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RESEARCH ARTICLE

Development of an electrochemical DNA biosensor for the detection of vitamin B_{12} (cyanocobalamin) at a carbon paste modified electrode with a manganese(II) complex

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A simple, fast, sensitive and selective electrochemical detection of vitamin B_{12} (cyanocobalamin) has been developed using a DNA electrochemical biosensor and a modified carbon paste electrode. Carbon paste electrode was modified with electrochemically produced polymer of [Mn(thiophenyl-2-carboxylic acid)$_2$(triethylonamine)] using cyclic voltammetry with a scan rate of 0.01 V/s and three number of scans. Vitamin B_{12} was immobilized onto the modified electrode. Measurements were carried out using adsorptive transfer square wave voltammetry. Detection was achieved from 3.667 μg/L to 236.0 μg/L, presenting sufficiently low detection (i.e. 1.210 μg/L) and quantification (i.e. 3.667 μg/L) limits. The precision was tested showing excellent results (i.e. from 5.50 % and 5.35 %). The selectivity towards certain interferences was also investigated and revealed that none of them had significant effect on the detection of vitamin B_{12}. The electrode has been applied in the determination of Vitamin B_{12} in human urine sample.

Keywords: cyanocobalamin, carbon paste electrode, cyclic voltammetry, adsorptive transfer voltammetry, square wave voltammetry, electrochemical DNA biosensor.

Introduction

Vitamins of group B ensure the normal performance of human body through participation in the biosynthesis of proteins and functioning of the central nervous, cardiovascular and gastrointestinal systems [1,2]. In specific, vitamin B_{12} or cobalamin is an organometallic cofactor with a complex structure [1, 2]. Vitamin B_{12} exhibits a rich redox chemistry centered on the cobalt atom, where the Co^{III} in the vitamin B_{12} can be reduced reversibly to Co^{II} and further reduced to Co^{I} [3,4]. Vitamin B_{12} adsorbs on electrode surfaces and can be used for oxidation or reduction reactions [5–7]. Vitamin B_{12} maintains healthy the nerve cells and the red blood cells and is also needed in the DNA synthesis [8,9]. The human body stores several years' worth of vitamin B_{12}, so nutritional deficiency of this vitamin is extremely rare [10]. Most vitamin B_{12} deficiency symptoms are actually folate deficiency symptoms, since they include all the effects of pernicious anemia and megaloblastosis, which are due to poor synthesis of DNA when the body does not have a proper supply of folic acid for the production of thymine [10]. Electrochemical polymerization leads to the simple and reproducible formation of a polymer deposit with accurate spatial analysis on the electrode's surface [11,12]. These polymers, formed by electrochemical procedures, possess low ionization potential and great electrical affinity regardless theirs size and morphology [12]. Electrochemical polymerization produces deposits that are stable both in organic and inorganic solvents. Electrochemically produced polymers can also be used as signal transducers,

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since they are sensitive in recognition events of biological procedures [13]. Apparently, the immobilization of a biomolecule, such as DNA, on a modified electrode surface with an electrochemically produced polymer can be done by performing various techniques such as adsorption of the biomolecule on the modified surface [14], physical entrapment of the biomolecule to the on the modified surface [15,16], covalent linking between the biomolecule and the polymer [17,18], trough affinity interactions between the biomolecule and the polymer [19] and direct co-polymerization of the biomolecule and the monomer. Taking into account the great amount of vitamin, multivitamin, and multidrug formulations based on vitamin B_{12} and the other members of group B produced, the production of vitamins, quality control, validity periods, and use in medical practice are not possible without the careful control over their manufacture, storage, and use. Moreover, considering the biological importance of B_{12}, a real boom on the search for novel methods of analysis of group B vitamins was observed. What is more, the detection of water soluble vitamins, such as B_{12}, in different samples is relatively difficult; due to the instability and the complexity of the matrix that they are usually exist. Thus, a variety of analytical methodologies, determining vitamin B_{12}, has been reported [20-23]. These methodologies include microbiological assays, radioisotopical assays, immunoassays, mass spectrometry, electrochemiluminescence, spectrophotometry, atomic adsorption spectrometry, high liquid chromatography and fluorescence spectrophotometry [20-23]. On the other hand, voltammetric methods are highly sensitive and rather accurate, do not require expensive instrumentation, and are rather frequently used to determine group B vitamins and therefore B_{12}. Electrochemical techniques like cyclic voltammetry (CV), differential pulse voltammetry (DPV), square wave voltammetry (SWV) and amperometry have been employed to the determination of B_{12} [24-34]. Furthermore, chemical modifiers of electrode surfaces, particularly manganese complexes, give to the newly resulted electrodes excellent properties and can be utilized to the detection of B_{12} [35-39]. In concluding, most of them are time consuming, require complex sample pretreatment as well as theirs resulted accuracy is inadequate. Apart from that, the majority of them usually demand the destruction of cyanocobalamin and afterwards the redox system Co^{II}/Co^{III} is studied.

With reference to the above mentioned facts, the present study a DNA electrochemical biosensor is developed. This biosensor is based on the modification of carbon paste electrode (CPE) with an electrochemically produced deposit of a manganese(II) complex, i.e. [Mn(thiophenyl-2-carboxylic acid)$_2$(triethylonamine)] (A), which was found to intercalate with DNA [40,41]. The modified surface of carbon paste electrode with the polymer of (A) was used on the immobilization of calf thymus dsDNA. Firstly, (A) was electropolymerized directly on CPE's surface using CV (Mn-CPE).

