

Serum Creatinine Electrochemical Biosensor on Printed Electrodes Using Monoenzymatic Pathway to 1-Methylhydantoin Detection

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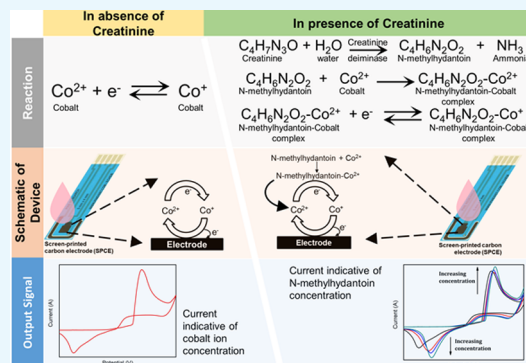


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ABSTRACT: The rising prevalence of Chronic Kidney Disease (CKD) has necessitated efforts towards the development of cost-effective and accurate biosensors for serum creatinine, which is a potent biomarker reflecting kidney function. This work presents a novel and cost-effective technique to estimate serum creatinine without any sample preprocessing. The technique involves the conversion of creatinine by a monoenzymatic pathway to 1-methylhydantoin. The concentration of 1-methylhydantoin is then quantified by utilizing its innate ability to form a complex with transition metals such as cobalt. The complex formation has been validated using optical spectroscopy and the transmittance at 290 nm wavelength is used to identify the optimum concentration of cobalt chloride in sensing chemistry. This chemical assay is shown to be robust against interference from serum albumin, the abundant plasma protein that can potentially influence the sensor response. The electrochemical biosensor developed using screen-printed electrodes thus provides highly selective creatinine estimation over the range of 0.2–4 mg/dL in a sample volume of 300 μ L with no preprocessing and hence can be easily translated into a viable point-of-care (POC) device.



INTRODUCTION

Chronic Kidney Disease (CKD) is associated with a significant decline in renal function, as reflected by the Glomerular Filtration Rate (GFR). According to the Kidney Disease Outcomes Quality Initiative (KDOQI), patients with GFR of <60 mL/min/1.73 m² or GFR of ≥ 60 mL/min/1.73 m², which is calculated based on the clearance of an established renal filtration marker over a period of at least 3 months, are diagnosed with CKD.^{1,2} The global prevalence of CKD is alarming, with developing countries facing a higher risk owing to increased population, lower socioeconomic status, and limited resources for optimal care. The prevalence of CKD in India is 17.2%, with an estimated age-adjusted incidence rate of End-Stage-Renal-Disease (ESRD) to be 229 per million population.^{3,4} The lack of community screening programs and asymptomatic progression of the disease leads to its diagnosis only in the advanced stages, whereby effective intervention involves either lifelong dialysis or renal replacement therapy. This reduces the quality of life with a considerable financial burden. Hence, efforts toward early detection of CKD are required for its effective management.⁵

There are several renal filtration markers that can be utilized to calculate GFR and monitor renal efficiency. These include creatinine, neutrophil gelatinase-associated lipocalin, urine protein, β -2-microglobulin, and cystatin C.⁶ However, the most commonly used exogenous biomarker is creatinine due to its analytical simplicity, relative susceptibility to changes in diet

and hydration, and exclusive clearance into the urine. The concentration of creatinine in serum is a more reliable indicator of renal dysfunction, as it should have trace levels under physiological conditions, owing to its excretion in urine. Its concentration in serum is 0.2–1 mg/dL in females and 0.4–1.2 mg/dL in males.

