

Dedicated to Professor Ionel Haiduc
on the occasion of his 70th anniversary

NADH ELECTROCATALYTIC OXIDATION AT GLASSY CARBON PASTE ELECTRODES MODIFIED WITH MELDOLA BLUE ADSORBED ON ACIDIC α -ZIRCONIUM PHOSPHATE

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The electrochemical behavior and the electrocatalytic activity for the NADH oxidation at glassy carbon paste electrodes (GCPEs) modified with Meldola Blue (MB) adsorbed onto acidic α -zirconium phosphate (ZP^{*}) have been investigated. Cyclic voltammetry and rotating disc electrode measurements, performed in Tris buffer solution (pH 7) in absence and in presence of different NADH concentrations, showed that: (i) the formal standard potential (E°) of MB was found pH dependent, according to the equation $E = E^{\circ} - 0.059 \text{pH} + 0.029 \log(1 + 10^{\text{pH} - \text{p}K_a})$, where the best fitting value for $\text{p}K_a$ was estimated to be 4.3 ± 0.4 ; (ii) the best electrocatalytic response was obtained for MB-ZP^{*}-modified GCPEs having a ratio between the glassy carbon powder (Sigradur K) and MB-ZP^{*} of 19:1; (iii) the presence of polyethyleneimine in the paste has a significant beneficial effect on the electrocatalytic activity of the investigated modified electrodes. As a general conclusion, it was stated that the particular features of the MB-ZP^{*}-GCPEs are related to the ZP^{*} crystallinity.

INTRODUCTION

The preparation of modified electrodes for NADH oxidation is of constant interest in developing new biosensors with higher sensitivity, selectivity and stability as well as efficient biofuel cells^{1–6} based on cofactors regeneration⁷.

For this reason, different mediators (quinines^{8, 9}, flavins¹⁰, or other quinoic type compounds such as phenoxazines, phenothiazines, and phenazines^{11–14}) were immobilised by simple adsorption on various support materials such as: zirconia, titania^{15, 16}, zirconium or titanium phosphates^{17–21}, silica gel^{12, 22–24}, and zeolites^{25, 26}. The use of these support materials, onto or into which the mediators are strongly bound avoiding their leaching under experimental conditions, is also important for electrocatalytic purposes in order to: (i) improve the mediator electrochemical activity and stability^{10, 12, 17, 27}, (ii) shift the mediator potential into the optimal potential range²⁸, (iii) improve the

rate of the electrocatalytic reaction^{17, 18}, and (iv) to decrease the effects of adsorption of products and other side reactions.

Meldola Blue (MB), a phenoxazine compound, is a two electron-proton acceptor type mediator, which has a high oxidation reaction rate with NADH^{29–32}. When immobilised onto an electronically conducting materials (such as graphite^{31, 32}) or onto amorphous homemade zirconium phosphate (ZP)^{11, 17} MB can be used to obtain highly efficient modified electrodes for electrocatalytic oxidation of NADH. In the last case, it was noticed that the formal potential (E°) of MB is apparently pH independent and has a value in the optimal potential region for amperometric detection²⁰.

Crystalline α -zirconium phosphate (α -Zr(HPO₄)₂·H₂O; α -ZP) was used in many research areas as a catalyst, ion exchanger, molecular sieve, electrode material, and chemical modified matrix³³. Taking into account that crystalline α -ZP is a good ion conductor, a good ion exchanger, and its structure (layered material)

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allows guest molecules to be adsorbed in the interlayer spaces^{34,35}, the previously used homemade amorphous ZP has been replaced for MB immobilisation by a better defined support material, constituted of α -ZP microcrystals connected by phosphoric acid molecules, (ZP*)³³. The electrochemical behaviour and the electrocatalytic activity for NADH oxidation at glassy carbon paste electrodes (GCPEs) modified with MB adsorbed onto ZP* (MB-ZP*) have been investigated with cyclic voltammetry and rotating disc electrode measurements, performed in Tris buffer solution (pH 7) in the absence and in the presence of different NADH concentrations. The influence of the ratio between glassy carbon powder and MB-ZP*, and the presence of a polycation (polyethyleneimine, PEI) on the electrochemical and electrocatalytic parameters of the modified electrodes were investigated. The particular features of the MB-ZP*-GCPEs in comparison with those observed for MB-ZP-GCPEs were related to the crystallinity of ZP*.

