Published in final edited form as:

Electroanalysis. 2006 February 16; 18(6): 559–572.

NADH Electrooxidation Using Bis(1,10-phenanthroline-5,6-dione) (2,2'-bipyridine)ruthenium(II)-Exchanged Zirconium Phosphate Modified Carbon Paste Electrodes

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Abstract

We present a carbon paste electrode (CPE) modified using the electron mediator bis(1,10-phenanthroline-5,6-dione) (2,2'-bipyridine)ruthenium(II) ([Ru(phend)₂bpy]²⁺) exchanged into the inorganic layered material zirconium phosphate (ZrP). X-Ray powder diffraction showed that the interlayer distance of ZrP increases upon [Ru(phend)₂bpy]²⁺ intercalation from 10.3 Å to 14.2 Å. The UV-vis and IR spectroscopies results showed the characteristic peaks expected for [Ru(phend)₂bpy]²⁺. The UV-vis spectrophotometric results indicate that the [Ru(phend)₂bpy]²⁺ concentration inside the ZrP layers increased as a function of the loading level. The exchanged [Ru(phend)₂bpy]²⁺ exhibited luminescence even at low concentration. Modified CPEs were constructed and analyzed using cyclic voltammetry. The intercalated mediator remained electroactive within the layers (*E*^{o'} = −38.5 mV vs. Ag/AgCl, 3.5 M NaCl) and electrocatalysis of NADH oxidation was observed. The kinetics of the modified CPE shows a Michaelis –Menten behavior. This CPE was used for the oxidation of NADH in the presence of Bakers' yeast alcohol dehydrogenase. A calibration plot for ethanol is presented.

Keywords

Zirconium phosphate; Modified carbon paste electrode; Ruthenium complex mediator; NADH electrooxidation

1. Introduction

Considerable research efforts have been devoted to combine enzyme-catalyzed reactions with electrochemical transducers to produce highly selective and sensitive biosensors [1–9]. These sensors are attractive for several reasons [10–17]: 1) complex organic molecules can be determined with the convenience, speed and ease that characterize amperometric measurements; 2) biocatalysts permit reactions to occur under mild conditions of temperature, pH and minimal substrate concentrations; 3) the sensor response is analyte dependent as dictated by the specificity of most enzyme reactions; and 4) the measured signal is inherently amplified by the enzyme catalytic cycle.

A common type of amperometric biosensors is based on pyridine-nucleotide dependent dehydrogenase enzymes. Many dehydrogenase enzymes use the pyridine nucleotide cofactor, nicotinamide dinucleotide (NAD⁺) or nucleotide adenine dinucleotide phosphate (NADP⁺)

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[5,18]. However, the electrochemical oxidation of NADH (or NADPH) requires a high overvoltage and suffers from side chemical reactions [18,19]. The use of redox mediators has been demonstrated to substantially overcome these limitations, particularly in dehydrogenase sensors [4,15,20]. Ideal mediators should exhibit chemical and electrochemical reversibility, stability under physiological conditions, a fast electron exchange reaction with the coenzymes, and a suitable redox potential, usually between -0.2 to 0 V vs. SCE to avoid interferences in biological determinations [5,18,21].

NADH oxidation using electron mediators follows a Michaelis – Menten mechanism [18]. Typical second order rate constants values are below $10^4 \text{ M}^{-1}\text{s}^{-1}$; very few mediators have second order rate constants in the range of 10^4 to $10^6 \text{ M}^{-1}\text{s}^{-1}$ [5]. These values suggest that the electron transfer mechanism is mass transfer controlled. Several mediators have shown large second order rate constants but have required large overpotentials [18]. Many attempts have been done to build more efficient amperometric NAD^+/NADH biosensors that exhibit large second order rate constants, including preparing self-contained sensors [6,17,22–26].

To build self-contained sensors, the mediator, the cofactor and the enzyme must be incorporated within the sensor body. Important aspects to consider for the immobilization of these molecules are the preservation of their redox properties, chemical stability and retention within the chosen support. Immobilization methods include adsorption, covalent attachment, polymer entrapment and inclusion of the mediator in the electrode material [22–36]. Adsorption and inclusion are the simplest procedures for mediator immobilization; however, other elaborate procedures have shown better stability [11].

