
Nano Conducting Polymer-Enzyme based Biosensor for Creatine and Creatinine

[No.F.41-352/2012 (SR)]

By

Dr.V.S.Vasantha
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Department of Natural Products Chemistry
School of Chemistry
Madurai Kamaraj University
Madurai-625 021

Submitted To
University Grants Commission (UGC)
Bahadur Shah Zafar Marg,
New Delhi
<table>
<thead>
<tr>
<th></th>
<th>UGC Reference No and Date</th>
<th>F.No.41-352/2012 (SR)</th>
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<tbody>
<tr>
<td>2</td>
<td>Name of the Principal Investigator</td>
<td>Dr. V.S. Vasantha</td>
</tr>
</tbody>
</table>
| 3 | Address | **Office**  
Dr. V.S. Vasantha  
Professor and Head  
Department of Natural Products Chemistry  
School of Chemistry,  
Madurai Kamaraj University, Madurai-21  
**Residential**  
Dr. V.S. Vasantha  
14-Sivasamy Street, Thendral Nagar,  
Thiruppalai Etn.  
Madurai-14 |
| 4 | Department and University College Where the project has undertaken | Department of Natural Products Chemistry  
School of Chemistry, Madurai Kamaraj University  
Madurai-21 |
| 5 | Title of the project | **NANO CONDUCTING POLYMER/ENZYME BASED BIOSENSOR FOR CREATIN AND CREATININE** |
| 6 | Date of Implementation | 1 July, 2012 |
| 7 | Tenure of the project | Three years, 1.07.2012 to 31.12.2015 (6 months extended by UGC) |
| 8 | Grants Received | 1<sup>st</sup> Installment: Rs.8,11,000/-  
2<sup>nd</sup> Installment: 1,31,446/- |
| 9 | Objectives of the Project | **Phase I**  
- To develop different types of single and dual enzymes entrapped nano-structured conducting polymer matrixes using hard and/or soft templates by electrochemical methods  
- To study the surface characteristics, particle size, electrochemical behaviour and conductivity of the enzymes entrapped nano-structured conducting polymer matrixes by employing SEM, XRD, AFM, electrochemical Analyzer and impedance analyser. |
<table>
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<th>Phase II</th>
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</thead>
<tbody>
<tr>
<td>➢ To study the catalytic behavior of the electrodes designed towards the redox reactions of creatin and creatinine using CV</td>
</tr>
<tr>
<td>➢ To fabricate a biosensor for the determination of creatin and creatinine</td>
</tr>
<tr>
<td>➢ To study their sensitivity, selectivity and stability towards creatin and creatinine using cyclic voltammetry and amperometry and</td>
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<tr>
<td>➢ To study the effect of inferences on sensitivity and selectivity of the sensors</td>
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<tr>
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<th>Methodology</th>
<th>The methodology of the proposed research work consists of the following steps:</th>
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<tr>
<td></td>
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<td>❖ Synthesis and characterization of a nano-structured carboxylic acid substituted poly-thiophenes / polypyrroles using self-assembled surfactants as templates</td>
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<tr>
<td></td>
<td></td>
<td>❖ Entrapment of enzymes simultaneously during electropolymerization Construction of the biosensor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>❖ Sensing of standard samples of creatine and creatinine and Sensing of creatine and creatinine in blood, and urine.</td>
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| 11 | Work was done so far                    | See Annexure- II                                                              |

| 12 | Has the project been according to the original plan of work and towards achieving objectives if not, state reasons | Yes, the project has been progressed slightly deviated from the original plan. That is, we had proposed enzymatic electrochemical biosensor for creatin and creatinine, however as we have faced problem in immobilization of tri-enzyme we could not achieve our original plan. But we have developed an enzymeless sensor and two optical biosensors with good sensitivity and selectivity for creatinine and they are published in peer reviewed journals. |

| 13 | Whether project work was delayed if yes, specify reasons | Yes, there is no delay in the project work for one year because of the delayed release of the second grant. |

| 14 | Please indicate the approximate time by which the project | 1.07.2015 |
|    |                                                             |

<p>| 16 | Please indicate the difficulties, if any, experienced in implementing the project | No |</p>
<table>
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<tr>
<th></th>
<th>Collaboration, if any</th>
<th>Dr.V. Kumaravel, Alpha Hospital &amp; Research Center, Institute of Diabetes and Endocrinology, 2B/2C, Gate Lock Road, Melaannupanady, Madurai-625 009.</th>
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| 18| Ph.D. enrolled, if any, details | Name: K.Balaji Viswanath, Research Scholar, Madurai Kamaraj University  
Title: “Detection of Proteins and Pathogens Using Electrochemical Immunosensors”  
Registration No: F9317 |
| 19| Details of the Publications resulting from the project work | “A New Route for the Enzymeless Trace Level Detection of Creatinine Based on Reduced Graphene Oxide/Silver Nanocomposite Biosensor”  
“Ultrasensitive Fluorescent Biosensor for Creatinine Determination in Human Biofluids Based on Water Soluble Rhodamine B Dye-Au$^{3+}$ ions Conjugate”  
“Michael Addition Based Chemodosimeter for Serum Creatinine Detection Using (E)-3-(Pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one Chalcone”,  
“Reply to the comments on Michael Addition Based Chemodosimeter for Serum Creatinine Detection Using (E)-3-(Pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one Chalcone”,  
<p>| 20| Any other information which could help in evaluation of the work done on the project | |</p>
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<td>1,00,040/-</td>
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<td>8,23,554/-</td>
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It is certified that the grant of Rs.1,31,446/- (Rupees one lakh thirty one thousand and four hundred and forty-six only) received from the University Grants Commission under the Scheme of support for Major Research Project entitled “Nano conducting polymer – enzyme-based biosensor for creatine and creatinine” vide UGC Letter No. F.No. 352/2012(SR) dated 23 July 2015 as a second grant and along with interest amount and amount deposited by the principal investigator of Rs. 17,643/-, the previous grant balance of Rs.10,814/- and hence the total amount is Rs.1,59,903/-, in which about Rs. 1,46,186.80/- has been utilized for the purpose for which it was sanctioned and the remaining balance amount of Rs.13,716.20/- has been returned to the funding agency in the account of “Secretary, UGC, New Delhi” via RTGS fund transfer mode in accordance with the terms and conditions laid down by University Grants Commission and the closing balance on the account is Rs.0.00/-.

Dr. V.S. Vasantha
Principal Investigator
UGC Project No.41-352/2012 (SR),
"Nano Conducting Polymer – Enzyme-Based Biosensor for Creatine and Creatinine",
School of Chemistry,
Madurai Kamaraj University,
Madurai-625 021, India
Utilization Certificate

Certified that the grant of Rs. 1,59,903/-[Rs.1,31,446/- (second grant aid) + Rs.3,443/- (interest amount)] + Rs.14,200/- (amount deposited by Principal Investigator) + Rs. 10,814/- (previous grant balance)] was received from the University Grants Commission under the Scheme of support for Major Research Project entitled “Nano conducting polymer – enzyme based biosensor for creatine and creatinine” vide UGC Letter No. F.No. 352/2012(SR) dated 22nd June 2015 in which about Rs. 1,46,186.80/- has been utilized for the purpose for which it was sanctioned and the remaining balance amount of Rs.13,716.20/- has been credited to the account of “Secretary, UGC, New Delhi” via RTGS fund transfer mode in accordance with the terms and conditions laid down by University Grants Commission and the closing balance on the account is Rs.0.00/-.

PRINCIPAL INVESTIGATOR
(Seal)

Dr. V.S. VASANTHA
Principal Investigator
UGC Project No.41-352/2012 (SR)
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Madurai-625 021, India

REGISTRAR
(Seal)

STATUTORY AUDITOR
(Seal)

DEPUTY DIRECTOR
Local Fund Audit Department
Madurai Kamaraj University Audit
MADURAL
State Bank Of India

Branch:......
Beneficiary A/c No.:..................Date: 25/01/2019
Name: Secretary...New Delhi.
Bank: Canara Bank...New Delhi.

IFS Code: CNR 82408627
RTGS/NEFT Amount: 71000
Commission: 50
Total: 7620

Rupees: Thirteen Thousand Seven Hundred Sixteen Rupees Only.

UTR No. SBINH.
Annexure-II

Part-I: “A New Route for the Enzymeless Trace Level Detection of Creatinine Based on Reduced Graphene Oxide/Silver Nanocomposite Biosensor”

Introduction

The measurement of creatinine and creatine in blood is an important clinical analysis because creatinine is important in the monitoring of kidney function while creatinine is a valuable index of muscle damage. The normal clinical range of creatinine in the human blood is ranging from 44 to 106 μM; however, it can exceed up to 1000 μM during nephrons malfunction. Blood levels >150 μM indicate the need to perform tests such as creatinine clearance. Values >500 μM indicate severe renal impairment, ultimately leading to dialysis or transplantation [2]; levels <40 μM indicate decreased muscle mass. Therefore, precise monitoring of creatinine in the blood is compulsory during a routine checkup.

The following techniques are proposed for the assay of creatine and creatinine: high-pressure liquid chromatography (HPLC) [3-5], mass spectroscopy [6], Nuclear Magnetic Resonance [7], and capillary zone electrophoresis [8]. However, the methods are affected by numerous metabolites and drugs found in biological samples, such as glucose, fructose, ketone bodies, ascorbic acid, and cephalosporins [9, 10]. But, by developing enzymes based biosensors have increased specificity and sensitivity.

Recently, there has been an upsurge of interest in the development of enzyme-based biosensors that utilize the direct electron transfer (DET) process of enzymes because the oxidative site of the enzymes tends to be buried within the large protein matrix [11]. Only a few reports are available to demonstrate the direct electron transfer of polyphenol enzymes used for the surface modifications [12]. Thus, it is necessary to develop a stable surface structure to load enzymes such as creatinase, sarcosine oxidase, creatininase, creatine amidinohydrolase demonstrating DET process successfully.

The conducting polymer films have shown promising applications in the field of biosensors and bioelectrochemistry by providing an active matrix with controlled morphology for immobilization of biological materials as well as transduction of the electrical signal [13, 14]. Conducting polymer-based biosensors come under third generation biosensors i.e. direct binding
of the biocatalyst to the electronic device that transduces and amplifies the signal [15]. Recently, nanoparticles based protocols have been exploited for the development of a biosensor application. This protocol can provide a good solution associated with the optimization of immobilized enzymes: minimum diffusion limitations, maximum active surface area and high effective loading [16].

(a) Preparation of RGO-Ag nanocomposite:

Graphene Oxide has been prepared by the modified Hummer’s method using previously reported literature [17]. Graphite powder was put into concentrated H₂SO₄ and then KMnO₄ was added gradually with stirring and cooling so that the temperature of the mixture was not allowed to reach 20°C. After 1 h, the reaction was terminated by the addition of a large amount of deionized water (2.8 L) and 30% H₂O₂ solution. The mixture was then stirred at 35°C for 2 hrs until the color of the suspension was changed to bright yellow. Then, the suspension was washed with a 1:10 HCl solution in order to remove metal ions. The paste collected was dried at 60°C, until it became agglomeration. The suspension is washed with much-deionized water at 5~7 times until the pH was reached nearly 7. The paste collected was dispersed into water by ultrasonication. The obtained brown dispersion was then subjected to 30 mins of centrifugation at 4000 rpm to remove any unexfoliated GO using a centrifuge. The GO platelets were obtained by dehydration at 60 °C in air.

To prepare GO/Ag ions modified electrode, about 0.02 wt% of GO and 2.5mM of AgNO₃ were mixed together in aqueous solution and sonicated for 30 minutes to get a uniform aqueous dispersion. The about 10µl of above suspension was drop casted onto a previously polished GC and dried at room temperature for overnight.

The above drop casted GO/Ag ions modified electrode was electrochemically reduced in phosphate buffer (pH 7.0) between 0.9 to -1.7 V for 15 cycles with a scan rate of 50mV/s for the reduction of oxygen functional groups of GO and to retain the lattice of graphene. The cyclic voltammograms in Fig.1 exhibit two peaks in the forward scan, first, a sharp peak at -0.155 corresponds to reduction Ag⁺ ions to Ag⁰ nanoparticles and second a broad peak at -1.335V corresponds the reduction of oxygen functional groups present on the surface of GO and formation of RGO. In the backward scan, there was a sharp oxidation peak at 0.553V corresponds to oxidation of Ag NPs formed in the previous scan. But, in the forward scan of the
second cycle, the reduction potential of Ag$^+$ ions to Ag$^0$ nanoparticles shifted to +0.031V due to the catalytic activity of RGO which was already formed on electrode surface during the first cycle. When the number of cycles were increased, the peak currents corresponding to reduction and oxidation of silver nano particles increased whereas the peak current corresponding to reduction GO decreased enormously and finally reached saturation. The RGO/Ag NPs composite was prepared through the electrochemical reduction of GO+AgNO$_3$ suspension. Since, GO contains plenty of epoxide and hydroxyl groups on its basal plane, and carbonyl and carboxyl groups on its edge plane, these functional groups act as anchoring sites for Ag$^+$ in AgNO$_3$ dispersion. Moreover, it was reported that Ag$^+$ could have strong interactions with $\pi$-orbital’s of alkenes, as a more direct chemical interaction in addition to the vander Waals interaction [18-19].

