

Research Article

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A rapid nanobiosensing platform based on herceptin-conjugated graphene for ultrasensitive detection of circulating tumor cells in early breast cancer

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Abstract: In this paper, we use a simple and cheap approach for the synthesis of herceptin-conjugated graphene biosensor to detect the HER2-positive breast cancer cells. The bifunctional graphene-herceptin nanosheets are prepared from graphite by a simple ultrasonic-mediated technique. The prepared protein-mediated graphene is fully characterized. The results show the exfoliation of graphene layers in herceptin solution. Moreover, herceptin is effectively conjugated into the surface of graphene nanosheets. The synthesized herceptin-conjugated graphene is applied for breast cancer detection. The linear range of this biosensor is 1–80 cells, which is significant. The biosensor shows an excellent selectivity performance for detection of HER2-positive cancer cells. Likewise, the stability and functionality of the biosensor is about 40 days.

Based on the results, this device is a promising candidate for rapid and selective detection of cancer cells.

Keywords: single cell, breast cancer, immunosensor, herceptin, graphene

1 Introduction

Cancer is one of the most dangerous diseases in the world [1–4] and the most common cancer among women is breast cancer [5]. Based on the existed challenges, there is still required a nonaggressive, safe, rapid, and easy method to detect breast cancer [5,6]. Electrochemical biosensing is one of those methods that can detect and quantify the biologic species without destroying the system [7–9] due to its high specificity, sensitivity, portability, low-cost preparation, and low response time [10].

Various nanomaterials have been used in biomedical engineering [11–16] as well as immunosensors and biosensors such as metal oxide nanoparticles [17], carbon-based nanomaterials [18,19], noble metal nanoparticles [20], and metal nitride nanoparticles [21]. Graphene is one of the most powerful 2D nanomaterials for biosensing applications [22]. Beside high surface area and π conjugation structure of graphene that allow a high capability for binding to other molecules, this material possesses cavities and also has a tunable structure which makes it suitable for electroanalysis [23]. Beside high electrical conductivity, graphene has more properties such as ultralight weight, superior mechanical strength, optical absorption properties, high thermal conductivity, superior elasticity, etc. [24–27], which make graphene a promising material for generating versatile signals in different biological matrixes [28]. Thus, graphene has been greatly used for several biomolecules stabilization [29,30]. Some recent successful cytosensors are based on several cell detections such as

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biosensors for detection of MCF-7 [31], MKN 45, MCF-7 and HT 29 [32], DU 145 [33], and LO2 [34] cells. A comparison of the research in different kinds of breast cancer biomarker detection methods based on graphene materials was recently reported and discussed [28].

As a brief report of recent research on circulating tumor cells (CTCs) counting in breast cancer, researchers used gold microelectrodes and Anti-EpCAM-modified LC-SPDP monolayer, which lead to a detection limit of 10^5 cells/mL for MCF-7 breast cancer cells [35]. Yang *et al.* [36] used DNA-labeled biosensor and reached a LOD 80 cells/mL. Also, Salahandish *et al.* [37] could fabricate a biosensor with a LOD of 2 cells/mL using silver nanoparticles/graphene/polyaniline. Beside, a biosensor based on peptide-aptamer/polyaniline was fabricated by Liu *et al.* [38], demonstrating a LOD of 20 cells/mL. Recently, Nasrollahpour *et al.* [39] could prepare a biosensor with a linear range of 20–2,000 cells/mL by an electrochemiluminescence cytosensor in 2021.

We already used a complex procedure for detection of breast cancer cells with minimum quantification of 2 cells [37]. In this work, we used a simple biosensor consisting of herceptin-conjugated graphene which utilized the special antibody of HER2 (turns this biosensor to an immunosensor) for single cell detection. Therefore, we used a graphene-based immunosensor on the surface of a glassy carbon electrode to quantify the amount of SK-BR-3 cancer cells. One of the advantages of this biosensor is simple and low-cost preparation of the electrode, beside an appropriate linear range for cancer cell level diagnosis. An exciting property of this biosensor is that this biosensor could quantify low concentrations of SK-BR-3 cells even to 1 cell number, which can be useful for real samples and early detection.

2 Materials and methods

2.1 Materials

Graphite prepared from Merck and Herceptin was obtained from Motamed Cancer Institute (Iran). Also, SK-BR-3, MCF-7, G-292, and HUVEC cell lines were cultured in Motamed Cancer Institute. The electrodes used in this project were glassy carbon electrodes and $\text{Fe}(\text{CN})_6$ solution was used as the electrolyte. Also, the water used in preparing aqueous solutions was deionized water.

