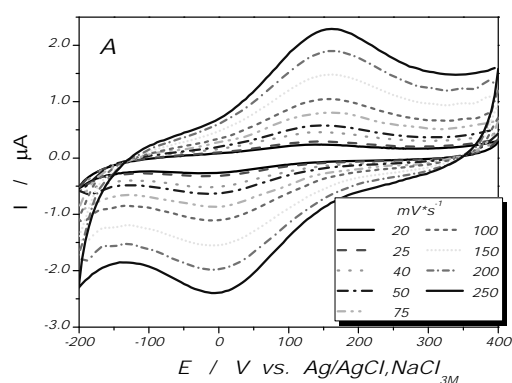# ELECTROCHEMICAL CHARACTERIZATION
OF Au/L-CYSTEINE/HEMIN MODIFIED ELECTRODE

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Iron(III) protoporphyrin IX (hemin, Hm) was successfully immobilized by simple adsorption on the surface of L-cysteine modified gold (Au/L-Cys) electrode. The electrochemical behavior of the resulting Au/L-Cys/Hm electrode was investigated by using cyclic voltammetry (CV) and square-wave voltammetry (SWV) under different experimental conditions (scan rate/ frequency and pH). The pH dependence of the formal standard potential, observed from SWV measurements, allowed estimation of the value of the acid dissociation constant of the adsorbed Hm ($pK_a = 6.25 \pm 0.09$), value which was found lower than that reported for dissolved Hm ($pK_a = 6.63$). Irrespective of the pH of the surrounding solution, CV and SWV measurements carried out at Au/L-Cys/Hm electrode gave similar values for the rate constant of the charge transfer process ($k_s = \sim0.6$ s^{-1}, at pH 6). The k_s values are significantly lower than those corresponding to Hm adsorbed onto the surface of a graphite electrode and finally covered with a chitosan matrix (~3.4 s^{-1}, at pH 5.7).
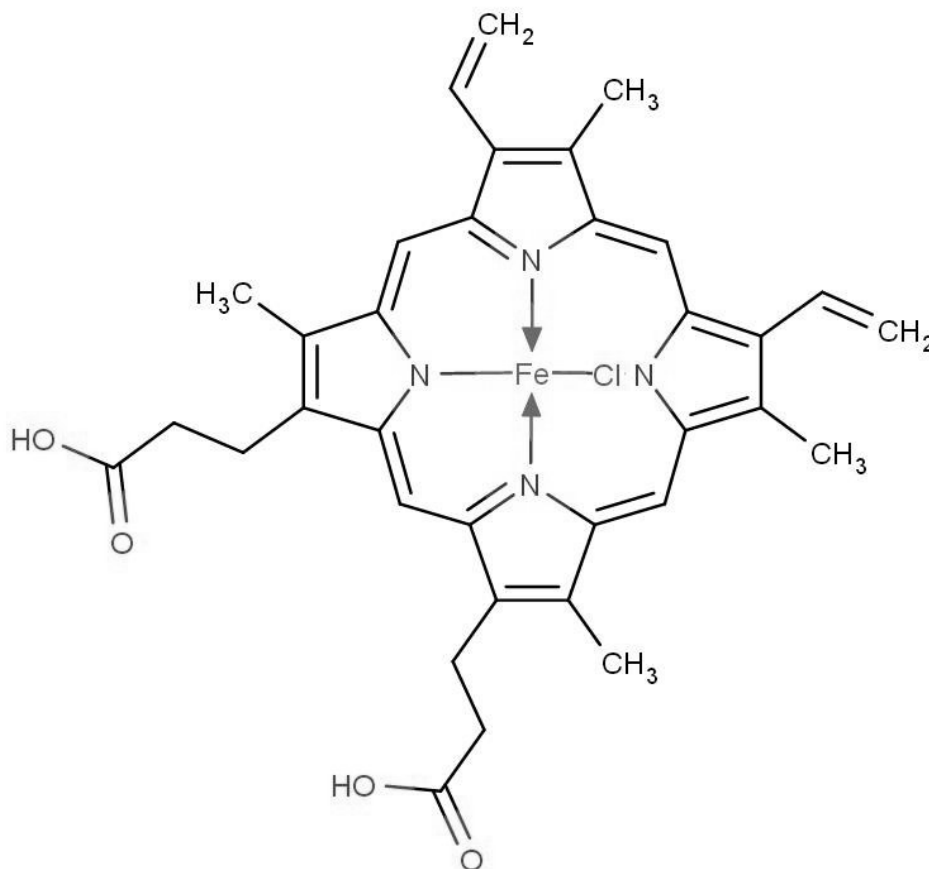
INTRODUCTION

