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Fast Determination of Vitamin B₂ Based on Molecularly Imprinted Electrochemical Sensor

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ABSTRACT

Under the condition of weak acidity of pH 5.2, a sensitive vitamin B2 electrochemical sensor based on molecularly imprinted non-conducting polymer of o-aminophenol by potentiostatic polymerization in the presence of template(vitamin B2) on a glassy carbon electrode was prepared, and its performance was studied. The sensor exhibited good sensitivity and selectivity to VB2. The detection limit went down to 2.3851nM, and a linear relationship between the current incremental and the concentration was found in the range of 10∼120nM. And the sensor could use in detection of VB2 real sample for a long time and show good reproducibility. The average recovery rate to VB2 was 98.41%.

1. Introduction

Vitamin B2(VB2), also named riboflavin(RF), is B-group water-soluble vitamin. Its structure[1] is exhibited in **Figure 1**. VB2 is stable in acidic aqueous solution and its isoelectric point(PI) is 6. Human beings as well as all animals requires a constant supply of VB2 for the optimal maintenance of large number of metabolic processes requiring the flavin coenzymes, such as flavin mononucleotide(FMN) and flavin adenine dinucletide(FAD)[2]. FMN and FAD are important cofactor of dehydrogenase in vivo. But VB2 can not be synthesized in vivo, only be obtained from vegetables, fruit, health care food and drug etc. What is worse, man and all animals have little ability to store VB2 and excess VB2 will be urinated out of body. Hence, VB2 should be intake and supplemented in time to avoid shortage. Recently, many counterfeit vitamin drugs (including veterinary medicine) that mainly consist of not enough quantity frequently appear on market. Thus, determination of VB2 is very significant in monitoring the amount of VB2 in vitamin drugs.
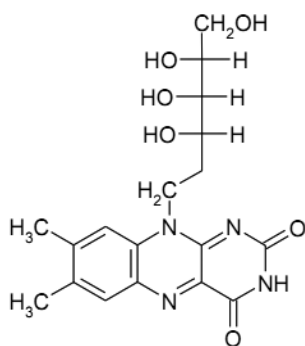

Figure 1. Chemical structure of VB2.

Currently, different methods and techniques have been used to determine concentration of VB2, including chemiluminescence[3-4], chromatography[5-7], voltammetry[8], fluorescence [9], flame atomic absorption spectrometry[10], and combination of these methods[11-13]. But these methods are not only sophisticated but nonspecific, worse still, some expensive and broad-scale equipments are needed. Molecularly imprinted electrochemical sensor, however, based on "molecular memory", combining the advantages of molecularly imprinted method(MIM) with electrochemical measurement technique, but has not been reported to used in determination of VB2 yet.

The synthesis of molecularly imprinted polymers(MIPs) and artificial recognition sites are key and core content in MIM. They are established by chemical and electrochemical methods. These sites tailor-formed in situ by copolymerization of functional monomers and cross-linkers around the template. The print molecules are subsequently removed from the polymer, living accessible complementary binding cavity[14]. According to the interaction between template and polymer, MIM can be divided into covalent imprinting and noncovalent imprinting. Noncovalent interaction consists of hydrogen bond, electrostatic force, metal ion coordination interaction, Van der Waals force etc.

Selection of functional monomer plays an essential role in preparing sensitive film in MIM. O-aminophenol(oAP), with important functional groups of –OH and –NH2, has been used to prepare kinds of sensitive films of biosensor[15]. In contrast to conducting polymers(polyaniline, polypyrrole, poly(3, 4-ethylenedioxythiophene) etc.), the electrosynthesis of non-conducting polymers, take PoAP as an example, is self-limited and inevitably ceased when the polymer film on the electrode grows thick enough to resist electron transfer between the electrode and monomer molecules[16]. Moreover, electrochemical polymerization is very easy to achieve in electrolyte mixed with

functional monomer and template. The thickness of films could be controlled precisely by adusting current density and goes down to nanometers scale[17]. PoAP film was selected in this study because of its relative cheapness, easy availability and preparation.