A material that shows promising for the immobilization of biological molecules is the layered α -zirconium phosphate ($\text{Zr}(\text{HPO}_4)_2 \cdot \text{H}_2\text{O}$, α -ZrP) [20,37]. Several authors have demonstrated the use of this material for the construction of NADH biosensors. Mediators previously immobilized in α -ZrP are phenoxazines, phenothiazines, phenazines, fluorenones and flavines [15,37–40]. These authors did not use enzymes in their studies nor provided X-ray powder diffraction (XRPD) evidence of the mediator intercalation into the layered material.

Ruan et al. showed that methylene blue-exchanged ZrP could be used for the electroreduction of hydrogen peroxide in the presence of horseradish peroxidase [39]. The authors presented XRPD and IR evidence that the mediator was intercalated between the layers of ZrP, not only exchanged on its surface. The interlayer distance of α -ZrP increased upon intercalation from $7.6 \text{ \AA}$ to $16.1 \text{ \AA}$, which demonstrates methylene blue incorporation within ZrP. The vibrational bands of α -ZrP at 3593 and 3508 cm^{-1} (assigned to the asymmetric and symmetric water stretching modes) disappeared upon methylene blue intercalation. This result indicates that methylene blue intercalation was accompanied by dehydration of α -ZrP. These authors also observed that the intensity of the $\text{P}=\text{OH}$ vibrational band at 3155 cm^{-1} for methylene blue-intercalated ZrP diminished compared to that of unintercalated α -ZrP [20]. This decrease in intensity occurs because some of the H^+ in ZrP are exchanged by methylene blue, decreasing the number of protonated phosphate groups.

In our laboratory, we are interested in the immobilization of luminescent polypyridine complexes and other luminescent molecules by direct exchange into the layers of a highly hydrated ZrP material known as the $10.3 \text{ \AA}$ phase of ZrP for their use in artificial photosynthesis [41]. Tris(2,2'-bipyridine)ruthenium(II) ($\text{Ru}(\text{bpy})_3^{2+}$), *fac*-tricarbonyl-chloro-1,10-phenanthroline-rhenium(I) ($\text{Re}(\text{phen})(\text{CO})_3\text{Cl}$), and 1-aminomethylpyrene have been intercalated into the $10.3 \text{ \AA}$ phase of ZrP [41,42]. Our current work involves the direct intercalation of bis(1,10-phenanthroline-5,6-dione)(2,2'-bipyridine) ruthenium(II) ($[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$) within these layers. This complex is known to be a good redox mediator for the oxidation of NADH [43–45]. We report here the construction of a CPE modified with $[\text{Ru}$

(phen)₂bpy]²⁺-exchanged ZrP (Ru-ZrP) for the mediated oxidation of NADH and the resultant electrode kinetics.

2. Experimental

2.1. Reagents

Mineral oil, Baker's yeast alcohol dehydrogenase (E. C. 1.1.1.1), β-nicotinamide adenine dinucleotide and β-nicotinamide adenine dinucleotide (reduced form) were obtained from Sigma Chemicals. Graphite powder and copper wire were obtained from Fisher Co. All other reagents, at least 98% pure, were obtained from Aldrich. Nanopure water was obtained using a Barnstead purification train (17.5 MΩ/cm). Capillary tubes were obtained from Kimble products.

