Research Article

Mahdi Sadeghi, Soheila Kashanian*, Seyed Morteza Naghib*, Esfandiyar Askari, Fateme Haghiralsadat, and Davood Tofighi

A highly sensitive nanobiosensor based on aptamer-conjugated graphene-decorated rhodium nanoparticles for detection of HER2-positive circulating tumor cells

<https://doi.org/10.1515/ntrev-2022-0047>

received November 15, 2021; accepted January 24, 2022

Abstract: Assessment of human epidermal growth factor receptor-2 (HER2) tumor marker status is an impressive factor in screening, diagnosing and monitoring breast cancer (BC). The electrochemical biosensor is a revolutionary method in cancer diagnosis, which is used in this research to detect HER2⁺ circulating tumor cells. The electrochemical activity, size, shape, and morphology of the synthesized nanomaterials were analyzed. The hybrid nanocomposite established by the coupling of reduced graphene oxide nanosheets (rGONs) and rhodium nanoparticles (Rh-NPs) on the surface of graphite electrode

resulted in improved surface area, electrochemical activity, and biocompatibility. The graphite electrode-based aptasensor (g-aptasensor) demonstrated exceptional performance against HER2-overexpressed SKBR3 cancer cells, with a linear dynamic range of 5.0 to 10.0×10^4 cells/mL, an analytical limit of detection (LOD) as low as 1.0 cell/mL, and a limit of quantification (LOQ) of 3.0 cells/mL. The G-rich DNA aptamers can fold into an intermolecular G-quadruplex, which specifically bind to the target molecule. Consequently, the advantages of this highly efficient nanocomposite platform include broad dynamic range, high specificity, selectivity, stability, reproducibility, and low cost. These characteristics indicate that the fabricated nanobiosensor has a high potential for use in detecting and monitoring HER2 level for the care of BC patients and clinical diagnosis.

* **Corresponding author: Soheila Kashanian**, Applied Chemistry Department, Faculty of Chemistry, Razi University, Kermanshah, Iran; Nanobiotechnology Department, Faculty of Innovative Science and Technology, Razi University, Kermanshah, Iran, e-mail: kashanian_s@yahoo.com

* **Corresponding author: Seyed Morteza Naghib**, Biomaterials and Tissue Engineering Research Group, Interdisciplinary Technologies Department, Breast Cancer Research Center (BCRC), Motamed Cancer Institute, ACECR, Tehran, Iran; Nanotechnology Department, School of Advanced Technologies, Iran University of Science and Technology (IUST), Tehran 1684613114, Iran, e-mail: naghib@iust.ac.ir

Mahdi Sadeghi: Nanobiotechnology Department, Faculty of Innovative Science and Technology, Razi University, Kermanshah, Iran

Esfandiyar Askari: Biomaterials and Tissue Engineering Research Group, Interdisciplinary Technologies Department, Breast Cancer Research Center (BCRC), Motamed Cancer Institute, ACECR, Tehran, Iran

Fateme Haghiralsadat: Department of Oral and Maxillofacial Surgery/ Oral Pathology, Amsterdam University Medical Centers-location VUMC and Academic Centre for Dentistry Amsterdam (ACTA), Amsterdam Movement Sciences, Amsterdam, The Netherlands.

Davood Tofighi: Department of Psychology and Biostatistics, Epidemiology, and Research Design Support (BERD), Clinical and Translational Science Center, University of New Mexico, Albuquerque, New Mexico, United States of America

Keywords: HER2⁺ circulating tumor cells, graphite-based electrochemical aptasensor, HER2 tumor marker diagnosis, breast cancer, rhodium nanoparticles, graphene-rhodium nanocomposite

1 Introduction

Cancer is an intricate morbidity that causes drastic changes in the levels of genes, proteins, and metabolites [1,2]. Breast, lung, and prostate cancers are the most common cancers worldwide with a prevalence of 2.26, 2.21, and 1.41 million cases, respectively [3]. The use of modern methods in the construction of biosensors to improve speed, sensitivity, and selectivity, and finally their application in advanced technologies such as point-of-care-testing (POCT) not only pave the way for more effective treatment but also have a significant impact on the prevention of a variety of cancers, especially BC [4,5]. Therefore, screening and early detection are essential to timely follow-up, enhance survival rate, and

reduce mortality in BC patients [6,7]. Definitive diagnosis techniques for detecting early stage of BC include mastography, tissue biopsy, ultrasound imaging, and magnetic resonance imaging (MRI). Also, the techniques of gene amplification and protein overexpression such as *in situ* hybridization (ISH) and immunohistochemistry (IHC) are practiced to diagnose BC [8]. However, most of these methods and techniques face many limitations such as high cost, complexity, and a significant reduction in sensitivity and specificity [9]. Electrochemical biosensors are one of the best analytical and diagnostic methods due to their unique features such as noninvasiveness, fast response, selectivity, specificity, sensitivity, ease of miniaturization, and cost-effectiveness [10–12].

Hybrid nanocomposites made by the synergistic combination of metal nanoparticles (NPs) supported on graphene sheets as nanoscale building blocks of new composites are promising for many technological applications ranging from gas sensing, bio-sensing, energy production and storage, catalysis to electronics, and so on. This is due to the benefits offered by the properties of both components, in particular, charge transfer effects and the unique properties of each of the nanomaterials used, which include excellent electrical and thermal conductivity, good electron mobility, high surface area-to-volume ratio, high elasticity and flexibility, good biocompatibility at graphene–nanoparticles interface [13,14]. Noble metal NPs (e.g., Au, Pt, Rh) are of particular interest due to their unique characteristics, and they have been frequently employed to adorn graphene sheets to generate a new class of composites and play a pivotal role in various scientific fields, including fabrication of medical biosensors [15].

Aptamers have an extensive variety of medical applications in different fields, including therapeutic solutions, diagnostic services, and biosensors [16]. They can even overcome physiological barriers, such as the blood–brain barrier, while maintaining high affinity and specificity for their target molecule [17]. Another major function of nucleic acid aptamers is to serve as the ligand-binding component of riboswitches [18]. A 3D structure is formed as the aptamers fold around the target molecule. Aptamers differ from other synthetic receptors in that they have a phenotype–genotype relationship [19]. Their recognition properties are determined not only by their specific sequences (genotype) but also by their ability to fold into distinct shapes with specified functions (phenotypes). Aside from structural complementarity, short-range noncovalent bonds such as hydrogen, van der Waals, and electrostatic interactions, as well as the arrangement of aromatic rings [20], help to stabilize the complex formed by the aptamers and the target molecule [21]. These outstanding features make the aptamers good

candidates for the biorecognition agents of electrochemical biosensors [22].

