Detection of GlycatedHemoglobin using 3-AminoPhenylboronic acid modified Graphene Oxide

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Abstract—**This paper presents the chemical synthesis of 3-Aminophenylboronic acid (APBA) modified graphene oxide (GO) and its application to the electrochemical detection of glycated hemoglobin (GHb). The compound (GO-APBA) was synthesized by forming an amide linkage between the amino group (-NH_2) of APBA and the carboxylic group (-COOH) of GO. The compound was characterized using IR spectroscopy. Detection of GHb was carried out using Electrochemical Impedance Spectroscopic (EIS) measurements with GO-APBA modified glassy carbon electrode as the working electrode.**

Keywords- Graphene Oxide; 3-Aminophenylboronic acid; Glycated Hemoglobin; IR Spectroscopy, Electrochemical Impedance Spectroscopy.

I. INTRODUCTION

Affinity of glycated proteins and saccharides towards boronic acid compounds is exploited quite extensively in designing sensors [1-6]. The cis-diol bonds of these compounds interact with boronic acids to form boronate esters [7]. D-glucose, the primordial saccharide in blood, undergoes a slow non-enzymatic irreversible reaction with hemoglobin (Hb) to form glycated hemoglobin. The reaction happens throughout the life cycle time of erythrocyte (RBC), which is 120 days. Measuring the concentration of glycated hemoglobin as a percentage of total hemoglobin gives the average value of glucose present in the blood over 120 days. The clinical reference range is between 4 and 20% with 4-6% being considered as normal. Glycated hemoglobin test is considered as gold standard for the long term monitoring of diabetes [8]. A disposable, low cost biosensor will be handy in achieving glycemic control, thereby reducing the risk of cardiovascular, retinal, renal complications that occur due to improper control of glycemia.

The present clinical methods of measuring GHb include immunoassay [9], ion-exchange chromatography [9], electrophoresis [10], boronate affinity chromatography [11], and high pressure liquid chromatography along with electrospray ionization mass spectrometry [12]. The equipments used in these techniques are bulky and the procedures are time consuming and are not cost effective. Frank Frantzen et al., have reported a table top GHb measurement unit based on boronate affinity and colorimetry [13]. This is also not cost effective due to the use of optics. Electroanalytical techniques have a unique advantage of having electrical signals as both input and output. A disposable sensor with a handheld electronic device similar to that of a glucometer can be easily achieved using this sensing methodology.

Liu et al., have used ferroceneboronic acid to detect GHb[5]. The whole process is based on adsorption of Hb/GHb onto Zirconia nanoparticles and is time consuming. Son et al., have fabricated a lab on chip for the determination of GHb [6]. They used m-aminophenyl boronic acid agarose beads to bind GHb. The chip involves a complex fabrication process. Park et al., formed a self assembled monolayer of thiophene-3-boronic acid to detect GHb. The substrate used was gold which is not suited for low cost applications. Carbon-based materials are biocompatible and cost effective. They have been extensively used in various electroanalytical techniques to detect bio-molecules [15]. Among these, graphene has received much attention due to its extraordinary electrical, thermal and mechanical properties [16-17]. Graphene and its oxidized form, graphene oxide (GO) have been extensively explored for sensor applications [18-21]. GO has hydroxyl and epoxy functional groups on the hexagonal network of carbon atoms with carboxyl groups at the edges. The presence of carboxylic group provides the possibility of amide bond formation with an amine group. In this work 3-aminophenylboronic acid (APBA) is chemically attached to carboxyl groups of GO as shown in the Fig. 1 and used in the detection of GHb.

II. EXPERIMENTAL

A. Chemicals

Natural graphite was obtained from Stratmin graphite co., USA., N-(3-Dimethylaminopropyl)-N`-ethylcarbodiimide hydrochloride (EDC), 3-AminoPhenylboronic acid (APBA) were procured from Sigma, USA. All other chemicals were

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procured from Merck, India and used without further purification. All the solutions and buffers were prepared in Millipore water with 18MΩ-cm resistivity.

