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Investigation of electrochemical properties of FMN and FAD adsorbed on titanium electrode

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Abstract

The electrochemical properties (such as the values of the formal potentials, the dependence of the formal potentials on solution pH, the reversibility of the electrochemical process) of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) adsorbed on a titanium electrode were dependent on the electrolyte. The formal potentials of adsorbed FMN and FAD in phosphate, HEPES and PIPES buffers at pH 7 were similar to those for dissolved flavins (− 460 to − 480 mV vs. SCE) and changed linearly with a slope of about 52 mV per pH unit in the pH region 3 to 8. In TRIS buffer, the formal potentials of adsorbed FMN and FAD were also pH-dependent, however, with invariance in the pH range 4.5 to 5.5. In non-buffered solutions (KCl, LiCl, NaCl, CsCl, $CaCl_2$, Na_2SO_4 at different concentrations), the electrochemical behavior of adsorbed FMN and FAD differed from that of dissolved flavins and was dependent on the electrolyte (especially at pH 4.5 and pH 5). Under certain conditions (electrolyte, concentration, pH), a two-step oxidation of FMN could be observed.

Keywords: Titanium; Adsorption; FMN; FAD; Riboflavin; Cyclic voltammetry

1. Introduction

Flavins such as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are coenzymes for many oxidoreductases and are known to undergo reversible redox conversions in biochemical reactions. The common electroactive moiety of FMN, FAD and related compounds, e.g., riboflavin (RF), (the structures are shown in Fig. 1) is the isoalloxazine group that can exist in three forms: the fully oxidized quinone form, the radical semiquinone (after the first electron transfer) and the fully reduced hydroquinone form (after the second electron transfer). Depending on the solution pH, each of these species can exist in either neutral or ionic forms [1,2]. The redox potentials of dissolved flavins are rather low (about − 460 mV vs. SCE at pH 7 for both FMN and FAD) and depend on solution pH. Both in acidic and basic solutions, the values of the formal potentials ($E^{\circ\prime}$) shift with a slope of 58 mV per pH unit. In neutral and weak basic media, the slope is 29 mV per pH unit [1,2]. The importance of the redox chemistry of flavin molecules in biological systems has led to the investigation of the properties of electrodes with adsorbed flavins as possible tools for bioanalytical purposes. For FAD adsorbed on graphite [3] or mercury electrode [4,5] the values of the $E^{\circ\prime}$ and the dependence of the $E^{\circ\prime}$ on solution pH followed that observed for dissolved flavins. For RF adsorbed on highly orientated pyrolytic graphite, the $E^{\circ\prime}$ changed with solution pH with a slope of 62 mV per pH unit at pH < 8.5 or 28 mV at pH>8.5 [6]. The electrochemical behavior of RF, FMN and FAD adsorbed onto silica gel modified with zirconium oxide and admixed to carbon paste was also similar to that in the solution with an exception in the case of RF when certain invariance of $E^{\circ\prime}$ was observed in the pH range 4 to 5 [7].

Investigation of the electrochemistry of FMN and FAD adsorbed on carbon fiber electrode modified with titanium dioxide obtained by titanium tetrachloride hydrolysis [8] revealed little change of the $E^{\circ\prime}$ of FMN and FAD in a pH range between 4 and 8. Besides, the $E^{\circ\prime}$ values of FMN and FAD were, respectively, − 245 and − 250 mV vs. SCE at pH 7, i.e., about 200 mV more positive than those obtained for FMN and FAD dissolved in aqueous solution or when adsorbed on graphite or mercury. Similar shifts of the $E^{\circ\prime}$s to

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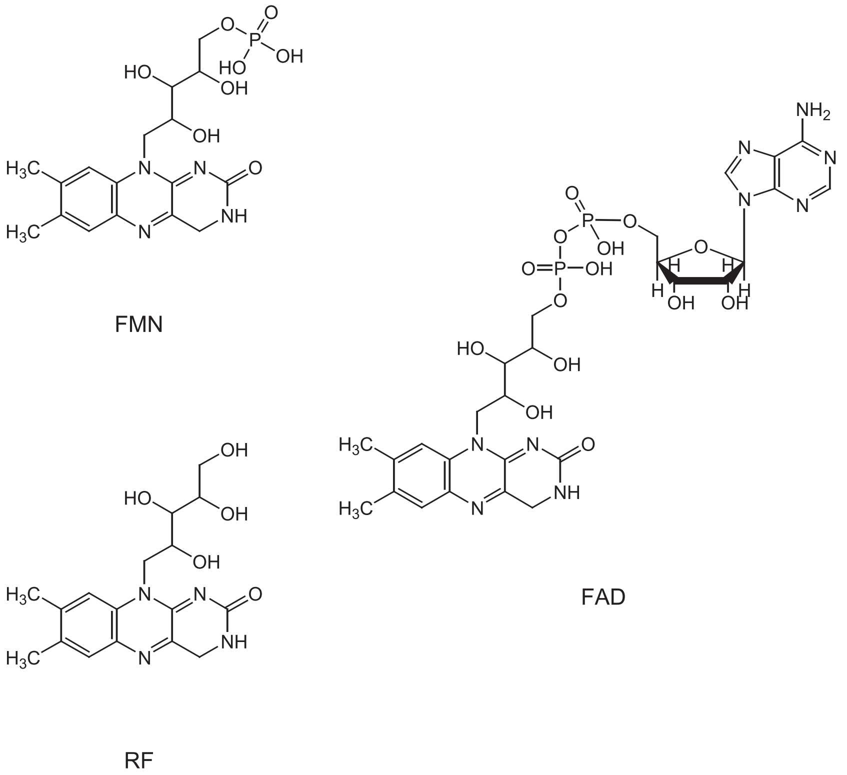

Fig. 1. Structures of RF, FMN and FAD.

more positive values and independence on solution pH were also observed for RF adsorbed on electrodes modified with zirconium phosphate [9,10] and titanium phosphate [11]. However, according to the results of the research on the electrochemical behavior of FMN and FAD adsorbed on textured titanium dioxide layer that was formed onto conductive glass by thermolysis of titanium ethoxide solutions [12], the $E^{\circ\prime}$ were found to be about -410 mV at pH 6.1, i. e, without a shift to more positive values (a detailed study of the dependence of the $E^{\circ\prime}$s on the solution pH was not presented). Thus, the mode of preparation of titanium dioxide-modified electrodes had influenced the electrochemical properties of adsorbed flavins.

The aim of this work was to investigate the electrochemical behavior of FMN, FAD and RF adsorbed onto a titanium electrode. Among numerous reports on adsorption on titanium, there are practically no electrochemical studies. This can be explained by passivation of the titanium surface with a dense layer of titanium dioxide formed spontaneously in the air or developed by anodization of titanium. Besides, titanium dioxide is known to be a semiconductor with the flat band potential of about 0 V (vs. NHE) at pH 0 and a shift of about -60 mV for each pH unit increase [13]. Cyclic voltammetry studies of the redox couples (with standard potentials more positive than the flat band potential) at titanium dioxide-modified electrodes showed only reductive currents [13]. The $E^{\circ\prime}$ values of FMN and FAD are close to the flat band potential of titanium dioxide, thus, the electrochemical reduction and oxidation of flavins adsorbed on titanium dioxide modified electrodes was possible [12]. The research was carried out using different surfaces, such as titanium covered with electrochemically grown and additionally electrochemically treated layer of titanium dioxide, titanium with etched surface (in order to remove titanium dioxide) and titanium treated with hydrogen peroxide to modify the oxide film and to produce titanium peroxy-gel [14–18]. FMN and FAD were expected to immobilize on the surface of Ti electrode by adsorption through phosphate group (due to electrostatic interaction between negatively charged phosphate and titanium oxide that was supposed to be positively charged at pH 4, i. e., below the isoelectric point of crystalline or colloidal titanium dioxide that was found to be from pH 4.6 to pH 6.4 depending on surface pretreatment [19–23]).

2. Experimental

FMN, FAD and RF were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. The supporting electrolytes were 0.1 M HEPES (2-[4-(2-hydroxyethyl)-piperazine 1-yl] ethane sulfonic acid), ICN Biomedicals, Costa Mesa, CA, USA), 0.05 M PIPES (2-[4-2-sulfoethyl)-piperazin-1-yl] ethane sulfonic acid), Sigma), 0.1 M TRIS (tris(hydroxymethyl)aminomethane hydrochloride, ICN Biomedicals), 0.1 M phosphate (KH_2PO_4, Merck, Darmstadt, Germany) buffers with or without KCl (Merck), Na_2SO_4 (Merck) and KCl, Na_2SO_4, LiCl (Merck), NaCl

(Merck), CsCl (BDH Chemicals, Poole, UK), $CaCl_2$ (Merck) solutions of various concentrations (as indicated). The pH of the solutions was adjusted by addition of either HCl or NaOH. All solutions were prepared using water purified by a Milli-Q system (Millipore, Bedford, MA, USA). The solutions were deoxygenated with Ar before use.

