Microfluidic Immuno-Biochip for Detection of Breast Cancer Biomarkers Using Hierarchical Composite of Porous Graphene and Titanium Dioxide Nanofibers

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1. INTRODUCTION

Point-of-care detection devices are highly desirable for early diagnosis of diseases, but they require high sensitivity and specificity with minimum sample consumption and easy operation.1,2 The quantification of protein biomarkers for the diagnosis and prognosis of breast cancer, inflammation, and bacterial, viral, and neurodegenerative diseases has received some attention recently.3 In particular, the detection of breast cancer biomarkers at an early stage is extremely important for clinical diagnosis and treatment and thus for patient survival.4

There are four members of the human epidermal growth factor receptor (ErbB) family: ErbB1, ErbB2, ErbB3, and ErbB4.5 ErbB2 (also known as EGFR2 and HER2) is considered a noninvasive tool for early breast cancer diagnosis.6,7 The ErbB2 gene belongs to the mammalian EGFR family and encodes a 185 kDa transmembrane glycoprotein and a receptor tyrosine kinase with intrinsic tyrosine kinase activity.8 Receptor tyrosine kinases have a strong affinity to cell surface receptors. Overexpression (~30%) of several receptor tyrosine kinases in ErbB2 is associated with increasing breast cancer metastasis.9,10 The excessive signaling of ErbB2 is a critical factor in the development of malignant cancerous tumors and also can cause other cancers such as ovarian, bladder, salivary, stomach, and lung carcinomas.10 Several methods, including X-ray

ABSTRACT: We report on a label-free microfluidic immuno-sensor with femtomolar sensitivity and high selectivity for early detection of epidermal growth factor receptor 2 (EGFR2 or ErbB2) proteins. This sensor utilizes a uniquely structured immuno-electrode made of porous hierarchical graphene foam (GF) modified with electrospun carbon-doped titanium dioxide nanofibers (nTiO2) as an electrochemical working electrode. Due to excellent biocompatibility, intrinsic surface defects, high reaction kinetics, and good stability for proteins, anatase nTiO2 are ideal for electrochemical sensor applications. The three-dimensional and porous features of GF allow nTiO2 to penetrate and attach to the surface of the GF by physical adsorption. Combining GF with functional nTiO2 yields high charge transfer resistance, large surface area, and porous access to the sensing surface by the analyte, resulting in new possibilities for the development of electrochemical immunosensors. Here, the enabling of EDC–NHS chemistry covalently immobilized the antibody of ErbB2 (anti-ErbB2) on the GF–nTiO2 composite. The sensor underwent differential pulse voltammetry and electrochemical impedance spectroscopy to quantify breast cancer biomarkers. The two methods had high sensitivities of 0.585 μM−1 cm2 and 43.7 kΩ μM−1 cm2 in a wide concentration range of target ErbB2 antigen from 1 × 10−15 M (1.0 fM) to 0.1 × 10−6 M (0.1 μM) and from 1 × 10−15 M (0.1 pm) to 0.1 × 10−6 M (0.1 μM), respectively. Utilization of the specific recognition element, i.e., anti-ErbB2, results in high specificity, even in the presence of identical members of the EGFR family of receptor tyrosine kinases, such as ErbB3 and ErbB4. Many promising applications in the field of electrochemical detection of chemical and biological species will derive from the integration of the porous GF–nTiO2 composite into microfluidic devices.

KEYWORDS: cancer immunodiagnosics, microfluidics, carbon-doped titanium dioxide, graphene foam, biosensor, electrochemical detection