Hemin (iron(III) protoporphyrin IX, Hm), an important natural metalloporphyrin (Scheme 1), is the active center for several families of heme-proteins such as peroxidases, catalases, hemoglobins, myoglobins etc.[1] Due to the presence of the Fe(III)/Fe(II) redox couple, hemin,[2-14] myoglobin[15,16] and hemoglobin[17,18] exhibits excellent electrocatalytic properties, which were intensively exploited for the mediated electrochemical reduction of several interesting molecules as for example: oxygen,[2-6] nitrite,[8,17] nitric oxide,[14] hydrogen peroxide,[3,7-12,17,18] glucose,[7] phenols,[11,19] tryptophan,[10] ascorbic acid,[10] uric acid,[10] dopamine,[10] and L-tyrosine.[13] That is why, Hm was intensively used as electrocatalyst for the construction of transducers able to detect amperometrically the above mentioned chemical species.

Various approaches were proposed for Hm immobilization onto the surface of conventional electrodes such as:

(i) Physical adsorption[3,5,8,10,12,17,20]

(ii) Incorporation within polymeric matrix,[14] carbon paste[2] or lipidic films;[9]

(iii) Covalent binding.[7]

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Scheme 1 – Structural formula of hemin.

The so obtained Hm modified electrodes exhibit relatively stable and efficient electrocatalytic activity. However, due to the intrinsic drawbacks of the electrocatalyst immobilization (as for example: Hm molecules are randomly deposited at the electrode/electrolyte interface and, consequently, they exhibit a non-uniform accessibility to the substrate; the interactions existing between the Hm redox centers within the electroactive matrix promote the electrochemical irreversibility of the Fe(III)/Fe(II) couple, etc.), an accurate electrochemical characterization of these modified electrodes is rather difficult.

In this context, we report here on a new approach to obtain Hm-modified electrodes, based on the Hm adsorption on a film of L-cysteine (L-Cys), self-assembled on Au electrodes. The electrochemical behavior of Au/L-Cys/Hm modified electrode was investigated by using cyclic voltammetry (CV) and square-wave voltammetry (SWV) measurements, performed under different experimental conditions. The immobilization of Hm on the monomolecular layer of L-Cys formed onto Au surface allowed the

estimation of its acid dissociation constant as well as the rate constant of the charge transfer process.

RESULTS AND DISCUSSION

Comparing the cyclic voltammograms recorded at low potential scan rate (25 mV/s) at bare Au, Au/L-Cys and Au/L-Cys/Hm electrodes, using phosphate buffer as supporting electrolyte (Fig. 1), in the case of the Au/L-Cys/Hm modified electrode it can be clearly noticed the characteristic wave of Hm.[14,21,22] Thus, it is proved without any doubt that Hm is adsorbed onto the L-Cys film deposited onto the Au/L-Cys electrode.

The stable and well-defined peaks pair observed for the immobilized Hm points out to a quasi-reversible charge transfer process (the width at half-height peak $> 90.6/n$ mV, $n = 1$; the peak current ratio, $I_{ap}/I_{cp} > 1$).[23] The value of the formal standard potential ($E^{0'}$) of the adsorbed Hm ($E^{0'} = -70$ mV *vs.* Ag/AgCl,NaCl$_{3M}$; pH 7), estimated as the half sum of the anodic (E_{pa}) and cathodic (E_{pc}) peak potentials, was found in good agreement with the already reported value.[12,21,22]

Voltammetric measurements carried out at different potential scan rates (CV) or frequencies (SWV) (Fig. 2) showed, in both cases, that the increase of the potential scan rate or frequency induces a progressive shift of the peak potentials towards higher absolute values. This feature reconfirms the quasi-reversible behavior of the charge transfer process involving the Fe(III)/Fe(II) couple from Hm, described by the following equation:[5,21,24]

$$Hm\text{-}Fe(III) + e^- + H^+ \leftrightarrows Hm\text{-}Fe(II) \quad (1)$$

As is expected for a surface confined redox couple[25] and irrespective of the surrounding pH, the anodic ($I_{p,a}$) and cathodic ($I_{p,c}$) peak currents depend linearly on the potential scan rate (v) in the investigated range of the potential scan rate (20 – 250 mV/s) (Fig. 3A). Similarly, the SWV measurements showed that the anodic ($I_{p,a}$) and cathodic ($I_{p,c}$) peak currents are linearly dependent on the square root of the frequency (Fig. 3B), confirming the above mentioned statement. The absence of a monotone variation of the pic currents

with the pH observed at Au/L-Cys/Hm electrode should be due to the complexity of the pH effect on the interaction involved in the Hm adsorption on L-Cys film.