The Ti electrode (Russia) was polished on fine emery paper and sonicated for 2 min to remove the dust. The titanium dioxide layer was further removed by etching the electrode for 30 s in a mixture of 0.5 M H_2SO_4 and 0.2 mg/ml NaF.

In order to obtain a TiO_2 layer of different thickness, the Ti electrode was anodized in 0.5 M H_2SO_4, 0.5 M Na_2SO_4, 0.1 M, 1 M or 2 M NaOH, either galvanostatically by applying various currents (1, 2, 20, 200 mA), potentiostatically by applying various potentials (1000, 1500, 2500 mV) or potentiodynamically by sweeping the potential from − 1200 to 1500 or 2500 mV at different potential scan rates (5, 10 mV/s). Further electrochemical treatment involved cycling in the potential region between − 1500 mV (or − 1000 mV) and 0 mV at a scan rate 50 mV/s in various electrolytes (0.1 M KOH, 0.1 M KCl, 0.1 M HCl) until constant voltammograms were obtained. For the experiments with hydrogen peroxide-treated titanium electrode, the electrode was polished on emery paper, sonicated for 2 min, etched in a mixture of H_2SO_4 and NaF, washed with water and immersed into 10% H_2O_2 for 14 h at room temperature. For immobilization of FMN, FAD or RF, the pretreated Ti electrode was carefully washed with water and immediately immersed into a solution of FMN (1 mg/ml), FAD (1 mg/ml) or RF (saturated solution obtained from 1 mg in 1 ml) in HEPES at pH 4.

Electrochemical measurements were carried out using a three-electrode electrochemical cell. The titanium rod (diameter 6 mm) was fitted into a Teflon tube leaving the flat front surface in contact with the solution. The modified titanium electrode was used as the working electrode, a platinum electrode as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode. All potential values are referred to this electrode. An electrochemical analyzer (BAS 100 W, Bioanalytical Systems, West Lafayette, IN) was used connected to a PC computer for potential control and data acquisition.

3. Results and discussion

3.1. Ti electrode with electrochemically grown titanium dioxide

Any attempt to observe the electrochemistry of the flavins adsorbed on the Ti electrode covered with an air-formed or anodically grown titanium dioxide failed totally. Despite the different conditions of anodization (electrolyte, galvanostatic, potentiostatic or potentiodynamic mode of anodization, potential sweep rate, limiting potential) performed in order to obtain the titanium dioxide layer with different properties [24] and subsequent additional electrochemical treatment by cycling in the cathodical potential region supposed to change the electrochemical behavior of the passive electrode [25,26], no electrochemistry of any of the flavins could be traced.

3.2. Ti electrode with etched surface

3.2.1. Electrochemical behavior of adsorbed FMN and FAD in buffered solutions

The surface of Ti is very active after the removal of the oxide layer and can be oxidized again both by oxygen and water [27]. When the etched electrode was cycled (immediately after the removal of the oxide) in 0.1 M HEPES at various pH, 0.1 M KCl or 0.1 M HCl in the potential region − 800 to 0 mV, oxidative currents attributed to titanium dissolution and formation of an oxide layer [28,29] appeared in the potential region − 700 to − 500 mV (depending on the electrolyte). The currents decreased with each subsequent scan. If the electrode was cycled in solutions at pH < 5, the development of a new broad anodic peak in the potential region − 800 to − 600 mV was observed. The position of this peak was pH-dependent and was shifted to more negative values with increasing pH. If the electrode was cycled in a solution at pH>5 in the potential region − 800 to 0 mV this peak was not observed, however, it appeared when the electrode was transferred to a more acidic solution. Constant voltammograms were obtained after 10 to 12 potential scans. Fig. 2 (dotted line) shows the 10th scan registered in 0.1 M HEPES buffer at pH 3. According to previous investigations of the electrochemical properties of Ti, the possible reactions in this potential region are: (1) oxidation of adsorbed hydrogen, (2) oxidation of non-stoichiometric titanium oxide species [30–32].

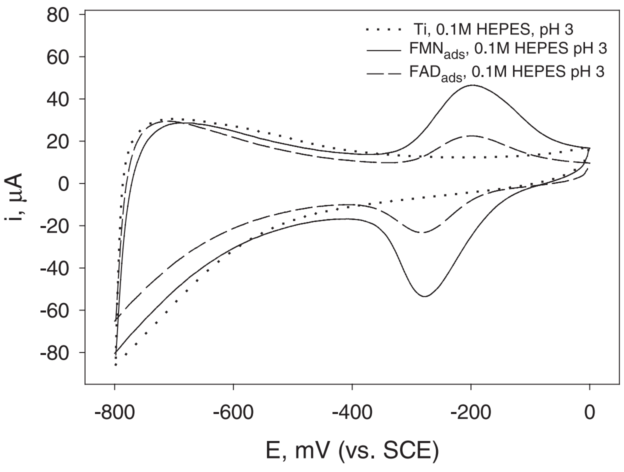

Fig. 2. Cyclic voltammograms of a non-modified Ti electrode (dotted line), a Ti electrode with adsorbed FMN (solid line) and FAD (dashed line) in 0.1 M HEPES buffer at pH 3. Potential scan rate: 50 mV/s.

The first potential cycles of Ti that has been immersed into HEPES buffer (without or with flavin) for 20 min differed from those of the Ti electrode described above. No oxidative currents were registered in the potential region − 700 to − 500 mV during the first scan in 0.1 M HEPES, 0.1 M phosphate or 0.1 M KCl at various pH values. Constant voltammograms with a broad anodic peak in the potential region − 800 to − 600 mV at pH 3 were achieved after three scans.

Constant peak currents in the voltammograms related to the electrochemistry of FMN and FAD were obtained after keeping the electrodes in the solutions of FMN and FAD for 15 min. Fig. 2 shows cyclic voltammograms of the Ti electrode with adsorbed FMN (solid line) and FAD (dashed line) in 0.1 M HEPES buffer at pH 3. In both cases the $E^{\circ\prime}$ values ($E^{\circ\prime}=(E_{pa}+E_{pc})/2$, where E_{pa} and E_{pc} are the anodic and cathodic peak potentials, respectively) were about − 240 mV. For RF, cyclic voltammograms with small peaks at similar peak potential values (not shown) were recorded when the electrode has been kept in RF solution for 2 h. The peak currents decreased with each subsequent cycle indicating very low stability of the RF-modified titanium electrode, thus confirming that phosphate moiety in the flavin was essential to obtain flavin immobilization on the titanium surface.

The stability of electrodes with adsorbed FMN or FAD was dependent on the pH of the solution. After keeping the electrode in a solution at pH 3 for 1 h, 30% to 40% of the peak current related to flavin electrochemistry was lost. If the electrode was kept in a solution at pH 8, the loss of electroactivity was much faster (85–90% after 1 h). Repetitive adsorption resulted in smaller currents, especially when the electrode was kept in phosphate buffer. Cyclic voltammograms were reproducible in terms of peak positions (the differences in the values of the $E^{\circ\prime}$ of repeated measurements did not exceed 5 mV), but not in peak height. Since it is not possible to avoid the contact with air, the air-formed Ti dioxide probably contributed to the loss of electroactivity. In the case of phosphate buffer, phosphate from solution probably competed for adsorption on the surface since it has been determined that phosphate adsorbed specifically on colloidal [33] or nanocrystalline [34] titanium dioxide. Compared with the stability of modified graphite [3] or mercury [4,5] electrodes onto which flavins were supposed to adsorb through the isoalloxazine ring and to that obtained when flavins were immobilized onto carbon fibers pretreated with titanium tetrachloride [8], the stability of the Ti electrode with immobilized FMN and FAD was significantly lower. The electrodes could be used for measurements only for about 2 days (after periodical immersion into a flavin solution in order to restore the lost amount of FMN and FAD).