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mammography, magnetic resonance imaging, computed tomography, and ultrasound imaging, are commonly used to monitor cancerous tumors.21 However, these technologies rely heavily on imaging to determine tumor status and thus provide limited information about the onset of cancer and limited monitoring of early stage cancerous cells. Other methods for cancer detection, such as enzyme-linked immunosorbent assay (ELISA),12 immunoblotting, and immunohistochemistry,15 require complex purification and pretreatment processes or are used as semiquantitative methods. The limits of state-of-art commercial immunoassays such as Abbott IMX,14 Roche Elecsys,15 and Hybritech Tandem R16 for detecting various magnetic beads to detect the cancer biomarker PSA. Yu et al.17 nanoparticles combined with multiple-enzyme-labeled antibody electrochemical immunosensor that uses densely packed gold platforms capable of detecting breast cancer biomarkers at a detection limit of 200 ng mL$^{-1}$ via DNA hybridization. Gao et al.26 reported on impedimetric detection of cancerous molecules using carbon nanotubes, modified with gold nanoparticles, as an electrochemical electrode for immobizalizing anticarcinoembryonic antigen, resulting in a detection limit of 0.06 ng mL$^{-1}$ in a range of 0.1–160 ng mL$^{-1}$.

Functional graphene has received considerable attention because of its large surface area, superior electroactivity, ease of functionalization with antibodies and other bioreceptors, and high mechanical strength.27,28 In addition, because of its excellent electrical conductivity, graphene can act as an “electron wire” whereby it establishes a conduction path for rapid electron transfer for selective detection of target species.28,29 Recently, 3D hierarchical porous graphene foam (GF) capable of rapidly transferring electrons along the continuously interconnected graphene building blocks was realized via a template-directed chemical vapor deposition method.30,31 Its large surface area (up to $\sim 850$ m$^2$ g$^{-1}$) and porosity make GF suitable for integration with nanostructured materials without agglomeration.32 With a pore diameter of 50–250 μm, GF is an ideal scaffold for the fabrication of monolithic composite electrodes. Nanostructure-based GF composites have been used in devices such as supercapacitors,33 field emitters, and photocatalysts.34 Deposition of vertically aligned ZnO nanowires on GF has resulted in the detection of uric acid, dopamine, and ascorbic acid with nanomolar sensitivity.35 Co$_3$O$_4$36 and Mn$_3$O$_4$36 nanostructures also have been grown in GF for electrochemical glucose biosensors with high sensitivity and improved detection limits. Thus, the synergistic effect from the integration of nanostructures with GF has shown that there is great potential for the improvement of biosensing efficacy.

Recently, highly sensitive chemical and biological sensors37–39 have become possible because of nanostructured metal oxide materials, particularly those in the form of 1D fiber or wire. Their reduced size, high aspect ratio, dimensions comparable to the Debye length, and fast transfer of electrons resulting from molecular recognition events along the entire

Figure 1. (a) Schematic of the microfluidic immunosensor with 3D porous GF electrode modified with carbon-doped TiO$_2$ nanofibers for the detection of breast cancer biomarkers. (b) Photo of two microfluidic immunosensors. (c) Schematic representation of the fabrication of the microfluidic sensor.
fiber make these materials good candidates for sensors. In addition, oxygen-vacancy-induced point defects in TiO$_2$ are within the bridging oxygen rows of the (110) surface, resulting in the potential to be stabilized with adsorbates on the surface. TiO$_2$ nanofibers (nTiO$_2$) have excellent charge transfer resistance, insignificant protein denaturation, high structural uniformity, long-term chemical stability, and biocompatibility. Thus, a TiO$_2$ nanowire-based immunosensor has been developed to rapidly detect Listeria monocytogenes with high sensitivity.

