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Direct Electrochemical Determination of Hemoglobin in Blood Using Iodine-Coated Platinum Polycrystalline Electrode

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A direct method for hemoglobin determination in blood was developed using iodine-coated platinum electrode. The electrochemical behavior of iron ions in hemoglobin within human red blood cells was investigated by cyclic voltammetry at iodine-coated platinum electrode. A well-defined peak assigned to oxidation of hemoglobin was observed at about 0.4 V. A more prominent peak for the oxidation of hemoglobin was observed at pH 5.5 in 0.9% NaCl solution compared to phosphate puffer solution of pH 3.5. Using hydroxylamine in the dry form with the hemoglobin in red blood cells enhanced the peak current for hemoglobin oxidation by 14 folds. A linear relationship between the hemoglobin oxidation peak current and the concentration of hemoglobin was illustrated within the concentration range from 1.53 g dl^{-1} to 9.2 g dl^{-1} with a correlation coefficient (R^2 = 0.9949). The estimated detection limit based on S/N = 3 ratio was 0.004 g dl^{-1}. The developed method was directly applied to determine hemoglobin without pretreatment of the blood sample. The developed method passed the tests for its liability towards interferences. The developed method was validated against the regular clinical analysis for hemoglobin. Analysis of real samples by the two methods indicated the absence of systematic errors as indicated by the confidence limits at 95% confidence level. Moreover, application of the null hypothesis to the results of two methods indicated the validity of the null hypothesis; no significant difference was observed between the two methods at p = 0.05.

Keywords: Hemoglobin analysis, Blood analysis, Iodine-coated platinum electrode, Cyclic voltammetry

INTRODUCTION

Assaying human blood for its hemoglobin content is one of the most important analyses conducted in clinical analysis because of its relevance to pathogenic disorders. Routine clinical methods demand accuracy, precision, rapidity, and simplicity for any proposed method for hemoglobin analysis. Electrochemical methods offer simple procedures, inexpensive instrumentation, simplicity of sample pretreatment in addition to accuracy, precision and rapidity. Therefore, the need for development of simple, fast and sensitive method is of great interest.

Many techniques such as cyanomethemoglobin, Sahli, Lovibond-Drabkin, and automated hematologytechniques are currently used for determination of hemoglobin. Most of these methods, however, are relatively expensive, and time-consuming in terms of sample pretreatment and data interpretation [1].

The presence of iron as an active redox center in hemoglobin molecule is the corner stone for electrochemical analysis. In the past years, several electrochemical methods have been reported for direct detection of hemoglobin using a various kinds of electrodes. For instance, direct electrochemical detection of hemoglobin using modified electrodes [2-15] and glassy carbon electrode has been reported [16,17].

The utilization of iodine-coated platinum electrode as a chemical sensor in chemical analysis was demonstrated for inorganic and organic species [18-22]. It has excellent electrochemical properties such as the high stability, the

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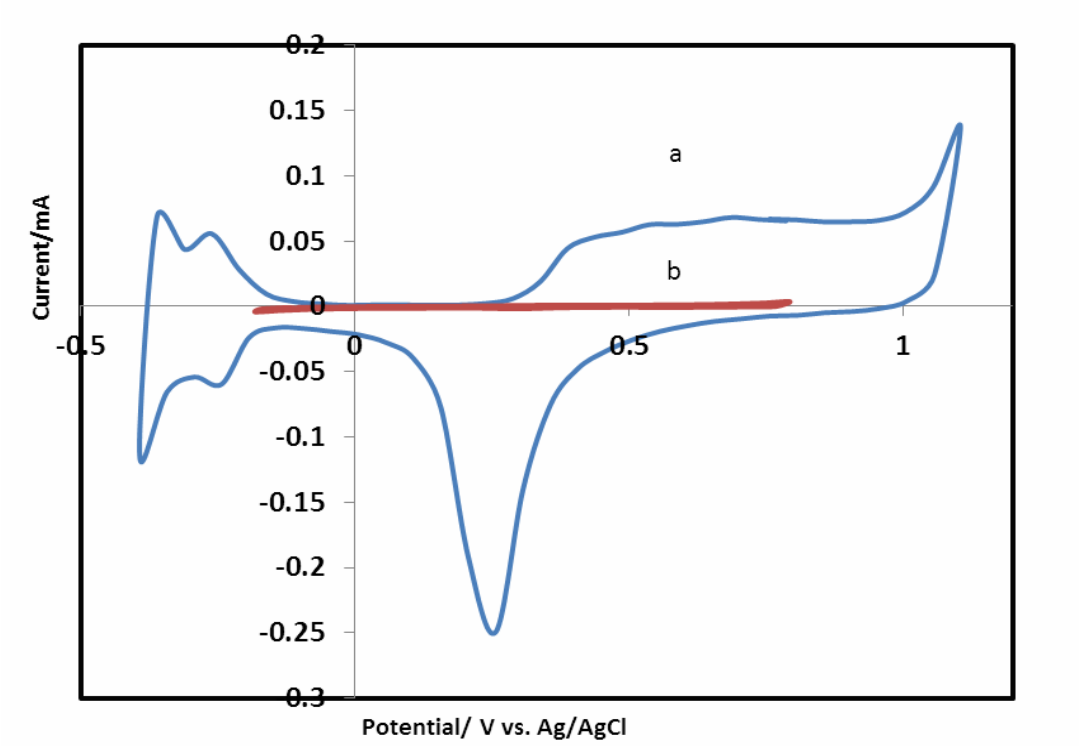