B. Apparatus and measurements

FTIR measurements were carried using Perkin Elmer FT-IR Spectrometer. UV-Vis spectroscopy was carried out using Perkin Elmer 35 UV-Vis spectrometer. EIS measurements were carried out using electrochemical workstation CHI660C, CH instruments, USA. The electrochemical system used consists of modified glassy carbon electrode as the working electrode, platinum foil as the counter electrode, standard calomel electrode (SCE) as the reference electrode and 0.15 mM phosphate buffer (pH 8.0) as the supporting electrolyte.

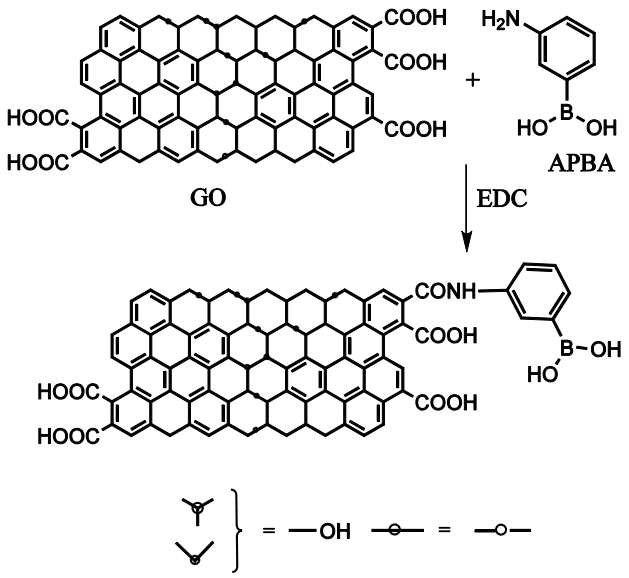

Fig. 1: Schematic representation of amide linkage formation enabled by coupling reagent EDC.

C. Synthesis

Graphene oxide was synthesized from exfoliated graphite using modified Hummers method [22]. Chemical modification of GO with APBA was carried out as follows. Required amount of GO and EDC were added to DI water and was stirred continuously for 36 hrs. EDC is a coupling reagent which aids in the formation of amide bond. After the stirring, of APBA was added to the mixture and stirred at room temperature for another 24 hrs. The suspension was filtered and washed several times with water and ethanol, to remove any physically adsorbed APBA. The material was then dried in vacuum using silicagel. The product was then characterized using IR spectroscopy. For the EIS measurements, the compound was dispersed in DI water and drop coated onto a cleanly polished glassy carbon electrode.

D. Electrochemical impedance spectroscopy:

A glassy carbon electrode (GCE) (3mm dia.) was polished with 0.05 µm alumina and washed thoroughly with DI water. Depending on the experiment GO or GO-APBA dispersion was drop cast on the electrode and was allowed to dry for 2 hours. Impedance measurements were carried out in 0.15 mM phosphate buffer (pH 8.0, 3mL volume) containing 2.5 mM each of $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$. The modified GCE was used as working electrode. A potential of 0.2V was applied between the working electrode and the SCE. The impedance offered by the electrode for the electron transfer was measured between the working and counter electrodes. Freshly prepared hemoglobin of known concentration was added in 30 µl steps to this solution. Nyquist plots and the extraction of electrical parameters were done using the software provided by the CH instruments.

E. Hemoglobin preparation

Ethylenediaminetetraacetic acid (EDTA) coated blood sample pool of diabetic patients were collected from Health Centre of Indian Institute of Science. Plasma and leukocytes were removed by centrifuging the sample at 5000 rpm for 5 min. The supernatant was discarded. The remaining erythrocyte cells were washed three times with 0.9% NaCl. The cells were then lysed by adding 8 volume of ice cold DI water to one volume of cell solution. After lysis, the mixture was centrifuged at 12000 rpm for 10 min. The cell debris settles at the bottom of the centrifuge tube. The supernatant was collected, labeled and stored at -20°C for further use.

UV-Visible spectroscopy was carried to find out the concentration of hemoglobin. Based on the absorbance at 541nm (Fig.2) and Beer-Lambart's law, the concentration of hemoglobin was found to be 240 µM [24]. Based on the measurements carried in the health centre, the glycated hemoglobin was found out be ~8% of the total hemoglobin.