Secondly, dsDNA was physiosorbed on the modified Mn-CPE by injecting the appropriate volume of dsDNA on Mn-CPE and letting the electrode to dry on air. Then the freshly constructed electrode (Mn-dsDNA-CPE) was used on the detection of B_{12} utilizing adsorptive transfer square wave voltammetry (SWAdSV) by monitoring the reduction peak of manganese complex.

To the best of our best knowledge, this is the first use of this complex as an electro-chemical modifier of electrode surfaces, in this case CPE, forming a stable polymer on its surface, and the first application of the resulted electrode to the determination of B_{12}. Evidently, manganese complex was found to both intercalate with DNA through π-π^* stacking interactions of thiophenyl groups, making the immobilization of dsDNA on the modified CPE with manganese complex easier [40,41]. By applying this novel electrode surface to the determination of B_{12}, the analytical features were improved, showing relatively broad linear range as well as low detection and quantification limits. More than that, this new approach is cheap, simple, fast, selective and sensitive. Under these conditions, the electrochemical determination vitamin B_{12} was also performed of in a pharmaceutical product. Moreover, the proposed method is the one of the limited approaches that a non destructive detection array was used, since the majority of the reported ones usually demand the destruction of cyanocobalamin and afterwards the redox system Co^{II}/Co^{III} is studied. The combination of these features renders this approach suitable as a general platform for the detection of other biomolecules or hybridization events in DNA biosensors without the need for labeled sequences.

Experimental section

Materials and methods

All reagents were of analytical grade unless stated otherwise and used as received. Dimethyl sulphoxide (DMSO) and tetra hydrate manganese(II) chloride ($MnCl_2 \cdot 4H_2O$) were purchased from Merck (USA). Thiophene-2-carboxylic acid was purchased from Aldrich (WI, USA). Triethanolamine and mineral oil were obtained from Sigma (MO, USA). Ethylene diamine tetraacetic (EDTA, ACS reagent, 99.4–100.06% mass to mass portion, m/m) and tris (hydroxymethyl) aminomethane (99.8 %

m/m, ACS reagent) were obtained from Sigma–Aldrich (USA). Graphite powder was purchased from Fluka (USA) (50870, p.a. purity 99.9% volume portion, v/v) and particle size 0.1 mm). Double-stranded calf thymus DNA (dsDNA) (Catalog No. D-1501, highly polymerized) was obtained from Sigma Chemical, Co. (St. Louis, MO, USA). [Mn(thiophen-2-carboxylic acid)$_2$(triethanolamine)] (A) was prepared as previously reported [40].

Stock solutions of 3 g/L of (A) were prepared after weighing a certain amount of the compound and dilution in dimethyl sulphoxide. All aqueous solutions were prepared with sterilized double-distilled water. Stock solutions of 1 g/L of dsDNA were pre-pared also after weighing a certain amount of the compound and dilution 0.001 mol/L Tris-HCl. In order to prepare the more diluted solutions of dsDNA, a 0.2 mol/L acetate buffer solution (pH 5.0) consisting of 20.0 mmol/L NaCl was applied. Stock solutions of 3 g/L of vitamin B_{12} were prepared after weighing a certain amount of the compound and dilution in sterilized double-distilled water. The more diluted solutions of vitamin B_{12} were prepared with double-distilled water. All the experiments were performed at ambient temperature in an electrochemical cell. The electrochemical cells were cleaned with diluted nitric acid and rinsed with sterilized double-distilled water. Ultrapure nitrogen was used to de-aerate the solutions by purging the dissolved oxygen for 15 min prior to each experiment.

For the determination of vitamin B_{12} in the real sample, the standard addition method was used. A human urine sample spiked with a known concentration of vitamin B_{12} served as real sample. Thus, the sample was diluted with electrolyte solution (acetate solution, pH 5.2, containing 0.01 mol/L NaCl) at a ratio 1:1 v/v. Voltammetric experiments were carried out using a μAutolab potentiostat/galvanostat and (Eco Chimie, the Netherlands) controlled by GPES 4.9.0005 Beta software. All electrochemical measurements were carried out at ambient temperature, using a conventional three-electrode cell containing a platinum wire as a counter and Ag/AgCl/3 mol/L KCl electrode as reference electrodes, respectively. A carbon paste electrode of 3 mm inner and 9 mm outer diameter of the PTFE sleeve was used as a working electrode. The pH of all solutions was measured using a Consort C830 pH meter (Consort bvba, Belgium). The carbon paste electrode was prepared by thoroughly mixing by hand adequate amounts of graphite powder and paraffin oil of 75/25 mass ratio. A portion of the resulting mixture was packed into the bottom of the PTFE sleeve. The surface was polished to a smooth finish manually on a piece of weighing paper before use. Electrical contact was established via stainless steel screws. Firstly, manganese complex (107 mg/L) was electrochemically polymerized on CPE's surface from a 0.1 mol/L phosphate buffer solution (pH 7.6) consisting of 30.0 mmol/L KCl. The polymer of (A) was formed on CPE by applying CV (potensiodynamically). Thus, the working electrode potential was scanned between the values +0.0 V to +1.2 V. The number of potential scan was equal to three, the potential scan rate was equal to 0.01 V/s and step potential was equal to 0.006 V. Secondly, the modified CPE with the electrochemically produced polymer of (A) (Mn-CPE) was washed with sterilized double-distilled water to remove the unbound manganese complex and dried to the air. The dsDNA immobilization on the surface of the Mn-CPE was achieved by physiorption of different volumes of dsDNA (Mn-dsDNA-CPE). Then, Mn-dsDNA-CPE was transferred into an acetate solution (pH 5.2), containing the appropriate amount of vitamin B_{12} and 0.01 mol/L NaCl. This solution was stirred for 180 s and the Mn-dsDNA-CPE was subsequently washed with acetate buffer (pH 5.0) for 5 s in order to remove the unbound B_{12} and dried to the air. Finally, the detection was achieved by monitoring the reduction peak of (A) and measurements was performed by adsorptive transfer stripping voltammetry using the square wave voltammetric mode in 0.2 mol/L of acetate buffer (pH 5.2) containing 0.01 mol/L NaCl and cathodically scanning the electrode potential between +1.2 V and 0.0 V with a frequency of 25 Hz, an interval time of 0.6 s, a step potential of 0.0003 V and a modulation amplitude of 0.1 V. The raw data were treated using the Savitzky and Golay filter (level 2) of the GPES software, followed by the GPES software moving average base-line correction using a peak width of 0.03. Repeated measurements were carried out following renewal of the CPE surface by cutting and polishing the electrode.