Fig. 1. The cyclic voltammogram of (a) polycrystalline platinum electrode and (b) the same electrode after adsorption of iodinefrom 0.50 M H_2SO_4 + 1.0 × 10^{-2} M KI solution. Both i-E scans were recorded in iodine-free 0.5 M H_2SO_4.Scan rate = 50 mV s^{-1}.

remarkable inertness towards molecular adsorption and suppression of surface processes. These properties dictate lowering the background and enhancement of S/N ratio in voltammetric analysis [23,24].

The present work has been undertaken with the main objective of method development for direct voltammetric analysis of blood for its hemoglobin content. The developed method is based on voltammetric analysis at iodine-coated platinum electrode. Fabrication of a prototype small electrochemical cell adapted for determination of hemoglobin is an added value to the present work.

MATERIALS AND METHODS

Instruments, Cell and Materials

A potentiostat (model 362, Pinceton Applied Research) interfaced to a computer *via* GPIB interface (IEEE) for data acquisition was used. A modified Labview® (IEEE) software was used for data acquisition. A home-made one-compartment, 0.50 cm^3 small electrochemical cell with one inlet/outlet for gas purging and blanketing with nitrogen gas was used in the present work. The working electrode, the reference, and the auxiliary electrode were inserted in a PTFE movable cover which can be easily placed and removed on the miniaturized cell. The used working electrode was a 0.5 mm polycrystalline platinum wire purchased from Aldrich (99.99% minimum purity certified reagent). The immersed end of the platinum electrode was a U-shape end to provide a mark for consistent surface area upon immersion under the surface of the fluid and touching the surface with the curved end of the wire. The reference electrode was silver wire quasi-reference electrode. The auxiliary electrode was a 0.5 mm polycrystalline platinum wire (Aldrich, certified 99.99% minimum purity).

All reagents used were analytical grade and used as received from the suppliers without further purification or