2.2 Graphene-herceptin synthesis

In order to synthesize the material, we first mixed a 30 mL of 20 mg/mL concentration of herceptin with 0.5 g of graphite to reach the primer solution. Then, we stir the solution using a magnetic stirrer for half an hour to disperse the graphites in the solution. After that, the stirred dispersion was ultrasonicated for 3 h in an ice bath. This was the major step to slice the graphites in order to reach the graphene sheets. The ice bath was crucial because of the presence of proteins in the dispersed solution. It should be then preserved in a steady and cold situation for 24 h in order to let remained and unreacted graphites sediment. After one day, we collected the upper parts of dispersion and centrifuged them two times in 3,000 rpm and one time in 4,000 rpm in order to wash the graphene sheets and reach the slightly graphene-herceptin. Before every biosensing test, the graphene-herceptin dispersion should be ultrasonicated for 15 min in ice bath [40].

2.3 Biosensing methods

This biosensing method was based on a three-electrode platform. Glassy carbon electrodes were used as working electrodes in this case. Before each step, in order to prepare the electrodes, the electrodes were wet-polished with alumina powder. Then, cyclic voltammetry (CV) analysis was carried out to characterize the materials and confirm the biosensor fabrication. In order to prepare electrodes for the tests, the dispersed graphene-herceptin solution was drop-coated on the electrodes and kept in the cold situation to dry for 24 h. For both selectivity and calibration tests, square wave (SQW) analysis was taken before and after putting cells on the electrodes. To make cells' attachment to the antibodies on the electrodes, the solution was again drop-coated and preserved in the refrigerator for an hour. The reference and counter electrodes used in the tests were Ag/AgCl and Platine, respectively. Finally, to complete the electrochemical cell, a solution was prepared using ferrocyanide and KNO_3 as the electrolyte.

2.4 Cell culture

SK-BR-3, a human breast cancer cell line, that overexpresses Her2 antibody (Neu/ErbB-2), human caucasian osteosarcoma (G-292), human breast adenocarcinoma (MCF-7), and

human umbilical vein endothelial cells (HUVECs) utilized here were provided from Pasteur Institute of Iran (IPI). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) at pH 7.4 supplemented with 10% (v/v) heat-inactivated at 50°C for 30 min from fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL of penicillin, and 100 mg/mL of streptomycin at 37°C and 5% CO₂ in a humidified incubator. Then, the cells were trypsinized (0.025% trypsin, 0.02% EDTA) after they were grown until 70–80% confluent [41].

2.5 Characterization and equipment

The graphene-herceptin was provided by the ultrasonic probe (Q 500 system with standard probe) and was dried in the Freeze Dryer GAMMA 1–16 LSC. Ultraviolet visible (UV) absorption of the material was obtained by T80 + UV-Vis spectrometer instruments Ltd. Morphology characteristics were investigated by a field emission scanning electron microscopy (TE-SCAN). Micro/nanostructure evaluations were studied by transmission electron microscopy

(TEM) 100 kV Philips TEM. Fourier-transform infrared spectra (FTIR) of graphene-herceptin were measured by IR spectrometer (8500S SHIMADZU) (400–4,000/cm). Raman spectra were tested in the range of 500–400/cm (excitation wavelength of 642 nm) by RFS-100/s Raman spectrophotometer. All electrochemical tests were scrutinized using a Potentiostat/Galvanostat model PGSTAT302N boards (Eco Chemie, Utrecht, Netherlands).

3 Results and discussion

3.1 Biosensor fabrication

The glassy carbon electrodes were cleaned with alumina powder and alcohol. The herceptin-conjugated graphene (bio-graphene) was synthesized by ultrasonic method and casted on the prepared electrodes. An electrochemical procedure based on the three-electrode platform was used to evaluate the biosensing performance and breast cancer detection (CTCs in blood). A schematic representation of the biosensor fabrication is shown in Figure 1.

