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An Enhanced Amperometric HER2 Immunosensor for Early Detection of Breast Cancer

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ABSTRACT

Human epithelium receptor 2(HER2) is the most important breast cancer biomarker, whose increased level in blood is very important for detection of breast cancer at early stage. We describe herein the enhancement of an HER2 antibody based amperometric immunosensor for detection of breast cancer, by graphene (Gr) sheets. The working electrode i.e. anti-HER2/Gr-sheets/PGE was characterized using cyclic voltammetry, scanning electron microscopy and electrochemical impedance spectra. The immunosensor had optimum activity at pH 7.0, 25°C, and antibody concentration of 18 µg/ml. It exhibited a linearity in the concentration range 1 fg/ml to 20 ng/ml, with a detection limit (LOD) of 0.85fg/ml. The immunosensor was employed to determine HER2 concentrations in sera of apparently healthy women and those women suffering from breast cancer at early stage. It was increased significantly ($p < 0.1$) in cancer patients compared to apparently healthy persons. The sensor lost 41% of its original activity within 75 days of its regular use, when stored dry at 4°C.

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Introduction

Breast cancer is extremely common in females of all ages and is the second leading cause of death worldwide. Breast cancer accounts for 30% or so of all cancers in women. Magnetic resonance imaging (MRI), mammography, sonography, thermography, biopsy, and molecular breast imaging are some of the methods that can be used to diagnose breast cancer [1]. In addition to these methods, breast cancer has also been diagnosed using biomarker-based expression methods such as immune histochemistry (IHC), radio immunoassay (RIA), and enzyme linked immunosorbent assay (ELISA) [2-4]. Even though they are extensively utilised, these approaches have some drawbacks, including low sensitivity, time commitment, frequent false positive outcomes, need for skilled personnel, and high cost. Thus, one of the main areas of study interest for scientists is the creation of sensitive and non-invasive cancer treatment methods. Point-of-care (POC) diagnostic tools offer a superior option in terms of quick, accurate, and identification of cancer indicators [5]. Given the rising instances of cancer being detected globally and the rising account of deaths from late diagnosis, biosensors are crucial for the early identification of cancer [6].

The most effective techniques for early cancer identification and accurate pre-treatment staging are likely cancer biomarkers [7].

One of the most important breast cancer biomarkers is human epithelium receptor 2(HER2), whose levels in blood are moderate but essential for early cancer detection and for initiating the proper course of treatment. The primary function of HER2 is to support excessive and out-of-control cell proliferation and cancer [8]. The HER2 gene functions as a receptor on the cell surface, and when more signals promoting cell growth and division are taken in, more HER2 proteins are produced [9]. HER2 is over-expressed in 25–30% of breast cancers, one of the most prevalent malignant tumour types in women. It is crucial to develop a rapid method that can detect low levels of the HER2 biomarker and lead to an early cancer diagnosis in order to improve survival rates, as well as to reduce costs and provide accurate disease prognosis [10].

It has been reported that several immunosensors can assess HER2 levels. PEMS (Piezo electric microcantilever sensors) were used by Loo et al. to create an immunosensor for the detection of HER2 [11]. These sensors had HER2 detection limits of >2 ng/mL. The gold nanoelectrode ensembles (NEE) were used to create another HER2 immunosensor [12]. In order to create an electrochemical immunosensor based on a sandwich assay, Lah et al. (2019) used lead sulphide quantum dots that were also coupled to a secondary HER antibody [13]. A microneedle array of high-density silicon was used by Dervisevic et al. (2021) to create an electrochemical immunosensor with a detection limit of 4.8 ng/ml and a linear range of 10-250 ng/ml for discovering breast cancer biomarker HER2 [1]. Electrochemical immunosensors based on a variety

of nanomaterials have also been demonstrated to detect HER2. Gold nanoparticles (AuNPs) were used as a supporting matrix by Li et al. (2018) to create an immunosensor that immobilised polycytosine DNA sequences [14]. Using an AuNPs-coated electrode with 3-mercaptopropionic acid and ss-DNA aptamer created an impedance aptasensor [15]. Ehzari et al used multi-walled carbon nano-tubes (MWCNT), Fe₃O₄NPs, and methacrylic acid (MAA) to create a label-free electrochemical immunosensor [16].