Therefore, in this paper, VB2 molecularly imprinted electrochemical sensor was prepared through electropolymerizaton of oAP with VB2 under pH 5.2. Meanwhile, the effects of percentage of oAP on polymerization were investigated by UV-vis spectrophotometry, and elution time of template, performance of this sensor, etc. were also investigated in detail.

2. Experimenta

Materials and Methods

1) *Chemicals and reagents*

O-aminophenol(oAP) was from Tianjin recovers the fine chemical research institute after appropriate recrystallization wherever necessary. Perchloric acid was purchased from Aladdin(Shanghai, China). Riboflavin(RF or VB2), ascorbic acid(VC), vitamin B6(VB6) came from Tianjin Ruijinte Chemical Co., Ltd. Flavin mononucleotide(FMN) was from Beijing dingguo changsheng biotech Co. Ltd. Acetic acid, sodium hydroxide, potassium hexacyanoferrate, sodium phosphate dibasic dodecahydrate, sodium dihydrogen phosphate, sulfuric acid and other reagents were of analytical reagent grade. All solutions were prepared with deionized water(conductivity$\leq$0.06μS/cm). Phosphate buffer solution(PBS) with various pH values and concentration were prepared by mixing standard stock solutions of NaH_2PO_4 and Na_2HPO_4 and adjusting the pH with NaOH or HCl. Acetate buffer solution(HAc-NaHAc, pH 5.0) was made by mixing HAc and NaOH.

2) *Apparatus*

All electrochemical measurements were performed on LK2005A Electrochemistry Workstation (LANLIKE, Tianjin, China). A standard tree-electrode system, a glassy carbon(GC) electrode(3 mm in diameter) or its modified electrode, a Ag/AgCl with saturated KCl solution and a Pt001 disk electrode(2 mm in diameter) were used as working electrode, reference electrode and counter electrode, respectively. TU-1810 UV-Vis Spectrofluorimeter(PERSEE, Beijing, China), FA2004A Electronic Analytical Balance(JINGTIAN, Shanghai, China), SB3200DT Ultrasonic Cleaning Machine(SCIENTZ, Ningbo, China) and PHS3BW pH meter(LIDA, Shanghai, China) were utilized in this study.

3) *Pretreatment of glassy carbon electrode*

Prior to modification, GC electrode was polished with 0.1μm and 0.05μm alumina slurry successively. After rinsed with deionized water, the electrode was treated in Piranha solution(30% H_2O_2 mixed with H_2SO_4 at the volume rate of 3 to 7) for 15min, followed by rinsing and sonication in 1:1(V/V) HNO3 aqueous solution, 10%(W/V) NaOH solution, ethanol and acetone for 10min, respectively. Repeated above process triplicately. And then the three-electrode system was put into a single-compartment cell with 0.2M H_2SO_4 solution, cyclic scaning from potential of -0.5 to 1.5V several cycles at the rate of 0.1V/s to achieve steady state for activation.

4) *Preparation of VB$_2$ molecularly imprinted polymers modified electrode[18]*

VB2 molecularly imprinted polymers modified electrode(VB2-/PoAP/GCE) was prepared by traditional tree-electrode system. A oAP stock solution(0.1M) was prepared by dissolving the oAP in 0.5M HClO4 solution. A VB2 stock solution(1mM) was prepared by dissolving the VB2 in a 1%(V/V) acetic acid aqueous solution at refrigerator(4℃). A 10mL volume of polymerization solution was prepared by mixing 8 mL of oAP and 2 mL of VB2 and adjusting the pH to 5.2 with 1M NaOH solution, and then standing in dark place for 20min to prereaction. After that, three electrodes was inserted into this mixed solution. Polymerization was performed by potentiostatic polymerization technology. The parameters were set as follows: potential 0.65V, time 1200s, sampling time interval 0.1125s. After polymerization, the modified electrod was rinsed by deionized water and immersed in   0.8M H2SO4 for 8h to remove template molecules. Nonmolecularly imprinted modified electrode(PoAP/GCE) was prepared by the same procedure in the absence of VB2, 2mL of 1%(V/V) acetic acid instead, for comparison. When not in use the electrode was stored in 0.01M pH 7.0 PBS at room temperature(RT).