Various nanomaterials have been used to design and fabricate electrochemical biosensors to detect HER2 tumor markers. Some of the most recent ones that have been explored and compared are a modified screen printed carbon electrode (SPCE) with gold NPs [23], multiwalled carbon nanotube (MWCNT)/ionic liquid electrode adorned with gold NPs [24], functionalization of 3-aminopropyltrimethoxysilane-coated magnetic iron oxide NPs with antibody (Ab) and immobilization on a glassy carbon electrode (GCE) [25], GCE modified with reduced graphene oxide/chitosan film [26], fluorine-doped tin oxide glass plates modified with a silver NP-grafted functionalized graphene and polyaniline [27], Au electrode modified with Zr-Hf metal–organic framework (MOF) embedded with abundant carbon dots [28], magnetic beads magnetically attracted on the SPCE surface as a capture probe [29], the surface on the GCE covered with Fe_3O_4 @TMU-21 composite, MWCNT, and Ab [30], and a cobalt porphyrin binuclear framework and gold functionalized graphene quantum dots with Ab on the GCE surface [31].

Cancers with higher than normal levels of HER2 are called HER2 positive or HER2^+ . The purpose of HER2 checking is to determine whether cancerous cells have excessive copies of the *HER2* gene or are beyond a normal limit of the HER2 peptide. In this study, we developed a hybrid nanocomposite platform and used an advanced and efficient procedure to modify graphite electrode by the *in situ* eco-friendly electrochemical synthesis of rGONs. The oxidation of graphite to form graphene oxide nanosheets (GONs) and its reduction to rGONs is thought to be an efficient method for producing scalable qualitative graphene. The oxidative cleavage of graphite is followed by its reduction to rGONs in this process. However, due to van der Waals interactions, rGONs tend to form irreversible agglomerates and even restack into graphitic crystals. As a result, several organic compounds and inorganic particles have been chosen to functionalize the surface of graphene sheets. In comparison to organic species, inorganic particles not only prevent individual sheet restacking/agglomeration but also contribute to the creation of graphene-based hybrids with superior physicochemical features [32]. Noble metal NPs piqued the interest of researchers due to their fascinating remarkable physicochemical features. However, the self-aggregation tendency of these NPs (due to greater surface charge) decreases their surface-to-volume ratio, resulting in poor performance. The feasible answer to this problem is to bind the NPs to a suitable substrate. Graphene was chosen as a conductive substrate and a growth platform for these NPs forming

hybrids because of their exceptional physicochemical features, allowing them to take advantage of their high surface-to-volume ratio [33]. Accordingly, we decorated Rh-NPs as self-assembled, conductive, and stable monolayers on the surface of rGON-modified graphite electrode. Finally, this nanocomposite platform was functionalized with anti-HER2 aptamers and was used to detect HER2 levels in BC circulating tumor cells (Scheme 1).

2 Experimental

2.1 Materials and methods

The pencil graphite (type HB) with a 2.0 mm diameter was purchased from Rotring Co. Ltd, Germany. 3-Mercaptopropionic acid (MPA), *N*-hydroxysuccinimide (NHS), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and rhodium(III) chloride (RhCl_3 , 98%) were

procured from Sigma-Aldrich, USA. Potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) and potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$) were obtained from Merck, Germany. Anti-HER2 aptamer specifically bound with HER2 tumor marker was obtained from Bio Basic Inc, Canada. The sequence of amine-terminated HER2 DNA aptamer (Apt) consists of 54-mer oligonucleotide bases is 5'-(NH_2 -(CH_2)₆-GGG CCG TCG AAC ACG AGC ATG GTG CGT GGA CCT AGG ATG ACC TGA GTA CTG TCC)-3'. Sulfuric acid (H_2SO_4 98%), hydrochloric acid (HCl 37%), and ethanol (99.8%) were purchased from Merck, Germany. Other analytical grade reagents obtained were of the highest level of purity, and all required solutions were prepared from double-distilled water.

2.2 Pencil-graphite electrode polishing

Before analyzing each fabricated g-aptasensor, a 5 mm of the pencil-graphite electrode (PGE) was removed with a razor blade, and the exposed PGE tip was polished with

Scheme 1: Schematic presentation of fabrication process of g-aptasensor for detecting HER2 tumor marker: (i) the synthesis of rGONs, (ii) the synthesis of Rh-NPs, (iii) the immobilization of anti-HER2 aptamer strands, and (iiii) the detection of HER2⁺ BC cell using the proposed g-aptasensor.

2,000-grit sandpaper until a significant quantity of graphite was removed before being polished on a wetted microfiber cloth. The PGE was cleaned with anhydrous ethanol and then with distilled water before any electrochemical treatment [34].

2.3 Characterization of transducer nanomaterial

In this study, we used field emission scanning electron microscopy (FE-SEM) to observe morphological changes of samples and imaging biological surfaces coupled with energy-dispersive spectroscopy (EDS) and EDS elemental mapping by TESCAN Mira 3 XMU. Raman analysis was performed using TEKSAN instrument. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra were recorded using Thermo Fisher Scientific (Nicolet iS20) device. The ThermoFisher apparatus was used for X-ray diffraction (XRD) investigation of nanomaterials.

2.4 Electrochemical measuring equipment and instruments

All electrochemical measurements include cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS) were carried out using a potentiostat–galvanostat Palmsense BV PGSTAT, with a compliance voltage of 30 V (Echo chemie, Netherlands) by a standard three-electrode cell arrangement. The graphite was employed as a working electrode. The desired amplitude of potentials was measured against an Ag/AgCl in a solution containing saturated KCl, acting as the reference electrode. A platinum wire electrode was used, functioning as a counter electrode. A freshly polished and prepared electrode was used for each experiment. The measurements were performed exactly after using the three electrodes and immersing them in the probe solution. All electrochemical experiments were carried out in PBS (0.1 M, pH 7.4) containing 5.0 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 ratio) and 0.1 M KCl as ion redox probe couple. The ferri-/ferro-cyanide ionic redox pair in buffered media is frequently employed as a standard probe in electrochemical studies. The $[Fe(CN)_6]^{3-/4-}$ ionic redox process involves a single electron transfer and exhibits quasi-reversible kinetic characteristics under the condition that the electron transfer in a complete cycle is less resistant [35]. CV curves were recorded with a scan rate of 50 mV/s and within -0.2 to

$+0.6$ V vs Ag/AgCl. DPV was measured between -0.2 and $+0.6$ V, with an amplitude of 50 mV and a pulse width of 0.2 s. EIS measurements were carried out in the frequency range of 0.01–50 kHz with a direct potential of 0.22 V as a bias potential and an amplitude of 5.0 mV.

