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Studies on the interaction of DNA with vitamin B_{12} based on the immobilization of dsDNA on nano-scale hydroxyapatite coating

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ABSTRACT

Nano-scale hydroxyapatite (HAp) was prepared by combining co-precipitation with microemulsion method, which exhibited strong adsorption for DNA due to its excellent biocompatibility and particular adsorbability. DNA and HAp could be modified onto glassy carbon electrode (GCE) by the simple and convenient "tip-coating" method. Cyclic voltammetry was used to investigate the interaction of DNA immobilized on the HAp film with vitamin B_{12} (VB_{12}). The existence of DNA led to the decrease of reduction current of VB_{12}. Both the electron transfer coefficient (α) and the standard rate constant (k_s) were different obtained on GCE and dsDNA/HAp/GCE, which indicated the formation of an electrochemical inactive super molecular complex DNA-$n$$VB_{12}$. The equilibrium constant of this complex was calculated to be 5.35×10^5 mol·L^{-1} and the binding number between DNA and VB_{12} of the complex were determined to be one. Copyright © 2010 VBRI press.

Keywords: Vitamin B_{12}; DNA; hydroxyapatite; interaction; cyclic voltammetry

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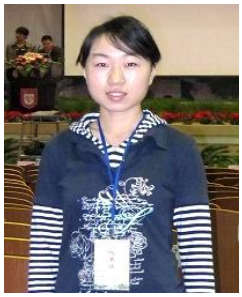

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Introduction

Nucleic acid offers the chemists a powerful tool in recognition and monitoring of many important compounds [**1, 2**]. DNA electrochemical sensors are of growing interest nowadays, and they have been applied in various fields, such as detection of medicine, additive in food, contamination in environment, DNA damage and so on [**3-6**], HAp [$Ca_{10}(PO_4)_6(OH)_2$], is widely used as implants or as coatings on prostheses, which is also a promising candidate material for reinforcing ceramic used in biomedical field. For the preparation of fine HAp powder, many chemical routes have been developed, including co-precipitation, hydrothermal reactions, sol-gel synthesis, pyrolysis of aerosols, and microemulsion etc [**7**]. Because of the excellent biocompatibility and particular adsorbability [**8**], HAp can be applied in the preparation of electrochemical biosensor.

The investigation of DNA interactions with small molecules is an important fundamental issue on life science, which is of great importance to understand the action mechanisms of some anti-tumor and anti-viral drugs [**9-11**]. Also it can provide guidance for exploring the origins of some diseases and designing new DNA-targeted drugs, thus it causes widespread interests [**12-16**]. The interaction of DNA with small molecules can cause the change of conformation as well as the charge distribution state of DNA. If these changes occurred on the surface of electrode, studies based on the cyclic voltammetry can reflect the functional phenomenon.

Vitamin is a class of necessary species for normal health and well-being of humans [**17, 18**]. It is now well-established that most vitamins act as coenzymes or co-factors in biocatalytic processes [**19**]. Vitamin B_{12} (VB_{12}) mainly exists in animal food, which can prevent pernicious anemia and plays an important role in the metabolism of protein, fat and carbohydrates [**20**]. Investigations on DNA interaction with VB_{12} can provide theoretical basis for further revealing the effect and mechanism of VB_{12}. Many methods have been reported to determine VB_{12} due to its featured electrochemical activity [**21-28**]. However, there are few investigations for detecting the interaction by DNA modified glassy carbon electrode (dsDNA/HAp/GCE), which is a surface electrochemical method.

Using solution electrochemical method to investigate the interaction of DNA with VB_{12} has imperfect reasoning. Formation of the electrochemical inactive super molecular complex is the uppermost reason for the decrease of the reduction peak currents of VB_{12}. The volume of the complex increases leading to the decrease of diffusion coefficient, which can also cause the decrease of the reduction peak currents. DNA in the solution has competitive adsorption with VB_{12}, which is another reasonable interpretation.

