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HEMIN MODIFIED ELECTROD FOR AMPEROMETRIC DETECTION OF H_2O_2

PhD Thesis Abstract

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Keywords: hemin, peroxidase, kitosan, L-cysteine, electrocatalisys, cyclic voltammetry, square wave voltammetry, self-assembled monolayers, biosensors, nitrite, hydrogen peroxide, electropolymerization, modified electrodes.

INRODUCTION

As defined by IUPAC, a chemically modified electrode (CME) “is an electrode made of a conducting or semiconducting material that is coated with a selected monomolecular, multimolecular, ionic, or polymeric film of a chemical modifier and that by means of faradaic (charge-transfer) reactions or interfacial potential differences (no net charge transfer) exhibits chemical, electrochemical, and/or optical properties of the film” (Durst R. A. *et al.* 1997). Usually, the chemically modified electrodes can be obtained by one of the following approaches: (i) chemisorption, (ii) covalent bonding, (iii) polymer film coating, and (iv) entrapment in a conductive material. Among these, the simplest way which exploits the adsorption of the modifier on the electrode surface, generates a stable film. Usually, this approach yields monolayer modified electrodes with high electrocatalytic activity.

Biological materials, with hight selective properties were used in preparation of the biosensors.

PART I. THEORETICAL CONSIDERATIONS

IV.4. Hemin ($C_{34}H_{32}O_4N_4FeCl$)

Hemin (iron protoporphyrin IX; Hm) is one of the most important biological substances. It contains the active center common of several families of heme proteins, as for example C-type cytocromes, peroxidase and oxygen–carrying proteins such as hemoglobin (Hb) and myoglobin (Mb) (Sagara T. *et al.* 1995). In Fig. 10 it is presented the structure of the hemin according to the http://www.chemicalize.org/structure/#!mol=hemin (consulted in 27th of march 2014).

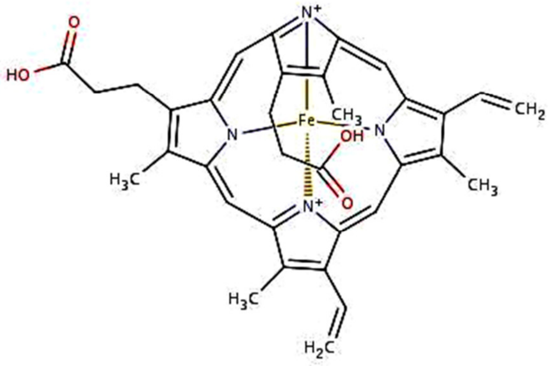


Figure 10. Structure of the hemin

Hemin dissolved in an aqueous solution and adsorbed on the electrode surface or incorporated within a polymeric film immobilized on the electrode surface maintains its electrochemical activity. Hemin is strongly adsorbed on the carbonaceous electrode materials, especially on pyrolytic graphite (PG). The obtained modified electrodes (Mares G. M. *et al.* 2013; Zheng N. *et al.* 2002; Ye J. S. *et al.* 2004; Gao Y., Chen J. 2005; Zejli H. *et al.* 2010; Chen J. *et al.* 2011; Xu J. B. *et al.* 2012; Hasebe Y., Wang Y. 2013; Aoki K. J. *et al.* 2014; Varma S. 2002; Guo Y *et al.* 2011; Valentini F. *et al.* 2012; Ma S.-H. *et al.* 2013; Zhang Y. *et al.* 2013; Sun R. *et al.* 2014) were used for the amperometric detection of H_2O_2 (Song H. 2013 ; Chen J. *et al.* 2011; Varma S. 2002; Guo Y *et al.* 2011; Valentini F. *et al.* 2012; Ma S.-H. *et al.* 2013; Zhang Y. *et al.* 2013; Sun R. *et al.* 2014; Zhang F.-T. *et al.* 2014; Han X. *et al.* 2002; Han X. *et al.* 2002; Dai Z. *et al.* 2004; Feng J. J. *et al.* 2006; Xu Y. *et al.* 2008; Kafi A. K. M. *et al.* 2007; Mares G. M. *et al* 2013) ***superoxide*** (H. Zejli *et al.* 2010) ***nitrit*** (Valentini F. *et al.* 2012; Dai Z. *et al.* 2004; Feng J. J *et al.* 2006; Xu Y., Hu C., Hu S., 2008) and other species.

PART II. *ORIGINAL CONTRIBUTIONS*

Objectives

The main objectives of the thesis were:

(i) Immobilization of hemin (Hm) on the graphite (G) and gold (Au) electrodes surface using different methods: chemical adsorption, covalent binding and incorporation in polymeric films such as chitosan, catechol, resorcinol and hydroquinone;

(ii) Preparation of an amperometric enzymatic biosensor based on horseradish peroxidase (HRP) and Hm, as mediator;

(iii) Electrochemical characterization of the obtained modified electrodes and estimation of their kinetic and analytic parameters;

(iv) Optimization and stability study;

(v) Use of the modified electrodes for the amperometric detection of chemical species of practical interests (H_2O_2 şi NO_2^-).

V. G/Hm MODIFIED ELECTRODE

V.2. Scan rate / frequency influence

The modified **G/Hm** electrode was investigated by using cyclic voltammetry (CV) and square wave voltammetry (SWV). The obtained voltammograms are presented in Fig. 19.

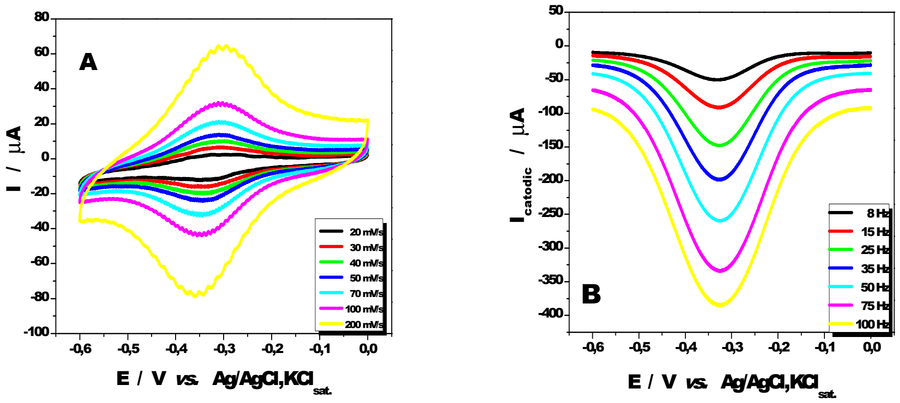


Figure 19. Cyclic (A) and square wave (B) voltammograms for modified G/Hm electrode. Experimental conditions: phosphate buffer solution (PB) 0.1M pH 7.02, deaerated solution (Ar); initial potential 0 V *vs*. Ag/AgCl,KCl$_{sat.}$; (A) *CV:* different scan rate (B) *SWV*: amplitude 20mV, different frequency.

