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Artificial molecular recognition material based biosensor for creatinine by electrochemical impedance analysis

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ABSTRACT

An electrochemical impedimetric sensor based on molecular imprinted polymer (MIP) has been developed for the detection of creatinine. Creatinine MIP was prepared by using the functional monomer methylacrylate and cross-linker ethylene glycol dimethacrylate in presence of the template creatinine. The resultant polymer was washed with water, methanol and 1 M HCl to extract the template from the polymer matrix. Formations of MIP, NIP (non-imprinted polymer) and template extraction were confirmed with vibrational Raman spectroscopic analysis. MIP incorporated carbon paste electrodes were used to develop a sensor for creatinine by applying electrochemical impedance spectroscopy (EIS) as transduction principle. Charge-transfer impedance (R_{ct}) of the sensor system was determined in the absence and presence of creatinine. Interestingly, magnitude of R_{ct} was found to increase with increasing concentrations of creatinine, which suggests the accumulation of creatinine into the polymer matrix. From the calibration plot, the low-detection-limit value was found to be ~20 ng mL^{-1}. The MIP electrode showed very good selectivity towards the specific recognition of creatinine in the presence of possible interferents like L-ascorbic acid and L-tryptophan. From the observations, we can conclude that the prepared imprinted polymer works successfully as an artificial biomolecular recognition element.

1. Introduction

Creatinine (2-amino-1-methyl-5H-imidazol-4-one) is the end product of creatine metabolism. During muscle contraction, creatine and creatine-phosphate spontaneously convert into creatinine. The level of creatinine in serum is a clinically important index of glomerular filtration rate, diabetic nephropathy, renal failure and muscular dystrophy. The physiological normal concentrations of serum/plasma creatinine and urine creatinine are 35–140 μM and 71–265 μmol d^{-1} kg^{-1}, respectively. High levels of creatinine are indicative of diabetic nephropathy, preeclampsia, glomerulonephritis, urinary tract obstruction and renal failure, while low levels are indicative of muscular dystrophy and myasthenia [1–3]. Due to its importance in biomedical diagnostics, specifically in monitoring kidney function and renal failure, the measurement of creatinine became critical in clinical analysis. From the literature, we came to know the importance of creatinine as the most requested analyte in the clinical laboratory.

Molecular imprinting is a generic technology which introduces recognition properties into synthetic polymers using appropriate template [4–6]. Molecular imprinted polymers (MIPs) are emerging as highly promising molecular recognition materials because of its vast versatility and functionality to incorporate binding sites for various kinds of analytes, starting from environmental toxins, pollutants, medical diagnostic markers, etc. MIPs found wide applications in separations and purifications because of its reversible binding with the template and thus extensive reusability. Artificial biosensors employing MIPs would be of great advantage because of their inherent stability under drastic conditions, long shelf-life and low-cost apart from selectivity and sensitivity [6–11].

The most common method for the detection of creatinine is based on Jaffe reaction which involves the reaction of picric acid in alkaline solution to form a red–yellow complex. The formed complex could be detected by a spectrophotometer at a wavelength of 490–520 nm range. The main drawback of this method is interference due to compounds such as protein, glucose or acetoacetate.

Molecular recognition materials are coupled to physicochemical transducers to transform the host–analyte interaction into a readable signal output. Among the various transduction methodologies, electrochemical techniques are widely investigated because of their potential advantages such as low-cost, miniaturization and portable instrumentation [12–22]. Till now most of biosensors developed are label-dependent sensors based on secondary functional electroactive/photoactive molecules. Label-free biosensors including QCM and SPR have attractive advantages with respect to speed and simplicity of operation [23–25]. Impedance is yet another rapid and inexpensive alternative technique to develop label-free biosensors for the detection of biomarkers [26,27], and

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even cost-effective hand-held impedance analyzers are also available.

In this investigation, electrochemical impedance sensor for detection of creatinine is developed based on molecular imprinted polymer electrode. MIP of creatinine is prepared with methylacrylate as monomer and ethylene glycol dimethacrylate as cross linker in the presence of creatinine template. It is incorporated into carbon paste to fabricate an easily renewable sensor surface for repeated cyclic use of the fabricated electrode. Surface morphologies of MIP with template, MIP and NIP have been studied by scanning electron microscopy (SEM). Formation of MIP and NIP, and template extraction from the MIP are confirmed by Raman spectroscopic analysis. The MIPs are incorporated into carbon paste electrode for impedimetric detection of creatinine, and the effect of creatinine on the charge-transfer impedance of the electrode interface is investigated for the detection of creatinine.