In the present paper, we prepared HAp by combination of co-precipitation and microemulsion. dsDNA/HAp/GCE with good stability was prepared by "tip-coating" method, which shows the evident advantages of simple preparation and good stability compared with other methods such as sol-gel method [**29**] and layer-by-layer immobilization method using sulfhydryl compounds [**30**] etc. The electrochemical behavior of the interaction between VB_{12} and DNA at pH value 4.87 was investigated by dsDNA/HAp/GCE, which was more convincing than solution electrochemical method and provided some new insights into the electrochemical behaviors for DNA interacting with vitamin.

Experimental

Reagents

VB_{12} (biochemical reagent) was purchased from China Medicine Company. VB_{12} (5.0×10^{-5} mol·L^{-1}) was dissolved in doubly distilled water as stock solution. Calf thymus DNA was purchased from Sigma Chemical Co. and used as received since the purity was sufficiently high as determined from optical measurements ($OD_{260}/OD_{280} > 1.8$, where OD represents the optical density). $Ca(NO_3)_2$, $(NH_4)_2HPO_4$ and other reagents were of analytical reagent grade.

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Instrumentation

Electrochemical experiments were carried out with a CHI-760D electrochemical workstation (Shanghai CH Instrument Company, China). A three-electrode system was employed with Pt wire as auxiliary electrode, saturated calomel electrode (SCE) as reference electrode and GCE or modified GCE as the working electrode. Hitachi model S-20500 scanning electron microscope and LinkISIS-300X energy spectrometer were used to characterize HAp.

Preparation of HAp and dsDNA/HAp/GCE

HAp was prepared in quaternary W/O microemulsion solution containing Triton X-100, cyclohexane, *n*-pentanol and water (the mass ratio was 1.0 : 0.5 : 1.0 : 45.0). 100.00 mL of 0.50 mol·L^{-1} $Ca(NO_3)_2$ and 60.00 mL of 0.50 mol·L^{-1} $(NH_4)_2HPO_4$ microemulsion were used respectively, and adjusted to pH 10.0 with ammonia. Then $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ were mixed fully, stirred for 30min, stood for more than 1 h, and underwent a process of air pump filtration and washing. Then the products were dried at 200 °C.

GCE was polished to a mirror-like surface with 1.0, 0.3 and 0.05 μm alumina slurry on microcloth pads, and sonicated in ethanol and water. The freshly pretreated GCE was modified by attaching HAp (~10 μL, ethanol was used as solvent) onto its surface, followed by drying at room temperature for 1 h. Then, ~10 μL of DNA stock solution (100 μg·mL^{-1}) was dropped onto the surface of the HAp film, also followed by drying at room temperature for 6 h. Finally, it was soaked in double distilled water for more than 3 h to remove dsDNA unabsorbed.

Electrochemical measurements

The electrochemical characteristics of the modified electrode were characterized by using cyclic voltammetry and electrochemical impedance spectroscopy (EIS). Both of the measurements are performed in 6.67 mmol·L^{-1} $K_3[Fe(CN)_6]$ solution containing KNO_3 supporting electrolyte. Cyclic voltammetry was also used to detect the electrochemical behaviors of the working electrode in HAc-NaAc buffer solution (0.10 mol·L^{-1}) with pH value 4.87 containing VB_{12}. Experiments were carried out at the room temperature (25°C).

Results and discussion

The characterization of HAp

Fig. 1a is the TEM image of HAp. It can be seen that HAp has club shaped structure, and nanometer structure. After the analysis of distributing size of the sample (**Fig. 1b**), it can be concluded that the sizes of HAp granule separated by ultrasonic are less than 1μm. Compositional analysis for HAp based on EDS is illustrated in **Fig. 1c**. It can be clearly observed that Ca, P and O elements were all involved in HAp. Compared with other methods to prepare HAp, this method is simple, effective and low-cost. Ethanol is a weak polar solvent and can reduce the aggregation degree of HAp. Therefore, HAp was dispersed in ethanol and the formed modified film can provide good reaction interface for electron transfer. It can also be used as coatings on modified electrode. Because of the high affinity of HAp for biomolecules, HAp can be used in DNA electrochemical biosensor. Ca^{2+} probably plays an important role as a bridge between HAp and DNA, both of which are negatively charged.