As expected for a surface confined redox couple, in the investigated range of potential scan rate (20 – 200 mV/s), the anodic (I_{ap}) and cathodic (I_{cp}) peak currents depend linearly on the potential scan rate (v) (Figure 20).

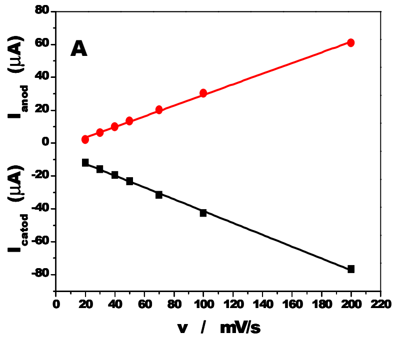


Figure 20. The I_p *vs.* potential scan rate (**A**) dependencies recorded at G/Hm modified electrode. Experimental conditions: see Figure 19.

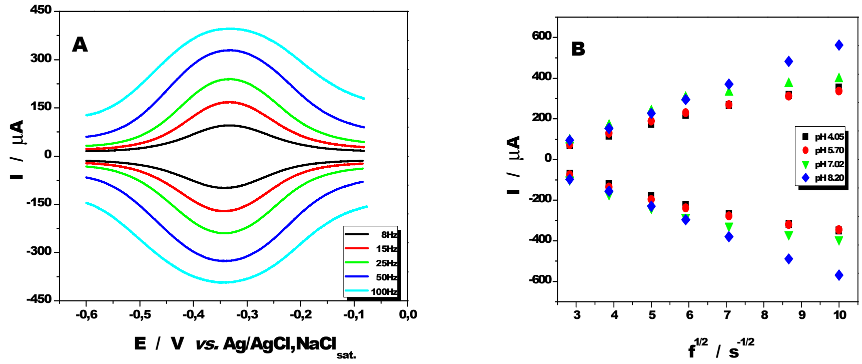


Figure 21. Square wave voltammograms (B) for modified G/Hm electrode (A); the I_p *vs.* frecvency (**A**) and I_{pc} *vs.* $v^{½}$ (**B**) dependencies recorded at G/Hm modified electrode. Experimental conditions: see Figure 19.

The peak separation $\Delta E_p = E_{p,a} - E_{p,c}$ was 0.034V and the peak current intensity ratio $I_{p,a} / I_{p,c}$ was 0.955 (closed to the theoretical value, 1); both values, calculated at the scan rate of 50 mV/s (pH 7.02), confirmed that the hemin is adsorbed on the electrode surface. The associated redox process has the characteristics of a quasi-reversible one (Santos R.M. *et al.* 2013; Bart J.A., Faulkner L.R. 1980).

The $\log(I_p)$ - $\log(v)$ dependence (Table 12) also demonstrated that hemin is immobilized on the electrode surface, taking into account that the slopes of these dependencies were closed to 1. At the same time, the SWV measurements confirmed this behavior (Fig. 21).

Table 12. Dependence of *log I_p vs. log v* for G/Hm modified electrode (CV) pH 7.02.

Redox process	Slope	R / Nr. points
anodic	1.18 ± 0.05	0.990 / 6
cathodic	0.82 ± 0.02	0.997 / 7

V.3. pH influence

Figures 22A and 22B show the pH influence on the voltammetric response of the G/Hm electrode. Table 15 contains the values of the peak parameters obtained at different pH values (CV and SWV).

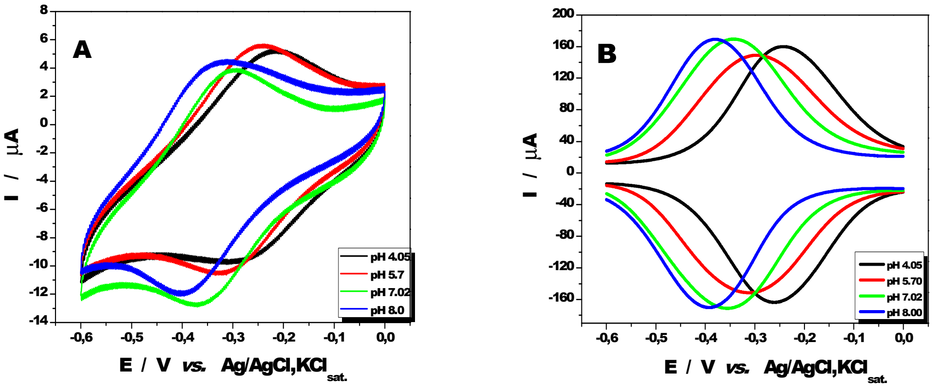


Figure 22. pH influence on the voltammetric response of the G/Hm modified electrode: (A) CV and (B) SWV measurements, respectively. Experimental conditions: PB solution 0.1M, deaerated solution (Ar); initial potential, 0 V *vs*. Ag/AgCl,KCl$_{sat}$; scan rate, 20 mV/s (A); frequency 25Hz, amplitude 20 mV (B).

In the pH range from 4 up to 8, the values of the slopes calculated for these dependencies [CV: (-0.041 ± 0.005) V/pH, with R = 0.962 and n = 4; SWV: (-0.033 ± 0.001) V/pH, with R = 0.99 and n = 4], do not agree with the theoretical value (0.059 V/pH) expected for a redox process involving the transfer of $1e^{-}/1H^{+}$.

Table 15. Values of the anodic and cathodic peak parameters obtained from CV and SW voltammograms for the G/Hm electrode at different frequency and pH.

	pH	**E_c (V)**	**E_a (V)**	**ΔE_p (V)**	**$E^{0'}$ (V)**
CV 20mV/s	4.05	-0.3131	-0.2179	0.0952	-0.2655
	5.70	-0.3346	-0.2408	0.0938	-0.2877
	7.02	-0.3703	-0.2921	0.0780	-0.3310
	8.0	-0.3960	-0.3104	0.0856	-0.3530
SWV 20mV 25Hz	4.05	-0.2626	-0.2417	0.0209	-0.25215
	5.70	-0.3142	-0.2957	0.0185	-0.30495
	7.02	-0.3566	-0.3447	0.0119	-0.35065
	8.0	-0.3896	-0.3793	0.0102	-0,38445

This peculiar behavior was already reported for adsorbed hemin and was attributed to the protonation states of the *trans* ligands of the heme iron, combined with the protonation of the amino acids surrounding the heme or the protonation of the water molecule coordinated to the iron atom (Sun J.-J. *et al.* 2011; Baron C. P., Andersen H. J. 2002)**.**

The SWV measurements, performed at pH 7 (amplitude 50 mV), allowed the calculation of the rate constant for the eterogen proces of electron transfer [k_s, = (0.3612 ± 0.02) s^{-1}].