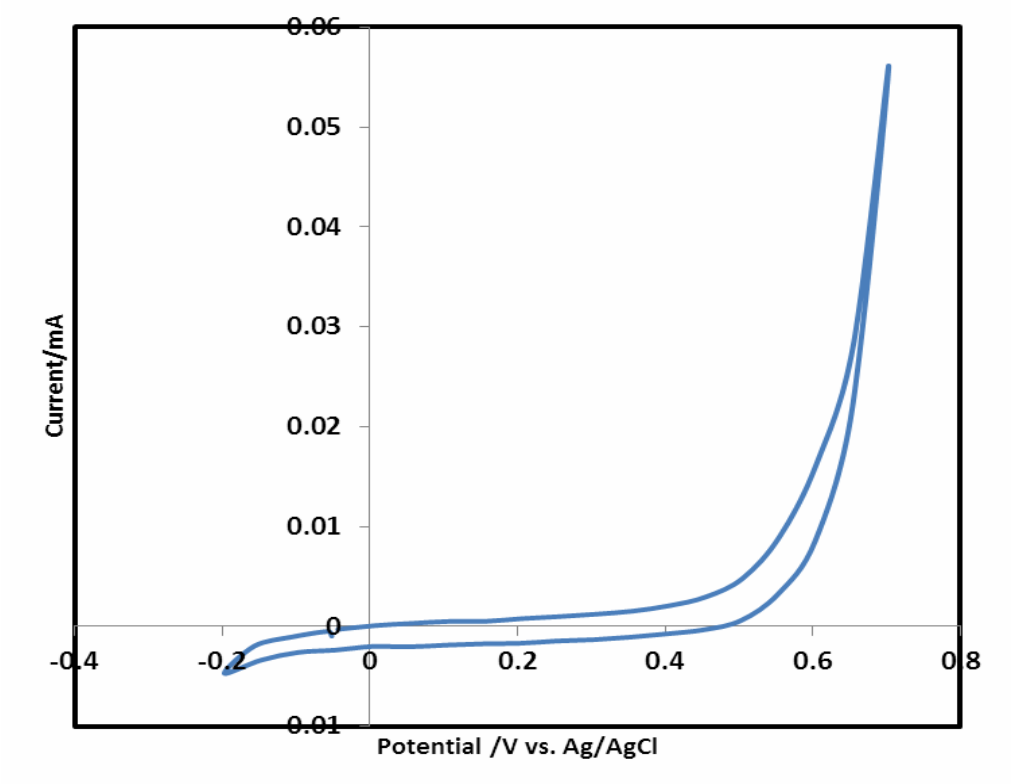

Fig. 2. The cyclic voltammogram of iodine-coated electrode recorded in 9.2 g dl^{-1} of standard hemoglobin. Scan rate = 50 mv s^{-1}.

pretreatment. Hemoglobin standard (9.2 g dl^{-1}) was supplied from Arab Company for Medical Diagnostics, Jordan. Sulfuric acid (95-97%) was supplied from Merck, nitric acid (69.5%) from Scharlau, and potassium iodide from Sigma-Aldrich. Hydroxylammonium chloride was an AnalaR Normapur®ACS reagent supplied by BDH. The nitrogen gas was a G5 grade, 99.999% minimum purity supplied from the International Jordanian Gases Company (Amman, Jordan).

Procedures

Preparation of iodine-coated platinum electrode. The cleanliness of the solid electrode surface is a critical step prior to surface coating with iodine. The platinum electrode was cleaned in fresh chromic acid solution and placed in the electrochemical cell where the electrode potential was cycled between the hydrogen discharge limit and oxygen discharge limit. Reproduction of the well-known voltammogram of the polycrystalline platinum electrode was taken as a criterion for cleanliness of the platinum surface and its readiness for dosing with iodine.

Dosing the platinum electrode with iodine was accomplished by dipping the platinum electrode in the freshly prepared (0.5 M H_2SO_4 + 0.01 M KI) solution for few minutes at the double layer region (~+0.2 V). The electrode was extensively *in situ* rinsed with iodine-free 0.5 M H_2SO_4. The cyclic voltammogram for iodine-coated platinum electrode was recorded between -0.2 V and 0.80 V. The absence of voltammetric peaks for hydrogen adsorption and desorption is a manifestation of complete coating of the electrode surface with iodine (Fig. 1).

Sample Preparation

Sixteen blood samples of different hemoglobin content were brought from Princess Haya Bint Al-Hussein Military