Additionally, using the data provided by CV measurements, the immobilization of Hm on the Au/L-Cys modified surface was confirmed again. Thus, irrespective of the pH value, the slopes values of the log – log dependences of peak currents on the potential scan rate (Table 1) are close to 1. This parameter is considered a relevant criterion to distinguish between the voltammetric response of a surface confined redox couple (slope close to 1) and that corresponding to a dissolved one (slope ~0.5).[25] Furthermore, it is worth to mention that, in their turn, SWV measurements point out that hemin behaves as a redox couple strongly immobilized on the graphite surface. Thus, as expected for a strongly adsorbed quasi-reversible redox couple,[26] the peak currents depend linearly on the square root of the applied frequency(ν) (Fig. 3B).
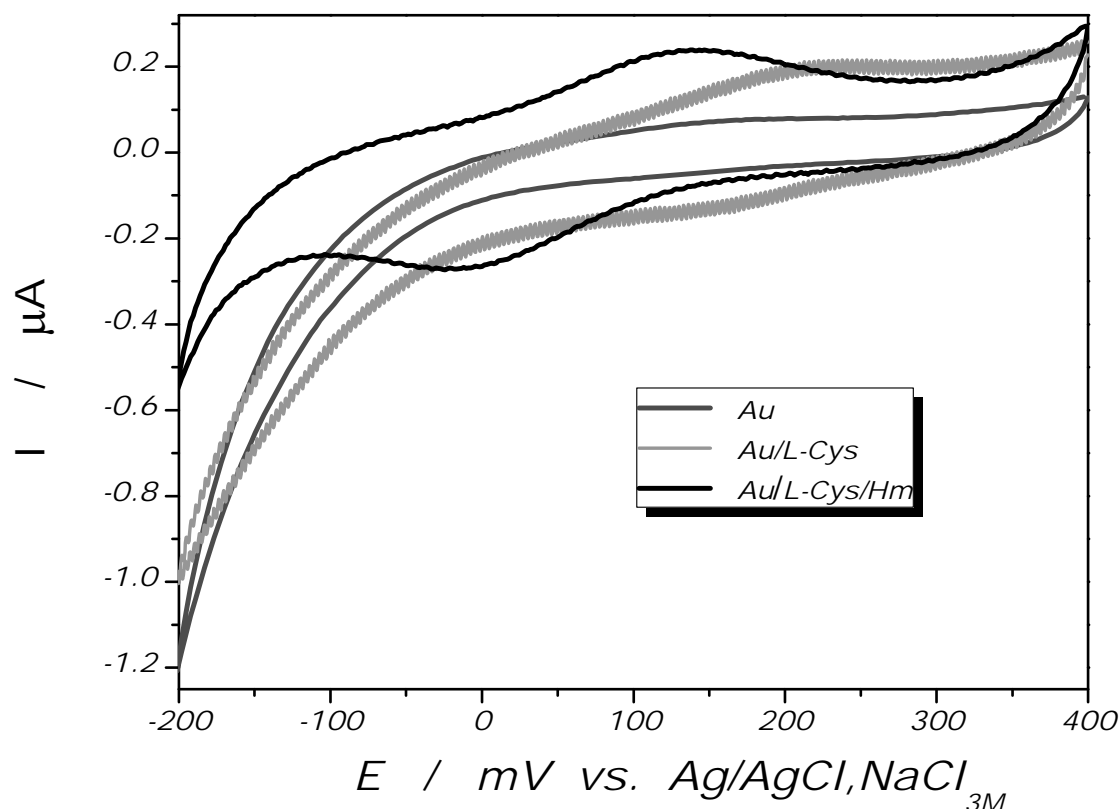

