CHAPTER 3

Peroxidase Mimetic Brominated Graphene for Sulphide Ion Recognition
3.1 Introduction

In this chapter, the functionalization of graphene oxide (GO) with bromine has been made, characterized and explored the properties taking into consideration of artificial enzymes and sensing application.

Peroxidases, a heme containing oxidoreductases, have various applications in the field of diagnostics, sensors, catalysis and bioremediation.\(^1\)-\(^7\) Horseradish peroxidase (HRP), a widely familiar enzyme, has been explored as sensor for H\(_2\)O\(_2\) detection,\(^8\) tracer in enzyme linked immunosorbent assay (ELISA)\(^9\)-\(^10\) and detection of some peroxidase inhibitors.\(^11\) In recent years, numerous studies have been reported on making artificial enzymes mimetic of HRP. Artificial enzymes which mimic natural ones, have numerous advantages like low cost, ease of mass production, robustness to harsh environment, high stability, long term storage and tunable activity.\(^12\)-\(^16\) So far, various materials, which mimic HRP have evolved, e. g. Au nanoparticles (NP),\(^12\) Ag NP,\(^13\) modified graphene,\(^14\) graphene quantum dots,\(^15\) tungsten oxide,\(^16\) and their nanoparticle hybrids\(^17\)-\(^20\) etc., and have been widely used as sensors for the detection of H\(_2\)O\(_2\), cholesterol and glucose via electrochemically, fluorometry and colorimetry.

Reduced graphene alone is incapable to mimic peroxidase\(^21\)-\(^24\) but its nanoparticle-based hybrids exhibit peroxidase mimetic activity via enhanced exfoliation, dispersibility, adsorptivity and easy facilitation of electron transfer in between substrates; e. g. Co\(_3\)O\(_4\) reduced graphene oxide nanocomposites as an effective peroxidase mimetic for glucose detection via colorimetry,\(^21\) CoFe\(_2\)O\(_4\) ferrite nanocubes on graphene as a peroxidase material for H\(_2\)O\(_2\) detection via colorimetry,\(^20\) CuS graphene nanosheets composites with peroxidase activity for H\(_2\)O\(_2\) detection via colorimetry,\(^22\) Fe\(_3\)O\(_4\) nanoparticles loaded graphene oxide dispersed carbon nanotubes with peroxidase activity for H\(_2\)O\(_2\) and glucose detection via colorimetry and electrochemically,\(^23\) 3D graphene network @WO\(_3\) nanowire composites as
peroxidase for H$_2$O$_2$, dopamine and ascorbic acid detection via electrochemically,$^{16}$ dual functional Pt and Pd supported on reduced graphene oxide hybrid as peroxidase for H$_2$O$_2$ detection via electrochemically$^{24}$ and palladium nanoparticles decorated magnetic graphene nanosheets as peroxidase mimetics for H$_2$O$_2$ via colorimetry.$^{19}$ Here, a novel cost effective peroxidase mimetic material made up of bromine functionalized graphene, brominated graphene (GBR), without loading of any nanoparticles have been developed.

GBR has been well-characterized by XRD, UV-Vis, XPS, EDX, Zeta Potential, TGA, SEM, and TEM techniques. It’s peroxidase mimetic activity has been studied via colorimetry using 3,3',5,5'-tetramethylbenzidine (TMB) as chromogen and H$_2$O$_2$. Further, the realistic application of as prepared peroxidase material has also been explored.

Sulphide ion is extensively released by tanneries, paper and pulp manufacturing plants, food processing plants and petroleum refinery industries as a by-product of industrial and manufacturing process.$^{25}$ The continuous exposure of sulphide ion at a high concentration leads to physiological and biological problems such as Alzheimer’s,$^{26}$ Down’s syndrome$^{27}$ and hyperglycemia.$^{28}$ Various techniques like voltammetry, gravimetry, fluorometry, colorimetry etc. for the detection of sulphide ion have been evolved and widely used nanoparticle-based materials$^{29-37}$ e.g. gold nanoparticles via colorimetry,$^{29}$ DNA templated gold/silver nanoclusters via fluorescence technique,$^{31}$ Cu@Au nanoparticles via colorimetry$^{32}$ and glutathione-modified gold nanoparticles probe via colorimetry$^{33}$ etc. Very few studies have also been reported using graphene derivatives. Qi et al.$^{38}$ reported the use of modified reduced graphene sheets electrode for sulphide detection via electrochemical technique. Later Hsu et al.$^{39}$ reported the peroxidase like activity of iron hydroxide immobilized graphene oxide which has been exploited for sulphide ion detection via fluorescence technique. Recently, Cao X. et al.$^{40}$ reported the sulphide ion determination in fruit using modified alizarin-reduced graphene oxide nano-sheets electrode via
electrochemical technique. Very recently, same group also reported the sulphide ion
detection using glassy carbon electrode modified with hemin functionalised graphene via
electrochemical technique. None is based on colorimetry. Still the researchers are interested
to develop simple, facile and rapid method for the detection of dangerous S²⁻.