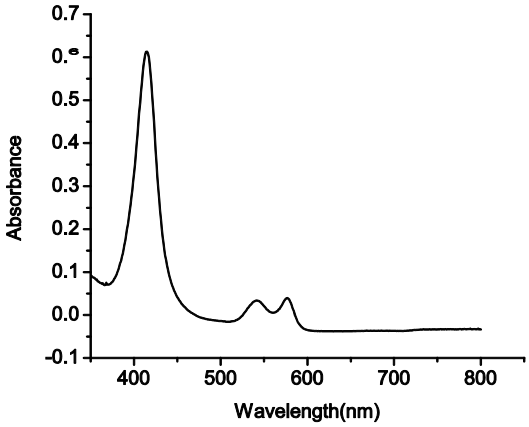

Fig.2: UV-Visible Spectrum of Hb

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III. RESULTS AND DISCUSSION

A. IR spectroscopy

IR spectroscopy was carried out to confirm the formation of amide bond between the amine group of APBA and carboxylic group of GO. Fig 3 Shows the IR spectra of all the compounds.

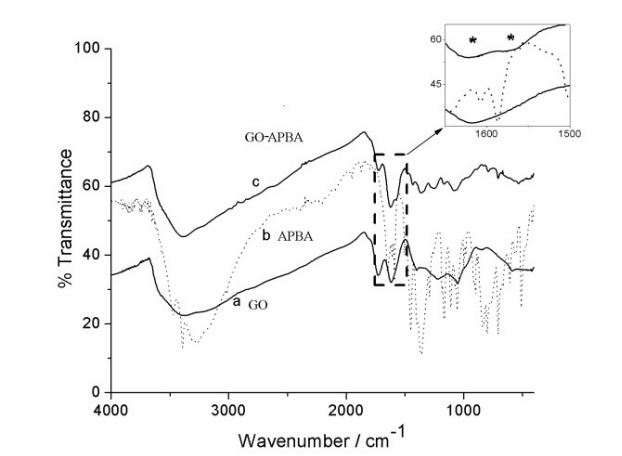

Fig. 3: IR spectrum of (a) GO , (b) APBA (c) GO-APBA; (Inset: Expanded region 1650 cm^{-1} to 1500 cm^{-1} The amide bands are labeled with a '*')

The region of interest is between 1640 cm^{-1} and 1580 cm^{-1}. In the case of GO, the band at 1617 cm^{-1} corresponds to aromatic C=C stretching [24]. In APBA the bands at 1608 cm^{-1} and 1586 cm^{-1} correspond to C=C stretching as well as N-H stretching[24,25]. In the case of the synthesized compound new bands appearing at 1625 cm^{-1} and 1580 cm^{-1} correspond to amide-I band and amide-II stretching respectively [25]. This confirms the covalent modification of GO with APBA. The other bands also match with the existing literature values [24, 25, 26].

B. Electrochemical impedance spectroscopy- Detection of GHb

The interaction of GHb with GO-APBA compound is shown in Fig. 4. When GO-APBA modified glassy carbon is used as working electrode GHb gets immobilized on to the electrode surface through affinity of cis-diol bonds of glucose to boronic acid moiety. The chemical adsorption of GHb inhibits the electron transfer rate of the redox couple $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ thereby increasing the charge transfer resistance (R_{ct}). The Nyquist plots are shown in Fig. 5. The diameter of the semicircle which is R_{ct}, increases with increase in the concentration of Hb. In order to eliminate the possible reason that physisorption may play a role in increase R_{ct}, an experiment was perfomed using GO modified GCE. The corresponding Nyquist plots are shown in the Fig.6. The variation in R_{ct} is small and is not systematic in this case. This proves that the increase in the charge transfer resistance is only due to the chemisorption of GHb onto the electrode surface. Hence this method can be used for detecting GHb.

