

Comparison Between The Experimentally and Numerically Modeled and Simulated Kinetic Parameters Corresponding to Michaelis-Menten Equation for NADH Sensors Based on Polymeric Phenothiazine Modified Electrodes

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The paper presents a comparison between the experimentally and numerical modeled and simulated kinetic parameters (Michaelis-Menten constant, K_M and I_{max}) obtained by fitting the Michaelis-Menten equation, for NADH amperometric measurements at electrodes modified with polymeric phenothiazines. The modified electrodes were obtained by two different approaches: (i) adsorption of the poly-phenothiazine formaldehyde (PPF) on graphite electrodes (G/PPF) and (ii) electro polymerization of *bis*-phenothiazin-3-yl-methane (BPhM) on glassy carbon electrodes (GC/poly-BPhM). It was obtained a good correlation of the simulated kinetic parameters with the experimental values. GC/poly-BPhM electrodes present a higher sensitivity for NADH detection than G/PPF electrodes, at an applied potential of +200 mV vs. SCE and +50 mV vs. SCE, respectively.

Key Words: Phenothiazine derivatives, NADH, Numerical modeling, Simulation, Michaelis-Menten equation.

INTRODUCTION

Recent efforts to prepare modified electrodes that can be used as amperometric sensors for NADH detection have focused on the use of polymeric films as new materials. Mediators can be immobilized using different procedures: (i) direct covalent attachment to the electrode, of a polymer film deposited electrochemically onto the electrode surface¹, or coupled with a polymer backbone mixed in carbon paste²; (ii) electro polymerization of a mediator on the electrode surface^{3,4}, into graphite-epoxy composite electrodes⁵ and into a random block methyl-siloxane polymer⁶; (iii) direct chemisorption or adsorption of a polymer containing the mediating functional group; (iv) just simply mixing into carbon paste.

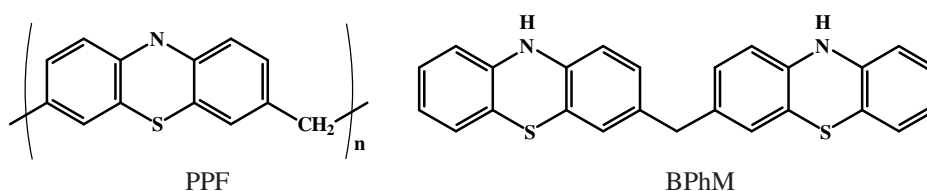
Phenothiazine, phenoxazine and phenazine derivatives (called azines) represent one of the most interesting groups of monomers providing a new group of electro active polymers.

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In this paper, electrodes were modified with polymeric films based on phenothiazine derivatives, using: (i) adsorption of the poly-phenothiazine formaldehyde (PPF) on graphite electrodes (G/PPF) and (ii) electro polymerization of *bis*-phenothiazin-3-yl-methane-BPhM on glassy carbon electrodes (GC/poly-BPhM). The amperometric detection of NADH at these modified electrodes was realized in order to determine the kinetic parameters (Michaelis-Menten constant, K_M and I_{max}). The calculated kinetic parameters were numerical modeled and simulated, using MATLAB program^{7,8}. The mathematical model was fitted to the experimental data. The calculated data were compared with the experimental data, recalculated in the optimization routine and fitted again until a minimal error between the experimental and integrated data was achieved.

EXPERIMENTAL

The phenothiazine derivatives, poly-phenothiazine formaldehyde (PPF) and *bis*phenothiazin-3-yl methane (BPhM) (**Scheme-I**) were synthesized and characterized according to previously published procedures^{9,10}.



