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# Semi-quantitative colorimetric and supersensitive electrochemical sensors for mercury using rhodamine b hydrazide thio derivative

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## Abstract

Mercury is a toxic heavy metal which pollutes air, land, and water posing a serious threat to the environment and human health. For a first-hand estimate, a qualitative method (colorimetric) for detection of mercury could suffice. However for precise measurement, below the threshold value, a quantitative method (electrochemical) is needed. The colorimetric strips were produced using mercapto-propanoic acid derivatized rhodamine hydrazide (RS). RS developed a pink color when in contact with mercury (Hg<sup>2+</sup>) and the shade became darker with an increase in concentration. A new polymeric hydrogel was used to entrap RS to fabricate sensing strips for quasi-quantitative detection of Hg<sup>2+</sup> through the naked eye. An electrochemical sensor was also developed for accurate quantification of Hg<sup>2+</sup> at ppt level by modified glassy carbon electrode with the same RS and green reduced graphene oxide (RS-gRGO/GCE) via differential pulse anodic stripping voltammetry (DPASV). The dipstick exhibited high sensitivity, good selectivity and low detection limit (0.29 ppb) whereas metal free, label-free modified electrode performed very well with a high sensitivity (70.22 µA.ppb.cm<sup>-2</sup>), low detection limit (57 ppt), good selectivity, and reproducibility. These sensors will serve the purpose of common people for onsite estimation as well as scientific laboratories requiring precision measurements.

#### **Graphical Abstract**



Keywords: Rhodamine b, Mercury, colorimetric-dipstick, electrochemical, green reduced graphene oxide

#### **1. Introduction**

The presence of heavy metals even at trace level has an adverse effect on biotic components in the ecosystem including humans. Among toxic metals, mercury is considered as the most potent hazard on the ground of its ability to diffuse through biological membrane [1] and also the formation of non-labile bond with thiol group in protein [2]. The World Health Organisation (WHO) and US Environmental Protection Agency (EPA) have considered permissible limit mercury in drinking water as 2 ppb [3]. The regular consumption of mercury-contaminated water leads to its accumulation in the human cell permanently. In the long run, this leads to "infamous" Minamata disease, and also Hunter-Russell syndrome, alzheimer, kidney failure [4]. In order to mitigate this, periodic monitoring of mercury is essential for all drinking sources. Several methods are already available for mercury detection. These include UV-Vis spectrophotometry [5], fluorescence spectroscopy [6], atomic absorption spectrometry [7], cold vapour atomic absorption spectrometry [8], induced coupled with plasmon mass spectrometry [9], atomic emission spectrometry [10], X-ray fluorescence spectrometry [11], atomic fluorescence spectrometry [12], gas chromatography [13] and aggregation induced emission spectrometry [14]. These methods are however insignificant for daily monitoring by common mass since these needs bulky immoveable expensive instrumentations along with extensive sample pre-treatment. On the other hand, a colorimetric dip stick method can be used by layman and makes monitoring convenient.

There are many reported attempts for colorimetric detection and these are based on rhodamine [15], chitosan [16], benzothiazole [17], nano-particles [18] and nanohybrid [19]. Onsite applications of these techniques face a formidable challenge due to their complicated aliquot preparations. Das and Sarkar [20] developed a hydrogel improvised dipstick sensor for arsenate detection in aqueous solution using polyacrylamide and polyvinyl chloride hydrogel network. The effective immobilization of colorimetric detector i.e. RS, on the solid support, was quite a challenge due to its leachable property in aqueous solution. The problem was resolved by using polymeric hydrogel, whose caged network structure could firmly entrap the detector element along with facile access to aqueous due to its swelling property. The polymeric hydrogel was prepared with polyethylene glycol and sodium alginate cage for dipstick preparation. The detection limit of these methods is nearly equal to the maximum contaminant level (MCL). In order to detect very low concentration of Hg<sup>2+</sup>, electrochemical method was chosen as the method is simple, cost effective and can by handled by semiskilled person. In electrochemical detection, working electrode surface modification step is important to get superior sensitivity for the detection of Hg<sup>2+</sup>. There are few reports on the use of modified carbon paste [21], platinum [22] and TiO<sub>2</sub> [23] [24] electrodes for Hg<sup>2+</sup> detection in aqueous samples. Fluorescent sensors [25-27] have been studied by a number of researchers. Chen et al. [26] used 1,4,8,11-tetraazacyclotetradecane (Cyclam)-functionalized carbon dots for the estimation of metal ( $Cu^{2+}$ ). Wang et al. [25] developed a fluorescent probe using polymer-nanoparticle- 4-ethoxy-9-allyl-1,8-naphthalimide detector element, and the probe had a low detection limit of 75 nM Hg<sup>2+</sup>. The probe showed high selectivity for  $Hg^{2+}$ . Chen et al. [27] developed a fluorescent as well as colorimetric sensor for  $Hg^{2+}$  using rhodamine B and 4-chloro-7-nitro-2,1,3-benzoxadiazole and achieved a detection limit of 120 nM. Hg<sup>2+</sup> has a tendency to amalgamate with gold, platinum and silver electrodes and changes the surface morphology which causes a decrease in response and hence a nonreproducible performance [28]. Interestingly, the same detector (RS) could also be used for accurate quantification of  $Hg^{2+}$ , even in ppt level, with the help of differential pulse anodic stripping voltammetry (DPASV). The thio derivatized rhodamine b hydrazide (RS) showed redox activities due to the presence of thio group along with the Hg<sup>2+</sup>complexing ability. The immobilization of RS on the electrode however required a supporting adsorbent [29] to provide RS a facile access to metal ions for their reduction and oxidation during the time of pre-concentration and anodic stripping respectively. The reduced graphene oxide (RGO) was chosen as adsorbent due to its tuneable conductive nature, high surface area, and adhesive property. Though the conductive nature and adhesive property had opposing effects,