Fig. 1 – Cyclic voltammograms recorded at Au (**red**), Au/L-Cys (**green**) and Au/L-Cys/Hm (**black**) electrodes. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer (pH 6.9); starting potential, 0.4 V *vs.* Ag/AgCl,NaCl$_{3M}$; potential scan rate, 25 mV/s; deaerated solutions.
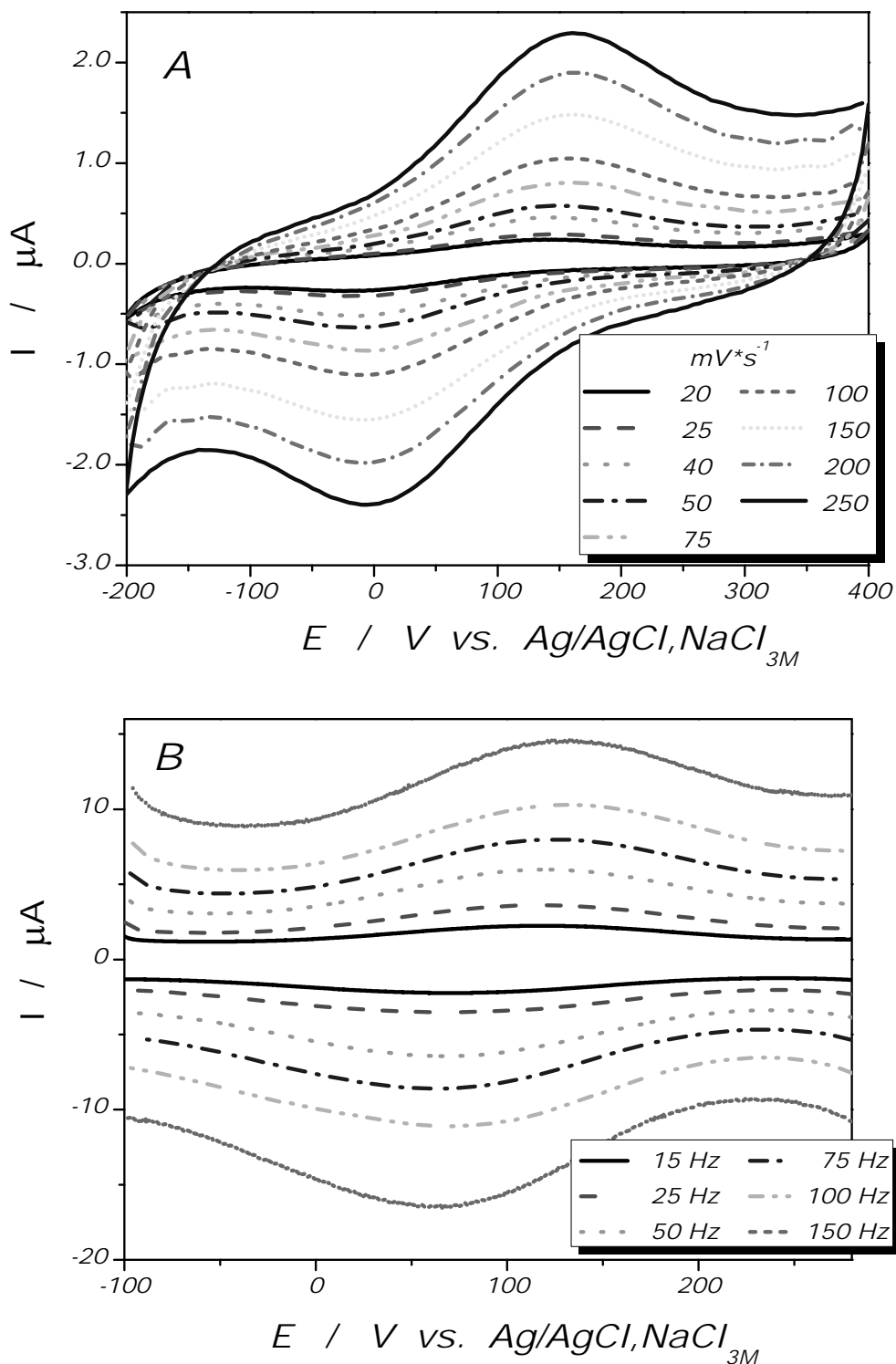
Fig. 2 – Influence of the potential scan rate on the voltametric response recorded at Au/L-Cys/Hm modified electrode: **(A)** CV; **(B)** SWV. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer (pH 6.9); starting potential, 0.4 V *vs.* Ag/AgCl,NaCl$_{3M}$; SWV amplitude, 50 mV; deaerated solutions.
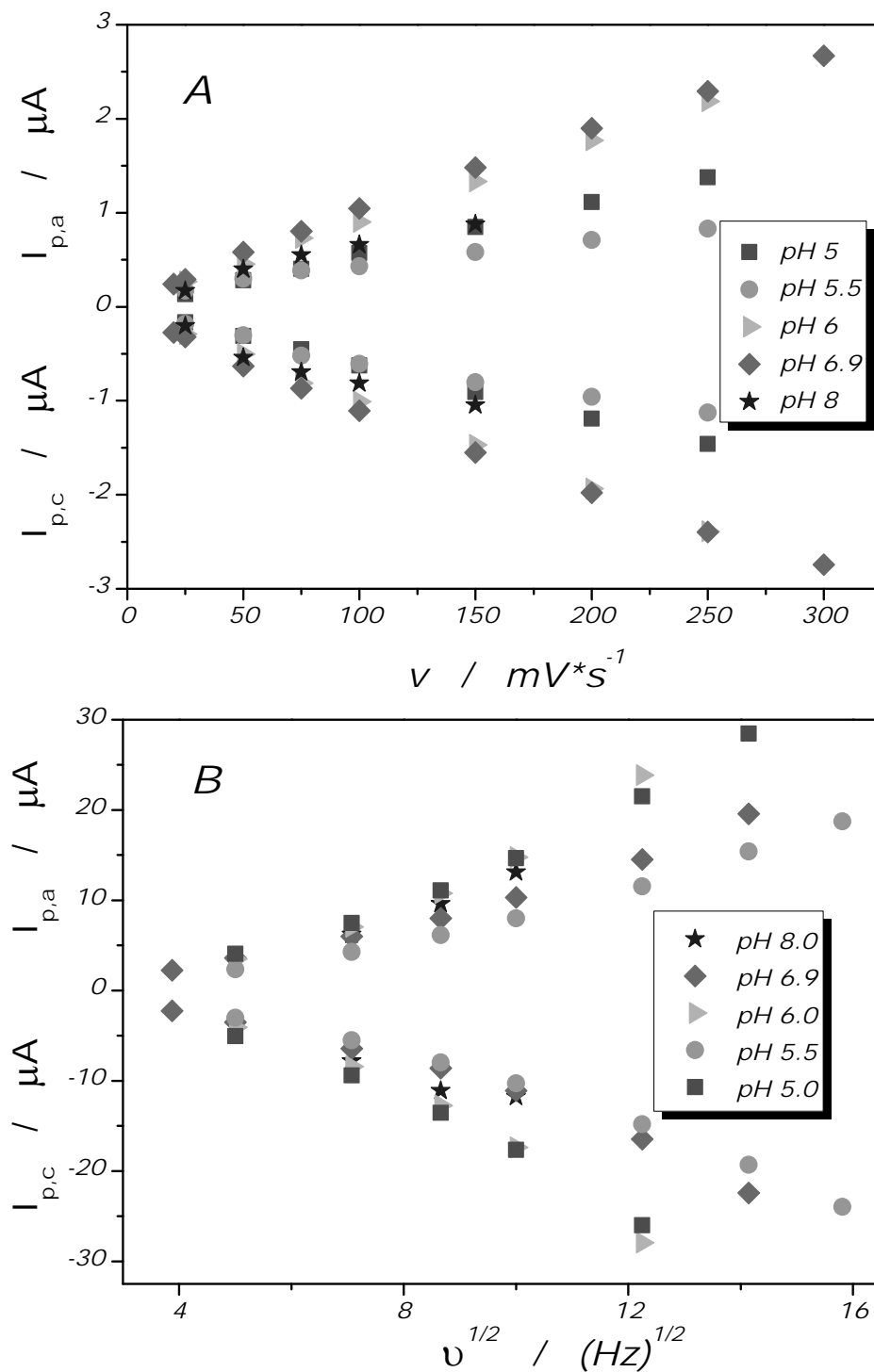
Fig. 3 – Potential scan rate (**A**) and frequency (**B**) dependences of the peak currents measured at Au/L-Cys/Hm modified electrode: (**A**) CV and (**B**) SWV measurements. Experimental conditions: see Fig. 2.