2.5 Synthesis of rGONs

We used a versatile and novel *in situ* oxidation and reduction electrochemical approach to directly produce rGONs-modified graphite electrodes. Briefly, CV sweeping the potential from 0.0 to $+3.0$ V was achieved for a series of four successive sweeps in PBS (pH 6.5) to form few-layer GONs on the surface of the electrode. Later, CV sweeps were carried out from 0.0 to -1.60 V in PBS (pH 6.9) for electrochemical reduction of exfoliated GONs in five successive cycles to obtain rGON-modified graphite electrode [36].

2.6 Synthesis of rhodium nanoparticles on the surface of graphite electrode

Rh-NPs were electrochemically synthesized using an electrolyte solution containing 0.01 M $RhCl_3$ and 0.01 M KCl. CV technique was employed in the potential range from 0.0 to -800.0 mV for four successive sweeps [37]. The electrolyte solution was provided in double-distilled water to a final volume of 10 mL. The cathodic reduction of rhodium ions at room temperature occurs as follows: $Rh_{aq}^{3+} + 3e^- \rightarrow Rh$.

2.7 Preparation of the graphite electrode-based aptasensor

To prepare the electrode used in g-aptasensor, the Rh-NPs/rGONs-modified graphite electrode was suspended in 20 mM MPA solution dissolved in ethanol/water (3:1 V/V) at pH 6.8 under shaking (75 rpm) at room temperature for 18 h. Thus, MPA/Rh-NPs/rGONs/graphite electrode was rinsed with double-distilled water. The optimal concentration of the MPA was used as a linker to bind the modified electrode to the anti-HER2 aptamer. The carboxyl-functionalized MPA as a linker can immobilize the aptamer by amide bond on the surface of the modified electrode and also improve the electrochemical activity and behavior of

the g-aptasensor [38]. The MPA/Rh-NPs/rGONs/graphite electrode was incubated in PBS containing 0.05 M NHS and 0.20 M EDC for 1 h at 8°C to activate its surface. For covalent conjugation of the activated surface with the amine group ($-\text{NH}_2$) of functionalized anti-HER2 aptamer, the modified electrode was dipped in PBS buffer containing 50 nM anti-HER2 aptamer at room temperature for 1 h for amide bond formation. Furthermore, Apt/MPA/Rh-NPs/rGONs/graphite electrode was immersed in BSA solution with the concentration of 5% at the temperature of 37°C under 5% CO_2 atmosphere and humidity with 95% for 5 min to block the activated carboxyl functional groups on the surface of the modified electrode that are not bonded with the amino functional groups of anti-HER2 aptamer. The functionalized graphite electrode was rinsed with PBS for several times. Finally, g-aptasensor composed of BSA/Apt/MPA/Rh-NPs/rGONs/graphite electrode was employed to detect cancerous cells.

2.8 Preparation of solutions

8.0 g NaCl, 200 mg KCl, 1.44 g Na_2HPO_4 , and 245 mg KH_2PO_4 were combined to make a 1.0 L phosphate-buffered solution (PBS; 0.1 M, pH 7.4). In the aforementioned PBS, a stock solution of aptamer (100 M) was produced and kept at 4°C. In addition, the solution was diluted with PBS to achieve the appropriate concentration. For the synthesis of rGONs, the pH of the 0.1 M PBS was adjusted by 0.01 M HCl.

2.9 Cell culture medium and detection of cells

SKBR3 cell is a human cancerous breast cell line in which the *HER2* gene product is overexpressed. This cell line and its products are exploited often as a positive control in HER2^+ tumor marker detection. Moreover, this cell line is a substantial preclinical model to screen for therapeutic agents targeting *HER2* tumor marker and also to describe precise mechanisms of resistance to *HER2*-targeted therapies. The *HER2* proteins are stained with a chemical dye in the IHC test. The IHC scores the number of *HER2* proteins on the surface of cells in a BC tissue sample from 0 to 3+. If the score ranges from 0 to 1+, it is deemed as *HER2* negative. If the score is 2+, it is deemed borderline. A score of 3+ indicates that you are *HER2* positive. If the IHC test findings are inconclusive, a

FISH (fluorescence *in situ* hybridization) test on a sample of cancer tissue will be performed to identify whether the malignancy is *HER2* positive [39]. *HER2*-expressing SKBR3 human BC cell line (as a cell with an expression score of 3+ is considered HER2^+), BT-474 human BC cell line (as a cell with an expression score of 2+), JIMT1 human cell line (*HER2*-negative cancerous cells with an expression score of 0–1+), human oral or KB cancerous mouth cell line, and human umbilical vascular endothelial cell (Huvec) line as a normal breast cell line were maintained in Dulbecco's modified Eagle's Medium (DMEM) complemented with 1% antibiotic (containing penicillin/streptomycin) and 10% FBS (fetal bovine serum), in the atmosphere of incubator chamber (5% CO_2 , 95% humidity at 37°C) for 72 h [40]. Then, cells were harvested with 1% EDTA/trypsin.

To image the SKBR3 cancer cells, the cells were fixed with a glutaraldehyde solution at a concentration of 2.5% for 6 h before being rinsed and cleansed with PBS buffer for 15 min. The probes were dehydrated with alcohol and then dried in the presence of airflow.