Results and discussion

Firstly, the electrochemical behavior of Mn-CPE was studied using CV in 0.2 mol/L acetate buffer (pH 5.2) containing 0.01 mol/L NaCl. For this reason the effect of scan rate on the oxidation and reduction peaks of Mn-CPE was investigated, **Figure 1**.

As it can be seen from **Figure 1a** the oxidation (curve a on **Figure 1a**) and the reduction (curve b on **Figure 1a**) peak current of Mn-CPE, respectively, was linearly increased with the square root of the scan rate, suggesting that the oxidation and reduction were diffusion controlled reactions. Meanwhile, the oxidation (curve a on **Figure 1b**) and the reduction (curve b on **Figure 1b**) peak current of Mn-CPE was exponentially increased

with the scan rate. These facts are indicative that diffusion is the only force that governs the redox procedure of Mn-CPE [42]. The peak potential of oxidation peak of Mn-CPE (curve a on **Figure 1c**) was directly proportional to the logarithm of scan rate, while the reduction peak of Mn-CPE (curve a on **Figure 1c)** was almost unaffected. From the slope of this diagram the electron transfer coefficient (αn) was calculated equal to 0.54. The Laviron's equation (**equation 1**) was used to estimate the standard rate constant (ks) value and was found to be equal to 5.32/s [43]. Generally, large values of ks indicate high ability of (A) for promoting electron transfer at the electrode surface.

$$Ep = Eo' + (RT/\alpha nF)\,[\ln(RTks/\alpha nF) - \ln\upsilon] \qquad \textbf{(1)}$$

Secondly, dsDNA was physiosorbed on Mn-CPE. Preliminary investigations, using CV in 0.2 mol/L acetate buffer (pH 5.2) containing 0.01 mol/L NaCl, have shown that that dsDNA was successfully immobilized on the Mn-CPE, **Figure 2a**. As it can be seen from **Figure 2a** and curve 1, manganese complex was oxidized at +0.827 V vs. Ag/AgCl and reduced at +0.618 V vs. Ag/AgCl, due to the oxidation and reduction of Mn^{2+} [41]. On the other hand, a low intensity oxidation peak was evident at +1.024 V vs. Ag/AgCl in the presence of dsDNA (**Figure 2a** and curve 2), which could be assigned to the oxidation of guanine residues of dsDNA. Furthermore, the current intensity of reduction peak of Mn^{2+} was decreased, while the peak potential of it shifted towards more negative values, in the presence of dsDNA (compare curves 1 and 2 in **Figure 2a**). What is more, the oxidation peak of Mn^{2+} was vanished, in the presence of dsDNA (compare curves 1 and 2 in **Figure 2a**). Additionally, after the dsDNA was physiosorbed on Mn-CPE, it was found that the current during the anodic scan was dramatically increased (compare curves 1 and 2 in **Figure 2a**). The results indicate that dsDNA was successfully immobilized on Mn-CPE.

Thirdly, in order to verify that dsDNA was indeed physiosorbed onto Mn-CPE, square wave voltammetric experiments were performed in 0.2 mol/L acetate buffer (pH 5.2) containing 0.01 mol/L NaCl, **Figure 2b**. As it can be seen from **Figure 2b** and curve 4, manganese complex was oxidized giving two oxidation peaks at +0.754 and +1.275 V vs. Ag/AgCl. On the other hand, when dsDNA was immobilized on CPE two low intensity oxidation peaks were evident at +0,914 V and +1.186 V vs. Ag/AgCl, which could be assigned to the oxidation of guanine and adenine residues of dsDNA, respectively (**Figure 2b** and curve 2). Furthermore, two oxidation peaks