In this paper, we report on a microfluidic electrochemical sensor for the detection and immunodiagnosis of breast cancer biomarkers via antigen–antibody interactions. The sensor contains a unique 3D immunoelectrode consisting of a T-shaped porous GF structure modified with carbon-doped nTiO$_2$ and anti-ErbB2 molecules. This immunoelectrode, the working electrode of the sensor, is hung from the upper side of a microfluidic channel above the counter electrode positioned on the bottom of the channel (Figure 1). The GF–nTiO$_2$ composite-based electrode integrated in the microfluidic device has a large specific surface area, 3D conductive pathways, and porous structure and provides remarkable detection of breast cancer biomarkers. In general, direct immobilization of biomolecules on the nTiO$_2$ surface via physical adsorption due to the low isoelectric points of nTiO$_2$ (~5.5; negatively charged) and anti-ErbB2 (~5.8; negatively charged) is a major limitation. The carbon contents available in the GF–nTiO$_2$ composite allow for covalent binding with antibody molecules via the EDC–NHS coupling mechanism. The amidation coupling reactions can cause the formation of amide bonds between the antibody and the GF–nTiO$_2$ matrix, resulting in an immunosensor with high stability and enhanced loading capacity for anti-ErbB2. In general, microscale sensors can provide superior performance compared with their large-scale counterparts. A miniaturized microfluidic device often allows for reduced consumption of agent and reagent, fast detection, well-controlled microenvironment, and high portability. In addition, microfluidic integration with various sensing mechanisms may provide compact platforms for multiplexed detection of biological analytes from complex mixtures to realize high-throughput assays. Specifically, the integration of the T-shaped porous immunoelectrode into a microfluidic channel and other microelectrodes results in a miniaturized sensor that requires reduced volumes of target fluids and chemical reagents. The porous nature of the immunoelectrode provides necessary microscale passages for efficient handling of solutions during functionalization and detection inside a microfluidic channel.

To detect interfacial changes originating from biorecognition events at the proposed working electrode, we used electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) with this microfluidic sensor. The incorporation of the semiconductor nTiO$_2$ into the porous GF improves the impedance signal in the EIS method for detecting the ErbB2 antigen. However, the low conductivity of nTiO$_2$ yields an inadequate response for DPV. Thus, a coating of nTiO$_2$ on highly conductive GF scaffolds can obviate this problem. Our microfluidic immunosensor with the unique GF–nTiO$_2$ structure is able to detect ErbB2 in a wide concentration range (1.0 fM to 0.1 μM) and provides high stability, reproducibility, and selectivity, even in the presence of nonspecific antigen molecules such as ErbB3 and ErbB4.

2. EXPERIMENTAL SECTION

2.1. Chemicals.

3D multilayer GF (99% carbon content; thickness = 1.2 mm; density = 4.0 mg/cm$^3$; pore size = 580 μm) was purchased from Graphene-Supermarket (Graphene Laboratories, Calverton, NY, USA). Titanium(IV) isopropoxide ([Ti(OiPr)$_4$]$_2$) was the sol–gel precursor material. This improved process has been described previously. In brief, 1.8 g of PVP was mixed with 30 mL of ethanol and stirred for 5–7 min. Separately, 6 g of Ti(OiPr)$_4$ was added to 24 mL of a solution of glacial acetic acid and ethanol, 1:1 in a volume ratio of 1:1. The mixture was stirred and then mixed with the PVP solution. Then, 60 mL of this combined solution was magnetically stirred for 3.5 h and immediately loaded into a plastic medical syringe with a 26-gauge metallic needle. The needle was attached to a high-voltage dc power supply (Gamma High Voltage, Ormond Beach, FL, USA) that could produce voltages up to 30 kV. The solution feeding rate was controlled by a syringe pump (Harvard Apparatus, Holliston, MA, USA). Fiber spinning began upon immediate application of voltage. The fibers were electrosprinn on a rotating cylindrical electrode that was covered with aluminum foil and positioned horizontally ~5–7 cm from the needle tip. The electrosprun Ti(OiPr)$_4$/PVP polymeric composite nanofibers were then collected and stabilized overnight in an air oven at 50 °C. Finally, the fibers were placed in a furnace with a 2 °C/min ramp rate and subjected to controlled calcination at 400 °C for 3 h to selectively eliminate the PVP component from the nTiO$_2$.