In addition to being affordable, carbon-based electrodes like pencil graphite electrodes (PGE) have exceptional electrochemical properties that set them apart from other electrodes like glassy carbon (GC), gold electrodes and platinum. PGE is a useful substrate for biosensor studies due to a number of factors, including its decreased baseline current flow, higher sensitivity, faster conductivity, affordable price, being disposable, flexible electroactive surface area, ease of use, reproducibility, and commercial accessibility [17]. We have reported HER2 targeted amperometric immunosensor based on pencil graphite electrode (PGE) for detection of breast cancer [18]. Recently we have classified various types of HER2 targeted biosensors depending on types techniques used, electrochemical, piezoelectric and optical fluorescence [19]. Because graphene sheets (GrS) have a high surface-to-volume ratio, superior electrical conductivity, and a tiny band gap that is responsible for electrical sensitivity, PGE has been adorned with them to create more sensitive biosensors [20]. Hence in the present work, GrS were electrodeposited onto PGE before immobilizing HER2 antibody to generate the improved/enhanced immunosensor.

Materials and Methods

Materials

A 2-millimetre diameter 6B graphite lead was acquired from a local store. Both the HER2 antigen and the HER2 antibody were purchased from Sigma Aldrich in the USA. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich, USA, and sera samples of female breast cancer patients were obtained from Pt. Bhagwat Dayal Sharma Post-Graduate Institute of Medical Sciences (PGIMS), Rohtak. For the creation of various dilutions and chemicals, Nirma Ltd., India (Nirlife Healthcare) supplied sterile pyrogen-free water was used.

Major Instruments

Potentiostat/Galvanostat, made by Eco Chemie Netherland (Make: Autolab, model: AUT83785), was used combined with NOVA 1.4 software to measure the Cyclic Voltammograms. In order to investigate the electrode's surface, scanning electron microscopy (SEM; Zeiss EV040, USA) had been used. For the investigation of electrochemical impedance spectra (EIS) in the frequency range of 0.1 Hertz to 1x10⁵ Hertz, frequency response analysis (FRA) software was employed.

Preparation of HER2/Gr- Sheets/PGE

Utilising a silica/alumina slurry, the outer surface area of the pencil graphite electrode (PGE) was cleared before being rinsed with distilled water. Following a 20-minute soak in piranha solution, it was cleared with a slurry of silica or alumina, second time. PGE had been immersed in ethanol for 5–6 hours, after which the absorbed particles were cleared out by employing sonication. Hummer's method was used to create the Gr-sheets with a few modifications. A 6B pencil's graphene stick was crushed in a pestle and mortar to create the powdered form. In a 1L conical flask, sodium nitrate (NaNO₃) and graphite powder (1g) were blended

properly after addition. In cold circumstances at 4°C, 75 ml of concentrated H₂SO₄ were added dropwise. 3g of KMnO₄ flakes were gradually put into this solution while maintaining a 15°C temperature. The flask was constantly stirred for 5–6 days while being wrapped with aluminium foil paper. The flask received 200 ml of 5% H₂SO₄ before being heated to 98 °C in a water bath for two hours. After letting it to cool to 60°C, 30% H₂O₂ (6 ml) was added drop by drop and the mixture was constantly stirred for the next two hours. Centrifuging the suspension for 15 minutes at 10,000 rpm, produced the pellet, which is composed of graphene sheets. The produced graphene sheets were sprayed with a 0.5% H₂O₂ and 5% HCl solution. In order to get the pH of the supernatant to 7.0, it was then rinsed with distilled water. To get the powdered form, the purified graphene sheets were oven dried. Subsequently 1 mg of these Gr-sheets to 25 ml of 0.1 M KOH and the resulting mixture was continuously stirred for 2 hours at a temperature of 91°C. Then, utilising cyclic voltammetry (CV) and 40 iterative polymerization cycles at 0 to 1.2 V at a scan rate of 20 mVs⁻¹ in a potentiostat/galvanostat, these dispersed Gr-sheets were electrodeposited onto PGE. To remove the unattached Gr-sheets, the PGE adorned with graphene sheets was thoroughly rinsed with distilled water. To deliver covalent immobilisation of HER2 onto graphene sheets modified PGE, the electrode was placed into an Antibody-HER2(Anti-HER2) slurry and gently stirred at 4°C for 12 hours. In order to clean the Anti-HER2/Gr- sheets/PG electrode, 50 mM sodium phosphate (SP) buffer (pH 7.4) was used for washing. After which, non-specific active sites were blocked for two hours at 4°C using 3 ml of BSA at a concentration of 10 mg/ml. Phosphate buffer saline (PBS, pH 7.0) was then used to soak the electrode's surface. Subsequently, for surface characterisation, cyclic voltammograms (CVs) of the bare PGE, anti-HER2/Gr-sheets/PGE, and HER2/anti-HER2/Gr-sheets/PGE was monitored. With the usage of a distinct auto lab/ potentiostat/ galvanostat/ cyclic voltmeter, the Anti-HER2/Gr-sheets/PGE, as a working electrode; silver/ silver chloride (Ag/AgCl) as the reference electrode and platinum (Pt) wire as the counter electrode were linked. The reaction mixture for the aforementioned three-electrode setup consisted one millilitre of HER2 antigen solution and 20 ml of 0.05M SP buffer with a pH value of 7.5. The current generated was subsequently monitored at various voltages. SEM and EIS were used for characterization of the electrode.