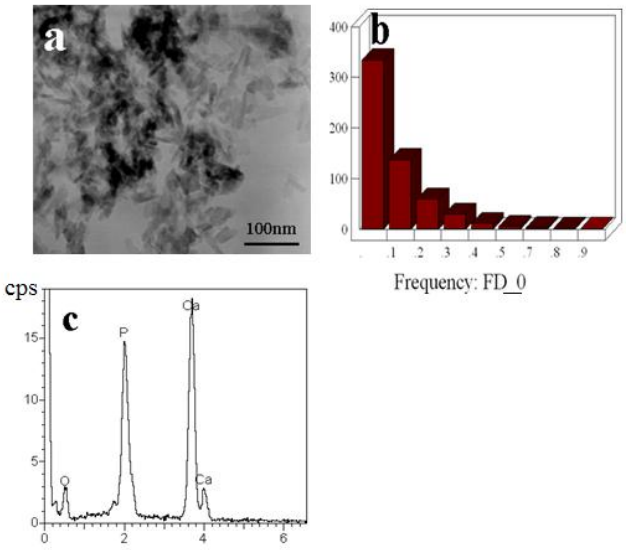

Fig. 1. (a) TEM image of HAp, (b) The distributing size of granule separated by ultrasonic, (c). Compositional information of HAp obtained through EDS analysis.

Electrochemical characterization of GCE modified by HAp and dsDNA

Fig. 2a presents the cyclic voltammogram (CV) of GCE, HAp/GCE, and dsDNA/HAp/GCE in $K_3[Fe(CN)_6]$ solution containing KNO_3 supporting electrolyte. The potential difference (ΔE) of 78 mV could be obtained on GCE. It can be also seen that the peak currents decreased, and ΔE dramatically increased to 197 mV on HAp/GCE. With DNA adsorbed on the HAp film, HAp and DNA were both negatively charged, which showed a repellency towards $[Fe(CN)_6]^{3-/4-}$, leading to a further decrease of peak currents with ΔE increased markedly to 318 mV on dsDNA/HAp/GCE. These electrochemical behaviors indicated that GCE surface had been covered with HAp and DNA. After numbers of sweeps and placed for at least 20 days under 4 °C, CV of dsDNA/HAp/GCE in $K_3[Fe(CN)_6]$ solution almost unchanged, indicating its good structural stability.

During the process of modification for GCE, EIS can reflect the change of surface impedance, as shown in **Fig. 2b**. For GCE, EIS showed a straight line both in high and low frequency region. With the electrode modified, EIS manifested a semicircular form in high frequency region and linearity in low frequency region. With DNA adsorbed on the HAp film, diameter of the semicircle increased, which indicated that the electron transfer was further hindered. Furthermore, the slope change of curves a, b and c was not appreciable in low frequency region. Therefore, HAp and DNA had little effect on charge transfer in the modified layers.

CV and EIS demonstrated that dsDNA/HAp/GCE was successfully prepared. Stable DNA modified electrode

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couldn't be obtained by directly dropping DNA solution onto the surface of GCE because that DNA was easy to leak without adsorption force. Compared with sol-gel method and layer-by-layer immobilization method using sulfhydryl compounds, this method is simple and convenient. Optical measurements also indicated that HAp had no evident effect on the biological properties of DNA. So using HAp film as medium can make ideal DNA modified electrode.

