (ChOx) (from Streptomyces species), cholesterol, Triton X-100 (t-octylphenoxypolyethoxyethanol), Horseradish peroxidase (HRP), carboxyl methyl cellulose (CMC), sodium phosphate dibasic, sodium phosphate monobasic, potassium hexacyanoferrate, potassium ferricyanide, potassium chloride were purchased from Sigma-Aldrich and used as received. A stock solution of cholesterol was prepared by dissolving cholesterol in 5 mL isopropanol and 5 mL Triton X-100 and finally diluted by phosphate buffer (pH 7.0). All solutions were prepared with ultra-pure water(18.2 MΩ cm) from a Milipore Mili-Q system.

2.2. Preparation of the Cholesterol Biosensor

Prior to the use, a GCE was polished with 0.3 and 0.05 mm alumina, then rinsed with deionized water and ethanol in ultrasonic bath, and dried at room temperature. Firstly, we dissolved 1.5 mg CMC in 110 μL phosphate buffer (pH 7.0) by vortex dispersion, and then added 5 μL potassium hexacyanoferrate (5 mg/mL) mixed completely and dried in the air. Secondly, dropped the amount of HRP to the surface of the modified electrode and dried in the air. Lastly, dropped the amount of ChOx to form ChOx/HRP/$K_3[Fe(CN)_6]$/GCE. All prepared biosensors were stored at 4°C when not in use. Scheme showed the schematic diagram of the fabrication of the cholesterol biosensor (Scheme 1).



Scheme 1. The schematic diagram of the fabrication of the cholesterol biosensor.

2.3. Instruments or Apparatus

All electrochemical experiments (such as the cyclic voltammetric (CV) experiments and impedance experiments) were performed with Metrohm AUTOLAB electrochemical system (AUT 84875) containing with an in-house-built, three-electrode glass cell. All experiments were performed with a conventional three-electrode system: a platinum coil as the counter electrode, an Ag/AgCl (saturated KCl) electrode as the reference electrode, the modified ChOx/HRP/$K_3[Fe(CN)_6]$/GCE electrodes as the working electrode. The assembling interface was characterized by electrochemical impedance spectroscopy and cyclic voltammetry. The pH measurements were conducted by a pH meter (FE 20, Mettler-Toledo Switzerland). Cyclic voltammetric measurements of cholesterol were carried out in a 0.1 M phosphate buffer solution (pH 7.0) at selected potential ranges. All measurements were conducted in a 2 mL solution and at room temperature (20 ± 2°C).

3. RESULTS AND DISCUSSION

3.1. Characterizations of the Assembling Process of ChOx/HRP/$K_3[Fe(CN)_6]$/GCE

It can be seen from Fig. 1 that bare GCE (curve *1*), CMC/GCE (curve *2*) and HRP/CMC/GCE (curve *4*) in cholesterol exhibit very low current, and no redox peaks could be observed in the CV spectrum, respectively. Corresponding to the reversible redox reaction of ferricyanide ions, $K_3[Fe(CN)_6]$ modified GCE(curve *3*) exhibits a redox current. When the ChOx and HRP are successively loaded onto CMC membrane (curve *5*), a little peak current is obtained. This is due to CMC hindered the electron transfer between ChOx/HRP and bare GCE. The ChOx/HRP immobilized $K_3[Fe(CN)_6]$/GCE electrode (curve *6*) presents a clear oxidation (0.37 V) and reduction (0.15 V) peaks, owing to the reduction of produced H_2O_2 by ChOx and HRP catalytic reaction. And indicates that $K_3[Fe(CN)_6]$ act as a bridge of electron-transfer and promote the electrontransfer. The inset