EXPERIMENTAL

Synthesis of acidic α -zirconium phosphate (ZP*). ZP* was prepared according to a previously reported method³³. An amount of 25 g of zirconyl chloride was dissolved in 430 ml of 2 M HCl solution, and the mixture was slowly added under continuous stirring to 400 ml of 1.25 M H₃PO₄ solution. The formed precipitate was centrifuged and washed with 0.3 M H₃PO₄ until free of chloride ions. The obtained solid was dried in air at room temperature. The resulting material (ZP*) is a mixture of two phases: α -Zr(HPO₄)₂·H₂O and an important amount of phosphoric acid (~15 % w).

XRD spectra. The XRD spectra for ZP*, MB, and MB-ZP* were recorded with a X-ray diffractometer (Philips, model PW1729/1720) using the CuK α ($\lambda = 1.5418 \text{ \AA}$) radiation.

Microanalytical measurements. Microanalytical data were obtained with an elemental analyser (Perkin-Elmer, model 2400B).

Modified carbon paste electrodes preparation. The procedure for immobilisation of Meldola Blue (MB; Sigma, St. Louis, MO, USA) on ZP* was the following: 1 g of ZP* was added to 100 ml of an aqueous solution of MB (0.2 g/l) (25°C). After 1 h of mechanical stirring the mixture was filtered. The resulting solid was washed with deionised water and dried in air at 50°C.

The MB-ZP*-modified GCPEs (MB-ZP*-GCPEs) were obtained by thoroughly mixing (in an agate mortar) 100 mg of glassy carbon powder and various amounts of the MB-ZP* mixture (prepared in different ratios such as: 19:1, 9:1, 5.6:1, 4:1, and 3:1 (w/w)), with 25 μ L of paraffin oil (Fluka, Buchs, Switzerland). The glassy carbon powder used was Sigradur K (SK; Hochtemperatur-Werkstoffe GmbH, Bonn, Germany), because, as was shown before^{20,36}, SK is the best choice for electrocatalytic oxidation of NADH at glassy carbon paste electrodes modified with MB adsorbed on amorphous ZP.

Different amounts of a solution of polyethyleneimine (PEI, 4.7% w/w, Chemicon, Malmö, Sweden), neutralised in 0.1 M Tris buffer (pH 7), were added as an additional modifier to the SK/MB-ZP* mixture. The resulting mixture (of specified composition) was dried at room temperature according to previous work²⁰.

Electrochemical measurements. All electrochemical measurements were carried out using a conventional three-electrode electrochemical cell. The MB-ZP*-modified glassy carbon pastes were put into a cavity of an in-house made Teflon holder, in the bottom of which a piece of pyrolytic graphite was used for electric contact. The Teflon holder was screwed onto a rotating disc electrode device (model 616, EG-G PAR, Princeton, USA), and was used as working electrode (geometrical area of 0.14 cm²) for cyclic voltammetry (CV) and rotating disc electrode (RDE) measurements. A platinum ring was used as counter electrode, and an Ag|AgCl (NaCl, 3M) as the reference electrode. An electrochemical analyser (BAS 50W Bioanalytical Systems, West Lafayette, IN, USA) was connected to a PC microcomputer for potential control and data acquisition for all electrochemical measurements.

In all experiments, a buffer solution of 0.1 M Tris (Sigma) was used as supporting electrolyte. The pH of the buffer solution was adjusted to the desired value by adding HCl or KOH (Merck, Darmstadt, Germany).

For each modified electrode, the surface coverage (Γ , mol/cm²) was estimated by integration of the cathodic peak area for MB, and supposing that two electrons participate in the overall redox process. Because the real electrochemically active surface for carbon paste electrodes remains unknown^{37,38}, the surface coverage was calculated with respect to the geometric electrode area.