The controlled synthesis of carbon-doped nTiO$_2$ has been previously discussed. To dope the synthesized nTiO$_2$ with carbon, the electrosprun Ti(OiPr)$_4$/PVP polymeric nanofibers were heat-treated at 350–400 °C for 2.5 h in the presence of air. The heat treatment converted the Ti(OiPr)$_4$/PVP polymeric composite nanofibers into pure anatase nTiO$_2$. The selective removal of PVP by the controlled heating produced residual carbon, which acts as the dopant, the amount of which was optimized via the calcination process. Raman studies have confirmed the existence of amorphous carbon in nTiO$_2$ owing to inadequate removal of the polymer (Figure S1). In Raman spectra, the appearance of 1353 and 1598 cm$^{-1}$ peaks in carbon-doped nTiO$_2$ indicates D and G bands, respectively. Raman spectra also confirm the presence of anatase nTiO$_2$ and four robust peaks at 139.5, 392, 511, and 632 cm$^{-1}$. The peaks at 139.5 and 632 cm$^{-1}$ correspond to the E$_g$ mode of phonon vibration, and the peaks at 392 and 511 cm$^{-1}$ correspond to the A$_1g$ and E$_g$ modes, respectively. These peaks are indicative of the existence of the anatase phase in nTiO$_2$.

2.3. Instruments.

The morphologies of the synthesized nTiO$_2$, GF, and GF–nTiO$_2$ composite were characterized using scanning electron microscopy (SEM, Supra 400VP, Zeiss, Oberkochen, Germany). The morphology of the nTiO$_2$ was further characterized.
Figure 2. Functionalization of anti-ErbB2 molecules on the surface of GF and GF–nTiO2 electrodes using EDC–NHS chemistry followed by oxygen plasma treatment.

using transmission electron microscopy (TEM, Tecnai G2, FEI, Hillsboro, OR, USA). X-ray photoelectron spectroscopy (XPS) measurements were performed on nTiO2, GF, GF–nTiO2, and anti-ErbB2/GF–nTiO2 samples using an Amicus/ESCA 3400 instrument (Kratos, Manchester, UK). These samples were irradiated with 240 W unmonochromatized Mg Kα X-rays, and the energy of the photoelectrons emitted at 0° normal to the surface was analyzed using a DuPont analyzer (DuPont, Wilmington, DE, USA). The pass energy was set at 75 eV, a Shirley baseline was subtracted from all reported spectra, and CasaXPS was used to process the raw data files. A Zive Potentiostat SP1 (eDAQ, Colorado Springs, CO, USA) measured EIS and DPV in PBS (pH 7.4) containing 5 mM of both [Fe(CN)6]3− and [Fe(CN)6]4− and KCl (0.1 M) for 2 min to obtain the Ag/AgCl electrode.

2.4. Device Fabrication. Soft lithography was used to fabricate a PDMS channel (10 mm long, 1.5 mm wide, and 1.5 mm deep, Figure 1c). The channel pattern was designed by AutoCAD software (Autodesk, San Rafael, CA, USA), and then a master mold was formed in a poly(methyl methacrylate) (PMMA) slab using a high-precision milling machine (CNC Masters, Irwindale, CA, USA). Two-part cured PDMS precursor solution (A/B weighing ratio = 10:1) was mixed and then degassed in a homemade chamber for 30 min under vacuum (10−3 Torr). The degassed PDMS solution was then poured over the master mold in a disposable polystyrene Petri dish (diameter = 100 mm; Sigma-Aldrich) and baked on a hot plate at 80 °C for 2 h. After heat curing, the PDMS channel device was peeled off the master mold. The channel was utilized to direct various solutions to the sensing area and further to the waste reservoir external to the device.

Meanwhile, a Au counter electrode and a Ag/AgCl reference electrode were fabricated on a glass slide (50 mm × 75 mm; thickness = 0.9 mm; Dow Corning). In this step, an 80 nm thick Au thin film was deposited on a glass slide by e-beam evaporation and then patterned by conventional photolithography using a photoresist (AZ 5214 E). The patterned photoresist was baked on a hot plate at 90 °C for 3 min. The wafer was then immersed in AZ photoresist developer solution (Microchem, Westborough, MA, USA) for 2 min. Next, the Au thin film was selectively etched using Au etchant (GE-8148), and the photoresist was stripped off with acetone. The wafer was washed off with DI water and then air-dried. The Ag electrode was formed using the same fabrication procedure as that for the Au electrode, except for the deposition of a 700 nm thick Ag film onto the glass slide containing the fabricated Au electrode and etched with Ag etchant (Silver Etchant TFS). The final product was treated with KCl (0.1 M) for 2 min to obtain the Ag/AgCl electrode.