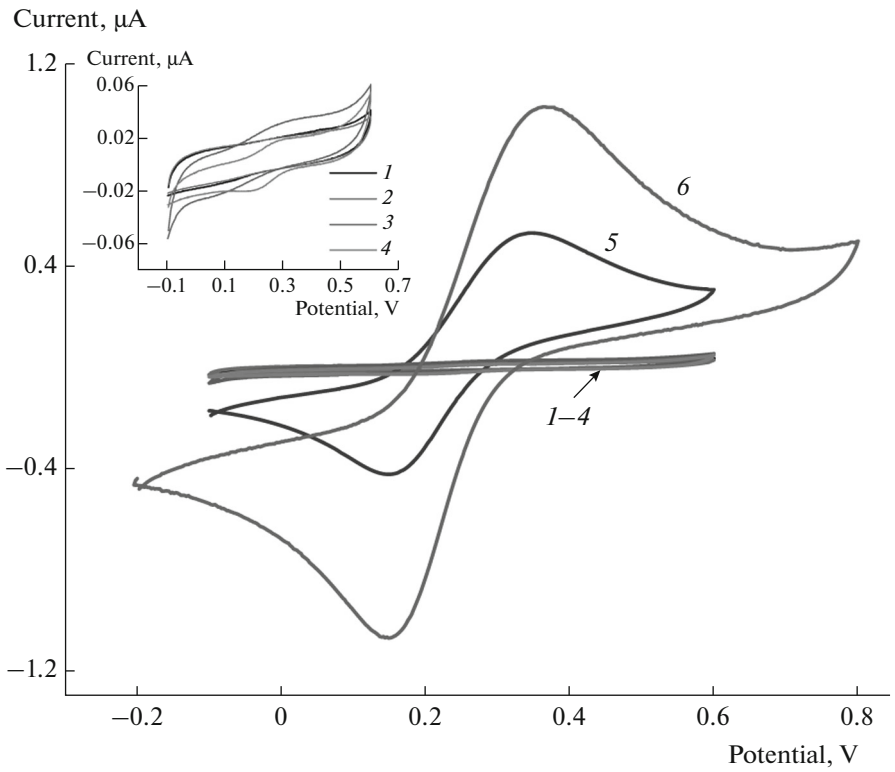


Fig. 1. Cyclic voltammetric response of the different modified electrode measured in 200 mg dL^{-1} cholesterol solution with buffer (pH 7.0). curve *1*: nake GCE, curve *2*: CMC/GCE, curve *3*: $K_3[Fe(CN)_6]$/CMC/GCE, curve *4*: HRP/CMC/GCE, curve *5*: ChOx/HRP/CMC/GCE, curve *6*: ChOx/HRP/$K_3[Fe(CN)_6]$/GCE. Potential scan rate: 50 mV s^{-1}. The inset shows the enlarge part of the curve near the zero current field.

shows the enlarge part of the curve near the zero current field (Fig. 1b). Furthermore, electrochemical impedance spectroscopy (EIS) is used to evaluate the assembling interface of the modified ChOx/HRP/$K_3[Fe(CN)_6]$/GCE. The EIS measurement was carried out after immersing ChOx/HRP/$K_3[Fe(CN)_6]$/GCE in the solution for 10 minutes later. In Fig. 2, when $K_3[Fe(CN)_6]$ attached on GCE surface by using CMC (Fig. 2, curve *2*), the Nyquist semicircle is smaller than bare GCE (Fig. 2, curve *1*). As can be seen in Fig. 2, curve *3*, the Nyquist semicircle is a little larger than $K_3[Fe(CN)_6]$/GCE. It reveals that HRP is poor electrical conductors. After the ChOx is immobilized onto the electrode, the Nyquist semicircle further increase (Fig. 2, curve *4*) due to hindrance the electron transfer between HRP and $K_3[Fe(CN)_6]$/GCE. From the impedance spectrum of each modified stage, modified materials are immobilized onto the electrode successfully.

3.2 Electrochemical Characterization of ChOx/HRP/$K_3[Fe(CN)_6]$/GCE

As seen in Fig. 3, both the anodic and cathodic peak currents increase linearly with the scan rates, indicating that the pair of redox waves originates from the surface confined molecules. The anodic and cathodic peak potentials also change as a function of the scan rate (50–200 mV s^{-1}). With increasing the scan rate, the oxidation peak shifts to more positive potentials, while the reduction peak shifts to more negative potentials. It is agreement with Laviron theory (Fig. 3).