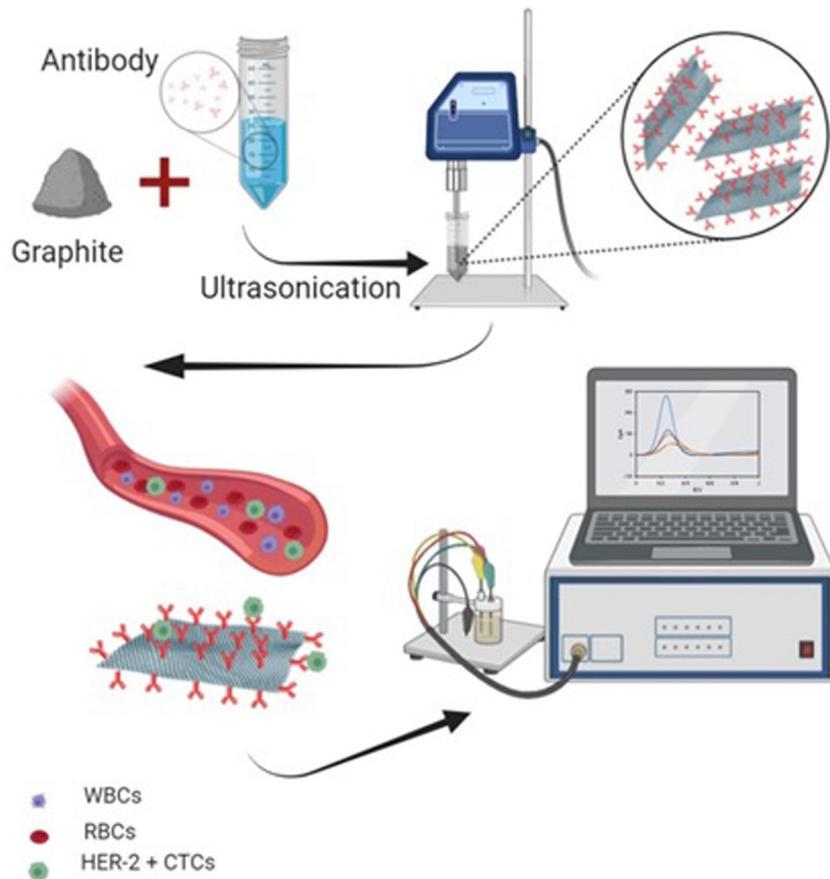


Figure 1: Schematic illustration of the herceptin-conjugated graphene synthesis and the immunosensor fabrication.

3.2 Morphological and physicochemical characteristics

The morphologies and microstructures of the simple herceptin-conjugated graphene nanosheets are presented in Figure 2. The FESEM results of the herceptin-conjugated graphene showed that graphene was the main material in the sample and the side dimensions of the herceptin-conjugated graphene nanosheets were in the range of 10 nm to several micrometers (Figure 2a). The non-dispersion method covered the strategy for the economically and environmentally friendly synthesis of graphene nanostructures.

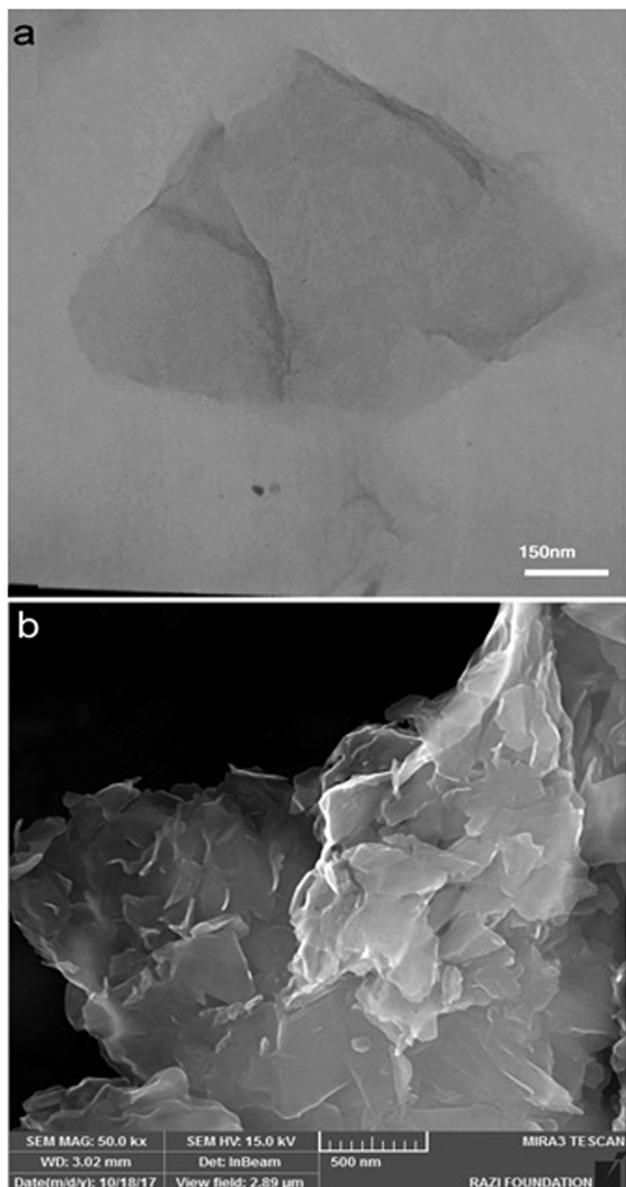


Figure 2: Micro/nanostructure and morphology of the herceptin-conjugated graphene prepared by sonication process: (a) TEM and (b) FE-SEM images.