a controlled reduction of graphene oxide (GO) was used to achieve an optimum ratio of the two. This enhanced proper immobilization of nonhomogenous RS gave better sensitivity to the sensor.

In this work, the thio-derivative of rhodamine b hydrazide (RS) was synthesized from rhodamine b hydrazide and 3 mercaptopropionic acid and used as a chelating ligand for both colorimetric (color change of RS, present in acetonitrile and water mixture, from colorless to pink by reaction with  $Hg^{2+}$ ) and electrochemical detection of mercury (preconcentration of mercury on electrode by complexation with graphene-bound RS followed by anodic stripping resulting in a current response). A practically applicable colorimetric polymeric hydrogel dipstick for semi-quantitative Hg<sup>2+</sup> detection was developed through the entrapment of RS in poly-ethylene glycol and alginate hydrogel network, the principle of color change being the same as that in the colorimetric sensor. For accurate detection of the very low concentration of  $Hg^{2+}$ , the same RS was immobilized on glassy carbon electrode [30] with green reduced GO (gRGO), produced by green reducing agent i.e. phytochemical (*Calendula officinalis* ) extract. The RS-gRGO-GCE composite was used to detect and measure mercury through DPASV for accurate, sensitive analysis. Scheme 1 shows the principle of colorimetric/dipstick and electrochemical estimation of mercury which has been described latter in details. This study gave a new direction of research considering demands from common people and scientists by offering both qualitative and quantitative methods for all practical purposes.



**Colorimetry/ Dip-stick** 

Scheme1. Principle of mercury estimation by different methods used in this work

#### 2. Experimental section

2.1. Materials and reagents

Rhodamine b, hydrazine hydrate, 3-mercaptopropionic acid, ethanol, di-cyclo carbodiimide (DCC), acetonitrile, needed for synthesis of RS were supplied by E. Merck (Germany) and

used as received. The  $Hg^{2+}$  stock solutions were prepared using mercuric chloride (HgCl<sub>2</sub>) purchased from Sigma (India).

The co-active metals (Fe<sup>2+</sup>, Fe<sup>+3</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Mn<sup>2+</sup>, Cd<sup>2+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>) ion solutions were prepared from their chloride salt (Purchased from Merck, India).

Poly-ethylene glycol-12000 (PEG) and sodium alginate (SA) were used to prepare polymeric hydrogel matrix for binding RS. Calcium chloride was used as a cross-linker. All these reagents were purchased from Merck, India and used as received.

Ultrapure water (18.2 M $\Omega$ ) was used for all the experiments and was obtained from arium®pro ultrapure water system (Sartorius, India).

Graphite powder, potassium permanganate (KMnO<sub>4</sub>), sodium nitrate (NaNO<sub>3</sub>), sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were procured from Merck, India and used to prepare graphene oxide (GO).

*Calendula officinalis* (COL) plant leaves, commonly named as marigold plant were collected from Uluberia, Howrah, WestBengal (22.4744° N, 88.1000° E). The leaves of the plant were cleaned with ultrapure water (18.2 M $\Omega$ ) from arium®pro ultrapure water system (Sartorius, India). These were chopped finely and soaked in hot (80 °C) water (2:5) for 30 min to extract most of the antioxidants (such as polyphenols) [31] into the aqueous phase. After filtration, the liquid extract was collected and used directly for reduction of GO.