**Fig.1:** Cyclic voltammograms of electrodeposition of Ag at GO/GCE in PB solution (pH 7) for 15 cycles.

**Fig. 2 (A)** shows the UV-visible spectrum for the RGO/Ag NPs film and RGO film. For the film RGO, one can be observed an absorption peak at 268 nm. For the film RGO/Ag NPs, there is a clear broad absorption peak centered at 392 nm, corresponds to the surface plasmon resonance absorption of Ag nanoparticles which indicates the formation of RGO/Ag NPs composite. Fig 2 (B) shows the cyclic voltammograms of the RGO/Ag NPs and RGO films in
neutral PB. The modified electrodes were electrochemically cycled between 0.9V to -0.5V with a scan rate of 50 mV/s. The RGO/Ag NPs film modified electrode exhibited a reduction peak at -0.28V and oxidation peak at 0.23V, corresponds to reduction and oxidation of silver nanoparticles. The silver nanoparticles were well distributed on RGO sheets and no particles scattered out of RGO, indicating a strong interaction between RGO support and Ag nanoparticles. SEM image of RGO/Ag NPs film (Fig 3AC) shows the uniform decoration of silver nanoparticles on the graphene nanosheets and the size of silver nanoparticles was calculated to be 50:30 nm from the TEM image (Fig 3B). Fig 3C shows the FT-IR spectrum for the films such as GO, RGO, RGO/Ag NPs composite and RGO/Ag NPs with creatinine. In the case of GO, the broad and intense peak centered at 3416 cm⁻¹, which is related to the OH groups, and the strong peak at 1727 cm⁻¹ corresponds to the stretching vibrations of C=O carboxylic moieties[20]. The peak at 1615 cm⁻¹ is associated with the skeletal vibrations of aromatic C=C bond or intramolecular hydrogen bonds [21]. Similarly, bands at 1375, 1207, and 1074 cm⁻¹ correspond to C–O–H deformation, C–H stretching (epoxy groups), and C–O stretching vibrations (alkoxy groups), respectively [22]. This confirms the presence of an abundance of hydroxyl groups and oxygen groups on the surface of GO. The intensity of all the above peaks was decreased for the electrochemically reduced graphene oxide and thus confirms the reduction of hydroxyl, carboxyl, carbonyl and epoxide groups on GO basal planes. In the case of RGO/Ag NPs film, the intensity of C=O carbonyl stretching (1736 cm⁻¹) decreased, whereas the aromatic C=C vibrations (1656cm⁻¹) of RGO sheets increased. This change can prove that there is an interaction between AgNPs and the oxygen-containing functional groups (i.e., −COOH) of RGO nanosheets by forming a chemical bond or electrostatic attraction. The RGO/Ag NPs film after the adsorption of creatinine doesn’t show any change in intensity or peak shift. This confirms the adsorption of creatinine only on the Ag NPs present on the electrode surface and not in RGO moiety.
Fig. 2: (A) UV-visible spectra for ERGO/Ag NPs and ERGO films; (B) Cyclic voltammograms for ERGO/Ag NPs and ERGO films and (C) FT-IR spectrum for the films GO, ERGO, ERGO/Ag NPs composite and ERGO/Ag NPs with creatinine.
(b). **Determination of creatinine using RGO/Ag NPs composite film**

Fig. 4 shows the cyclic voltammograms (CVs) RGO/Ag NPs film in neutral PB solution. When creatinine was added to the electrolyte, the oxidation and reduction peak currents of silver decreased to certain concentrations and finally became saturated. The bare GC electrode does not show any response for creatinine. The advantage of pulse techniques offers high sensitivity, low detection limit, responses to faraday current and increases signal to noise ratio.
Fig. 4: Various concentrations of creatinine adsorption on ERGO/Ag NPs in PB solution by cyclic voltammetric technique.

Fig. 5: Square wave voltammograms at RGO/Ag NPs in absence (a) of and presence of creatinine from 10 pM to 120 pM (b-m). Calibration plot between $I_{pc}$ vs [creatinine]
Thus, we have studied the adsorption of creatinine by Square wave voltammetry [Fig 5 (A)] shows very high sensitivity and low detection limit of 0.743 pM towards RGO/Ag NPs film which is lower than that of the earlier proposed methods (Table 1) [23-32]. The linear plot calculated from SWV reveals that this modified electrode can work well in creatinine solution with a sensitivity of 0.04 μA pM⁻¹ [Fig 5 (B)]. The proposed method has a lower detection limit than the other electrochemical methods reported so far in the literature and does not require enzymatic modification on the electrode surface. Hence, it can be concluded that the proposed method is reliable and simple to determine creatinine successfully. Based on the results, we have given the possible mechanism for the interaction of creatinine and Ag NPs in scheme 1.

Scheme.1 schematic representation for the interaction of silver and creatinine.
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<td>Upto 5mM</td>
<td>[24]</td>
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<td>Carbon paste electrode</td>
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<td>[25]</td>
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<tr>
<td>PbO2 film</td>
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<td>1-1000 µM</td>
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Table 1. A comparison of the various analytical performance of present creatinine RGO/Ag NPs biosensor with previously reported creatinine biosensors.

(d) Conformation studies for adsorption of creatinine at RGO/Ag NPs/GCE

The peak currents of RGO/Ag NPs composite decreased with the addition of creatinine gradually. This phenomenon may be due to the adsorption of creatinine on the silver nanoparticles. Since the creatinine containing nitrogen centers have a greater affinity to act as a binding site for the Ag⁺ ions according to Pearson’s classification [54-56]. After the adsorption of creatinine by RGO/Ag NPs film, its surface gets aggregated which is confirmed by the SEM and HRTEM images [Fig 6 (A) and (C)]. Fig 6 (B) shows the conformation and interaction of RGO/Ag NPs film with creatinine in UV-visible spectroscopy. The adsorption of the Surface Plasmon resonance peak of Ag NPs from 390 nm to 442 nm decreases with the increase in the
concentration of creatinine. This confirms the adsorption of creatinine by silver nanoparticle on RGO/Ag NPs film. **Fig 6 (D)** shows the RGO/Ag NPs interaction with creatinine in Electro impedance spectroscopy. The EIS were recorded in the presence of 5mM Fe(CN)₆³⁻/⁴⁻ with 0.1M KCl in PB at a frequency range between 0.1 Hz and 100 kHz. The electron transfer resistance (Rct) at the electrode surface is equal to the semicircle diameter, which can be used to describe the interface properties of the electrode. The bare GCE has Rct value of ~475Ω while the RGO film shows low Rct value of ~270 Ω and RGO/Ag NPs film has Rct value of ~50Ω. Thus the conductivity of the film is increased after the Ag NPs formation on RGO. It is a well-known fact that most organic molecules such as creatinine are poor electrical conductors. Hence, the linear increase in Rct should be due to the electron transfer hindrance by the adsorption of creatinine on and RGO/Ag NPs film. From the Nyquist plots, the change in Rct values for bare GCE and surface modified electrodes are illustrated in Table 2. The addition of creatinine about 1 nM shows a high increase in Rct values of ~1275Ω while comparing with RGO/Ag NPs composite film, on further increase in creatinine concentration up to 5 nM the Rct values of ~30 to 150Ω only. After the addition of 5 nM concentration of creatinine, impedance spectra overlayed on it which shows that the adsorption on RGO/Ag NPs film is saturated.
Fig. 6: SEM (A) and TEM (B) images of RGO/Ag NPs after adsorption of 1 nM creatinine. (C) UV-Visible spectroscopy of RGO/Ag NPs before (a) and after adsorption of 1 (b), 3 (c), 5 (d) and 7 nM (d) creatinine. (D) EIS RGO/Ag NPs before (a) and after adsorption of 1 (b), 3 (c), 5 (d) and 7 nM (d) creatinine.
Table 2. Tabulated values of Charge transfer resistance of modified electrode surfaces.

(e) Stability, repeatability and reproducibility studies

The electrode stability is most significant factor for all newly developed sensors. Fig 7 shows the cathodic peak current response of 100 pM creatinine at RGO/Ag NPs modified electrode was examined up to 50 days by Square Wave Voltammetry and stored in PB when not in use. The modified electrode retains about 88% of its initial current response after 50 days, which indicates the excellent storage stability of the sensor. To evaluate the repeatability and reproducibility of the developed sensor, Square Wave Voltammetry performed for the cathodic peak current response of RGO/Ag NPs modified electrode on 100 pM creatinine in PB. The acceptable repeatability with the RSD of 4.30% is found for single electrode in 5 different measurements. The fabricated sensor shows a satisfactory reproducibility with the RSD of 3.80% for determination of creatinine using 5 different electrodes.
Fig. 7: Square wave voltammetric responses obtained at RGO/Ag NPs modified electrode with 100 pM creatinine in PB solution for long term stability of the electrode.

(f) Interference Study:

The response of the modified film RGO/Ag NPs film was examined in the presence of possible interfering molecules such as urea, uric acid, creatine and ascorbic acid of concentration 50 µM along with creatinine (10 µM) in neutral PB at their physiological conditions. Fig 8 shows the results that creatine and urea interfere while the ascorbic acid, uric acid, and glycine does not show any interference. The percentage of the relative activity on the interference molecules with the known concentration of creatinine is shown in Table 3.
Fig. 8: Voltammogram for the interference studies on RGO/Ag NPs composite with creatinine (10 µM) along with other interfering molecules.

<table>
<thead>
<tr>
<th>Interference</th>
<th>Relative activity of creatinine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No interferent</td>
<td>100</td>
</tr>
<tr>
<td>Creatine</td>
<td>97</td>
</tr>
<tr>
<td>Urea</td>
<td>98</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>100</td>
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<td>Uric acid</td>
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</tr>
<tr>
<td>Glycine</td>
<td>100</td>
</tr>
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</table>

Table 3. Effect of potential interferents on RGO/Ag NPs film based creatinine biosensor in the presence of 10 µM.
(g) **Real sample analysis**

To demonstrate the feasibility of using RGO/Ag NPs composite for practical samples, the detection of creatinine was performed using urine samples. The samples were collected from three healthy persons. The observed good recoveries of three different concentrations implied that the sensor could be used for the detection of creatinine in urine samples. Each experiment has been repeated thrice and the mean% recovery was calculated. The results are summarized in Table 4. The 100% recovery of creatinine was achieved indicating the reliability of the present method for the detection of creatinine under practical physiological conditions without interferences.

<table>
<thead>
<tr>
<th>Samples code</th>
<th>Creatinine spiked concentration</th>
<th>Found</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.40</td>
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<td>50 pM</td>
<td>50.41</td>
<td>100.82</td>
<td>1.73</td>
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<td></td>
<td>100 pM</td>
<td>100.17</td>
<td>100.17</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>10 pM</td>
<td>10.448</td>
<td>100.44</td>
<td>0.43</td>
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<tr>
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<td>49.78</td>
<td>99.56</td>
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<td>100 pM</td>
<td>100.22</td>
<td>100.22</td>
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<td></td>
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<td>Sample 3</td>
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<td>100 pM</td>
<td>99.85</td>
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<td>1.99</td>
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</tbody>
</table>
Table 4. Determination of creatinine in a urine sample by the proposed method.

(h) Conclusions

We have successfully fabricated an enzymeless creatinine biosensor using RGO/AgNPs. The adsorption of creatinine on the surface of silver was studied and confirmed by CV, SEM, TEM, EIS, and UV-Vis spectroscopy. SWV was used to determine creatinine based on the decrease in the cathodic peak of AgNPs. The proposed sensor shows very good sensitivity and very low LOD for the quantification of creatinine as compared to the other analytical methods reported previously. The simple preparation procedure and the new proposed method for the detection of creatinine indicate that our sensor holds good for the promising applications in the future.

Acknowledgement

The corresponding author, V. S. Vasantha, thanks the University Grants Commission (UGC), New Delhi, India by providing the necessary funds to carry out this work.

References


Part-II: “Ultrasensitive Fluorescent Biosensor for Creatinine Determination in Human Biofluids Based on Water Soluble Rhodamine B Dye-Au$^{3+}$ ions Conjugate”

Introduction

Renal function plays a vital role to filter numerous metabolic wastes from human blood through glomerular filtration. This glomerular filtration rate (GFR) is used to determine the number of waste products especially creatinine and urea which is directly filtered from human blood. Actually, creatinine is a metabolic waste molecule from our muscle metabolism. It is actually produced from creatine, which is used to produce major energy in our muscles. Almost 2% of the precursor creatine is converted to creatinine at every day and then it is transported to the kidneys through the bloodstream. When the kidney is not properly functioned, the level of creatinine is increased in the blood.$^{[1]}$ The optimum physiological concentration range of creatinine in blood is 40–150 µM, but under extreme pathological conditions, its level could exceed 1000 µM. when the level of creatinine exceeds >500 µM it clearly indicates severe renal impairment. When the levels reach <40 µM, it indicates decreased muscle mass.$^{[2]}$ The earlier and frequent detection of creatinine in human blood could improve the quality of human life, especially for peritoneal dialysis patients.$^{[3-6]}$ In general, Jaffé’s reaction is most commonly used for the quantification of creatinine based on the color change of alkaline solution of creatinine and picric acid.$^{[7]}$ Some enzymic colorimetric methods are also available for the detection of creatinine.$^{[8]}$ But, it has some adverse effects like the affection to numerous metabolites and drugs found in biological samples, more time-consuming, complicated and expensive. Meanwhile, some costly chromatographic methods have also been using for the relatively fast creatinine determination in human biofluids. $^{[9-11]}$ Recently, electrochemical creatinine sensors are emphasizing its specific advantages like sensitivity, selectivity, less time consuming, cost effective etc. For creatinine biomolecule concern, there were lot of potentiometric biosensors,$^{[12-15]}$ conductometric biosensors,$^{[16]}$ enzymatic $^{[17-20]}$ and enzyme less $^{[21-23]}$ creatinine biosensors, Dissolved oxygen amperometric creatinine biosensors,$^{[24-27]}$ nanoparticles based amperometric biosensors $^{[28-30]}$ and capacitive creatinine biosensors$^{[31]}$ reported with a good limit of detection (µM range). Very recently a new digital camera based technology was imposed for creatinine detection in urine
with a LOD of 89 & 111 µM. Very recently, a TURN ON fluorescent sensor has reported using thioglycolic acid (TGA) capped ZnS: Mn/ZnS quantum dots (QDs) for creatinine detection within the linear range of 0.07 and 3.4 µM along with LOD of 7.25 nM and 0.0242 µM.