Several optical and electrochemical biosensors for creatinine estimation have been developed.^{7,8} However, they do not exhibit the required selectivity in the complex matrix of whole blood or serum. There are nine point-of-care (POC) devices in the market for creatinine estimation that rely on cascaded enzymatic conversion followed by an optical readout signal in most cases. The use of multiple enzymatic pathways has an inherent disadvantage of multiple reaction kinetics governed by different enzymes, thereby affecting creatinine estimation in point-of-care settings, which is necessary over a wide range of ambient temperature and humidity variations. Also, the electrochemical techniques focus on converting liberated ammonia into hydrogen peroxide through cascaded enzymatic

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reactions, which can be affected by endogenous ammonia concentration. Finally, the use of multiple enzymes makes it cost-prohibitive and poses an issue in its utilization for community screening programs or rural healthcare awareness programs. In this work, we have developed an enzymatic amperometric sensor for creatinine by enzymatic hydrolysis of creatinine by creatinine deiminase to produce 1-methylhydantoin, and then complexing it with the transition metal to produce the redox signal. The sensing chemistry has been optimized on disposable screen-printed electrodes that require a small sample volume of 300 μL and a reaction time of 3 min. This highlights the advantages of low cost, due to the use of the widely adopted electrochemical platform and mono-enzymatic detection pathway, simplicity of analytical instrumentation, and high specificity due to the involvement of enzyme. The measurement of creatinine has been performed in saline and in the presence of physiological levels of serum proteins. We have been able to accurately quantify creatinine in serum from 0.2 to 4 mg/dL within 3 min without any sample preprocessing.

PRINCIPLE OF DETECTION

Creatinine is hydrolyzed to form *N*-methylhydantoin (primarily 1-methylhydantoin) by the enzyme creatinine deiminase as shown in Figure 1. The reaction also releases ammonia as a

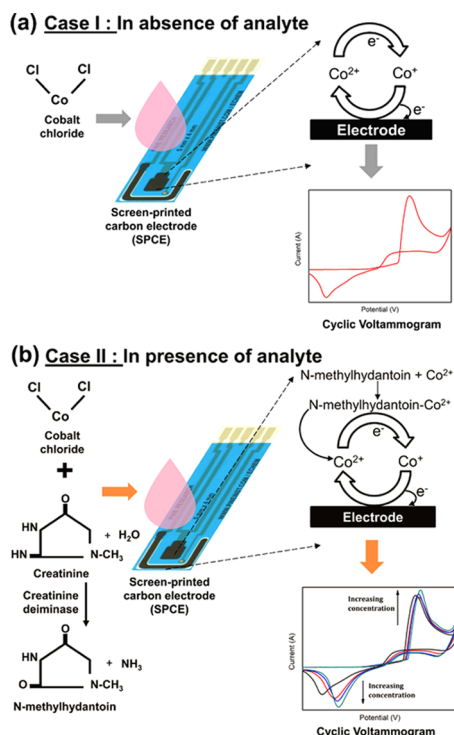


Figure 1. Principle of electrochemical detection of 1-methylhydantoin by cobalt ions. (a) Case I: In the absence of analyte and (b) Case II: In the presence of analyte.

byproduct, which has been used in the past as an electrochemical probe for creatinine estimation. However, the ammonia detection technique is adversely affected by the influence of endogenous ammonia present in the body. In this work, we focus on the detection of 1-methylhydantoin, which overcomes the disadvantages of ammonia detection. This has been achieved by using the ability of 1-methylhydantoin to

bind transition metals such as cobalt. Cobalt ions would exhibit a current signal on application of potential due to a change in its oxidation state as shown in Figure 1a. However, in the presence of 1-methylhydantoin, cobalt forms a complex with it that increases the intensity of the current signal as shown in Figure 1b.

The concentration of 1-methylhydantoin is then used as an indirect measure of the concentration of creatinine, as shown in Figure 1b. This is possible by its redox labeling with a transition metal, cobalt as mentioned before.^{9–13} Creatinine in the test sample undergoes conversion to 1-methylhydantoin in the presence of creatinine deiminase. The generated 1-methylhydantoin binds with cobalt ions to yield an electroactive cobalt–hydantoin complex. This complex generates a current signal that is dependent on the concentration of the analyte, as indicated in Figure 1b.