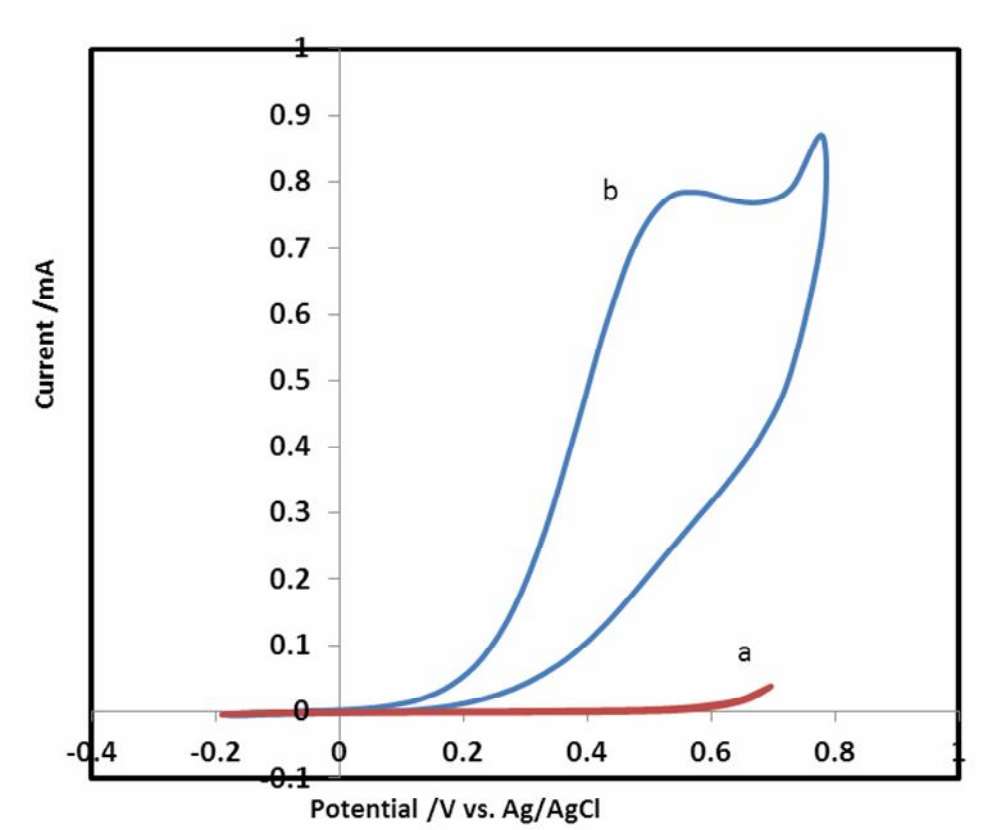

Fig. 3. (a) The cyclic voltammogram of iodine-coated platinum electrode recorded in 9.2 g dl^{-1} of standard hemoglobin (—), (b) the cyclic voltammogram of iodine-coated platinum electrode recorded in 9.2 g dl^{-1} of standard hemoglobin with few crystals of hydroxylamine (—), Scan rate = 50 mV s^{-1}.

hospital, Jordan. The samples were directly analyzed after collection.

A volume of 0.2 ml of human blood sample was injected in the miniaturized small scale electrochemical cell followed by adding few crystals of hydroxylamine (NH_2OH).The cell was swirled to homogenize the solution and the cyclic voltammogram of the iodine-coated platinum electrode was recorded.

Separation of Red Blood Cells

A fresh blood sample was obtained from human donor and directly collected in vacutainer plastic blood collection tubes with K2-EDTA to prevent blood coagulation, washed with 0.9% NaCl solution and centrifuged three times, the supernatant saline was discarded and the red blood cells were collected and analyzed by scanning the potential between -0.2 and 0.8 V *vs*. Ag/AgCl electrode.

RESULTS AND DISCUSSION

Method Analytical Parameters

Initially the cyclic voltammogram of a hemoglobin standard solution (9.2 g dl^{-1}) at iodine-coated platinum electrode was recorded without addition of any chemical reagent (Fig. 2). The voltammogram shows a large anodic peak centered at about 0.6 V without a counter peak upon scan reversal. This peak is most likely assigned to oxidation of iron(II) in hemoglobin. While the counter cathodic peak is not observed due to some following coupled reaction. This peak however couldn't be used for quantification of hemoglobin in blood because it is very close to the threshold of iodine stripping from the electrode surface.

Figure 3 shows a representative voltammogram of the iodine-coated electrode in blood with addition of hydroxylamine. A prominent peak centered at 0.5 V for