The fluorimetric technique is a very simple analytical tool for various metal ions, biomolecules and pathogens detection based the fluorescent change of the molecules. By adopting this simple and less time-consuming technique, one could achieve a very good linear range and finally end with an excellent Low limit of detection (LOD) on particular analytes what we want to detect. The solubility of fluorescent molecules is the main and notable demerits on fluorescent based sensors. Nowadays, the solubility of the fluorescent molecules in an aqueous medium has been improved by introducing the suitable functional groups in it. Recently, rhodamine dye-based optical sensors have been allured for designing various metal ions and biosensors owing to their high photo stabilities, high extinction, high fluorescent quantum yields and low degree of triplet formation. Among all other rhodamine dyes, rhodamine b dye has views as a notable dye because of its complete solubility in water. It also has been used as fluorescent markers for labeling proteins, nucleic acids, lipids, carbohydrates, toxins, hormones, and another biomolecule. A fluorescent sensor so far reported has adopted a particular mechanism after a specific analyte binding with it. Among these mechanisms, ICT has been widely exploited in metal ion based chemosensors. In which either blue shifts or red shifts of both the absorption and fluorescence spectra will be expected which is purely relying on the electron-donating character of the electron donating group and cation binding.

Peer review of literature on creatinine biosensor has precisely evinced that there is no fluorescent biosensor using water-soluble organic dye reported for detection of creatinine at different disease status. In our current project, we have designed a very simple and quick response biosensor platform based on rhodamine b dye and Au³⁺ ions for the selective recognition of creatinine in presence of all other interfering biomolecules and metal ions in neutral aqueous medium. The developed sensor could also act as a ratiometric metal ion sensor for Au³⁺ ions. The developed biosensor has successfully detected Au³⁺ ions and creatinine based on ICT mechanism within a very good sensitivity range. Finally, the biosensor was applied for
the detection of creatinine in human urine. All those results were made a very good agreement with our emission data and suggesting its applicability in clinical diagnosis. The linear range implies that the developed biosensor can be applied for clinical diagnosis of all muscle disorder (creatinine level < 40µM), renal problems (creatinine level > 500µM) and even for the extreme renal problem (creatinine level = 1000µM).

(a) Development of biosensor

Typically, at room temperature 2 mL of RHB dye solution(0.5 x10^{-5} M) was mixed with 300µL (5 x 10^{-6} M) of Au^{3+} ions solution and then the solution was incubated for 10mins. After that, the above mixture was mixed with different concentrations of creatinine molecule and then further it was incubated for 15 mins. The fluorescence intensities of the mixture in the presence (I) and absence (I_0) of creatinine were recorded at 575 nm with the excitation wavelength of 535 nm and the calibration curve for creatinine was drawn between Intensity (I) versus the concentration of creatinine. By utilizing the developed biosensor platform, the detection of creatinine in human urine was determined by the standard addition method using Cary Eclipse spectrofluorimeter.

(b) UV –Visible spectra for RHB dye and RHB-Au ^{3+} ion conjugate:

Under optimized condition, the RHB dye has exhibited three characteristic peaks at 553, 295& 352 nm, which is mainly attributed to n- π* and π-π* transitions of RHB dye, respectively (Figure 1). While varies the concentration of Au^{3+} ions from 0 to 5 µM, a ratiometric response was observed. i.e. peak at 553 nm gradually diminished whereas the peak at 295 nm gradually enhanced and finally, the peak at 352nm completely diminished. There were two isosbestic points are observed at 483 & 576 nm (Figure 2 & 3). Initially, the dye is pale pink in color under the naked eye. After binding with Au^{3+} ions, the color is visually changed from pale pink to magenta color immediately. This was mainly due to the internal charge transfer (ICT) mechanism while absorbing the light. Meanwhile, the selective recognition of Au^{3+} ions were further confirmed by varying the different metal ions like Ag+, Fe^{2+}, Na^+, K^+, Ca^{2+}, Hg^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} ions (Figure 4). From these studies, it is clearly revealed that other monovalent and divalent metal ions do not show any remarkable shift or absorbance
change rather than Au$^{3+}$ ions. These results utterly show that RHB dye has selectively detected the Au$^{3+}$ ions.

**Figure 1.** UV-vis Spectra of RHB dye in water. [Concentration of RHB dye is 5 μM, under neutral aqueous medium.]

**Figure 2.** Absorption spectra of RHB dye – Au$^{3+}$ ion conjugates while adding various the concentration of Au$^{3+}$ ions from 0 - 5 μM [in a neutral aqueous medium, the concentration of RHB dye is 5 μM, Inset: corresponding linear plot].
Figure 3. Linear spectra of RHB dye in while varying the concentration of $\text{Au}^{3+}$ ions from 0 - 5 μM [Concentration of RHB dye is 5 μM, under neutral aqueous medium].

Figure 4. Selectivity studies: Absorption spectra of RHB Dye while adding the different metal ions [in a neutral aqueous medium, the concentration of probe and metal ions is 5 μM].
(c) Competitive observations among other metal ions:

The competitive titrations were also carried out to reveal the selectivity of the sensor towards Au\(^{3+}\) ions in presence of various biologically important and heavy metal ions. In these particular studies, the absorbance change of RHB-Au\(^{3+}\) ions conjugate has monitored while varying the concentration of other metal ions. Under optimized condition, the RHB- Au\(^{3+}\) ions conjugate has shown a ratiometric response at 295 and 553nm. When two equivalents (20µL) of various biologically important and heavy metal ions were added to the solution, no remarkable absorbance changes are observed (Figure 5). From these studies, it is firmly understood that the developed sensor could detect Au\(^{3+}\) ions selectively.

![Figure 5. Competitive studies: (a) Bar diagram of RHB Dye –Au\(^{3+}\) ions conjugate (one equivalent, 10µL) in the presence of two equivalents (20µL) of other interfering metal ions (b). Corresponding absorption spectra [in a neutral aqueous medium, the concentration of probe is 5 µM and interfering metal ions are 1 mM].](image)

(d) Recognition of Creatinine using RHB Dye-Au\(^{3+}\)ions conjugate sensor platform

The ultimate idea of our work is to recognize the biomolecule creatinine using the same RHB Dye -Au\(^{3+}\)ions conjugate as a sensor platform. This developed biosensor has selectively recognized creatinine rather than other biomolecules like glucose, creatine, ascorbic acid, uric acid, urea, L-dopamine, bilirubin (Figure 6).
Figure 6. Absorbance spectra for the selective recognition of creatinine in presence of other biomolecules. [Concentration of RHB dye -Au$^{3+}$ ion conjugate and other biomolecules are 5 μM, in neutral aqueous medium].

When the concentration of creatinine is gradually increased from 100 nM - 15 μM, the initial absorbance was quenched at two characteristic wavelengths (295 & 553 nm). This is mainly attributing to the inhibition or gradual relaxation of internal charge transfer mechanism (ICT) which was previously observed in the RHB- Au$^{3+}$ions Conjugate (Figure 7 & Figure 8).

Figure 7. Absorption spectra for the RHB dye –Au$^{3+}$ ion conjugate while varying the concentration of creatinine from 100 nM – 15 μM [in a neutral aqueous medium, the concentration of probe is 5 μM, Inset: corresponding linear data at a lower concentration range of creatinine].
(a) & (b) Linear graphs of RHB - Au$^{3+}$ ions conjugate dye while varying the concentration of creatinine from 100 nM - 15 μM [Concentration of RHB dye is 5 μM, under neutral aqueous medium.]

From the linear graph, the LOD was calculated for creatinine using above formula as 54 nM and linear range was from 100 nM to 15 μM. [LOD=$3S_a/b$]

(e) Competitive recognition among other Biomolecules
Our human blood plasma and urinary excrete products consist of so many biomolecules like glucose, urea, dopamine, ascorbic acid, creatine, uric acid etc. So the developed sensor must show a specific recognition of creatinine among other competitors as mentioned above. Herein, we have crosschecked the selectivity of creatinine with most relevant competitors like urea, uric acid, ascorbic acid, dopamine, bilirubin, glucose, creatine with the absorbance change in the UV-Spectral data. Remarkably, there were no obvious absorbance changes and it clearly evinced that the developed colorimetric sensor (RHB-Au$^{3+}$ ion conjugate) could selectively detect the creatinine molecule in the presence of all other interfering biomolecules.

(f) Fluorescence spectral analysis of RHB dye and RHB –dye -Au$^{3+}$ion conjugate

Firstly, the dye RHB dye been has excited at 553 nm wavelength and it has exhibited an intensive emission peak at 650 nm with very good fluorescence intensity. After the addition of Au$^{3+}$ ions, TURN - OFF response was observed i.e. a new peak was observed at 575 nm along with a quenching in fluorescent intensity. While increasing the concentration of Au$^{3+}$ ions from 1
- 12 µM, a gradual decrease in the fluorescent intensity was observed along with a good blue shift of 75 nm from the initial dye wavelength (Figure 10). This clearly evinces that the developed sensor has followed the Internal Charge Transfer mechanism (ICT) in which the electron charge density has transformed from the dye to the Au$^{3+}$ ions.

**Figure 10.** (a) Emission spectra of the RHB dye while varying the concentration of Au$^{3+}$ ions from 1-12uM. [in neutral aqueous medium, concentration of probe is 5µM].

Where the $K_{SV}$ was calculated as $2.6 \times 10^9 \text{M}^{-1}$. This large quenching constants has suggested that the strong Au$^{3+}$ ions are a kind of very satisfying the ICT Mechanism. Generally the $K_{SV}$ value around the $2.6 \times 10^9 \text{M}^{-1}$ support ICT Mechanism.$^{56}$ The stoichiometry of the probe- Au$^{3+}$ ions was confirmed through a continuous variation method i.e. the Job’s Plot. This study (Figure 11), is clearly revealed the formation of 1:1 stoichiometry of RHB dye: Au$^{3+}$ ions with 0.5 molar ratio. In addition to that it is also confirmed via $^1$H NMR titrations. And it was clearly understood that when one equivalent of Au$^{3+}$ ions was added to the RHB dye, the entire characteristic peak at $^1$HNMR was shifted to download with decrease in intensity (Figure 12). When 2 equivalents of Au$^{3+}$ ions were added to the RHB dye, the precipitate was obtained. All these studies were very confirms that formation of 1:1 conjugate. From the linear graph, the
LOD was calculated for creatinine using above formula as 0.95µM and linear range is from 1 µM to 12 µM (Figure 13.)

**Figure 11.** Job’s plot of the RHB dye (1.0 equiv.) in presence of Au^{3+} ions (1.0 equiv.) [Concentration of RHB dye and Au^{3+} ions are 5 µM.]
Figure 12. $^1$H NMR of RHB Dye (a) 400 MHz, [D$_6$] DMSO and D$_2$O, 298 K): $\delta$ = 8.06 (s, 1H), 7.64 (d, 1H), 7.08 (m, 2H), 6.83 (m, 2H), 6.61 (m, 2H), 6.44 (m, 2H), 3.36(s, 8H), 1.04 (m, 12H) ppm. (b) Comparative $^1$H NMR spectra of RHB dye, when one equivalent of Au$^{3+}$ ions is added.

Figure 13. Linear fluorescence emission spectra of RHB dye in the presence of increasing concentrations of Au$^{3+}$ ions from 1 to 12 µM. [in neutral aqueous medium, Concentration of dye is 5 µM]

Figure 14. Competitive studies: (a) Bar diagram of RHB Dye –Au$^{3+}$ ions conjugate/Creatinine (one equivalent, 10 µL) in presence of two equivalents (20 µL) of other interfering biomolecules (b). Corresponding absorption spectra [in neutral aqueous medium, the concentration of RHB dye, Au$^{3+}$ ions are 5 µM, creatinine is 15 µM and other biomolecules are 1mM].
(f) Recognition of Creatinine using RHB dye -Au\(^{3+}\) ions conjugate sensor platform

As the RHB dye did not interact directly with Creatinine, RHB dye -Au\(^{3+}\) ion conjugate was used as a sensing platform for creatinine. Under optimized condition, the developed biosensor (RHB dye -Au\(^{3+}\) ion conjugate) has utilized to detect the creatinine molecule. For this study, the initial wavelength of 575nm for RHB-Au\(^{3+}\) ion conjugate was fixed. Under optimized condition, the developed biosensor has shown a remarkable increase in fluorescent intensity for creatinine in the presence of other biomolecules like glucose, creatine, L-dopamine, urea, uric acid, ascorbic acid, bilirubin (Figure 15).

![Emission spectra for the selective recognition of creatinine in the presence of other interfering biomolecules.](image)

**Figure 15.** Emission spectra for the selective recognition of creatinine in the presence of other interfering biomolecules. [Inset: Corresponding bar diagram]

When the concentration of creatinine molecule was increased from 10 nM to 1.2 \(\times\) 10\(^{-3}\)M, a TURN – ON response i.e. a gradual increment in fluorescent intensity was observed which was mainly attributed to the partial/slow release of dye from RHB- Au\(^{3+}\) ions conjugate (Figure 16 & Figure 17). In addition to the old peak at 575nm, a new peak was also observed at 627 nm which corresponds to the RHB dye emission range (600-750nm). Initially, after the addition of Au\(^{3+}\) ions to the RHB dye a gradual blue shift was observed. But when the creatinine is added, this RHB- Au\(^{3+}\) ions conjugate was gradually releasing the dye to the solution. It doesn’t attain the initially fluorescent intensity of RHB dye at 650nm, because now the solution containing
both the RHB dye along with Creatinine –Au$^{3+}$ ions conjugate. So this study clearly reveals that the increasing the concentration of creatinine leads the partial/slow release of RHB dye molecule from the RHB- Au$^{3+}$ ions conjugate. It’s also showed that the creatinine has a very good binding efficiency with Au$^{3+}$ions while comparing with dye. Now the dye molecule is now free from the Au$^{3+}$ ions. So, the fluorescent intensity of the gradually enhances and its initial fluorescent color is retained.