V.4. Stability of the G/Hm modified electrode

The stability of the modified electrode is a very important characteristic because it provides information on the electrode life-time, a decisive parameter for its future applications. For this reason, the short-time stability of G/Hm was evaluated by continuous cycling of the electrode potential when it was in contact with the supporting electrolyte (0.1 M PB, pH 7). Both cathodic and anodic peak currents showed no significant variation in time after 30 of repetitive full potential scans.

The variation in time of the electrode surface coverage [$\Gamma(\mathrm{mol/cm^2}) = Q/(nFA)$, where Q(Coulomb) is the amount of charge corresponding to the cathodic or anodic under-peak area estimated after the background current correction; n (=1) is the number of electrons transferred during the redox process, generating the voltammetric peak; F (Coulomb) is the Faraday's constant; A($\mathrm{cm^2}$) is the electrode geometric area] confirms that hemin is strongly adsorbed on the graphite surface.

The tables 16 and 18 summarized the data concerning the short and long time variation of the surface coverage of the voltammetric response recorded at G/Hm modified electrode.

Tabelul 16. Short time stability of the G/Hm electrode.

Time (s)	**Γ_c (nmol/cm^2)**	**Γ_a (nmol/cm^2)**	**$\Delta\Gamma_c$ (%)**	**$\Delta\Gamma_a$ (%)**
240	76.6	128	0.53	0.08
480	76.8	135	0.79	4.65
720	76.5	130	0.39	0.08

$\Delta\Gamma_c = 100(\Gamma_{c,t} - \Gamma_{c,t=48})/\Gamma_{c,t=48}$; $\Delta\Gamma_a = 100(\Gamma_{a,t} - \Gamma_{a,t=48})/\Gamma_{a,t=48}$

Tabel 18. Long time variation of the peak parameters for the voltammetric response of **G/Hm** modified electrode.

ΔI_{ap} (%)	**ΔI_{cp} (%)**	**$\Delta\Gamma_c$ (%)**	**$\Delta\Gamma_a$ (%)**	**ΔE_p (mV)**	**I_{ap}/I_{cp}**
7.6	2.4	6.5	9.3	23	1.15

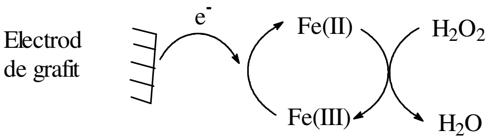
$\Delta I_{cp} = 100(I_{cp,\,t=305} - I_{cp,\,t=0})/I_{cp,\,t=0}$; $\Delta I_{ap} = 100(I_{ap,\,t=305} - I_{ap,\,t=0})/I_{ap,\,t=0}$

$\Delta\Gamma_c = 100(\Gamma_{c,t=305} - \Gamma_{c,t=0})/\Gamma_{c,t=0}$; $\Delta\Gamma_a = 100(\Gamma_{a,t=305} - \Gamma_{a,t=0})/\Gamma_{a,t=0}$

Between measurements the G/Hm electrodes were stored at 4°C, in a water saturated atmosphere. All data proved the good stability of the G/Hm modified electrode. This behavior was related to the hemin insolubility under acidic and neutral conditions, associated with the strong π-π interaction between hemin and graphite (Zheng N. *et al.*2002; (Wang X. *et al.* 2002). After 2 hours the cathodic current decreased with 2.01%, while after 5 hours the decrease was ~2.74% related to the initial value.

V.5. Amperometric detection of H_2O_2 using the G/Hm electrode

Taking into account that H_2O_2 is a product of the biochemical reactions catalyzed by oxidases its detection is of considerable importance in clinical, food, pharmaceutical and environmental analysis. In this context, the electrocatalytic behavior of the G/Hm modified electrode was investigated for H_2O_2 electrocatalytic reduction by using CV and SWV measurements (Varvari L., Popescu I. C. 2010):



As can be seen from figure 27 and table 19, the SWV measurements provided better analytical and kinetic parameters than those obtained from CV measurements. Probably, this difference is due to the higher resolution of SWV technique, associated with an easier and better correction of the background current in the case of SWV measurements (Mares G.M. *et al.* 2013).

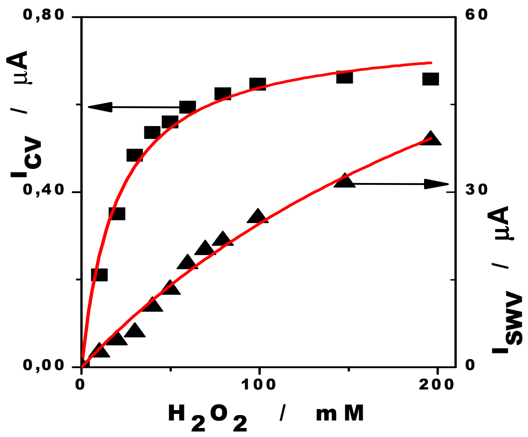


Figure 27: Calibration curve of G/Hm modified electrode for H_2O_2 detection using CV and SWV measurements. Experimental conditions: supporting electrolyte, 0.1 M PB (pH 7.0); starting potential, -0.6V *vs.* Ag/AgCl,$KCl_{sat.}$(CV) and 0 mV *vs.* Ag/AgCl,$KCl_{sat.}$ (SWV); scan rate, 20 mV/s; amplitude, 20 mV; frequency, 25 Hz; deaerated solution (Ar).

Table 19. Analytical and kinetic parameters for G/Hm modified electrode.

Parameters	**CV**	**SWV**
Michaelis-Menten fitting		
I_{max} (μA)	0.77 ± 0.02	101 ± 16.1 311 ± 68.3
K_M^{app} (mM)	20.2 ± 2.5	
Sensitivity (mA/M)	0.038	0.325
R / N	0.9836 / 11	0.9861 / 12
Linear Fitting		
Slope(A/mM)	16.0 ± 1.3	276.3 ± 11.9 0.012
Detection Limit / mM	0.011	
Linear range (mM)	10 - 40	10 - 80
R / N	R = 0.979 / n = 5	R = 0.993 / n = 9

***Sensitivity= I_{max}/K_M^{ap}**

VI. MODIFIED ELECTRODES BASED ON Hm INCORPORATION INTO ELECTROPOLYMERIZED PHENOLIC POLYMERIC FILMS

VI.4. Preparation of G/Hm-Res, G/Hm-Cat, and G/Hm-HQ electrodes

Figures 28-30 showed the cyclic voltammograms recorded during the polymerization process in absence (Figure 28 A-30A) and in presence of Hm (Figure 28B- 30B):