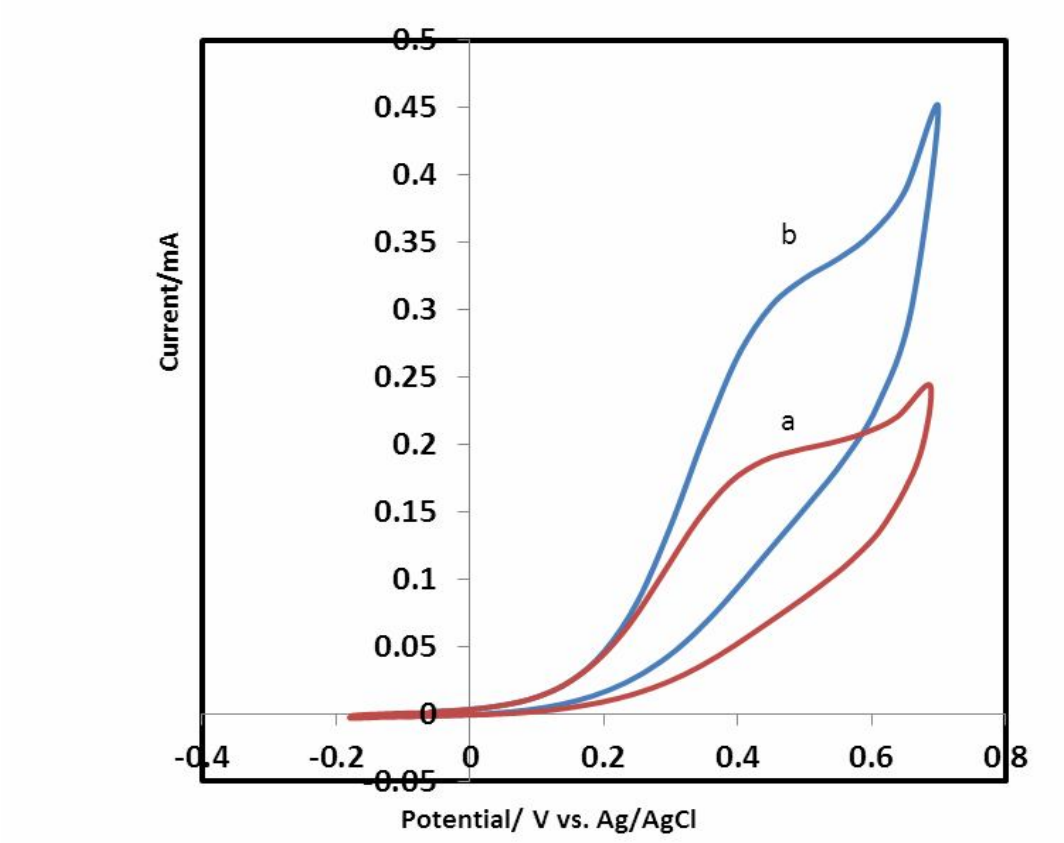

Fig. 4. Cyclic voltammograms of iodine-coated platinum electrode were obtained for 3.07 g dl^{-1} standard hemoglobin in a) aqueous 0.1 M phosphate buffer at pH 3.5 (—) and (b) for 0.9% NaCl solution at pH 5.5 (—), scan rate = 50 mv s^{-1}.

oxidation of hemoglobin with complete absence of a cathodic peak was observed. Iron(III) in porphyrins is relatively easily reduced and undergoes nitrosylation reaction when acted on by hydroxylamine [25].

The experimental conditions were optimized by variation of the pH of the blood specimens. Two pH values were tested, pH 5.5 (0.9% NaCl solution) and pH 3.5 (0.1 M phosphate puffer solution). Figure 4 shows the voltammograms for the iodine-coated platinum electrode reproduced at pH 5.5 and 3.5. The voltammograms show a peak centered at 0.50 V at both pH values. The sensitivity, however, is higher at pH 5.5 more than at pH 3.5. The observed pH effect on sensitivity of the electrochemical oxidationis is attributed to the involvement of hydrogen ions in the electrochemical oxidation process at the electrode [26,27],

as described by the following equation

$$HbFe(III) + H^+ + e^- \leftrightarrows HHbFe(II)$$

The effect of scan rate on the cyclic voltammogram of hemoglobin at the iodine-coated platinum electrode was investigated for a scan rate that ranged from 10-100 mV s^{-1}. Figure 5 shows a set of cyclic voltammograms reproduced at various scan rates in the presence of hydroxylamine. A linear relationship between the hemoglobin oxidation peak current and the square root of the scan rate, $(dE/dt)^{1/2}$ is demonstrated (R^2 = 0.9984). This finding indicates that the observed peak is due to a diffusion controlled process.

The calibration curve was constructed by establishing the relationship between the hemoglobin concentration and the anodic peak current for oxidation of hemoglobin. The

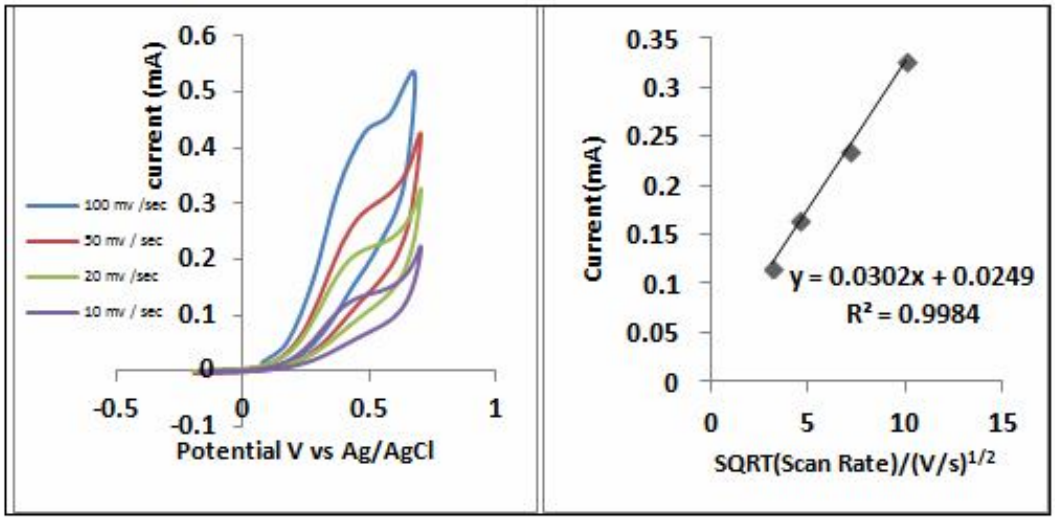

Fig. 5. a) Cyclic voltammograms of an iodine-coated platinum electrode in 0.9% NaCl solution and 4.6 g dl^{-1} standard hemoglobin recorded at 10,20, 50 and 100 mV s^{-1}. b) The least square line for the hemoglobin oxidation peak current vs. the square root of scan rate.