#### 2.2. Synthesis

Rhodamine-NHNH<sub>2</sub> was prepared following a literature method [32]. Then 3-mercapto-propionic acid (0.010 g) was dissolved in 6 mL CH<sub>3</sub>CN. After complete dissolution, DCC (0.4 g, 0.0195 mol) was added under stirred condition [33] for 15 min. Rhodamine hydrazide (0.00108 mmol) was added and stirred overnight. After removing dicyclohexylurea (DCU) by filtration, CH<sub>3</sub>CN was evaporated out to give crude product. Purification of crude product was done by re-precipitation using diethyl ether and methanol. The crude product was subjected to NMR analysis and hydrogel film preparation. <sup>1</sup>H NMR  $(300 \text{ MHz}, \text{CDCl}_3)$  (Fig. S1) :  $\delta$  8.16 (d, J = 8.9 Hz, 2 H), 7.80 (s, 1 H), 7.42-7.40 (m, 2 H), 7.04 (d, J = 8.0 Hz, 1 H), 6.51-6.49 (m, 2 H), 5.40-5.39 (m, 1 H), 3.34 (q, J = 8.0 Hz, 8 H), 3.1 (m, 2 H), 2.44 (m, 4 H), 1.15 (t, J = 8.0 Hz, 12H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) (Fig. S2): δ 165.13, 152.53, 152.41, 149.79, 148.78, 133.24, 128.14, 128.03, 127.71, 123.43, 123.41, 107.97, 105.50, 97.97, 65.16, 44.29, 33.54, 30.65, 15.17, and 12.51 (Supporting information, S1).



Scheme2. Schematic presentation for rhodamine b hydrazide thio-derivative synthesis

2.3. Synthesis of graphene oxide (GO) and green reduced graphene oxide

#### (gRGO)

The modified Hummers and Offeman method [34] was used to prepare GO from graphite powder. GO was prepared as per our previous reported literature [35]: 1 g of graphite powder and 0.6 g of NaNO<sub>3</sub> were mixed in ice-cold concentrated H<sub>2</sub>SO<sub>4</sub> (0 °C, 23 mL). Then KMnO<sub>4</sub> solution (30% in water) was added dropwise until the color of the solution turned greenish black. The greenish-black solution was then heated to 80-90 °C until it turned brown. The brown solution was cooled to room temperature followed by addition of H<sub>2</sub>O<sub>2</sub> (9 mL). This produced the typical yellow color of the GO. After the settlement, the supernatant was removed by centrifugation. The solid product was dried at 50 °C to obtain GO powder. This GO powder was dispersed in 50 mL water by ultra-sonication and 30 mL of COL extract was added (1 drop/min) with constant stirring. The mixture was kept for 12 h. The color of the mixture turned black due to the formation of gRGO which was filtered out and dried. This black gRGO was ready for RS immobilization on the surface of the electrode. (Characterisation: supporting information S2-S4)

#### 2.4. Preparation of gRGO-RS composite and electrode modification

The gRGO-RS composite was prepared by first dispersing the RGO (1 mg/mL) in 1 mL acetonitrile by ultrasonication. Then 500  $\mu$ L (1 mg/mL) of RS was added to the dispersed gRGO and continued the sonication process for next 10 minutes. 6  $\mu$ L of the prepared gRGO-RS composite was drop cast on the surface of the precleaned GCE and dried at ambient temperature. The modified GCEs thus prepared were stored at 4 °C.

#### 2.5. Preparation of Polymer-RS test strip

A measured proportion of 12.5% PEG-12000 and 2.5% SA were mixed in 20 mL hot water (80 °C) with constant stirring. A homogeneous clear solution was obtained and cooled down to room temperature.

100  $\mu$ L of RS reagent was mixed with 1 mL of a PEG-SA polymer hydrogel mixture and 20  $\mu$ L of 20% (w/v) calcium chloride to make a stable reagent–polymer mixture. Plastic detector strips were dip coated (HWTL 0.01 Dip Coater, MTI Corporation, USA) with this mixture and dried in an oven at 45 °C for 30 min.

#### 2.6. Characterisation and instrumentation

Fourier transform infrared spectra were recorded on Perkin Elmer instrument using KBr pellets in case of the solid sample. <sup>1</sup>H NMR characterization was studied by Bruker-300 spectrophotometer at 400 MHz. Energy-dispersive X-ray analysis (EDX) spectra of the synthesized product were recorded by Joel JSM 6100. UV–visible absorption spectra were recorded using a double beam, UV–vis spectrophotometer (CE7200 CECIL, Thermo Spectronic, UK) and the color developed was photographed by Sony digital camera. The RGB analysis was accomplished using software (Adobe Photoshop 6). The voltametric study was performed on Autolab analyzer (PGSTAT 12/30/302) and the data were recorded and analyzed through GPES 4.9 software.

#### 2.7. Method of detection of Mercury

A stock solution of RS (1 mg/mL) and Hg<sup>2+</sup> (10 ppm) in 1:1 mixture of H<sub>2</sub>O: CH<sub>3</sub>CN were diluted to 100 ppm and 1- 60 ppb respectively. A colorimetric aliquot of 2 mL was prepared by mixing equal quantities of RS and Hg<sup>2+</sup> solution and finally distinct shades of color variation with variable Hg<sup>2+</sup>concentration could be identified by naked eye. The same set of experiments was performed in presence of other co-active metal ions (Fe<sup>+2</sup>, Fe<sup>+3</sup>, Co<sup>+2</sup>, Ni<sup>+2</sup>, Mn<sup>+2</sup>, Cd<sup>+2</sup>, Ca<sup>+2</sup>, Mg<sup>+2</sup>) present in 100 times that of mercury to determine the selectivity of the detector element. This method of developing color was transformed into paper strips for ease of application. The prepared hydrogel dipsticks were immersed into mercury solutions of various strengths to prepare mercury detection visual calibration chart. The RGB analysis was also performed on the developed shades for quantifying Hg<sup>2+</sup>. UV-vis spectra were also studied to ensure the binding mechanism of RS with Hg<sup>2+</sup>. In all the experiments, RS concentration was maintained at 100 ppm with variable Hg<sup>2+</sup> concentrations.