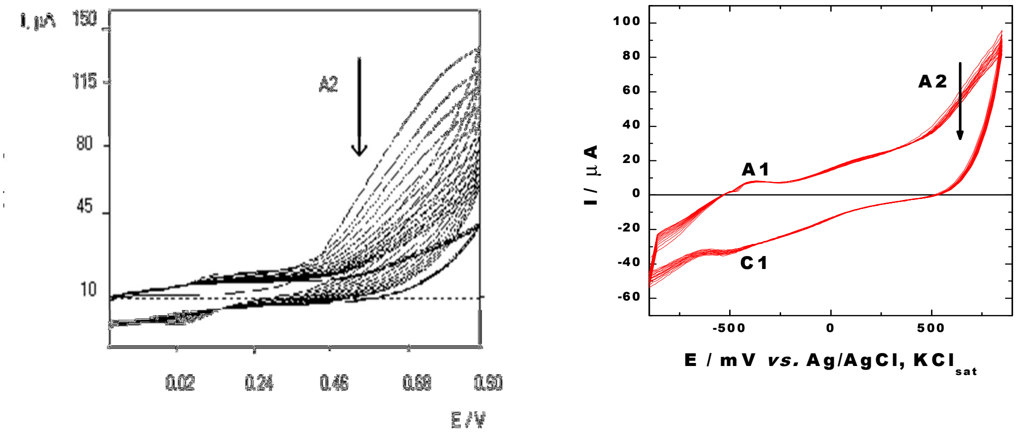


Figure 28. Cyclic voltammograms showing the preparation of the G/Res (black) and G/Hm-Res (red) modified electrodes. Experimental conditions: 10 ml solution 5 mM Res in TRIS buffer (black); 10 ml solution containing: 5 mM Hm + 5 mM Res in TRIS buffer 5 mM (pH 10), pH final 8.6 (adjusted with) (rred); potectial scan rate 100 mV/s, 30 cicles between –0,9 – +0,85 V *vs.* Ag/AgCl,KCl$_{sat.}$; deaerated solution (Ar); temperature 25±2ºC.

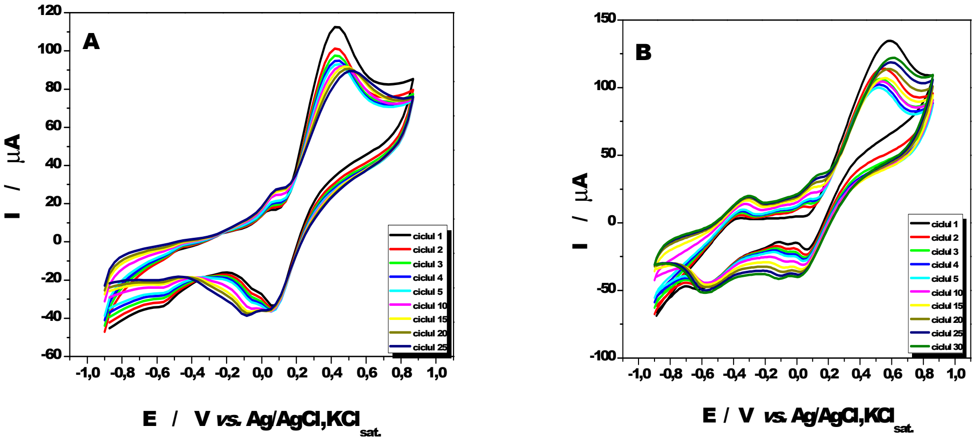


Figure 29. Cyclic voltamograms recorded during the preparation of the G/Cat (A) and G/Hm-Cat (B) modified electrodes. Experimental conditions: 10 ml solution 5 mM Cat in TRIS buffer (A); 10 ml solution containing: 5 mM Hm + 5 mM Cat in TRIS buffer 5 mM (pH 10), pH final 8.6 (adjusted with) (rred); potectial scan rate 100 mV/s, 30 cicles between –0,9 – +0,85 V *vs.* Ag/AgCl,KCl$_{sat.}$; deaerated solution (Ar); temperature 25±2ºC.

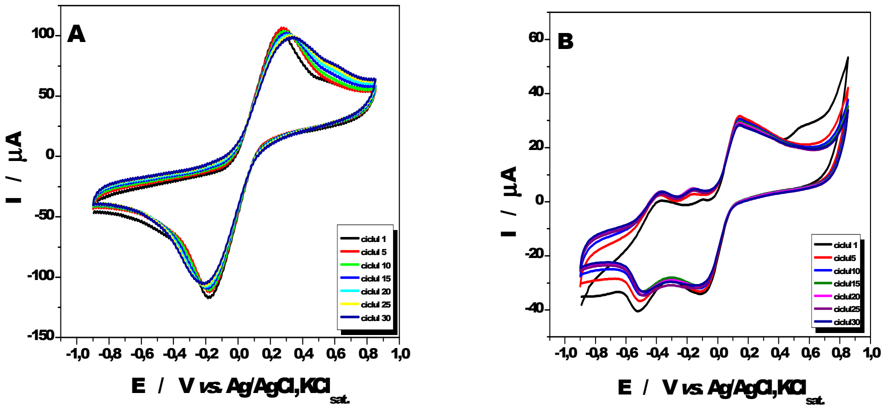


Figure 30. Cyclic voltamograms recorded during the preparation of the modified G/HQ (A) şi G/Hm-HQ (B). electrodes. Experimental conditions: 10 ml solution 5 mM HQ in TRIS buffer (A); 10 ml solution containing: 5 mM Hm + 5 mM HQ in TRIS buffer 5 mM (pH 10), pH final 8.6 (adjusted with) (rred); potectial scan rate 100 mV/s, 30 cicles between –0,9 – +0,85 V *vs.* Ag/AgCl,KCl$_{sat.}$; deaerated solution (Ar); temperature 25±2ºC.

As can be seen from figures 28A-30A the anodic peak intensity decreases during the potential cycling, denoting the deposition on the graphite electrode surface of a nonconducting polymeric film. In presence of hemin (Fig. 28B-30B), its characteristic peak pear can be observed on the recorded voltammograms.

VI.5. Mechanisms for the Hm incorporation of into polymeric films

Two possible mechanisms for the electropolimerization of the phenolic derivates were reported. Due to the electrostatic interaction between the positive charge of hemin and the negative charge of the phenolic compounds a self-assembled (layer-by-layer) architecture is probably formed on the electrode surface (Turdean G. L. *et al.* 2002; Cheng L., Dong S. 2000; McCormac T. *et al.* 2001; http://www.uni-regensburg.de/Fakultaeten/; Zen J. M., Chung H.-H., Kumar A.S. 2002; Nakabayashi Y., Wakuda M., Imai H. 1998; Chen X. *et al.* 200; http://intel.ucc.ie/sensors/Electropolym.htm; Hrapovic S., Luong J. H. T. 2003; Sayyah S.M., *et al.* 2011; www.intechopen.com). The position of the peak pear characteristic to the hemin depends of the relative position of the –OH groups (Table 21).

Table 21. The influence of the –OH posision from the phenolic compound on the values of the peak potential.