2.2. Procedures

We used a hydrated form of ZrP, which has approximately six water molecules per formula unit and an interlayer distance of 10.3 Å [41,46]. This material was synthesized as described previously [47]. A volume of 120 mL of 0.05 M ZrOCl₂ · 8H₂O was mixed with 85 mL of 6 M H₃PO₄ with constant stirring at 94°C for 48 hours. The precipitated solid was filtered and washed three times with nanopure water. The wet precipitate was characterized using XRPD; the first diffraction peak gave the expected interlayer distance of 10.3 Å for this phase of ZrP. [Ru(phen)₂bpy](PF₆)₂ was synthesized as reported in the literature [43,44] and the expected spectroscopic bands were observed (UV-vis (water) λ_{max} (nm): 288, 438; IR (cm⁻¹): ν_{CO} = 1695, ν_{C=C} = 1424, ν_{CN} = 1296; ¹H-NMR (δ/ppm, CD₃CN, 300 MHz: δ 8.55 (4 H, d), 8.15 (2 H, t), 8.08 (4 H, d), 8.00 (2 H, d), 7.87 (2 H, d), 7.64 (4 H, t), 7.46 (2 H, t))) HHH. Several suspensions with different solution molar concentration ratios (1:30, 1:20, 1:10, 1:1, and 5:1 [Ru(phen)₂bpy]²⁺:ZrP) were prepared from a 1.0 × 10⁻³ M [Ru(phen)₂bpy]²⁺ aqueous stock solution adding different amounts of ZrP. These suspensions were maintained with constant stirring during five days until the pH stopped dropping. Afterwards, they were filtered, washed with water until a clear supernatant was obtained, and dried in air for four days. The obtained [Ru(phen)₂bpy]²⁺-intercalated ZrP materials are referred as Ru-ZrP. Elemental analysis was done by QTI Enterprises. The [Ru(phen)₂bpy]²⁺ loading levels in ZrP obtained were: 1:21 (1:30), 1:12 (1:20), 1:10 (1:10), 1:2.3 (1:1), 2.2:1 (5:1), where the first ratio corresponds to the experimental loading level obtained from the elemental analysis of the intercalated materials and the second ratio (in parenthesis) is the [Ru(phen)₂bpy]²⁺:ZrP solution molar concentration ratios used in the intercalation experiments. XRPD measurements were performed to determine the interlayer distance after intercalation. Suspensions of the intercalated material in water were used for UV-vis absorption and luminescence measurements. IR spectroscopy was done on dried KBr pellets.

Modified CPEs were prepared mixing thoroughly 15 mg of Ru-Zr, 150 mg of graphite powder and 75 μL of mineral oil to form a uniform paste suitable for molding the CPEs. Capillary tubes (2 mm diameter) were used to construct the modified CPEs. The electrical contact was done with a copper wire.

2.3. Instrumentation

UV-vis absorption spectra were obtained using a HP 8453 diode array spectrophotometer. XRPD measurements were performed using a Siemens D5000 X-ray with Cu Kα radiation (λ = 1.5406 Å) with a filtered flat LiF secondary beam monochromator. The XRPD patterns were run on a 2θ range of 1.2 – 45°. IR spectroscopy measurements were performed using a MAGNA-IR Spectrometer model 750 from Nicolet with a deuterated triglycine sulfate (DTGS) detector, and the spectra were analyzed using OMNIC software, version 4.2. All electrochemical measurements were done with a BAS Model CV-50W potentiostat in a 10 mL

cell containing the modified CPE, a Ag/AgCl (3 M NaCl) reference electrode and a Pt wire auxiliary electrode. Most of the electrolyte solutions were prepared using a phosphate buffer solution (PBS, $\mu = 0.1$) at pH 8.0. When analyzing the dependence of the formal potential as a function of pH, PBS solutions with pHs of 4.0, 6.0, 7.0 and 8.0 were used. The electrochemical results were reported as the average of three electrodes. Kinetics studies were performed using a modified carbon paste rotating electrode using a constant sweep rate of 5 mV/s at different frequencies.

2.4. Construction of the Calibration Plot for Ethanol Sensing

The current response of $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$:ZrP modified CPE was measured using the following procedure. A stock solution of γ -ADH and NAD^+ (7.5 U/mL of γ -ADH and 10 mM of NAD^+ in PBS) was prepared. Known concentrations of ethanol were added and the current measured after each addition. All measurements were done at steady state.

3. Results and Discussion

3.1. Characterization of Ru-ZrP

The elemental analyses results show that the ruthenium complex loading level in Ru-ZrP increases upon increasing the $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$:ZrP solution molar concentration ratio used in the intercalation experiments. Figure 1 shows XRPD data of Ru-ZrP at several loading levels. The diffractograms show that the ZrP interlayer distance increases from 10.3 Å to 14.2 Å upon proton exchange with $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$. This is a first indication that $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ was intercalated into the ZrP interlayer space. This distance is smaller than that reported by Martí and Colón for $[\text{Ru}(\text{bpy})_3]^{2+}$ intercalation into ZrP (15.1 Å) [41]. From the dimensions of $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ the minimum distance that $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ ions can separate the ZrP layers is 9.5 Å. The thickness of an α -ZrP type layer is 6.6 Å [48,49]; adding the expansion produced by $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ ions would give an expected interlayer distance of 16.1 Å. There is a difference of 1.9 Å between the calculated and observed Ru-ZrP interlayer distance. Aside from the presence of water molecules, a possible explanation for this difference is that while the α -ZrP is made of nearly perfect planes of Zr atoms having bridging O_3PO^- groups, above and below the Zr plane [46], nothing precludes intercalating molecules to penetrate slightly between the phosphate groups.