5) *Electrochemical measurements*

All electrochemical measurements were performed in 10mL single-compartment cell with 4mM K3[Fe(CN)6] as electrochemical probe at RT and potential range from -0.1V to 0.6V. All parameters of cyclic voltammetry(CV) were scan rates of 0.05V/s. All parameters of differential pulse voltammetry(DPV) were potential incremental of 0.004V, pulse amplitude of 0.05V, pulse width of 0.06V. All parameters of square wave voltammetry(SWV) were potential incremental of 0.004V, square-wave frequency of 15Hz, square-wave amplitude of 0.025V. All potentials refered to Ag/AgCl with saturated KCl solution. The tested solutions were deoxygenated by high pure nitrogen bubbling for 20min just before experiments and the nitrogen atmosphere was kept over the cell during all measurements.

3. Results and Discussion

3.1. Current-time curve of forming VB2 imprinted membrane electrode

Figure 2 is Current-time(I-t) curve of copolymerization of oAP and VB2 on GC electrode. As shown in **Figure 2,** at the beginning of electrochemical polymerization(0s) the value of current decreases sharply from 80μA which indicates the fast formation of nonconducting polymer. As increasing of electrolysis time the current decreases gradually slow, and then down to a platform. Finally the current decreases close to zero. This is the result of electrochemical oxidation of oAP to form PoAP on GC electrode, which is demonstrated that a layer of dense polymer membrane blocking electrons transferring from bulk solution to surface of electrode is formed and VB2 molecular is freezed in PoAP at the same time. This thanks to "self-limited" electrodeposition process of nonconductive PoAP film[16]

3.2. Selection of Polymerization Time

Here three modified electrode were prepared in 600s, 1200s and 1800s, respectively. CV was performed in PBS with 4mM
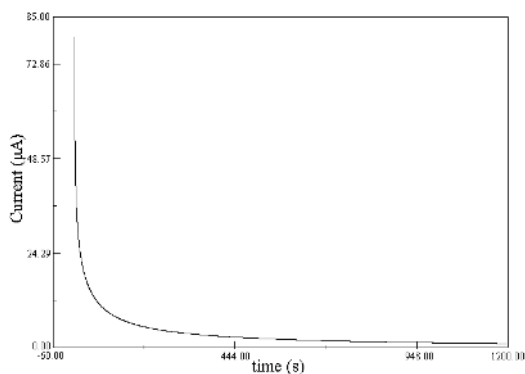
Figure 2. I-t curve of molecularly imprinted copolymerization process of oAP and VB2 on GC electrode.

K3[Fe(CN)6] to characterize their properties. It was indicated that the formed polymer modified electrode of polymerization of 600s and 1200s show no redox peak in K3[Fe(CN)6] solution, but the response current of the former is larger than the latter. When polymerization of 1800s, however, there are a couple of weak redox peaks which indicates the conductivity of this polymer. This result is corresponding to Ref. [19], i.e., PoAP can transfer between insulator and conductor. Therefore 1200s(equivalent to 15 cycles in CV) is selected in this study which is closed to the result of Chi et al[20](14 cycles in CV polymerization on carbon paste electrode) .

3.3. Electrochemical Characterization of VB2 Imprinted Sensor

CV was performed in PBS with 4mM K3[Fe(CN)6] to characterize property of this sensor. [Fe(CN)6]3- can successfully diffuse from the bulk solution to the surface of electrode and quasi-reversible redox reaction take place on naked GC electrode(**Figure 3a**). After elution of the template molecular(VB2-/PoAP/GCE), though there is redox peak the peak is very weak, the redox current is very small too(**Figure 3b**). Moreover, the CV curve shape is poor, which indicates the template has been eluted by H2SO4 living imprinted sites that pave the way for a portion of [Fe(CN)6]3- transferring. But uneluted electrode(VB2+/PoAP/GCE) and nonimprinted electrode(PoAP/GCE) have no response to [Fe(CN)6]3-(**Figure 3c**, **3d**, respectively), which also demonstrated that PoAP thin film is very dense and nonconducting, blocking diffusion of [Fe(CN)6]3- completely.