Optimization of Immunosensor

The effects of pH, incubation duration, temperature, time, and concentration of antigen on the response of the biosensor were taken into consideration when optimising the anti-HER2/Gr-sheets/PG electrode for its operating circumstances. The buffer solution's pH was adjusted from 5.0 to 10.0 to get the ideal pH. Similar to this, the ideal temperature range was investigated by incubating the reaction mixture at various temperatures between 15 and 50 degrees Celsius with a 5-degree gap in a heat-controlled water bath. Immunosensors' time of response was tracked between 2 to 90 seconds. Current measurements were recorded as the amount of HER2 antigen in the reaction buffer changed.

Applications of Immunosensor

One millilitre of blood serum was taken from apparently healthy women and women diagnosed with breast cancer at an early stage at hospital of Pt. Bhagwat Dayal Sharma Post-Graduate Institute of Medical Sciences (PGIMS), Rohtak, Haryana, India. HER2 antigen in sera were detected using the current immunosensor.

Storage Reliability and Working Electrode Regeneration

The performance of the immunosensor was evaluated succeeding 25,50 and 75 days of storage at 4°C in order to assess its stability.

Correlation Analysis

The ELISA technology was employed to conduct correlation investigations. For covering the wells of the microtiter plate with the HER2 antigen, carbonate-bicarbonate buffer (pH 7.4) was employed for 12 to 15 hours at a temperature 4°C and then discarded. PBS was added to the mix. 200 µl of blocking solution was introduced with 5% BSA in PBS to eliminate unspecific proteinaceous binding regions. The next step was to cover it with plastic tape that was adhesive and re-incubate for a period of 12 to 15 hours at 4 degrees Celsius. Anti-HER2, primary monoclonal antibody was attached to each well, and it was let to sit there for around 2 hours at room temperature. The wells were treated with PBS three times. Horseradish peroxidase (HRP) labelled; secondary antibody (anti-mouse) was subsequently immersed in each well for an additional hour at room temperature. PBS was applied to the plate four times. The signals were produced utilising 3,3',5,5'-tetramethylbenzidine (TMB), the substrate of HRP. The signals were recorded using an ELISA reader.

Results and Discussion

Characterization of Anti-HER2/Graphene Sheets/PG Immunosensor

The procedure for the preparation of the anti-HER2/Gr-sheets/PGE is shown in Figure 1. Figure 2A displays the scanning electron microscopy (SEM) images of the bare PGE, graphene sheets/PGE, and anti-HER2/Gr-sheets/PGE. The SEM image shows that there is no adhesion to the surface of the bare electrode; however, graphene fragments can be identified on the Graphene sheets/PG electrode and some globular deposits can be recognized on the anti-HER2/Gr-sheets/PGE. This indicates that antibodies were

incorporated onto modified anti-HER2/Gr-sheets/PG electrode surface.