**Figure 16.** Emission spectra for RHB dye-Au$^{3+}$ion conjugate while varying the concentration of Creatine from 10nM to 1µM. [Inset: Linear graph at lower concentrations of creatinine]
Figure 17. (a) & (b) Linear emission spectra of RHB dye- Au\(^{3+}\) ions conjugate in the presence of increasing concentrations of creatinine from 10 nM to 1.2 mM. [In neutral aqueous medium, concentration of RHB dye is 5μM]

From the linear graph, the LOD was calculated for creatinine using above formula as 5 nM and linear range is from 10 nM to 1.2 mM. When compare to the previously reported creatinine electrochemical biosensors, this firstly developed fluorescent biosensor possessed both LOD and linear range are excellent compared with earlier reports i.e no early reported sensor platform possesses both LOD and linear range of detection excellent. Hence, our sensing platform can be applicable for all kind creatinine based disease. (Table 1.)

<table>
<thead>
<tr>
<th>Sensor platform</th>
<th>Technique</th>
<th>LOD (µM)</th>
<th>Linear range (µM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
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<td>Phosphomolybdic-polypyrrole film modified glassy carbon</td>
<td>Cyclic voltammetry</td>
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<td>[21]</td>
</tr>
<tr>
<td>c-MWCNT/PANI</td>
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<td>10-750</td>
<td>[28]</td>
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<tr>
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<td>10-650</td>
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<tr>
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<td>1-800</td>
<td>[30]</td>
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<tr>
<td>Picric acid-Jaffe reaction</td>
<td>Digital Camera Based</td>
<td>89 &amp; 111</td>
<td>160 – 1600</td>
<td>[32]</td>
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<tr>
<td>ZnS:Mn/ZnS quantum dots</td>
<td>Fluorimetric</td>
<td>0.00725 &amp; 0.0242</td>
<td>0.07 - 3.4</td>
<td>[33]</td>
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</table>
Table 1. Comparison of LOD and Linear range with previous creatinine biosensor reported.

<table>
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<td>0.054</td>
<td>0.1 - 15</td>
<td>Present work</td>
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<tr>
<td></td>
<td>Fluorimetric</td>
<td>0.005</td>
<td>0.01 – 1200</td>
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</table>

(g) Competitive fluorescent titrations in presence of other interfering biomolecules

Competitive analysis has also been done for the selective recognition of the creatinine in presence of other biomolecules. We have observed two kind of competitive responses in both higher as well as lower concentration. Under optimized condition, two equivalents (40 uL) of other interfering biomolecules like glucose, creatine, ascorbic acid, urea, uric acid were added to the one equivalent (20 uL) of RHB-Au<sup>3+</sup> ions at both lower (Figure 18.) and higher concentrations (Figure 19). Results are clearly supports that the developed biosensor platform could selectively sense the creatinine biomolecule in presence of other interferents (Figure 20).
Figure 18. Competitive studies: Emission Spectra of RHB Dye –Au$^{3+}$ ions conjugate -in presence of Creatine (one equivalent, 20μL) along with two equivalents (40μL) of other interfering biomolecules (5μM) at lower concentration range of creatinine. [in neutral aqueous medium, the concentration of RHB dye is 5 μM creatinine is 10 nM and other biomolecules are 1 mM, Inset: Corresponding bar diagram]
Figure 19. Competitive studies: (a) Bar diagram of RHB Dye –Au$^{3+}$ ions conjugate in presence of creatinine (one equivalent, 20μL) along with two equivalents (40μL) of other interfering biomolecules (5μM) at higher concentration range of creatinine (b) & (c) Corresponding emission spectra for competitive studies at both high and lower concentrations of creatinine respectively. [in neutral aqueous medium, the concentration of RHB dye is 5μM, creatinine and other biomolecules are 1 mM].

![Figure 19](image)

Figure 20. Bar diagram for the selective recognition of creatinine in presence of other biomolecules based on the fluorescence changes of RHB dye – Au$^{3+}$ ions conjugate [in neutral aqueous medium, Concentration of dye is 5 μM and other interferents are 1mM].

(h) Responses of RHB dye towards creatinine in the presence of other biologically metal ions.

Our developed biosensor could detect the creatinine molecule in presence of RHB dye - Au$^{3+}$ ions conjugate. Under optimized condition, the fluorescent studies of RHB dye were carried in the presence the biologically important metal ions like Au$^{3+}$, Na$^+$, K$^+$, Ca$^{2+}$, Hg$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, Co$^{2+}$, Ni$^{2+}$ and Cu$^{2+}$ ions along with creatine. There is no change in the fluorescent property of the dye in the presence of above metal ions except Au$^{3+}$ions. Addition of Au$^{3+}$ions causes a quenching effect with blue shift. Moreover, when the addition of creatine to the RHB
dye - Au\(^{3+}\) ions conjugate the fluorescent intensity of RHB dye - Au\(^{3+}\) ions conjugate increases with slight red shift. Remaining metal ions - RHB dye platform didn’t show any increment in fluorescent intensity when the creatine molecule is added till (Only they over lay on the initial spectrum of the dye) It is strongly supporting that the developed biosensor could sense the creatinine biomolecule in presence of Au\(^{3+}\) ions only (Figure 21).

Figure 21. Emission spectra for RHB dye-Au\(^{3+}\)ions conjugate while adding the different metal ions under optimized condition. [in neutral aqueous medium, the concentration of RHB dye, Au\(^{3+}\) ions, other biomolecules and metal ions are 5μM]

(i) Proposed ICT (Internal Charge Transfer) Mechanism for the developed biosensor

The ICT mechanism has been widely exploited for cation sensing. If the electron-donating character of the electron donating group is reduced; blue shifts of both the absorption and fluorescence spectra are expected. Conversely, if a cation promotes the electron-donating character of the electron donating group, the absorption and fluorescence spectra should be red-
shifted. In our case, we have successfully designed a biosensor based on this ICT mechanism. From the emission data, it is clearly understood that the developed biosensor has followed the ICT mechanism. In which, the electron donating group in the dye molecule has arrested by the Au$^{3+}$ ions. So that a gradual blue shift along with decrease in the emission intensity was observed (Scheme 1). After the addition of creatinine biomolecule, the initial ICT mechanism was gradually relaxed and releases the dye molecule slowly which results the increase in fluorescence intensity of the resulting solution.

**Scheme 1.** Proposed scheme for the developed biosensor RHB -Dye/Au$^{3+}$ions/Creatinine.

(j) **Naked eye as well as UV-Lamp detection**

Initially the RHB dye was pink color under naked eye and emits pinkish orange fluorescence color under UV Lamp. After binding with Au$^{3+}$ ions, the color was changed from pink to
magenta under naked eye. But in UV Lamp, the initial fluorescence color was quenched. When the creatinine molecule is added to the above solution, the initial color of dye was regenerated both under naked as well as UV lamp (Figure 22). So the designed biosensor could be applicable for the detection of creatinine molecule in real sample by both colorimetric as well as fluorimetric method.

**Figure 22.** Color changes observed after the addition of Au$_{3}^{3+}$ions and creatinine biomolecule to RHB dye under naked eye (a) and UV vis-Lamp (b).
(k) Real Sample analysis

The biological application of the developed biosensor RHB dye–Au$^{3+}$ion conjugate is demonstrated by the quantitative estimation of creatinine in human urine sample (Figure 23). The unknown concentrations of creatinine were determined by the standard addition method using. Briefly, some known concentration of the creatinine molecule was spiked with human urine sample. The results obtained by this method were compared with the previous emission data using creatinine as analyte in water. From these results, it was clearly showed the developed biosensor could be used as very good sensor for the detection of creatinine in human urine sample. The corresponding recovery of creatinine molecule was shown in Table 2.

![Emission spectra for the determination of creatinine in human urine sample under optimized condition.](image)

**Figure 23.** Emission spectra for the determination of creatinine in human urine sample under optimized condition.
Table 2: Standard addition method for the detection of creatinine in human urine sample under optimized condition.

(1) Conclusions

A very simple and cost effective biosensor has developed in neutral aqueous medium by using water soluble Rhodamine B dye as a fluorescent probe for the simultaneous detection of Au\(^{3+}\) ions and creatinine. The developed biosensor has showed “Turn-off” ratiometric response for the Au\(^{3+}\) ions and “Turn –on” response for the creatinine molecule in both colorimetric and fluorimetric techniques. Meanwhile, the selectivity and competitive studies were strongly supports the detection of Au\(^{3+}\)ions and creatinine molecule in presence of interfering metal ions and biomolecules. As a colorimetric sensor, the developed sensor could detect Au\(^{3+}\)ions from 1-5 µM within the LOD (Limit of Detection) of 0.47 µM; simultaneously for creatine the linear range of detection is from 100 nM to 15 µM within the LOD of 54 nM. Meanwhile as a fluorimetric sensor, this biosensor has showed linear range of 1-12 µM for the detection of Au\(^{3+}\) ions within the LOD of 0.95 µM and for creatinine the linear range is varied from 10 nM to 1.2 \(\times\) 10\(^{-3}\) M. The limit of detection (LOD) was found to be 5 nM under optimize condition. The real sample analysis was also being carried out in normal human urine sample by using standard addition method.
Abbreviations
RHB- Rhodamine B Dye
ICT-Internal Charge Transfer

Acknowledgement
The corresponding author, V. S. Vasantha, thanks the University Grants Commission (UGC), New Delhi, India by providing the necessary funds to carry out this work.

References


Introduction

Creatinine is a routine biomarker which plays a crucial role in renal and muscular dysfunctions because of its direct secretion from muscle as a side product. The variation in creatinine level in blood and urine is an important parameter in clinical diagnostics. When the kidney is not properly functioned, the level of creatinine is increased in the blood. In normal human fluid like urine creatinine levels are varied in the range from 0.45 to 1.69 mg/dL but under extreme pathological conditions, its level could exceed to 11.31mg/dL and this abnormal level >5.65 mg/dL can indicate either renal malfunction in situation where creatinine concentration increases or can be a sign of muscular dystrophy for lower creatinine values. Due to its medical relevance the development of a fast and accurate assay for the determination of creatinine in human serum or urine is required. Although several techniques have been proposed for a fast and accurate quantification of creatinine in human serum or urine, most of them require expensive or complex apparatus, advanced sample preparation or skilled operators.\textsuperscript{1-2} The earlier and frequent detection of creatinine in human blood could improve the quality of human life especially for peritoneal dialysis patients.\textsuperscript{3-4} Generally, Jaffé’s reaction has been used commonly for the quantification of creatinine based on the color change of alkaline solution of creatinine and picric acid.\textsuperscript{5} Apart from this, a few enzymic colorimetric protocols and chromatographic tools could also be used for the detection of creatinine.\textsuperscript{6} These protocols have been generally affected by numerous of metabolites and drugs found in biological samples and it requires high time. It is also more complicated and expensive.\textsuperscript{7} With selectivity concern, some electrochemical creatinine sensors have also reported and having specific advantages like sensitivity, selectivity, less time consuming, cost effective etc. For example, there are a lot of potentiometric biosensors, conductometric biosensors,\textsuperscript{8-9} some enzymatic\textsuperscript{11-12} and enzymeless\textsuperscript{13-14} creatinine biosensors and dissolved oxygen amperometric creatinine biosensors,\textsuperscript{15-16} nanoparticles based amperometric biosensors\textsuperscript{17-18} and capacitive creatinine biosensors\textsuperscript{19} are also reported with a good limit of detection. In the current sensor era, the fluorimetric technique has been acting as a very simple
analytical platform for various heavy metal ions, biomolecules, and disease-causing pathogens. Using this simple and less time-consuming technique, one could achieve a very good linear range along with an excellent Low limit of detection (LOD) towards a particular analyte. A new digital camera based technology was developed and reported for creatinine detection in urine with LOD of 1 & 1.25 mg/dL. Recently, thioglycolic acid (TGA) capped ZnS:Mn/ZnS quantum dots (QDs) based fluorescence sensor has been reported for creatinine detection within linear range of 0.0008 and 0.0385 mg/dL along with LOD of 0.0001 and 0.0003 mg/dL. Meanwhile, our group has reported a simple fluorescence-based biosensor for the creatinine detection using Rhodamine B dye - Au\(^{3+}\) ion conjugates. Currently, a novel turn-on fluorescent sensor has been reported for creatinine based on gluten stabilized gold quantum clusters along with picric acid as a quencher. And the detection limit was found to be 0.00002 mg/dL within the linear range from 0.2262 to 5.8822 mg/dL. And also these reports utilized Picric acid as a quencher and they have adopted the basic principle of Jaffe reaction indirectly. So, the direct detection of creatinine using an organic fluorophore/dye will be a cost effect sensitive method to substitute for clinical diagnostics of creatinine.

Chemodosimeter is molecular probes used to achieve the recognition of analyte with the irreversible process. They have been intensively studied in the anion sensing area, because they have the advantage of high selectivity by the minimized interference of other anions. There was plenty of the fluorescent sensor so far reported which has adopted a particular mechanism like ICT, FRET, PET, ESIPT etc after the binding with a specific analyte. In these mechanisms, ICT has been noted as a frequent mechanism in fluorescent-based chemo or biosensors. In which, based on the electron-donating character of the electron donating group and analyte binding, either blue shifts or red shifts will be expected in the absorption or fluorescence spectra. Peer review of above said literature on creatinine biosensor has precisely evinced that there is no chemodosimeter based fluorescent biosensor has been reported using a simple organic fluorophore for direct detection of creatinine at normal and different disease states. Moreover, nobody has compared their experimental data with commercially available clinical reports so far. To address these valid key notes, in our current work, we have synthesized a very simple chalcone (fluorophore) as a chemodosimeter through fissile route for the selective detection of
creatinine in human serum. The structure was elucidated by NMR and ESI-Mass and its photophysical properties were analyzed using UV-Vis and fluorophotometer. As a colorimetric biosensor, the probe detected creatinine ratiometrically through ICT mechanism. This mechanism was further supported by DFT (Density Functional Theory) calculations. Under the optimized conditions, the biosensor detected the creatinine within the linear range of 1.13 to 11.31 mg/dL along with the LOD of 0.3959 mg/dL. As a fluorimetric biosensor, it could detect creatinine from 0.00000113 to 15.8 mg/dL. The performance of the biosensor implies that it could be applied for clinical diagnosis of muscle disorder (creatinine level <0.45 mg/dL) and kidney male function (creatinine level = 11.31 mg/dL) as well. To the best of our knowledge, we are the first group applied our protocol for the determination of creatinine in clinical samples and proved as our protocols equally good as a commercial clinical protocol for the same samples. The biosensor is also seems to be cost-effective for the existing clinical methods.