In Figure 2b, the TEM result of the herceptin-conjugated graphene exhibited that the prepared graphene was effectively exfoliated and one of the main features of the synthesized herceptin-mediated graphene was high surface area. TEM result showed the sonicating procedure for reducing the flake size (the flake dimensions are scaled as $t^{-1/2}$). Moreover, TEM and FE-SEM results demonstrated large graphene nanosheets on the surface and their resemblance of crumpled silk veil waves scrolled and corrugated was the nature of graphene nanosheets. These typical characteristics of graphene and its derivatives were already confirmed by many researchers [42–45]. The protein-mediated graphene nanosheets were transparent and demonstrated an excellent stability under the electron beam. The distinct diffraction marks approved the structural properties (crystallites) of graphene nanosheets synthesized with a simple protein-mediated procedure. In the ultrasonic condition, ultrasonic waves establish acoustic cavitation, leading to creation, growth, and collapse of bubbles in the solution [46]. This event leads to shock waves onto the graphite and graphene layers, leading to exfoliation and isolation of graphene layers. This process relied on low-power sonicating procedure for long time. Stabilizer helps forming the few-layer graphene sheet without sheets aggregation which may be established because of the hydrophobic nature of graphene surface. Therefore, herceptin (protein) was used as a stabilizer with ultrasonic procedure to address this critical challenge. Moreover, herceptin could diffuse into the graphene layers and act as a bioreceptor for biosensing. Herceptin is a biological element that can establish selectivity in the graphene biosensor. Dong *et al.* reported a water-phase and non-dispersion exfoliation approach for producing large scale of high concentration graphene [47]. Khan *et al.* investigated exfoliation of graphite dispersions and preparation of graphene at high concentrations [48]. This technique facilitated graphene synthesis for many applications including biosensor, drug delivery, tissue engineering, and lab on a chip. The prepared hybrid material was deposited on the glassy carbon electrodes. The electrodes were cleaned completely. Then, the biosensing activity, selectivity, and calibration tests were established.

Fourier-transform infrared (FTIR) spectroscopy of the herceptin-conjugated graphene was analyzed to scrutinize the *in situ* forming of the novel functional groups existed in bio-graphene under ultrasonication (Figure 3). The peaks at 1,098 and 1,550/cm were related to C–N and C=N stretching vibration, respectively. It seems that the nature of these peaks is ascribed to the hydrophobic residue of herceptin that reacts with the graphene layers. 1,720, 1,200, and 3,430/cm peaks are attributed to the C=O, C–OH, and O–H stretching vibration, respectively.

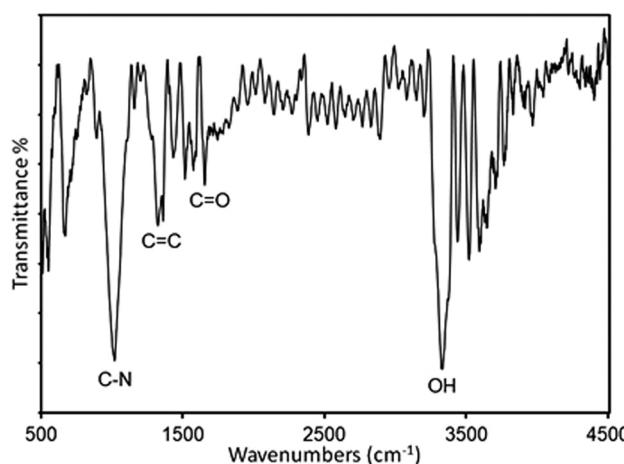


Figure 3: Fourier-transform infrared (FTIR) spectrum of the herceptin-conjugated graphene synthesized under ultrasonication in the presence of the antibody, herceptin.

Also, the peak at 1,600/cm was ascribed to C=C aromatic vibration [40].

The UV-Vis spectroscopy analysis of the herceptin-conjugated bio-graphene is shown in Figure 4. As seen, a peak is observable at 270 nm, which is ascribed to the bio-graphene characteristic [49]. An increment was observed in the intensity of this peak which was attributed to concentration of the pristine antibody, herceptin. Therefore, the herceptin-conjugated bio-graphene was successfully synthesized.