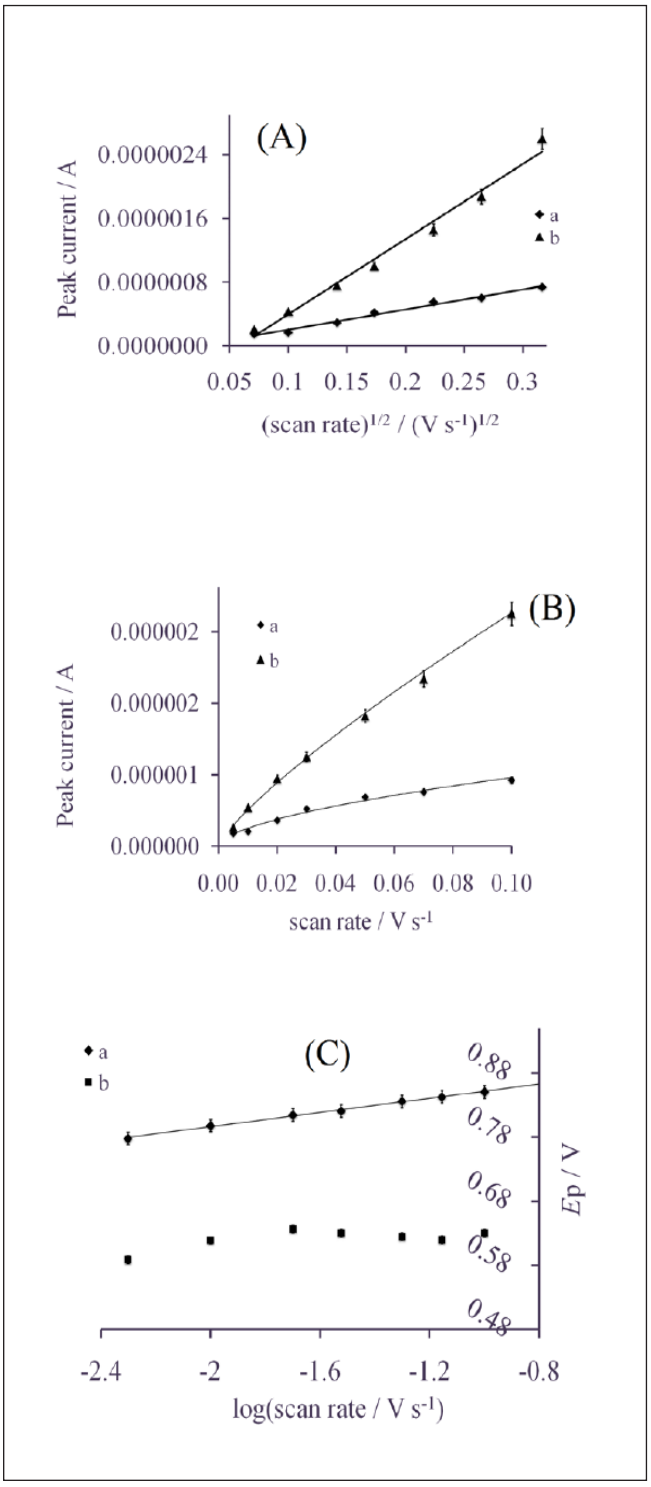

Figure 1. Cyclic voltammetric study of the electrochemical behavior of Mn-CPE. (A) Effect of the square root of scan rate on the (a) oxidation peak and (b) reduction peak current of Mn-CPE, (B) effect of the scan rate on the (a) oxidation peak and (b) reduction peak current of Mn-CPE and (C) effect of the logarithm of scan rate on the (a) oxidation peak and (b) reduction peak potential of Mn-CPE (experimental conditions and voltammetric are mentioned in the material and methods section).

were evident at +1.035 V and +1.273 V vs. Ag /AgCl, which could be assigned to the oxidation of quinine residues of dsDNA and Mn^{2+}, respectively (**Figure 2b** and curve 3), when Mn-dsDNA-CPE was studied. The results show that, the oxidation signal of guanine residues is significantly increased when the Mn-CPE (compare curves 2 and 3 in **Figure 2b**). Additionally, the peak potential of quinine residues on Mn-CPE is positively shifted compared to CPE (compare curves 2 and 3 in **Figure 2b**). Moreover, the oxidation peaks of Mn^{2+} were disappeared when dsDNA was physiosorbed on Mn-CPE (compare curves 3 and 4 in **Figure 2b**). The results indicate that dsDNA was successfully immobilized on Mn-CPE.

The electrochemical behavior of vitamin B_{12} on Mn-dsDNA-CPE was then investigated with CV and SWV in 0.2 mol/L acetate buffer (pH 5.2) containing 0.01 mol/L NaCl, **Figure 3a and b**, respectively. The CV results made clear that Mn-CPE gave an oxidation peak at +0.836 V vs. Ag/AgCl and a reduction peak at +0.569 V vs. Ag/AgCl Mn-CPE (**Figure 3a**, curve b), ascribed to the oxidation and reduction of the Mn^{2+} [42]. However, only one oxidation peak at +0.310 V vs. Ag/AgCl was found; while there aren't any reduction peaks on CVs, when B_{12} was studied CPE (**Figure 3a**, curve c). In addition, B_{12} was oxidized giving an oxidation peak at +0.320 V vs. Ag/AgCl and a reduction peak at +0.560 V vs. Ag/AgCl, when Mn-CPE was used (**Figure 3a**, curve d). This oxidation peak could probably be assigned on the monoelectronic oxidation reaction of B_{12s} σε B_{12r}, representing the redox reaction of the couple Co^{3+}/Co^{2+} [3]. Finally, an oxidation peak at + 1.021 V vs. Ag/AgCl and a low intensity reduction peak at + 0.610 V vs. Ag/AgCl were found, when Mn-dsDNA-CPE was utilized (**Figure 3a**, curve e). In other words, on Mn-dsDNA B_{12} is either oxidized at potential near the oxidation potential of guanine residues or the resulted oxidation peak is very large that masks B_{12} oxidation peak. It must be noted that, the reduction peak is ascribed to the reduction of Mn-CPE. Conclusively, the results are representative that B_{12} was successfully immobilized on Mn-dsDNA-CPE. To further investigate the electrochemical behavior of B_{12} on Mn-dsDNA-CPE, SWV experiments were carried out (**Figure 3b**). Thus, an oxidation peak at +0.770V vs. Ag/AgCl was evident on SWVs (**Figure 3b**, curve b), because of the oxidation of Mn^{2+} ions [42], in the absence of B_{12} when CPE was used. In the presence of B_{12} an oxidation peak at +1040 V vs. Ag/AgCl was obvious, when also CPE was used (**Figure 3b**, curve c), which probably could be ascribed to the oxidation of B_{12}. Furthermore, when Mn-CPE was used an oxidation peak at +1.038 V vs. Ag/AgCl was obvious in the presence of B_{12} (**Figure 3b**, curve e), which probably could be ascribed to the oxidation of B12. Finally, in the presence of B12 an oxidation peak at +1.026 V vs. Ag/AgCl was in present on SWVs, when Mn-dsDNA-CPE was used (**Figure 3b**, curve f). Thus, B12 is either oxidized at potential near the oxidation potential of guanine residues or the resulted oxidation peak is very large that masks B12 oxidation peak. What is more, the peak potential of quinine residues or B12 on Mn-dsDNA-CPE is negatively shifted compared to CPE and Mn-CPE (compare curves c, d, e