Table 1

Slopes of the $\log(I_p)$ *vs.* $\log(v)$ dependences observed for Au/L-Cys/Hm modified electrode (pH = 5.0, 6.0, 6.9, 8.0)

pH	Slopes of the $\log(I_p)$ – $\log(v)$ dependences			
	anodic	R^2 / N	cathodic	R^2 / N
5.0	1.01 ± 0.01	0.9989 / 5	0.95 ± 0.02	0.9994 / 7
6.0	0.92 ± 0.02	0.9972 / 7	0.92 ± 0.01	0.9976 / 7
6.9	0.89 ± 0.01	0.9991 / 6	0.86 ± 0.01	0.9992 / 9
8.0	0.91 ± 0.08	0.9681 / 4	0.90 ± 0.12	0.9265 / 5

Qualitatively, both CV and SWV measurements used to investigate the electrochemical behavior of Au/L-Cys/Hm modified electrode showed a positive shift of the formal redox potential ($E^{0'}$) when the pH of the supporting electrolyte decreases (Fig. 4).

Quantitatively, in the investigated domain of pH (from 5 up to 8), the slope value corresponding to the E_0' *vs.* pH dependence, obtained from CV measurements [(-0.040 ± 0.004) V/pH, with R^2 = 0.9751, N = 4; Fig. 4A), is relatively close to the theoretical value (-0.059 V/pH), expected for a redox process involving the transfer of $1e^-/1H^+$ [see eqn. (1)].
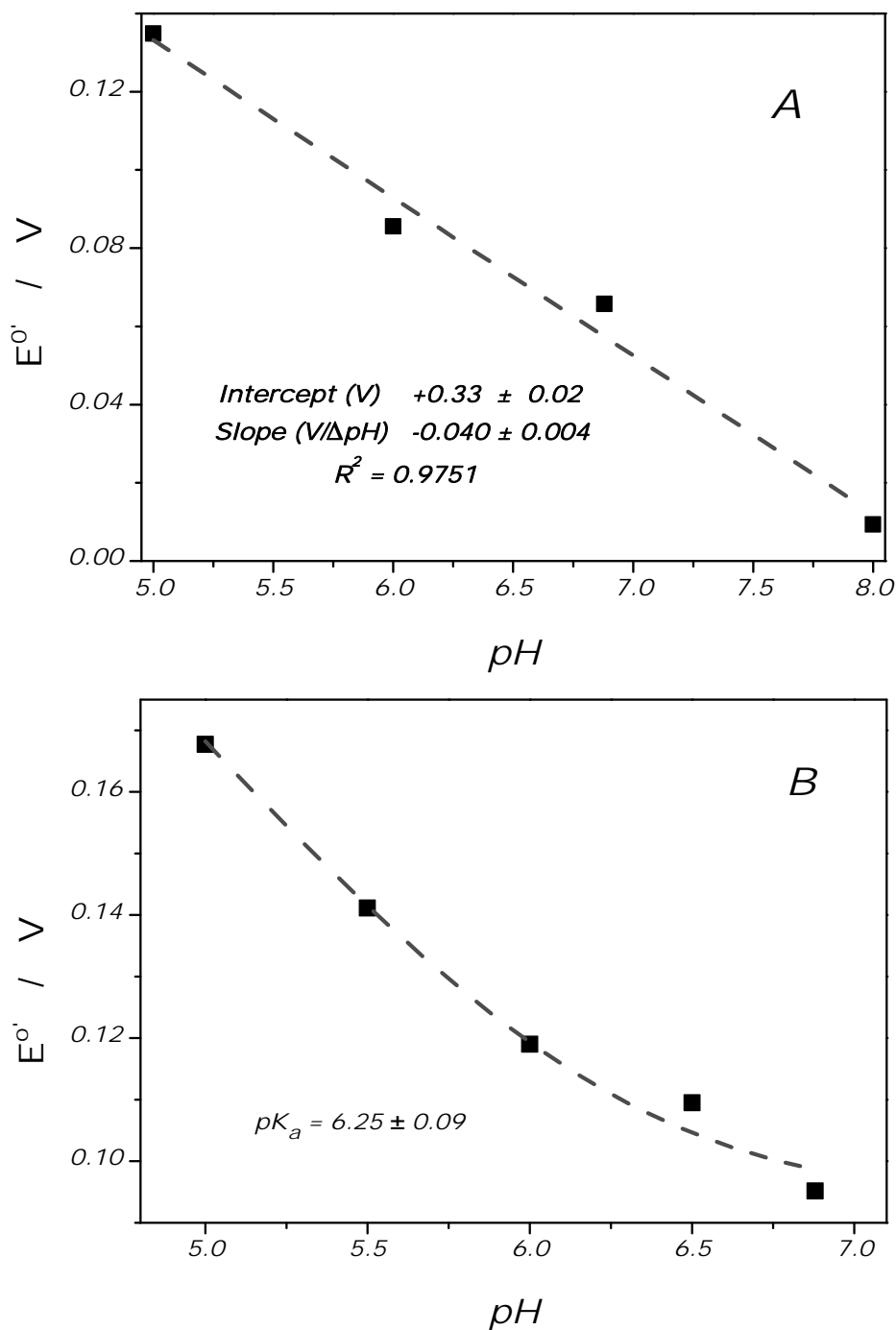

Fig. 4 – pH dependence of the formal redox potential ($E^{0'}$) of Hm adsorbed on the cysteine modified Au surface: (**A**) CV and (**B**) SWV. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer. CV measurements: potential scan rate, 25 mV/s. SWV measurements: frequency, 75 Hz; amplitude, 50 mV.

This peculiar behavior was already reported for Hm[27] and was attributed to the protonation states of the *trans* ligands of the heme iron, overlapped on the protonation of the amino acids surrounding the heme or the protonation of the water molecule coordinated to the iron atom.[28]

Surprisingly, SWV measurements, carried out within the same pH domain (from 5 up to 8), revealed a nonlinear dependence of $E^{0'}$ *vs.* pH (Fig. 4B). The nonlinear fitting of the experimental data allowed estimation of the pK_a value for the acid dissociation constant of the adsorbed Hm on the Au/L-Cys surface ($pK_a = 6.25 \pm 0.09$). This value was found slightly lower than that reported for dissolved Hm ($pK_a = 6.63$),[29] probably due to the interaction between the adsorbed Hm and the L-Cys film deposited on the Au surface. Unfortunately, the experimental data available at this moment cannot be used to elucidate the nature of the interaction involved in the Hm adsorption on L-Cys film, and the cause of the pK_a shift observed for adsorbed Hm.