Electro-chemical impedance spectra (EIS) is an efficient and sensitive technique for demonstrating the change in the electrode's surface's charge transfer resistance. Therefore, an EIS was employed to validate the immune response between the HER2 antigen and anti-HER2/Graphene sheets/PG immunosensor. The Nyquist plot was constructed to represent the surface of anti-HER2/Gr-sheets/PGE and also HER2/anti-HER2/Gr-sheets/PGE in a frequency spectrum of 0.1 Hz-105Hz. Rct value was seen to increase as a result of the HER2 antigen association with the anti-HER2/Gr-sheets/PGE (Figure 2B). Because the link between the surface of electrode and the redox probe has been disintegrated, the resistance has increased. This break in communication between electrons and mass transfer happened as a result of the creation of immune complexes. The antigen-antibody immobilization is validated by the increased resistance. These findings further demonstrate the suitability of the created immunosensor for detecting HER2.

The CVs were analysed in order to confirm the manufacturing of the anti-HER2/Gr- sheets/PG immunosensor. The findings of the CV for the immunosensor in Zobell's solution with a 5 mM molarity, at 20 mV/sec scan rate are shown in Figure 2C. Anti-HER2/Gr- sheets/PGE had a greater recorded current and redox crest than bare electrode. Anti-HER2/Gr-sheets/PGE showed an oxidation current at a potential of 0.37 V. The results indicated a decrease in the redox current peak of the immunological response between antigen (HER2) and antibody (Anti-HER2) when contrasted with the anti-HER2/Gr- sheets/PG immunosensor only. Higher surface resistance of electrode explains this change.

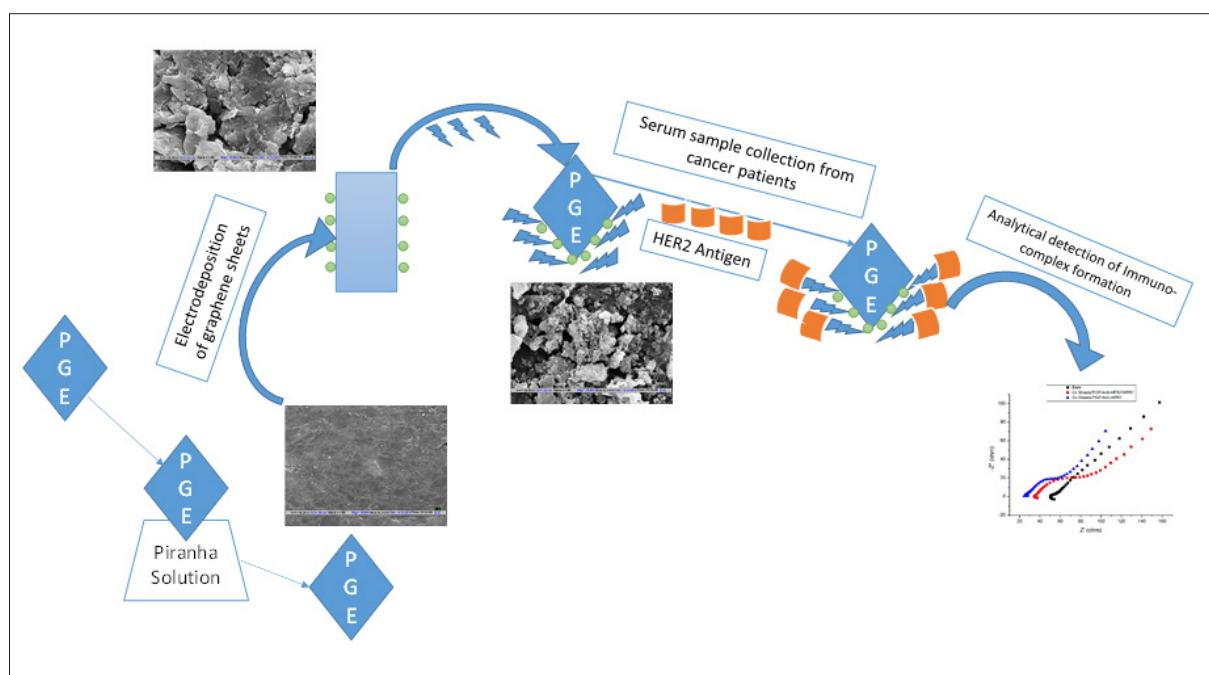


Figure 1: Diagrammatic Representation of Anti-HER2/Graphene Sheets/PG Immunosensor Fabrication and Analytical Detection Process