The electrocatalytic behaviour of MB-ZP*-GCPEs for NADH oxidation was investigated using a freshly prepared NADH (Sigma) aqueous solution. The NADH concentration was evaluated by monitoring the absorbance of its solution at 340 nm, taking its molar extinction coefficient as 6600 cm⁻¹M⁻¹³⁹. The electrocatalytic oxidation of NADH at these modified electrodes was investigated using CV and RDE techniques. The RDE experiments were carried out within a rotating speed range between 100 and 1000 rpm at an applied potential 100 mV more positive than the E^{o'} of adsorbed MB.

RESULTS AND DISCUSSION

Powder XRD patterns for MB-ZP* system

α -ZP is known to be a good ion exchanger because it is a strongly acidic material^{40,41}, and a good H⁺-conductor due to its phosphate groups⁴². These properties make it an attractive material to be employed as a rigid matrix for immobilisation of mediators used for NADH electrocatalytic oxidation^{17,18,43}.

The ZP* is a homogeneous mixture of α -ZP and H₃PO₄, *i.e.* a layered crystalline phosphate with well defined basal spacing. Consequently, a clear XRD pattern attributed to Zr(HPO₄)₂·H₂O was observed for ZP* (Fig. 1A). After MB adsorption onto ZP*, the XRD spectrum for MB-ZP*

(Fig. 1C) shows that, in comparison with the starting material, the interlayer distance remains quasi-constant and the crystalline structure of α -ZP remains practically unaltered. This result suggests that contrarily to other similar compounds adsorbed on layered crystalline phosphates^{13, 33, 44-51} the MB molecules are not intercalated between the α -ZP layers. Therefore, the mediator retention is only superficial, and mainly associated to the strong acid centres of H_3PO_4 . Additionally, it is interesting to notice that MB-ZP* was much more coloured than MB-ZP²⁰, pointing out that its organic load is bigger. Indeed, the experimental data indicated a carbon content of $\sim 0.91\%$ for MB-ZP*, while for amorphous ZP this value was 0.17% .

Electrochemical behaviour of MB-ZP*-GCPEs

Cyclic voltammetric measurements recorded at MB-ZP*-GCPEs, obtained with different ratios between the Sigradur K GC powder and MB-ZP*, were used to compare the voltammetric response

of MB-ZP*-GCPEs in 0.1 M Tris buffer solution of pH 7 (results not shown). The best electrochemical behaviour was observed for the ratio of 19:1 (Figure 2). Obviously, the ZP*-modified GCPEs does not reveal any redox process, while for the MB-ZP*-modified GCPEs a well shaped voltammetric response for MB redox couple was observed.

The $E^{\circ'}$ value of MB adsorbed on ZP* (evaluated as the average of the potentials for the oxidation and the reduction peaks) was around -100 mV vs. Ag|AgCl (NaCl 3M) (Tris buffer, pH 7). This value shows a positive shift compared with the $E^{\circ'}$ value observed for MB adsorbed directly onto graphite (-175 mV vs. SCE)^{31, 52}, and a negative potential shift compared with the value observed for MB adsorbed on ZP ($+40$ mV vs. Ag|AgCl (NaCl 3M))²⁰. This behaviour suggests that, for the compared materials, the negative charge density decreases in the sequence graphite > ZP* > ZP, which corresponds to the increase in the $E^{\circ'}$ values.