3 Results and discussion

3.1 Synthesis and preparation of the transducer nanomaterials

We synthesized rGONs on the graphite electrode by an *in situ* electrochemical method. To produce multilayers of GONs on the graphite electrode surface, electrochemical oxidation was performed for four sequential sweeps in PBS (pH 6.5) at anodic potentials ranging from 0.0 to +3.0 V. As shown in the CV curves in Figure 1a, electrochemical oxidation of graphite structure eventuated during the positive sweep at around +1.4 V. During the electrochemical oxidation process, the Van der Waals and other cohesive forces in the midst of graphene sheets decreased and then allowing the layers to be separated and spaced apart with the help of gas evolution. Thus, intercalation and exfoliation of graphite to GONs occurred as a result. Intercalating compounds comprising a diverse spectrum of hydrophilic oxygenated functional groups were produced, with the majority of them positioned at the edges of basal planes [41]. CV scanning in the cathodic range potentials ranging from 0.0 to –1.60 V for five sequential sweeps in PBS (pH 6.9) was used to electrochemically reduce the GONs (Figure 1b). The curve in the first cyclic voltammogram of electrochemical reduction shows a significant and broad cathodic peak current at

Figure 1: (a) CV curves of GONs production on graphite electrode for four successive sweeps in PBS (pH 6.5) at a scan rate of 50 mV/s and (b) cyclic voltammograms of the GON-modified graphite electrode recorded for five consecutive sweeps to synthesize rGONs in PBS (pH 6.9) at a scan rate of 50 mV/s.

about -1.350 V. This significant peak current at about -1.350 V in the first cycle is most likely due to the reduction of the oxygenated functional groups on the GON-modified graphite electrode surface because the reduction of H_2O molecules to hydrogen occurs at greater negative potentials. In the second CV, the reduction current peak decreases dramatically at negative potentials and almost disappears after the second scan. This finding suggests that a significant drop in surface-oxygenated functional groups at GONs occurs and rGONs are converted irreversibly and rapidly. As a result, the exfoliated GONs can be reduced using the electrochemical approach at negative potentials [42]. Accordingly, scan rate, number of voltammetric cycles, and pH of the supporting electrolyte are among the characteristics that are effective in converting graphite to rGONs. This technique is more efficient when

performed at modest scan rates and more voltammetric cycles, allowing adequate time for oxidation and exfoliation of graphene sheets in the graphite structure. Also at slightly acidic pH, the conditions are more favorable for the oxidation of graphene layers. As a result, the electrochemical approach is the most practical and cost-effective way for producing graphenic nanomaterials from graphite.

Noble metal NPs are easily synthesized by reducing the equivalent metallic salt in a water environment [43]. By using an electrochemical method, Rh-NPs were produced and reduced. Rh-NPs were produced on the surface under optimal conditions using the CV technique in the potential range of 0.0 to -800.0 mV for four sequential sweeps at a scan rate of 30 mV/s. Therefore, the rGON-modified graphite electrode was decorated with Rh-NPs.

The oxygenated functionalities of electrochemically modified graphene (*i.e.* GONs or rGONs) provide an abundance of nucleating sites for the synthesis of noble metal NPs. As a result, various methodologies for the synthesis of graphene hybrids with noble metals such as Au, Ag, Rh, Pd, and Pt have been developed and investigated for use in the design of electrochemical sensors/biosensors, catalysis, fuel cells, magnetism, optoelectronics, and so on [44]. The 2D nanosheets of graphene in these hybrid nanocomposites not only provide a perfect framework for anchoring the metal nanostructures but also improve the electrical conductivity and interfacial electron transport unleashed by these adherent NPs, limiting nanoparticle aggregation. Furthermore, the incorporation of metal NPs on the surface of graphene may result in new hybrid nanocomposite features and capabilities, expanding its application in a variety of domains of electrocatalysis, electro analysis, and sensor [45].

3.2 Morphology and spectroscopy characterizations

The ATR-FTIR spectra of graphite, GONs, and rGONs are shown in Figure 2a. ATR-FTIR spectroscopy is commonly utilized to investigate material surface-specific molecular information. When a highly collimated synchrotron IR beam is coupled with an ATR hemispherical crystal in a microscopic arrangement, the chemical distribution of complex solid materials can be examined at a higher spatial resolution while maintaining good spectrum quality [46]. The characteristic peak at $1,460/\text{cm}$ corresponds to unoxidized graphitic domain's $\text{C}=\text{C}$ skeletal vibration. Several peaks in the GONs spectrum show oxygen-containing

Figure 2: (a) ATR-FTIR spectra of bare, GON-modified, and rGON-modified graphite electrodes, (b) SEM image of unmodified graphite electrode, (c) SEM image of rGON-modified graphite electrode, (d–f) EDS mapping analysis of oxygen element on the bare, GON-modified, and rGON-modified graphite electrodes.

functional groups. The characteristic peaks of O–H ($3,391/\text{cm}$), C=O ($1,732/\text{cm}$), C–O ($1,556/\text{cm}$), C–OH ($1,317/\text{cm}$), and C–O ($1,162/\text{cm}$) are thought to be assigned to stretching of hydroxyl, carboxyl groups, carboxylic acid, and carbonyl groups. The presence of oxygen functional groups in ATR-FTIR of GONs spectra indicates that the flake graphite has been oxidized to GONs. These peaks do not appear in the graphite spectra, indicating the existence of a significant number of oxygen-containing functional groups (–COOH and C=O near the sheet edge, –OH and C–O groups in epoxy on the GONs sheet's basal planes) added during the oxidation step. There was no noticeable peak after reducing and converting GONs to rGONs, indicating that rGONs were electrochemically reduced. Carbon-oxygen functional groups, such as carboxyl groups, persisted in the rGONs structure, but with faint peaks.

The morphology of various bare and modified graphite electrodes was analyzed by scanning electron microscopy (SEM). SEM pictures of graphite and rGONs-modified graphite electrodes are shown in Figure 2b and c. As illustrated in Figure 2b, the multilayer graphitic flakes are closely and firmly stacked and expanded into a 2D structure with large and thin-sized graphitic sheets, showing that the graphite possessed a platelet-like crystalline form of carbon. In contrast, after the *in situ* electrochemical treatment, a wrinkled, crumpled, and flake-like structure of rGONs was clearly evident on the graphite electrode surface in Figure 2c. Under electron beam irradiation,

the structure of flake-like rGONs reveals a relatively stable nature consisting of a few layers. Thus, the synthesis of graphenic nanomaterials by the electrochemical method has enough energy to prevail the van der Waals forces and produce a stable exfoliated graphene [47]. The produced rGONs were properly fixed on the surface of the graphite electrode, with outstanding stability, durability, and reproducibility. Figure 2d–f illustrates the elemental mapping images of oxygen atoms in bare, GON-modified, and rGON-modified graphite electrodes, respectively. The amount of oxygenated surface species grows remarkably during the electrochemical oxidation of graphite to GONs (Figure 2e). The relative contribution and the distribution of the oxygen-containing components and sp^3 hybridized atoms are significantly reduced after deoxygenation and reduction of GONs during the electrochemical reduction (Figure 2f).