2. Experimental

2.1. Reagents

Creatinine and methylacrylate were procured from Sisco Research Laboratories and Loba Chemie, India, and they were of high pure analytical grade. Ethylene glycol dimethacrylate, azobisisobutyronitrile (AIBN), human serum and n-eicosane were purchased from Sigma–Aldrich, St. Louis, USA, dimethyl sulphoxide from SD Fine Chemicals Limited, India, and graphite fine powder from Loba Chemie, India. All other chemicals were of analytical grade and were used as received. Artificial urine was prepared according to the literature [28] and it contains urea, lactic acid, citric acid, glycine, NaCl, $MgSO_4$, KH_2PO_4, Na_2SO_4 and NH_4Cl. De-ionized double-distilled water purified by passage through Millipore 0.2 μm filtration system was used throughout the experiments.

2.2. Preparation of molecular imprinted polymers

MIP of creatinine was prepared by the polymerization of methylacrylate monomer in presence of the template creatinine similar to a literature procedure reported elsewhere [29]. Methylacrylate (MA; 1.0 g) and ethylene glycol dimethacrylate (EGDMA; 15.8 g) were dissolved into 40 mL DMSO, and AIBN (0.1 g) was added to the mixture. The resultant mixture was heated for 24 h at 60–70 °C, and the polymer was collected as solid mass. To extract the template molecule from the polymer matrix, the polymer product was treated sequentially with water, methanol and 1 M HCl each for 2 h and centrifuged each time to collect the polymer. The resultant imprinted polymer of creatinine based on MA is denoted as Cre-MA-MIP. For control experiments, a non-imprinted polymer (NIP) was prepared similarly except in the absence of creatinine and was treated sequentially with water, methanol and 1 M HCl, similar to the MIP. The resultant non-imprinted polymer is denoted as Cre-MA-NIP.

2.3. Electrode preparation

Carbon paste electrode (CPE) based on MIP was prepared by mixing 0.3 g of MIP and 1.2 g graphite powder using n-eicosane as binder molecule. The resultant carbon paste was filled into a hollow Teflon tube of 10 mm inner diameter. The other end of the Teflon tube was connected to a copper wire for electrical contact. The exposed surface is polished with a butter paper before the experiment. After a set of electrochemical impedance measurements, a new surface is exposed by removing a thin-layer of carbon paste followed by polishing.

2.4. Raman, SEM and electrochemical impedance experiments

Raman spectra of the imprinted polymers were recorded using a HR800 LabRAM confocal Raman spectrometer operating at 20 mW laser power using a peltier cooled CCD detector. The spectra were collected using a He–Ne laser source having an excitation wavelength of 633 nm and with an acquisition time of 10 s using a 5× objective. The Raman spectral data were acquired in a mixture (4:1, v/v) of acetonitrile and water using a quartz cuvette. SEM images of MIP with target, MIP and NIP were recorded using TESCAN VEGA3 scanning electron microscope, Czech Republic. These polymer samples were coated with a thin layer of gold by sputtering method to avoid charging during SEM studies.

Electrochemical impedance measurements were performed with the electrochemical workstation, Zahner-Elektrik (Model IM6e), GmbH, Germany in the frequency range from 60 kHz to 10 mHz and the results were analyzed with Thales 3.08 USB software. A conventional two-compartment three-electrode system is used for impedance analysis. The working carbon paste MIP electrode is held at the open circuit potential with small excitation amplitude of 10 mV peak-to-peak. Pt spiral wire and Ag/AgCl (3 N KCl) act as counter and reference electrodes, respectively. The electrochemical experiments were carried out in aqueous phosphate buffer solution (PBS) of pH 7.4, and prior to start EIS experiment, the solution was purged with nitrogen gas for 15 min to de-aerate the solution.

3. Results and discussion

3.1. Synthesis and Raman spectroscopic analysis of molecular imprinted polymers

Raman spectra of Cre-MA-MIP recorded before and after the extraction of template molecule are shown in Fig. 1(A) and (B). Characteristic peaks of creatinine were observed in the Raman spectrum of MIP at 677 cm^{-1}, 709 cm^{-1}, 1420 cm^{-1}, 2920 cm^{-1} and 3076 cm^{-1}. The prominent peak at 2920 cm^{-1} is relevant to the stretching vibration of imino N—H group, and the peak at 709 cm^{-1} could be attributed to the out-of-plane bending vibration of N—H group. The peak at 3076 cm^{-1} is relevant to the cyclic N—H group, and 1420 cm^{-1} peak to the semi-circle C=N stretching vibration. All these peaks are characteristic to the presence of creatinine [30–32]. The Raman spectrum of MIP recorded after the removal of the template creatinine (Fig. 1(B)) was completely free from those characteristic peaks, and it clearly reveals that the