Raman spectroscopy analysis was used to validate carbon structure in the nanosheets of the herceptin-conjugated graphene (Figure 5). The specific bands of the functionalized bio-graphene in Raman analysis are D,

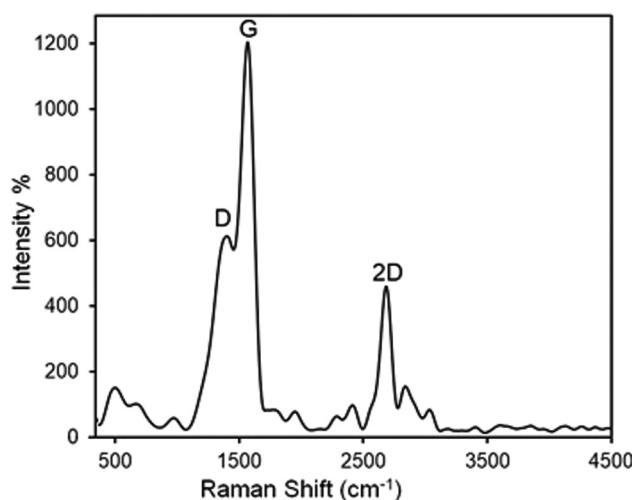


Figure 5: Raman spectrum of the herceptin-conjugated graphene synthesized *in situ* under ultrasonication in presence of the antibody, herceptin.

G, and 2D. These bands are located at ~1,350, ~1,570, and ~2,680/cm for D, G, and 2D, respectively. The shape and position of these bands in the Raman analysis include critical information in association with the quality of the bio-graphene. The intensity ratio of D/G for the herceptin-conjugated graphene and graphite was 0.32 and 0.02, respectively, revealing that the aromatic domain number was increased in the bio-graphene micro/nanostructure. The intensity ratio of 2D/G was 0.42 for the herceptin-conjugated graphene, demonstrating successful synthesis of the bio-graphene.

3.3 Electrochemical characteristics

The CV and Nyquist analysis were conducted for the bare electrode and the functionalized electrode (herceptin-conjugated graphene) in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) containing 5 mM $K_3Fe(CN)_6$ (these electrochemical analyses were commonly applied to the investigation of the characterization of sensors and biosensors). These two tests were used to explore the electrochemistry and electrochemical features of the herceptin-conjugated graphene biosensor (Figures 6 and 7).

As can be seen in Figure 6, herceptin-conjugated graphene substrate decreased the charge transfer resistance (R_{ct}) in comparison with the bare electrode and enhanced the conductivity and surface area of the conventional electrode (glassy carbon), presenting a highly conductive and inexpensive substrate for biosensing applications. The herceptin-conjugated graphene substrate enriched the

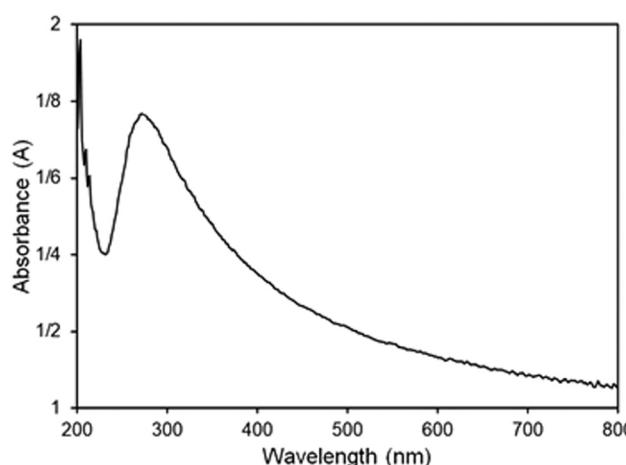


Figure 4: UV-Vis spectrum of the herceptin-conjugated graphene synthesized under ultrasonication in presence of the antibody, herceptin.

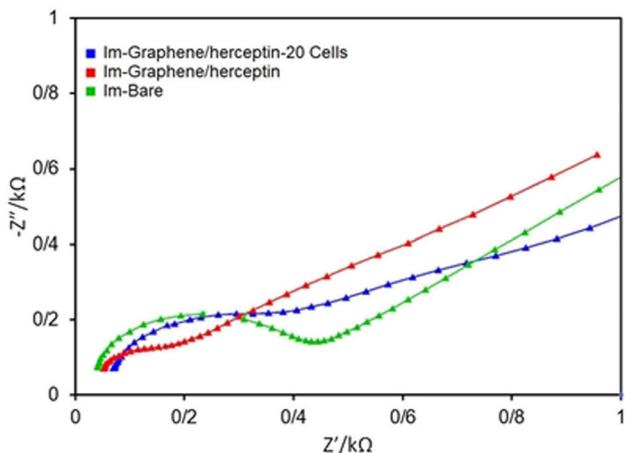


Figure 6: Nyquist plots for the bare electrode (green), herceptin-conjugated graphene (red), and herceptin-conjugated graphene with 20 cells (blue), respectively. All tests were done in 0.01 M PBS (pH = 7.4) containing 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$.