Type of the electrode	**Hm peak**			**Polymer peak**		
	Ea (V *vs.* ER)	**Ec (V *vs.* ER)**	**E°' (Ea+Ec)/2**	**E_a (V *vs.* ER)**	**E_c (V *vs.* ER)**	**E°' (Ea+Ec)/2**
G/Cat	-	-	-	+0.42	+0.055	0.2375
G/Cat-Hm	-0.35	-0.560	-0.455	+0.48	+0.06	0.270
G/Res	-	-	-	+0.80	-	-
G/Res-Hm	-0.44	-0.51	-0.475	-	-	-
G/HQ	-	-	-	+0.30	-0.20	0.050
G/HQ-Hm	-0.40	-0.50	-0.450	+0.10	-0.10	0

VI.6. Scan rate influence on the voltammetric response of G/Hm-Res, G/Hm-Cat and G/Hm-HQ modified electrodes

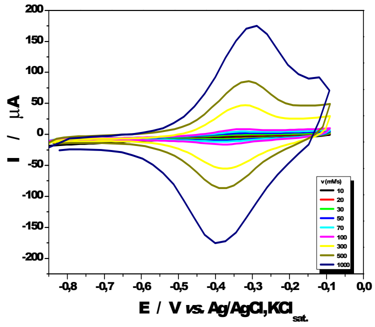


Figure 36. Scan rate influence on the electrochemical behavior of G/Hm-Cat electrode. Experimental conditions: PB solution 0.1 M, pH 8, start potential–0.85 V *vs.* Ag/AgCl, KCl_{sat}; deaerated solution (Ar); temperature, 25±2ºC.

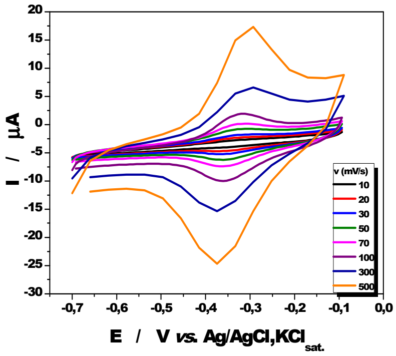


Figure 37. Scan rate influence on the electrochemical behavior of G/Hm-HQ electrode. Experimental conditions: PB solution 0.1 M, pH 8, start potential–0.85 V *vs.* Ag/AgCl, KCl_{sat}; deaerated solution (Ar); temperature, 25±2ºC.

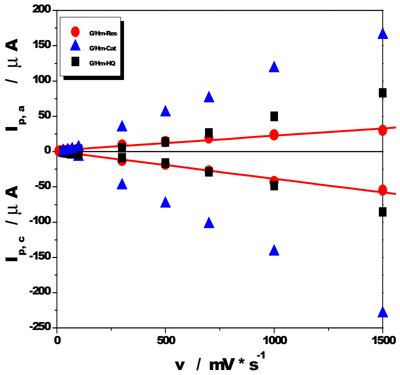


Figure 38. The I_p *vs.* potential scan rate (**A**) dependencies recorded at G/Hm-Res(●), G/Hm-Cat (▲) şi G/Hm-HQ (■) modified electrodes. Experimental conditions: PB0.1 M, pH 8; start potential –0.85 V *vs.* Ag/AgCl, $KCl_{sat.}$; Deaerated solution (Ar); temperature, 25±2ºC.

For all type of the investigated modified electrodes, the log I *vs.* log v dependence was linear (Table 22) and the slope value was between 0.8 ÷ 1.3, nearby the theoretical value (1). The dependence I *vs.* v confirmed that the redox species it was adsorbed on the electrode surface (Figure 38; table 22).

Table 22. The slopes of the log(I_p) *vs.* log(v) dependencies for G/Hm electrode.

Type of electrodes	Log I *vs.* Log v	R	Number of points
G/Hm-Res	log $I_{p,a}$ = -1.03 + 0.8 log v	0.997	11
G/Hm-Cat	log $I_{p,a}$ = -7.9 + 1.3 log v	0.995	9
G/Hm-HQ	log $I_{p,a}$ = -7.9 + 1.2 log v	0.986	9

VI.8. Electrocatalytic activity of the G/Hm-Res, G/Hm-Cat and G/Hm-HQ modified electrodes for H_2O_2 and nitrites electroreduction

The electrochemical properties of Fe(III) from hemin included into the polymeric films, were investigated in the reduction of two species of practical interest, e.g. hydrogen peroxide and nitrite.

VI.8.1. Electroreduction of H_2O_2

In the literature was reported a possible mechanism for H_2O_2electroreduction (Figure 47; Li Y., Goodwin D. C. 2004; Laszlo J. A, Compton D. L. 2002).

$$\mathrm{Fe(III)PC} + \mathrm{HOOH} \xrightarrow{k_1} \text{compound I} + H_2O \qquad (1)$$

$$\text{compound I} + AH \xrightarrow{k_2} \text{compound II} + A^{\bullet} \qquad (2)$$

$$\text{compound II} + AH \xrightarrow{k_3} \mathrm{Fe(III)PC} + A^{\bullet} + H_2O \qquad (3)$$

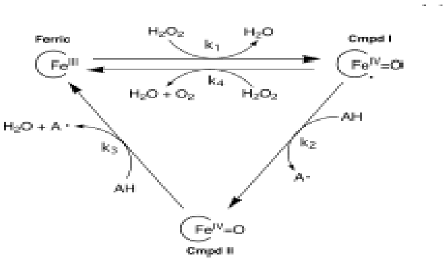


Figure 47. The catalytic cycle for hydrogen peroxide reduction and the corresponding reaction mechanism.

VI.8.2. Electroreduction of the nitrites

The peak potential corresponding to the reduction process of nitrite ions was found around –0.70 V *vs.* Ag/AgCl, KCl_{sat}, in agreement with the reported mechanism (Mimica D., Zagal J. H., Bedioui F. 2001).

In neutral aqueous solutions, the first step of the catalytic process involves an iron-nitrosil complex, which is followed, in the second step, by the formation of an intermediate compound (peak at –0.45 V vs. Ag/AgCl, KCl_{sat}), according to the next reactions:

$$\mathbf{[Fe(III)PC]^+ + e^- \rightarrow [Fe(II)PC]} \tag{R4}$$

$$\mathbf{[Fe(II)PC] + HONO + H^+ \rightarrow [Fe(II)(NO^+)PC]^+ + H_2O} \tag{R5}$$

where [FePC] symbolizes the redox center of hemin. The last step is the reduction of the $[Fe(II)(NO^+)PC]^+$ at a potential situated around –0.750 V *vs.* Ag/AgCl, KCl_{sat}):

$$\mathbf{[Fe(II)(NO^+)P]^+ + e^- \rightarrow [Fe(NO^\bullet)]} \tag{R6}$$

The values of the peak parameters for H_2O_2 and NO_2^- reduction, obtained from cyclic voltammetric measurements, are presented in the Table 29.

Tabelul 29. Peak parameter values in the electrocatalysis of the H_2O_2 and NO_2^-.