In the electrochemical cell, creatinine diffuses from the bulk solution and undergoes enzymatic conversion to form 1-methylhydantoin, which is then subsequently quantified by its ligand formation with cobalt as illustrated in Figure 2. The

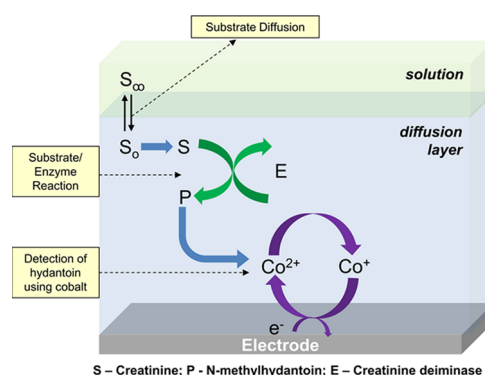


Figure 2. Principle of electrochemical estimation of creatinine using enzymatic conversion into 1-methylhydantoin.

system exhibits a current signal in proportion to the formation of the electroactive complex, which is correlated to the concentration of 1-methylhydantoin and in turn, creatinine.

RESULTS AND DISCUSSION

Optical Characterization of Cobalt–Hydantoin Complex. Transmittance spectra of cobalt chloride were observed in the presence of increasing concentration of 1-methylhydantoin as shown in Figure 3a. The inset figure expands the spectra as recorded from 300 to 600 nm. It is observed that cobalt exhibits its characteristic peak at ~ 500 nm (Figure 3d), while the peak at ~ 209 nm (Figure 3c) is associated with 1-methylhydantoin.

The intensity of the cobalt peak did not indicate any correlation with the increasing concentration of 1-methylhydantoin when cobalt ions were present in a ratio of 1:1 to 15:1. However, when cobalt ions were present in excess (50:1), i.e., 50 times the concentration of 1-methylhydantoin, the transmittance of cobalt peak indicated a linearly decreasing correlation with hydantoin concentration. It is also observed that the intensity of the hydantoin peak remains unaltered in the presence of the increasing concentration of cobalt chloride.

In addition to the characteristic peaks of cobalt and 1-methylhydantoin, a new shoulder peak at ~ 290 nm (Figure 3b) was observed in the cobalt–hydantoin mixtures. This may be attributed to the possible ligand formation between cobalt

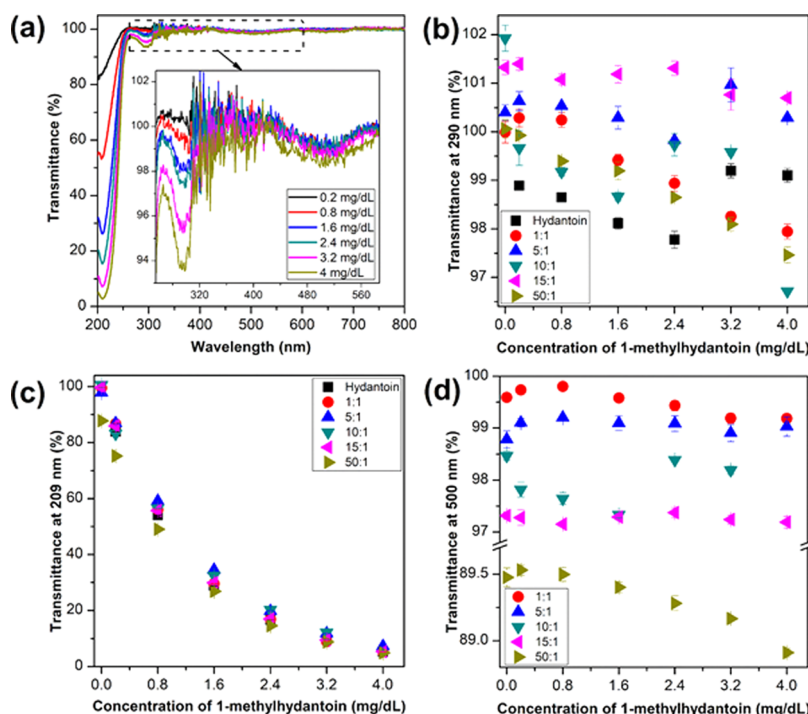


Figure 3. (a) Transmittance spectra of cobalt chloride in the presence of increasing concentration of 1-methylhydantoin. Variation in transmittance at (b) 290 nm, (c) 209 nm, and (d) 500 nm with increasing concentration of 1-methylhydantoin.