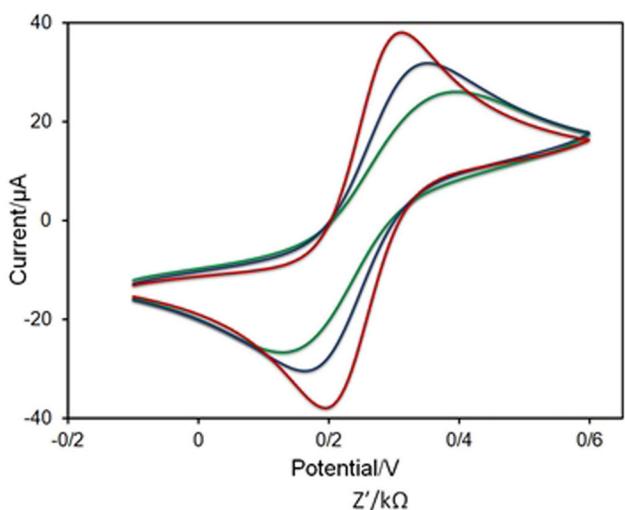


Figure 7: Cyclic voltammograms (CVs) for the bare electrode (red), herceptin-conjugated graphene (blue), and herceptin-conjugated graphene with 20 cells (green), respectively. All analyses were established in 0.01 M PBS (pH 7.4) containing 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$.

reaction kinetics and decreased the R_{ct} because of great surface area and porosity that were established by its pellet/flake-like graphene structure. Owing to the rise in the surface conductivity, the electron transfer and conductivity were increased with the $\text{Fe}(\text{CN})_6^{3-}$ probe and subsequently formed the diffusion layer.

As can be seen in Figure 7, there is a satisfactory agreement between Nyquist plot and CV where the peak height in CV was decreased with the functionalization of the electrode with herceptin-conjugated graphene substrate.

The redox peaks of the herceptin-conjugated graphene and herceptin-conjugated graphene with 20 cells were reduced in comparison with the bare electrode which could ascribe to the good electron mediation of herceptin-conjugated graphene and the glassy carbon electrode.

Impedance spectroscopy technique is used for electrochemical characterization. However, the use of impedance technique is semi-qualitative for biosensing evaluation and detection. Moreover, this technique uses resistance to sense the analyte, which allows the interferences to affect the results decreasing the selectivity. The square wave voltammetry was used as a strong technique for biosensing measurements [50]. Actually, we established a single cell detection platform for early breast cancer.

3.4 Selectivity analysis

The main analysis for sensing performance of a biosensor is the selectivity test and response of the sensor in real biological samples containing electroactive species. In order to perform this analysis, we used different kinds of cell lines including MCF-7, SK-BR-3, HUVEC, and G-292. In every step, we drop-coated a solution of cells with a 2,000 cells/mL concentration. In each one, SQW analysis was performed before and after putting cells on the electrode surface. In order to remove the parameter of difference of the electrodes from the selectivity results, we divided the difference of the SQW peaks intensity on the primitive intensity (before depositing the cells) as the parameter $\Delta I/I$. The concerned curves and final comparison of the data are shown in Figure 8.

Apparently, the parameter $\Delta I/I$ for SK-BR-3 cells is more than 0/45 which is more than these data for other cell lines. This means in the SK-BR-3, cells adhered to the electrodes were more than other cells which showed the excellent selectivity and good affinity of the herceptin antibodies existed in the synthesized graphene to HER-2 receptors of SK-BR-3 cells (Figure 9).