The dependences of the peak currents on the potential scan rate were investigated in 0.1 M HEPES buffer at pH 3. Both for FMN and FAD, both anodic peak (i_{pa}) and cathodic (i_{pc}) currents depended on the scan rate (10 to 200 mV/s) linearly (Fig. 3, solid and dashed lines for adsorbed FMN and FAD, respectively) as expected for electroactive species immobilized onto the electrode surface. The increase in peak separation (ΔE_p) with scan rate was in both cases similar: from about 30 to 130 mV for scan rates 10 and 200 mV/s, respectively, indicating lower reversibility of the electrochemical processes with increasing scan rate. (The variation of the peak current at about − 700 mV with both scan rate and square root of scan rate was, however, not linear indicating a complex behavior of the electrochemistry of titanium oxide.)

According to the theory, for the ideal case the peak width at half height (E_{fwhm}) should be $90.6/n$ [35], where n is the number of electrons participating in the reaction. For FMN and FAD adsorbed onto titanium, the values of E_{fwhm} depended both on buffer composition and solution pH and were significantly higher (125 to 250 mV) than the theoretical values for one- or two-electron processes. The highest values (200 to 250 mV) were observed for FMN-modified electrode in TRIS buffer at pH 4 to 6.

The dependence of the $E^{\circ\prime}$ values of adsorbed FMN and FAD on buffer pH is shown in Fig. 4a and b, respectively. The values of the $E^{\circ\prime}$ of FMN and FAD in phosphate, HEPES and PIPES buffers differed 10 to 20 mV and changed with a slope of about 52 mV per pH unit. (For FAD, the dependence of the $E^{\circ\prime}$ on solution pH in phosphate buffer is shown from pH 3 to 6 (Fig. 4b, dotted line) since the peaks in cyclic voltammograms at higher pH were poorly defined and the evaluation of the $E^{\circ\prime}$ was not possible.) The effect of pH on the $E^{\circ\prime}$ of the adsorbed flavins was similar to that observed for these species in aqueous solution.

Both for adsorbed FMN and FAD, the dependences of the $E^{\circ\prime}$ on pH in TRIS buffer were not linear with certain invariance of the $E^{\circ\prime}$ in the pH region 4.5 to 5.5 (Fig. 4a, solid line and Fig. 4b, solid line, for FMN and FAD, respectively). Similar exception (an invariance of the $E^{\circ\prime}$

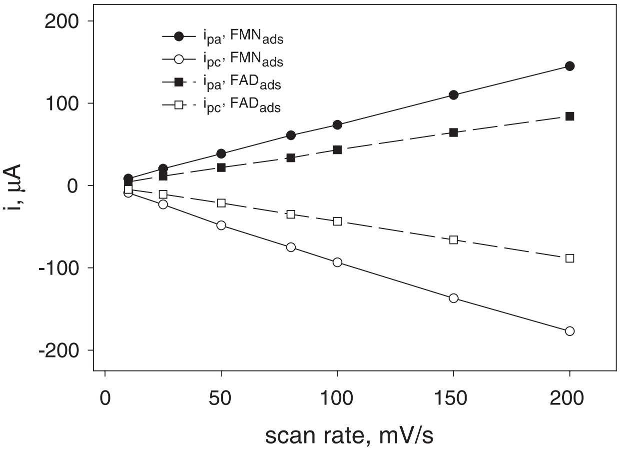

Fig. 3. Dependences of anodic and cathodic peak currents of FMN (solid lines) and FAD (dashed lines) adsorbed on a Ti electrode on potential scan rate.

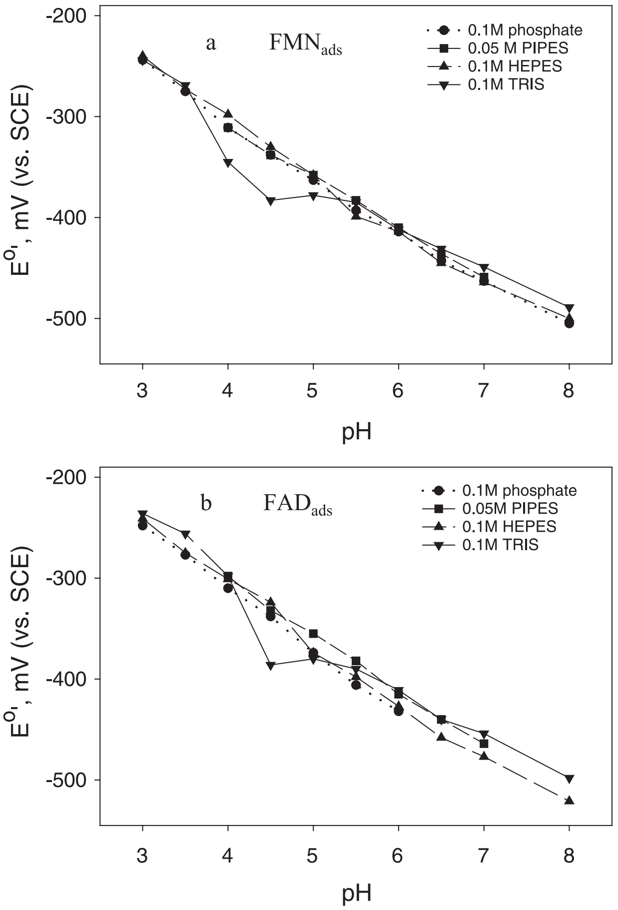

Fig. 4. (a) Dependence of the $E^{\circ\prime}$ of FMN adsorbed on a Ti electrode on solution pH in 0.1 M phosphate buffer (dotted line), 0.05 M PIPES (long-dashed line), 0.1 M HEPES (short-dashed line) and 0.1 M TRIS (solid line). (b) Dependence of the $E^{\circ\prime}$ of FAD adsorbed on a Ti electrode on solution pH in 0.1 M phosphate buffer (dotted line), 0.05 M PIPES (long-dashed line), 0.1 M HEPES (short-dashed line) and 0.1 M TRIS (solid line).

values also only in TRIS buffer at pH between 4 and 5) in the electrochemical properties of carbon paste electrodes containing FMN, FAD and RF adsorbed onto silica gel modified with zirconium oxide was observed only for RF-containing electrodes [7]. This was explained by immobilization of RF onto silica gel–zirconium oxide matrix through the isoalloxazine ring. In the case of Ti electrode, stable immobilization of RF through the isoalloxazine ring was not obtained, however, similar invariance of the $E^{\circ\prime}$ of both adsorbed FMN and FAD was observed. Probably some interaction of zirconium and titanium oxides with TRIS (different from that with phosphate, HEPES or PIPES) should not be excluded.

Addition of KCl to phosphate buffer had little effect on the $E^{\circ\prime}$ values of adsorbed FMN and FAD (Fig. 5a, dotted line and Fig. 5b, dotted line, for FMN and FAD, respectively). For PIPES (Fig. 5a, long-dashed line and Fig. 5b, long-dashed line for FMN and FAD, respectively) and HEPES (Fig. 5a, short-dashed line and Fig. 5b, short-dashed line, for FMN and FAD, respectively), addition of KCl resulted in a shift of the $E^{\circ\prime}$ of 10 to 20 mV to more positive values. The dependence of the $E^{\circ\prime}$ on pH remained the same (with a slope of 52 mV). In TRIS, a shift of the $E^{\circ\prime}$ (about 40 mV) to more positive values was observed only at pH 4 to 5 for FMN and at pH 4.5 (about 70 mV) for FAD. The dependence of the $E^{\circ\prime}$ on pH in TRIS changed with the addition of 0.1 M KCl but still was not linear (Fig. 5a, solid line and Fig. 5b, solid line, for FMN and FAD, respectively).