The IR and UV-vis spectra of Ru-ZrP (Supplemental Information) show no new bands. The IR bands observed are almost identical to those observed for unintercalated ZrP and for $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ in solution, except for the “lattice” water band at 1630 cm^{-1} in unintercalated ZrP [50] which is dramatically reduced in the Ru-ZrP IR spectrum. This reduced intensity is expected since water is displaced in the intercalation process. In addition, $\pi - \pi^*$ and MLCT bands are observed (at 288 nm and 438 nm, respectively) in the UV-vis spectra, as expected [43, 44], with an intensity proportional to the loading level.

3.2. Ru-ZrP Modified CPE

The cyclic voltammetry of Ru-ZrP modified electrodes at a 1:2.3 loading level was evaluated at different scan rates. The voltammograms show the dione/diol redox process indicating that the complex remains electroactive upon intercalation. The formal potential (E^0) of $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ exchanged into ZrP is -38.5 mV compared to -32.4 mV in buffered solution at pH 8.0 [43]. When compared to other loading levels we observe that the E^0 shifts to more negative potentials as the concentration of $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ increases inside ZrP (see Table 1). Since the dione/diol redox process is pH dependent, this behavior could be due to differences in the pH within the ZrP layers. ZrP layers are acidic ion exchangers. The intercalation process in ZrP materials is known to occur primarily by ion-exchange when cations from the bulk solution exchange with interlayer protons within the ZrP matrix [41,51,52]. When the

concentration of $[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$ is low, the microenvironment inside the layers is highly acidic. When the concentration of $[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$ is high, most of the protons have been exchanged with $[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$ and a slightly alkaline environment is expected. It is well known that the E^0 of this complex (quinones in general) becomes more negative as the solution pH increases [43–45].

To determine the number of electrons responsible for the electron transfer process we evaluated the Ru:ZrP formal potential as a function of pH. Figure 2 shows the formal potential at two loading levels of Ru-ZrP; the potential drops as the solution pH increases. This result is different to that observed by Gorton et al. [15,37,38,53]. These authors immobilized indophenol and *o*-quinones derivatives in amorphous ZrP to study their electrocatalytic properties for NADH oxidation. Gorton et al. showed that the organic compounds retained the electrocatalytic behavior upon immobilization. However, with many of the dyes, such as methylene blue [37, 53], Nile blue [38], brilliant cresyl blue [38], lumichrome [38], toluidine blue [38], riboflavin [38,53,54], Meldola's blue [53], and indophenol and *o*-quinones derivatives [15], the formal potential was pH independent, contrary to our results. Gorton et al. attributed the pH-independent formal potential to the effect of having the organic dye bound to ZrP. Our pH-dependent E^0 suggests that the dione functionality does not interact directly with the ZrP layer. The charge on the metal complex (from Ru(II)) is responsible for the electrostatic binding of the complex to ZrP. No binding occurs through the dione ligand. This explanation is further supported by the observation of the electrochemistry of the dione functionality. It is well known that the dione electroactivity is diminished or suppressed by direct interaction with other chemical species such as metal ions and protons [43,45,55].

In Figure 2, the slope of the linear plot is 31.8 mV/pH for the 1:2.3 Ru-ZrP loading level. As reported previously, between pH 2.0 and 4.0 the dione/diol redox process is a $2e^-$, $3H^+$ process [43]. However, between pH 4.0 thru 7.0 it is a $2e^-$, $1H^+$ process and from 7.0 to 9.0 it is a $1e^-$, $1H^+$ process. The slope value of 31.8 mV/pH shown in Figure 3 is consistent with a $2e^-$, $1H^+$ process, where the ideal slope should be 29.5 mV/pH.