3.4. Mechanism of Interaction between oAP and VB2

Reference[16] reported that PoAP thin film formed by electrochemical oxidation polymerization was electric inert when pH > 3 and in form of oxidized state in the absence of Cl- in pH 5. In this case, PoAP was insulated completely and showed no redox activity itself. Hence, PoAP, in weak acid environment, can not transfer electrons through redox process to be conducting.

Reference[21] reported that the first and the second proton binding constant of VB2 were 10^{10} and 10^6, respectively. Therefore, when VB2 in solution of pH < 6, it positively charged(RFH22+), balance state equation defined as:

$$RF + H^+ \rightleftharpoons RFH^+ \qquad (1)$$

$$RFH^+ + H^+ \rightleftharpoons RFH_2^{2+} \qquad (2)$$
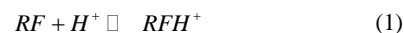
Hence, it is speculated that hydrogen bond must be formed between oAP and VB2.

In this text, UV-vis spectrometry was performed to analyze mechanism of interaction between oAP and VB2 due to its low cost, ease of operation, cheapness of instrument and so on. Volume percentage of oAP changed from 0 to 100%(0, 10%, 20%, 40%, 80% and 100%, respectively) and scanning curve from 300nm to 700nm was recorded as shown in **Figure 4**. From these scanning curve, adding of oAP did not alter the absorbance peak shape of VB2 and it was found the value of maximum absorption wavelength shifted to long-wave direction(redshift, from 374nm to 384nm) with absorbance value increasing(from 2.245 to 2.816) as increasing percentage of oAP from 0 to 80%. This was due to hydrogen bond has impact on the first π-π* absorption band of chromophore as a proton donor molecule. It was conformation of hydrogen bond formation between oAP and VB2.

Thus imprinted process of VB2 was sketched in **Figure 5**. When mixing oAP with VB2 and standing for 20min, there were numerous hydrogen bonds producing(process of prepolymerization). And then electrochemical oxidation polymerization reaction was performed to form PoAP and VB2 was imprinted in PoAP thin film simultaneously. Followed by elution process, i.e. under the diluted sulfuric acid(0.8M) condition the hydrogen bond was destroyed, which lead to separation of VB2 from the film, living cavity memorized for VB2.
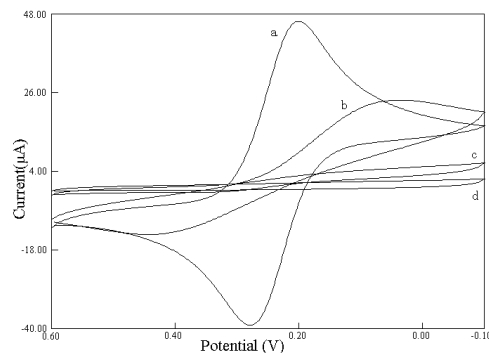
a: nGCE; b: VB$_2^-$-PoAP/GCE; c: VB$_2^+$-PoAP/GCE; d: PoAP/GCE

Figure 3. Cyclic voltammograms of different modified electrode in potassium ferricyanide.
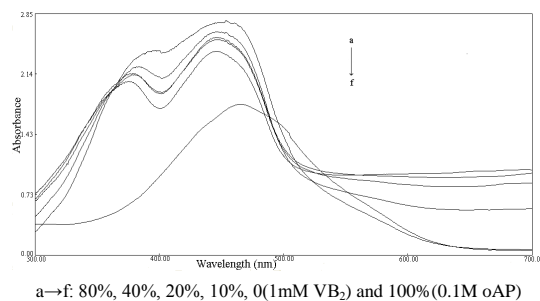


a→f: 80%, 40%, 20%, 10%, 0(1mM VB$_2$) and 100%(0.1M oAP)

Figure.4. UV-vis scanning curve of different percentage of oAP in VB$_2$.

3.5. Selection of Elution Time

Diluted strong acid solution is a powerful tool to destroy the hydrogen bond. 0.8M H2SO4 was used in this study and its elution kinetics was also studied. It was indicated that the reduced peak current incremental value(ΔI) increased with dilution time at the range from 0 to 80min. Up to 140min ΔI achieved a platform. After elution of 480min(8h) ΔI was almost unchanged which indicated elution came to equilibrium. Hence 8h of elution was selected in this study.