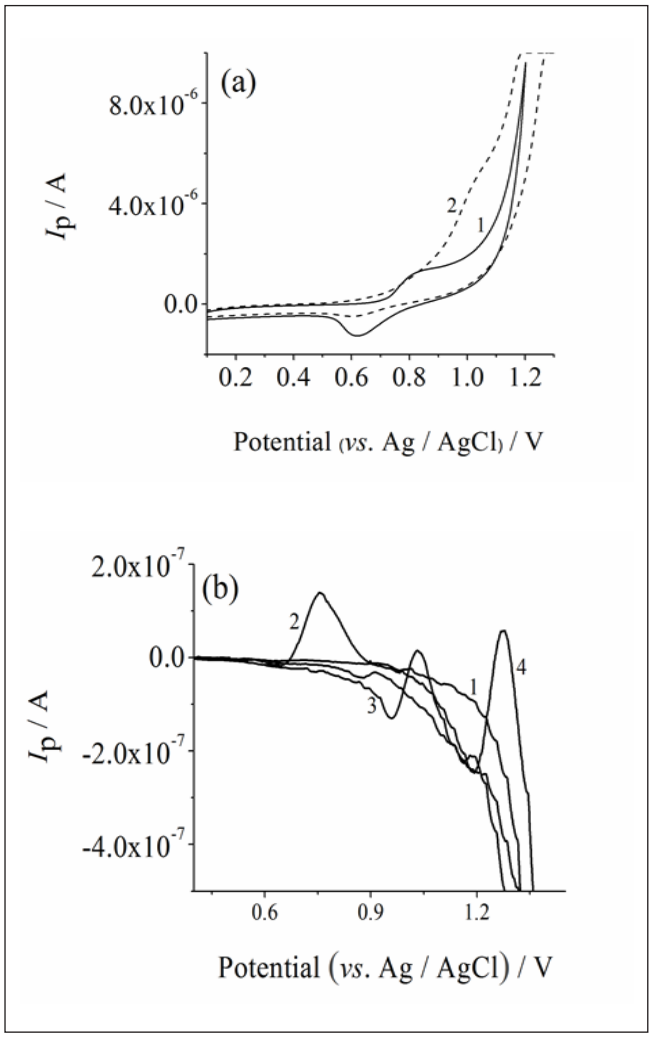

Figure 2. (a) Cyclic voltammograms of (1) Mn-CPE and (2) Mn-dsDNA-CPE (5 μg/L dsDNA and 107 mg/L manganese complex, voltammetric conditions: start potential = first vertex potential = +0.0 V, second vertex potential = +1.2 V, step potential = 0,006 V, scan rate = 0.01 V/s and number of scans = 1) and (b) Square wave voltammograms of (1) CPE, (2) dsDNA guanine residues on CPE, (3) Mn-dsDNA-CPE and (4) Mn-CPE (5 μg/L dsDNA and 107 mg/L manganese complex and others experimental or voltammetric conditions are mentioned in the material and methods section).