#### 2.8. RGB analysis

Any color is a combination of three basic colors as red (R), blue (B) and green (G). The intensity of the paper strip was calculated as follows

$$A_{R} = -\log(Rs/Rb)$$
(1)  

$$A_{G} = -\log(Gs/Gb)$$
(2)  

$$A_{B} = -\log(Bs/Bb)$$
(3)

Where Rs, Gs, Bs and Rb, Gb, Bb are the average red green and blue values in the RGB analysis. In the symbols "s" denotes sample while "b" denotes blank. For analyzing color values in the RGB system, digital images were fitted into Adobe Photoshop 6 software, three representative areas on each test strip were analyzed and the RGB values were calculated. The effective intensity of the color was obtained by subtracting the RGB value of blank experiment.

#### 2.9. Electrochemical study

Initially, bare glassy carbon electrode [30] was polished by 0.3  $\mu$ m alumina powder on rayon pad to remove the previous deposition. Then GCE was washed by ultrapure water, followed by ultra-sonication in ethanol for 15 minutes. Cyclic voltammograms (CVs) were generated in the potential range from -1 V to 0.5 V, scan rate 50 mV/s in triethyl amine-HCl (Tris-HCl) of pH 7 until they reached a constant current profile. The optimised electrochemical conditions for differential pulse voltammetry (DPV) measurement using pulse amplitude: 50 mV, pulse period: 2 s, pulse width: 0.05 s and pre-deposition time for metal ions: 180 s. The DPV was performed in the potential range scanning from -1 to 0.5 V. The main adsorption sites for Hg<sup>2+</sup> are the sulfur in the RS because the sulfur atom has a lone pair of electrons that can efficiently bind with Hg<sup>2+</sup> metal ions by soft-soft combination (HSAB theory) and forms a stable metal complex. The gRGO was used as a supportive adsorbent with RS to provide more active sites and surface area for the enhanced detection of Hg<sup>2+</sup>.

#### 3. Results and discussion

#### 3.1. Characterisation of rhodamine b hydrazide thio derivative (RS)

The sensitive and selective color response depends upon the opening of the spirolactam ring which was directly synthesized by coupling reaction of Rhodamine-NHNH<sub>2</sub> with DCC activated MPA. The detailed mechanism is shown in scheme 2. In FTIR (Fig. 1a) study, a sharp amide peak at 1698 cm<sup>-1</sup> was observed and also, peaks at 2576 cm<sup>-1</sup> for S-H stretching vibration [36] and 643 cm<sup>-1</sup> for C-S vibration [16] confirmed the presence of thiol group in RS. In NMR spectroscopy, RS derivative showed the presence of an additional intensive signal at 2.5–2.8 ppm for the –SH group (Fig. 1b) [16]. A sharp intense peak due to the high density of the –SH functional group in the RS suggested the competence the second amidation reaction. Additionally the cyclic hydrogen bonding between –C=O and H-S- at 3260 cm<sup>-1</sup> in RS was observed after interaction with Hg<sup>2+</sup>. A similar observation was found by Ueyama et al. between –C=O and H-N- [37] The detailed <sup>1</sup>H NMR and <sup>13</sup>C NMR characterisations were included in supporting information.



Figure 1 Spectroscopic characterisation (a) FTIR and (b) <sup>1</sup>H NMR

#### 3.2. Colorimetric determination of Hg<sup>2+</sup>

The sensitivity of RS derivative was studied for qualitative  $Hg^{2+}$  detection by UV-vis spectroscopy. Fig. 2a showed the change of absorbances in addition to  $Hg^{2+}$  in RS in aqueous media. The gradual increase of  $Hg^{2+}$  concentration (0-60 ppb) registered the increased intensity of the pink (Fig. 2b: inset). The photograph was taken after the 30 s upon addition of  $Hg^{2+}$ . The visual color change of the RS solution by  $Hg^{2+}$  was confirmed by UV-vis spectroscopy. The intensity of the peak at 555nm increased with the gradual increase of  $Hg^{2+}$  concentration. The intensity of absorption was plotted against the concentration of  $Hg^{2+}$  (Fig. 2b). The plot shows a linear response in the concentration of 0-10 ppb (Fig. 2c). The

limit of detection was calculated from three times the standard deviation of UV-vis absorption/slope of the calibration curve and found to be 0.078 ppb which is well below the permissible limit of  $Hg^{2+}$  in drinking water guided by WHO and EPA. The stability of the probe RS was studied in different pH (Fig. S5, see supporting information), the results clearly explain that this probe can be used in a broad range of pH 5.0-8.0.