3.5 Cancer biosensing analyses

The linear range of a biosensor usually shows the analyte concentration range in which the biosensor exhibits a good linear correspondent response. The amended electrochemical cell and bio-graphene electrode were applied to achieve the linear dynamic range of the immunosensor

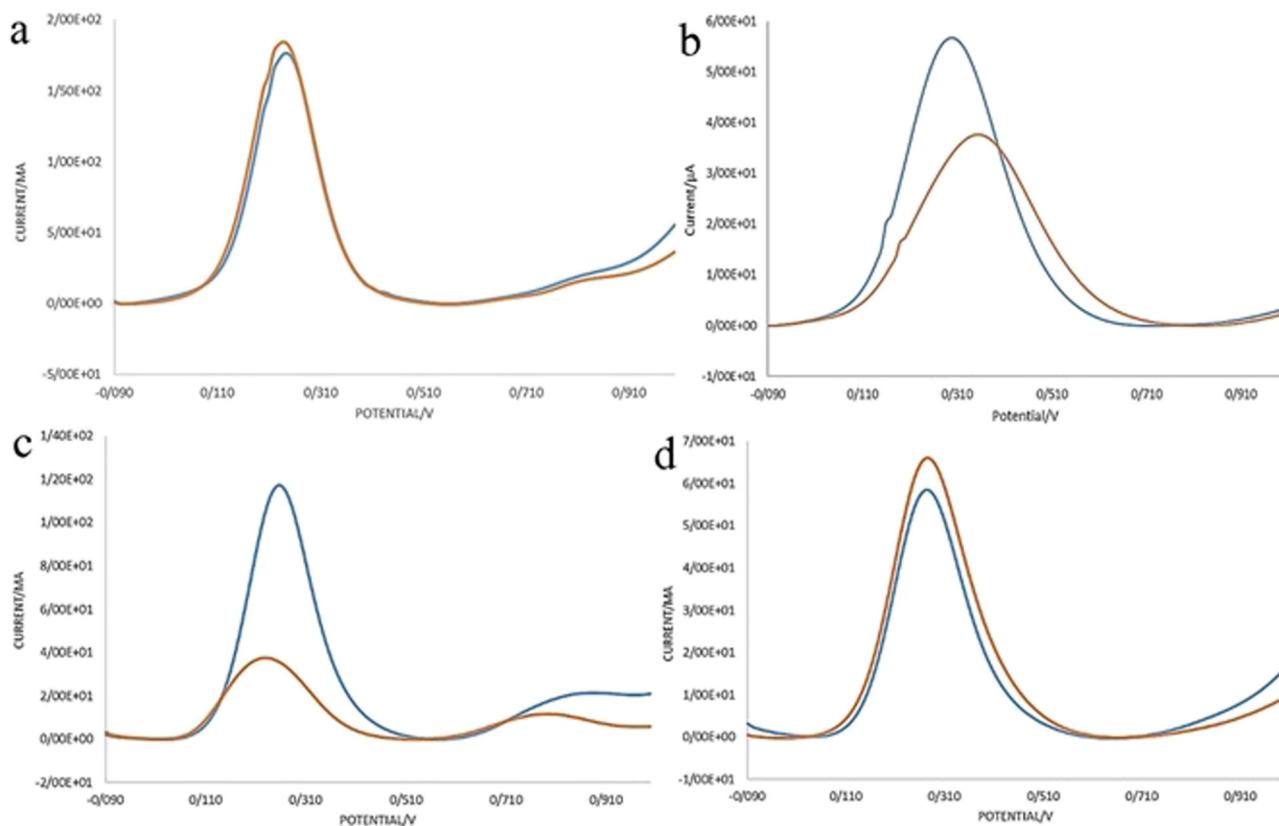


Figure 8: Selectivity of the herceptin-conjugated graphene biosensor and the comparison of different cell lines on the sensing performance of the bioelectrode: (a) MCF-7, (b) G-292, (c) SK-BR-3, and (d) HUVEC. In each graph, the blue and red curves are related to electrodes before and after depositing cells on them, respectively.

for detection of HER²⁺ overexpressed on the surface of SK-BR3 cell line (as a sample of breast cancer cells). To find the linear range of the biosensor, we put different concentrations of SK-BR3 cells on the electrode surface

so that the following results were reached by comparing the SQW graphs (Figure 10).

As can be seen in Figure 10, this biosensor can detect cancer cells linearly with a $R^2 = 0.9957$ in the range of 1–80 cells which is a very successful result for developing this biosensor in real sample analysis (because in real samples (blood) of patients, there is low concentration of cancerous cells). Also, this biosensor had the potential to be tested in more concentrations above 80 cells.

Beside, in the case of reproducibility of this biosensor, six results in the concentration of 20 cells were compared. As a result, we found that the parameter $\Delta I/I$ is more than the numerical quantity of 0/45 for four of six tests. Moreover, the functionality or durability of this synthesized material was estimated for 40–50 days. As a result, beside simple and low-cost synthesis of the used hybrid material, this biosensor was able to be used in real sample detections and developments for better performances.