The characterization of graphite, GONs, and rGONs was investigated by Raman spectroscopy (RS). RS is a useful tool for studying defects and disorders in the crystal structure [48]. Raman spectra are sensitive to even minor (chemical or structural) perturbations within chemical bonds in (even amorphous) solids, liquids, and gases and can thus aid in the identification, characterization, and differentiation of individual minerals, fluid inclusions, glasses, carbonaceous materials, solid solution phases, strain in minerals, and dissolved species in multicomponent solutions [49]. Therefore, RS is one of

the more fascinating technologies for characterizing carbon compounds. This procedure provides a wealth of information regarding the testing materials and, more significantly, is nondestructive [50].

Figure 3a depicts the Raman spectroscopic characterization of graphite, GONs, and rGONs. The standard Raman spectrum of carbon compounds has the main and distinguished D, G, and 2D vibrational bands [51]. The D band is caused by vacancies or dislocations in the graphene layer and at its edges. This band is also related to the existence of flaws in the material. The following band, the G peak, is associated with the in-plane vibration of sp^2 hybridized carbon atoms. The last peak (2D) is proportional to the number of graphene layers. This band is also known as G' . Additional information about the carbon compounds can be gathered from the Raman spectra data by analyzing the ratio of the intensities of the different peaks. For example, the I_D/I_G ratio (intensity of the D peak to the intensity of the G peak) is

related to the number of defects present in the material, whereas the I_{2D}/I_G ratio (intensity of the 2D peak to the intensity of the G peak) is related to the number of graphene layers in the material; when the G band increases and the 2D band decreases, the number of layers in the material is assumed to increase [52].

The D vibrational band at 1,362/cm is pertained to the formation of local vibration of the sp^3 disorder of the graphene structural band during the oxidation process and species with the mentioned hybridization, which is primarily situated at the margins of graphene flakes. The G strong vibrational band at 1,588/cm is also related to the presence of the C–C bond stretching, which is common in all sp^2 carbons. As a result, the intensity of D and G vibrational bands ratio (I_D/I_G), which reflects the sp^3/sp^2 hybridization carbon ratio, is an influential defining property for the sp^2 domain size in the graphenic structure of carbon including sp^2 and sp^3 hybridization atoms [53]. The D vibrational band can be observed at 1,362, 1,360,

Figure 3: (a) Raman spectra of bare, GON-modified, and rGON-modified graphite electrodes and (b) XRD pattern carbon-supported Rh-NPs.

and 1,351/cm, and the G vibrational band can be observed at 1,588, 1,599, and 1,586/cm for graphite, GONs, and rGONs, respectively. The D vibrational band in GONs is enlarged because of the reduction in the size of sp^2 domains caused by distortion, defects, and vacancies created during the oxidation process. The presence of oxygenated functional groups causes the G vibrational band in GONs to shift slightly to a higher wavenumber, resulting in the formation of the sp^3 hybridized atoms. The increase in the I_D/I_G ratio of GONs to graphite affirms the grafting of oxygenated functional groups to graphite planes. When GONs are reduced and transformed to rGONs by the electrochemical process, the intensity of the D vibrational band increases. Defects produced during the synthesis of rGONs may be responsible for this change. The I_D/I_G ratio in rGONs is larger than that of graphite due to the latterly formed sp^2 domains during the electrochemical reduction [54]. Moreover, Raman spectra imaging of carbon compounds include a well-known 2D (G') vibrational band that is reactionary in the stacking of graphene sheets. When rGONs are compared to graphite, there is a significant difference in their 2D vibrational band. The formation and the number of layers of graphene sheet can be determined using data derived from the intensity, shape, and position of the 2D vibrational band. The presence of an exceptionally faint 2D vibrational band in the rGONs Raman spectra implies that the exfoliated graphene sheets are properly formed [55].

The X-ray diffractogram of the synthesized Rh-NPs is shown in Figure 3b. XRD spectroscopy is a nondestructive test method used to analyze the structure of crystalline materials. XRD analysis, by way of the study of the crystal structure, is used to identify the crystalline phases present in a material and thereby reveal chemical composition information. The wavelength of X-rays is on the atomic scale, so XRD is a primary tool for probing the structure of nanomaterials [56]. Peak intensity shows the position of atoms within a lattice structure, and peak width shows crystallite size and lattice strain. As shown in Figure 3, well-defined polycrystalline peaks were observed for carbon-supported Rh NPs. The graphite shows a sharp and narrow peak ($2\theta = 28.385^\circ$) that corresponds to the diffraction line C (002) with the intercellular spacing in the crystal. The presence of metallic rhodium NPs is showing diffraction peaks positioned at the Bragg angles (2θ) 40.605° , 42.369° , 66.445° , and 77.540° . The observed peaks correspond to the lattice planes (111), (200), (220), and (311). All diffraction peaks can be well indexed to a face-centered cubic (fcc) lattice system [57]. The Rh peaks are rather broad in general, indicating small particle sizes. By using the Scherrer equation, the mean crystallite size, which was calculated from the full width at half maximum

of the crystalline plane (111), was found to be 38.5 nm for Rh-NPs.

Figures 4a and b show the FE-SEM pictures that extremely dispersed and spherical morphology of Rh-NPs with nanoscale sizes that cover practically the whole electrode. Also, the elemental mapping image of the rhodium atoms shown in Figure 4c confirm its presence. The elemental composition of the surface of Rh-NPs/rGONs/graphite electrode was also investigated by the EDS analysis (Figure 4d). The EDS profile of carbon, oxygen, and rhodium in turn corroborates the successful formation of high-purity components. All results indicate that the rGONs and Rh-NPs were successfully synthesized.