Further experiments were done using the 1:2.3 Ru-ZrP loading level ($\Delta E_p = 62$ mV and $i_{pc}/i_{pa} = 1.18$), because at this loading level the intercalated $[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$ electro-chemical parameters resemble those of the complex in solution. The shape of the cyclic voltammograms suggests that the electron transfer is diffusion controlled. This result was further analyzed by looking at the dependence of the peak current on scan rate. Figure 3 shows that a plot of current as a function of the square root of the scan rate is linear. A slope of 0.6 was obtained from a $\log i_p$ vs. $\log \nu$ plot indicating that the process is mainly diffusion controlled with a small nondiffusional component.

To investigate the origin of the diffusional process, we studied whether leaching was occurring. No leaching of the electroactive species to the electrochemical cell was observed as monitored by UV-vis spectrophotometry (results not shown). We corroborated this result using a glassy carbon electrode to detect if $[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$ was present in the buffer solution after withdrawing the modified CPE using Osteryoung square wave voltammetry (OSWV). Leaching of $[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$ to the buffered solution was not detected with OSWV before and after 125 cycles between -300 to 300 mV using cyclic voltammetry (see Supplemental Information). The only signal detected was the oxidation of the phosphate groups around 0.5 V. No leaching of $[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$ was observed, as anticipated since leaching would occur in acidic solutions ($\text{pH} < 3$), but not at the moderate pHs of our experiments ($\text{pH} 4 - 8$), as has been shown for other mediators in ZrP by Gorton et al. [56].

The binding process of this metal complex to the ZrP matrix is controlled by electrostatic forces. Pessôa et al. suggested that when methylene blue is strongly adhered to ZrP, the mechanism

of heterogenous electron transfer is affected by the transport of counter ions to and from the electrode interface to attain charge neutrality [37]. We observed (Supplemental Information) that upon an increase in the electrolyte concentration, the peak separation decreased and the peak current increased, demonstrating that charge transport is assisted by counter ion diffusion.

While counter ion diffusion is important in these materials, it is probable that the redox species in the modified CPE couple electronically through an electron-hopping mechanism. Electron hopping occurs via “electron self-exchange” whereby a reduced redox species simply gives an electron to its oxidized counterpart of the same species [34]. If this mechanism is occurring here a counter ion must come into the film from the contacting electrolyte solution to maintain electroneutrality. This type of mechanism has been proposed for electroactive species covalently bound to a polymer chain or entrapped within zeolites [57–59].

3.3. Electrocatalysis of NADH

Guadalupe et al. [43] and Abruña et al. [44] have previously shown that $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ is a mediator for the electro-oxidation of NADH with and without the presence of lactate dehydrogenase. Figure 4a shows the cyclic voltammetry of Ru-ZrP modified CPE without NADH present. When a known concentration of NADH is added to the electrolytic cell a catalytic current is observed (Figure 4b). As can be observed the onset of the catalytic current began at -100 mV and reached a potential plateau above 50 mV. Also, the anodic peak current increases almost 5 times in the presence of NADH while almost no cathodic current is observed. These observations are consistent with a catalytic EC mechanism as illustrated in Scheme 1.

We prepared carbon paste rotating disk electrodes for the determination of the second order rate constant for the Process 2 in Reaction Scheme 1. To accomplish this process the NADH must reach the electrode surface. The formation of a complex between NADH and the surface is modeled as a Michaelis – Menten-like mechanism. This approach was introduced by Gorton and coworkers but criticized by Lyons [60]. Lyons indicated that this approach is only valid if the current is much less than the mass transport value. The kinetic data was fitted in the linear response range of the catalytic current as a function of NADH concentration. The 1:2.3 Ru-ZrP loading level material shows the highest catalytic current (Supplemental Information Figure S5) and therefore we model its behavior in the kinetics studies with a Michaelis – Menten-like mechanism (Scheme 2).

To determine the Michaelis – Menten constant (K_M in mM) we combine the rate constants to obtain:

$$K_M = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad (1)$$

The second order rate constant (k_{obs} , in $\text{M}^{-1}\text{s}^{-1}$) can be obtained as a function of K_M :

$$K_{\text{obs}} = \frac{k_{+2}}{K_M + C_{\text{NADH}}} \quad (2)$$

where C_{NADH} is the NADH concentration in mol/cm^3 .