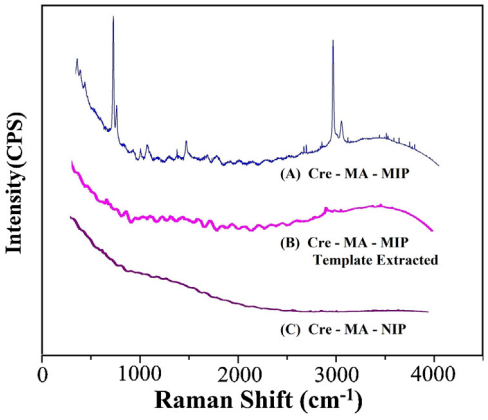

Fig. 1. Raman spectra of Cre-MA-MIP before (A) and after template extraction (B) and of Cre-MA-NIP (C) recorded in a mixture of 4:1 (v/v) acetonitrile and water.

template creatinine has been eluted entirely from the polymer matrix.

Raman spectrum of the non-imprinted polymer (Cre-MA-NIP) was recorded and is shown in Fig. 1(C). Raman spectra of Cre-MA-NIP obtained both before and after the extraction protocol were completely identical to each other (only one spectrum was shown). It shows no characteristic peaks which suggest that in the polymer form either the monomer or cross-linker give no specific peak and further confirm that the NIP does not have any recognition sites for creatinine in the polymer matrix. These resultant polymers were used for the binding of creatinine in electrochemical impedance measurements.

3.2. Surface morphology by SEM analysis

SEM images of MIP with target, MIP and NIP at two different magnifications for each sample are shown in Fig. 2. SEM images of Cre-MA-MIP with target (Fig. 2(A) and (B)) show flaky plate-like