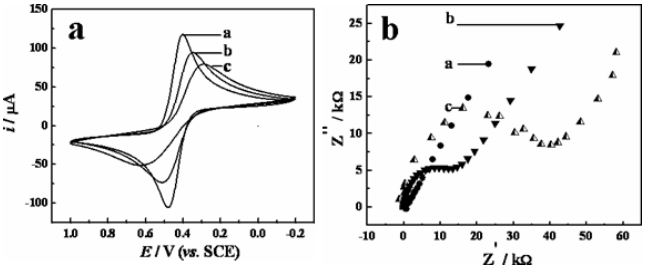

Fig. 2. CV (a) and EIS (b) of GCE (curve a), HAp/GCE (curve b) and dsDNA/HAp/GCE (curve c) in 6.67 mmol·L^{-1} $K_3[Fe(CN)_6]$ solution containing KNO_3 supporting electrolyte; scan rate, 100 mV·s^{-1}.

Electrochemical behavior of VB_{12} on dsDNA/HAp/GCE

Fig. 3 shows the CV curves of GCE and dsDNA/HAp/GCE in 5.0 × 10^{-5} mol·L^{-1} VB_{12} and 0.10 mol·L^{-1} HAc-NaAc buffer solution with pH value 4.87. As can be clearly found, there were two obvious reduction peaks appeared at -0.20 V (E_{p1}) and -0.61 V (E_{p2}) on GCE, while the reduction peak currents decreased significantly on dsDNA/HAp/GCE. The evident difference in current intensity demonstrated the existence of the interaction between DNA and VB_{12}. It is considered that the only reason is the formation of the electrochemical inactive super molecular complex as demonstrated by using surface electrochemical method.

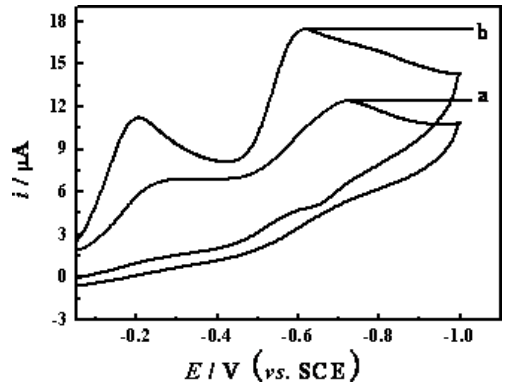

Fig. 3. CV curves of dsDNA/HAp/GCE (curve a) and GCE (curve b) in 5 × 10^{-5}mol·L^{-1} VB_{12} solution; scan rate, 100 mV·s^{-1}

Spectroscopic evidence of the interaction between DNA and VB_{12}

Fig. 4 displays the absorption spectra of DNA (**a**), VB_{12} (**b**), and their mixture (**c**). It can be seen that VB_{12} had three absorption peaks located in 285.4、357.1 and 546.8 nm, while DNA had one absorption peak in 260.8 nm. After adding DNA in the VB_{12} solution, the absorption peak of VB_{12} located in 285.4 nm was blue shifted 28.9 nm with a significant hyperchromic effect, which verified the interaction of DNA and VB_{12}.

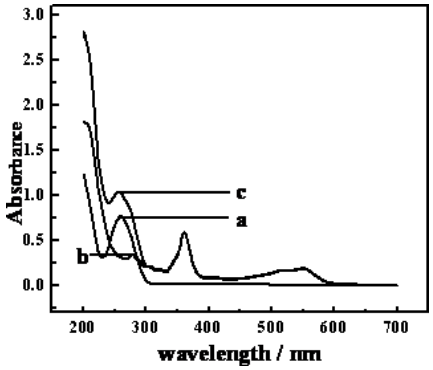

Fig. 4. The absorption spectrum of DNA (a), VB_{12} (b), and their mixture (c).