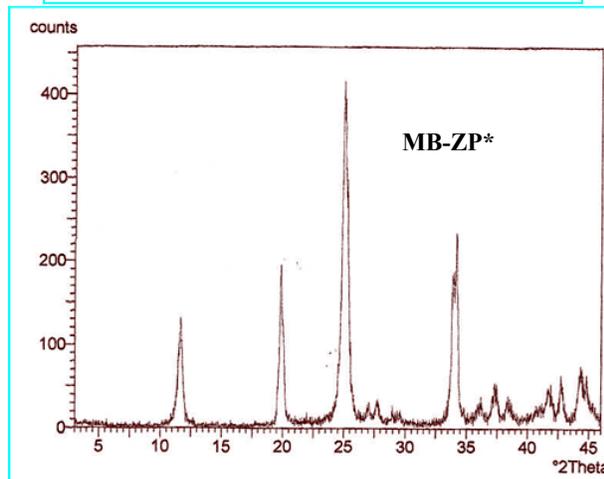
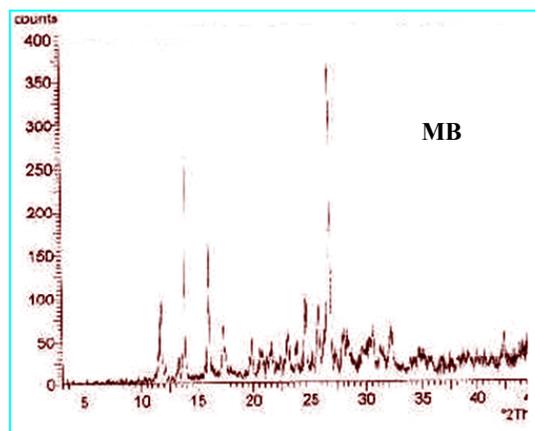
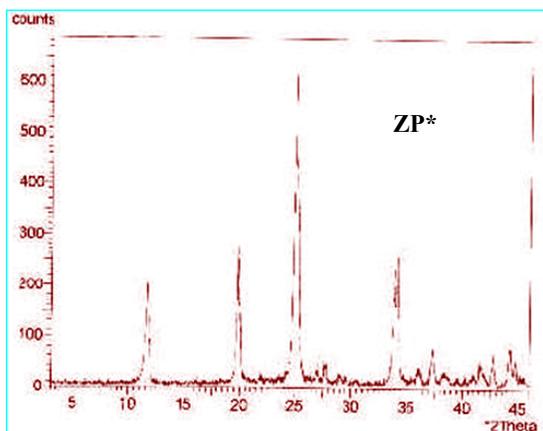


Fig. 1 – XRD spectra of ZP* (A), MB (B) and MB-ZP* (C).

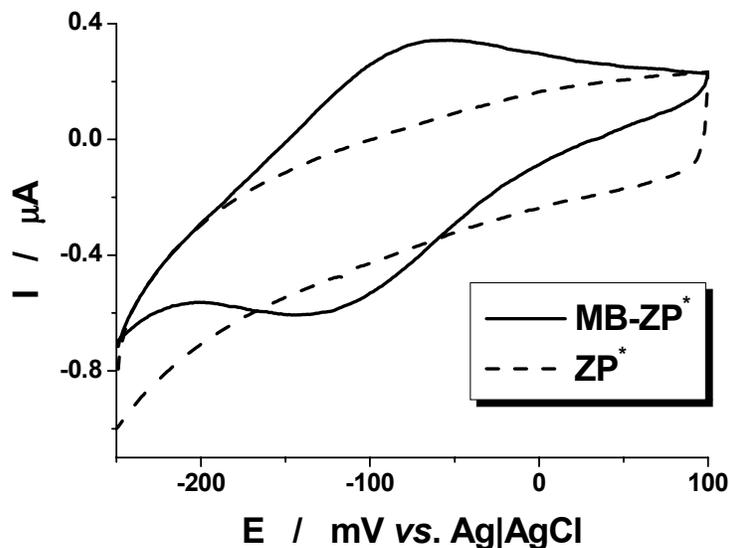


Fig. 2 – Cyclic voltammograms recorded at MB-ZP*-GCPEs (SK : MB-ZP* = 19:1 (w/w)) (A), and at ZP*-GCPEs (SK : ZP* = 19:1 (w/w)). Experimental conditions: potential scan rate, 20 mV/sec; supporting electrolyte, 0.1 M Tris buffer (pH 7).