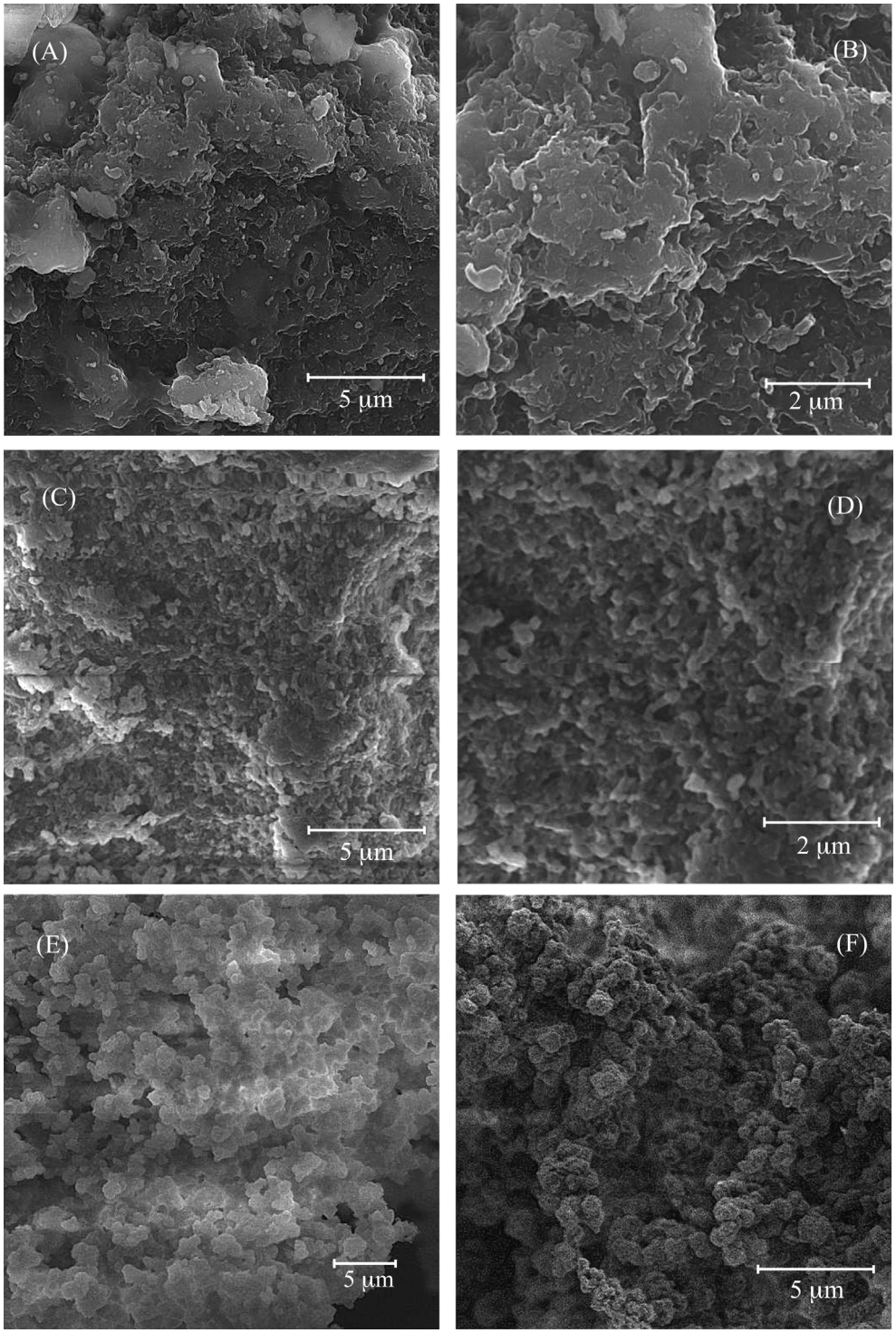

Fig. 2. SEM images of Cre-MA-MIP with template (A, B), Cre-MA-MIP (C, D) and Cre-MA-NIP (E, F) at two different magnifications. Gold was coated on these polymer samples by sputtering for 90 s.

structure uniformly all over the surface. In the case of Cre-MA-MIP (Fig. 2(C) and (D)) also, similar plate like structure is observed but the surface is of more porous nature and the size of the particles are relatively smaller. Size of the particles (Fig. 2(C) and (D)) are widely at sub-micrometer levels in the range of 0.4–0.8 μm. SEM images of Cre-MA-NIP (Fig. 2(E) and (F)) show very different surface morphologies compared to the MIP samples. Images of Cre-MA-NIP reveal uniform globular structure all over the surface and the size of the particles is widely in the region of 1–1.5 μm. SEM studies clearly reveal that Cre-MA-MIP without template has relatively more porous surface structure with smaller particles which could promote effective interaction with the target molecules.

3.3. Electrochemical impedance analysis of carbon paste electrodes

Electrochemical impedance spectroscopy (EIS) is an effective tool for analysis of the changes that take place at an interface during a recognition event. The impedance of an electrode is calculated by applying a sinusoidal potential of small peak-to-peak amplitude and measuring the resultant sinusoidal current. The EIS data can be represented by Bode and Nyquist plots. Nyquist plot, also known as Cole–Cole plot, comprises a semi-circle region lying on the axis followed by a straight line as the frequency approaches lower values. The semicircle portion in the higher frequency region corresponds to the charge-transfer process whereas the straight line part in the lower frequency region represents diffusion controlled process. Bode plot, a plot of absolute impedance and phase shift of the impedance each as a function of frequency, is a useful alternative to the Nyquist plot. Since frequency appears as one of the axes, it is easy to understand from the plot how the impedance depends on the frequency.

EIS analysis of the carbon paste electrode comprising Cre-MA-MIP is carried out in PBS (pH 7.4) in the absence and presence of different concentrations of creatinine in the frequency range of 60 kHz to 10 mHz. The Bode diagrams and Nyquist plots of the electrochemical impedance analysis were recorded. Results of the electrochemical impedance measurements were analyzed based on the best-fitting equivalent circuit analysis. They were found to best fit with a simple Randles equivalent circuit. As seen in Fig. 3(B), there are three regions in the Bode plots of the impedance spectrum which correspond to three types of elements in the equivalent circuit. Fig. 3 shows the Nyquist (A) and Bode (B) plots of Cre-MA-MIP electrode in PBS in the absence and presence of creatinine (0, 50, 140 and 666 ng mL^{-1}).

From the Nyquist plots (Fig. 3(A)), the interfacial charge-transfer impedance (R_{ct}) of the electrode was determined from the diameter of the semi-circle. The R_{ct} of the electrode in the absence of creatinine is 507 Ω. The observed small value of the impedance can be attributed to the trouble-free electron transfer from the electrode to the solution. The R_{ct} value increases by the presence of creatinine, as the creatinine molecule present in the solution get absorbed into the recognition sites of the polymer matrix. The magnitude of the change in R_{ct} increases further with increasing concentration of creatinine.