The effect of pH on the electrochemical behavior of VB_{12} on dsDNA/HAp/GCE

CV of dsDNA/HAp/GCE in VB_{12} solution changed with different pH value. The two reduction peak potentials shifted negatively with the increase of pH value in the range from 3.5 to 5.6, which indicated that both electron and proton participated in the reduction process of VB_{12}. The current difference (Δi_p) of VB_{12} between dsDNA/HAp/GCE and GCE had relationship with the pH value, as shown in **Fig. 5**. Therefore, HAc-NaAc buffer solution (0.10 mol·L^{-1}) with pH value 4.87 was the best choice. Considering the peak shape of VB_{12}, the second reduction peak located at -0.61 V was investigated in the following experiments.

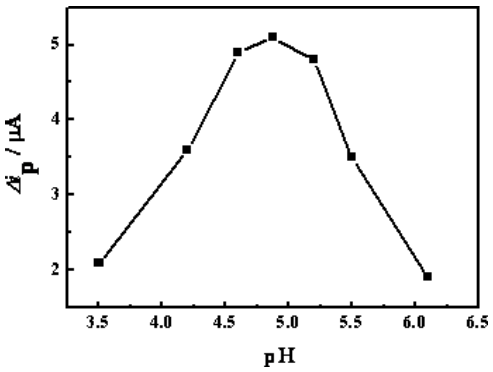

Fig. 5. The relationship between Δ*i* and pH

Electrochemical studies of the interaction between DNA and VB_{12}

The effect of scan rate on the electrochemical behavior of VB_{12} on dsDNA/HAp/GCE was investigated. We illustrated the relationship between the reduction peak current (i_{pc}) and the scan rate (v), as shown in **Fig. 6**. It can be seen that the reduction peak currents of VB_{12} increased

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with scan rate in the range from 50 to 350 mV·s^{-1}. The good linearity of these plots indicated that the electrode reaction was surface controlled reaction process. The linear regression equation was established as followed.

$$i_{pc} = 9.635 + 0.0256v \ (r = 0.9989) \qquad (1)$$

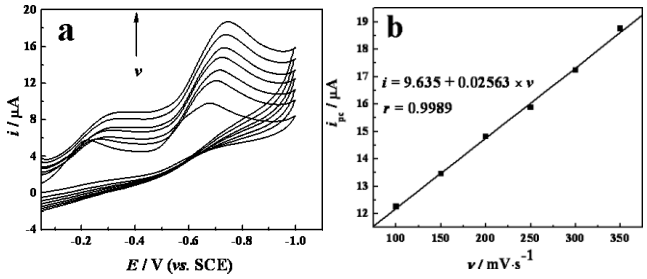

Fig. 6. (a) CV curves of dsDNA/HAp/GCE in 5.0 × 10^{-5}mol·L^{-1} VB_{12} solution with different scan rates of 50, 100, 150, 200, 250, 300 and 350 mV·s^{-1} respectively, (b). The dependence of the peak currents on scan rates.

According to *Laviron* theory [**31**], the peak potential (E_p) of irreversible adsorption wave has relationship with scan rate (v) as followed:

$$E_p = E^0 + \frac{RT}{\alpha nF} \ln \frac{RTk_s}{\alpha nF} - \frac{RT}{\alpha nF} \ln v \qquad (2)$$

E^0 was calculated to be -0.70 V for GCE using the extrapolation method. **Fig. 7** was obtained from E_p as a function of ln$v$, which showed good linearity. According to the slope and intercept, αn and the standard rate constant (k_s) could be calculated. If the value of the electron transfer number (n) was one, the electron transfer coefficient (α) value was calculated to be 0.71 and k_s was 2250 s^{-1}. In the same way, α value and k_s were calculated to be 0.53 and 603 s^{-1} for dsDNA/HAp/GCE respectively. Therefore, DNA immobilized on the HAp film had significant influence on the electrochemical parameters. The intercalation mode between VB_{12} and DNA leaded to the formation of inactive super molecular complex, which caused the decrease of the reduction peak current.