3.1 Synthesis of (E)-3-(pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one [PTP] Chalcone

Briefly, 1-Pyrenecarboxaldehyde (1.0 g, 5 mmol, 1 equiv.) was dissolved in 5 mL of absolute ethanol (solution–I) and mixed with another solution (solution (II)) which was prepared by dissolving 3, 4, 5- trimethoxyacetophenone (1.3 g, 10 mmol, 2 equiv) in 5 mL of ethanol and then, in 0.1M of 3 mL of NaOH. A pale yellow color precipitate thrown out immediately and it was shaken for 1 min and filtered. The resulting crude yellow product (III) was washed thrice with absolute ethanol and diethyl ether and dried over MgSO₄ under vacuum. The completion of product formation was confirmed by Thin Layer Chromatography (appearance of single yellow spot). The percentage of yield was found to be 99% [1.98 g] The NMR data for the synthesized PTP chalconeare given below. ¹H NMR (400 MHz, CDCl₃, 298): δ = 3.99 (s, 9H), 9.00 (d, 1H), 8.19 (d, 1 H), 7.39 (d, 1H), 8.13 (d, 1 H), 8.02- 8.96 (m, 9H) and ¹³C NMR (300 MHz, CDCl₃, 298 K): δ = 189.08(C7), 61.04, 61.05, 56.42 (C28, C30 &C32), 142.60 (C2), 141.52 (C3), 153.25 (C4), 106.23 (C1), 125.60 (C6), 130.75(C10), 128.80 (C9) , 122.60 - 127.34 (C11-C26) C₂₈H₂₂O₄(calculated mass 422.15): observed mass [M + H]⁺=423.20 [Figure S1, S2 & S3].
Scheme 1. Instant synthesis of PTP chalcone via hand shaking.

Figure 2. $^1$H NMR Spectra of the synthesized PTP Chalcone.
Figure 3. $^{13}$C NMR Spectra of the synthesized PTP Chalcone.
Figure 4. ESI- Mass spectra of the synthesized PTP Chalcone.

3.2 Development of biosensor using (E)-3-(pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one

Typically, 2 mL of 5 µM of (E)-3-(pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (PTP)solution (Ammonium buffer, pH=10) was mixed with different concentrations of creatinine (from 1.13 to 11.31 mg/dL for UV studies and from 0.00000113 to 15.8 mg/dL for emission studies) at room temperature. The changes in absorbance were recorded at 297 and 407 nm and the changes in emission were observed at 473 and 587 nm. The linear calibration curve for creatinine was drawn between absorbance/fluorescence intensity versus the concentration of creatinine molecules.

3.3. Analysis of human blood serum by our protocol

Under optimized conditions, 2 mL of 5 µM (E)-3-(pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (PTP)solution at pH=10.0 (Ammonium buffer) was thoroughly mixed with 10 µL of different blood serum samples from different persons and incubated for 10 minutes. As per report of Alpha hospital, the clinical samples were contained in between concentration of creatinine (in mg/dL). In our case, the fluorescence was linearly varied at 527 nm for this particular concentration range. So, the we have recorded the fluorescence changes at 527 nm with the excitation wavelength of 407 nm.

3.4. Analysis of human blood serum by Clinical protocol

To 10 µL of different blood serum samples, 180 µL of Reagent R1 and 60 µL of Reagents R2 was added and allowed to react for 10 minutes. And then amount of creatinine from the reaction mixture was recorded at primary wavelength of 550 nm and secondary wavelength of 700 nm using Medica EasyRA Chemistry Analyzer programmed on the RFID chip on the reagent wedge. And the calibration interval time and reagents – onboard stability is maximum for 20 days.
3.5. Photophysical studies

3.5.1. Effect of pH on photochemical properties of PTP Chalcone

In general, the protons in the probe/fluorophore could play curial role in the absorbance or fluorescence response of the fluorescent probe. From the response, one could design a sensor based on the absorbance or fluorescence changes after binding with particular analytes. So, it is necessary to investigate the effect of pH. At low pH, the probe showed two major absorption peaks at 297 & 407 nm (Figure 5).

Figure 5. (a) UV-vis absorption spectra and (b) Fluorescence emission spectra of PTP chalcone [Concentration of PTP chalcone is 5 μM, at pH10, Ammonium buffer]

While, the pH was being adjusted from 2 to 10, there was a gradual increment in the absorbance at both the wavelengths along with a small blue shift which is mainly attributed to the charge transfer mechanism within the PTP Chalcone (Figure 6). When, the pH was further increased from 10 to 13.5, no absorbance change was observed. The PTP chalcone consists of –OCH₃ and –C=O groups and no effective functional groups like –OH or –NH₂ generally which are responsible for the change in pH of the solution. So, under acidic pH from 2 to 5, the
protonation of –OCH₃ and –C=O groups could be taken place whereas under basic pH, the PTP chalconemight be existed in neutral formso thatthe absorbance intensity of the probe was high due to charge transfer mechanism. Based on aboveobservation, the pH change didn’t show any remarkable changes in peak position, but a drastic change in absorption intensity was observed in the pH range from 7 to 10. Further increase of pH from 10-14, no change in the peak position and intensity of the probe. Since, our ultimate aim was to develop the chemodosimeter for the detection of creatinine based on the simple Michael Addition reaction; we have chosen pH-10 for our study to detect the creatinine. In this aspect, our synthesized PTP chalcone could act as Michael acceptor and creatinine molecules as Michael Donor.

3.5.2. UV spectral analysis of synthesized PTP Chalcone and PTP-CRT Conjugates

The UV studies were carried out under different conditions for PTP Chalcone and PTP-CRT Conjugates. Initially, the PTP Chalcone showed two major peaks around 297 & 407 nm which are mainly because of π-π* and n-π* transitions, respectively. (Figure 5) These two peaks were fixed for designing chemodosimeter based the colorimetric sensor for the detection of creatinine at pH 10. There was a ratiometric response was observed while varying the concentration of creatinine from 1.13 to 11.31 mg/dL with blue shift in both spectral wavelength and the limit of detection (LOD) is calculated as 0.39 mg/dL. (Figure 6a & 6b). The blue shift in both spectra clearly supports the Internal Charge Transfer (ICT) mechanism during the interaction between creatinine and probe.
3.5.3. Response of PTP chalcone towards creatinine in the presence of interfering molecules

Our human serum consists of so many biomolecules like glucose, urea, dopamine, ascorbic acid, cretin, uric acid, various amino acids, proteins etc. So, the selectivity study has to be done to show the specific recognition of creatinine by PTP chalcone in the presence of other competitive biomolecules. Herein, we have checked the selectivity of PTP chalcone for creatinine in both and diseased (Figure 6c) and normal (Figure 7b) physiological concentration (mg/dL) levels in the presence of interfering molecules like urea, uric acid, ascorbic acid, dopamine, bilirubin, glucose, creatin, serotonin, amino acids like L-Arginine, L-Cystine, L-Cystin, reduced glutathione and BSA in same physiological standards level. A very good ratiometric absorbance change with blue shift in spectra was observed only for creatinine, i.e. the peaks at 297 nm and 407 nm were blue shifted by 13 nm and 23 nm, respectively. No remarkable change was observed for other biomolecules and it is clearly evinced that the developed colorimetric sensor has absolute selectivity towards creatinine.

Figure 6. (a) Change in Absorbance while varying the concentration of creatinine from 1.13 to 11.31 mg/dL and (b) Corresponding linear plot.
Figure 7. (b) Selectivity study based on the absorbance change in presence of other interfering biomolecules and metal ions [For selectivity study: Concentration of creatinine and other interferents are 11.31 mg/dL in pH 10.0 ammonium buffer medium].

Based on the blue shift, it is concluded that the interaction between creatinine and PTP chalcone must be ICT (Intramolecular Charge Transfer) mechanism. Further, the selective recognition of the PTP chalcone for creatinine was confirmed by adding different biologically relevant metal ions like Ag⁺, Fe²⁺, Na⁺, K⁺, Ca²⁺, Zn²⁺, Cd²⁺, Co²⁺, and Cu²⁺ ions and the results clearly reveals that the above metal ions do not show any interference. The corresponding color change is shown in (Figure 8a).

Figure 8. (a) Visible Color changes observed during naked eye visualization (a) and fluorescent color changes observed under UV Lamp emission.

Notably, we have separately done the selectivity study for sulfur containing amino acids. Because these kind of sulfur nucleophile can also form Michael adduct by means of 1, 4 conjugate addition. But the sulfur nucleophile can form Michael adduct in very strong basic catalyst condition like amine nucleophilic catalyst like DBU, DBN, phosphates etc. But we use only
simple ammonium buffer pH 10.0. So that other sulfur related biomolecules cannot form Michael adduct. So the PTP chalcone showed didn’t show any responses to these amino acids.

3.5.4. Competitive studies in presence of all other interfering molecules and metal ions.

The selectivity recognition of creatinine by the PTP chalcone chemodosimeter was further confirmed by performing the absorbance competitive titrations in presence of various biologically important metal ions and biomolecules along with creatinine in the same experimental conditions. To this one equivalent of PTP chalcone, one equivalent of creatinine (10 µL) and two equivalents (20µL) of various biomolecules and metal ions (concentration is 11.312 mg/dL) were added to the same solution one by one and there was no obvious absorbance change. The developed sensor has a ratiometric response at 297 and 407 nm only for creatinine (Figure 6). The relevant competitive emissions spectra for all those interferents are given in supporting information (Figure 9&10). From these studies, it is clearly understood that the developed sensor can detect creatininemolecules selectively even in presence of double the concentration of interferents.
Figure 9. Bar diagram based on the emission responses for the competitive analysis of PTP chalcone along with one equivalent of creatinine (10 μL) and 2 equivalents (20 μL) of other biomolecules (a) and metal ions (b). (c) Corresponding emission spectra for competitive studies. [Concentration of PTP chalcone and creatinine are 5 μM and 11.3 mg/dL respectively and other biomolecules and metal cations are 11.3 mg/dL in pH 10.0 ammonium buffer medium. (A) Bilirubin, (B) Creatine, (C) Glucose, (D) Ascorbic acid, (E) Dopamine, (F) Urea, (G) Uric acid, (H) Zn$^{2+}$, (I) Cd$^{2+}$, (J) Ca$^{2+}$, (K) Ag$^+$, (L) Na$^+$, (M) K$^+$, (N) Fe$^{2+}$, (O) Fe$^{3+}$, (P) Co$^{2+}$, (Q) Cu$^{2+}$].

Figure 10. (a) Bar diagram based on the emission responses for the competitive analysis using PTP chalcone along with 1 equivalent of creatinine (10 μL) in presence 2 equivalents (20 μL) of other aminoacids and protein in the same solution. (b) Corresponding emission spectra for competitive studies. [Concentration of PTP chalcone is 5 μM, 11.3 mg/dL creatinine and other aminoacids, protein are 11.3 mg/dL in pH 10.0 ammonium buffer medium].
3.5.5. Effect of pH on fluorescent property of PTP chalcone

In UV-Vis study, the PTP chalcone shows two absorption maxima at 297 & 407 nm, while exciting the PTP chalcone at 407 nm, two emission peaks appeared around 473 and 587 nm under optimized conditions (Figure 5b). While adjusting the pH from 2 to 12.5, no remarkable peak shift was observed as observed in UV spectra whereas gradual increase in fluorescent intensity was observed at 473 and 587 nm from pH 2.0 to 8 whereas a drastic increase in intensity was observed from pH = 8 to 10 (Figure 11) and then remained constant. So, pH 10.0 was chosen as an optimum pH for further studies. The quantum yield PTP chalcone was calculated from the fluorescent intensity at pH = 10 and found as 0.85. The above value is very close to the quantum yield of Rhodamine 6G (0.91) and this excellent emitting property of PTP has been exploited for the development of fluorescent biosensor for creatinine. In addition, the stability of the PTP chalcone was tested once in a week for 5 weeks and no change in fluorescence property was observed (Figure 12). The results depict that the PTP chalcone is stable for five weeks [Details of quantum yield calculations and stability data are given in supporting information].
Figure 11. Effect of pH on fluorescent property of PTP chalcone while adjusting the pH from 2 to 12.5. [The concentration of PTP chalcone is 5 μM in different buffer solutions. The stock solution was prepared by using 0.001 M of PTP chalcone in DMSO and then diluted to 5 μM by using various buffers].

![Figure 11](image1.png)

Figure 12. (a) Bar diagram: Emission spectra for the stability studies of synthesized PTP chalcone at various time interval. (b) Corresponding emission diagram [Concentration of PTP chalcone is 5 µM, in pH 10.0 ammonium buffer]

3.5.6. Selective fluorescent response of PTP chalcone towards creatinine

In sensors, selective detection towards a particular target analyte is one of the essential criteria to extend its application for real sample analysis. Our human biofluids consist of so many biomolecules and metal ions. So, we have already carried out the selectivity study of the PTP chalcone for creatinine in the presence different interfering biomolecules and metal ions (Figure. 6b) using UV-Vis spectroscopy. The same experiments were repeated using Fluorimetry technique. We do not observe any fluorescent change of PTP chalcone in presence of interfering biomolecules like urea, uric acid, creatine, glucose, ascorbic acid, dopamine, bilirubinserotonin, amino acids like L-Arginine, L-Cysteine, L-Cystin, reduced glutathione, BSA and metal ions like Na⁺, K⁺, Ag⁺, Ca²⁺, Cd²⁺, Zn²⁺, Co²⁺, Fe²⁺, Fe³⁺, and Cu²⁺ ions at diseased (Figure. 13a & 13b) and normal (Figure 7b & 7c), physiological concentration level of creatinine. For creatinine, a very good blue shift (60 nm) has been observed from the original wavelength of PTP chalcone i.e. from 587 nm to 527 nm. From this scrutiny, the selective response of the PTP chalcone for
creatinine has once again been proved. The corresponding fluorescent color changes were shown in supporting information (Figure 8b).