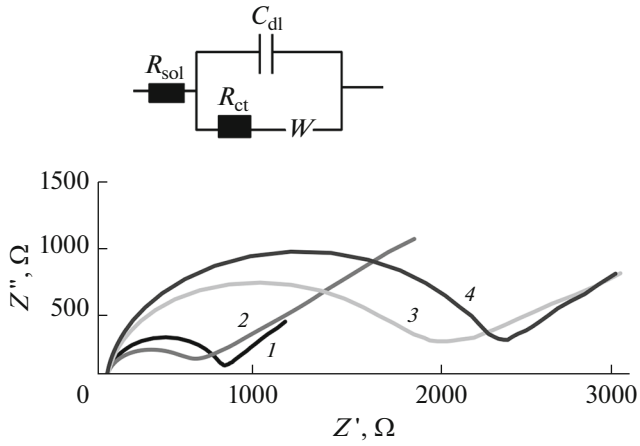


Fig. 2. Electrochemical impedance spectroscopy of the different modified electrode. Curve *1*: nake GCE, curve *2*: $K_3[Fe(CN)_6]$/CMC/GCE, curve *3*: HRP/$K_3[Fe(CN)_6]$/CMC/GCE, curve *4*: ChOx/HRP/$K_3[Fe(CN)_6]$/CMC/GCE.

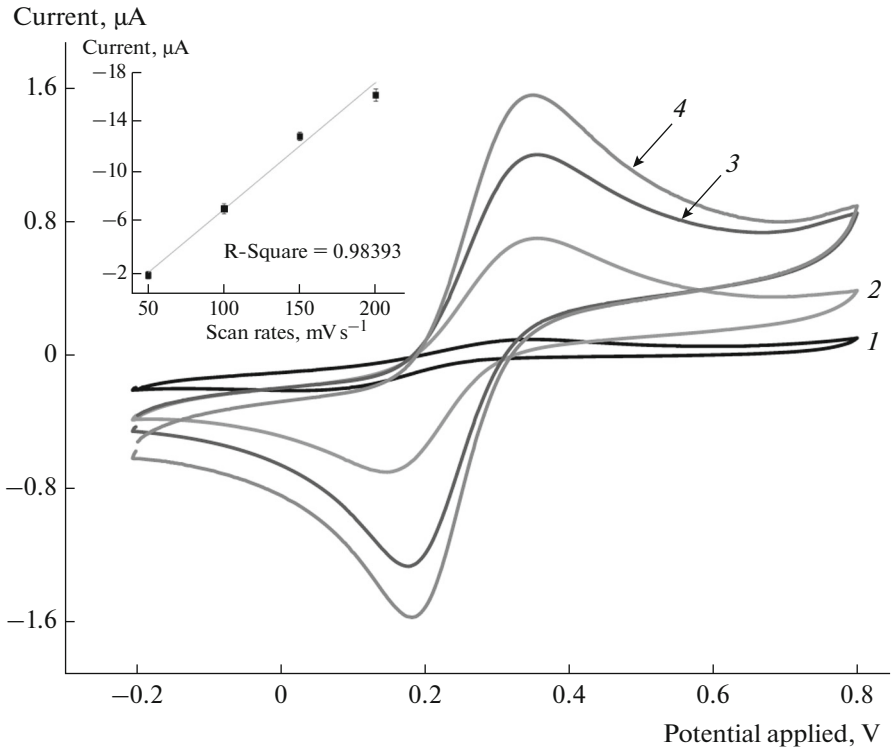


Fig. 3. Cyclic voltammamograms (curves *1–4*, scan rates = 50, 100, 150, 200 mV s^{-1}) of the ChOx/HRP/$K_3[Fe(CN)_6]$/GCE electrode at different scan rates in a phosphate buffer (pH 7.0).

As seen in Fig. 4, The effect of pH on the performance of the ChOx/HRP/$K_3[Fe(CN)_6]$ /GCE is investigated in 0.1 M phosphate buffer solutions with the pH varied from 5.8 to 8.0. The maximum current response at pH 7.0 indicats the immobilized enzymes retain their natural structure on GC electrode. Thus, all the performance tests of the biosensor are detected at pH 7.0.