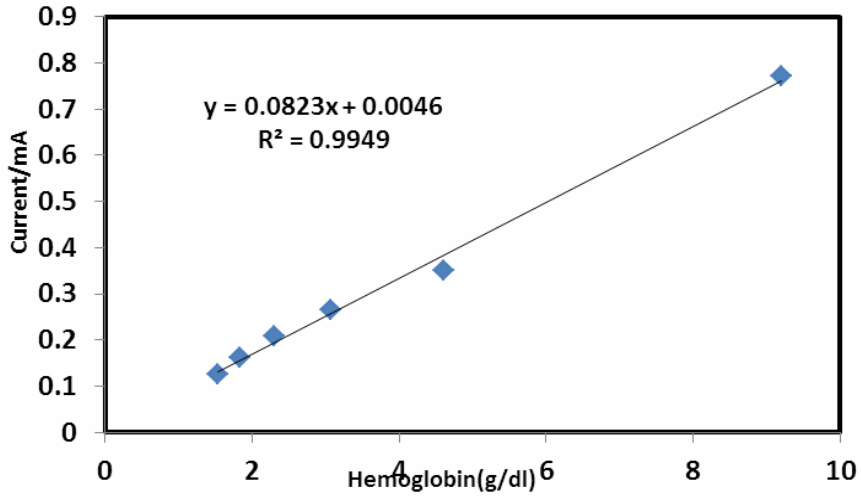

Fig. 6. A calibration curve plot for the relationship between hemoglobin standard concentration and oxidation peak current of hemoglobin measured in 0.9% NaCl solution at iodine-coated platinum electrode. Scan rate = 50 mv s^{-1}.

concentration was established by decremental dilution of standard hemoglobin (9.2 g dl^{-1}) with 0.9% NaCl solution. The hemoglobin concentration in the solutions ranged from 9.2-1.53 g dl^{-1}. Three voltammograms were recorded for each solution and the peak currents were measured from the voltammograms. Figure 6 indicates that the anodic peak current for hemoglobin oxidation is linearly proportional to the hemoglobin concentration with an excellent linearity