Figure 13. Bar diagram: Selective recognition of creatinine in presence of other interfering biomolecules (a). Inset of (a): Corresponding emission spectra for selectivity study. Selective recognition of creatinine in presence of other interfering metal ions (b) [For selectivity study: Concentration of PTP chalcone and creatinine is 5 µM and 15.8 mg/dL; other biomolecules and metal ions are expressed in 11.31 mg/dL in pH 10.0 ammonium buffer medium].
**Figure 14.** (b) and metal ions(c). Inset of (b): Corresponding emission spectra for selectivity study. [Concentration of PTP chalcone and creatinine is 5 µM and 1.13 mg/dL (100 µL 100 µM), respectively. Concentration of other biomolecules(100 µL of each) is of 1.13 mg/dL for creatine, bilirubin and 8 mg/dL Uric acid; 5 mmol/L for glucose, urea, other amino acids, and BSA; 1 nM for dopamine and ascorbic acid; and other metal ions are 100 µ M, at pH 10 [ammonium buffer].

**Figure 15.** Visible Color changes observed during naked eye visualization (b) during creatines detection. (A) Bilirubin, (B) Creatine, (C) Glucose, (D) Ascorbic acid, (E) Dopamine, (F) Urea, (G) Uric acid, (H), Arginine, (I) Cystine, (J) Cystin, (K) Serotonin, (L) Glutathione, (M) BSA, (N) Zn²⁺, (O) Cd²⁺, (P) Ca²⁺, (Q) Ag⁺, (R) Na⁺, (S) K⁺, (T) Fe²⁺, (U) Fe³⁺, (V) Co²⁺, (W) Cu²⁺.

3.5.7. Sensing of Creatinine using PTP chalcone by Fluorimetry

The fluorescent change of the PTP chalcone was studied during the addition of different concentration of creatinine. As we have already discussed in the introduction, the detection for creatinine was focused in the normal and diseased states so that the sensor could be applied to identify the various health issues associated with creatinine in clinics. So, the concentration of creatinine was varied from 0.000000113 to 15.8 mg/dL(Figure 14). Very interestingly, the peak
intensity at 587 nm gradually decreased whereas the peak intensity at 473 nm gradually increased for 0.00000113 to 0.0001 mg/dL concentration range of creatinine in a ratiometric manner. When the creatinine level was increased from 1.13 to 15.8 mg/dL the peak at 587 nm was blueshifted (60 nm) from 587 nm to 527 nm. This might be due to the formation of Michael adduct at higher concentration of creatinine under basic condition. This remarkable blue shift is also may be due to the creatinine induced ICT mechanism between donor and acceptor part of PTP chalcone. The formation of Michael adduct was further confirmed by observing the emission peak for isolated adduct and the emission was recorded at the excitation wavelength of 407 nm and the similar emission spectra was obtained at 524 nm (Figure 15). Due to the solubility problem, NMR spectra for this Michael adduct could not be measured. Under optimized condition, the developed biosensor could detect creatinine with a broad detection range as mentioned above. It would be very useful to diagnosis various diseases associated with creatinine level in our human body when compare to the previously reported optical and electrochemical creatinine biosensors (Table 1). The reproducibility experiment was carried out for three different concentrations of creatinine from different diseased range and our chemodosimeter shows a very good reproducibility even for 6 trails towards creatinine at three different concentrations ranges 0.00000113, 5.65 & 15.8 mg/dL. The related standard deviation values were calculated as 0.73 %, 0.13 % and 0.85 % at respective concentration ranges of creatinine, respectively. (Calculations’ were given in supporting information). Corresponding data are given in supporting information (Figure 16).
Figure 16. (a) Fluorescence emission change of the PTP chalcone while adding different concentrations of creatinine from 0.00000113 to 15.8 mg/dL. (b), (c) & (d) corresponding linear plots for different concentration levels respectively. [Concentration of PTP chalcone is 5µM and different concentration solutions of creatinine were made from stock creatinine solution of 11.312 mg/dL using ammonium buffers, pH 10.0]

Figure 17. Fluorescence emission spectra of PTP –Creatinine Michael adduct [Concentration of Michael adduct is 5µM, at pH10, ammonium buffer]
<table>
<thead>
<tr>
<th>Sensor platform</th>
<th>Technique</th>
<th>LOD (mg/dL)</th>
<th>Linear range (mg/dL)</th>
<th>References</th>
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<tr>
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<td>0.0000011-15.8</td>
<td>Present Work</td>
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**Table 1.** Comparison of performance of our biosensor with previous biosensors.
Figure 18. Bar diagram for the reproducibility experiments based on the emission response of PTP chalcone along with different concentration levels creatinine 0.0000011 mg/dL(a), 5.6 mg/dL(c) & 15.8 mg/dL(e) under optimized condition. (b), (d) & (f) Corresponding emission spectra for six successive trials at different concentration levels of creatinine in pH 10.0 [Concentration of PTP chalcone is 5 µM in pH 10.0 ammonium buffers].

3.5.8. Fluorescent spectral changes of PTP chalcone in presence of competitive analytes

Before going for real sample analysis, it is mandatory to check the competitive response of the PTP chalcone in presence of all interfering molecules in the same solution. We have followed the same protocol as in the case UV competitive studies. From the data analysis, it’s clearly evincing that the PTP chalcone has absolute selectivity for creatinine even in presence of all
the interferents (Figure 17). The corresponding emission spectra for competitive experiments of biomolecules and metal ions are given in supporting information (Figure 18 & 19).

Figure 17. Bar diagram: Emission spectra for the competitive studies of PTP chalcone containing 1 equivalent (nM concentration) of creatinine along with 1 equivalent of biomolecules (a). Metal ions (c). And 1 equivalent (µM concentration) of creatinine along with 1 equivalent of biomolecules (b) and metal ions (d). [Concentration of PTP chalcone is 5 µM; creatinine was made into two different concentration levels 0.0000011 mg/dL and 15.8 mg/dL respectively. Concentration of other biomolecules is 0.0000011 mg/dL and 15.8 mg/dL, metal cations are 0.001 M in pH 10.0 ammonium buffers. (A) Bilirubin, (B) Creatine, (C) Glucose, (D) Ascorbic acid, (E) Dopamine, (F) Urea, (G) Uric acid, (H) Zn$^{2+}$, (I) Cd$^{2+}$, (J) Ca$^{2+}$, (K) Ag$^{+}$, (L) Na$^{+}$, (M) K$^{+}$, (N) Fe$^{2+}$, (O) Fe$^{3+}$, (P) Co$^{2+}$, (Q) Cu$^{2+}$]
Figure 18. Emission spectra for the competitive studies using PTP chalcone along with 1 equivalent of creatinine at lower (0.0000011 mg/dL) and higher concentration (15.8 mg/dL) in presence 2 equivalents of other biomolecules (a) & (b) metal ions (c) & (d). [Concentration of PTP chalcone is 5 µM, creatinine was made into two different concentration ranges 0.0000011 mg/dL and 15.8 mg/dL and respectively and concentration of other biomolecules is 0.0000011 mg/dL and 15.8 mg/dL; Metal cations are 0.001 M in pH 10.0 ammonium buffers]
Figure 19. Bar diagram: Emission spectra for the competitive studies using PTP chalcone along with 1 equivalent of creatinine at lower (nM) and higher concentration (µM) in presence 2 equivalents of other amino acids (a) & (c). Corresponding emission diagram (b) & (d) [Concentration of PTP chalcone is 5 µM, creatinine was made into two different concentration ranges (0.0000011 mg/dL and 15.8 mg/dL and respectively and concentration of other aminoacids is 0.0000011 mg/dL and 15.8 mg/dL; Metal cations are 11.3 mg/dL in pH 10.0 ammonium buffer].
3.5.9. Mechanism involved during sensing of creatinine by PTP chalcone

Based on all the UV and Emission data, it is understood that the chemodosimeter has adopted ICT mechanism. In general, if a probe with a conjugated couple of electron-donating/electron-withdrawing groups (donor/acceptor, D/A), undergo ICT mechanism it will normally display large Stokes shift, visible light excitability and metal coordination-induced emission shift. Modification of D or A by any analyte will be induced blue or red shift in excitation/emission spectra along with ratiometric responses by means of altering the photo-induced internal charge transfer excited state. In our present study also we have observed a very good blue shift (23 nm) in UV-vis absorbance spectra as well as in fluorescence emission spectra (60 nm) along with a ratiometric response. From this, we clearly suggest that the chemodosimeter has followed the ICT mechanism. After the addition of creatinine at 1,4 position of PTP chalcone there may be electronic charge cloud repulsion would arise and it may increase the energy levels in PTP chalcone and finally may lead to blue shift. Hence the developed chemodosimeter has adapted ICT mechanism which is further supported by DFT calculations. In general, Michael acceptors are α,β-unsaturated compounds which having electron deficient center at which the donor will be added to form Michael adduct. Since Michael donors have acidic hydrogen atoms or having delocalization of electrons, they produce electrons to form Michael adduct. In our sight, the creatinine could act as Michael donor due to the presence of acidic hydrogen atom and PTP chalcone could act as a Michael acceptor because of the α,β-unsaturated carbonyl part. Hence, under basic condition, the creatinine could form a very stable active methylene carbanion[37] due to its resonance structure with the nearby carbonyl group present in the five member ring (Scheme 1a)
Scheme 1. Plausible Mechanism for the formation of PTP-CRT adduct (a) and energy level diagram for proposed biosensor (b).

This active methylene resonance stabilization is not possible in other above biomolecules. Even creatinine and other biomolecules like urea, uric acid, dopamine and aminoacid contains amino group, the possibility of Aza Michael Reaction doesn’t occur, because it need high basic or acidic condition.\textsuperscript{[43-44]} Hence, this stable carbanion nature has making the creatinine to interact more strongly and specifically with the PTP chalcone via normal Michael Addition reaction pathway when comparing with the above biomolecules. This is the main reason for high selectivity of our biosensor. The stoichiometry of the PTP chalcone with CRT was found to be 1:1 approximately by continuous variation/Jobs method.\textsuperscript{[45-47]} (Figure 20)
Figure 20. Determination of stoichiometry of PTP: CRT [For this study both the PTP and CRT were made at same concentration i.e. 5 µM, in pH 10.0 ammonium buffer. Firstly, the emission responses of PTP alone were examined. Followed by emission changes for the mixture of PTP and CRT (From 0.1 to 0.9 mole fraction) were determined. Based on the intersect position (~ 0.5 mole fraction) of these two lines, the stoichiometry PTP: CRT of developed chemodosimeter was determined as 1:2]

3.2.0. DFT-calculations

In order to analyze the charge transfer mechanism between PTP chalcone and creatinine, DFT calculations were performed at B3LYP/6-31G and 6-311G levels by using Gaussian 09 programme. The corresponding energy level diagram is shown in (figure 21).
The geometries of PTP chalcone optimized using the above said basis sets. TD-DFT calculations of PTP chalcone reveals that HOMO is spread over the pyrene unit and the LUMO spread over from the pyrene to acetophenone moiety with energy difference of 3.6357 eV. After binding of creatinine molecule, HOMO is only localized on the pyrene moiety whereas the charge density at LUMO was spread from acetophenone moiety to pyrene moiety with energy difference of 3.8229 eV. From the data it is well understood that the initial charge transfer has been occurred between the donor pyrene moieties to the acceptor acetophenone moiety which is responsible for an intense peak at 587 nm in emission spectra. In which, α and β unsaturated carbonyl group could act as a spacer which assist the charge transfer between the two moieties. In the excited state, after the addition of creatinine molecule, the α, β unsaturated double bond, the initial charge transfer among the molecule was insisted and now the charge was distributed highly towards the pyrene moiety which result the increased in the energy levels and finally showed a very good blue shift of 60 nm. At this stage, creatinine could acts as donor and Pyrene could act as acceptor. From this DFT visualization, it clearly supports that the developed
chemodosimeter has followed the intra molecular charge transfer mechanism and the proposed energy level diagram was shown in (scheme 1b).

3.2.1. Analysis of Human Blood Creatinine

As already mentioned in the introduction itself elevation in creatinine level in our human body will results so many health issues like renal malfunction, muscle disorder etc. Till date costlier clinical methodology has been used in order to quantify the creatinine in our human blood serum and urine. There is no simple tool available as a substitute for clinical diagnosis. Keeping this need in mind, we have developed a simple chemodosimeter based biosensor for the quantification of creatinine. To highlight the outcome of this current project, we have made collaboration with Alpha Hospital & Research Center, Institute of diabetes & Endocrinology, Madurai and collected 10 human blood serum samples of different aged peoples. The creatinine level in these serum samples has been analyzed based on the fluorescence responses of the PTP chalcone. Meanwhile in the hospital, the serum creatinine for the above same blood samples has also been analyzed simultaneously using Medica instrument model name of Esyra. The detailed clinical protocol was given in supporting information. The data have been comparable clinical results for the same serum sample and given in table. (Table. 2) To further support our method, we have also cross checked our data for the neat creatinine of different concentrations with the help of clinical method also and these results were also showed a very good agreement (Table. 3) and corresponding emission data were given in supporting information (Figure 22).