Analyt	**Concentrație (mM)**	**G/Hm-Res**		**G/Hm-Cat**		**G/Hm-HQ**	
		E_c (V)	**I_c (µA)**	**E_c (V)**	**I_c (µA)**	**E_c (V)**	**I_c (µA)**
H_2O_2	0	-0.182	-4,337	-0.157	-2,9053	-0.157	-4,229
	0.2	-0.207	-6,0155	-0.157	-5,505	-0.157	-5,264
	0.4	-0.266	-8,005	-0.157	-5,841	-0.157	-5,883
	0.8	-0.296	-1,0567	-	-	-	-
	0.9	-	-	-0.157	-8,133	-0.157	-8,157
NO_2^-	0	-0.753	-5.987	-0,710	-7.5043	-0.708	-4.1374
	0.01	-	-	-0,7104	-3.7475	-0.706	-5.0349
	0.1	-0.760	-6.9723	-0,7104	-9.5397	-0.709	-9.6460
	1	-0.784	-9.6147	-0,7104	-25.381	-0.716	-14.518

VII. AMPEROMETRIC BIOSENSOR BASED ON HRP and Hm

VII.2. Peroxidases

Peroxidases are a group of oxidoreductase able to catalyze, efficiently, different substrates (AH_2-reducer substrate) oxidation by peroxo compounds (ROOH):

$$\mathbf{ROOH + AH_2 = A + ROH + H_2O} \qquad (1)$$

Peroxidase are present in the body of animals, plants, microorganisms playing an important physiological role (Bartonek-Roxa E., Holm C. 1999; Cherry J. R. *et al.* 1999; Duroux L., Welinder K. G., 2003; Folkes L. K., Wardman P. 2001).

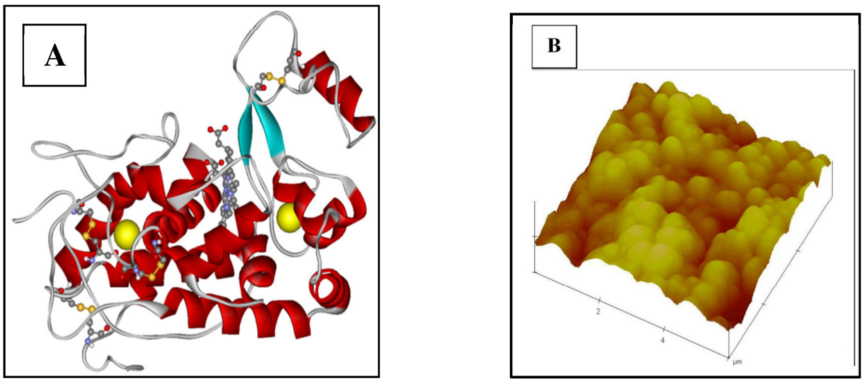


Figure 53. Structure of HRP (A) according to Protein Data Bank (PDB); film of HRP (B) (AFM) (Armstrong F. A. *et al.* 1997; Rusling J. F., Z. Zhang 2003).

Horseradish peroxidase, **HRP** is a very important and studied enzyme (Ahuja T. *et al.* 2007; Smeller László 2007; Smith A.T. 1990; Passardi F. *et al.* 2007; Folkes L. K. *et al.* 2002; Gaspar T. *et al.* 1982; Gaspar T. *et al.* 1992; Mittler R. 2002; Welinder K. G., 1992; Welinder K. G., Gajhed M. 1993; Welinder K.G. *et al.* 2002). At the pH between 5 ÷ 10, the native HRP has a remarkable stability for the long period of time. From this point of view, HRP is an ideal enzyme for analytical applications (Nagy G., Pungor E., 1988; Smeller L. 2007; Adányiné dr. Kisbódi N. 2003; Smeller L., Fidy J. 2002; Schlichter J. *et al.* 2001; Szigeti K. 2008).

Modified electrodes with peroxidase were used for amperometric detection of H_2O_2, and different other chemical species (Nagy G., Pungor E. 1988; Ahuja T. *et al.* 2007).

VII.3. Amperometric biosensor containing Hm, Chit, HRP and Ga

The main objective was to obtain an amperometric biosensor for H_2O_2 detection based on Hm, Chit, HRP and Ga (glutaraldehide).

VII.5.1. Three steps preparation of G/Hm/HRP/Chit/Ga and G/Hm/Chit/HRP/Ga biosensors

VII.5.1.2. Amperometric detection of H_2O_2

The Table 31 contains the values of the kinetic parameters corresponding to the obtained modified electrodes. It can be observed that when the order of immobilization was changed (HRP was placed and Chit) the value of sensitivity decrease ~30%. (Mares G.M. *et al.* 2013).

Tabelul 31. The values of the kinetic parameters estimated for the **G/Hm/Chit/HRP/Ga** (A) **G/Hm/HRP/Chit/Ga** (B) biosensors.

Method of estimation		$I_{max.}$ / µA	K_M / mM	$S = I_{max.}/K_M$ (µA/mM)	R / n
(A)	**MM fitting**	0.82 ± 0.05	0.23 ± 0.03	3.6 ± 0.6	0.99 /16
	Lineweaver-Burk	0.9 ± 0.1	0.25 ± 0.04	3.6 ± 0.1	0.99 /23
	Eadie-Hofstee	0.84 ± 0.06	0.24 ± 0.03	3.5 ± 0.6	0.97 /12
	Hanes-Woolf	0.83±0.06	0.23 ± 0.03	3.6 ± 0.2	0.99 /13
(B)	**MM fitting**	1.1 ± 0.1	0.6 ± 0.2	1.9 ± 0.8	0.98 / 20
	Lineweaver-Burk	0.8 ± 0.1	0.30 ± 0.06	2.7 ± 0.1	0.99 / 24
	Eadie-Hofstee	0.57 ± 0.06	0.20 ± 0.03	2.9 ± 0.8	0.94 / 9
	Hanes-Woolf	0.74 ± 0.09	0.30 ± 0.07	2.5 ± 0.3	0.96 / 13

VII.5.2. Two steps preparation of G/Hm/HRP-Chit/Ga and G/Hm-HRP /Chit/Ga biosensors

VII.5.2.2. Amperometric detection of H_2O_2

Tables 32 and 33 show the kinetic parameters characteristic for **G/Hm/HRP-Chit/Ga** and **G/Hm-HRP/Chit/Ga** biosensors, obtained from CV and amperometric measurements.

Table 32. The values of the kinetic parameters for **G/Hm/HRP-Chit/Ga** biosensor.

G/Hm/HRP-Chit//GA	I_{max} (µA)	K_M (mM)	$S = I_{max}/K_M$ (µA/mM)	R/n
M-M fitting	1,07 ± 0.02	0.31 ± 0.01	3,48 ± 0.21	0.99 / 40
L-B	1,13 ± 0.03	0.38 ± 0.01	3,34 ± 0.05	0.99 / 42
E-H	1,07 ± 0.02	0.31 ± 0.01	3,45 ± 0.19	0.99 / 36
H-W	1,08 ± 0.02	0.31 ± 0.01	3,43 ± 0.01	0.99 / 38

Table 33. The values of the kinetic parameters for **G/Hm-HRP/Chit/Ga** biosensor.