So, herein, we have fabricated a sensor combining TMB, H₂O₂ and GBR visibly
appeared as bluish green owing to the formation of oxidised form of TMB (OxyTMB) and
used the same for the novel detection of different analytes. Out of the studied analytes, S²⁻ has
shown enormous sensitivity. Considering all these, a well calibrated curve for S²⁻ has been
prepared with the linearity range in the order of 0.04 - 0.4 mM and the limit of detection of ~
25.3 μM. Water samples collected from different origins have also been analysed using our
fabricated sensor and calibration curve. Standard addition experiment has validated the
process. Using the fabricated sensor solutions, we have finally developed a paper strip based
sensor for S²⁻ detection.

3.2 Experimental section

3.2.1 Materials

Hydrobromic acid (HBr; Sd fine chemicals, India), phosphoric acid (H₃PO₄; Qualigens,
India), 95% sulphuric acid (H₂SO₄; Fischer Scientific, India), potassium permanganate
(KMnO₄; Qualigens, India), 30% hydrogen peroxide (H₂O₂) (Merck, India), hydrochloric acid
(HCl; Fischer Scientific, India), hydrazine mono hydrate (NH₂NH₂.H₂O; Loba chemie,
India), ammonia (NH₃; Merck, India), 3,3’/5,5’-tetramethyl benzidiamine (TMB; Merck,
India), methanol (CH₃OH; CDH, India), acetone (CH₃COCH₃; CDH, India), sodium
hydrogen phosphate (Na₂HPO₄; Merck, India) and potassium hydrogen phosphate (K₂HPO₄;
Merck, India) were used as received. Analytes of analytical grades were used as received.
Deionised water (DDW) was prepared by redistillation of the double distilled water in a glass
distillation apparatus. Dry ethanol was prepared by leaving ethanol (Saraya Distillery, India) over CaO for overnight followed by distillation over fresh CaO. Graphite was provided as a gift by Dr. Sanjay Dakate of National Physical Laboratory, New Delhi. Synthesis and characterization of graphene oxide (GO)\textsuperscript{42,43} was discussed in details in chapter 2. Water samples for S\textsuperscript{2–} detection was collected from Ganga River, and Sewage.

3.2.2 Characterization

FT-IR spectra were recorded using a PERKIN ELMER Spectrum version 10.03.05. spectrometer in the range of 400–4000 cm\textsuperscript{-1}. The scattering pattern of the powder samples were recorded in a Bruker D8 advance X-Ray diffractometer. Samples were grinded well before measurement, and the diffractogram was recorded from 5° to 80° using CuK\textalpha radiation. Raman spectra of samples were taken by Agitron micro-Raman spectrometer from 800 to 3000 cm\textsuperscript{-1} using 514 nm excitation laser sources. The XPS measurement was made in an ultra high vacuum AMICUS photoelectron spectrometer equipped with MgK\textalpha X-ray as a primary excitation and a KRATOS VISION 2 SOFTWARE. The curve fitting of high resolution spectra was performed with combined Gaussian–Lorentzian functions. Zeta potentials were measured using HORIBA-Nanoparticles analyzer SZ-100, Japan at 25 °C for reference samples dispersed in deionised water (0.1 mg/mL) after ultra-sonication for 15 min. Morphology studies were performed using Scanning Electron Microscope (EVO 18 Research, Zeiss) along with energy dispersive X-Ray diffractogram Oxford instrument (X-act) and TEM (Technai G2 20 twin, FEI, USA). UV-Vis spectra of composites and sensing activity were recorded using JASCO V 650 spectrophotometer operating in the spectral range 200–1100 nm.

3.2.3 Synthesis of Reduced Graphene Oxide (RGO)
Chemical conversion of GO to reduced graphene oxide (RGO) was carried out according to the reported method.\textsuperscript{44} Typically, 50 mg of GO dispersed in 100 mL of deionized water was sonicated for 6 h to get its colloidal solution. To it, hydrazine hydrate (34.3 µL, 35 mg) was added and the reaction mixture was placed in an oil-bath pre-heated at 95 °C and kept under stirring for 2 h. The resultant reduced graphene oxide was collected by filtration, washed with deionized water several times and finally with acetone and dried under vacuum for 12 h at 40 °C.