For all previously investigated mediators, adsorbed on amorphous ZP made according to Gorton *et al.*¹¹, the $E^{\circ\prime}$ value was found practically independent on the buffer solution pH^{11, 20, 53-58}. Surprisingly, the $E^{\circ\prime}$ value for MB adsorbed on ZP* was found pH dependent (Fig. 3), in a similar manner to MB adsorbed on spectrographic graphite in the sense that $E^{\circ\prime}$ varies with pH and that at more acidic solutions the slope of the $E^{\circ\prime}$ vs pH plot varies with 60 mV per pH equivalent to a $2 e^- 2 H^+$ redox process, whereas above pH 5 the slope changes to around 30 mV per pH equivalent to a $2 e^- 1 H^+$ redox process^{27, 31, 32}. A similar behaviour was reported for New Methylene Blue (NMB)⁵⁹, Toluidine Blue^{60, 61}, Methylene Blue⁶² adsorbed on crystalline α -ZP.

The experimental data, obtained in the pH range from 1 to 11, were best fitted by a nonlinear regression described by the following equation:

$$E = E^{\circ\prime} - 0.059 pH + 0.029 \log(1 + 10^{pH - pK_a})$$

[$\chi^2 = 0.00026$; $R^2 = 0.9866$; $N = 11$), with the $pK_a = 4.3 \pm 0.4$ (Fig. 3). This calculated pK_a value is lower than that reported for MB adsorbed on spectrographic graphite ($pK_a = 5$)³¹. The difference between the pK_a values suggests that, as a consequence of the MB-ZP* interaction, the mediator adsorbed onto ZP* becomes more acidic than when adsorbed onto graphite.

The difference between the mediators behaviour adsorbed on ZP^{11, 17, 20, 55-58} and ZP*, regarding the dependence of $E^{\circ\prime}$ on pH, is not yet

fully understood. However, a connection between the crystallinity of the support and the influence of pH on the redox process involving adsorbed MB cannot be excluded. Thus, taking into account that less crystalline compounds have a smaller particle size and, in addition, the van der Waals forces between layers are decreased as the crystallinity is reduced⁶³, it can be stated that both of these features facilitate adsorption of MB in the case of ZP, making it less sensitive to the pH variation in the adjacent solution.

Electrocatalytic NADH oxidation

MB-ZP*-GCPEs, prepared with various SK: MB-ZP* ratios, showed pronounced electrocatalytic activity for NADH oxidation. Thus, the cyclic voltammograms recorded at these electrodes in the presence of NADH (Fig. 4, dashed lines) present all the features of an electrocatalytic process: the oxidation peak is drastically increased, simultaneously with the disappearance of the reduction peak.

The efficiency of the electrocatalytic oxidation of NADH at MB-ZP*-GCPEs (Eff) was calculated according to the following relation:

$$Eff = \frac{I_{cat} - I_{MB}}{I_{MB}}$$

where I_{cat} is the oxidation current intensity in the presence of 5 mM NADH and I_{MB} is the oxidation current intensity observed in its absence. The electrocatalytic efficiency increases with the increase of the SK : MB-ZP* ratio (Table 1).

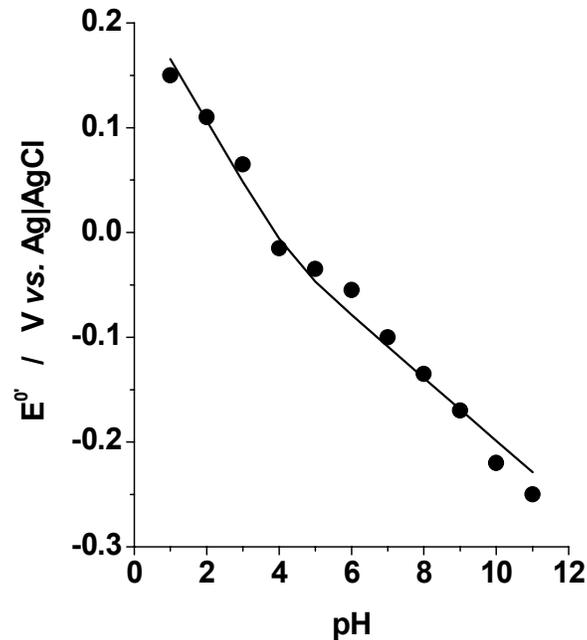


Fig. 3 – pH influence on the E° -value of MB-ZP*, entrapped in GCPEs (SK : MB-ZP* = 19:1 (w/w)). Experimental conditions: supporting electrolyte, 0.1 M Tris buffer.