G/Hm-HRP/Chit/GA	**I_{max} (μA)**	**K_M (mM)**	**S = I_{max}/K_M (μA/mM)**	**R/n**
M-M fitting	1,1 ± 0.1	0.39 ± 0.07	2,7 ± 0.8	0.98 / 25
L-B	1,2 ± 0.1	0.22 ± 0.04	5,4 ± 0.4	0.98 / 14
H-W	1,5 ± 0.1	0.33 ± 0.06	4,5 ± 0.5	0.99 / 18

When the biosensor contains HRP in direct contact with Hm it exhibits a ~ 40% higher sensitivity (~**5,0 μA/mM**; estimated as the average between the L-B and H-W values) than that corresponding to the case when HRP was not in direct contact with Hm (~**3,4 μA/mM**; estimated as the average between the L-B, E-H and H-W values). One possible explanation could be based on a higher efficiency of the electrical connection between HRP and the electrode in the first case.

VII.5.3. One step preparation of the G/Hm-HRP-Chit/Ga biosensor

VII.5.2.2. Amperometric detection of H_2O_2

For the **G/Hm-HRP-Chit/Ga** amperometric biosensor the kinetic parameter values are listed in Table 34.

Table 34. The values of the kinetic parameters for **G/Hm- HRP-Chit/Ga** biosensor

G/Hm-HRP-Chit/Ga	**I_{max} / μA**	**K_M / mM**	**S=I_{max}/K_M (μA/mM)**	**R / n**	
M-M fitting	2.74 ± 0.12	0.31 ± 0.07	8.78 ± 2.9	0.99 / 18	**TI**
L-B	3.02 ± 0.48	0.35 ± 0.08	8.59 ± 0.59	0.98 / 19	
E-H	2.82 ± 0.22	0.33 ± 0.04	8.50 ± 1.7	0.95 / 14	
H-W	2.85 ± 0.18	0.34 ± 0.04	8.42 ± 0.41	0.98 / 14	
FM-M fitting	3.15 ± 0.53	0.35 ± 0.09	9.0 ± 3.9	0.99 / 13	**TII**
L-B	5.89 ± 0.97	0.75 ± 0.15	7.86 ± 0.24	0.99 / 28	
E-H	5.93 ± 0.15	0.69 ± 0.04	8.56 ±0.68	0.989 /17	
H-W	5.94 ± 0.17	0.69 ± 0.04	8.56 ± 0.68	0.998 /17	

TI and TII symbolized the first (in the first day after the biosensor preparation) and the second testing (in the fifth day after the electrode preparation). The sensitivity has practically the same value in both of case. That confirms the good stability of the **G/Hm-HRP-Chit/Ga** biosensor.

VIII. Au/L-Cys/Hm MODIFIED ELECTRODE

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).

VIII.5.1. Scan rate / frequency influence

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, $I_{ap}/I_{cp} > 1$). 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. (Zejli H. *et al.* 2010; Zhang F.-T. *et al.* 2014; Turdean G. L. *et al.* 2006; Mares G. M. *et al.* 2013).

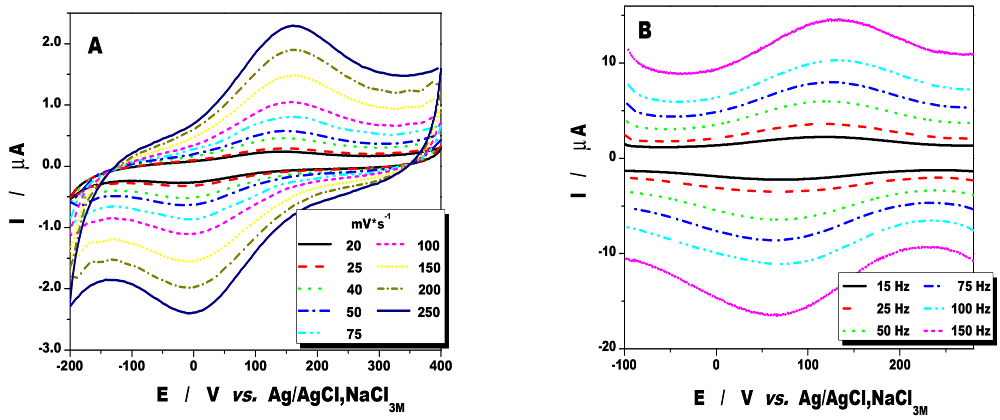


Figure 70. Influence of the potential scan rate on the voltametric response recorded at Au/L-Cys/Hm modified electrode: **(A)** CV; **(B)** SWV. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer (pH 6.9); starting potential, 0.4 V *vs.* Ag/AgCl,$NaCl_{3M}$; SWV amplitude, 50 mV; deaerated solutions.

Voltammetric measurements carried out at different potential scan rates (CV) or frequencies (SWV) (Figure 70) 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: (Hasebe Y., Wang Y. 2013; Dai Z. *et al.* 2004; Turdean G. L. *et al.* 2006; Pilloud D. L. *et al.* 2000; Wang Q. *et al.* 2004):

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

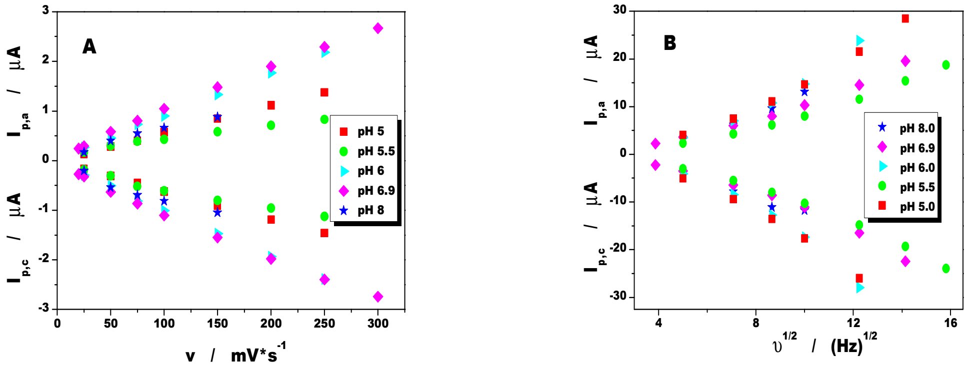


Figure 71. Potential scan rate (**A**) and frequency (**B**) dependences of the peak currents measured at Au/L-Cys/Hm modified electrode: (**A**) CV and (**B**) SWV measurements. Experimental conditions: see figure 2.

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)(Figure 71A). 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 (Figure 71B).

The slopes values of the log –log dependencies of peak currents on the potential scan rate (Table 37) are close to 1.