Scheme-I: Structures of phenothiazine derivatives

β -Nicotinamide adenine dinucleotide, reduced form (NADH), was obtained from Sigma (St. Louis, USA) and dimethyl sulfoxide was obtained from Aldrich (Steinheim, Germany). Tetrabutylammonium tetrafluoroborate (TBA-TFB) was provided by Jansen Chimica (Denmark). All other chemicals were of analytical grade. The solutions throughout this work were always prepared using distilled water from a Milli-Q (Millipore, Bedford, USA) device. NADH solutions were freshly prepared in phosphate buffer (pH 7.0). The concentration of NADH in the solutions was checked monitoring the absorbance of the solution at 340 nm and considering an extinction molar coefficient¹¹ of $6600 \text{ cm}^{-1} \text{ M}^{-1}$. Phosphate buffer solutions (0.1 M) were prepared with proper amounts of NaH_2PO_4 (ACS-Scharlau) and $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ (Merck).

Electrodes preparation: The graphite modified electrodes with PPF (G/PPF) were obtained by spreading onto the electrode surface 2 μL of 1mM phenothiazine derivative solution in dimethyl sulfoxide and leaving them for 1 d at room temperature to evaporate the solvent.

Glassy carbon modified electrodes with BPhM (GC/poly-BPhM) were prepared by potential cycling (20 scans) from -1000 to +1200 mV vs. SCE, at 50 mV s^{-1} , in

1 mM BPhM containing 0.1 M TBA-TFB dissolved in DMSO, as was reported for redox dyes dissolved in water^{12,13}. Prior to all electro polymerization experiments, the DMSO solutions containing BPhM and TBA-TFB were de-oxygenated with nitrogen gas for 10 min.

All presented results are the average of at least three identically prepared electrodes, if not otherwise mentioned.

Electrochemical measurements: Amperometric experiments were performed in a traditional three electrode system. A platinum wire was used as counter electrode, a saturated calomel electrode (SCE) as reference electrode and the modified electrode (3 mm diameter) as working electrode. All experiments were carried out using a BAS 100A (Bioanalytical Systems, West Lafayette, IN, USA) electrochemical analyzer. An Eg&G rotator (Princeton Applied Research, Princeton, NJ, USA) was used for rotation the solution. A Kontron Instruments, 930 UVIKON spectrophotometer was used for measuring absorbance of the NADH solutions at 340 nm.

RESULTS AND DISCUSSION

Amperometric calibration for NADH, with modified electrodes based on phenothiazine derivatives, performed at constant applied potential (+200 mV vs. SCE for GC/poly-BPhM electrodes and +50 mV vs. SCE for G/PPF electrodes) is according to Michaelis-Menten kinetics. Thus, the kinetic parameters Michaelis-Menten constant, K_M and I_{max} were calculated by using fitting of experimental data based on Michaelis-Menten equation¹⁴:

$$I = \frac{I_{max}[S]}{[S] + K_M} \quad (1)$$

where: I_{max} (A) is the maximum current and [S] (mM) is NADH concentration.

By fitting the amperometric calibration curves to the Michaelis-Menten equation for both modified electrodes, the experimentally kinetic parameters were found (Table-1).

In order to obtain a model based on experimental values we used the method of least squares for fitting data. The fitting process requires a model that relates the response data to the predictor data with one or more coefficients. The result of the fitting process is an estimation of the "true" but unknown coefficients of the model. For obtaining the coefficient estimation, the least squares method minimizes the summed square of residuals. The residual for the $I_{theoretical}$, data point r_i is defined as the difference between the observed response value, I_i and the fitted response value $I_{theoretical i}$ and is identified as the error associated with the data:

$$r_i = I_i - I_{theoretical i} \quad (2)$$

The summed square of residuals is given by:

$$R = \sum_{i=1}^n r_i^2 = \sum_{i=1}^n (I_i - I_{theoretical i})^2 \quad (3)$$

where n is the number of data points included in the fitting and R is the sum of squares error estimation.

The numerical calculus and the graphic representation of the experimental data have been achieved in MATLAB program, where the kinetic equations have been implemented.

The intensity I as a monotonically increasing function of time is a polynomial approximation:

$$I_{\text{theoretical}_i} = p_N c_{\text{theoretical}}^N + p_{N-1} c_{\text{theoretical}}^{N-1} + \dots + p_1 c_{\text{theoretical}} + p_0 \quad (4)$$

The coefficients $p_i, (i = 1, N)$ are determined by fitting the values of I obtained from experimental measurements with the values returned by the function (4).