Differential pulse voltammetry (DPV) technique is used for biosensing measurements. However, the results of DPV technique are very similar to square wave

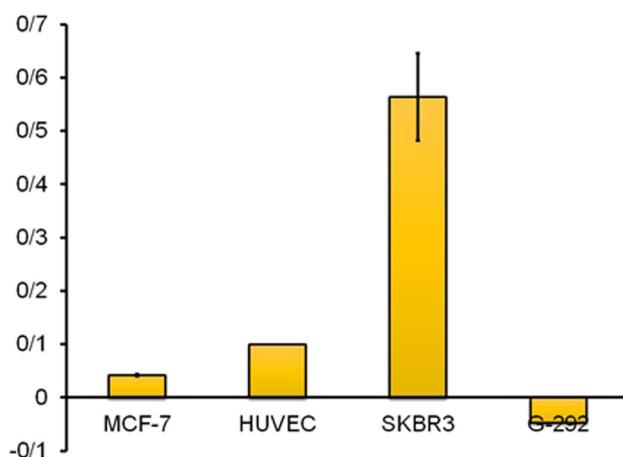


Figure 9: Effect of several cells on the electrode response and a comparison of the biosensor activity in the presence of the cells: MCF-7, G-292, SK-BR-3, and HUVEC.

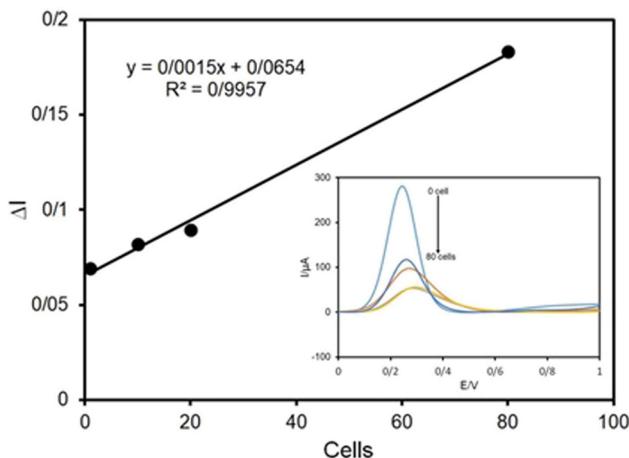


Figure 10: Calibration graph of the graphene-herceptin biosensor in the range of 1–80 cells.

voltammetry for biosensing evaluation and detection. Moreover, both of these techniques use the same procedure to sense the analyte. As mentioned, square wave voltammetry was used, which is a noble technique for biosensing measurements [50]. In biosensors, linear range is very important. In most biosensors such as glucometer, the maximal limit of quantification is very critical, because the amount of the analyte (glucose) is very vital to consider the diet and medication. But, in cancer detection, only minimal limit of quantification is very important for medical diagnosis and this platform is more sensitive than other devices in literature (the ability to detect single cell). Moreover, there is almost 15–20 cell/mL in blood of metastatic breast cancer (the worst case of cancer) [51]. Our platform can detect more than 80 cells, which is suitable for clinical applications.

4 Conclusion

The quick advances of nanotechnology and nano-science facilitate the fabrication of nanostructures-mediated biosensing devices with amended biosensing features for cancer diagnosis. Because of the increasing need to detect and quantify the chemical and biological species, electrochemical biosensors have been widely developed. These devices have been used for diagnosis and therapy of cancer owing to their properties such as portability, high sensitivity, specificity, fast response, and being user-friendly. Additionally, graphene, due to its unique features such as high surface area, conductivity in room temperature, and brilliant optical, mechanical, and thermal properties, is one of most used materials in electrochemical biosensors,

which significantly increases the sensitivity of biosensors. However, detecting the single cell needs a new class of nanostructure-based device with excellent sensitivity and specificity. Biosensors with low limit of detections and fast responses can make this result feasible. In this project, we used a facile *in situ* synthesis of graphene-herceptin hybrid for applying this material for a SK-BR-3 cell biosensor. Selectivity tests were successfully carried out which showed excellent results over the potential electroactive cells presented in real samples. The calibration test exhibited a linear dynamic detection in the range of 1–80 cells. Moreover, it was observed that for SK-BR-3 cells, the parameter $\Delta I/I$ was more than 0/45. Reproducibility of the biosensor was estimated about 66% which can be passed by programming the processor to ignore data with $\Delta I/I$ less than 0/45. Also, stability and functionality of this material were about 40–50 days. The obtained results proved that this material is a promising candidate for rapid and selective detection of cancer cells.

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