Table 37. Slopes of the log(I_p) *vs.* log(v) dependencies 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) dependencies**			
	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

VIII.5.2. pH influence

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 peak potential ($E^{0'}$) when the pH of the supporting electrolyte decreases.

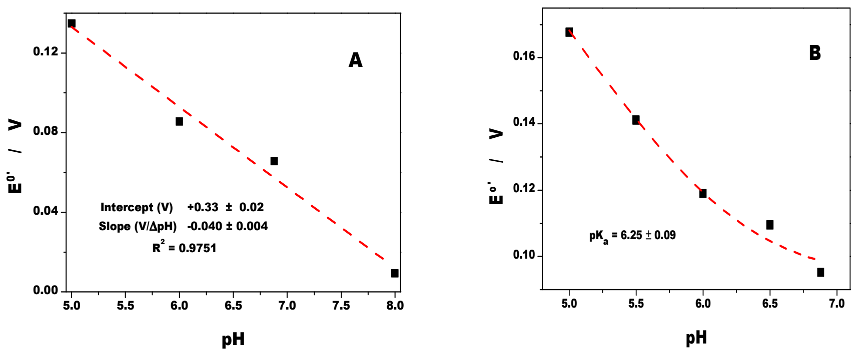


Figure 4. pH dependence of the formal redox potential ($E^{0'}$) of Hm adsorbed on the cysteine modified Au surface: (**A**) CV and (**B**) SWV. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer. CV measurements: potential scan rate, 25 mV/s. SWV measurements: frequency, 75 Hz;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

Quantitatively, in the investigated domain of pH (from 5 up to 8), the slope value corresponding to the $E^{0'}$ *vs.* pH dependency, obtained from CV measurements [(-0.040 ± 0.004)V/pH, with $R^2 = 0.9751$, N = 4; Figure 73B), is relatively close to the theoretical value (0.059 V/pH), expected for a redox process involving the transfer of $1e^-/1H^+$ [see equation 1].

This peculiar behavior was already reported for Hmand 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.

VIII.5.3. Determination of pK$_a$ and k$_s$

SWV measurements, carried out within the same pH domain (from 5 up to 8), revealed a nonlinear dependency of $E^{0'}$*vs.* pH (Figure 73B). 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 ± 0.09). This value was found slightly lower than that reported for dissolved Hm (pK$_a$ = 6.63), probably due to the strong interaction existing between the adsorbed Hm and the negatively charged Au/L-Cys surface (Hasinoff B. B. *et al.* 1969).

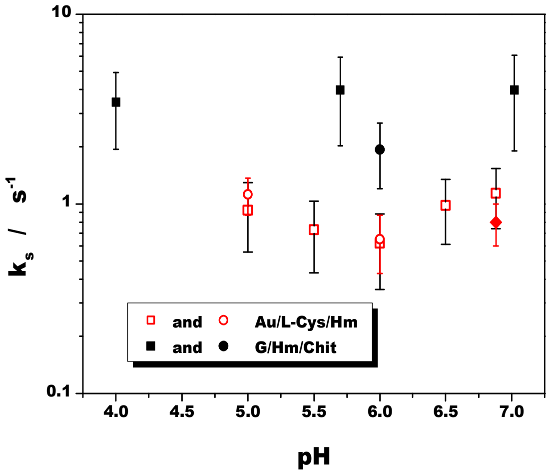


Figure 74. pH effect on the rate constant of charge transfer (k_s) corresponding to Au/L-Cys/Hm (□,○) and G/Hm/Chit (■,●) modified electrodes, estimated from CV (□,■) and SWV (○,●).

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, involving Hm immobilized on the Au/L-Cys/Hm modified electrode. Thus, the k_s values were calculated from CV measurements by using the Laviron's treatment (Laviron E. 1979), (while from SWV data the k_s values were obtained by using the treatment elaborated by Reeves (Reeves H. J. *et al.* 1993) for an adsorbed quasi-reversible redox couple. As can be seen from Figure 74, within the limits of the experimental errors and irrespective the value of the surrounding pH, CV and SWV measurements gave similar values of k_s.

At the same time, it can be noticed that the rate constants of the charge transfer process involving Hm immobilized on Au/L-Cys self-assembled film [$(k_s)_{CV}$ =(0.65± 0.22) s^{-1}; $(k_s)_{SWV}$ = (0.62 ± 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 ± 0.7) s^{-1}; $(k_s)_{SWV}$ = (3.4 ± 1.5) s^{-1}; pH 6)] (Mares G.M. *et al.* unpublished results).

GENERAL CONCLUSIONS

According to the objectives of this thesis, the following general conclusions can be stated:

1. Modified electrodes with Hm immobilized on the G and Au electrode surfaces by adsorption, by incorporation within a polymeric film and self-assembled on the gold were obtained;
2. The modified electrodes were investigated by using CV, SWV and amperometric measurements, performed under various experimental conditions (scan rate/ frequency; pH etc.). At the same time, it was analyzed the electrochemical response for H_2O_2 and nitrite reduction, as well as the electrode stability (for short and long time). The standard formal potential values, *ks* and pKa. were calculated;
3. The electrochemical parameters confirmed that Hm is strongly adsorbed on the electrode surface and participates to a quasi-reversible electrochemical process, involving $1H^+/1e^-$.
4. Amperometric biosensors, based on Hm and HRP with different architectures, were obtained and applied for H_2O_2 detection;
5. The bioelectrochemical parameters I_{max} and K_m^{app} were calculated by using three different approaches (fitting to the Michaelis-Menten equation, and Lineweaver-Burk, Eadie-Hoffstee and Hanes-Woolf linearizations) and were found in good agreement with the already reported values.

Participation at national and international conferences

1. **Mares Georgeta Maria**, Turdean Graziella Liana, Popescu Ionel Cătălin, *Hemin-modified Graphite Electrode: Electrochemical Characterization and Hydrogen Peroxide Detection Using Cyclic Voltammetry and Square Wave Voltammetry*, 18th International Conference on Chemistry, Băile Felix, România, 22-25 Noiembrie, **2012**, prezentare poster.

2. **Mares Georgeta Maria,** Turdean Graziella Liana, Popescu Ionel Cătălin, *Hemin-modified Gold Electrode: Electrochemical Characterization and* k_s *Determination Using Cyclic Voltammetry and Square Wave Voltammetry*, 19th International Conference on Chemistry, Baia-Mare, România, 21-24 Noiembrie, **2013**, prezentare poster.

3. **Mares Georgeta Maria,** Turdean Graziella Liana, Popescu Ionel Cătălin, *Biosenzor amperometric conținând hemină pentru detecția peroxidului de hidrogen*, Oradea, România, mai, **2013**, prezentare poster.

Papers

1. **Georgeta Maria Mares**, Graziella Liana Turdean, Ionel Cătălin Popescu, **2013**, *Electrochemical behavior of the hemin modified graphite electrode for* H_2O_2 *detection*, Studia UBB Chemia, *58*, 105-114, (ISSN 1224-7154).

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