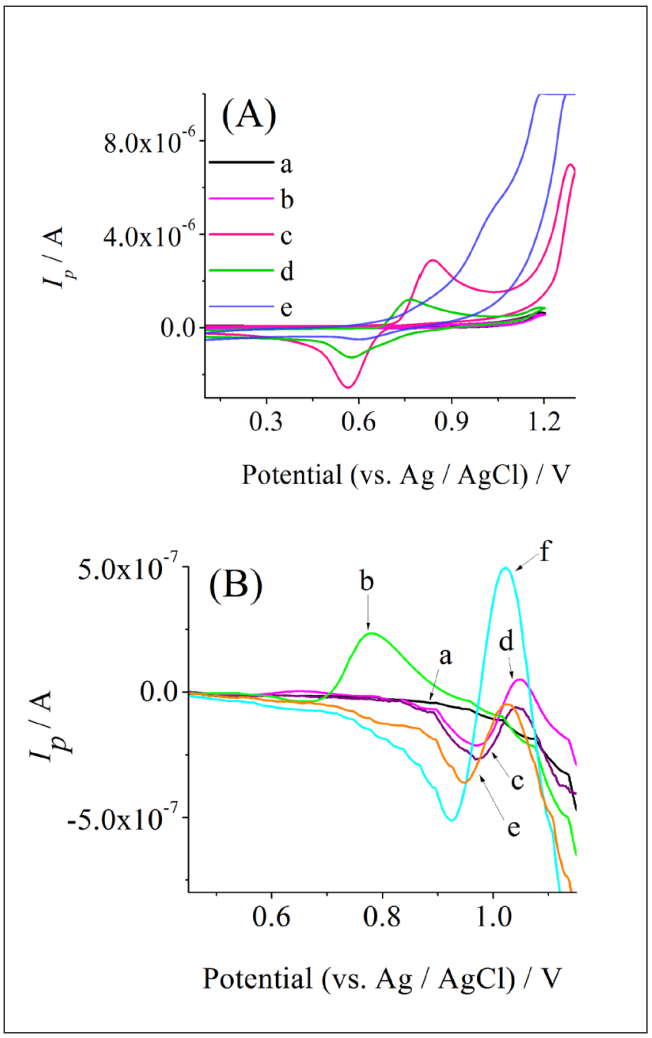

Figure 3. (A) Cyclic voltammograms of (a) CPE, (b) Mn-CPE, (c) B_{12}-CPE, (d) B_{12}-Mn-CPE and (e) B_{12}-Mn-dsDNA-CPE (100 μg/L B_{12}, 5 μg/L, dsDNA and 107 mg/L manganese complex, voltammetric conditions: start potential = first vertex potential = -0.6 V, second vertex potential = +1.3 V, step potential = 0,006 V, scan rate = 0.015 V s-1 and number of scans = 1) and (B) Square wave voltammograms of (a) CPE, (b) Mn-CPE, (c) B_{12}-CPE, (d) dsDNA guanine residues on CPE, (e) B_{12}-Mn-CPE and (f) B_{12}-Mn-dsDNA-CPE (5 μg/L dsDNA and 107 mg/L manganese complex and others experimental or voltammetric conditions are mentioned in the material and methods section).

and f in **Figure 3b**). Moreover, the oxidation peaks of B12 were disappeared when Mn-CPE and Mn-dsDNA-CPE were used (compare curves c, d, e and f in **Figure 3b**). On the whole, the results show that B_{12} was successfully immobilized on Mn-dsDNA-CPE. Obviously, due to the complexity of oxidation of B_{12} on Mn-dsDNA-CPE, the reduction peak of manganese was used to monitor the behavior of B_{12} on Mn-dsDNA-CPE on the forthcoming experiments.

The effect of the pH of the solution that B_{12} was dissolved on the detection of B_{12} was tested, **Figure 4a**. As it can be seen from **Figure 4a**, the peak current increased with the incensement of pH up to the 5.2 afterwards it decreased until pH 6.2 and then remained almost constant. Thus, pH 5.2 was chosen as dissolution pH for the subsequent experiments. Furthermore, at this pH value the interaction of B_{12} with Mn-dsDNA-CPE was the maximum, while the response of Mn-CPE itself was the mini-mum. Moreover, the measurement pH solution was investigated, **Figure 4b**. As it can be seen from Figure 4b, the peak current increased with the incensement of

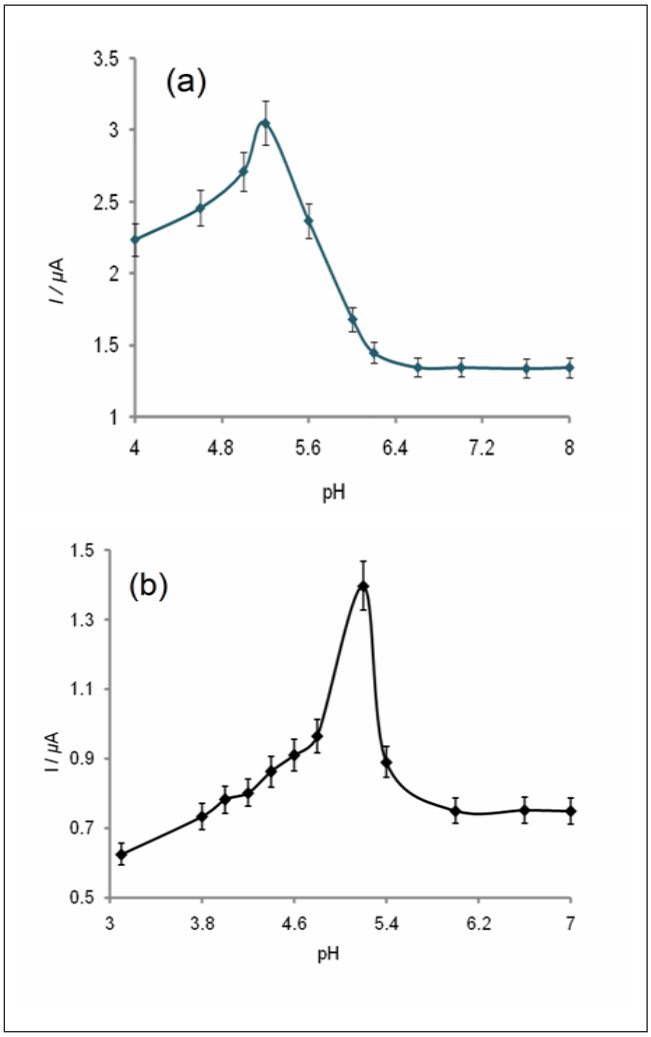

Figure 4. (a) Effect of pH the dissolution solution B_{12} on the detection of B_{12} at Mn-dsDNA-CPE at accumulation time of B_{12} of 90 s and (b) Effect of pH the measurement solution on the detection of B_{12} at Mn-dsDNA-CPE accumulation time of B_{12} of 90 s (Other experimental and voltammetric conditions were as described in the material and methods section).

pH up to the 5.2 afterwards it decreased until pH 6.2 and then remained almost constant. Thus, pH 5.2 was chosen as measurement pH for the subsequent experiments. Furthermore, at this pH value the interaction of B_{12} with Mn-dsDNA-CPE was the maximum, while the response of Mn-CPE itself was the minimum.