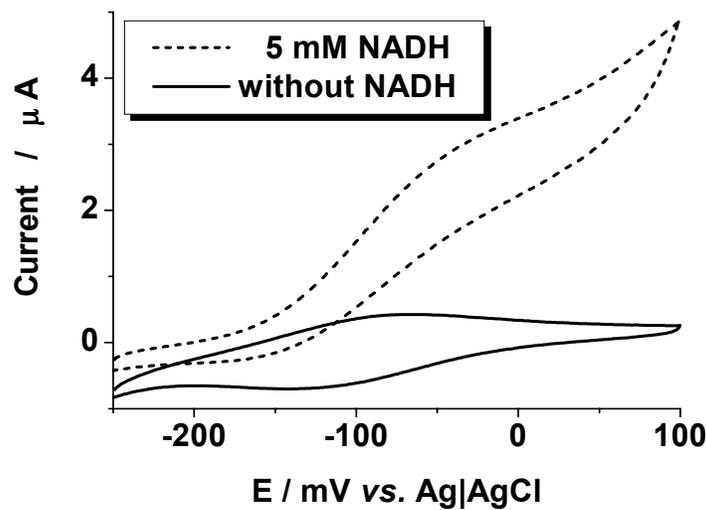


Fig. 4 – NADH electrocatalytic oxidation at MB-ZP*-GCPEs (SK : MB-ZP* = 19:1 (w/w)): no NADH (a); 5 mM NADH (b). Experimental conditions: potential scan rate, 20 mV/sec; supporting electrolyte, 0.1 M Tris buffer (pH 7).

Table 1

Electrochemical and kinetic parameters for NADH electrooxidation at MB-ZP*-GCPEs made from different ratio of glassy carbon powder SK : MB-ZP* (0.1 M Tris buffer pH 7).

Parameters	MB-ZP* : SK (w/w)			
	1:19	1:9	1:5.6	1:3
$\Gamma / \text{mol}\cdot\text{cm}^{-2} (*10^{11})$	3.27 ^b	40 ^b	95.1 ^b	814 ^b
Eff	5.7 ^b	4.6 ^b	2.7 ^b	1.1 ^b
K_M / mM	1.22 ^a	1.32 ^a	1.44 ^a	5.29 ^a
$k_{\text{obs}[\text{NADH}] = 0} / \text{M}^{-1}\cdot\text{s}^{-1} (*10^{-3})$	4.5 ^a	2.7 ^a	1.9 ^a	0.1 ^a
k_{+2} / s^{-1}	5.6 ^a	3.58 ^a	2.85 ^a	0.59 ^a