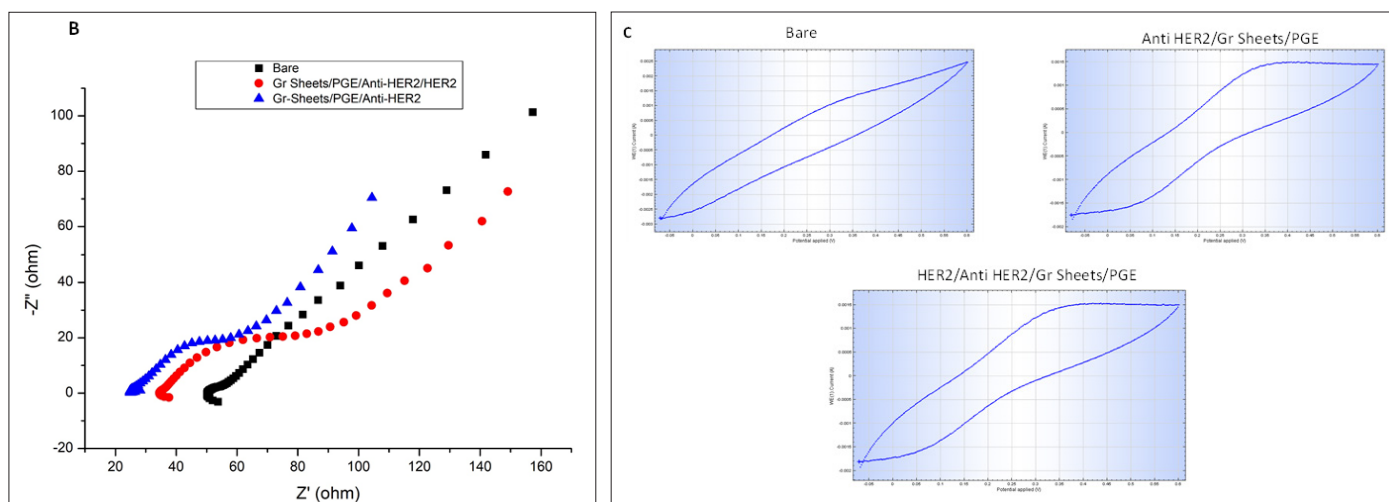
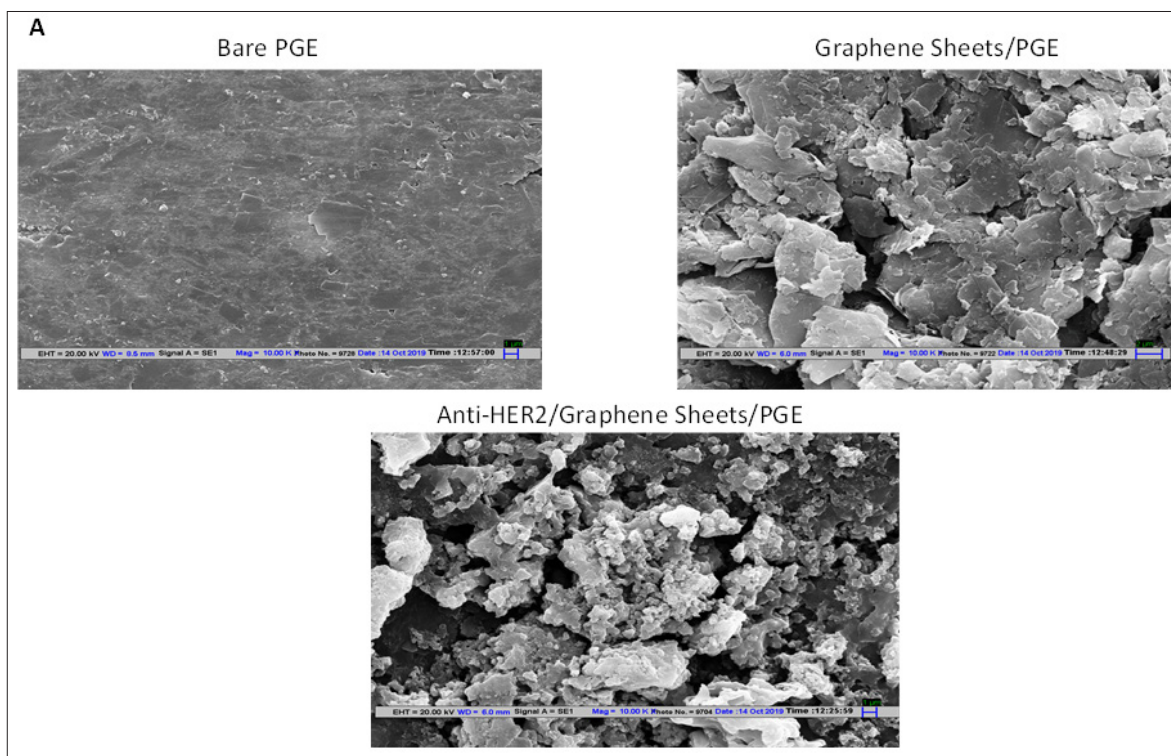