Finally, the CV and SWV data collected for different potential scan rates and frequencies, respectively, were used to estimate the rate constants (k_s) of the charge transfer process for Hm immobilized on Au/L-Cys/Hm and G/Hm/Chi modified electrodes. The k_s values were calculated from CV measurements by using the Laviron's treatment,[30] while from SWV data the k_s values were obtained by using the treatment proposed by Reeves[26] for an adsorbed quasi-reversible redox couple. As can be seen from Fig. 5, within the limits of the experimental errors and irrespective of the value of the surrounding pH, CV and SWV measurements gave similar values of k_s for both investigated electrodes. However, it can be noticed that the k_s values corresponding to Hm immobilized on Au/L-Cys self-assembled film [$(k_s)_{CV} = (0.65 \pm 0.22)$ s^{-1}; $(k_s)_{SWV} = (0.62 \pm 0.27)$ s^{-1}; pH 6] are significantly lower than the corresponding values estimated for Hm immobilized on G/Hm/Chi modified electrode [$(k_s)_{CV} = (1.9 \pm 0.7)$ s^{-1}; $(k_s)_{SWV} = (3.4 \pm 1.5)$ s^{-1}; pH 6].[31]
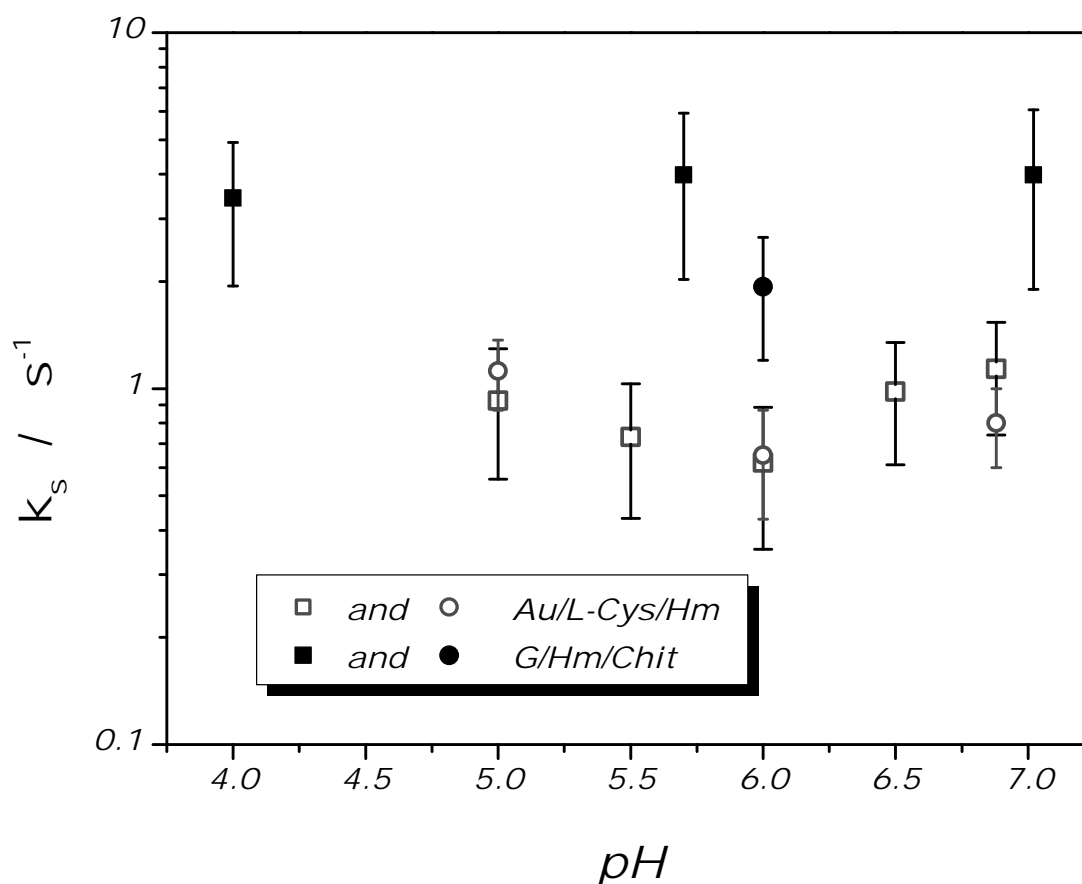

Fig. 5 – pH effect on the rate constant of charge transfer (k_s) corresponding to Au/L-Cys/Hm (□,○) and G/Hm-Chit (■,●) modified electrodes, estimated from CV (□,■) and SWV (○,●).

EXPERIMENTAL

Reagents

Hemin [iron (III) protoporphyrin (IX) chloride, Hm], L-cysteine (L-Cys) and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Fluka, Merck and Sigma, respectively. A stock solution of 5 mM Hm was prepared by dissolving the appropriate amount of Hm salt in 0.05 M TRIS chloride buffer solution (pH 9.4).