Figure 2 (a) UV-vis titration spectra of RS (100 ppm) on incremental addition of 0 to 60 ppb Hg2+ in CH<sub>3</sub>CN/H<sub>2</sub>O (5:5, v/v). (b) UV-vis binding isotherm : absorbance vs concentration of Hg<sup>2+</sup> (2-60 ppb), Inset: the color changes of RS due to Hg<sup>2+</sup> (c) Calibration curve: A/A<sub>0</sub> vs concentration of Hg<sup>2+</sup> (2-10 ppb) (d) UV-vis absorption spectra of RS (100 ppm) observed upon addition of 100  $\mu$ M ions (Na<sup>+1</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Mn<sup>2+</sup>, Cd<sup>2+</sup> Ca<sup>2+</sup>, Mg<sup>2+</sup>), red line and with Hg<sup>2+</sup>, black line

#### 3.3. Selectivity of colorimetric sensor

Selectivity of RS was evaluated using colorimetric response in presence of other co-active metal ion (Na<sup>+1</sup>, Fe<sup>+2</sup>, Fe<sup>+3</sup>, Co<sup>+2</sup>, Ni<sup>+2</sup>, Mn<sup>+2</sup>, Cd<sup>+2</sup> Ca<sup>+2</sup>, Mg<sup>+2</sup>). Fig. 2d shows that the colorimetric sensitivity was not affected in presence of other ions. However, the addition of

 $Hg^{2+}$  produced a pink color. The binding interaction and mechanism of the reaction with  $Hg^{2+}$  was studied by UV-vis and FTIR spectroscopy.



Figure 3 (a) UV-vis absorption spectra of before (black line) and after(red line)  $Hg^{2+}$  (1 ppb) addition in RS (100 ppm) (b) UV-vis binding isotherm :absorbance vs concentration of  $Hg^{2+}$  (2-60 ppb) (c) Job's plot of RS and  $Hg^{2+}$  complexation (d) Benasi-Hidelbrand plot:  $1/A_{RS-complex}$  vs  $1/C_{Hg^{2+}}$ 

In Fig. 3a, RS exhibited a maximum peak ( $\lambda_{max}$ ) 540 nm, on the addition of Hg<sup>2+</sup>,  $\lambda_{max}$  shifted to 555 nm. The blue shift (bathochromic shift ~15 nm) is an evidence of strong interaction of Hg<sup>2+</sup> with RS (Fig. 3a). In FTIR (Fig. 3b) binding interaction was confirmed by shifting of stretching frequency of amide bond from 1698 cm<sup>-1</sup> to 1637 cm<sup>-1</sup> (Fig. 3b) [37]. All the above fact indicated that a metal chelate complex was formed between the RS and Hg<sup>2+</sup>. FTIR spectrum of the RS–Hg complex reveal a large shift of S-H stretch mode from 2576 cm<sup>-1</sup> RS to 2380 cm<sup>-1</sup> in the Hg<sup>2+</sup>-impregnated RS, suggesting the formation of the complex through the – SH group of RS [36]. Further confirmation of Hg<sup>2+</sup> binding with the RS can be observed through the shift in (C–S) vibration from 643 cm<sup>-1</sup> to a lower wave-

number due to Hg-S bond [16]. Similarly, the strong effects are also derived for other peaks (N-H vibrations) at 1607 and 1557  $\text{cm}^{-1}$  [16]. The presence of Hg<sup>2+</sup> was indicated by shifting of stretching frequency of amide bond from 1698 cm<sup>-1</sup> to 1637 cm<sup>-1</sup>. Additionally the cyclic hydrogen bonding between -C=O and H-S- at 3260 cm<sup>-1</sup> in RS was not observed after interaction with Hg<sup>2+</sup>. A similar observation was found by Ueyama et al. between –C=O and H-N- [37]. To further investigate the interaction between synthesized thiomer sensor and Hg<sup>2+</sup>, the <sup>1</sup>H NMR spectrum was recorded before and after addition of Hg<sup>2+</sup> and is displayed in Figure (Fig. S6, supporting information). Fig. S6 shows a significant down-field shift of the <sup>1</sup>H NMR spectra for thiomer-Hg in comparison to RS. The results can be accounted for the  $Hg^{2+}$  coordination to -C=O and -SH groups with the loss of hydrogen [37]. The coordination through covalent bond may result in the decrease in electronegativity of the siprolactum ring, which can be attributed to significant upfield shift. The d orbital of Hg<sup>2+</sup> was available for forming  $\pi$  bonding and a complex spirolactam ring opening procedure, thereby regenerating the pink color. In conclusion, the FTIR and <sup>1</sup>H NMR experiments suggested the probable binding mode of sensor and Hg<sup>2+</sup>, and the same is demonstrated in Scheme 3.