The numerical procedure used in this case was one similar to previous approach. The main goal was to minimize the error between the analytical expression given by (4) and the measured data.

The order of the approximating polynomial was obtained by trial-and-error and some sensitivity of the results was observed due to these changes.

The resulted mathematical models for obtained modified electrodes are:

For G/PPF electrode:

$$I_{\text{theoretical}} = 0.012c_{\text{theoretical}}^3 - 0.11c_{\text{theoretical}}^2 + 0.5c_{\text{theoretical}} + 0.0066 \quad (5)$$

For GC/poly-BPhM electrode:

$$I_{\text{theoretical}} = -0.15c_{\text{theoretical}}^4 + 0.82c_{\text{theoretical}}^3 - 1.7c_{\text{theoretical}}^2 + 1.9c_{\text{theoretical}} + 0.0056 \quad (6)$$

The coefficients from polynomial eqns. 5, 6 are very small for high powers of concentrations. A simple conclusion would be that a smaller order polynomial was sufficient for the approximation.

In the next step, I (μA) values have been generated with respect to NADH concentration, c (mM). The theoretical curves obtained from the eqns. 5 and 6 were represented for each NADH concentration (Fig. 1,+), in comparison with the experimentally obtained curves (Fig. 1,o).

The optimization has been achieved with the help of much iteration so that the average square deviation will be as small as possible, for the best superposition of the simulated line with the experimental one.

Difficulties in making accurate determinations of K_M and I_{max} from simple graphs of $I = f(c)$ led to the extensive use of the double reciprocal transformation of the equation^{15,16}:

$$\frac{1}{I} = \frac{1}{I_{\text{max}}} + \frac{K_M}{I_{\text{max}}} \cdot \frac{1}{[S]} \quad (7)$$

A plot of $I_{\text{theoretical}}^{-1}$ (μA^{-1}) vs. $c_{\text{theoretical}}^{-1}$ (mM^{-1}) will give a straight line if a complex is involved in the reaction between NADH and the mediator (Fig. 2), according to simple Michaelis-Menten reaction mechanism, proposed by Gorton^{17,18}:



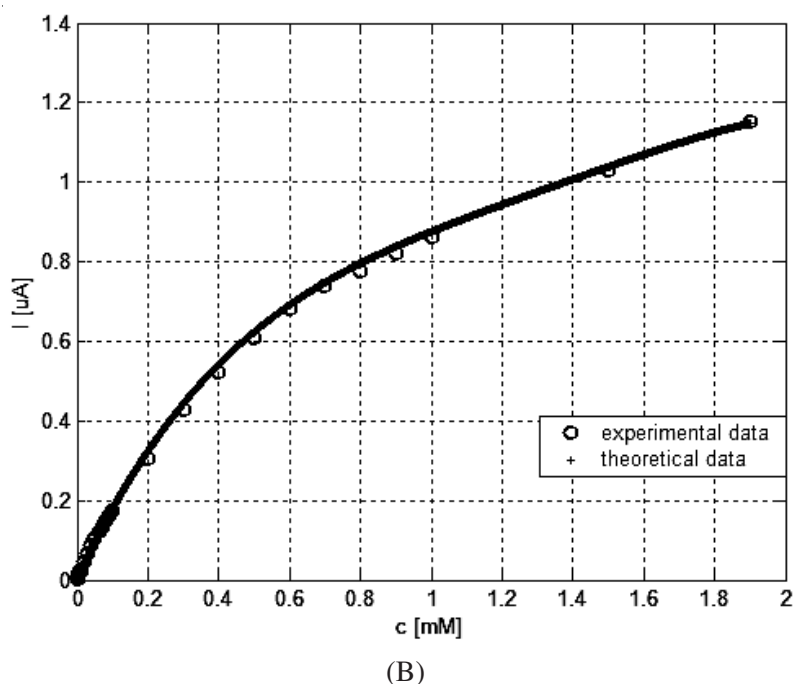
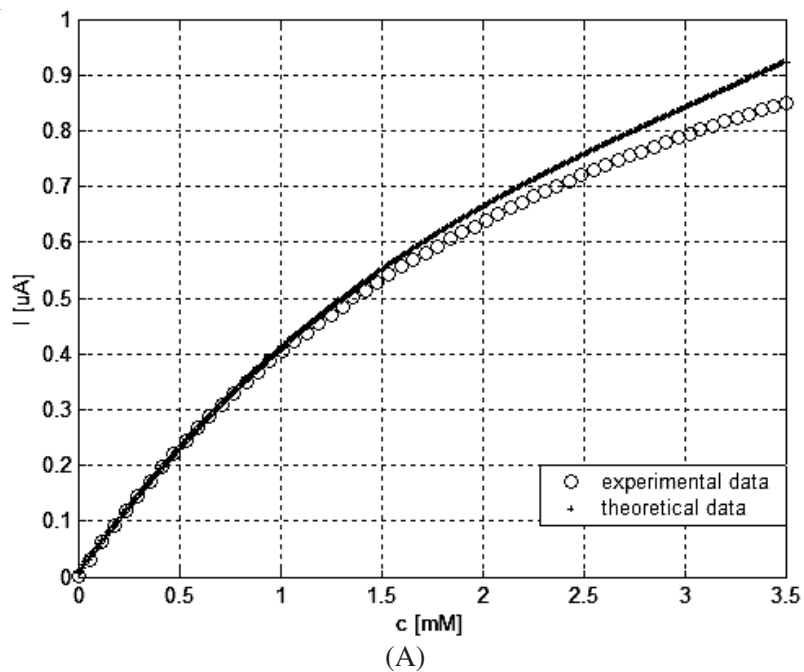