Koutecky-Levich has developed an equation where we can determine the reaction rate by studying the reciprocal current as a function of the reciprocal square root of the angular frequency of rotation of the electrode [13–15,38,45,60,61]:

$$\frac{1}{i_{\text{cat}}} = \frac{1}{nFAk_{\text{obs}}\Gamma C_{\text{NADH}}} + \frac{1}{0.620nFAD_{\text{Nadh}}^{2/3}v^{-1/6}C_{\text{NADH}}} \cdot \frac{1}{\omega^{1/2}} \quad (3)$$

where Γ is the surface coverage in mol/cm², F is the Faraday constant in C/equiv, A is the area of the electrode in cm², D_{NADH} is the NADH diffusion coefficient in cm²/s, ν is the hydrodynamic viscosity of the medium (0.010 cm²/s) and ω is the angular frequency of rotation of the electrode in s⁻¹.

If Equation 1 and 2 are substituted in the Koutecky-Levich equation we obtain:

$$\frac{1}{i_{\text{cat}}} = \frac{1}{nFAk_{+2}\Gamma} + \left\{ \frac{K_M}{nFAk_{+2}\Gamma} + \frac{1.61\nu^{1/6}}{nFAD_{\text{NADH}}^{2/3}\omega^{1/2}} \right\} \times \frac{1}{C_{\text{NADH}}} \quad (4)$$

Equation 5 shows that a plot of $1/i_{\text{cat}}$ vs. $1/C_{\text{NADH}}$ will give a straight line if the [NADH-(Ru-ZrP)] complex is involved in the mechanism. From the intercept k_{+2} can be evaluated and from the slope K_M . Figure 5 shows the plot obtained for this mechanism at different NADH concentrations. The k_{+2} and K_M values from the intercept and the slope are 503.3 s⁻¹ and 1.98 mM, respectively.

The second order rate constant k_{obs} , at different rotation frequencies was evaluated from the intercept using Equation 3. The k_{obs} value obtained, $5.67 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, shows that the electron transfer kinetics is much faster than that of the complex in solution ($2.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) at the same experimental conditions [43, 45]. The difference in the catalytic activity observed between the dissolved and the immobilized mediator occurs since the immobilized mediators is an integral part of the electrode surface and therefore facilitates electron hopping between mediators inside the zirconium phosphate layers, compared to mediators diffusing in the bulk solution. This result along with the potential shift to positive potentials in the presence of NADH shows that the thermodynamic driving force is enhanced for the immobilized complex. This observation was also observed by Gorton et al. when they studied the immobilization of dyes in amorphous ZrP [4, 5]. These authors obtained higher k_{obs} values at pH 6 and pH 7. Table 2 summarizes the electrochemical and kinetic parameters of our modified CPE at different pHs. This table shows an increase in k_{obs} with an increase in the formal potential indicating that the thermodynamic driving force is responsible for the charge transfer and not the pH [15]. This result is in concordance with Marcus theory for an heterogeneous electron transfer mechanism [13 – 15, 22, 23, 25, 43, 53, 61].

These results suggest that a charge transfer (CT) complex is formed on the electrode surface. These findings also demonstrate that immobilizing inorganic complexes into ZrP can improve the kinetic parameters in comparison with the complex in solution.

3.4. Calibration Curve for Ethanol Sensing

Having characterized our modified CPE we performed experiments where NAD⁺ and Bakers' yeast alcohol dehydrogenase (γ -ADH) were present in the electrochemical cell. In the absence of ethanol no electrocatalytic current was observed indicating that no NADH has been formed. When ethanol is added to the electrochemical cell, the monoenzymatic reaction cycle presented in Scheme 3 is completed and a catalytic current dependent on ethanol concentration is observed.