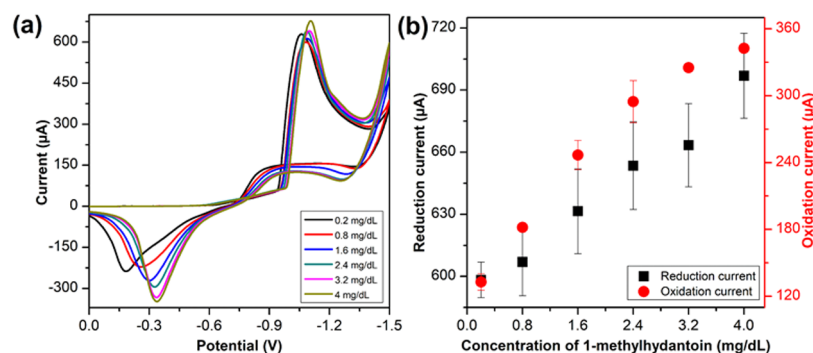


Figure 4. (a) Cyclic voltammogram of 0.6 mg of cobalt chloride in the presence of increasing concentration of 1-methylhydantoin. (b) Variation in oxidation and reduction current of cobalt chloride with different concentrations of 1-methylhydantoin.

and 1-methylhydantoin. The hypothesis was confirmed by monitoring the intensity of the shoulder peak with different concentrations of cobalt chloride to increasing concentration of 1-methylhydantoin. It was observed that the transmittance of the possible complex peak decreased with the increasing concentration of 1-methylhydantoin in most cases. This is attributed to the increased formation of the cobalt–hydantoin complex in the presence of increasing concentration of 1-methylhydantoin. Maximum sensitivity was observed when cobalt chloride was present in a ratio of 50:1 with 1-methylhydantoin concentration.

Electrochemical Detection of 1-Methylhydantoin.

Cyclic voltammograms of 0.6 mg of cobalt chloride were recorded in the presence of varying concentrations of 1-methylhydantoin, as shown in Figure 4a. The potential was varied from 0 to -1.5 V, and the corresponding current signals were recorded as shown in the voltammograms. Cobalt indicates a reduction peak at ~ -1.1 V, indicative of the transition of Co (II) to Co(I) ions and an oxidation peak at -0.35 V due to conversion of Co(I) to Co(II) ions. A positive

reduction peak at -0.9 V was also observed as the potential sweep reversed from -1.5 to 1 V. This is attributed to the reduction of cobalt ions localized near the electrode surface by virtue of sluggishness in the diffusion of cobalt ions away from the electrode surface. This residual reduction current in the reverse sweep of cyclic voltammogram is characteristic of cobalt ions, as it exhibits a tendency to deposit on the electrode surface due to the application of potential gradient.

The reduction current and the oxidation current indicated a linearly increasing correlation with the concentration of 1-methylhydantoin in saline, as indicated in Figure 4b after 30 s. Triplicated measurements were performed for each concentration and the coefficient of variation was within 10%. The oxidation current was lower compared to the reduction current owing to the irreversibility of electron transfer by cobalt ions. Further, the oxidation current signal indicated a higher sensitivity and reduced variability as compared to the reduction current signal.