3.2.4 Synthesis of Brominated Graphene (GBR)

GBR was synthesised using method reported by Jankovsky et al.\textsuperscript{45} Typically, GO (150 mg) was added to HBr (7 mL, 48 % extra pure) taken in a round bottom flask during sonication, then transferred to an oil bath maintained at 122 °C and kept under reflux for 5 h. The resultant product was then precipitated in water, filtered and washed with double distilled water (250 mL) and finally with methanol (250 mL). Resultant product was dried under vacuum at 50 °C for 24 h.

3.2.5 Peroxidase activity by GO, RGO and GBR

GO (50 µL, 1mg/mL) was dispersed in 400 µL of double distilled water (pH 6-7). To it, TMB (50 µL, 1mM) added followed by the addition of H₂O₂ (100 µL, 1 mM). After keeping the mixture for 30 min in dark, absorbance spectra at 652 nm were recorded. A similar procedure was performed for RGO and GBR at the set temperature of 27 °C.

3.2.6 pH dependent and time dependent absorbance studied using GBR peroxidase

pH dependent study was performed using GBR (100 µL, 1mg/mL), TMB (50 µL, 1 mM) and H₂O₂ (200 µL, 1 mM) added onto different pH solutions of phosphate buffer (400 µL) and after keeping the mixture for 30 min in dark, absorbance spectra at 652 nm was recorded.
Time dependent absorbance study was performed using H$_2$O$_2$ (200 µL, 1 mM), TMB (50 µL, 1 mM) and GBR (100 µL, 1mg/mL) in 400 µL phosphate buffer solution (pH 4.48). Absorbance spectra at 652 nm were recorded at different time intervals.

3.2.7 Sensing ability of fabricated sensor towards different analytes

Analytes (150 µL, 1mg/mL) cations or, anions were added into the fabricated sensor containing H$_2$O$_2$ (200 µL, 1 mM), TMB (50 µL, 1 mM) and GBR (100 µL, 1mg/mL) in phosphate buffer solution (400 µL, pH 4.48), and kept for 1 h in dark to reach equilibrium simultaneously and the absorbance spectra were monitored.

3.2.8 Sulphide assay using fabricated sensor

Stock solution containing different concentrations of sulphide ion (in the range of 0.001 - 1 mM, 150 µL) was added into the fabricated sensor containing H$_2$O$_2$ (200 µL, 1 mM), TMB (50 µL, 1 mM) and GBR (100 µL, 1mg/mL) in phosphate buffer solution (400 µL, pH 4.48), and kept for 1 h in dark to reach equilibrium simultaneously and the absorbance spectra were monitored at 652 nm.

3.2.9 Water Sample analysis

Water samples (150 µL) collected from different origins were added into the fabricated sensor containing H$_2$O$_2$ (200 µL, 1 mM), TMB (50 µL, 1 mM) and GBR (100 µL, 1mg/mL) in phosphate buffer solution (400 µL, pH 4.48), and kept for 1h in dark to reach equilibrium simultaneously and the absorbance spectra were monitored at 652 nm.
3.3 Results and discussion

3.3.1 Material Characterization

Brominated graphene (GBR) has been synthesised using Jankovsky et al.’s method.\textsuperscript{45} It contains around ~ 77, 20, and 3 weight % of C, O and Br, respectively, as revealed by SEM-EDX study (Table 3.1). XPS study indicates that apart from the peaks corresponding C(1s) and O(1s), the peaks corresponding to Br (3d) and Br (3p) are also present [Figure 3.1(a)] within it and the Br-content is estimated to be 3.6% as calculated by dividing the area of Br by total area comprised by C, O and Br. This ‘Br’ content result supports the results of SEM-EDX. To determine the nature of the linkages by which bromine is attached to the sheets of GBR, we have performed the curve fitting of the Br (3d) peak in its XPS spectrum. Fitted and deconvoluted XPS spectra of Br (3d) peak [Figure 3.1(b)] has revealed that Br (3d) spectrum is composed of two components: one is attributed to covalently bonded Bromine (C-Br) at 70.10 eV and the another one is comprised of Br\textsuperscript{-} and Br\textsubscript{2} species at 67.90 eV.\textsuperscript{45,46} The ratio between the C-Br and Br\textsuperscript{-}/Br\textsubscript{2} component is nearly about 3:1 as obtained by dividing the area of each component by total area of Br (3d). Hence, we have concluded the presence of C-Br in large extent in the formed GBR.

Table 3.1. SEM-EDX (Energy Dispersive X-Ray Diffraction pattern) results of GO, RGO and GBR.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Carbon</th>
<th>Oxygen</th>
<th>Bromine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight % of Element</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>43.88</td>
<td>54.67</td>
<td>0.00</td>
</tr>
<tr>
<td>RGO</td>
<td>79.12</td>
<td>20.88</td>
<td>0.00</td>
</tr>
<tr>
<td>GBR</td>
<td>77.21</td>
<td>19.86</td>
<td>2.94</td>
</tr>
</tbody>
</table>
Figure 3.1. (a) XPS spectra of GO, RGO and GBR (b) Fitted and deconvulated XPS spectra of Br (3d) in GBR.