^a estimated from K-L interpretation of RDE measurements

^b estimated from CV

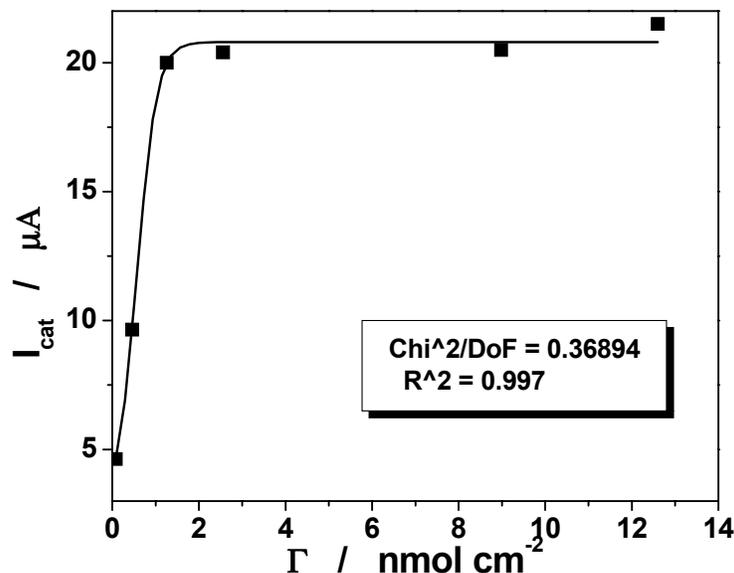


Fig. 5 – The dependence of the current for NADH electrooxidation on the surface coverage for MB-ZP*-GCPEs. Experimental conditions: 0.1 M Tris buffer (pH 7), $w = 300$ rpm; potential scan rate, 2 mV/sec.

Based on this observation, in order to establish the optimum surface coverage ensuring the highest electrocatalytic efficiency, the dependence of the catalytic current intensity on the surface coverage was investigated (Fig. 5). Above of a critical value (1.2×10^{-9} mol/cm²), the catalytic response becomes practically independent on the surface coverage. Below this value the catalytic current is almost linearly dependent on the surface coverage. Taking into account that for a surface coverage higher than what could be expected for a monolayer, additional kinetic restrictions may prevail other than the mass transfer of NADH from the solution or the reaction rate between NADH and the immobilised mediator, all RDE experiments were performed in the range of surface coverage, where the catalytic current intensity is linearly dependent on the MB surface concentration^{12, 19}.

For quantitative evaluation of the kinetic parameters of the NADH electrocatalytic oxidation at MB-ZP*-GCPEs, RDE measurements were performed at different NADH concentrations. In order to have the global electrode process controlled by the chemical reaction between NADH and MB, the applied electrode potential was set with 120 mV more positive than the E° of MB adsorbed onto the particular support material^{17, 31, 52}.

According to previous findings³¹, the reaction mechanism between NADH and MB involves an intermediate charge transfer complex, being closely related to the Michaelis-Menten kinetics.

Using the Koutecky-Levich approach (K-L), for the interpretation of the experimental results obtained using the RDE^{29, 31}, the apparent rate constant of the NADH electrocatalytic oxidation (k_{obs}) and the apparent Michaelis-Menten constant (K_M) values were estimated.

For all investigated GCPEs it was found that k_{obs} strongly depends on the NADH concentration^{29, 64}. A linear relation between the reciprocal value of k_{obs} and [NADH] was observed in the concentration range from 1 mM to 5 mM, proving that indeed the electrocatalytic reaction proceeds via an initial formation and a concurrent rate limiting decomposition of a complex between MB and NADH (results not shown). From the intercept and the slope of $1/k_{obs}$ vs. [NADH] linear regression, described by the equation

$$k_{obs} = \frac{k_{+2}}{K_M + [NADH]}$$

the extrapolated value of the apparent rate constant ($k_{obs,[NADH]=0}$) and the turnover number (k_{+2}), respectively, were calculated (Table 1).

The values of the kinetic parameters obtained for MB-ZP*-GCPEs (Table 1) are adversely influenced by the increase of the surface coverage. Thus, when the Γ values increase, the k_{+2} , as well as $k_{obs,[NADH]=0}$ values decrease, suggesting a hindered access of NADH to the bound MB molecules. At the same time, the increase in K_M values with the increase in Γ reflects a decreased affinity of MB to bind NADH. Finally, comparing the $k_{obs,[NADH]=0}$ values, obtained for the different

ratios used for MB-ZP*-GCPEs preparation, it can be concluded that electrodes prepared with an SK : MB-ZP* of 19:1 exhibit the best activity for NADH electrooxidation (Table 1).