The Nyquist and Bode plots of the non-imprinted polymer (Cre-MA-NIP) based carbon paste electrodes have been recorded for control experiments in the absence and presence of creatinine. The Nyquist plots of Cre-MA-NIP (Fig. 4(A)) show simply linear plots with no semicircular pattern, relevant to an interfacial charge-transfer resistance, and this observation suggests that only a diffusion controlled process occurs at the electrode. Similarly, the Bode plots (Fig. 4(B)) show only two regions which correspond to only two types of elements in the equivalent circuit. The frequency range 1–60 kHz shows a linear plot with an almost negligible change in the impedance against the frequency, depicting the

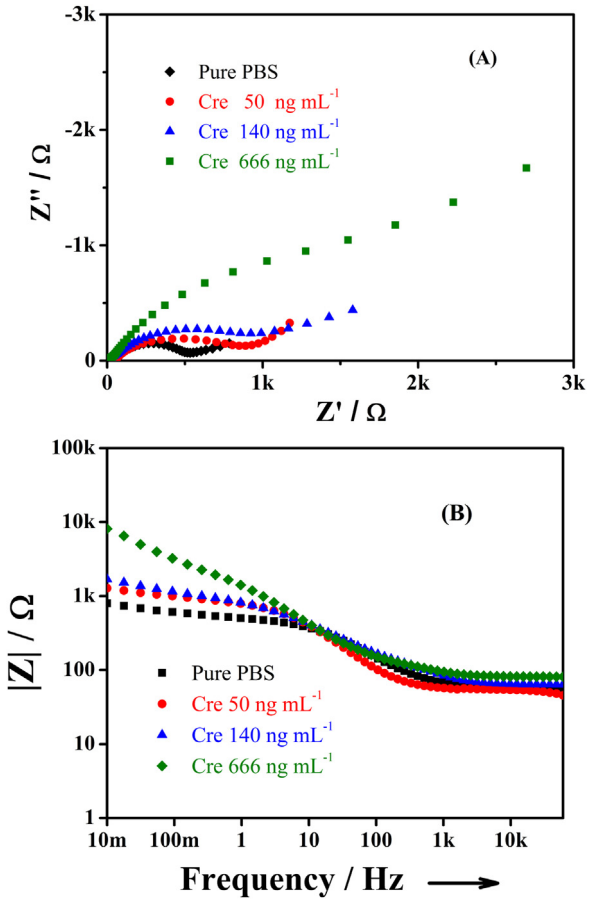

Fig. 3. The Nyquist (A) and Bode (B) plots of EIS analysis of Cre-MA-MIP carbon paste electrode in phosphate buffer (pH 7.4) with the presence of creatinine (0, 50, 140 and 666 ng mL^{-1}).

conduction of the ions in the electrolyte. In the frequency range of 10 mHz to 100 Hz, the observed linear plot with a negative slope clearly reveals that the double layer capacitance of the interface with only Warburg impedance dominates the observed signal in this region. Thus, there is no charge-transfer impedance with the non-imprinted polymer electrode. These observations clearly show that there is no accumulation of creatinine in the Cre-MA-NIP polymer matrix of the electrode.

The Nyquist and Bode plots of bare carbon paste electrode have been recorded in the absence and presence of creatinine (Supplementary Fig. S-1). The Nyquist plots of bare carbon paste electrode show simply linear plots with no semi-circular pattern both in the absence and presence of creatinine. Bare carbon paste electrode did not show any significant change in the EIS response to the addition of creatinine. All these observations clearly indicate that both the non-imprinted polymer (Cre-MA-NIP) based carbon paste electrode and bare carbon paste electrode did not respond at all to the presence of creatinine.

Fig. 5 shows the calibration plot drawn between the concentration of creatinine and the change in R_{ct} values at the Cre-MA-MIP based carbon paste electrode. The results of the electrochemical impedance measurements from the imprinted and non-imprinted polymer electrode clearly show that the Cre-MA-MIP electrode interacts and accumulates creatinine into the polymer matrix selectively. Further the obtained results were found to be reproducible in three independent experiments, and the magnitude of the change in R_{ct} is only a little with a relative standard deviation (RSD) of <4%. Considering that the change of impedance by three RSDs as the detection limit, the lowest detection limit of