Scheme3. Schematic presentation for thio-derivative mercury complex after mercury addition

The selectivity of RS could be explained by hard-soft acid-base theory. The presence of SH group in RS, a soft coordination site for soft Hg<sup>2+</sup> only (as soft-soft interaction is stable), could be an explanation for the spirolactam ring opening process. The binding stoichiometry between RS and Hg<sup>2+</sup> was calculated from Jobs plot (Fig. 3c) and found to be 1:1 mole ratio of ligand to the metal complex. Subsequently, Benesi-Hillebrand (B-H) plot also gave a confirmation of the binding mole ratio of 1:1 by showing linearity (Fig. 3d). Further, the binding constant was calculated from the slope of B-H plot and found to be logK=7.38 which was comparable to values reported in the literature [38-40]. Mass spectra was conducted to speculate the mechanism of mercury triggered ring opening of RS and different products

formed in the process mass spectrometry. The original RS solution and the pink colored solution (after one day waiting) in acetonitrile/water mixture were analyzed by mass spectroscopy. Fig. S7 confirmed presence of nearly 100% RS in the solution. However the pink solution, wherein Hg<sup>2+</sup> triggered ring opening occurred, gave three prominent peaks (Fig. S8). The peak corresponding to molecular weight (MW) 443.5 was maximum followed by those for MW 457.5 and 544.7. This mass spectroscopy revealed Scheme 4 as the possible ring opening mechanism. The final hydrolyzed product was the carboxylic acid (MW 443.5) which might have been formed through direct hydrolysis of RS (MW 544.7) or through the formation of hydrazide (MW 457.5) intermediate. The carboxylic acid being the final product registered higher amount compared to RS and the intermediate. The amount of RS was found to be minimum as it hydrolyzed to form the two compounds (Fig. S8).



Scheme 4. Possible products in Hg<sup>2+</sup> triggered ring opening

# 3.4. Synthesis and characterization of thio-derivatized polymer hydrogel dipstick (RS-PEG-SA)

The polymeric hydrogel was prepared from PEG and SA mixture and chelated by CaCl<sub>2</sub>. Pure hydrogel exhibited characteristic peaks at 1620 cm<sup>-1</sup> and 1400 cm<sup>-1</sup> (Fig. 4a) for asymmetric and symmetric vibration of –COO group in SA and peak at 1100 cm<sup>-1</sup> for bending vibration of C-O group in PEG. The other absorption peaks at 2890 cm<sup>-1</sup> and 3421 cm<sup>-1</sup> were attributed to C-H stretching vibration and O-H bending vibration, respectively.

After incorporation of RS to hydrogel matrix, a distinct amide stretching peak at 1665 cm<sup>-1</sup> in association with –COO group stretching peak at 1620 cm<sup>-1</sup> were observed [41]. On close inspection, peak at 2576 cm<sup>-1</sup> was also found and could be attributed to S-H stretching vibration [42]. All of the above findings established a successful entrapment of RS to PEG-SA matrix. After sorption of Hg<sup>2+</sup> (1 ppb), RS-PEG-SA coated polymeric hydrogel dipstick showed a color change from colorless to pink. FTIR analysis of used dipstick showed a shift in peaks at 2676 cm<sup>-1</sup> (S-H) to 2360 cm<sup>-1</sup> and 1666 cm<sup>-1</sup> (O=C-N) to 1612 cm<sup>-1</sup>, respectively. This clearly indicated Hg<sup>2+</sup> interaction with amide and thio bonds of RS in RS-PEG-SA hydrogel. The peak at 631 cm<sup>-1</sup> was observed due to the presence of Hg<sup>2+</sup> in RS-PEG-SA polymeric hydrogel.



Figure 4 (a) FTIR analysis of hydrogel (black), RS+hydrogel (red), Hg<sup>2+</sup>+RS+hydrogel (blue) : Fe-SEM images of (b) hydrogel (c) RS with hydrogel (d) Hg<sup>2+</sup> adsorbed RS-hydrogel

Additionally, SEM and EDS analyses were performed to support the above findings. SEM analysis showed that pristine RS-PEG-SA hydrogel surface was crumpled and wrinkled due to amorphous nature of hydrogel (Fig. 4b) whereas on the addition of RS, the SEM image depicted the hydrogel morphology more furrowed and craggy (Fig. 4c). After sorption of  $Hg^{2+}$ , the surface turned rough and lumpy due to mercury sorption (Fig. 4d). EDS results (Fig. 5a) suggested 0.54 atomic% sulfur atoms in RS loaded PEG-SA hydrogel and confirmed the presence of RS in the hydrogel. Fig. 5a also depicted the presence of  $Hg^{2+}$  (0.05 atomic %) giving a strong evidence of adsorbed mercury by the RS loaded hydrogel.