<table>
<thead>
<tr>
<th>Serum Sample Code</th>
<th>Our value (mg/dL)</th>
<th>Clinical value (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0.9615</td>
<td>0.92</td>
</tr>
<tr>
<td>P2</td>
<td>0.9050</td>
<td>0.90</td>
</tr>
<tr>
<td>P3</td>
<td>0.6262</td>
<td>0.71</td>
</tr>
<tr>
<td>P4</td>
<td>0.4751</td>
<td>0.51</td>
</tr>
<tr>
<td>P5</td>
<td>0.55</td>
<td>0.57</td>
</tr>
</tbody>
</table>
Table. 2 Analysis and comparison of blood creatinine level in serum samples collected from different persons by the proposed method and clinical methods.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Serum Creatinine level (mg/dL)</th>
<th>Our value</th>
<th>Serum Creatinine level (mg/dL)</th>
<th>Clinical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.5656</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.6787</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.905</td>
<td>0.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1.018</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>1.131</td>
<td>1.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table. 3 Analysis and comparison of neat creatinine level by proposed as well as clinical methods.
Figure 22. Emission spectra for serum creatinine analysis for person P1(a), P2(b), P3(c), P4(d), P5(e), P6(f), P7(g), P8(h), P9(i) and P10(j) [Concentration of PTP chalcone is 5 µM, and 10 µL of each blood serum was used as such for analysis]

3.6. CONCLUSIONS

Since creatinine is a main biomarker for renal malfunction, our group has given much more intension and more clinical sound to quantify the creatinine by using a very simple and first fluorescence chemodosimeter. The synthesizing of chalcone PTP is highly facile as it can be synthesized by simple hand shaking method. Its quantum yield is 0.85. It could able to quantify the creatinine from 1.13 to 11.31 mg/dL with LOD of 0.39 mg/dL based on absorption changes. For clinical quantification of creatinine, we have successfully achieved two the linear ranges is varying from 0.11–1.13 mg/dL and 2.26 – 15.8 mg/dL for normal as well as renal infection state
along with an excellent LOD of 0.0000065 mg/dL. This is the very first broad detection clinical range for detection of creatinine. To stand the outcome of this developed bioassay with clinical creatinine tools, the human blood serum samples were collected and analyzed. Results were firmly supported with clinical methods and it has turned into a new opening/substitute for clinical channels for creatinine bioassay.

3.7. ABBREVIATIONS

PTP - (E)-3-(Pyren-2-yl)-1-(3,4,5-Trimethoxyphenyl)Prop-2-en-1-one
CRT –Creatinine
BR – Bilirubin
DOP –Dopamine
GLU- Glucose
UR –Urea
URA –Uric acid
CR – Creatine
ARG – Arginine
CYS - Cystin
CYST- Cystine
GLUTO- Glutathione
SERO –Serotonin
BSA – Bovine Serum Albumin
MWCNT – Multiwall Carbon Nanotube
PANI – Polyaniline
CHIT – Chitosan
HRP- Horse Radish Peroxidase
ELISA – Enzyme Linked Immunosorbent Assay
QD- Quantum Dots
QC - Quantum Clusters
LOD – Limit of Detection
RSD – Relative Standard Deviation
TGA -Thioglycolic acid
DMSO – Dimethyl Sulfoxide
ICT – Intramolecular Charge Transfer
FRET – Fluorescent Resonance Energy Transfer
PET – Photo Induce Electron Transfer
ESIPT-Excited State Internal Proton Transfer
DFT - Density Functional Theory
HOMO – Highest Occupied Molecular Orbital
LUMO – Lowest Unoccupied Molecular Orbital

3.8. Determination of Quantum Yield

Fluorescence quantum yields (Φ) for PTP chalcone molecules was estimated by integrating the area under the fluorescence curves using the following equation. The absorbance value of probe was chosen from the absorption spectra Figure S4a. The concentration of probe was 5 μM and the integrated area was calculated from the emission spectra of PTP chalcone Figure S4b. with excitation the wavelength of 407 nm in ammonia buffer pH=10.0 For the standard Rhodamine 6G, all the values were taken from the previous report.48-49

\[ \Phi_u = \frac{\Phi_s I_u A_u \lambda_{exu} \eta_u}{I_s A_s \lambda_{exs} \eta_s} \]

Where, \( \Phi \) is the quantum yield; I is integrated area under the corrected emission spectra; \( A \) is absorbance at the excitation wavelength; \( \lambda_{ex} \) is the excitation wavelength; \( \eta \) is the refractive index of the solution; the subscripts u and s refer to the unknown and the standard, respectively. For this study, Rhodamine 6G in ethanol was taken as a standard, which has the quantum yield of 0.91. Based on the above formulae the quantum yield of PTP chalcone was calculated as 0.8500

\( \Phi_u = 0.91; \ I_u = 130766.3676; \ A_u = 1; \ \lambda_{exu} = 480 \text{ nm}; \ \eta_u = 1.33; \ I_s = 80673.1240; \ A_s = 2; \ \lambda_{exs} = 407 \text{ nm}; \ \eta_s = 1.361 \)
\[ \phi_u = \frac{0.91 \times 130766.3676 \times 1 \times 480 \times 1.33}{80673.1240 \times 2 \times 407 \times 1.361} \]

\[ = \frac{75967936.6590}{89374043.1282} \]

\[ \phi_u = 0.8500 \]

3.9. Determination of Limit of Detection (LOD)

The Low Detection Limit was calculated using the following formula.

\[ \text{LOD} = \frac{3 \times s_a}{b} \]

Where, \( s_a \) is the standard deviation of the response and \( b \) is the slope of the calibration curve. The standard deviation of the response can be estimated by the standard deviation of either y-intercepts, of regression lines. \(^{50-52}\)

The Linear equation for the variation of creatinine concentration at pH 10.0

\[ y = 0.20845 + 0.21503x \]

\( S_a = 0.00421 \) (nM); \( b = 0.21503 \)

\[ \text{LOD} = 3 \times 0.00421 \]

\[ = \frac{0.01263}{0.21503} \]

\[ \text{LOD} = 0.0587 \text{ nM} \]

3.10. Calculation of Relative Standard Deviation (RSD)

\[ \text{RSD} = \frac{\text{S.D}}{\bar{x}} \]

Where SD is the standard deviation and \( \bar{x} \) mean value of the sample data set.

At lower concentration of CRT (0.1 nM)

S. D = 0.00164

\( \bar{x} = 0.2245 \)

No of trials (N) = 6

\[ \text{RSD} = \frac{0.00164}{0.2245} = 0.0073 \text{ or } 0.73\% \]

At medium concentration of CRT (500 µM)
S. D = 1.0327
\( \bar{x} = 786.3333 \)
No of trials (N) =6
RSD = \( \frac{1.0327}{786.3333} = 0.00131 \) or 0.13 %

At Higher concentration of CRT (1400 µM)
S. D = 7.9665
\( \bar{x} = 935.6666 \)
No of trials (N) =6
RSD = \( \frac{7.9665}{935.6666} = 0.0085 \) or 0.85 %

3.11. Clinical Protocol for creatinine detection
REF 10203-4 4 x 29 mL/10mL

Principle and Procedure

The EasyRA creatinine reagent is intended for the quantitative determination of Creatinine (CREA) in human serum, using the MEDICA “EasyRA Chemistry Analyzer” in clinical laboratories.

This method uses two reagents to perform the enzymatic reaction.

\[
\begin{align*}
\text{Creatinine} + \text{H}_2\text{O} & \xrightarrow{\text{Creatinine amidohydrolase}} \text{creatinine} \\
\text{Creatine} + \text{H}_2\text{O} & \xrightarrow{\text{Creatinine amidinohydrolase}} \text{sarcosine} + \text{urea} \\
\text{Sarcosine} + \text{H}_2\text{O} + \text{O}_2 & \xrightarrow{\text{Sarcosine oxidase}} \text{glycine} + \text{HCHO} + \text{H}_2\text{O} \\
2 \text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{ESPMT}* & \xrightarrow{\text{Peroxidase}} \text{quinoneimine dye} + \text{H}_2\text{O}
\end{align*}
\]

*where ESPMT is N-ethyl-N-sufopropyl-m-toluidine
**Regents Used**

**Creatinine Enzyme Buffer Reagent (R1):**
- Good buffer (pH 7.4) 25 mmol/L
- Creatineamidinohydrolase >25 KU/L
- Sarcosine oxidase > 7 KU/L
- Ascorbate oxidase > 4 KU/L
- ESPMT 140 mg/L

**Creatinine Enzyme Color Reagent (R2):**
- Good buffer (pH 7.3) 100 mmol/L
- Creatinineamidohydrolase >250 KU/L
- Peroxidase > 5 KU/L
- 4-aminoantipyrine 600 mg/L

The reagent is ready to use as supplied. Unopened reagent is stable until the expiration date listed on the label if stored at 2º-8ºC. The reagent is stable on-board in the refrigerated reagent area of the MedicaEasyRA Chemistry Analyzer for the number of days programmed on the RFID chip on the reagent wedge. If the analyzer does not have the refrigeration option, the reagents need to be recapped and stored at 2º-8ºC after use. Do not use the reagent if it is turbid or cloudy or if it fails to recover known serum control values.

**EasyRA Assay Parameters (CRT)**
- Primary Wavelength (nm) 550
- Secondary Wavelength 700
- Reaction Type Endpoint, sample blank corrected (2)
- Reagent Direction Increase
- Reagent Blank Yes (with each calibration)
- Sample Blank Yes
- Blank high Abs. limit 0.10
- Reaction Time 10 min
Calibration interval (maximum) 20 days
Reagent on-board stability 20 days

**Serum**

Sample volume (μL) 10
Diluent 1 Volume (μL) 10
Diluent 2 Volume (μL) 10
Reagent Volume R1 (μL) 180
Reagent Volume R2 (μL) 60

DecimaPlaces (default values) 2
Units (default values) mg/dL

Dilution Factor to extend measuring range 1:1
Linearity 0.20 to 15 mg/dL

**Calibration**

MedicaEasyCal Chemistry, REF 10651 is recommended for the calibration of the assay. The calibration interval (20 days maximum) is programmed on the RFID chip on the reagent wedge. Recalibration is required whenever there is a change in reagent lot number or if a shift in quality control values occurs.

**Quality Control**

It is recommended that two levels of human serum based controls (normal and abnormal) be run with the assay at least once every 8 hours and with each reagent lot change. Failure to obtain the proper range of values in the assay of control material may indicate reagent deterioration, instrument malfunction, or procedural errors. The laboratory should also follow local, state, and federal quality control guidelines when using quality control materials.
Results

After completion of the assay, the MedicaEasyRA Chemistry Analyzer calculates the Creatinine concentration from the ratio of the corrected unknown sample’s absorbance to the corrected absorbance of the calibrator multiplied by the calibrator value.

\[
\text{CRT (mg/dL)} = \frac{[\frac{\text{A}_{\text{U550}} - \text{A}_{\text{U700}}}{\text{A}_{\text{C550}} - \text{A}_{\text{C700}}}] - [\frac{\text{A}_{\text{RBlk550}} - \text{A}_{\text{RBlk700}}}{\text{A}_{\text{C550}} - \text{A}_{\text{C700}}}] - [\frac{\text{A}_{\text{SBlk550}} - \text{A}_{\text{SBlk700}}}{\text{A}_{\text{C550}} - \text{A}_{\text{C700}}}] - [\frac{\text{A}_{\text{RBlk550}} - \text{A}_{\text{RBlk700}}}{\text{A}_{\text{C550}} - \text{A}_{\text{C700}}}] \times \text{dF} \times \text{SBlk} \times \text{Cal Value}}{[\frac{\text{A}_{\text{U550}} - \text{A}_{\text{U700}}}{\text{A}_{\text{C550}} - \text{A}_{\text{C700}}}] - [\frac{\text{A}_{\text{RBlk550}} - \text{A}_{\text{RBlk700}}}{\text{A}_{\text{C550}} - \text{A}_{\text{C700}}}] - [\frac{\text{A}_{\text{SBlk550}} - \text{A}_{\text{SBlk700}}}{\text{A}_{\text{C550}} - \text{A}_{\text{C700}}}] - [\frac{\text{A}_{\text{RBlk550}} - \text{A}_{\text{RBlk700}}}{\text{A}_{\text{C550}} - \text{A}_{\text{C700}}}] \times \text{dF} \times \text{SBlk} \times \text{Cal Value}}
\]

Where \( A_U \) and \( A_C \) are the absorbance values of the unknown and the calibrator, respectively; \( A_{RBlk} \) is absorbance of the reagent blank; \( SBlk \) is sample blank; and “Cal Value” is the concentration of Creatinine in the calibrator (mg/dL). Since the volume of the reaction is changed with the delayed addition of the R2 reagent, there is a dilution correction factor (dF) included in the calculation.\(^{53-54}\)

References


[50] Lurie, J. Hand Book of Analytical Chemistry, Mir Publishers, Moscow, **1975**.


STATEMENT EXPENDITURE INCURRED ON FIELD WORK

Name of the Principal Investigator: Dr. V.S. Vasantha

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Certified that the above expenditure is in accordance with the UGC norms for Major Research Projects.

SIGNATURE OF PRINCIPAL INVESTIGATOR

REGISTRAR
A New Route for the Enzymeless Trace Level Detection of Creatinine Based on Reduced Graphene Oxide/Silver Nanocomposite Biosensor

K. Balaji Viswanath,[a] Rajkumar Devasenathipathy,[b] Sea-Fue Wang,[b] and V. S. Vasantha*[a]

Abstract: Renal insufficiencies and muscle diseases can be easily identified from the concentration of creatinine in blood and urine. Although various chemical sensors have been developed to detect creatinine, selectivity and robustness of chemical sensors are the main obstacles for many researchers. To overcome these difficulties, finding a suitable chemical biosensor with long-term stability, low cost, high sensitivity and selectivity for the detection of creatinine is immensely desirable. Herein, we have developed a novel enzymeless creatinine biosensor for the trace level detection of creatinine using reduced graphene oxide (RGO)/silver nanoparticles (AgNPs) which was prepared by simple one step electrochemical potentiodynamic method. The anodic peak current of AgNPs gradually decreased when the concentration of creatinine was increased. Based on the decrease of anodic peak current, we have introduced a new platform for the detection of creatinine. The adsorption of creatinine on AgNPs was confirmed by various techniques. The newly proposed biosensor exhibited a very low detection limit of 0.743 pM with linear range from 10 pM to 120 pM. The demonstrated sensor can detect creatinine even in the presence of other interfering biomolecules such as glucose, ascorbic acid, uric acid, urea and creatine.