Fig. 1. Overlap of the experimental data (o) with the modelled ones (+) for concentration dependence of intensity, corresponding to G/PPF electrodes (A) and GC/poly-BPhM electrodes (B)

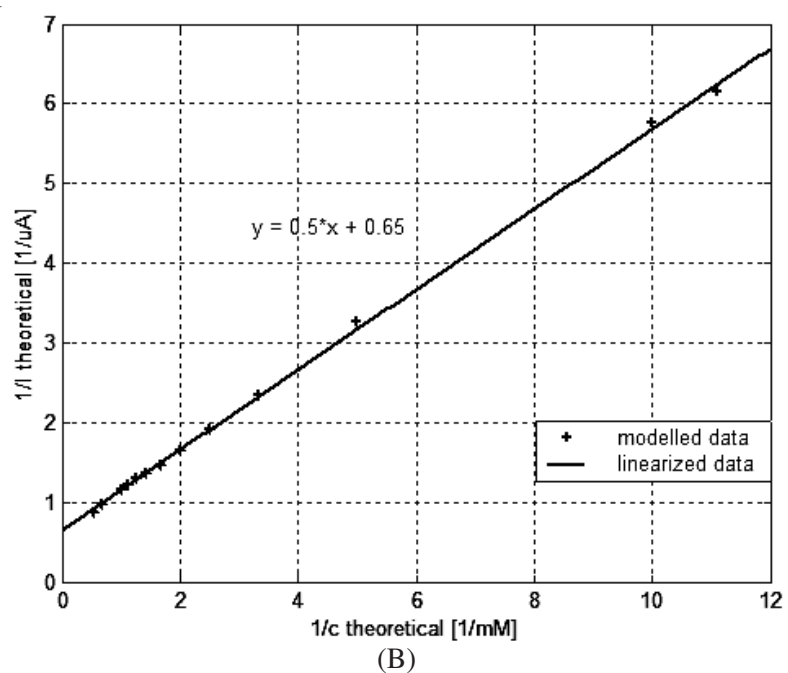
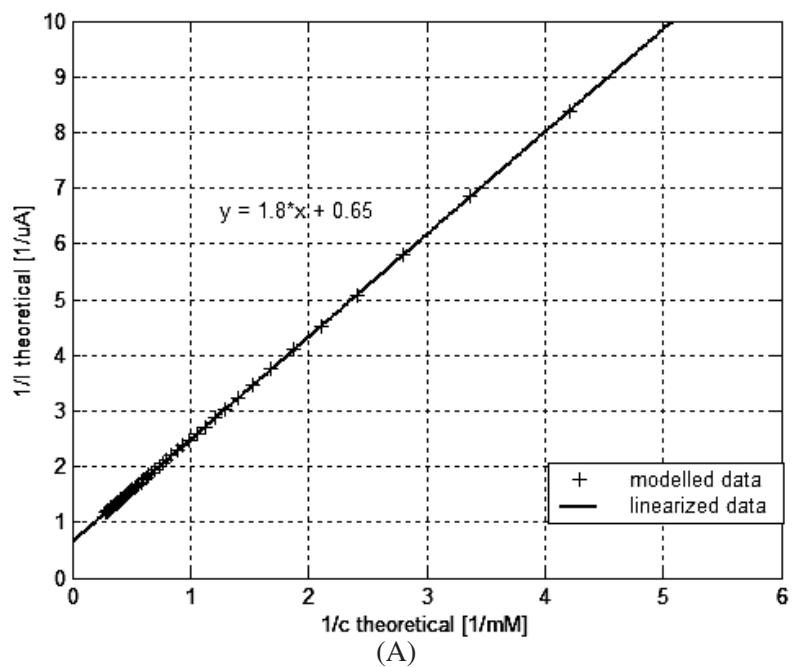