Figure 5 (a) EDS spectra of  $Hg^{2+}$  adsorbed RS-hydrogel (b)Standard color chart of  $Hg^{2+}$  by naked eye observations (c) Calibration curve: RGB vs. concentration of  $Hg^{2+}$  (2-40 ppb); Inset: RGB analysis color chart and interference study (d) interference study

Standard  $Hg^{2+}$  solutions of 2- 40 ppb together with blank were quantified using this method. The intensity of the pink color increased with the increase in the concentration of  $Hg^{2+}$  in water. A standard color chart was prepared based on the variation of the intensity of the pink color (Fig. 5b: inset and 5c). The proof of concept was analyzed by plotting of color intensity versus concentration of  $Hg^{2+}$  (Fig. 5b). A linear calibration curve was obtained indicating the

good sensitivity of R-value ( $R^2 = 0.99$ ). The detection limit was calculated from the slope of the calibration curve and found to be 0.29 ppb.

Selectivity of new mercury hydrogel sensor was evaluated using the colorimetric response in presence of another co-active metal ion (Na<sup>+1</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Mn<sup>2+</sup>, Cd<sup>2+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>). Fig. 5d gave evidence of almost no cross-reactivity in the colorimetric response of mercury.



3.5. Characterisation of gRGO-RS composite and electrochemical mercury sensing

Figure 6 Fe-SEM images of (a) gRGO (b) RS with gRGO (c)  $Hg^{2+}$  adsorbed RS-gRGO (back scattered) (d) EDS spectra of  $Hg^{2+}$  adsorbed RS-hydrogel.

The gRGO-RS composite was characterized by SEM, FTIR and EDS. The surface morphology of gRGO seemed nanosheets like structure (Fig. 6a) having lamella sheaths in layer by layer configuration. Some of the nano-sheets are oriented like marigold flower structure. This orientation gave a high exposed surface area with surface edge defects by green reduction. After electrodeposition on the same electrode (Fig. 6b), the nano-sheet

surface was uniformly covered by a white layer due to deposition of mercury also viewed in backscattered image (Fig. 6c). EDS analysis (Fig. 6d) also confirmed presence of mercury after electrodeposition by a characteristic  $Hg^{2+}$  peak.



Figure 7 (a) FTIR spectra for (black)GO, (red) gRGO, (blue) RS with gRGO and (pink) after mercury pre-concentration of 180 s (b) CV response of bare GCE and RS-gRGO composite modified GCE in pH 7.0 tris-HCl buffer; optimisation of RS-gRGO/GCE by (c) optimization by pH (d) optimization by scanning potential.

In FTIR (Fig. 7a), the gRGO sample exhibited peaks corresponding to –OH stretching (3432 cm<sup>-1</sup>), C=O stretching (1710 cm<sup>-1</sup>), C=O bending (1628 cm<sup>-1</sup>), OH bending (1394 cm<sup>-1</sup>), C-OH stretching (1261 cm<sup>-1</sup>), C-O stretching (1046 cm<sup>-1</sup>), C-C (2931 cm<sup>-1</sup>) and C=C (2960 cm<sup>-1</sup>). After adsorption of RS on the surface of gRGO, a distinct amide stretching peak at 1665 cm<sup>-1</sup> [43] and –COO group stretching peak at 1620 cm<sup>-1</sup> were observed [41]. A peak at 2576 cm<sup>-1</sup> was also found and it could be attributed to S-H stretching vibration [42]. After

pre-concentration of  $Hg^{2+}$  (1 ppb), RS-gRGO showed a shift in peaks at 2676 cm<sup>-1</sup> (S-H) to 2360 cm<sup>-1</sup> and 1657 cm<sup>-1</sup> (O=C-N) to 1642 cm<sup>-1</sup>, respectively. The observations predicted  $Hg^{2+}$  interactions with amide and thio bond of RS. The detailed GO and gRGO characterisation was included in supplementary information.

Cyclic voltammetry (CV) was performed to confirm redox property of the composite. In CV, redox peaks were observed at the potential -0.49 V and 0.39 V respectively (Fig. 7b). The presence of redox peak infers the success of coupling reaction and also thio group immobilization. The same observation was reported earlier using glutathione [44].

Before electrochemical detection of  $Hg^{2+}$ , some parameters such as accumulation time, accumulation potential and pH were optimized for analysis by DPV. Fig. 7c showed the plot of DPV response at different pH (2-9) containing 1 ppb  $Hg^{2+}$ . The RS-gRGO/GCE electrode showed a maximum response at pH 7. At lower pH, the availability of a lone pair of RS functional group (N and S) decreased by protonation which reduced the coordination ability to form a stable complex with  $Hg^{2+}$ . On the other hand at higher pH, amidolysis affected the coordination by  $Hg^{2+}$ adsorption. The method of detection has been explained in scheme 5.



Scheme 5. Schematic presentation for electrochemical detection of mercury in RS-gRGO composite.

When accumulation potential (Fig. 7d) was increased from -0.4 V to -1 V, current rating increased up to -0.6 V and subsequently decreased. Thus, -0.6 V was chosen to be optimum for the reduction of  $Hg^{2+}$  to Hg(0). The time for accumulation was optimised by varying time and the desired value was found to be 180 s (Fig. 8a).