In addition, the influence of deposition time and potential of B_{12} was explored, **Figure 5**. From **Figure 5** it can be seen that the deposition potential was increased up to +0.5 V and then decreased and finally became constant. Therefore +0.5 V was chosen for the subsequent experiments. Meanwhile, deposition time was studied after the accumulation of B_{12} on Mn-dsDNA-CPE. The reduction signal of B_{12} after the accumulation of B_{12} on Mn-dsDNA-CPE achieved its maximum value at 180 s, while the reduction peak current of Mn-CPE had its minimum value. Then the reduction signal decreased and remained almost constant. Hence, 180 s was chosen for further measurements.

Meanwhile, the diagnostic performance of the proposed DNA electrochemical biosensor was studied under the selected conditions. Hence, the variation in the difference between the reduction peak current of the accumulated B_{12} on Mn-dsDNA-CPE prior to and post to the interaction with B_{12} versus the B_{12} mass concentration was studied and a calibration graph was plotted. The calibration graph was linear between 3.667 μg/L and 236.0 μg/L with a correlation coefficient of 0.9998. The limits of detection and quantification (cL and cQ) were calculated by means of $3s_b/a$ and $10s_b/a$ respectively, and the regression equation (in μg/L): *y(i) = (0.0133 ±0.0001)γ B12 + (0.017 ±0.005)* where s_b and a represent the standard deviation of the intercept and the slope of the calibration plot, respectively. The limit of detection was 1.210 μg/L, while the limit of quantification was calculated and found to be equal to 3.667 μg/L. The relative standard deviation measured at two level mass concentrations, i.e. 40.00 μg/L and 200 μg/L of B_{12} was 5.50 % and 5.35 %, respectively, indicating good reproducibility of the detection method.

There is always the possibility of the interference of other ionic metallic species to the detection of B_{12}. Therefore, the effect of foreign substances to the current response of the modified electrode was tested and no interference was caused until the addition of 100-fold excess of the interfering substance in a pH 5.2 buffer containing Vitamin B_{12}, as shown in **Table 1**.

In order to verify the applicability of the Mn-dsDNA-CPE, the determination of vitamin B_{12} in human urine sample was carried out using the SWAdSV technique. Aliquots of vitamin B_{12} standard solution were added to the human urine sample. The human urine sample spiked with a known concentration of vitamin B_{12} served as real sample. Thus, the sample was diluted with electrolyte solution (acetate solution, pH 5.2, containing 0.01 mol/L NaCl) at a ratio 1:1 v/v, in order to achieve concentrations in the range of those used in the calibration curve. A low concentration (1 $x10^{-6}$ μg/L) was intentionally used for the preparation of the samples. It is well known that the precision and accuracy of analytical determinations are

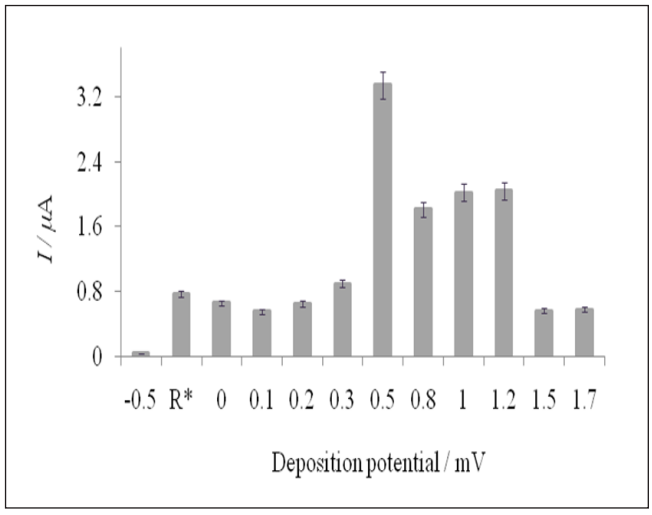

Figure 5. (a) Deposition potential effect on the detection of B_{12} with the proposed biosensor (R* corresponds to the condition where there isn't any potential applied). (Experimental and voltammetric conditions were as described in the material and methods section).

Table 1. Interference study of the proposed biosensor (mass ratio:100:1)

Interference	Recovery
Riboflavin	98.1
Biotin	99.9
Pyridoxal (B_6)	100.9
Niacin	104.0
Pantothenic acid	98.9
Thiamin (B_1)	100.7
Folic acid	99.8
Ascorbic acid	101.4
Glucose	100.7
Mg	100.4
Fe	99.7
Cu	99.8
Al	100.7
Zn	101.4

Table 2. Comparison of the efficiency of various electrodes in the determination of vitamin B_{12}.