The linkage nature of Br in GBR is also supported by FT-IR (Figure 3.2) as evidenced by the presence of the peak corresponding to C-Br at ~500 cm\(^{-1}\). The presence of electronic conjugation like reduced graphene (RGO) is supported by the observation of an absorption peak at 263 nm in UV-visible spectroscopy (Figure 3.3). XRD study has revealed the presence of a broad peak at 2\(\theta\) = 24.2\(^{\circ}\) indicating its randomly ordered exfoliated sheets form like RGO (2\(\theta\) = 22.7\(^{\circ}\)) [Figure 3.4(a)].\(^{47}\) Indeed, the crumpled, and large aggregated sheet form of GBR is visualized in its SEM and TEM micrographs (Image 3.1). ID/IG ratios of GO, RGO and GBR calculated from the corresponding raman spectra [Figure 3.4(b)] were 0.949, 1.029 and 1.044 cm\(^{-1}\), respectively. This result indicates that more graphitic domains are formed and the sp\(^2\) cluster number (domain) has been increased on conversion from GO to RGO and then to GBR as expected. Interestingly, dispersibilty of GBR (- 47.85 mV) in water is close to GO (- 50.27 mV) as revealed by Zeta potential measurement (Figure 3.5). It is to be noted that observed zeta potential of RGO is - 40.82 mV. The better dispersibilty of GBR compared to RGO may be due to the incorporation of bromine.
Figure 3.2. FT-IR spectra of GO, RGO and GBR.

Figure 3.3. UV-Vis spectra of GO, RGO and GBR.
Figure 3.1. SEM (a) and TEM (b) Images of GBR.

Figure 3.4. (a) XRD and (b) Raman spectra of GO, RGO and GBR.

Figure 3.5. Zeta potentials of GO, RGO and GBR.
3.3.2 Peroxidase activity studies of GO, RGO and GBR

Graphene based materials have widely been used as peroxidase mimicking material. Here, we have established the same for GBR. We have studied first the oxidase-like activity of the substrates: GBR, the parent graphene oxide (GO), and reduced graphene (RGO) towards the oxidation of 3,3′,5,5′-tetramethyl benzidiamine (TMB) into its oxidised form Oxy-TMB. It is monitored by UV-Vis spectroscopy as TMB, on oxidation, gives a characteristic peak of Oxy-TMB at 652 nm (Figure 6). GO has not oxidised TMB while RGO has shown little efficacy. But GBR has been highly active towards TMB oxidation. It may be mainly due to the incorporated bromine in the graphene sheets as C-Br and Br-Br bonds, which can easily dissociated by accepting electrons from TMB and convert it into Oxy-TMB (bluish green colour). It is to be noted here that no peak at 652 nm is observed in the absence of TMB.

We then studied the peroxidase activity of the substrates (GO/RGO/GBR) (50 µL, 1 mg/mL) towards TMB (50 µL, 1 mM) in the presence of H₂O₂ [Figure 3.7(a)-(c)]. Only GBR (Figure 3.6) has shown the H₂O₂ concentration (up to 200 µL, 1mM) dependent enhanced peroxidase activity. As the concentration (0, 100 and 200 µL, 1mM) of H₂O₂ increases, the conversion of TMB to Oxy-TMB increases as evidenced by the increase in the absorbance intensity of Oxy-TMB at 652 nm (visibly deepens the colour of bluish green). All these results have confirmed GBR’s novel peroxidise mimetic activity (Scheme 3.1) owing to the incorporation of bromine which facilitates radical formation as well as electron transfer by restoration of π-π conjugation along with enhancing dispersibility.
**Figure 3.6.** UV-Vis absorbance spectra of TMB (50 $\mu$L, 1 mM) in water after 30 min addition of GO, RGO and GBR (50 $\mu$L, 1 mg/mL).