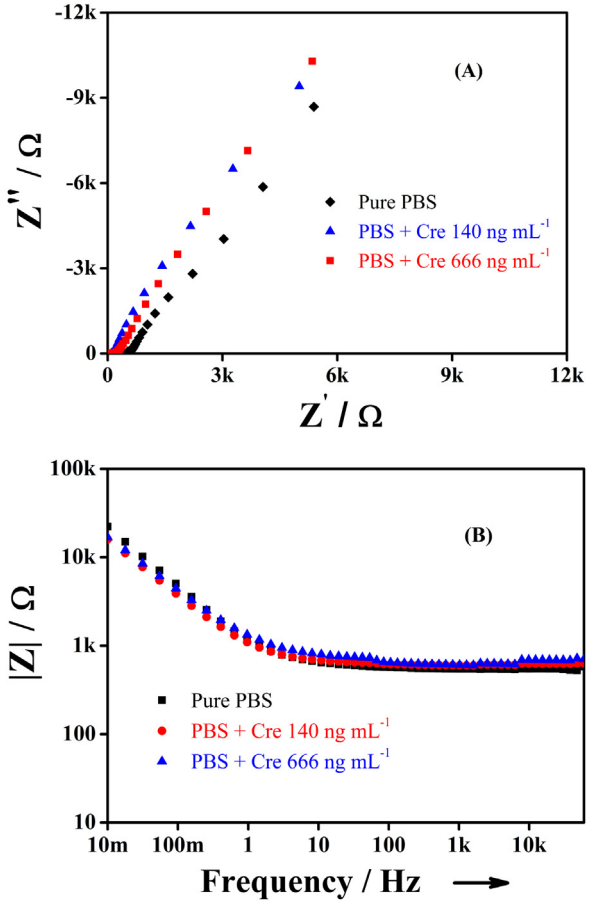

Fig. 4. The Nyquist (A) and Bode (B) plots of EIS analysis of Cre-MA-NIP carbon paste electrode in phosphate buffer (pH 7.4) with the presence of creatinine (0, 140 and 666 ng mL^{-1}).

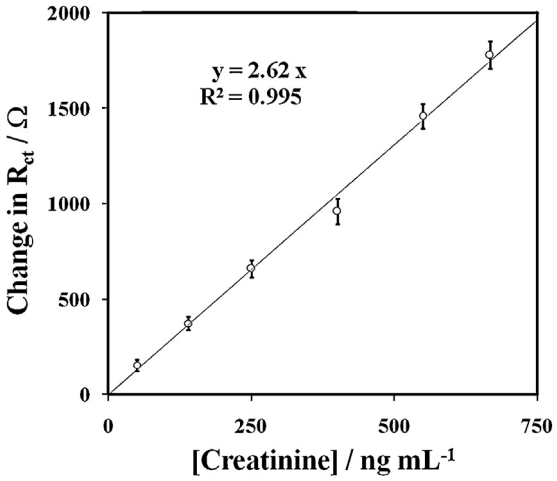

Fig. 5. Standard calibration plot between the change in charge-transfer impedance (R_{ct}) of the Cre-MA-MIP carbon paste electrode against the concentration of creatinine present in the experimental solution.

the impedance analysis could be determined from the slope and intercept of the plot to be 23 ng mL^{-1}. The calibration plot shows a linear determination range of 20–670 ng mL^{-1}, which is equivalent to 0.18–5.9 μM. The determination range of the sensor system is very much superior, considering the clinical range of creatinine in serum samples (35–140 μM), and thus the developed sensor could be applied even in 20–50 times diluted serum samples so as to reduce interferences from other physiological compounds. The observed low-detection-limit of the current electrochemical impedance analyzer is highly significant compared to the low-detection-limits reported for creatinine and other analytes (e.g., bilirubin, p-nitrophenol, etc.) by amperometric, chromatographic, electrophoretic and fluorescence methods using enzymes, MIPs etc. [9,14,18–21,33–35], and the performances of the present biosensor are compared with those reported in the literature in Table 1. The low-detection-limit reported in these investigations ranges from ~2 ng mL^{-1} to as high as 972 ng mL^{-1}. Fluorescence analysis using a creatinine MIP based on 4-methylimino-N-allyl naphthalimide monomer showed a low detection-limit of 6.4 μg mL^{-1} [11]. With chromatographic analysis based on hydrophilic interaction [20], a low-detection-limit of 60 ng mL^{-1} creatinine was established. Recently an amperometric enzyme biosensor fabricated using multi-walled carbon nanotubes and enzymes showed a low-detection-limit of 15 ng mL^{-1} creatinine [13]. Biosensors based on MIP have shown rarely detection limits as low as 2 ng mL^{-1} for creatinine and p-nitro phenol by amperometric analysis [9,33]. The low-detection-limit achieved in this investigation, 23 ng mL^{-1}, is highly comparable (Table 1), and the electrochemical impedance analysis method used here would pave way to the development of biosensors applicable for non-electroactive analytes also.

3.4. Interference studies and analysis in serum and urine samples

Electrochemical impedance responses of the Cre-MA-MIP based carbon paste electrodes to the presence of L-ascorbic acid (AA) and L-tryptophan (Trypt) were examined to investigate interferences due to possible reactive compounds coexisting in physiological samples. Fig. 6 shows the Nyquist and Bode plots of the EIS analysis to the absence and presence of L-ascorbic acid (0, 140 ng mL^{-1}) with

Table 1
Comparison of the performance of various electrochemical biosensors employing MIP, enzyme, MWCNT, nanoparticles, etc. with the current MIP-CPE based creatinine sensor.