The electrochemical measurements for the quantification of 1-methylhydantoin were then performed in the presence of

physiological levels of serum albumin, i.e., 3–5 g/dL albumin to ascertain its impact on the signal resolution. A linearly increasing oxidation current signal was observed even in the presence of albumin with varying concentrations of 1-methylhydantoin from 0.2 to 4 mg/dL, as shown in Figure 5. The sensitivity of measurement was independent of the

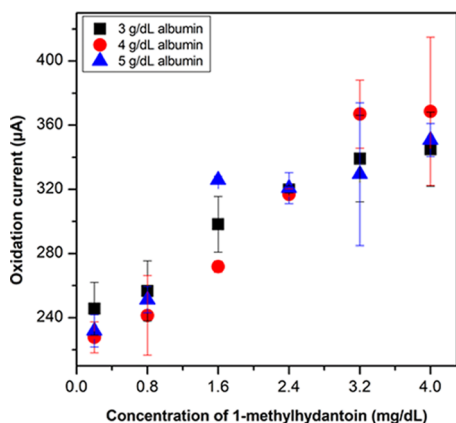


Figure 5. Variation in oxidation current of 0.6 mg cobalt chloride for different concentrations of 1-methylhydantoin in the presence of 3–5 g/dL albumin.

protein concentration. The coefficient of variation was within 10% at all concentrations of 1-methylhydantoin with varying concentrations of serum proteins.

Detection of Creatinine Using Mediated Enzymatic Reaction. Cyclic voltammograms of cobalt ions along with creatinine deiminase were recorded in the presence of varying concentrations of creatinine, as indicated in Figure 6a. In this case, the oxidation current signal does not indicate a reliable trend. This may be attributed to insufficient reaction kinetics for the complete oxidation of the cobalt–hydantoin complex after enzymatic conversion. The consistently lower intensity of the oxidation current in Figures 4b and 6b highlights an inherent sluggishness in the oxidation of the cobalt–hydantoin complex. Furthermore, the presence of the enzyme and unconverted creatinine also increases the diffusive resistance. This resistive component is amplified in the oxidation current due to greater accumulation as a result of relatively higher time (reduction peak appears at ~13 s, while the oxidation peak appears at ~27 s).

However, a new reduction peak is observed at −1.4 V, whose current signal exhibits a better correlation to the concentration of the analyte. Hence, the reduction current signal at −1.4 V is monitored henceforth. On the introduction of creatinine deiminase, the increasing concentration of creatinine is converted to 1-methylhydantoin, which then binds with cobalt ions and results in an increasing intensity of the reduction current signal with comparable sensitivity, as shown in Figure 6b. It is to be noted that the linearly correlated current signals for the detection of creatinine via 1-methylhydantoin required an assay time of 3 min for synergistic enzymatic conversion of creatinine into detectable 1-methylhydantoin and its subsequent electrochemical estimation.

Optimization of Parameters of Mediated Enzymatic Reaction. The influence of various parameters, such as reaction time and concentrations of enzyme and mediator, on the electrochemical quantification of creatinine, was evaluated. The concentration of cobalt chloride was not varied, as it has been optimized for the estimation of the maximum concentration of 1-methylhydantoin produced in the reaction.

Variation of Reaction Time. The effect of reaction time on the electrochemical sensing of creatinine subsequent to enzymatic conversion was investigated in detail, as shown in Figure 7a. For 1 min reaction time, the intensity of the reduction current signals did not show any trend. On the other hand, the reaction time of 3 min resulted in linearly correlated current signal as a function of creatinine concentration, indicative of a simultaneous occurrence of enzymatic conversion and electroactive metal complex formation. Hence, a reaction time of 3 min has been used for subsequent electrochemical measurements.

Variation of Concentration of Enzyme. As indicated in Figure 7b, on the introduction of 0.12 units of creatinine deiminase, the current levels decreased as creatinine concentration increased from 0.2 to 0.8 mg/dL, thereby indicating a nonlinear correlation. With 0.06 units of the enzyme, a linear correlation reduction current signal was observed over the targeted range of creatinine concentration, i.e., 0.2–4 mg/dL. In addition, the intensity of the current signals increased with 0.06 units of the enzyme. This appears counterintuitive as a higher enzyme concentration should translate into a higher conversion of creatinine into 1-methylhydantoin, which would bind with cobalt and indicate a current signal. However, an increased quantity of enzyme (0.12 units) would also facilitate diffusive resistance and