**Figure 3.7.** UV-Vis absorption spectra of (a) GO-TMB (b) RGO-TMB and (c) GBR-TMB [GO, RG and GBR -50 $\mu$L, 1 mg/mL, TMB- 50 $\mu$L, 1 mM) mixture in water after 30 min addition of $\text{H}_2\text{O}_2$ (100 $\mu$L, 1 mM)].
3.3.3 Effect of pH on the peroxidase activity of GBR

In order to understand the effect of pH on the peroxidase activity of GBR, fixed amount of stock solutions containing H$_2$O$_2$ (200 µL, 1 mM), TMB (50 µL, 1 mM) and GBR (100 µL, 1 mg/mL), have been added into each phosphate buffer solutions of different pHs (range 3.33 - 9.12) and the mixtures are kept for 30 min in dark, and then the absorbance spectra at 652 nm has been monitored. At pH 4.48, quick and best peroxidase activity is observed in comparison to other pHs as evidenced by the appearance of intense bluish green colour (Image 3.2) and UV-Vis absorption spectrum [Figure 3.8(a)-(b)]. Moreover, dormant nature of GBR peroxidase activity has been observed at pH 7 and beyond it. Time dependent absorption studies in phosphate buffer solution of pH 4.48 at 27 °C have indicated that ~30 min is required for the complete conversion of TMB to Oxy-TMB in equilibrium condition [Figure 3.9(a)-(b)].
Image 3.2. Effect of pH on GBR activity [GBR (100 µL, 1 mg/mL), TMB (50 µL, 1 mM) and H$_2$O$_2$ (200 µL, 1 mM) were added onto different pH solutions of phosphate buffer (400 µL) and kept in dark for 30 min].

Figure 3.8. (a) UV-Vis absorbance spectra and (b) corresponding curve of absorbance (at 652 nm) vs. pH showing the effect of pH on GBR activity [GBR (100 µL, 1 mg/mL), TMB (50 µL, 1 mM) and H$_2$O$_2$ (200 µL, 1 mM) were added onto different pH solutions of phosphate buffer (400 µL) and after keeping each mixture for 30 min in dark, absorbance spectra at 652 nm was recorded].
Figure 3.9. (a) UV-Vis absorbance spectra and (b) corresponding curve of absorbance (at 652 nm) vs. time curve showing catalytic conversion of TMB to Oxy-TMB by GBR (100 µL, 1 mg/mL) added onto fabricated system [(H₂O₂ (200 µL, 1 mM), TMB (50 µL, 1 mM) in 400 µL phosphate buffer solution of pH 4.48].

3.3.4 Sensor fabrication

Considering all these, we have fabricated a sensor combining TMB (50 µL, 1 mM), H₂O₂ (200 µL, 1 mM) and GBR (100 µL, 1 mg/mL) in 400 µL phosphate buffer of pH 4.48, which takes ~30 min to complete the quantitative conversion of TMB into Oxy-TMB.

It is to be noted here that the UV-Vis study (intensity at 652 nm corresponding to the TMB-Oxy) evidently reveals that GBR can itself oxidise TMB to ~ 24.0 % [with respect to that of the standard sensor solution: GBR (100 µL, 1 mg/mL), TMB (50 µL, 1 mM) and H₂O₂ (200 µL, 1 mM), in phosphate Buffer (400 µL, pH 4.48) kept 1h in dark to reach equilibrium] and this extent of oxidation is almost remained constant even after 10 min and there is no change afterwards (Figure 3.10). In the standard solution, maintained at equilibrium, the observed intensity of bluish green color will be the same and it is mainly due to the added H₂O₂, and the remaining constant value is due to GBR. Now, concentration of analytes can be detected only by the gradual proportional change in the intensity of the bluish green colour of the fabricated sensor. So, there will be no problem in the control of background.
3.3.5 Sensing ability of fabricated sensor towards various analytes

In order to evaluate sensing ability towards specific anions and cations, different analytes (150 µL, 1 mg/mL) have been added to the fabricated standard sensor solution [GBR (100 µL, 1 mg/mL), TMB (50 µL, 1 mM) and H$_2$O$_2$ (200 µL, 1 mM) in phosphate buffer (400 µL, pH 4.48) and kept for 1 h in dark to reach equilibrium] (Image 3.3) and monitored the change in absorbance spectra at 652 nm for $\frac{1}{2}$ h [Figures 3.11(a)-(b)]. Out of all anions (each sodium salt) studied, remarkable changes are visibly observed for iodide (to dark blue), nitrite (to dark yellow) and sulphide anions (to colourless) with respect to standard solution leaving other anions ineffective. However, time required to observe considerable change is minimum ($\sim$ 2 min) for $S^{2-}$ with respect to $\Gamma$ ($\sim$ 30 min) and NO$_2^-$ ($\sim$ 10 min) [Figure 3.12(a)-(c)]. Moreover, detection in lower (0.04 – 1 mmol) concentration range is observed only for $S^{2-}$. Nevertheless, the detection of $S^{2-}$ is relatively independent of cation types [Figure 3.9(b)]. So, the duration of sensing and detection limit by $\Gamma$ and NO$_2^-$ are significantly poor (Figure 12a-c) in our proposed and fabricated sensor.
**Image 3.3.** Showing visual sensing ability of fabricated sensors [GBR (100 µL, 1mg/mL), TMB (50 µL, 1 mM) and H$_2$O$_2$ (200 µL, 1 mM) in Phosphate Buffer (400 µL, pH 4.48) kept 1 h in dark to reach equilibrium] towards various analytes (anion and cation, 150 µL, 1mg/mL).