3.3 Electrochemical characterization of the synthesized nanomaterial

EIS, DPV, and CV are the most essential techniques utilized in the development of biosensors and the evaluation of their performance [58]. The CVs of the different graphite electrodes are shown in Figure 5a. The electrochemical output of the modified graphite electrodes was compared to that of the unmodified graphite electrode. The CV technique is extensively implemented for exploratory purposes. The use of this technique in biosensor development is common since the CV technique provides significant information such as types of redox processes present in the analysis and the process reversibility in reactions [59]. Redox peaks in the cyclic voltammogram correlated with the oxidation of ferrocyanide ion and reduction of ferricyanide ion on the graphite electrode surface were seen at 0.26 and 0.17 V, respectively. The peak current for the Rh-NPs/rGONs/graphite electrode was proportionately higher than those of the rGONs/graphite and bare graphite electrodes. This amplification in electrochemical signal could be attributed to increased conductivity in the presence of Rh-NPs and enhanced diffusive mass transport of the anion $[Fe(CN)_6]^{4-}$ reactant to the Rh-NPs/rGONs/graphite electrode surface when compared to other modified and unmodified graphite electrodes. All electrochemical characterizations of the Rh-NPs/rGONs/graphite electrodes were carried out in PBS containing the 5.0 mM Potassium ferri-/ferro-cyanide (+0.1 M KCl) as a solution of the standard electrochemical probe. Figure 5b demonstrates the peak currents in cyclic voltammogram for redox states in the presence of the standard electrochemical probe at the scan rates from 5.0 to 400.0 mV/s. As shown in Figure 5c, there is a relationship of linearity between the square root of the sweep rate and the redox of peak current

Figure 4: (a) and (b) FE-SEM images Rh-NPs/rGONs/graphite electrode with nanometer sized, (c) EDS mapping analysis of rhodium (Rh) element on the rGON-modified graphite electrode, and (d) EDS elemental analysis obtained from Rh-NPs/rGONs/graphite electrode.

density. When the scan rate increases, the anodic and cathodic peak current densities are elevated significantly at the same time with an average correlation coefficient (R^2) value of 0.990, stating that the redox reaction is diffusion controlled [60]. Accordingly, the Rh-NPs/rGONs/graphite electrode was very stable and reproducible. Electrochemical methods based on the pulse techniques like DPV are more sensitive than the linear sweep methods due to possible minimization of the interference capacitive current. The differential pulse voltammograms of bare and modified graphite electrodes are shown in Figure 5d. The pulse techniques are mostly employed for quantitative determinations since DPV has a significantly larger detection limit than other well-known electrochemical techniques due to a greater signal-to-noise ratio.

EIS is a sensitive and precise electrical resistance measurement technique generally employed for the characterization of

modified electrode surfaces or significant change of bulk properties [61]. The electron transfer characteristics between electrolyte and electrode interfacial surface were checked using EIS in a solution of standard electrochemical probe. The charge transfer resistance (R_{ct}) is responsible to control the kinetics of electron transfer of potassium ferri-/ferro-cyanide ionic redox reaction at the electrolyte–electrode interface. As shown in Figure 5e, when the graphite electrode is modified by rGONs and Rh-NPs (denoted as Rh-NPs/rGONs/graphite electrode), it exhibits a semicircle at high frequencies including a small R_{ct} value, which is the result of a process with fast electron transfer [62]. Finally, among the different fabricated graphite electrodes with various modifications, Rh-NPs/rGONs/graphite electrode was used as an optimal platform of g-aptsensor due to its high performance compared to others.

Figure 5: (a) Cyclic voltammograms of different bare and modified graphite electrodes, (b) CV graph characterization of Rh-NPs/rGONs/graphite electrode based on various scan rates of 5, 15, 30, 50, 100, 150, 200, and 400 mV/s, (c) calibration curves of the redox peaks current density against the square root of the sweep rate based on cyclic voltammogram characterization of Rh-NPs/rGONs/graphite electrode, (d) DPV curves of different modified and bare graphite electrodes, and (e) EIS Nyquist plots of different modified and bare graphite electrodes in PBS containing 5.0 mM ferri-/ferro-cyanide (1:1) ion redox as an electrochemical probe.

3.4 Optimization and immobilization of anti-HER2 aptamer on the modified electrode

The predicted secondary structure of anti-HER2 aptameric strand shows double stem-loops and a random sequence is situated in its primary stem-loop structure, anticipated to be bound to the particular place of HER2. The transducer layer is extremely important for immobilization of the aptamer strand and finally stabilization of the formed G-quadruplex, in addition to affecting the sensitivity and selectivity of the aptasensor. It seems that nanomaterials are ideal platforms for immobilization of anti-HER2 aptamer strands due to their privileged biocompatibility as well as the extremely large surface area-to-volume ratio [28]. The interaction between HER2 tumor marker and aptameric strand led to the opening of the anti-HER2 hairpin duplex and the formation of aptamer/HER2 complex (Tabasi *et al.* [26]). Moreover, aptamers are insulating compounds that the phosphate groups in their structures would be ionized into plenty negative charges in the aqueous solution, which barricade the transfer of electron on the surface of the electrode owing to the intense electrostatic repulsion with ferri-/ferro-cyanide ionic redox available in electrochemical probe [63]. As a result, a

remarkable decrease in CV and DPV peak current measurements in g-aptasensor was seen. The difference of electrochemical current density obtained by changing cell concentration was used compared to BSA stabilization step as the measurement system, which is displayed as Δj . The effect of two important experimental parameters, *i.e.*, anti-HER2 aptamer concentration and incubation binding time for HER2⁺ cancer cells were explored. First, the MPA/Rh-NPs/rGONs/graphite electrodes that have already been activated by EDC/NHS were submerged in different concentrations of anti-HER2 aptamer (5, 15, 30, 50, and 100 nM) and dipped in 5% BSA solution and then incubated with 5.0×10^4 cells/mL of SKBR3 cells. As shown in Figure 6a, an increase in the concentration anti-HER2 aptamer up to 50 nM increased the current density. However, no significant change was observed for aptamer concentration greater than 50 nM since the modified surface of the electrode was thoroughly saturated with anti-HER2 aptamer [64]. Accordingly, a concentration of 50 nM anti-HER2 aptamer was selected as the optimal concentration. Second, to gain the most effective of incubation binding time of the HER2⁺ cancer cells, the g-aptasensor for 10, 20, 40, 60, 90, and 120 min in 5.0×10^4 cells/mL SKBR3 cells was incubated. The intensity of current density (*j*) increased

Figure 6: (a) Aptamer concentration and (b) incubation binding time in the presence of 5.0×10^4 cells/mL SKBR3 cells.

with increasing the incubation binding time and then stayed almost steady after 40 min incubation because the surface of the electrode was saturated. Hence, the most favorable incubation time was determined to be 40 min (Figure 6b).