Keywords: Creatinine - Enzyme free - Graphene - Silver - Square Wave Voltammetry

1 Introduction

Creatinine (2-amino-1-methyl-5H-imidazol-4-one) is a polar molecule which is transported through the blood stream to the kidneys and disposed in the urine. Determination of the amount of creatinine in biological fluids is an increasingly important clinical measurement for the evaluation of renal dysfunction, thyroid malfunction and muscle damage [1]. The amount of creatinine in the urine is proportional to the amount of creatine and creatine phosphate present in the body and also to muscle mass. In addition, creatinine concentration in the urine is measured during standard drug tests, and normal levels range from 40 to 300 mg dL⁻¹ (3.6–27 mmol L⁻¹) in male and 37–250 mg dL⁻¹ (3.3–22.5 mmol L⁻¹) in female. However, values below 20 mg dL⁻¹ (1.8 mmol L⁻¹) have been observed in rare instances, which can indicate that the urine sample was adulterated. The routine clinical laboratory method using spectrophotometry on the Jaffe reaction suffers interference study and lacks specificity. However, existing biological compounds in the samples, such as ascorbic acid, glucose, and uric acid, can seriously interfere with the results of the above method [2–4]. Fluorimetry [5–6], chemiluminescence [7] and chromatography [8–11] methods require a preliminary derivatization step, which results in longer analysis times. Electrochemical creatinine biosensors include amperometry using monoenzyme and trienzyme systems and potentiometry based on ion-sensitive or gas-sensitive electrodes and ion-selective field-effect transistors (ISFET) [12–14]. Enzymatic methods are apparently costly and time consuming. The thickness of the enzyme layer can limit the analytical signal and response time because of the impeded transport of the analyte through the modifier layer. A method using a molecularly imprinted polymer (MIP) electrochemical sensor for creatinine detection requires the laborious construction of a template molecule and an accurate printing of a model on a substrate to obtain the desired specificity, making it a complex and expensive technique. However, the main problem in the development of the MIP-based sensors for creatinine is associated with the effective detection of the MIP-analyte binding event and registration of the sensor response by the available equipment [15–17]. Therefore, numerous efforts have been made to develop enzyme free sensors for clinical diagnosis of creatinine to achieve low cost, good sensitivity, selectivity, low detection limit and high stability.

Graphene, the mother of all graphitic material comprises a single layer of carbon atoms joined together by sp² covalent bonds. It is described as an individual of graphite

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Ultrasensitive Fluorescent Biosensor for Creatinine Determination in Human Biofluids Based on Water Soluble Rhodamine B Dye-Au$^{3+}$ ions Conjugate

S. Ellairaja, K. Shenbagavalli, and V. S. Vasantha[a]

Simple, ultrasensitive and selective diagnostic tool is required for the quantification of creatinine in human biofluids. To the best of our knowledge, we are reporting the second fluorescent as well as colorimetric biosensor for creatinine with a very good linear range. We have utilized a simple water soluble Rhodamine B dye as a probe because of its excellent emitting properties and quantum yield. As a colorimetric biosensor, the dye has selectively detected the Au$^{3+}$ ions in a ratiometric aspect through ICT mechanism among all other interfering metal ions. When the creatinine was introduced, the Au$^{3+}$ ions were gradually released from the dye and bound with the creatinine which finally enhanced the fluorescent intensity. The Stern Volmer quenching constant $K_{sv}$ was found to be $2.6 \times 10^{6}$ M$^{-1}$. As a colorimetric biosensor, the limit of detection and linear range for creatinine are found to be 54 nM and 100 nM to 15 μM, respectively. Under the optimized condition, the fluorescent biosensor has detected the creatinine within the linear range of 10 nM to 1.2 × 10$^{7}$ M along with the LOD of 5 nM. The linear range of developed biosensor implies that the biosensor can be applied for clinical diagnosis of muscle disorder (creatinine level < 40 μM), extreme renal problem (creatinine level = 1000 μM). We have extended the application of this developed biosensor for the determination of creatinine in human urine sample and achieved a very good recovery.

Renal function plays a vital role to filter numerous metabolic wastes from human blood through glomerular filtration. This glomerular filtration rate (GFR) is used to determine the amount of waste products especially creatinine and urea which is directly filtered from human blood. Actually creatinine is a metabolic waste molecule from our muscle metabolism. It is actually produced from creatine, which is produced to produce major energy in our muscles. Almost 2% of the precursor creatine is converted to creatinine at every day and then it is transported to the kidneys through the bloodstream. When the kidney is not properly functioning, the level of creatinine is increased in blood.[1] Optimum physiological concentration range of creatinine in blood is 40–150 μM, but under extreme pathological conditions, its level could exceed to 1000 μM. When the level of creatinine exceeds >500 μM it's clearly indicates severe renal impairment. When the levels reaches <40 μM, it indicates the decreased muscle mass.[2] The earlier and frequent detection of creatinine in human blood could improve the quality of human life especially for peritoneal dialysis patients.[3–6] In general, Jaffe's reaction is most commonly used for the quantification of creatinine based on the color change of alkaline solution of creatinine and picric acid.[7] Some enzymatic colorimetric methods are also available for the detection of creatinine,[8] but, it has some adverse effects like the affection to numerous metabolites and drugs found in biological samples, more time-consuming, complicated and expensive. Meanwhile some costly chromatographic methods have also been used for the relatively fast creatinine determination in human biofluids.[9–11] Recently, electrochemical creatinine sensors are emphasizing its specific advantages like sensitivity, selectivity, less time consuming, cost-effective etc. For creatinine biomolecule concern, there were lot of potentiometric biosensors,[12–15] conductometric biosensors,[16] enzymatic[17–20] and enzymeless[21–23] creatinine biosensors, Dissolved oxygen amperometric creatinine biosensors,[24–25] nanoparticles based amperometric biosensors[26–28] and capacitive creatinine biosensors[29] reported with a good limit of detection (μM range). Very recently a new digital camera based technology was imposed for creatinine detection in urine with a LOD of 89 & 111 μM. Very recently, a TERN ON fluorescent sensor has reported using thioglycolic acid (TGA) capped ZnS:Mn/ZnS quantum dots (QDs) for creatinine detection within in linear range of 0.07 and 3.4 μM along with LOD of 7.25 nM and 0.0242 μM.[30]

Fluorimetric technique is a very simple analytical tool for various metal ions,[31–35] biomolecules,[36] and pathogens[37–42] detection based on the fluorescent change of the molecules. By adopting this simple and less time consuming technique, one could achieve a very good linear range and finally end with an excellent limit of detection (LOD) on particular analytes what we want to detect.[43–44] Solubility of fluorescent molecules

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Michael Addition Based Chemodosimeter for Serum Creatinine Detection Using (E)-3-(Pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one Chalcone

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Supporting Information

ABSTRACT: First, a simple and highly emissive fluorescent chalcone (E)-3-(pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (PTP) was synthesized via simple shaking along with an excellent quantum yield of 0.85, and proved as a stable, highly sensitive, and selective biosensor for creatinine. Owing to its unique photophysical interaction with creatinine through Michael adduct formation, PTP was utilized as a Chemodosimeter for the selective recognition of creatinine in blood serum. Under optimized conditions, a broad range of creatinine detection was achieved from 0.000000113 mg/dL to 15.8 mg/dL along with an excellent limit of detection of 0.00000065 mg/dL (0.058 nM). This biosensor is highly reproducible even for different concentration levels of creatinine. It is the very first creatinine biosensor possessing a wider linear range for clinical applications for creatinine. To ensure its clinical application, blood serum samples of people of different age groups were collected from Alpha Hospital and analyzed for creatinine by using our chemodosimeter method and compared with data obtained using a commercial method in the Alpha hospital. Our data show very good agreement with clinical data. Because clinical protocol involves trienzymes and tedious sample preparation, no doubt, our chemodosimeter will be a cheap and sensitive option compared to the existing clinical methods.

KEYWORDS: chalcone, chemodosimeter, creatinine, fluorescence, biosensor, blood serum

Creatinine is a routine biomarker which plays a crucial role in renal and muscular dysfunctions because of its direct secretion from muscle as a side product. The variation in creatinine level in blood and urine is an important parameter in clinical diagnostics. When the kidney is not properly functioning, the level of creatinine is increased in blood. In normal human fluid such as urine, creatinine levels are varied in the range from 0.45 to 1.69 mg/dL, but under extreme pathological conditions, its level could reach 11.31 mg/dL and abnormal level >5.65 mg/dL can indicate either renal malfunction in the situation where the creatinine concentration increases or can be a sign of muscular dystrophy for lower creatinine values. Due to its medical relevance the development of a fast and accurate assay for the determination of creatinine in human serum or urine is required. Although several techniques have been proposed for a fast and accurate quantification of creatinine in human serum or urine, most of them require expensive or complex apparatus, advanced sample preparation, or skilled operators.1,2 The earlier and frequent detection of creatinine in human blood could improve the quality of human life especially for peritoneal dialysis patients.3 Generally, Jaffé’s reaction has been used commonly for the quantification of creatinine based on the color change of the alkaline solution of creatinine and picric acid.4 Apart from this, a few enzymatic colorimetric protocols and chromatographic tools could also be used for the detection of creatinine.5 These protocols have been generally affected by numerous metabolites and drugs found in biological samples and require extensive time. It is also more complicated and expensive. With selectivity as a concern, some electrochemical creatinine sensors have also been reported having specific advantages such as sensitivity, selectivity, being less time-consuming and cost-effective, etc. For example, there are many potentiometric biosensors6,7 conductometric biosensors,8 some enzymatic9,10 and enzymeless11,12 creatinine biosensors as well as dissolved oxygen amperometric creatinine biosensors13,14 nanoparticles-based amperometric biosensors15,16 and capacitive creatinine biosensors17,18 which have also been reported with a good limit of detection. In the current sensor era, a fluorimetric
Reply to the Comment on “Michael Addition Based Chemodosimeter for Serum Creatinine Detection Using (E)-3-(Pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one Chalcone”

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ABSTRACT: A very simple chemodosimeter has been developed for creatinine biomolecules based on Michael addition reaction by using (E)-3-(Pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one Chalcone. The photophysical properties of the chalcone were thoroughly analyzed using UV-vis and emission techniques. The chalcone has exhibited two absorption maxima at 297 and 407 nm which are due n−π* and π−π* transitions, respectively. This property was further confirmed by repeating UV-vis absorbance studies of the chalcones in different solvents having different polarity. The PTF chalcone has originally exhibited ICT mechanism and it is arrested while creatinine is added. However, a ratiometric response is observed due to the creatinine induced ICT mechanism and it is also clearly supported with DFT studies. In our original work, we did DFT studies for only one isomer of the creatinine. Currently, we have extended our DFT studies for another isomer also. The relative quantum yield of the PTP chalcone was calculated in sensing and standard conditions as 0.85 and 0.45, respectively.

This reply complements the comment of Krishnamoorthy et al. on our recent work entitled, Michael Addition Based Chemodosimeter for Serum Creatinine Detection Using (E)-3-(Pyren-2-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one Chalcone: The creatinine exists in two isomeric forms. We have considered only one of the isomers and now we have also performed calculations on another isomer. The results obtained from DFT calculations by Krishnamoorthy and group and our group are similar (Figure 1). The HOMO and LUMO of PTP:CRT are mainly located on the pyrene group only, which indicates that intramolecular charge transfer is not feasible. The CRT induced ICT shown scheme is purely based on emission interpretations and was confirmed by blue shift that is a well-reported mechanism. The FMO results produced in the main manuscript also indicate the same. There is a slight interpretation misconception that in the PTP molecule the LUMO is predominantly located on the α,β-unsaturated carbonyl. Moreover, a significant contribution is also found on pyrene as well as the phenyl ring. That is why we have written the contribution up to the phenyl ring. This is the idea behind our interpretation. At this point, we agree with Krishnamoorthy et al. that the initial ICT was initiated from pyrene to α,β-unsaturated carbonyl predominantly and then a small contribution from phenyl ring. We are thankful to the explanation by Krishnamoorthy et al. regarding FMO analysis.

The probe has initially adopted ICT mechanism, in which charge transfer occurs from pyrene to α,β-unsaturated carbonyl part and small contribution to phenyl ring. When CRT is added to the α,β-unsaturated carbonyl part, the delocalization is arrested and the initial ICT is inhibited and then CRT induces charge transfer, which is confirmed by the emission analysis synthesized Michael adduct. Herein, DFT studies are only used to support the occurrence and inhibition of the ICT mechanism before and after addition of CRT. CRT induced ICT was supported purely based on the emission results only. Generally, molar extinction coefficient for n−π* is less than that of π−π*. However, the transitions assigned for our chalcone are the characteristic absorption maxima for all chalcones. This kind of behavior is mainly due to the presence of extended conjugation −C=O−C=C−O chromophore system in the chalcone structure which resulted in greater delocalization of π electrons along the molecule. In our case, it is well understood that π−π* occurred at lower wavelength and n−π* occurred at higher energy and thus lower wavelength. To confirm this property, UV-vis spectral studies for the chalcone in solvents having different polarity were carried out (Figure 2). The results indicate that π−π* shows red shift and n−π* shows a blue shift with increasing polarity of the solvent (Table 1).

The mechanism is proposed based on the UV-vis and emission results. Initially, ICT was operated from donor pyrene to the acceptor part mostly at α−β, unsaturated carbonyl and then significantly at the phenyl part. After binding of creatinine at α−β, unsaturated double bond, it further enhances the donating ability of pyrene, but conjugation between donor and acceptor parts is disconnected after the binding of CRT, and hence ICT from the pyrene moiety to acceptor is arrested. The CRT induced ICT is...