Although the $E^{\circ\prime}$ values of adsorbed FMN and FAD in HEPES, phosphate and PIPES buffers were similar, cyclic voltammograms in HEPES remarkably differed from those recorded in phosphate or PIPES in terms of the shifts of E_{pa} and E_{pc} values with increasing buffer pH. In HEPES buffer, two regions of pH could be observed, i.e., pH < 5 and pH>5 (Fig. 6, full symbols). In the pH region 3 to 4.5, the E_{pa} (Fig. 6a, full triangles down) of adsorbed FMN decreased from about − 200 to − 265 mV (a shift of 65 mV) whereas the shift of the E_{pc} (Fig. 6a, full triangles up) from about − 275 to − 395 mV was significantly higher (120 mV). At pH 5, both E_{pa} and E_{pc} were sharply shifted: E_{pa} to more positive

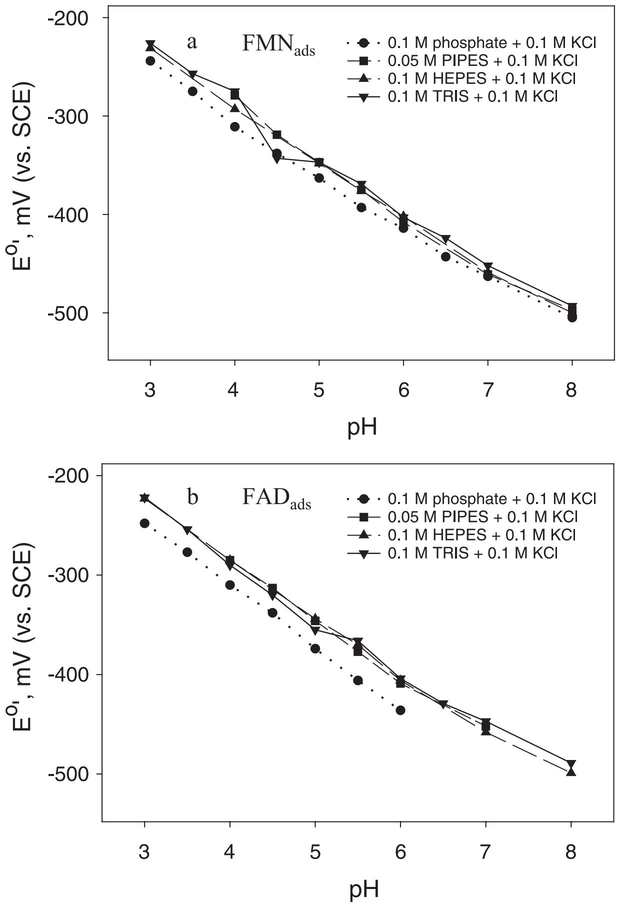

Fig. 5. (a) Dependence of the $E^{\circ\prime}$ of FMN adsorbed on a Ti electrode on solution pH in 0.1 M phosphate buffer + 0.1 KCl (dotted line), 0.05 M PIPES + 0.1 M KCl (long-dashed line), 0.1 M HEPES + 0.1 M KCl (short-dashed line) and 0.1 M TRIS + 0.1 M KCl (solid line). (b) Dependence of the $E^{\circ\prime}$ of FAD adsorbed on a Ti electrode on solution pH in 0.1 M phosphate buffer + 0.1 M KCl (dotted line), 0.05 M PIPES + 0.1 M KCl (long-dashed line), 0.1 M HEPES + 0.1 M KCl (short-dashed line) and 0.1 M TRIS + 0.1 M KCl (solid line).

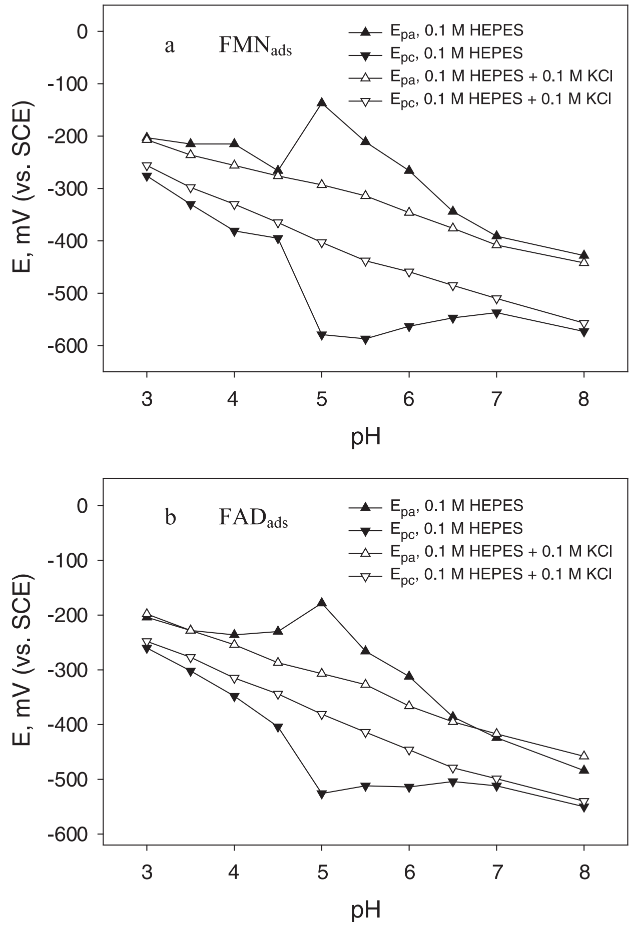

Fig. 6. (a) Dependence of the anodic (full triangles up) and cathodic peak potentials (full triangles down) of cyclic voltammograms of a Ti electrode with adsorbed FMN in 0.1 HEPES and anodic peak potentials (blank triangles up) and cathodic peak potentials (blank triangles down) in 0.1 M HEPES + 0.1 M KCl on solution pH. (b) Dependence of the anodic (full triangles up) and cathodic peak potentials (full triangles down) of cyclic voltammograms of a Ti electrode with adsorbed FAD in 0.1 HEPES and anodic peak potentials (blank triangles up) and cathodic peak potentials (blank triangles down) in 0.1 M HEPES + 0.1 M KCl on solution pH.

value of about − 140 mV and E_{pc} to more negative value of − 580 mV. In the pH region 5 to 7, E_{pa} decreased from − 140 to − 390 mV (a shift of 150 mV) while the E_{pc} increased from − 580 to − 540 mV with a lower shift (about 40 mV).

For adsorbed FAD, the dependences of the E_{pa} (Fig. 6b, full triangles up) and E_{pc} (Fig. 6b, full triangles down) on pH of HEPES buffer were very similar. The absolute values of the shifts of the E_{pa} also were lower compared with those of the E_{pc} at pH 3 to 5 and higher at pH 5 to 7 probably reflecting different interactions of the oxidized and reduced forms of FMN and FAD with the electrode surface (probably positively charged at pH < 5 and negatively charged at pH > 5). After addition of 0.1 M KCl (or 0.5 M KCl or 0.1 M Na$_2$SO$_4$), the shifts of both the E_{pa} (Fig. 6, blank triangles up) and E_{pc} (Fig. 6, blank triangles down) with pH became similar and did not differ from those in phosphate or PIPES (both with and without KCl).

Addition of KCl to HEPES buffer had a large effect on the reversibility of the redox processes of the adsorbed FMN and FAD in HEPES. At pH 5, the dependences of ΔE_p on pH of HEPES buffer had maximum values (about 440 mV for FMN and about 280 mV for FAD). After addition of 0.1 M KCl, the ΔE_p decreased indicating that the reversibility was significantly improved. The dependence of the ΔE_p on pH became almost linear with the increase from 50 mV at pH 3 to 115 mV at pH 8 and was similar to those obtained in phosphate and PIPES buffers (with and without 0.1 M KCl). If the electrodes were transferred back to HEPES buffer without any additional electrolyte again poor reversibility at pH 5 was obtained.

In TRIS (both with and without KCl), the dependence of the ΔE_p on solution pH was not linear, the lowest reversibility being at pH 4.5 to 5.5 (ΔE_p about 200 mV).

By comparing the cyclic voltammograms in the potential region of − 800 to − 600 mV, it could be seen that the current related to the electrochemistry of titanium oxide was higher after addition of KCl to HEPES buffer (Fig. 7, dashed lines) compared to those in HEPES (Fig. 7, solid lines). In

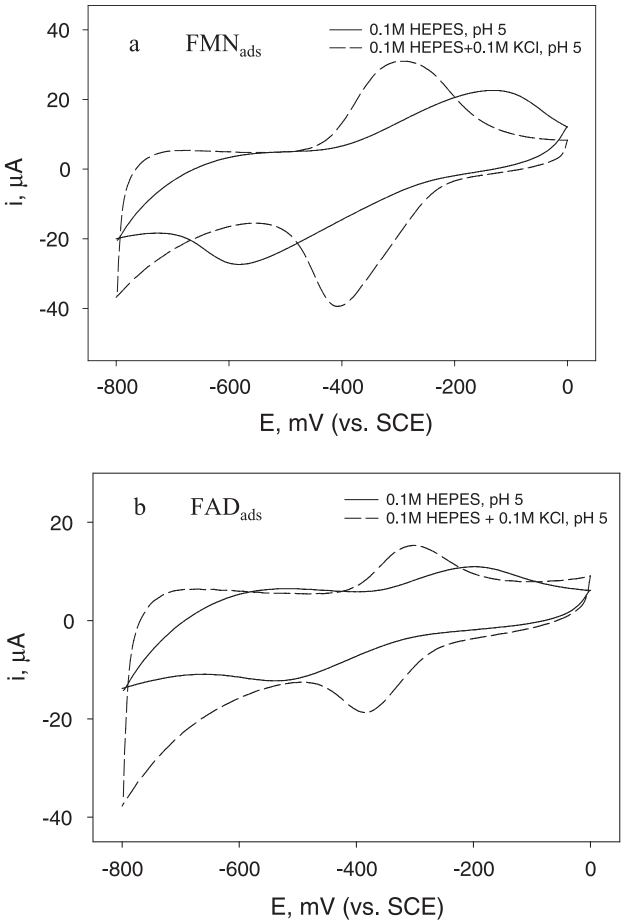

Fig. 7. (a) Cyclic voltammograms of a Ti electrode with adsorbed FMN in 0.1 M HEPES (solid line) and 0.1 M HEPES + 0.1 M KCl (dashed line) at pH 5. Potential scan rate: 50 mV/s. (b) Cyclic voltammograms of a Ti electrode with adsorbed FAD in 0.1 M HEPES (solid line) and 0.1 M HEPES + 0.1 M KCl (dashed line) at pH 5. Potential scan rate: 50 mV/s.