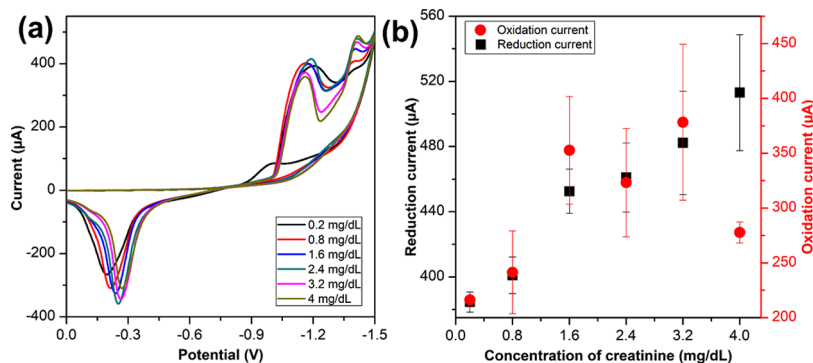


Figure 6. (a) Cyclic voltammograms of 0.6 mg of cobalt chloride with 0.06 unit of creatinine deiminase in the presence of varying concentrations of creatinine. (b) Variation in the current of 0.6 mg of cobalt chloride with 0.06 unit of creatinine deiminase to increasing concentration of creatinine.

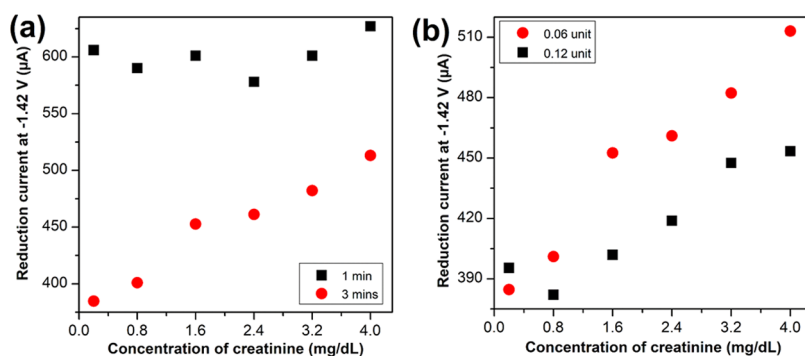


Figure 7. Effect of (a) reaction time and (b) concentration of enzyme on the detection of 1-methylhydantoin directly from creatinine using 0.6 mg of cobalt chloride and 0.06 units of creatinine deiminase in the presence of 3 g/dL albumin.

thereby negatively hinder mass transport. Further, a linear correlation between the quantity of enzyme and its action can be expected provided all of the enzyme sites are active and regenerated instantaneously, which is not necessarily guaranteed in this setup.

Hence, a higher concentration of enzyme increases the resistance and alters the reaction dynamics and hence it has to be closely optimized for each case. Here, a linearly correlated current signal was obtained with 0.06 units and has been employed hereafter. A lower quantity of enzyme also lowers the cost of the proposed assay.

Electrochemical Quantification of Creatinine in the Presence of Serum Proteins. Electrochemical measurements were then performed in the presence of serum proteins to evaluate the possible interference. A linearly increasing reduction current signal was still obtained as a function of increasing concentration of creatinine in the presence of 3–5 g/dL albumin (Figure 8). Triplicated measurements were

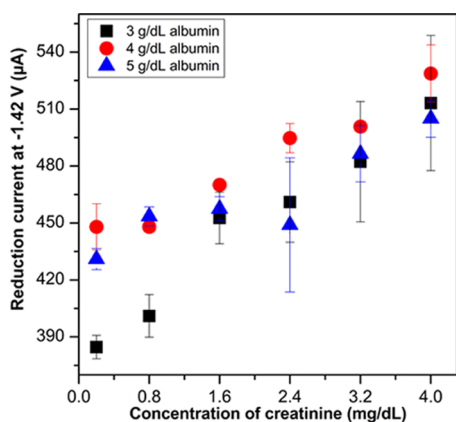


Figure 8. Variation in reduction current of 0.6 mg of cobalt chloride with 0.06 units of creatinine deiminase with increasing concentrations of creatinine in the presence of 3–5 g/dL albumin.

performed, and the average current values with the corresponding coefficient of variation are tabulated in Table 1. It was observed that the average coefficient of variation over the entire range of creatinine in the presence of physiological levels of serum protein was within 10%. This signifies the robustness of the proposed assay.