The steady state catalytic response of the modified CPE at various ethanol concentrations is shown in Figure 6. A linear range is observed from 10 to 80 mM ethanol concentration after which saturation occurs. The maximum catalytic current is dependent on enzyme and NAD⁺ concentration. Similar behavior was observed previously by Guadalupe et al. [43]. These authors reported that if we consider NAD⁺ as another enzyme substrate, the behavior can be explained as a saturation effect. A saturation effect occurs at NAD⁺ concentrations that are large enough so that NAD⁺ occupies all active sites reaching the maximum velocity at those

conditions. This behavior is expected for a Michaelis –Menten-like mechanism. The saturation effect on amperometric sensors has been observed previously in a wide range of sensors such as those for glucose [43], cholesterol [59], lactate [62].

4. Conclusion

We have intercalated $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ into the layers of ZrP increasing the interlayer distance from 10.3 Å in ZrP to 14.2 Å in Ru-ZrP. Ru-ZrP shows the spectral characteristics of intercalated complexes as shown by the UV-vis, luminescence and IR spectroscopic results. We have proven that $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ remains electroactive within ZrP/CPE composites. The charge transfer process in the modified CPE is dependent on the electrolyte concentration. In addition, we observed that the formal potential of the electrode remains pH dependent, indicating that the dione ligand is not directly interacting with the ZrP matrix. We are currently evaluating the use of these modified CPEs in ethanol biosensors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Antonio Martínez and the staff of UPR's Material Characterization Center for their help with the XRPD and IR spectroscopy measurements. The authors want to acknowledge Angel Martí for the molecular modeling analysis of $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$. This work was supported by the NIH-RISE (grant R25GM061151), NIH-SCORE (grant 5S06GM08102), and PR-AGEP (NSF grant HRD-0302696) programs.

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Fig. 1. XRPD patterns of the 10.3 Å phase of ZrP and of $[\text{Ru}(\text{phend})_2\text{bpy}]^{2+}$ -exchanged ZrP materials at various loading levels. The interlayer distance is indicated next to the first order diffraction peak.

Fig. 2. Formal potential of Ru-ZrP as a function of pH. The measurements were made in PBS, $\mu = 0.1$ at 100 mV/s.

Fig. 3.
Randles – Sêvcik plot for the modified CPE at different scan rates.

Fig. 4.

a) Cyclic voltammogram at 5 mV/s of Ru-ZrP modified CPE (1:2.3 Ru-ZrP loading level) in PBS ($\mu = 0.1$, pH 8.0). b) Cyclic voltammogram at 5 mV/s showing electrocatalysis of 0.69 mM NADH using the Ru-ZrP modified CPE (1:1 Ru-ZrP loading level) in PBS ($\mu = 0.1$, pH 8.0).

Fig. 5. Variation of $1/i_{\text{cat}}$ as a function of $1/[NADH]$ at pH 8.0 (PBS $\mu = 0.1$, $rpm = 200$, scan rate = 5.0 mV/s).

Fig. 6. Calibration plot for ethanol sensing using $[Ru(phen)_2bpy]^{2+}$ -exchanged ZrP modified CPE in the presence of 7.5 U/mL γ -ADH and 10 mM of NAD^+ in PBS ($\mu = 0.1$), at pH 8.0, 1 mV/s.

Scheme 1.

EC scheme for the reaction between NADH and $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ -exchanged ZrP.

Scheme 2.

Proposed Michaelis – Menten-like mechanism for the electrochemical reaction that occurs in the electrode surface.

Scheme 3.

A monoenzymatic reaction cycle for ethanol sensing.

Table 1

Electrochemical parameters for modified CPEs at different loading levels. Scan rate = 100 mV/s, $\mu = 0.1$ at pH 8.0 in PBS.

Ru:ZrP Loading level	$E^{0'}$ (mV)	ΔE_p (mV)	i_{pa}/i_{pc}
2.2: 1	-42.5	61.0	1.20
1: 2.3	-38.5	62.0	1.18
1: 10	-27.5	54.0	0.90
1: 12	-22.0	33.0	0.97
1: 21	-23.0	28.0	1.05

Table 2

Electrochemical and kinetic parameters of the modified CPE for NADH electrooxidation.

1: 2.3 modified CPE	pH values		
	6	7	8
$E^{\circ'}$	33	0.83	-38.5
K_M (mM)	3.40	5.27	1.98
K_{+2} (s ⁻¹)	586.3	1068.37	503.26
k_{obs} (M ⁻¹ s ⁻¹)	1.68×10^5	1.24×10^5	5.67×10^4