In Fig. 5, the reduction peak current increase strongly with increasing the concentration of cholesterol. It illustrates a typical electrocatalytic reduction process of H_2O_2 produced by the enzymatic reaction of cholesterol. Figure 5 displays electrochemical response of ChOx/HRP/$K_3[Fe(CN)_6]$/GCE upon successively injecting a known amount of cholesterol solutions. The achieded a dynamic linear range from 0.5 up to 300 mg dL^{-1} with the sensitivity of 13.28 μA mg^{-1} dL cm^{-2}.

It shows a low detection limit of 0.416 mg dL^{-1} (S/N = 3), which is better than in recently reported papers (see table). The value of the apparent Michaelis–Menten constant (K_M^{app}) means a catalytic efficiency of enzyme onto the GCE, which results in higher affinity of ChOx toward cholesterol. Based on Michaelis–Menten mechanism, we can obtain K_M^{app} as 2.728 mM.

Comparison of ChOx/HRP/$K_3[Fe(CN)_6]$/GCE biosensor with other ChOx based biosensors

Electrode	Linear range	Detection limit	References
ChOx/MWCNTs/GCE	48.6–279 μM	48.6 μM	36
ChOx/AgNPs/GCE	3.9–773.4 mg dL^{-1}	0.99 mg dL^{-1}	37
ChOx-HRP-ChE/AuNPs/Ti	0.97–7.8 mM	13 μM	38
Nafion/ChOx/α-Fe_2O_3/Ag	0.1–8 mM	18 μM	39
ChOx/HRP/$K_3[Fe(CN)_6]$/GCE	0.5–300 mg dL^{-1}	0.416 mg dL^{-1}	This work

MWCNT: multi-walled carbon nanotubes, AgNPs: silver nanoparticles, PAni: polyaniline, AuNPs: gold nanoparticles, ChEt: cholesterol esterase.

1 mM*38.67 = 1 mg dL^{-1}.

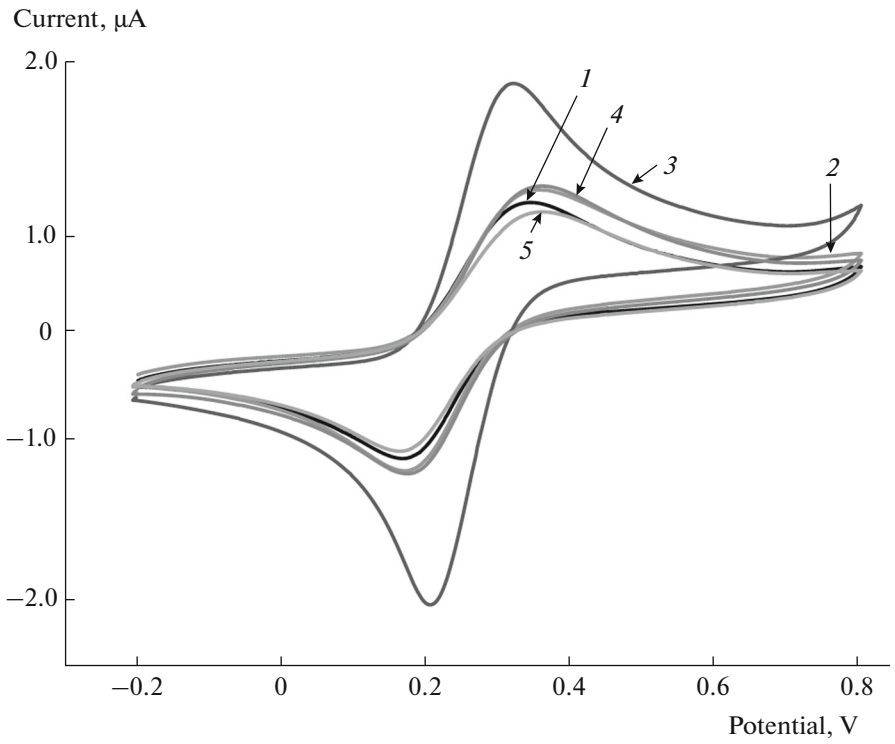


Fig. 4. Cyclic voltammamograms (curves *1*–*5*, pH 5.8,6.0,7.0,7.4,8.0) of the ChOx/HRP/$K_3[Fe(CN)_6]$/GCE electrode in a different phosphate buffer at 50 mV s^{-1} scan rates.