**Figure 3.11.** Graphs showing sensing ability of fabricated sensors [(GBR (100 µL, 1mg/mL), TMB (50 µL, 1 mM) and H$_2$O$_2$ (200 µL, 1 mM), in Phosphate Buffer (400 µL, pH 4.48) kept 1h in dark to reach equilibrium)] towards various analytes (150 µL, 1mg/mL): (a) anions and (b) cations.
Figure 3.12. Detection limit and duration of sensing by (a) \( \Gamma \) (b) \( \text{NO}^2^- \) and (c) \( S^2^- \) (150 µL, 1mg/mL) using fabricated system [GBR (100 µL, 1mg/mL), TMB (50 µL, 1 mM) and \( \text{H}_2\text{O}_2 \) (200 µL, 1 mM), in Phosphate Buffer (400 µL, pH 4.48) kept 1h in dark to reach equilibrium.]

3.3.6 Probable mechanism of Sulphide ion sensing

Therefore, further studies are focused on sulphide ion sensing. The following mechanism is proposed for the detection of sulphide ion by the fabricated sensor as shown in Scheme 3.2. \( S^2^- \) does not inhibit the activity of peroxidase mimetic material. It competes with TMB for common \( \text{H}_2\text{O}_2 \) and both the reactions (reactions between TMB and \( \text{H}_2\text{O}_2 \) or, between \( S^2^- \) and \( \text{H}_2\text{O}_2 \)) are catalysed by GBR peroxidase mimetic material.\(^{52-53}\) The competition generally triumphs over by more reactive \( S^2^- \) and major products formed from oxidation of \( S^2^- \) by \( \text{H}_2\text{O}_2 \) are \( S^0 \), \( \text{SO}_3^{2-} \), \( S_2\text{O}_3^{2-} \) and \( \text{SO}_4^{2-} \) \(^{52-53}\) which favour the conversion of OxyTMB to TMB by releasing excess electron to the system.
3.3.7 Sulphide ion detection

To determine the linearity range and limit of detection of $S^{2-}$ for the fabricated sensor, different concentrations (0.04 mM - 1.00 mM) of Na$_2$S (150 µL each) have been added to the standard fabricated sensor solution simultaneously and monitored via UV-Vis absorption study at 652 nm. The change in absorbance with respect to standard solution has been shown in Image 3.4 and Figure 3.13, which show that the bleaching (OxyTMB to TMB) increases with increase in concentration. The absorbance change ($\Delta A$) is the change in absorbance with respect to the standard solution shown in Figure 3.14(a). Using these results, a calibration curve for $S^{2-}$ has been obtained having linear correlation in the range of 40 - 400 µM, with an estimated limit of detection (LOD) of 25.3 µM as shown in the Figure 3.14(b). Here, LOD has been calculated according to the formula: LOD = 3 (SD/k) where, SD is the standard deviation of the absorbance intensity at 652 nm in the absence of $S^{2-}$ ion and k is the slope of the calibration curve.$^{53-54}$

Image 3.4. Visual response of different concentration of sulphide ion (150 µL, 0.04 -1.00 mM) towards fabricated sensors [(GBR (100 µL, 1mg/mL), TMB (50 µL, 1 mM) and H$_2$O$_2$ (200 µL, 1 mM), in Phosphate Buffer (400 µL, $pH$ 4.48) kept 1h to reach equilibrium].
Figure 3.13. UV-Vis Absorbance spectra at 652 nm showing response of different concentrations of $S^{2-}$ (150 $\mu$L, 0.04 -1.00 mM) towards fabricated sensor [GBR (100 $\mu$L, 1mg/mL), TMB (50 $\mu$L, 1 mM) and $H_2O_2$ (200 $\mu$L, 1 mM), in Phosphate Buffer (400 $\mu$L, pH 4.48) kept 1h in dark to reach equilibrium]

Figure 3.14. (a) curve showing absorbance changes at 652 nm on adding $S^{2-}$ ion (150 $\mu$L, 0.04 -1.00 mM) and (b) corresponding calibration curve of $S^{2-}$ ion using the fabricated sensor [GBR (100 $\mu$L, 1mg/mL), TMB (50 $\mu$L, 1 mM) and $H_2O_2$ (200 $\mu$L, 1 mM), in Phosphate Buffer (400 $\mu$L, pH 4.48) kept 1h in dark to reach equilibrium.
3.3.8 Interference study and Real sample analysis