3.5 HER2 sensing performance of aptasensor

Since the selected anti-HER2 aptamer strand is made up of 54 oligonucleotide bases (5'-(NH₂-(CH₂)₆-GGG CCG TCG AAC ACG AGC ATG GTG CGT GGA CCT AGG ATG ACC TGA GTA CTG TCC)-3), optimizing the sensitive layer for g-aptasensor to bind the aptamer strand and immobilize the generated G-quadruplex is very important [28]. This anti-HER2 DNA aptamer sequence was selected by serial evolution of ligands *in vitro* by the exponential enrichment

(SELEX) process. Given their high bio-affinity and intrinsic bio-compatibility, rGONs and Rh-NPs could be used as a nanocomposite platform for immobilizing HER2 aptamer strands, followed by HER2 recognition *via* G-quadruplex formation between aptamer strands and HER2.

Guanine (G)-rich sequences in nucleic acids can assemble into G-quadruplex structures that involve G-quartets linked by loop nucleotides [65]. The main component of a G-quadruplex (G4 motif) is the G-tetrad or G-quartet, a planar arrangement of four guanine bases associated through eight Hoogsteen-type (G:G) hydrogen bonds [66]. Stacked G-quartets generate a central cavity with a strong negative electrostatic potential, where cationic components can be accommodated and thus play an important role in the formation, topology, and stability of the G4 architectures [67]. The remarkable stability of G-quadruplexes owes to a combination of forces including hydrogen bonding, stacking, Van der Waals, ionic, and hydrophobic interactions. Some

G-quadruplex aptamers have shown great biomedical significance due to their potential as potent therapeutic and diagnostic agents [68]. A G-rich DNA aptamer can fold into an intermolecular G-quadruplex, which specifically bind to the target molecule. Therefore, this type of aptamer is considered as an advanced anticancer aptamer and a diagnostic aptamer. The G-quadruplex plays a crucial role in mediated analyte detection by aptamers, which are commonly referred to as aptasensors [69]. Noble metal NPs were extensively combined with aptamers for analyze various target biomolecules because of their unique chemical, physical, and electronic properties. In addition, the exceptional conductivity and catalytic properties of these metals have led them to act as “electronic wires” while increasing electronic transmission between redox centers in proteins and electrode surfaces [70]. To date, noble metal-based aptasensors offer high performance to improve both selectivity and sensitivity by enhancing the tuned signal. Hence, the special focus on these aptasensors has led to the development of many advanced analytical approaches used in

quality control programs for sensitively and selectively recognize diverse targets [71]. As a consequence, the g-aptasensor based on BSA/Apt/MPA/Rh-NPs/rGONs/graphite electrode was employed to evaluate each stage of the detection procedure of various cancer cells.

3.5.1 Selectivity, stability, and detection limit of g-aptasensor for living SKBR3 cells

Living HER2⁺ SKBR3 cancer cells have abundant HER2 tumor marker on their surface. Therefore, an obvious electrochemical signal was obtained when the g-aptasensor was in contact with SKBR3 cells. The DPV value decreased greatly when g-aptasensor was used to detect SKBR3 cells (Figure 7a). It represents that binding specifically takes place between aptamer strands and SKBR3 cells [72]. Concurrently, the cyclic voltammograms results also exhibited good compliance with those of DPV (Figure 7b). To calculate the LOD of fabricated g-aptasensors toward SKBR3

Figure 7: (a) DPV graphs of modified graphite electrode in various stages (10^3 cells/mL of SKBR3 cells), (b) CV plots of modified graphite electrode in various stages (10^3 cells/mL of SKBR3 cells), (c) DPV responses of the g-aptasensor with different concentrations of SKBR3 cells (5, 20, 200, 10^3 , 10^4 , 5.0×10^4 , 10^5 cells/mL), and (d) dependence of current density on concentration changes in the SKBR3 cells (inset: the linear portion of calibration curve).

Table 1: Comparison of the suggested electrochemical g-aptasensor with other biosensors to determine HER2⁺ BC cells

Materials	Technique	Cell type	Linear range (cells/mL)	LOD (cells/mL)	Ref.
Au electrode surface of 100 MHz LiTaO ₃ piezoelectric crystal	Label-free leaky surface acoustic wave (LSAW) aptasensor	MCF7 cells	10 ² –10 ⁷	32	[78]
polydA-aptamer functionalized GO/AuNPs hybrid	Label-free electrochemical aptasensor (LFEA)	MCF-7 cells	10–10 ⁵	8	[79]
GCE/poly(glutamic acid)/MWNT nanocomposite	Labeled electrochemical biosensor (LEB)	MCF-7 cells	10 ² –10 ⁷	25	[80]
Au nanocages (Au NCs)/(MWCNT-NH ₂)	LEB	MCF7 cells	10 ² –10 ⁶	80	[81]
NFG/AgNPs/PANI	LFEA	SKBR3 cells	10 to 5 × 10 ⁶	2	[27]
CDs@ZrHF-MOF	LFEA	MCF-7 cells	10 ² to 10 ⁵	23	[28]
SPCE/HOOC-MBs	LEB	SKBR3 cells	10 ² to 10 ⁵	3	[29]
MPA/Rh-NPs/rGONs/graphite electrode	LFEA	SKBR3 cells	5 to 10 ⁵	1	This work