TRIS at pH 4.5 to 5.5 where the invariance of the $E^{\circ\prime}$ was observed, the currents related to electrochemistry of titanium oxide were also suppressed (compared to those recorded in phosphate or PIPES buffers) probably confirming some difference in the interaction of surface oxide with TRIS and/or adsorbed FMN and FAD. After addition of 0.1 M KCl to TRIS, the currents in the potential region − 800 to − 600 mV were not developed, contrary to HEPES with KCl.

These buffer composition-dependent electrochemical properties of adsorbed FMN and FAD probably were related to different adsorption of the ions from the electrolyte on the electrode surface. It has been determined that phosphate adsorbed strongly [33,34], chlorides were also detected on the titanium dioxide surface [20] whereas no adsorption of HEPES was observed [36]. Any literature data concerning adsorption of PIPES and TRIS on titanium dioxide could not be found.

3.2.2. Electrochemical behavior of adsorbed FMN and FAD in non-buffered solutions

In non-buffered solutions of various concentrations, cyclic voltammetry revealed some differences in the electrochemical properties of adsorbed FMN and FAD, especially in pH region 4 to 6. Fig. 8a and b shows cyclic voltammograms recorded for FMN- (solid lines) and FAD- (dashed lines) modified Ti electrodes in, respectively, 0.1 M KCl and 0.1 M Na_2SO_4 at pH 4. The value of the $E^{\circ\prime}$ of FMN in 0.1 M KCl was about − 300 mV, i. e., shifted by 25 mV to more negative potential compared to that of FAD (− 275 mV) whereas in 0.1 M Na_2SO_4 at pH 4 the $E^{\circ\prime}$ for both adsorbed FMN and FAD were similar (about − 295 mV). The values of E_{fwhm} for FMN were higher (170 mV) compared to that of FAD (115 mV). Besides, broad anodic peaks in the potential region − 800 to − 600 mV were more developed for the electrodes with adsorbed FAD compared to those of the electrode with adsorbed FMN probably reflecting some difference in the interaction between the electrode surface with the species in the solution and/or the adsorbed flavin. (Similar suppression of the currents in the potential region − 800 to − 600 mV was also observed in buffered solutions of 0.1 M HEPES and 0.1 M TRIS at pH 5; however, similarly both for FMN- and FAD-modified electrodes).

With increasing pH, the differences in the electrochemical behavior of adsorbed FMN and FAD became more obvious. In 0.1 M KCl at pH 4.5, the $E^{\circ\prime}$ of FMN was shifted about 230 mV to more negative potential and varied little in the pH region between 4.5 and 8 (Fig. 8a, solid line). If this was connected only with a possible decrease of pH at the electrode surface (since during the reduction of flavins protons were consumed) one could expect that the shifts of the $E^{\circ\prime}$ of adsorbed FMN or FAD in various non-buffered solutions should be similar. However, sharp shifts to more negative potential values and small variation of the $E^{\circ\prime}$ values with solution pH in more concentrated KCl (Fig. 9a) and in solutions of Na_2SO_4 at various concentrations (Fig. 9b) were observed mostly for adsorbed FMN. The pH values of the shifts in more concentrated solutions were different, i.e., pH 4 for 1 M KCl (Fig. 9a, dotted line) and pH 5 for 0.5 M Na_2SO_4 (Fig. 9b, dashed line). The $E^{\circ\prime}$ values in Na_2SO_4 solution in the pH region 5 to 8 were about 70 mV higher compared to those in KCl. For adsorbed FAD, the $E^{\circ\prime}$ decreased almost linearly in the pH range 3 to 6.5 (Fig. 10) except in the case of 0.5 M KCl (Fig. 10a, dashed line) when at pH 4.5 the $E^{\circ\prime}$ was sharply (about 220 mV) shifted to a more negative value of about − 500 mV that practically did not vary in the pH region 4.5 to 8.

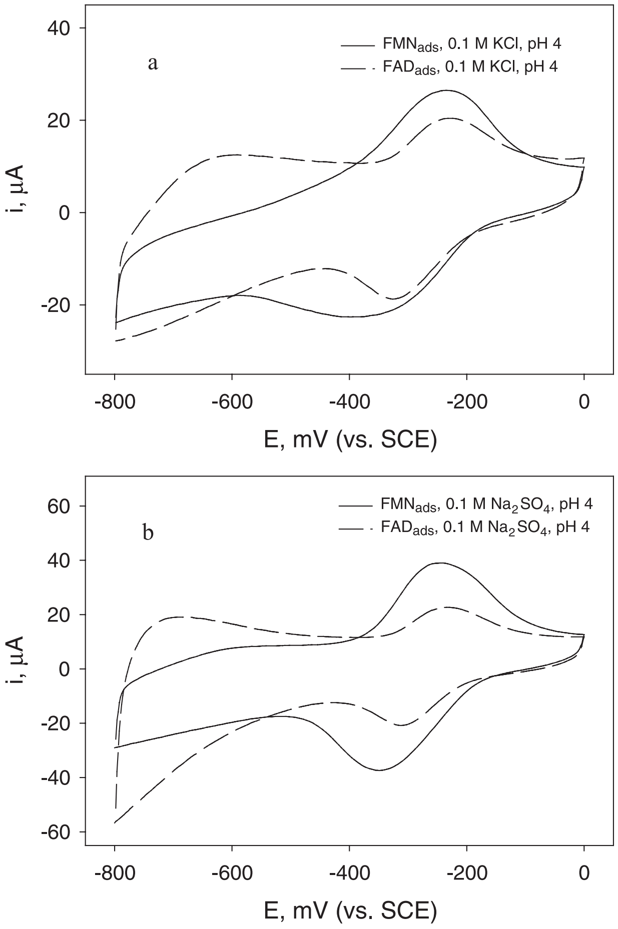

Fig. 8. (a) Cyclic voltammograms of a Ti electrode with adsorbed FMN (solid line) and FAD (dashed line) in 0.1 M KCl at pH 4. Potential scan rate: 50 mV/s. b: Cyclic voltammograms of a Ti electrode with adsorbed FMN (solid line) and FAD (dashed line) in 0.1 M Na_2SO_4 at pH 4. Potential scan rate: 50 mV/s.