Further, we have analysed the effect of mixture of all salts that have been studied previously upon the detection limit of sulphide ions. The effect has been minor (observed LOD = 32.4 μM) in the case of lower ion concentration of all (concentration of each ions has been kept at 30 μg/mL). On the other hand, it has been affected considerably (observed LOD = 395 μM) at higher concentration of mixtures (concentration of each ions has been kept at 150 μg/mL) [Figure 3.15(b)]. To check the application of the developed sensor, we have determined the concentration of S\(^2\)- in water samples collected from two different origins: i) Ganga river water and ii) sewage water. 150 μL of each water samples has been added to the fabricated sensor and the absorbance changes are monitored as shown in Figure 3.16. S\(^2\)- concentration in water samples is evaluated using calibration curve [Figure 3.14(b)]. The observed concentrations are ~ 0.06 mM for Ganga water, and 0.155 mM for sewage water. The higher concentration of S\(^2\)- found in sewage water is expected as biodegradation of pollutants in sewage leads to the production of sulphide in large quantity. Results of standard addition experiments (Table 3.2) also support the suitability of this method for the quantification of S\(^2\)- ions in water samples. A comparison of different graphene-based sensors for sulphide ion detection reported in the literature has been tabulated in Table 3.3. The present work is the first report of the colorimetric method for S\(^2\)- ion estimation in water samples using GBR and this method is superior to the earlier reported fluorimetric method using iron hydroxide/oxide on reduced graphene oxide system.
Figure 3.15. Calibration curve of $S^{2-}$ ion using the fabricated sensor [GBR (100 $\mu$L, 1mg/mL), TMB (50 $\mu$L, 1 mM) and $H_2O_2$ (200 $\mu$L, 1 mM), in Phosphate Buffer (400 $\mu$L, pH 4.48) kept 1h in dark to reach equilibrium] containing mixture of all ions (a) each ions concentration 30 $\mu$g /mL (b) each ions concentration 150 $\mu$g /mL.

Figure 3.16. $S^{2-}$ detection in water samples (150 $\mu$L) collected from different origins (Ganga water and sewage water) using our fabricated sensor [Standard - GBR (100 $\mu$L, 1mg/mL), TMB (50 $\mu$L, 1 mM) and $H_2O_2$ (200 $\mu$L, 1 mM), in phosphate buffer (400 $\mu$L, pH 4.48)].
Table 3.2. Validation of the developed strategy (S\textsuperscript{2-} ion detection) by standard addition experiment using deionized water (Each set has been repeated three times).

[Known concentrations of freshly prepared S\textsuperscript{2-} ion (0.015, 0.050, 0.150, 0.200, and 0.250 mM, 150 µL) have been added to the sensor solution [GBR (100 µL, 1mg/mL), TMB (50 µL, 1 mM) and H\textsubscript{2}O\textsubscript{2} (200 µL, 1 mM), in Phosphate Buffer (400 µL, pH 4.48) kept 1h in dark to reach equilibrium] and the absorbance are monitored at 652 nm].

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>S\textsuperscript{2-} spiked (mM)</th>
<th>S\textsuperscript{2-} measured (mM)</th>
<th>Recovery* (%)</th>
<th>Relative Standard* Deviation (%)</th>
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<tr>
<td>1</td>
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<td>2</td>
<td>0.050</td>
<td>0.032</td>
<td>66</td>
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<tr>
<td>3</td>
<td>0.100</td>
<td>0.111</td>
<td>111</td>
<td>1.63</td>
</tr>
<tr>
<td>4</td>
<td>0.150</td>
<td>0.154</td>
<td>103</td>
<td>1.70</td>
</tr>
<tr>
<td>5</td>
<td>0.200</td>
<td>0.207</td>
<td>104</td>
<td>1.09</td>
</tr>
<tr>
<td>6</td>
<td>0.250</td>
<td>0.276</td>
<td>110</td>
<td>0.72</td>
</tr>
</tbody>
</table>

\*Note: (i) Recovery = (standard concentration/ determined concentration) x 100 (ii) RSD = (Standard deviation/average value) x 100.

Table 3.3. Comparison of different graphene-based sensors for sulphide estimation reported in the literature.