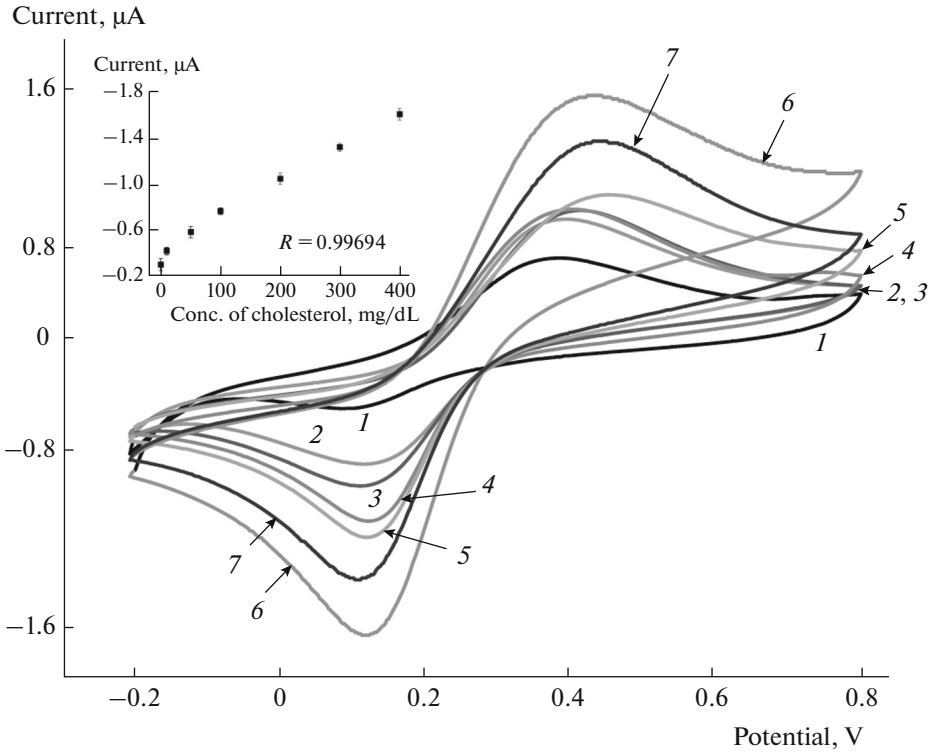


Fig. 5. Cyclic voltammograms ((*1*) 0 mg dL^{-1}, (*2*) 10 mg dL^{-1}, (*3*) 50 mg dL^{-1}, (*4*) 100 mg dL^{-1}, (*5*) 200 mg dL^{-1}, (*6*) 300 mg dL^{-1}, (*7*) 400 mg dL^{-1} cholesterol concentrations) and calibration curve of the biosensor upon addition of different concentrations of cholesterol in a phosphate buffer (pH 7.0) at scan rate: 50 mV s^{-1}.

4. CONCLUSIONS

We have successfully fabricated a high-performance cholesterol biosensor by co-immobilizing cholesterol oxidase and horseradish peroxidase. The immobilized cholesterol oxidase effectively catalyzes the oxidation of cholesterol, while, the immobilized horseradish peroxidase facilitates the detection of the H_2O_2 generated by the catalytic conversion of cholesterol to cholest-4-en-3-one. The developed ChOx/HRP/$K_3[Fe(CN)_6]$/GCE biosensor exhibited reliable cyclic voltammetric responses, high sensitivity and widely linear range under pH 7.0 detection condition. The high-performance along with the ease of fabrication and low costs makes the ChOx/HRP/$K_3[Fe(CN)_6]$/GCE biosensor promising for the detection of cholesterol in both clinical diagnostics and the food industry.

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