For a similar Γ value, the turnover number estimated for MB-modified electrodes incorporating amorphous ZP (SK | MB-ZP = 1:2.3), 18-s^{-1} ²⁰, was twice higher than the value corresponding to MB-ZP*-modified electrodes (5.6 s^{-1} ; Table 1). Taking into account that $(E^{\circ}_{\text{MB}} - E^{\circ}_{\text{NADH}})_{\text{ZP}} > (E^{\circ}_{\text{MB}} - E^{\circ}_{\text{NADH}})_{\text{ZP}^*}$, this difference could be due to a higher driving force in the case of electrocatalytic oxidation of NADH at the MB-ZP-GCPEs. Surprisingly, the K_{M} value (1.22 mM; Table 1) for MB-ZP*-GCPEs was found very close to that reported for MB adsorbed on graphite (1.1 mM^{29}), and lower than that obtained for MB-ZP-GCPEs (2.5 mM^{20}). This result suggests that in the case of MB-ZP-GCPEs a hindered NADH diffusion could be responsible for the increase of K_{M} value.

Influence of PEI on NADH electrooxidation

Based on our previous results concerning the beneficial effect of the addition of PEI into the

glassy carbon paste²⁰, the PEI influence on NADH electrooxidation at MB-ZP*-GCPEs was examined, using cyclic voltammetry and RDE techniques.

As expected, the obtained results (Table 2) show a significant increase of both k_{+2} and $k_{\text{obs},[\text{NADH}]=0}$ with an increase of the PEI content in the glassy carbon paste. The true reason for the enhanced rate between NADH and the positively charged mediator is not known, but can be due either to the increase of the number of catalytic sites, or the increase of the hydride transfer rate because of a more favourable orientation of the NADH molecule, or by some combination of these effects¹.

In contrast, when the content of PEI increases the K_{M} value decreases, probably due to the effect of the positive charge of PEI on the binding affinity of the MB for NADH. A similar explanation was suggested as the cause for higher $k_{\text{obs},[\text{NADH}]=0}$ but a lower K_{M} , observed in the case of neutral mediators in the presence of Ca^{2+} ⁶⁵⁻⁶⁸.

The highest amount of PEI (2.4%, w/w) brings the $k_{\text{obs},[\text{NADH}]=0}$ value to $1.56 \times 10^4\text{ M}^{-1}\text{s}^{-1}$, which is 120% higher than that obtained for MB-ZP-GCPEs²⁰.

Table 2

Influence of PEI addition on the analytical and kinetic parameters for NADH electrooxidation at MB-ZP*-GCPEs (SK : MB-ZP* ratio was 19:1).

Parameters	PEI / % (w/w)		
	0	0.6	2.4
$\Gamma / \text{mol}\cdot\text{cm}^{-2} (*10^{11})$	3.27 ^b	7.2 ^b	21 ^b
K_{M} / mM	1.22 ^a	2.22 ^a	0.78 ^a
$k_{\text{obs},[\text{NADH}]=0} / \text{M}^{-1}\cdot\text{s}^{-1} (*10^{-3})$	4.55 ^a	4.96 ^a	15.6 ^a
k_{+2} / s^{-1}	5.6 ^a	11.0 ^a	12.3 ^a

^a estimated from K-L interpretation of RDE measurements

^b estimated from CV

CONCLUSIONS

The present study reveals that the composite electrodes based on glassy carbon particles mixed with MB immobilised onto ZP* exhibit the best characteristics (seen as high catalytic current and high catalytic rate constant) for NADH electrooxidation, when the ratio between SK and MB-ZP* was 19:1. For these modified electrodes E° was pH dependent ($1 \leq \text{pH} \leq 11$) according to the following equation:

$$E = E^0 - 0.059\text{pH} + 0.029 \log(1 + 10^{\text{pH} - \text{p}K_a})$$
 It can be stated also, that MB-ZP*-GCPEs with a ratio of SK : MB-ZP* of 19:1 and containing 2.44% (w/w) PEI showed the best behaviour for electrocatalytic oxidation of NADH, expressed by a $k_{\text{obs},[\text{NADH}]=0}$ value with 120% higher than that obtained for MB-ZP-GCPEs.

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