Fig. 2. Overlap of the modelled data (+) and the extrapolated simulated data (continuous line) for the theoretical Lineweaver-Burk plot corresponding to G/PPF electrodes (A) and GC/poly-BPhM electrodes (B)

where: k_1 and k_{-1} are rate constants for association and dissociation of intermediate complex and k_{+2} is the turnover number. The Michaelis-Menten constant, K_M is expressed as:

$$K_M = \frac{k_{-1} + k_2}{k_1} \quad (9)$$

The first degree equations which are obtained by extrapolation of the line theoretically obtained by numerical simulation are:

For G/PPF electrode:

$$y = 1.8x + 0.65 \quad (10)$$

For GC/poly-BPhM electrode:

$$y = 0.5x + 0.65 \quad (11)$$

The double reciprocal plot continues to be used for routine determinations of K_M and I_{max} . Linear regression methods are now generally used to obtain lines of best fit and a very good correlation. K_M and I_{max} are determined indirectly as the y-intercept ($1/I_{max}$) and the slope (K_M/I_{max}) only estimates the errors of K_M and I_{max} .

Simulated data (from equations 10 and 11) were used to assess the accuracy of determinations for K_M and I_{max} of the Michaelis-Menten equation. Curves were fitted using a direct least squares fit and the unweighted double reciprocal plot¹⁹.

In Table-1 is presented a comparison between experimentally and simulated results for kinetic parameters. It is clear from Table-1, the experimental obtained data overlap almost perfectly with the mathematical and numerical simulated data, so the validity of the model has been proved.

TABLE-1
KINETIC PARAMETERS K_M AND I_{max} CORRESPONDING TO
MODIFIED ELECTRODES

Electrode	K_M (mM)		I_{max} (μ A)	
	Experimental	Simulated	Experimental	Simulated
G/PPF	2.83	2.77	1.53	1.54
GC/poly-BPhM	0.84	0.77	1.62	1.54

It can be observed that the GC/poly-BPhM modified electrode presents a better sensitivity (calculated as I_{max}/K_M ratio) than G/PPF modified electrodes. This can be due to the high polymeric film thickness deposited on glassy carbon electrode surface by using electro polymerization than by using adsorption on graphite electrodes.

Conclusion

The experimentally kinetic parameters for NADH detection, determined using the Michaelis-Menten equation were compared with the numerical modeled and simulation data, for electrodes modified with polymeric phenothiazines.

The results obtained by mathematical modeling and numerical simulation correspond well with the experimentally determined values. This method can present a

good compromise, between theoretic rigorous formalism and possibilities, sometimes very limited, of experimental methods of the evaluation.

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