Electrode	Linear range (mol/L)	Detection Limit (mol/L)	Ref.
SWCNT-PGE[a]	5×10^{-9}- 8×10^{-8}	2.1×10^{-9}	[25]
MAA/SAM/Au[b]	4×10^{-9} - 4×10^{-5}	1.5×10^{-9}	[26]
DBCH-CPE[c]	2×10^{-9} - 2×10^{-7}	0.85×10^{-9}	[27]
PGE-PNT[d]	2×10^{-7} - 95×10^{-7}	93×10^{-9}	[28]
HTP-MWCNT-CPE[e]	1.5×10^{-6} - 135.3×10^{-6}	0.2×10^{-9}	[29]
MWCNT-GCE[f]	1×10^{-8}- 4×10^{-7}	1.5×10^{-9}	[30]
BiFE[g]	1×10^{-8}- 1×10^{-6}	33.1×10^{-9}	[31]
GCE	1×10^{-4} - 12×10^{-4}	1.0×10^{-3}	[4]
CPE	2×10^{-7} - 1×10^{-6}	150×10^{-9}	[27]
GSPE[h]	1×10^{-10} - 8×10^{-9}	0.007×10^{-9}	[32]
BSA-AuNCs[i]	0.118×10^{-9} - 0.028×10^{-6}	0.074×10^{-9}	[33]
Mn-dsDNA-CPE	2.7×10^{-9} - 1.74×10^{-7}	0.97×10^{-9}	Present study

[a]Single walled carbon nanotube–chitosan modified disposable pencil graphite electrode; [b]Thin self-assembled monolayer of mercaptoacetic acid on a gold electrode; [c]trans-1,2-dibromocyclohexane modified carbon paste electrode; [d]pencil graphite electrode modified with peptide nanotubes; [e]4-hydroxy-2-(triphenylphosphonio) phenolate multi-walled carbon paste electrode; [f]Multi walled carbon nanotubes modified glassy carbon electrode nanotubes modified carbon paste; [g]Bismuth-film electrode; [h]Graphite screen printed electrodes; iBSA-stabilized gold nanoclusters.

dependent on the concentration. Thus, although the Mn-dsDNA-CPE provides precise and accurate results in the determination of low concentrations of vitamin B_{12} these will certainly be better for higher concentrations. The resulting voltammograms showed well-defined reduction stripping peaks and the standard addition plots were linear.

The selectivity, i.e. absence of interferents, was tested by comparing the slope of the standard addition plot with the slope of the calibration curve. For the tested real sample, the slope was 0.0136 A L/g. This value is very close to 0.0133 A L/g, the slope of the calibration curve. Thus, it can be concluded that the components of the sample matrix do not interfere with vitamin B_{12} determination. The accuracy provided by Mn-dsDNA-CPE-SWAdSV was evaluated by the recovery of vitamin B_{12} experiments. Three additions of vitamin B_{12} standard solutions yielded recoveries of vitamin B_{12} between 99.2% and 101.6%. These results confirm the accuracy of the measurements carried out using the Mn-dsDNA-CPE in vitamin B_{12} bioanalysis.

Conclusions

The square wave adsorptive stripping voltammetric detection of B_{12} on the modified CPE with a manganese complex and dsDNA using a novel, readily prepared in the laboratory by simple, low-cost and user-friendly materials, was found to be possible. The principle of the present strategy was based on the indirect detection of B_{12} exploiting the elevation of the peak current of (A) on the Mn-dsDNA-CPE modified electrode, due to the interaction of B_{12} with it. Hence, the principal positive aspect related to the Mn-dsDNA-CPE is certainly the use of non-toxic reagents and the intercalation ability of (A) with DNA through π-π* stacking interactions of thiophenyl groups, making the immobilization of dsDNA on the modified CPE easier, as well as the non destructive character of the assay, since the majority of the reported ones usually demand the destruction of cyanocobalamin. In addition, for electroanalytical determinations, the detection under acidic conditions, where the reversibility of the B_{12r}–B_{12s} couple is greater, probably decrease the detection limit using square-wave voltammetry, as could the destruction of the organic part of vitamin B_{12} by UV irradiation before of electroanalytical steps. In the meantime, this new biosensor is simple, sensitive and selective within a short manipulation time.

As a final point, the performance of Mn-dsDNA-CPE in the determination of vitamin B_{12} was compared to that of modified and unmodified electrodes. **Table 2** shows the results obtained in this study and others published elsewhere. The detection limit of the proposed assay was in the range of nmol/L. As it can be seen from **Table 2**, the linear range of the proposed detection of B_{12} is

competitive to the reported ones. Furthermore, the calculated limit of detection of the proposed methodology is lower compared to those reported on references [4, 26-26, 28-31]. In addition, the detection limit of the proposed methodology, comprising the modified CPE with (A) and dsDNA, is lower than that of the unmodified CPE [27]. On the other hand, the reported detection limits on references [27], [28], and [32] are lower that of the proposed biosensor. In spite of their advantages arising from low detection limits, generally modified electrodes are made of expensive materials like gold nanotubes [31] or screen printed electrodes [32]. Also these nanomaterials or screen printed electrodes have complex synthesis processes and electrode preparation is very difficult [27,31,32]. However, Mn-dsDNA-CPE has very simple preparation procedure. In comparison with other electrodes, carbon paste can be supplied extremely cheap. So we developed a cost-effective electrode, with a wide linear range and relatively low detection limit, which is feasible for the electroanalytical determination of vitamin B_{12} in real samples. Conclusively, this sensor can have future potential in its application as a general platform for the detection of other vitamins of group B in real samples and can serve as a useful tool in the diagnoses of infectious diseases. Moreover, a non destructive detection array was proposed.

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