Figure 8 (a) Optimization of pre-concentration time (b) DPV response of RS-gRGO composite modified GCE for various concentration of (1 to 40 ppb) of  $Hg^{2+}$  (c) Linear plots of current response vs.[Hg<sup>2+</sup>] (d) Interference study in presence of co-active ions containing 0.1 ppb Hg<sup>2+</sup>

The RS-gRGO/GCE electrode obtained using the above-optimized parameters were used for electrochemical detection of  $Hg^{2+}$ . A sharp anodic current was detected at 0.22 V and pH=7. The DPV response (Fig. 8b) increased linearly with the increase of  $Hg^{2+}$  concentrations (0.1 ppb to 1 ppb, again 1 ppb to 40 ppb with different slopes). Thus two linear calibration plots were obtained for very low and comparatively higher concentration ranges shown in Fig. 8c. The limit of detection (LOD) was calculated using 3 times standard deviation of blank/slope of the calibration curve and was found to be 0.057 ppb. The sensitivity of the RS-gRGO/GCE sensor was calculated as 70.22  $\mu$ A.ppb.cm<sup>-2</sup> using the slope of the calibration curve/geometrical surface area of the electrode. The results indicated that the developed RS-gRGO composite was capable of giving effective analytical performance towards  $Hg^{2+}$  detection. It was further noted that the LOD value was found to be much lower than the

recommended threshold value given by WHO and EPA. The selectivity of RS-gRGO/GCE electrode was evaluated by DPV measurement for detection of 1 ppb Hg<sup>2+</sup> in presence of 100 fold higher co-active metal ions (by consecutive addition) at parameters' values for DPV measurement described earlier. It was seen that an insignificant amount of current ( $0.2 \mu A$ ) was increased (Fig. 8d); thus, other coactive metal ion does not affect any significant current response for the detection of Hg<sup>2+</sup>. At such low potential, another metal ion might not deposit on the electrode surface. The results demonstrated that RS-gRGO/GCE electrode may be used for selective determination of Hg<sup>2+</sup>. RS-gRGO composite modified GCE was used to detect and measure mercury in different water samples and Hg<sup>2+</sup> spiked same samples to check its performance and percentage Hg<sup>2+</sup> recovery (Table-1). The water sample was prepared by adding a Tris-HCl buffer to adjust pH 7 and no other purification steps were necessary except filtration for the waste sample. Table-1 clearly showed that the RS-RGO-GC electrode could give consistent results even in real samples.

**Table 1:** Determination of Hg<sup>2+</sup> in different water samples using RS-gRGO modified GCE by DPASV; <sup>a</sup>RSD- Relative standard deviation, <sup>b</sup>ND- Not detected

Water labelled	Spilzed (pph)	Detected (nnh)	Decovery	DSDa
water labelled	Spiked (ppb)	Detected (ppb)	Kecovery	KSD
Waste water	ND <sup>b</sup>	ND	-	-
	10	9.3	92	5.2
	20	19.1	96	3.3
Tap water	ND <sup>b</sup>	ND	-	-
	10	9.6	96	2.9
	20	19.7	98.5	1.9
Drinking water	ND <sup>b</sup>	ND	-	-
	10	9.8	98	1.6
	20	19.9	99.5	1.3

#### 4. Conclusions

This paper presented two unique methods of mercury detection for rapid on-site field analysis e.g. colorimetric through RS mediated polymeric hydrogel and electrochemical analysis by RS-gRGO modified electrode via DPASV. The most interesting feature of this work was that the same RS was used for both the methods. The RS mediated polymeric hydrogel exhibited excellent sensitivity, selectivity towards mercury detection which was quite different from the existing colorimetric methods and by far the best with respect to sample preparation and detection procedure. A low detection limit of 0.29 ppb was achieved. The detection needed only 10 min to visualize by naked eyes.

RS-gRGO composite used for electrochemical detection of mercury by DPASA was successful. This label-free, metal free, practically viable electrode exhibited good analytical performance, high sensitivity (70.22  $\mu$ A.ppb.cm<sup>-2</sup>), low LOD (57 ppt or 0.285 nM), good selectivity, reproducibility, repeatability with a wide linear range of response. The LOD was found to be significantly less than some published works [25, 27]. The detailed characterizations by SEM, EDS and NMR confirmed mercury adsorption and ligand formation in the composite. There have been no interferences from common metals ions on the responses of both these sensors.

Since mercury pollution is detrimental to human health, these could be used by common people and scientists depending on the requirement and help protect human health.

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#### Highlights

- A rhodamine b thio derivative as detector element for both dipstick and electrochemical sensor for  ${\rm Hg}^{2+}$
- A very simple polymeric hydrogel dipstick developed with polyethylene glycol and alginate network
- Highly selective and sensitive Hg<sup>2+</sup>level indicator till 0.29ppb through easy color matching methodology for semi-quantification
- The differential pulse anodic stripping voltammetry used for ultra-sensitive Hg<sup>2+</sup>
- Green reduced graphene oxide used as an adsorptive electrode surface modifier

A CERTICAL SCRIPT