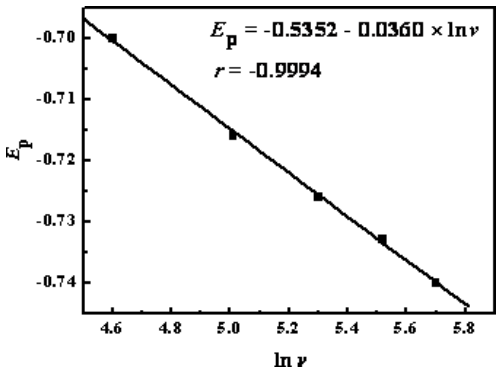

Fig. 7. The relationship between E_p and lnv.

The adsorption constant and the binding number between DNA and VB_{12} on dsDNA/HAp/GCE

The adsorption constant (β) and the binding number (n) between DNA and VB_{12} can be calculated according to literature [**32**]. Under the assumption that VB_{12} and DNA formed the compounds DNA-$n$$VB_{12}$, the following equilibrium equation existed.

$$DNA + nVB_{12} \rightleftharpoons DNA(VB_{12})_n \qquad (3)$$

The adsorption constant can be expressed as followed:

$$\beta = \frac{[DNA(VB_{12})_n]}{[VB_{12}]^n[DNA]} \qquad (4)$$

After derivation, the equation was obtained:

$$\frac{1}{\Delta I} = \frac{1}{\Delta I_{max}} + \frac{1}{\beta \cdot \Delta I_{max}} \times \frac{1}{[VB_{12}]^n} \qquad (5)$$

where ΔI and ΔI_{max} were the peak current difference and the maximum peak current difference of VB_{12} on GCE and dsDNA/HAp/GCE respectively.

When $\frac{1}{\Delta I}$ was plotted by $\frac{1}{[VB_{12}]^n}$ (n = 1, 2, 3), one linear should be obtained if n was the real binding number. According to the slope and intercept, β can be calculated.

The relationship between I_p and c (VB_{12}) on GCE and dsDNA/HAp/GCE and the relationship between ΔI_p and c (VB_{12}) are shown in **Fig. 8a**. **Fig. 8b** shows the relationship between ΔI_p and $[VB_{12}]^{-n}$ (a: n = 1; b. n = 2; c. n = 3), which was obtained from ΔI_p^{-1} as a function of c (VB_{12}). When n = 1, one linear was obtained, indicating that the compounds DNA-VB_{12} was formed. β value was calculated to 5.35 × 10^5 mol·L^{-1} accordingly.

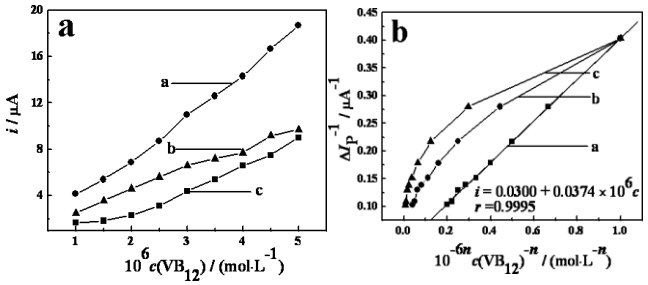

Fig. 8. (a) The relationship between I_p and c(VB_{12}) on GCE(a) and dsDNA/HAp/GCE(b) and the relationship between ΔI_P and c(VB_{12}); (b). The relationship between ΔI_p and $[VB_{12}]^{-n}$ a: n = 1; b: n = 2; c. n = 3

Conclusion

In this paper we prepared dsDNA/HAp/GCE with good performance and studied the electrochemical behavior of VB_{12} interactions with calf thymus DNA by cyclic voltammetry systematically. The method is characterized by simplicity, high-repeatability and stability. From the different electrochemical parameters obtained on GCE and dsDNA/HAp/GCE, it was inferred that an electrochemical inactive super molecular complex DNA-$n$$VB_{12}$ was formed. The equilibrium constant of this complex was calculated to

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be 5.35×10^5 mol·L^{-1}, and the binding number was determined to be 1 between DNA and VB_{12}.

Acknowledgements

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