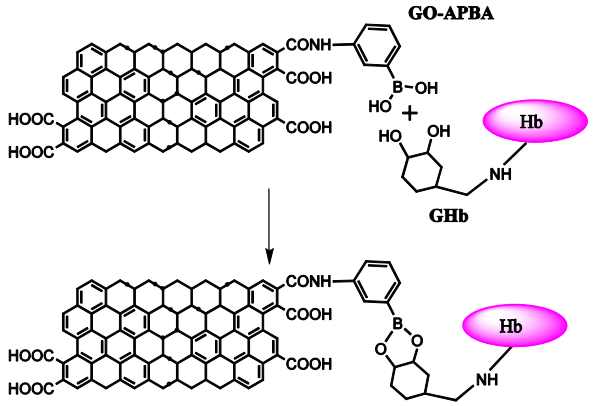

Fig.4: Schematic representation of GO-APBA and GHb interaction

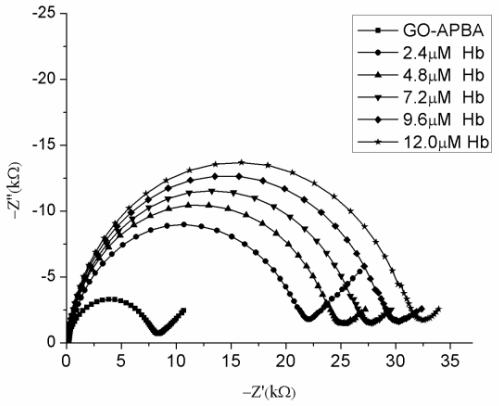

Fig. 5: Impedance data obtained for GO-APBA modified GCE before and after the addition of Hb

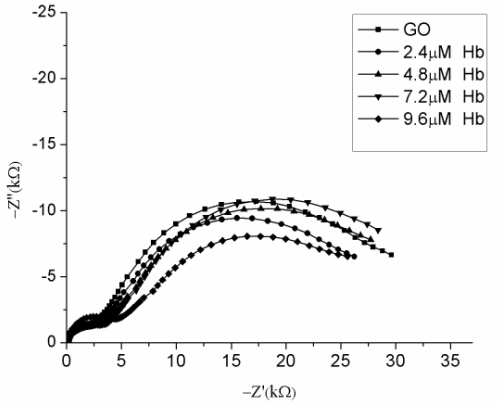

Fig. 6: Impedance data obtained for GO modified GCE before and after the addition of Hb

C. Linearity:

R_{ct} is calculated from Nyquist plots by fitting the curve with parameters in the Randles circuit shown in Fig.7 [27]. R_{Ω} is the solution resistance and C_{dl} is the double layer capacitance. The values of R_{ct} with Hb were normalized to R_{ct} without Hb and are denoted by R_{norm}. The variation of R_{norm}

with respect to the concentration of Hb is shown in Fig.8 and it is linear. The concentration of Hb used in the present studies are in μM range which implies that the concentration of GHb is in the range of nM (8% of Hb).

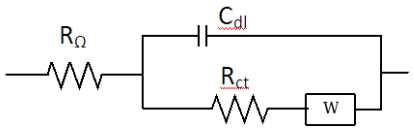

Fig.7: Randles Circuit

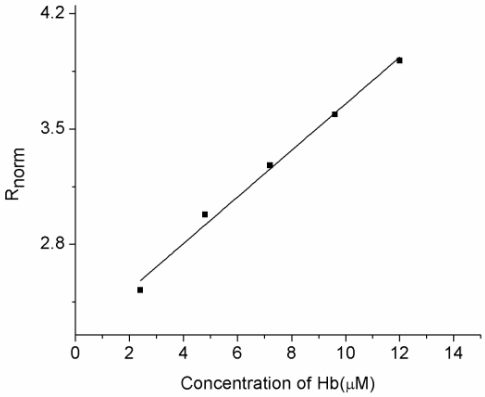

Fig.8: Variation of normalized charge transfer resistance with respect to concentration of Hb

IV. CONCLUSIONS

Chemical synthesis of APBA modified GO was carried out and was characterized with IR spectroscopy. GCE surface modified with GO-APBA complex was used to detect GHb using EIS which is compatible with low cost diagnostics.

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