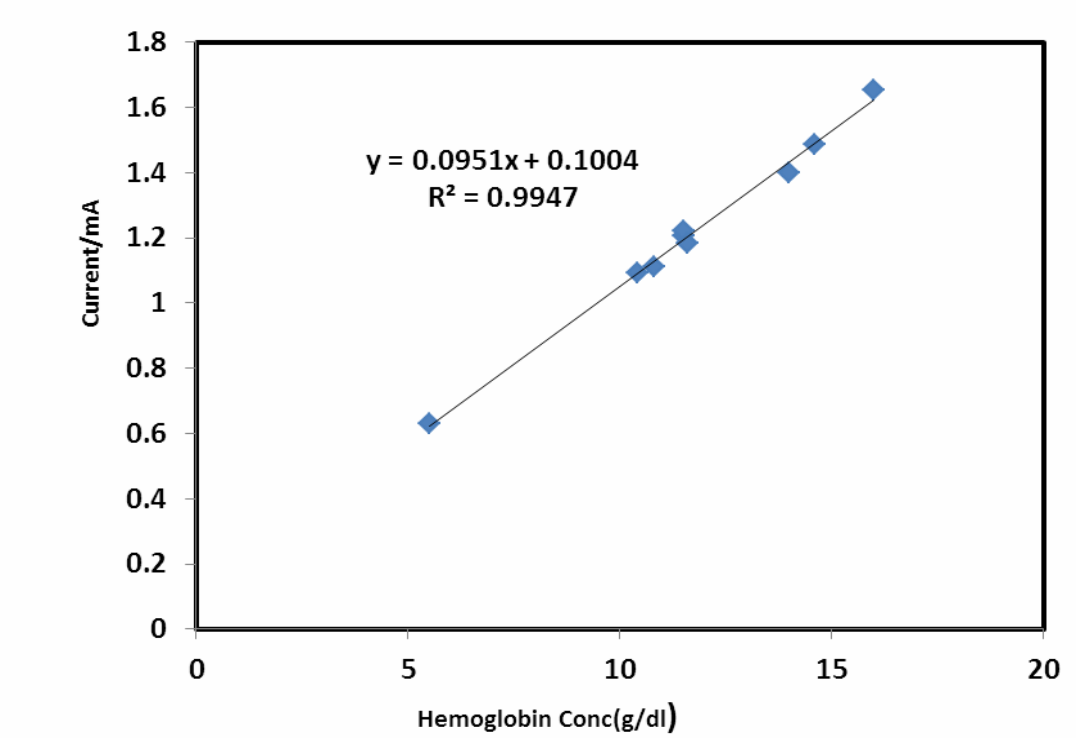

Fig. 7. A calibration curve plot for the relationship between clinical value of hemoglobin in actual blood and oxidation peak current of hemoglobin measured with the addition of a portion of hydroxylamine at iodine-coated platinum electrode. Scan rate = 50 mv s^{-1}.

(R^2 = 0.9949) and a calibration equation given by

$$i_{(mA)} = 0.0823C_{Hb} + 0.0046$$

The method detection limit (based on S/N = 3) is 0.004 g dl^{-1} and the limit of quantitation (LOQ) (based on S/N = 10) is 0.014 g dl^{-1}. The relative standard deviation in the measurements, RSD, is 5.01%. These data attest to the acceptable sensitivity of the method and its high precision. Higher sensitivity can be achieved by application of a more sensitive technique like differential pulse voltammetry. Differential pulse voltammetry, however, was not attempted because cyclic voltammetry provides satisfactory sensitivity for the concentration ranges of hemoglobin in human blood.

The inter- and intra-day precision of the developed voltammetric method was evaluated by analyzing four standard samples of hemoglobin. The concentrations of the samples were selected to cover the range from 0.53-9.2 g dl^{-1}. The relative standard deviation was found to be within 1.05% and 4.95% for six successive determinations for each concentration. The values of the relative standard deviation attest to the high repeatability for both inter- and intra-day measurements.

Method Validation

Figure 7 shows the excellent linearity (R^2 = 0.9947) between the hemoglobin anodic peak current and the concentration of blood hemoglobinin real blood samples. This finding attests to the applicability of the developed method to analyze the real samples and the negligible effects of the complex matrix in blood.

Since standard addition is meaningless for a method based on whole blood analysis, the developed method was validated by analysis of a set of eight real blood samples. The results of the developed method were compared with a well-established clinical methods for determination of hemoglobin in blood. The selected method for comparison was a photometric method on Sysmex auto-analyzer using

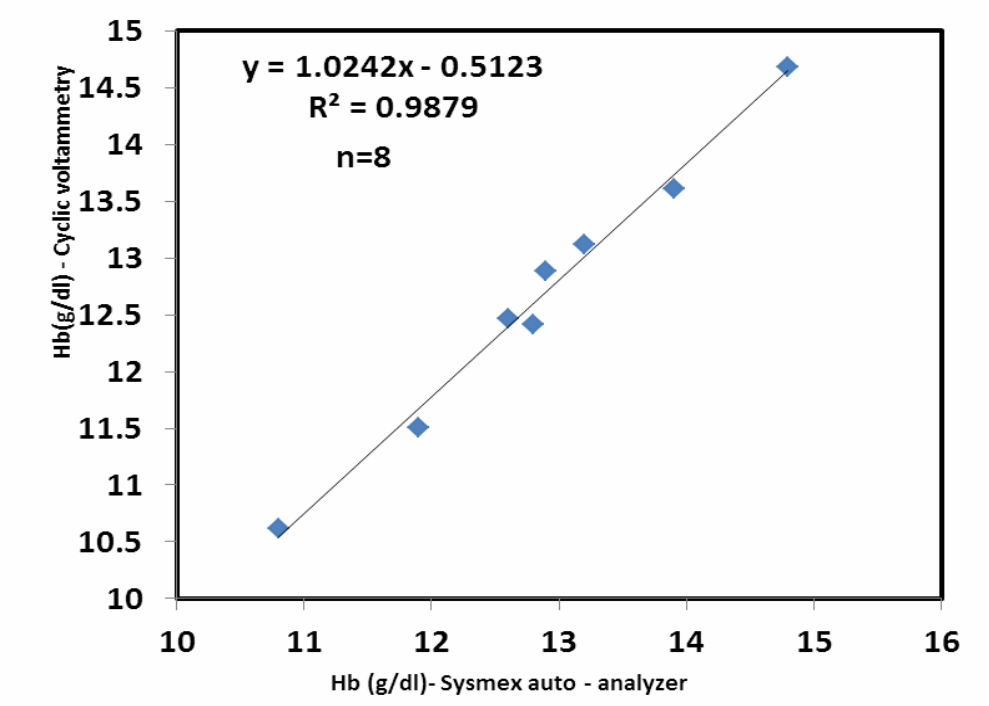

Fig. 8. Correlation between hemoglobin values from hematology lab of Princess Haya Bint Al-Hussein Military hospital using Sysmex auto-analyzer and the experimental value using dry hydroxylamine at iodine-coated platinum electrode.