Gold wire (99,99%) of 0.4 mm diameter was obtained from Engelhart-CLAL, The Netherlands. The graphite disc electrode wasmade from spectral graphite (3 mm diameter) obtained from Ringsdorff-Werke, Gmbh, Bonn-Bad Godesberg, Germany.

The 0.1 M phosphate buffer solution (PB) was prepared by mixing appropriate volumes of 0.05 M KH_2PO_4 and 0.05 M K_2HPO_4 (Sigma). The pH value of the PB solution was adjusted by using HCl and NaOH (Reactivul-Bucharest). Deionized water was used for preparing all solutions.

In order to remove the dissolved oxygen, highly purified argon gas was purged into the working solution for at least 15 minutes prior to each measurement. Additionally, the argon flow was kept over the solution during the measurement. All experiments were performed at room temperature (25 ± 2 °C).

Equipments

A standard single-compartment cell equipped with three electrodes (Au working electrode, Ag/AgCl,KCl$_{sat}$ reference electrode (Radiometer, France) and Pt counter electrode) was used. All electrochemical measurements were carried out using a computer controlled voltammetric analyzer (Autolab PGSTAT 302N EcoChemie, The Netherlands). A combined glass electrode connected to a digital pH meter (Hanna Instruments, HI 1230) was used for the pH measurements.

Preparation of Au/L-Cys/Hm modified electrodes

The cleaning procedure of Au electrodes started by keeping the Au wires for 1 hour in a hot "*piranha*" solution (98% H_2SO_4 / 33% H_2O_2, 1:3 v/v; ~100ºC). [ATENTION! Because the pirahna solution is corrosive and reacts violently with organic compounds, the precaution must be taken at all experiments invloving this mixture]. Then, the Au electrodes were rinsed and sonicated in distilled water for 10 minutes. Subsequently, the Au electrodes were boiled for 2 h in 2M KOH solution. Finally, the rinsing and ultra-sonication procedure was repeated. The so cleaned Au electrodes were kept in a 0.1M H_2SO_4 solution.

In the first step,Au/L-Cys modified electrodes were obtainedby keeping the cleaned Au electrodes, for ~12 h, into a L-Cys solution (1.8 mg L-cysteine chloride monohidrate were dissolved into 5 ml of absolute C_2H_5OH). In order to remove the physically adsorbed L-Cys, the Au/L-Cys modified electrodes were thoroughly rinsed with distilled water.

In the second step, the Au/L-Cys modified electrodes were immersed for 8 h in a solution of Hm (0.0325 g of Hm were disolved in 10 ml of 0.05 M TRIS solution, pH 9.4). Before use, the Au/L-Cys/Hm modified electrodes were rised using the same procedure as that mentionated above.

Preparation of G/Hm/Chi modified electrodes

The G/Hm/Chi modified electrode was prepared by dropping 5 µL of 5 mM Hm solution (Hm was dissolved in TRIS, pH 10) on the surface of a graphite electrode (3 mm diameter). Further, the G/Hm modified electrode was dried by keeping it in air during the night.

Finally, on the surface of G/Hm modified electrode were dropped 5 µL of 10.7 µg/µL chitosan solution (prepared in 0.1% acetic acid). The so obtained G/Hm/Chi modified electrode was dried in air during the night.

CONCLUSIONS

To the best of our knowledge, this work is reporting for the first time on a simple and reproducible way to prepare an electrode modified with Hm, using its adsorption on the L-Cyst film, self-assembled on the surface of Au electrode. This new approach exploits the electrostatic interactions existing between the negatively charged surface of the Au/L-Cys modified electrode (due to the dissociation of the carboxylic groups of L-Cys, occurring predominantly at pH > 5) and the positively charged regions of Hm (located around the Fe coordinated N atoms).

The electrochemical characterization of the resulting Au/L-Cys/Hm electrode, performed by using CV and SWV measurements, allowed establishing the influence of the experimental conditions (scan rate, and pH) on the electrode behavior. Thus, it was stated that the immobilized Hm is involved in a quasi-reversible $1e^-/1H^+$ redox process, keeping all features characteristic to a surface confined species.

Additionally, it can be supposed that the uniform structure characteristic for the adsorbed Hm film, spontaneously formed onto the surface of Au/L-Cys electrode, facilitates the first voltammetric estimation of the pK_a for the adsorbed Hm.

At the same time, due to the presence of L-Cys film on the Au electrode surface, the distance between the Hm active center (in fact, the Fe(III)/Fe(II) redox couple) and the Au surface is obviously higher than that between Hm and the graphite surface, resulting in a slower charge transfer process. It is worth to mention that, recently, it was reported that the π-π interactions involved in the Hm adsorption on the graphite surface contribute decisively to promote a fast charge transfer process.[32]

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