There are breaks in the dependences of the $E^{\circ\prime}$ of adsorbed FMN on solution pH in 0.5 M KCl (Fig. 9a, dashed line) at pH 4.5, in 0.1 M Na_2SO_4 (Fig. 9b, solid line) at pH 4.5 and in 1 M Na_2SO_4 (Fig. 9b, dotted line) at pH 5 since the cyclic voltammograms in 0.5 M KCl at pH 4.5 (Fig. 11a, solid line), in 0.1 M Na_2SO_4 at pH 4.5 (Fig. 11a, dashed line) and in 1 M Na_2SO_4 at pH 5 (Fig. 11a, dotted line) had two poorly defined peaks at about − 420 and − 225 mV during anodic scan, indicating a two-step course of FMN oxidation. During the cathodic scan, two peaks were not resolved. For

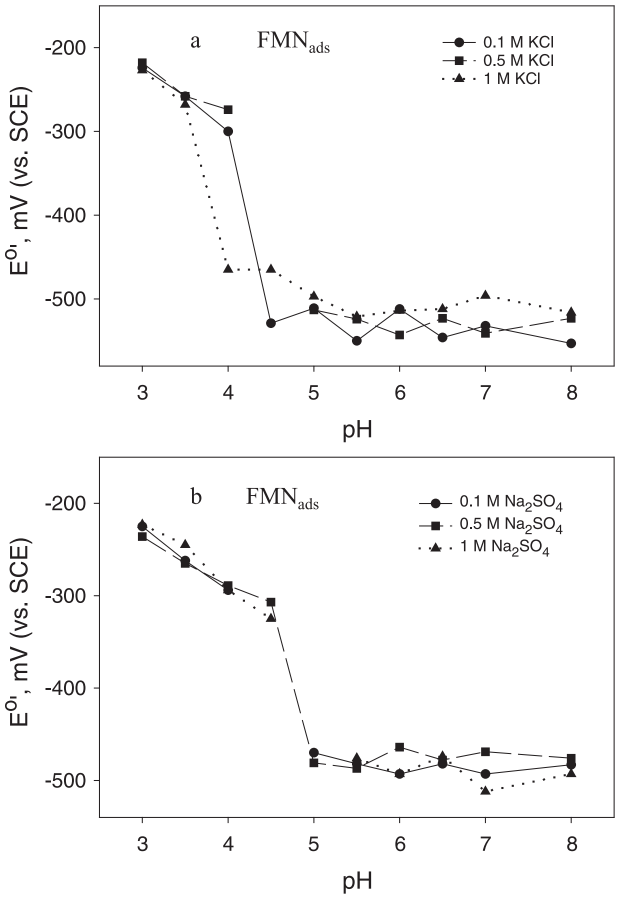

Fig. 9. (a) Dependences of the $E^{\circ\prime}$ of FMN adsorbed on a Ti electrode on solution pH in 0.1 M KCl (solid line), 0.5 M KCl (dashed line) and 1 M KCl (dotted line). (b) Dependences of the $E^{\circ\prime}$ of FMN adsorbed on a Ti electrode on solution pH in 0.1 M Na_2SO_4 (solid line), 0.5 M Na_2SO_4 (dashed line) and 1 M Na_2SO_4 (dotted line).

FAD, however, voltammograms recorded under the same conditions (and in other non-buffered solutions as well) had only one peak (Fig. 11b). Probably the orientation of the isoalloxazine ring of adsorbed FMN was favourable for the interaction with electrode surface in such a way that a semiquinone form was stabilized whereas the orientation of the isoalloxazine ring of adsorbed FAD was influenced by adenine moiety. In the case of electrodes with adsorbed FMN (Fig. 11a), the currents in the potential region − 800 to − 600 mV were suppressed at pH 4.5 in 0.5 M KCl (Fig. 11a, solid line) and 0.1 M Na_2SO_4 (Fig. 11a, dashed line) while for the electrodes with adsorbed FAD these currents were registered probably reflecting different interaction of surface oxide and immobilized flavin.

3.2.3. Effect of cation

In order to investigate the effect of cation of the electrolyte on the electrochemistry of the flavins in non-buffered chloride solutions (especially at pH 4.5 and pH 5), the experiments were also carried out in 0.1 and 0.5 M solutions of lithium, sodium, cesium and calcium chloride.

In LiCl, the electrochemical behavior of both adsorbed flavins was similar. At pH 4.5, the values of the $E^{\circ\prime}$ were sharply shifted to − 490 and − 450 mV for, respectively, FMN and FAD in 0.1 M LiCl and to lower values of − 540 and − 520 mV for, respectively, FMN and FAD in 0.5 M LiCl. In both cases the $E^{\circ\prime}$ varied little in the pH region 4.5 to 8. The differences were observed in NaCl, CsCl and $CaCl_2$. A two-step course of FMN oxidation was recorded in 0.1 M NaCl at pH 4.5 (Fig. 12a, solid line), in 0.1 M CsCl at pH 4.5 (Fig. 12a, long dashed line), in 0.5 M CsCl at pH 4.5 (Fig. 12a, medium dashed line) and in 0.5 M NaCl at pH 5 (Fig. 12a, short dashed line). In 0.1 M $CaCl_2$ at pH 4.5 (Fig. 12a, dotted line), the position of the cyclic voltammogram was very similar although two peaks were not resolved. Voltammograms of FAD-modified electrode recorded under the same conditions (Fig. 12b) did not show a similar two-step oxidation. In all these solutions the values of the $E^{\circ\prime}$ for both FMN and FAD varied little in the pH range 5 to 8 and were in the potential region − 470 to − 500 mV.

Cyclic voltammograms with double peaks obtained in various non-buffered electrolytes at different concentrations

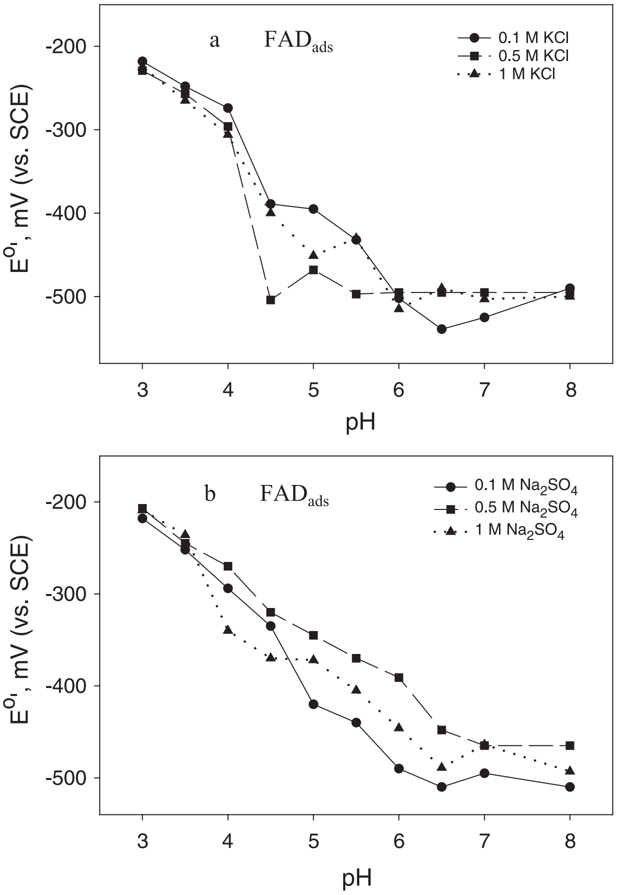

Fig. 10. (a) Dependences of the $E^{\circ\prime}$ of FAD adsorbed on a Ti electrode on solution pH in 0.1 M KCl (solid line), 0.5 M KCl (dashed line) and 1 M KCl (dotted line). (b) Dependences of the $E^{\circ\prime}$ of FAD adsorbed on a Ti electrode on solution pH in 0.1 M Na_2SO_4 (solid line), 0.5 M Na_2SO_4 (dashed line) and 1 M Na_2SO_4 (dotted line).

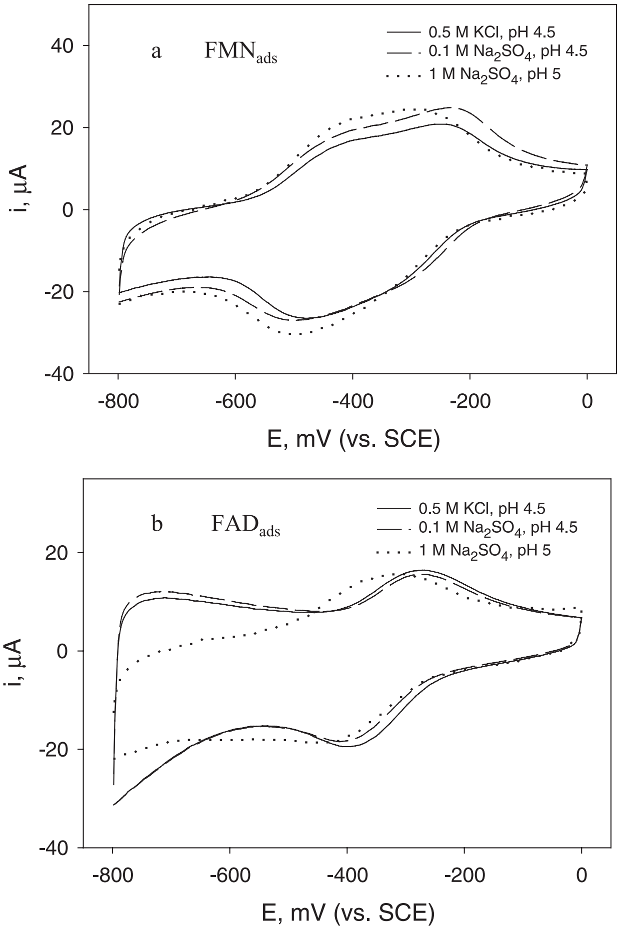

Fig. 11. (a) Cyclic voltammograms of a Ti electrode with adsorbed FMN in 0.5 M KCl at pH 4.5 (solid line), 0.1 M Na_2SO_4 at pH 4.5 (dashed line) and 1 M Na_2SO_4 at pH 5 (dotted line). Potential scan rate: 50 mV/s. (b) Cyclic voltammograms of a Ti electrode with adsorbed FAD in 0.5 M KCl at pH 4.5 (solid line), 0.1 M Na_2SO_4 at pH 4.5 (dashed line) and 1 M Na_2SO_4 at pH 5 (dotted line). Potential scan rate: 50 mV/s.