The effect of albumin is only evident in the lower concentration range of albumin and creatinine. However, it exhibits no significant variation with varying albumin at higher concentrations. Although different patients have different

albumin concentrations, it generally lies between 3.5 and 5 g/dL, and the minor variation due to varying albumin is compensated by the larger variation due to varying creatinine concentrations of the patient sample. Hence, it may not affect the clinical measurements of creatinine significantly.

CONCLUSIONS

An electrochemical technique to estimate the concentration of 1-methylhydantoin, using cobalt chloride as the chemical receptor, has been explored with screen-printed carbon electrodes. The optical absorption spectroscopy results validate the complex formation between 1-methylhydantoin and cobalt. The technique is indifferent to physiological concentrations of serum proteins, highlighting its sensitivity. It has then been utilized to quantify the concentration of creatinine in serum over the range of 0.2–4 mg/dL via enzymatic conversion by creatinine deiminase. The present work provides a reliable enzymatic approach to quantify creatinine in the serum by focusing on the quantification of 1-methylhydantoin produced, which signifies its novelty. We can reliably measure creatinine in 300 μL of serum without any preprocessing within a time span of 3 min.

MATERIALS AND METHODS

All of the chemicals such as creatinine, 1-methylhydantoin, human serum albumin, sodium chloride, cobalt chloride, creatinine deiminase (1 mg contains 21 active units of the enzyme) was procured from Sigma-Aldrich. All of the solutions were prepared in deionized water. Cobalt chloride was prepared in 0.085 M sodium chloride solution. The analyte, i.e., creatinine and 1-methylhydantoin along with albumin and creatinine deiminase, was prepared in physiological levels of saline, i.e., 0.154 M sodium chloride.

The experimental setup involves a potentiostat connected to disposable screen-printed electrodes that were procured from Pine Research Instrumentation. These electrodes include carbon as the working and counter electrodes and silver chloride as the pseudoreference electrode and have an electrode area of 20 mm². The electrochemical measurements were performed on a CHI 660E electrochemical workstation. The total volume dispensed on the electrode was maintained at 300 μL. The sensing chemistry was mixed with the analyte in the solution for all of the electrochemical measurements. Optical measurements were performed on a Shimadzu MPC3600 spectrophotometer. The optical analysis of liquid samples was performed in quartz cuvettes with a sample volume of 3 mL.

Table 1. Repeatability of Measurements for Creatinine Detection^a

creatinine (mg/dL)	concentration of albumin (g/dL)					
	3		4		5	
	average current (μ A)	coefficient of variation (%)	average current (μ A)	coefficient of variation (%)	average current (μ A)	coefficient of variation (%)
0.2	384.7	1.60	448.0	2.71	431.0	1.31
0.8	401.1	2.80	448.0	0.19	453.5	1.09
1.6	452.6	3.0	470.1	0.02	457.5	1.39
2.4	461.1	4.59	494.8	1.56	449.0	7.87
3.2	482.3	6.57	500.8	0.52	486.5	3.05
4	513.2	6.93	528.8	2.85	505.0	1.96

^aTripllicated measurements were performed for electrochemical estimation of creatinine from 0.2 to 4 mg/dL in the presence of different concentrations of albumin, 3–5 g/dL. The average current intensity along with the coefficient of variation is indicated for each concentration.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All the authors contributed equally.

Notes

The authors declare the following competing financial interest(s): The authors are inventors on two patent applications related to this work filed by Indian Institute of Science (Indian Patent Application No: 201941007991, 24 February 2020; US 16/802,179, 26 February 2020). The authors declare no other competing interests regarding the publication of this paper.

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