Figure 2: Characterization of Anti-HER2/Graphene Sheets/PG Immunosensor (a) Scanning Electron Micrograph Showing Surfaces of Bare PGE, Graphene Sheets/PGE and Anti-HER2/Graphene Sheets/PGE. (b) Electrochemical Impedance Spectra Showing Changes in Rct Value Before and After Binding of HER2 Antigen with Anti-HER2/Graphene Sheets/PG Immunosensor. (c) Cyclic Voltammograms Showing Variations in Current Before and After Binding of HER2 Antigen with Anti-HER2/Graphene Sheets/PG Immunosensor.

Optimization of Anti-HER2/Graphene Sheets/PG Immunosensor

Antibody concentration, time, pH and temperature were the four key testing variables that were optimized. Analyzing the impact of concentration of antibody ranging from 1 to 30 g/ml, it was shown that the response decreased from 1 to 18 g/ml as a result of persistent immobilization of antibody, and subsequently rose beyond 18 g/ml due to saturated sites of activation. Therefore, 18 g/ml of the optimal antibody concentration was applied. (Figure 3A).

Over the range of 5.0 to 9.0, the pH impacts on immuno-reactions had been optimised. The signal strength declined from pH 5.0 to 7.0. However, starting at pH 7, the signals started to rise. Thus, pH 7.0 was determined to be the ideal pH for the ensuing studies (Figure 3B). This optimised pH value of 7.0 was the same as a previously published biosensor's pH 7.0 [16]. This pH is slightly lower than the HER2/PGE immunosensor (pH 7.5) and pH 7.4 [18, 21].

The biosensor was tested at temperatures ranging between 5 and 35 °C to establish the optimal temperature. The detected immune-reaction responses appeared to be diminishing between 5 and 25 °C, and the signal didn't appear to diminish after 25 °C. This resulted in a determination that the highest interactions between antigen and antibody occurred at 25 °C (Figure 3C). Therefore, all of the trials that follow were carried out at 25 °C, which is equal to that but lower than the 30 °C that reported in ref. [18].

The most efficient experiments for the ongoing immunoreactions of the biosensor's incubation time were run. From 0 to 12 minutes, the biosensor was submerged in Anti-HER2 for a fixed but variable amount of time. Up to the first 10 minutes, there was a decline in the observed responses; after that, the signals remained steady and stable. However, a maximal antigen-antibody interaction was seen within 10 minutes to produce a minimal reaction. So, in the subsequent studies, the incubation time was 10 minutes (Figure 3D), similar to that our earlier report [18].