Electrode material	Analyte	Detection method	Detection limit (ng mL^{-1})	Linear range	Reference
Screen printed electrode	Creatinine	SqWV	972	41.8–407 μg mL^{-1}	Chen et al. [34]
Enzymes co-immobilized Pt electrode	Creatinine	Amperometry	509	0.5–56.5 μg mL^{-1}	Walsh and Dempsey [18]
Enzymes co-immobilized Pt electrode	Creatinine	Amperometry	600	0.6–17 μg mL^{-1}	Tombach et al. [19]
Enzymes co-immobilized MWCNT/polyaniline/Pt	Creatinine	Amperometry	11.3	1.12–84.8 μg mL^{-1}	Yadav et al. [13]
Enzymes co-immobilized nanoFe_3O_4/Chitosan-polyaniline/Pt	Creatinine	Amperometry	113	0.1–90.4 μg mL^{-1}	Yadav et al. [21]
MIP/Hg	Creatinine	DPCSV	2.5	0.0025–84.0 μg mL^{-1}	Lakshmi et al. [9]
MIP-CPE	p-Nitrophenol	Voltammetry	1.1	1.1 ng mL^{-1} to 69.5 μg mL^{-1}	Alizadeh et al. [33]
MIP-CPE	Creatinine	EIS	20	20–670 ng mL^{-1}	Present work

SqWV, square wave voltammetry; DPCSV, differential pulse cathodic stripping voltammetry.

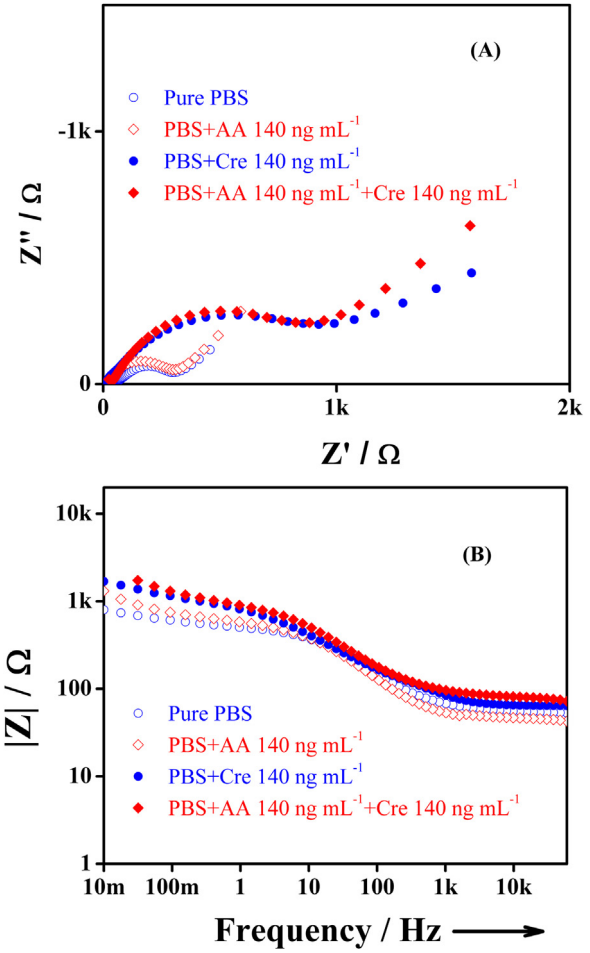

Fig. 6. The Nyquist (A) and Bode (B) plots of EIS analysis of Cre-MA-MIP carbon paste electrode in phosphate buffer (pH 7.4) for the possible interferent L-ascorbic acid (0 and 140 ng mL^{-1}) in the absence and presence of creatinine (0 and 140 ng mL^{-1}).

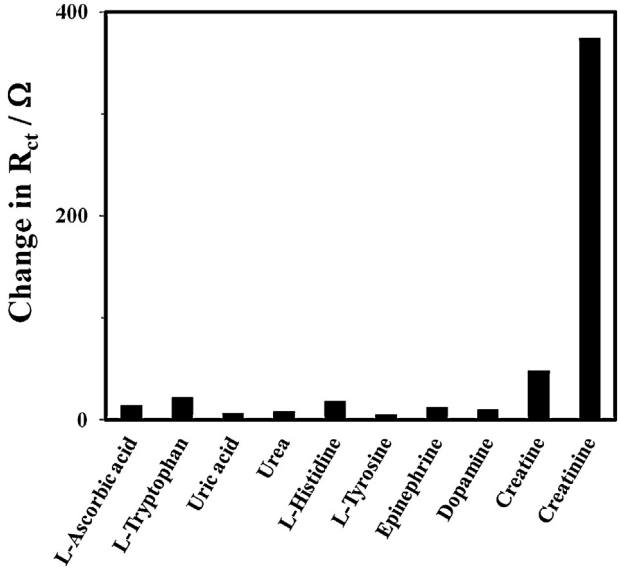

Fig. 7. The change in R_{ct} values observed at Cre-MA-MIP based carbon paste electrode to the presence of creatinine and other interferents individually each at 140 ng mL^{-1}.