<table>
<thead>
<tr>
<th>Sensing Element</th>
<th>Method</th>
<th>Linear Range (µM)</th>
<th>Limit of Detection (µM)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced graphene oxide</td>
<td>Cyclic Voltametry</td>
<td>5-7400</td>
<td>4.2</td>
<td>38</td>
</tr>
<tr>
<td>Iron hydroxide/oxide on reduced graphene oxide</td>
<td>Fluorometry</td>
<td>0.1-1.5</td>
<td>0.050</td>
<td>39</td>
</tr>
<tr>
<td>Alizarin-reduced graphene nanosheets</td>
<td>Cyclic Voltametry</td>
<td>6-3280</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Hemin functionalised reduced graphene oxide</td>
<td>Amperometric</td>
<td>2-212</td>
<td>1.3</td>
<td>41</td>
</tr>
<tr>
<td>Brominated Graphene (GBR)</td>
<td>Colorimetry</td>
<td>40 - 400</td>
<td>25.3</td>
<td>This work</td>
</tr>
</tbody>
</table>

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3.3.9 Fabrication of paper strip sensor

In order to explore the efficacy as paper strip sensor, the same has been fabricated by Axiva ashless filter paper (no. 420 R pore size - 5 µm), soaked with standard fabricated sensor solution and dried for 10 min. Then, a drop of $S^{2-}$ (10 mM, 2 µL) solution has been added on to the detection zone. Instantly, the standard blue colour of the detection zone has turned colourless in the presence of $S^{2-}$ as shown in Image 3.5.

![Image 3.5. Paper strip sensor showing successful $S^{2-}$ detection](image)

Image 3.5. Paper strip sensor showing successful $S^{2-}$ detection

We have also studied the effect of the interference of other ions, concentration of $S^{2-}$ ions and fabrication time of sensor onto the activity of paper strip sensor (Images 3.6 - 3.8). The results have showed that paper strip sensor (i) is suitable to detect $S^{2-}$ ion even in the presence of higher concentrations of other ions (containing each ion of 150 µg/mL concentration) (Image 3.6); (ii) is not suitable to detect below 2 mM $S^{2-}$ ion concentration (Image 3.7) and (iii) has shown activity even after 40 min of fabrication time (Image 3.8). Further standardization may improve its sensing activity. So, the cost effective GBR-based fabricated paper strip sensor can also be useful for quick and reliable detection of $S^{2-}$.

![Image 3.6. Activity of paper sensor in presence and absence of mixture of all other ions](image)

Image 3.6. Activity of paper sensor in presence and absence of mixture of all other ions

[2 µL of (i) pure $S^{2-}$ ion (10 mM), (ii) mixture containing $S^{2-}$ ion (10 mM, 2 µL) and
concentrated mixture of other ions (2 μL of stock solutions containing each ion of 150 μg/mL concentration), (iii) concentrated mixture of all other ions (containing each ion of 150 μg/mL concentration) and (iv) diluted mixture of all other ions (containing each ion of 30 μg/mL concentration) have been added on to the detection zone]

**Figure Image 3.7.** Activity of paper sensor using different concentrations of S²⁻ ions (2 μL, 0.4 - 10.00 mM).

**Image 3.8.** Activity of paper sensor at varying fabrication time (min) (using 2 μL, S²⁻ ion conc., 10 mM).
3.4 Conclusions

GBR having ~ 3% bromine content has shown novel peroxidase mimetic activity towards TMB (50 µL, 1 mM) in the presence of H₂O₂. pH dependence study has revealed best mimetic activity at pH 4.48. Time dependence study has exposed the requirement of at least ~ 30 min for the quantitative conversion of TMB into its oxidised form OxyTMB. A sensor combining TMB (50 µL, 1 mM), H₂O₂ (200 µL, 1 mM) and GBR (100 µL, 1 mg/mL) in 400 µL phosphate buffer of pH 4.48 has been fabricated for the sensing of different analytes. Short duration of sensing (~ 2 min) and detection range (0.04 – 1 mM) are selectively observed for S²⁻ ion. Calibration curve for S²⁻ ion estimation has shown linearity in 40 – 400 µM range with the limit of detection at 25.3 µM. In the mixture of others ions at comparatively higher concentration (30 µg/mL concentration of each ions) very minor change in LOD has been observed, however, at very much higher concentration (150 µg/mL concentration of each ions), the LOD is considerably affected as expected. Use of our fabricated sensor and the calibration curve for S²⁻ has shown satisfactory results for water samples collected from different origins. We have also successfully validated the S²⁻ ion detection by standard addition experiment. Initial study of paper strips sensor fabricated by absorbing sensor solution has also shown equally successful for the detection of S²⁻ ion. Activity of paper strip sensor has not been affected by the presence of all other ions and remained active for long duration with minor demerit that sulphide ions at lower concentration (below 2 mM [S²⁻]) cannot be detected. It needs more standardization as adsorption tendency significantly controlled the activity of paper sensor. Thus, we have successfully developed a novel peroxidase mimetic material GBR without loading any nanoparticles. Using this, we have fabricated a facile and rapid colorimetric method for the detection of dangerous sulphide ion.
3.5 Reference