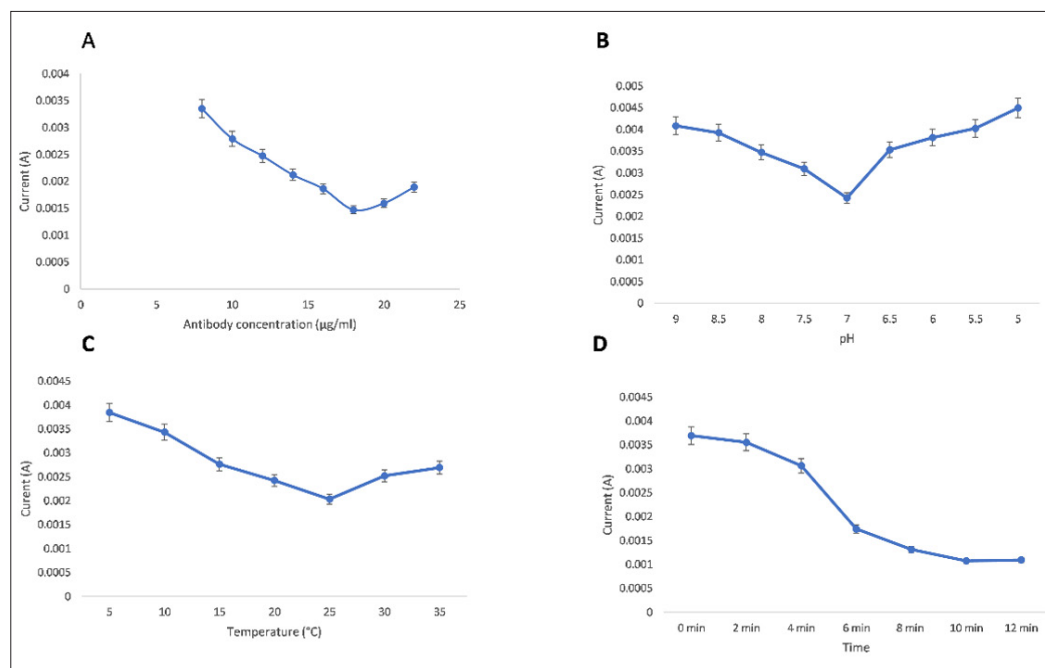


Figure 3: Optimization of Experimental Parameters of Anti-HER2/Graphene Sheets/PG Immunosensor (a) Effect of Antibody Concentrations (b) Effect of Various pH on Immunosensor. (c) Effect of Temperature (d) Effect of Incubation Time.

Evaluation of Analytical Productivity of Anti-HER2/Graphene Sheets/PG Immunosensor

Under the ideal test conditions, the created immunosensor's performance was assessed. Using an ideal range between 1 fg/mL and 20 ng/mL, the substrate concentration for the Anti-HER2/Gr-sheets/PGE biosensor was measured. Anti-HER2/Graphene sheets/PG immunosensor and HER2 were found to be linearly related (Figure 4A). This linear range was bigger than the ranges of 10-250 ng/ml, 15-100 ng/ml, and 0.01-100 ng/ml and but close to the range of 1 pg/ml to 100 ng/ml. Due to the immune response, a decrease in current was seen along with hike in concentration of antigen (HER2). It was discovered that there no immune reaction occurred at a concentration of 20 ng/mL. It was also seen that the detection limit was 0.8 fg/ml, which is smaller than 4.8 ng/ml, 2.5 mg/ml, 4.4 ng/ml, 0.01 ng/ml and 1.0fg/mL [1, 16, 18, 20, 21]. The correlation experiments with the current immune-sensor were conducted using a conventional procedure called ELISA, and the results showed a correlation coefficient of 0.9734 (Figure 4B).

Application of Anti-HER2/Graphene Sheets/PG Immunosensor

To investigate the bio-medical significance of anti-HER2/Graphene sheets/PG immunosensor, the procured serum samples were employed. Serum samples were acquired from the hospital of PGIIMS, Rohtak women diagnosed with breast cancer and those who were in apparently good health. The HER2 antigen amount in sera, as determined by this particular immunosensor, ranged from 7.30.17 to 22.80.3 ng/mL (n=15) in apparently healthy people and 38.60.4 to 119.40.23ng/mL in breast cancer patients, demonstrating that it is significantly higher ($p < 0.01$) in breast cancer patients in contrast to reportedly healthy individuals (Table 1).

Storage Reliability and Regeneration Studies of Anti-HER2/Graphene Sheets/PG Immunosensor

An assessment of the immunosensor's storage reliability was carried out after 25, 50, and 75 days. The activity offered by the immunosensor dropped by 12%, 31%, and 41%, respectively, from starting performance, which is better than reported earlier (41.2% decrease) [18]. Anti-HER2/Gr-sheets/PG electrode were maintained dry at 40C all throughout the experiment (Figure 4C).