SLS-Hb complex [28]. The developed electrochemical method involved using a 0.5 cm^3 miniaturized cell that works on 200 µl-sample only. The measurement time takes only about 30 s. An excellent correlation (R^2 = 0.9879, n = 8) has been noticed between the results of the two methods as noticed in Fig. 8. Table 1 shows the statistical parameters for the results of the developed electrochemical method. The data in Table 1 demonstrate that the nominal values are within the 95% confidence interval, indicating no determinate error in the results with probability of 95%.

The relative error values for the eight blood samples were less than 5%, a manifestation for the accuracy of the developed method. The coefficient of variation values from 1.16% to 6.75% add an obvious evidence for the precision of the developed voltammetric method. Moreover, the paired t-test was applied to test the significant difference between the values of voltammetric analysis results and the results of the clinical method using Sysmex auto-analyzer. The calculated t value is -0.328 compared to a critical value of 2.365 at p = 0.05 [33]. This indicates that the null hypothesis is true validating that there is no significant difference between the results of the two methods. The methods mentioned in Table 2 were used for hemoglobin determination, however, these methods have the disadvantage of complicity of analytical procedures, sophisticated instrumentations, time consuming and expensive compared to the green, simple, fast and inexpensive developed electrochemical method.

CONCLUSIONS

In the present work, an iodine-coated platinum electrode was successfully applied as a potential sensor in a miniaturized cell for direct assay of whole blood for its hemoglobin content. The developed voltammetric method is simple, fast, accurate, and can be developed to a hand held device for determination of hemoglobin. The developed method was validated according to the well-known

Table 1. Comparison of Various Chemical Methods for Hemoglobin Determination

Method	Linear range	LOD	Matrix	Pretreatment	Ref.
Drabkin's test	17.3-25.58 mM	-	Whole blood	Yes	[29]
Chemiluminescence	7.35×10^{-3}-2.5 µM	1.8×10^{-3} µM	Whole blood	Yes	[30]
Colorimetry	6.21×10^{-2}-2.48 µM and 6.21-12.41 µM	6.21×10^{-3} µM	-	-	[31]
Fluorimetry	10 nM to 10 µM	10 nM	-	-	[32]
Cyclic voltammetry	949.5-5709.3 µM	2.48 µM	Whole blood	No	This work

Table 2. The Results of Assaying Human Blood Samples for their Hemoglobin Content by Voltammetric Analysis Based on Iodine-coated Electrode Compared with the Results of Standard Clinical Method

Human blood Sample No.	Hb concentration ($g\ dl^{-1}$)		Standard deviation	95% Confidence limits	95% Confidence interval	Percent bias	Coefficient of variation
	Standard clinical method[a]	Cyclic voltammetry[b]					
1	10.8	10.62±0.66	±0.66	10.62±0.552	10.068-11.172	-1.67	6.21
2	11.9	11.51±0.68	±0.68	11.51±0.567	10.943-12.077	-3.28	5.91
3	12.8	12.41±0.81	±0.81	12.41±0.677	11.733-13.087	-3.05	6.53
4	12.9	12.88±0.87	±0.87	12.88±0.728	12.152-13.608	-0.155	6.75
5	13.9	13.61±0.77	±0.77	13.61±0.644	12.966-14.254	-2.086	5.66
6	14.8	14.68±0.17	±0.17	14.67±0.142	14.528-14.812	-0.811	1.16
7	12.6	12.46±0.68	±0.68	12.46±0.569	11.891-13.029	-1.111	5.46
8	13.2	13.12±0.45	±0.45	13.12±0.376	12.744-13.496	-0.606	3.43

[a]These values were obtained from the hematology lab in Princess Haya Bint Al- Hussein Military Hospital (Jordan) using Sysmex auto-analyzer.

analytical procedures. The analytical signal, the anodic peak current was proved to be proportional to the concentration of hemoglobin in blood. Moreover, the developed method was proved to be applicable to real analysis of blood samples. Eight samples were analyzed by the developed method and a well-established clinical method. Comparison of the results indicated that there is no significant difference between the two methods.

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