the coexistence of 140 ng mL^{-1} creatinine. The Nyquist plot shows no much difference between the plots obtained in the absence and presence of L-ascorbic acid of 140 ng mL^{-1}. It clearly indicates that the fabricated MIP sensor surface shows no response to the presence of L-ascorbic acid. Further the Nyquist plot observed in the presence of 140 ng mL^{-1} creatinine is same to that observed in the presence of both creatinine and L-ascorbic acid 140 ng mL^{-1} each, and this observation confirms clearly that the recognition (binding) sites of MIP are specific to creatinine. Further, the Bode plots also confirm that the MIP sensor surface has no response to the presence of L-ascorbic acid.

The Nyquist and Bode plots of Cre-MA-MIP carbon paste electrode observed to the absence and presence of the interferent L-tryptophan with the coexistence of creatinine are given as supplementary data (Fig. S-2). The results indicate that the MIP electrode has no response to the presence of L-tryptophan, very similar to L-ascorbic acid.

Interference studies with the presence of L-ascorbic acid and L-tryptophan establish that the charge-transfer impedance of the fabricated MIP sensor surface did not respond to the presence of the interferents either L-ascorbic acid or L-tryptophan. Electrochemical impedance responses for creatinine and different interfering compounds such as uric acid, urea, histidine, tyrosine, epinephrine, dopamine and creatinine were studied and the results are summarized in Fig. 7. The results show that all these physiological compounds had practically no interference, except creatine which showed nearly a response of ~13% to that of the template, creatinine. From all these observations, we can conclude that the MIP electrode works specifically in recognizing the template creatinine molecule even in the presence of interferents.

Analytical applicability of the present method was assessed by applying the present biosensor to the determination of creatinine in human serum and artificial urine samples. Considering the clinical range of creatinine in serum (35–140 μM) and urine (3.5–15 mM) samples [36] and the determination range of the present biosensor (0.18–5.9 μM), the developed sensor could be applied in 20–100 times dilute serum samples and >100 times dilute urine samples. Dilute human serum and artificial urine samples were spiked with creatinine at relevant concentrations, and the results for the determination of creatinine are summarized in Table 2. Analytical recovery of exogenously added creatinine in

Table 2
Determination of creatinine in spiked human serum and artificial urine samples using the Cre-MA-MIP based carbon paste electrode.

Sample[a]	Spiked creatinine concentration (ng mL^{-1})	Determined value (ng mL^{-1})[b]	Recovery %	RSD %
Dilute serum (50 times)	–	163 ± 4 (72.1 μM)[c]	–	2.5
Dilute serum (50 times)	150	149 ± 5	99.3	3.4
Dilute serum (50 times)	250	247 ± 7	98.8	2.8
Dilute serum (20 times)	150	143 ± 5	95.3	3.5
Dilute serum (20 times)	250	242 ± 8	96.8	3.3
Artificial urine (50 times)	150	150 ± 4	100.0	2.7

[a] Dilution factor is given in the parenthesis.
[b] Each value is the average of four independent determinations.
[c] Value in parenthesis indicates creatinine concentration in undiluted serum sample.

20 times dilute serum samples was at least 95.3%, and the recovery limits as high as 99.3% and 100% were observed in 50 times dilute-serum and–urine samples, respectively. The observed good recovery limits of spiked creatinine concentrations from human serum and artificial urine samples demonstrate that the present biosensor is highly promising for determination of creatinine in real samples.

4. Conclusions

A novel molecular imprinted polymer (MIP) based electrode was developed for electrochemical impedimetric detection of creatinine. Magnitude of impedance was measured in a frequency range of 60 kHz to 10 mHz in PBS with the absence and presence of creatinine. Based on the best-fitting equivalence circuit analysis, it was found that the charge-transfer impedance of the electrode increased by the addition of creatinine and increases with increasing concentration. Control experiments with non-imprinted polymer showed that the sensor is totally non-responsive to creatinine. The lowest detection limit of the fabricated impedance sensor was 23 ng mL^{-1}. The surface of the fabricated MIP electrode is easily re-generable for repeated cyclic use in multiple analyses. The MIP electrode shows a specific and selective recognition towards the target analyte in the presence possible interferents and in serum and urine samples. Thus the fabricated MIP electrode could be used for the detection of creatinine in real-world physiological samples. The present methodology based on electrochemical impedance analysis could be extended as well to the fabrication of biosensors for non-electroactive analytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2013.04.015.

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