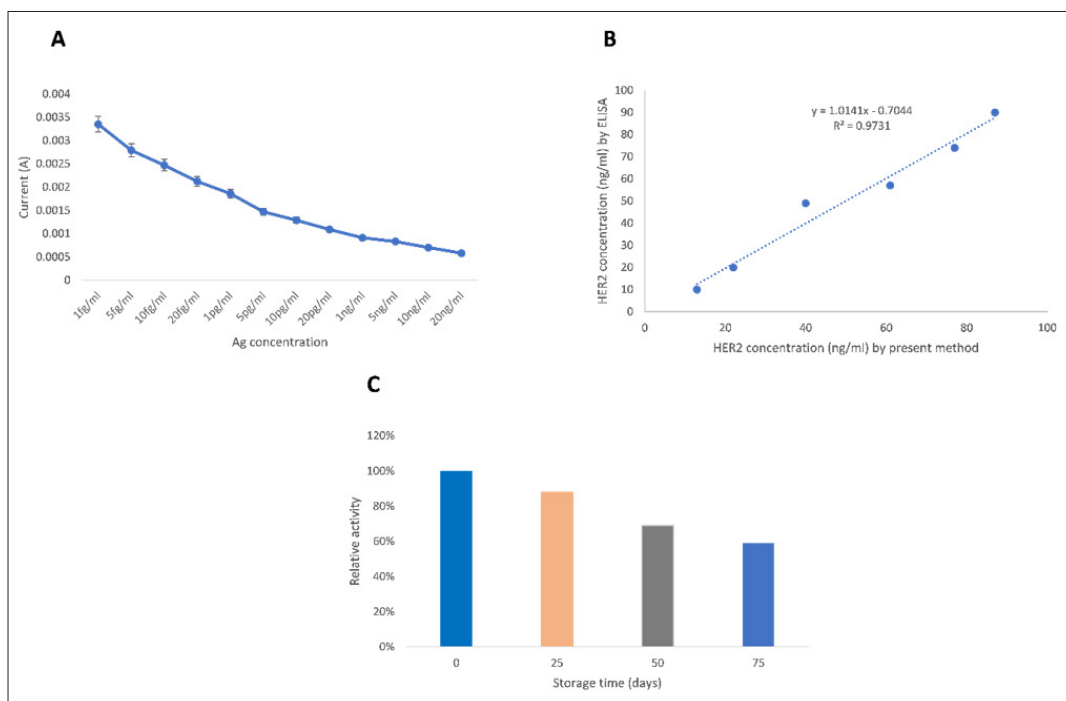


Figure 4: Analytical Performance of Anti-HER2/ Graphene Sheets/PG Immunosensor (a) Current Responses of Anti-HER2/Graphene Sheets/PG Immunosensor After Detection of HER2 Antigen. (b) Correlation Plot Between Anti-HER2/Graphene Sheets/PG Immunosensor and ELISA Method with a Correlation Coefficient 0.9731. (c) Relative Activity of Immunosensor Representing Storage Ability. All Experiments were Repeated Three Times. Data were as Expressed Mean ±sem.

Table 1: Measurement of HER2 Antigen Levels by Anti-HER2/ Graphene Sheets/PG Immunosensor in Serum Samples from Apparently Healthy Persons and Breast Cancer Patients

Age (Year)	HER2 concentrations (ng/mL) in serum of apparently healthy persons
38	7.3±0.17
64	9.8±0.3
53	11.3±0.12
43	22.8±0.3
47	13.3±0.14
Age (Year)	HER2 concentrations (ng/mL) in serum of breast cancer patients
49	38.6±0.4
63	68.4±0.14
57	103.2±0.2
67	119.4±0.23
52	84.5±0.15

Conclusions

A pencil graphite electrode customized with Gr-sheets and the HER2 biomarker was created as an electrochemical amperometric immunosensor for breast cancer. Utilizing SEM, EIS, and CV, the developed breast cancer immunosensor was characterized. The current sensor had a limit of detection of 0.8 fg/ml and responded well in the linear range of 1 fg/ml to 20 ng/ml. The created biosensor was employed to monitor and diagnose HER2 in breast cancer patients. Future research might concentrate on creating lab-on-a-chip microdevices for breast cancer patients' bedside early diagnosis.

Competing interest: Authors declare no competing interests.

Ethics Approval

The research work carried out in the manuscript has been approved by the ethical committee constituted by the institute for applications of blood for diagnosis of different disease in OPD of the hospital.

Consent to Participate: All authors have given their consent for the manuscript.

Consent for Publication: All authors agree for the publication of this paper if accepted.

Availability of Data and Materials: All relevant data are available.

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Authors' Contributions: Prof. C.S. Pundir and Dr. Reeti Chaudhary planned the research and supervised/checked the whole work. Practical work and initial writing of research was carried out and written by Dr. Bhawna Nohwal and Himani Guliya.

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