3.6. Evaluation of the Imprinting Effect

PoAP/GCE and VB_2^+-PoAP/GCE had no response to target molecule(VB_2) in 0.2M pH 5.0 HAc-NaHAc with 1mM VB_2 and 4mM $K_3[Fe(CN)_6]$(**Figure 5d**, **5e**). VB_2^--PoAP/GCE had good response to VB_2 and the cathode peak current to target molecule decreased from 14.183μA to 12.786μA (**Figure 6b**, **6c**). Compared to nGCE(**Figure 6a**) response current decreased significantly which demonstrated VB_2 molecule binded to imprinted cavity by hydrogen bond and closed the empty site, blocking $[Fe(CN)_6]^{3-}$ diffused from bulk solution to the surface of electrode. Thus concentration of $[Fe(CN)_6]^{3-}$ on the surface of electrode decreased significantly, leading to reduced current decreased significantly.
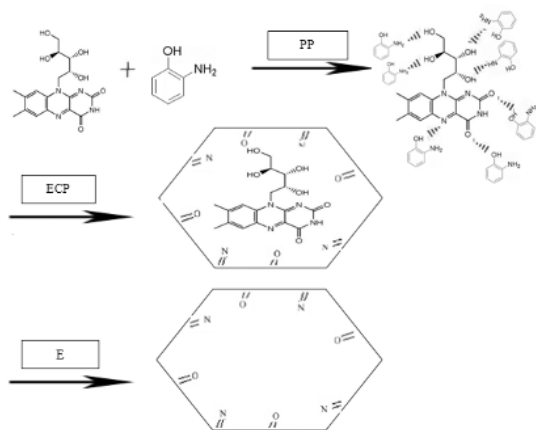
PP: Prepolymerization; ECP: Electrochemical Polymerization; E: Elution

Figure 5. Schematic illustration of imprinted process of VB₂ on GC electrode.
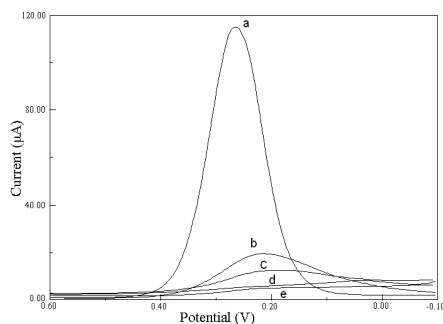
a: nGCE; b: before rebinding of VB_2; c: after rebinding of VB_2 d: PoAP/GCE； e: VB_2^+-PoAP/GCE

Figure 6. DPV curve of different modified electrode in potassium ferricyanide with VB₂.

3.7. Drawing of Calibration Curve of VB2 Rebinding

SWV with high sensitivity was performed to draw calibration curve of VB_2 rebinding as shown in **Figure 7**. The curve showed nonlinear overall from 20nM to 80μM, and the curve tended to steady at 80μM with increasing concentration of VB_2, which indicated there was saturation effect of the sensor responding to VB_2. To obtain good linear relationship between the concentration and ΔI, low VB_2 concentration was selected to do rebianding test again. The result was shown in **Figure 8**. The linear response range of this imprinted sensor to VB_2 concentration was from 10nM to 120nM with a correlation coefficient of 0.9989(n=7). The linear regression equation was $\Delta I=-0.03381[VB_2]+15.7694$. The detection limit was calculated according to equation[22] as follows:

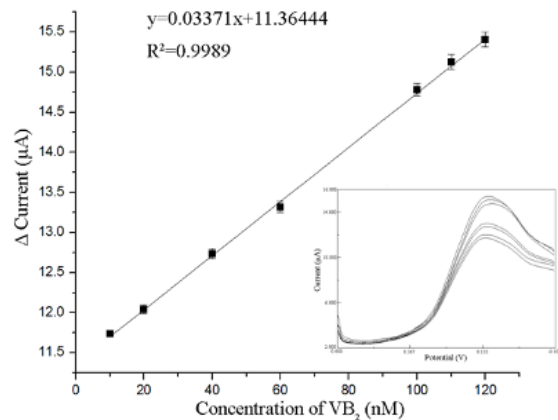$$LOD = \frac{3SD}{B} \tag{3}$$

where SD and B were standard deviation and slope of linear regression equation, respectively. LOD of VB_2 was 2.3851nM.
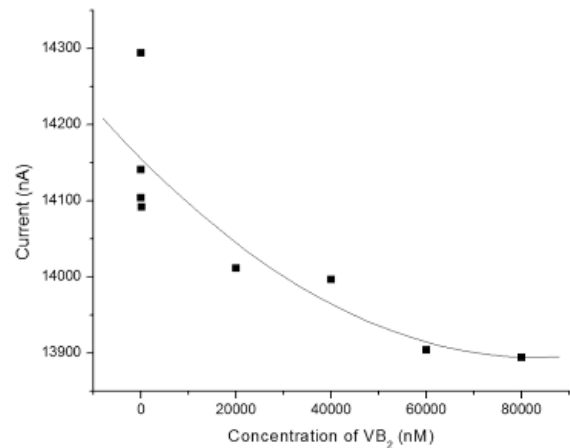
VB_2 concentration from 20nM to 80μM(20nM, 40nM, 60nM, 80nM, 20μM, 40μM, 60μM and 80μM, respectively)

Figure 7 Relationship between VB₂ concentration and current of cathode.

VB_2 concentration from 10nM to 120nM(10nM, 20nM, 40nM, 60nM, 100nM, 110nM, and 120nM, respectively). Inset: SWV testing curve of different concentration of VB_2

Figure 8. Calibration curve of VB₂ rebinding to imprinted sensor.

Table 1. The determination and recovery result in vitamin B$_2$ tablet.

Real amount/mg	Adding amount of standard/mg	Detected value/mg	Recover rate /%	Average recover rate/%	RSD/%
4.98	5.18	4.89	94.42		
4.97	10.32	9.30	90.12		
5.01	15.12	14.93	98.75		
4.95	20.34	21.04	103.45		
4.95	25.20	26.60	105.00	98.41	4.75
5.02	30.15	29.33	97.28		
4.96	35.40	35.30	99.72		
4.99	40.44	41.05	101.52		
5.04	45.70	43.61	95.43		

3.8. Selectivity of Imprinted Sensor

Water-soluble vitamin, such as VB$_6$ and VC, were selected to be disruptors to rebind to imprinted cavity site competitively due to their consist of a large number of hydroxy group. And FMN was also selected because of its structure similarity with VB$_2$. The absorption isotherm was made in absorption equilibrium time of 100min. It was found that VB$_6$, VC and FMN could rebind to imprinted cavity site by hydrogen bond , but compared to VB$_2$, the quantity was very low showed in terms of ΔI. Hence the sensor has good selectivity.

3.9. Real Sample Analysis

VB$_2$ tablets were grinded to powder in a mortar and then dissolved by 1%(V/V) acetic acid solution. A quarter of above solution was tested by SWV according to the methods as mentioned before. As shown in **Table 1**, the average recovery rate to VB$_2$ was 98.41%.

3.10. Reproducibility and Stability of the VB2 Imprinted Sensor

Reproductive testing of 8 times in 40μM successively(absorbed, tested and eluted , repeated 8 times), showed an acceptable reproducibility with a RSD of 4.1%. The sensor could retain 94.8% of acivity to VB$_2$ after a storage of 5 weeks in 0.01M pH 7.0 PBS at RT, which indicated good stability.

4. Conclusion

On the surface of naked GC electrode , oAP could electrochemical oxidation polymerization with VB$_2$ by potentiostatic method. Trough controlling polymerization parameters of potential, time, pH of solution and percentage of monomer, oAP as monomer, VB$_2$ as template molecule, electrons as initiator, VB$_2$ electrochemical molecular imprinting sensor was established. The sensor showed good recognition to VB$_2$, LOD went down to 2.3851nM   and exhibited good selectivity, reproducibility and stability. Hydrogen bond plays important role betreen the host(PoAP) and the guest(VB$_2$). This sensor was used to test VB$_2$ tablet with good recovery rate.

5. Acknowledgements

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