cells, the current density measurements were carried out at concentrations ranging from 5.0 to 1.0×10^5 cells/mL of SKBR3 cells (Figure 7c). In the linear range from 5.0 to 1.0×10^5 cells/mL, the current density increased in proportion to the concentration of SKBR3 cells. The maximum amount of current density was obtained when the concentration of SKBR3 cells reached 1.0×10^5 cells/mL, and afterward, the current density did not increase considerably, showing that the binding of SKBR3 cells and anti-HER2 aptamer has achieved balance. A linear relationship can be established between the Δj ($I_{(\text{SKBR3})} - I_{(\text{BSA})}$) values and the logarithm of SKBR3 cells (Figure 7d). The linear relationship between Δj and $\log[C_{\text{Cell}}]$ were as follows: Δj ($\mu\text{A}/\text{cm}^2$) = $26.469 + 14.663 \log[C_{\text{Cell}}]$ (cells/mL) and $R^2 = 0.9937$, where $[C_{\text{Cell}}]$ and R^2 are the concentration of SKBR3 cells and correlation coefficient, respectively. The LOD of SKBR3 cells was obtained to be 1.0 cell/mL as determined by the equation of $3.3\sigma/m$, where σ in statistics is a standard deviation of the blank measurement and m is the slope of the calibration curve [73]. LOQ was also obtained 3.0 cells/mL by the equation of $10\sigma/m$ [74]. These significant results were mainly originated from the high stability and sensitivity of g-aptasensor as well as strong bio-affinity and specificity of aptamer. All quantification measurements were repeated at least four times with relative standard deviations (RSD) less than 4.8%. Table 1 lists various types of biosensors for detecting of HER2⁺ BC cells, and the fabricated g-aptasensor outperforms the other biosensors in terms of analytical efficiency, low LOD, and highly good linear range. The superior biosensing performance of g-aptasensor is primarily attributed to the design and the synthesis of high-quality nanomaterials, particularly graphene family and noble metal NPs with high electrical conductivity, large specific surface area, good biocompatibility, chemical inertness, strong capacity to promote electronic transmission, high sensitivity, and selectivity in the electrochemical analysis, ability to form vigorous bonds with thiol moieties in organic compounds, and the ability to form self-assembled monolayers (SAMs) with functional groups in exposed interface layers, all of which are essential parameters for sensitive detection and determination of nonelectroactive compounds such as cells and other biomolecules [75]. Furthermore, because nanomaterials utilized in aqueous solutions have a high stability, it can improve the consistency and conformity of the HER2/aptamer complex interaction [76]. To detect different cancerous and normal cells using the constructed g-aptasensor, the functionalized graphite electrodes were suspended in a determined concentration of cells examined and incubated for 40 min. Afterward, the surface of electrodes was washed by PBS containing 0.05%

Figure 8: (a) The selectivity of g-aptasensor in contact with 5.0×10^4 cells/mL from SKBR3, BT-474, JIMT1, Huvec, and KB cell lines, individually, (b) stability of g-aptasensor for detecting of 5.0×10^4 cells/mL SKBR3 cells within 14 days, (c) reproducibility of g-aptasensor for detecting of 5.0×10^4 cells/mL SKBR3 cells with 4 electrodes, separately; each measurement was repeated at least 3 times. (d) FE-SEM images of SKBR3 cells on the surface of g-aptasensor.

Tween-20 for effective clearance nonspecific adsorbed cells. Then, DPV technique was exerted to measure current density (j) in the operational potential area of -0.2 to $+0.6$ V (scan rate: 50 mV/s) as a consequence of the modality of interaction between specific anti-HER2 aptamer and target cells. The current density against the concentration of different cells was considered as an analytical signal. The selectivity of the g-aptasensor for accurate detection of SKBR3 cell and other types of cell lines including BT-474, JIMT1, Huvec, and KB was examined in the presence of 5.0×10^4 cells/mL for each cell line. The g-aptasensor successfully distinguished between SKBR3 overexpressed HER2⁺ BC cells and BT474 middle expressed HER2⁺ BC cells where the intensity of current density peak for BT474 was measured to be nearly half of the intensity of current density peak in SKBR3 cells with the same concentration (Figure 8a). These results suggested that the selectivity and sensitivity of the fabricated g-aptasensor for HER2 overexpressed and middle expressed BC cells can be predominantly ascribed to the specific binding between anti-HER2 aptamer and HER2⁺

cancer cells [77]. This feature makes the g-aptasensor both sensitive to HER2 tumor marker expression level and selective to HER2⁺ cancer cells. In contrast, the expression of HER2 tumor marker was low in JIMT1 cell line. Thus, unsteady bond between the anti-HER2 aptamer and these HER2-negative (cancer cells with normal HER2 level) cancer cells was observed, and the current density decreased significantly. The same result was achieved for Huvec as a normal cell line. However, the current density was negative for KB cell line primarily owing to the fact that there is no binding between anti-HER2 aptamer and HER2-negative KB cancer cell. This negative current density can be due to the cells and other biomaterials such as BSA and aptamer, which were probably detached in the presence of mild detergent (0.05% Tween-20) and led to a noticeable jump in the RSD value for HER2-negative cancer cells [27]. The long-term stability is an important parameter in clinical applications. As shown in Figure 8b, the signal changes after 14 days are minimal, and this phenomenon indicates that the g-aptasensor has maintained its detection function and its stability

compared to the first day with RSD 4.3%. Moreover, to assess the reproducibility, four electrodes were constructed for the detection of SKBR3 cells, and current density was recorded independently (Figure 8c). The RSD of 5.3% reveals that the reproducibility is adequate. This result implies that the developed g-aptasensor has a high level of reliability and efficiency. FE-SEM picture of fixed SKBR3 cells collected on the electrode surface is shown in Figure 8d.

4 Conclusion

In summary, a novel and well-organized g-aptasensor was constructed for detection and determination of various cancer cells. The collaborative effect of rGONs and Rh-NPs can boost electrochemical signal and sensitivity by increasing conductivity and specific surface area. Also, the stability of the formed G-quadruplex increased through strong covalent interactions between the aptameric strand and HER2 tumor marker. The fabricated of g-aptasensor revealed the excellent LOD value of 1.0 cell/mL toward SKBR3 cells. The results of this study confirmed the successful performance of the constructed aptasensor. In particular, graphene and Rh-NPs hybrid nanocomposites can attract tremendous attention in the fields of electrocatalysis and biosensors. This promising electrochemical aptasensor could feasibly be applied to as a platform for diagnosis and monitoring of a wide variety of cancer cells in different cancers.

Acknowledgements: The authors are much grateful and thank Dr Keivan Majidzadeh-A, and Dr Ramin Sarrami-Forooshani from BCRC, Motamed Cancer Institute, ACECR, Tehran, Iran, for their support in laboratory facilities and providing various cell lines.

Funding information: The authors state no funding involved.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Conflict of interest: The authors state no conflict of interest.

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