and pH values were very similar, probably indicating that under those conditions the electrode surface state was very similar and favourable for the stabilization of the semioxidized form of FMN. Probably those pH values (pH 4.5 for 0.5 M KCl, 0.1 M Na_2SO_4, 0.1 M NaCl, 0.1 M CsCl, 0.5 M CsCl and pH 5 for 1 M Na_2SO_4, 0.5 M NaCl when double peaks of adsorbed FMN in the cyclic voltammograms were recorded) were very close to the isoelectric point of titanium oxide where titanium oxide became deprotonated.

Investigation of adsorption of ions on crystalline titanium dioxide in various concentrated alkali halides [37] revealed that the isoelectric point started to shift to higher pH values with increasing ionic strength and the effect of potassium salts was more pronounced than that of corresponding lithium salts. At higher electrolyte concentrations (about 1 M), there was no isoelectric point in solutions of lithium and sodium salts (i. e., the surface titanium dioxide remained positively charged). However, for potassium and cesium salts, the isoelectric point shifted back to lower pH values. This complicated situation was related to different concentration-dependent interactions of lithium and sodium with water molecules at the interface titanium dioxide/solution (compared to those of potassium and cesium) and to their different adsorption properties. The increase of the amount of adsorbed lithium and sodium with an increase in concentration was higher than that of adsorbed potassium or cesium (depending also on the nature of the anion). The conclusion was that lithium showed a high affinity to titanium dioxide and was accumulated at the surface. The fact that the electrochemical behavior of FMN and FAD adsorbed on the titanium electrode was similar in LiCl solution (with large shift of the $E^{\circ\prime}$ to the negative direction at pH 4.5 and further invariance with solution pH) and differed from that in sodium, potassium, cesium or calcium chloride was probably somehow related to co-adsorption of ions from the electrolyte on the electrode. (Phosphate probably also was co-adsorbed on the electrode surface. The properties of both

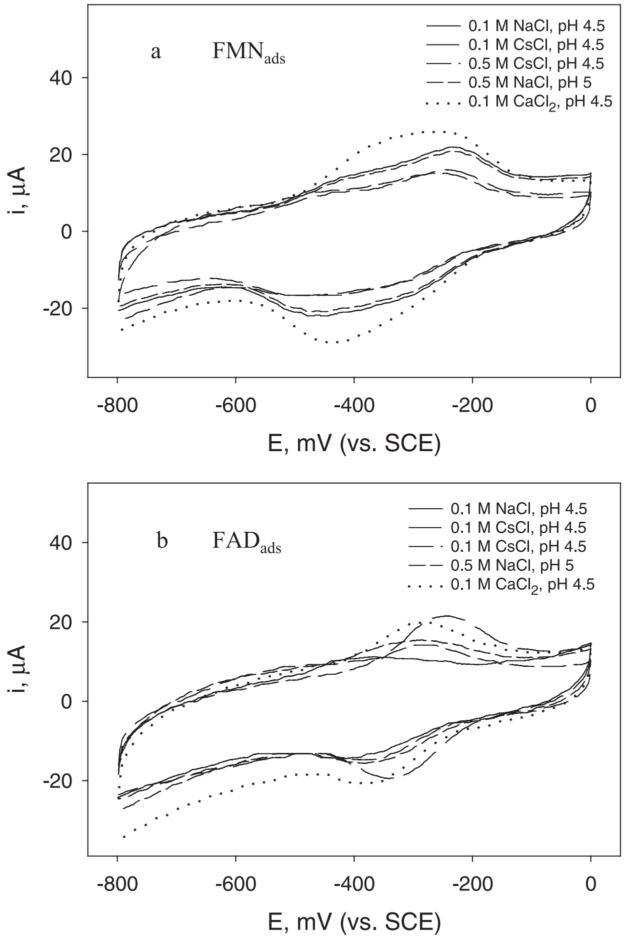

Fig. 12. (a) Cyclic voltammograms of a Ti electrode with adsorbed FMN in 0.1 M NaCl at pH 4.5 (solid line), 0.1 M CsCl at pH 4.5 (long dashed line), in 0.5 M CsCl at pH 4.5 (medium dashed line), in 0.5 M NaCl at pH 5 (short dashed line) and in 0.1 M $CaCl_2$ at pH 4.5 (dotted line). Potential scan rate: 50 mV/s. (b) Cyclic voltammograms of a Ti electrode with adsorbed FAD in 0.1 M NaCl at pH 4.5 (solid line), 0.1 M CsCl at pH 4.5 (long dashed line), in 0.5 M CsCl at pH 4.5 (medium dashed line), in 0.5 M NaCl at pH 5 (short dashed line) and in 0.1 M $CaCl_2$ at pH 4.5 (dotted line). Potential scan rate: 50 mV/s.

FMN and FAD in phosphate buffer were very similar and were practically not influenced by addition of other electrolyte.) Probably different pH of the shifts of the $E^{\circ\prime}$ values in concentrated electrolyte solutions (pH 4 for 1 M KCl and pH 5 for 0.5 M Na_2SO_4) reflected the shifts of the isoelectric points.

3.3. Hydrogen peroxide-treated Ti electrode

Cyclic voltammograms of hydrogen peroxide-treated Ti electrode with adsorbed flavins were very similar to those recorded for the etched Ti electrode with an exception that the height of the peak at about 720 mV (in 0.1 M HEPES buffer at pH 3) varied with repetitive electrode preparations indicating that the reproducibility of the electrode surface was poor. This was confirmed by different colors of the electrode surface (blue, golden, dark gray) that appeared after a few potential scans although initially all surfaces had the same gray-yellowish color. (Electrode surfaces with anodically grown titanium dioxide also had different colors due to different thickness of titanium dioxide. However, in those cases the electrochemistry of FMN or FAD was not observed.) Titanium peroxy-gel produced during the reaction of titanium with hydrogen peroxide [15–18] probably possessed more porous structure compared to that of anodically grown titanium dioxide and this allowed observing the electrochemistry of adsorbed FMN and FAD. In the case of RF, the adsorption was not stable.

The electrochemical properties of FMN and FAD adsorbed on peroxide-treated titanium electrode were similar (in terms of the $E^{\circ\prime}$ values and the dependence of the $E^{\circ\prime}$ on buffer pH and composition) to those of FMN and FAD adsorbed on the etched Ti surface, with the exception that double peaks in the cyclic voltammograms of adsorbed FMN in 0.5 M KCl, 0.1 M Na_2SO_4, 0.1 M CsCl at pH 4.5 or 0.5 M NaCl at pH 5 were not resolved (although broadening of anodic peaks in voltammmograms was observed in all these cases) and ΔE_p values were 10 to 20 mV higher. The electrodes could be used for measurements for about 2 days.

4. Conclusions

The electrochemical behavior of immobilized FMN and FAD could be observed in those cases when the passivating layer of titanium dioxide was removed from the electrode surface by etching or by treating Ti surface with hydrogen peroxide. Phosphate moiety in the flavin molecule was essential for obtaining flavin immobilization onto Ti electrode. Solution composition-dependent electrochemical behavior of adsorbed flavins probably reflected different interaction of species in solution with surface oxide and/or adsorbed flavin. The differences in the electrochemical behavior of FMN- and FAD-modified Ti electrodes in non-buffered solutions suggested that adenine moiety in FAD molecule influenced the orientation of the isoalloxazine ring since under certain conditions (electrolyte, concentration, probably at pH close to isoelectric point of titanium oxide) a two-step oxidation could be observed only for adsorbed FMN. Probably the orientation of the isoalloxazine ring in FMN molecule was affected by relatively large HEPES, PIPES, TRIS molecules; thus, in buffered solutions, a two-step oxidation was not observed. The values of the $E^{\circ\prime}$, the dependence of the $E^{\circ\prime}$ on buffer pH and the reversibility of the electrochemical processes were similar both for FMN and FAD and were dependent on buffer composition especially in pH range 4.5 to 5.5 (probably at pH values around the isoelectric point of the surface oxide) in HEPES and TRIS buffers.

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References

[1] G. Dryhurst, Electrochemistry of Biological Molecules, Academic Press, London, 1977, pp. 365–391, Chap. 7.

[2] O.S. Ksenzhek, S.A. Petrova, Electrochemical properties of flavins in aqueous solutions, Bioelectrochem. Bioenerg. 11 (1983) 105–127.

[3] L. Gorton, G. Johansson, Cyclic voltammetry of FAD adsorbed on graphite, glassy carbon, platinum and gold electrodes, J. Electroanal. Chem. 113 (1980) 151–158.

[4] V.I. Birss, H. Elzanovska, R.A. Turner, The electrochemical behavior of flavin adenine dinucleotide in neutral solutions, Can. J. Chem. 66 (1988) 86–96.

[5] M.M. Kamal, H. Elzanovska, M. Gaur, D. Kim, V.L. Birss, Electrochemistry of adsorbed flavin adenin dinucleotide in acidic solutions, J. Electroanal. Chem. 318 (1991) 349–367.

[6] J. Zhang, Q. Chi, S. Dong, E. Wang, Orientation and electrocatalysis of riboflavin adsorbed on carbon substrate surfaces, J. Chem. Soc., Faraday Trans. 92 (1996) 1913–1920.

[7] M. Yamashita, S.S. Rosatto, L.T. Kubota, Electrochemical comparative study of riboflavin, FMN and FAD immobilized on the silica gel modified with zirconium oxide, J. Braz. Chem. Soc. 13 (2002) 635–641.

[8] L.T. Kubota, L. Gorton, A. Roddick-Lanzilotta, A.J. McQuillan, Electrochemical behaviour of FAD and FMN immobilized on TiO_2 modified carbon fibers supported by ATR-IR spectroscopy of FMN on TiO_2, Bioelectrochem. Bioenerg. 47 (1998) 39–46.

[9] L.T. Kubota, L. Gorton, Electrochemical investigations of the reaction mechanism and kinetics between NADH and riboflavin immobilised on amorphous zirconium phosphate, J. Solid State Electrochem. 3 (1999) 370–379.

[10] A. Malinauskas, T. Ruzgas, L. Gorton, Tuning the redox potential of riboflavin by zirconium phosphate in carbon paste electrodes, Bioelectrochem. Bioenerg. 48 (1999) 21–27.

[11] L.T. Kubota, F. Munteanu, A. Roddick-Lanzilotta, A.J. McQuillan, L. Gorton, Electrochemical investigation of some aromatic redox mediators immobilized on titanium phosphate, Quim. Anal. 19 (2000) 15–27.

[12] H. Shinohara, M. Grätzel, N. Vlachopoulos, M. Aizawa, Interfacial electron transfer of flavin coenzymes and riboflavin adsorbed on textured TiO_2 films, Bioelectrochem. Bioenerg. 26 (1991) 307–320.

[13] R.N. Noufi, P.A. Kohl, S.N. Frank, A.J. Bard, Semiconductor electrodes XIV. Electrochemistry and electroluminescence at n-type TiO_2 in aqueous solutions, J. Electrochem. Soc. 125 (1978) 246–252.

[14] J. Pan, D. Thierry, C. Leygraf, Electrochemical impedance spectroscopy study of the passive oxide film on titanium for implant application, Electrochim. Acta 41 (1996) 1143–1153.

[15] P. Tengvall, H. Elwing, I. Lundstrom, Titanium gel made from metallic titanium and hydrogen peroxide, J. Colloid Interface Sci. 130 (1989) 405–413.

[16] P. Tengvall, T.P. Vikinge, I. Lundstrom, B. Liedberg, FT-Raman spectroscopic studies of the degradation of titanium peroxy gels made from metallic titanium and hydrogen peroxide, J. Colloid Interface Sci. 160 (1993) 10–15.

[17] P. Tengvall, B. Walivara, J. Westerling, I. Lundstrom, Stable titanium superoxide radicals in aqueous Ti-peroxy gels and Ti-peroxy solutions, J. Colloid Interface Sci. 143 (1991) 589–592.

[18] M.R. Ayers, A.J. Hunt, Titanium oxide aerogels prepared from titanium metal and hydrogen peroxide, Mater. Lett. 34 (1998) 290–293.

[19] G.D. Parfitt, J. Ramsbotham, C.H. Rochester, An electrophoretic investigation of the effect of chloride and of solanol groups on the properties of the surface of rutile, J. Colloid Interface Sci. 41 (1972) 437–444.

[20] R. Sprycha, Surface charge and adsorption of background electrolyte ions at anatase/electrolyte interface, J. Colloid Interface Sci. 102 (1984) 173–185.

[21] M. Kosmulski, J.B. Rosenholm, Electroacoustic study of adsorption of ions on anatase and zirconia from very concentrated electrolytes, J. Phys. Chem. 100 (1996) 11681–11687.

[22] D.E. MacDonald, B. Markovic, A.L. Boskey, P. Somasundaran, Physico-chemical properties of human fibronectin binding to well characterized titanium dioxide, Colloids Surf., B Biointerfaces 11 (1998) 131–139.

[23] P.A. Connor, K.D. Dobson, A.J. McQuillan, Infrared spectroscopy of the TiO_2/aqueous solution interface, Langmuir 15 (1999) 2402–2408.

[24] Y.T. Sul, C.B. Johansson, Y. Jeong, T. Albrektsson, The electrochemical oxide growth behaviour on titanium in acid and alkaline electrolytes, Med. Eng. Phys. 23 (2001) 329–346.

[25] T. Clark, D.C. Johnson, Activation of titanium electrodes for voltammetric detection of oxygen and hydrogen peroxide in alkaline media, Electroanalysis 9 (1997) 273–278.

[26] E.M. Oliveira, C.E.B. Marino, S.R. Biaggio, R.C. Rocha-Filho, Reactivation of passive titanium: the enhancement of O_2 evolution after potentiodynamic cyclings, Electrochem. Commun. 2 (2000) 245–258.

[27] A.M. Shams El Din, H.A. El Shaeb, F.M. Abd El Wahab, Stability of titanium tubes towards acid wash, J. Electroanal. Chem. 214 (1986) 567–587.

[28] T.R. Beck, Electrochemistry of freshly generated titanium surfaces: I. Scraped rotating disk experiments, Electrochim. Acta 18 (1973) 807–814.

[29] T.R. Beck, Electrochemistry of freshly generated titanium surfaces—II. Rapid fracture experiments, Electrochim. Acta 18 (1973) 815–827.

[30] C.K. Dyer, J.S.L. Leach, Reversible reactions within anodic oxide films on titanium electrodes, Electrochim. Acta 23 (1978) 1387–1394.

[31] C.P. De Pauli, M.C. Giordano, J.O. Zerbino, Elipsometric response of titanium electrodes in sulphate acid solutions under different potential perturbations, Electrochim. Acta 28 (1983) 1781–1788.

[32] O.R. Camara, C.P. de Pauli, M.C. Giordano, Potentiodynamic behaviour of mechanically polished titanium electrodes, Electrochim. Acta 29 (1984) 1111–1117.

[33] P.A. Connor, A.J. McQuillan, Phosphate adsorption onto TiO_2 from aqueous solutions: an in situ internal reflection infrared spectroscopic study, Langmuir 15 (1999) 2916–2921.

[34] B.P. Nelson, R. Candal, R.M. Corn, M.A. Anderson, Control of surface and ζ potentials on nanoporous TiO_2 films by potential determining and specifically adsorbed ions, Langmuir 16 (2000) 6094–6101.

[35] R.W. Murray, in: A.J. Bard (Ed.), Electroanalytical Chemistry, vol. 13, Dekker, New York, 1984, pp. 191–368.

[36] A.D. Roddick-Lanzilotta, P.A. Connor, A.J. McQuillan, An in situ infrared spectroscopic study of the adsorption of lysine to TiO_2 from aqueous solution, Langmuir 14 (1998) 6479–6484.

[37] M. Kosmulski, J.B. Rosenholm, Electroacoustic study of adsorption of ions on anatase and zirconia from very concentrated electrolytes, J. Phys. Chem. 100 (1996) 11681–11687.