The T-shaped GF electrode was modified with nTiO2 and functionalized with anti-ErbB2. nTiO2 powder (4 mg) was dispersed in 2 mL of pure ethanol, and the solution was sonicated for 1 h at 25 °C. The solution (50 µL) was then spread onto the surface of the GF electrode. Due to the high porosity of GF, the nTiO2 suspension easily penetrated the GF electrode. The nTiO2 attached onto the surface of the GF scaffold through physical adsorption. The modification of the GF with nTiO2 increased the total surface area of the GF, thus increasing the ability to load antibodies and potentially increase the electrochemical output signals. Before immobilization of anti-ErbB2, the GF–nTiO2 electrode was treated with oxygen plasma for 15 s to oxidize carbon and create carboxyl (–COOH) groups on its surface. These –COOH groups were then activated by EDC–NHS coupling. EDC is a cross-linking agent used to couple the –COOH groups on the GF–nTiO2 surface to primary amines of anti-ErbB2 (–NH2), and NHS is an activator used to increase the stability of the active esters formed during the coupling reactions. For EDC–NHS coupling, 10 µL of EDC (0.2 M) solution was mixed with 10 µL of NHS (0.05 M) and spread onto the surface of the GF–nTiO2 electrode. The electrode was kept in a humidity chamber (100% relative humidity) for 4 h and then was washed with PBS. Subsequently, 20 µL of anti-ErbB2 solution (0.24 µM) was spread on the surface of the GF–nTiO2 electrode by drop-casting. Again, the electrode was kept in a humidity chamber at 4 °C for 4 h and then was washed with PBS (pH 7.4).

Figure 2 is a schematic of the functionalization of anti-ErbB2 molecules on the surface of both the GF and the GF–nTiO2. The –NH2 amine groups of the anti-ErbB2 were bound covalently with the –COOH groups from the GF and GF–nTiO2 by forming C–N amide bonds. The nonspecific sites of anti-ErbB2/GF–nTiO2 were blocked by treatment with BSA (2 mg/mL). Thus, the T-shaped BSA/anti-ErbB2/GF–nTiO2 immunoelectrode was formed.

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To attach the anti-ErbB2/GF−nTiO$_2$ immunoelectrode (WE) to the top of the PDMS channel, we punched a hole through the top PDMS layer and inserted the upside-down T-shaped immunoelectrode into the hole so that the horizontal part was inside the channel and the vertical part was above the PDMS channel for external electrical connections (see Figure 1a). Next, a drop of degassed PDMS precursor solution was placed carefully in the hole and cured at room temperature. Oxygen plasma treatment permanently bonded the glass substrate containing the counter (CE) and reference (RE) electrodes and the PDMS slab containing the immunoelectrode. Thus, the WE hung from the top PDMS layer was parallel to the Au CE and ∼300 μm away. The parallel layout of the WE and CE inside the microfluidic channel reduces the size of the device and may also provide fast transport of electrons 48 due to redox reactions toward the WE resulting in increased electrochemical signals.

3. RESULTS AND DISCUSSION

3.1. Microscopic Analyses. Field-emission SEM (FESEM) imaging was used to study the surface morphologies of the nTiO$_2$, GF, and GF−nTiO$_2$ composite. Figure 3a shows the solid, rodlike nTiO$_2$, which have an average diameter of 72 nm and are a few micrometers long. The TEM image in Figure 3b shows that each nanofiber comprises a number of TiO$_2$ grains. The pore size of the GF ranges from 500 to 600 μm (Figures 3c), which is large enough for the nanofibers to penetrate (Figure 3d). Figure 3d–f show that the GF scaffold is almost entirely covered by randomly oriented nTiO$_2$. This coating increases the ability of the immunoelectrode to load antibodies via covalent interactions.

3.2. Spectroscopic Studies. XPS was used to confirm the presence of the functional groups in the carbon-doped nTiO$_2$, GF, and GF−nTiO$_2$, and the formation of amide between antibodies and GF−nTiO$_2$. The C 1s, O 1s, and Ti 2p peaks in the wide-scan spectrum of nTiO$_2$ indicate the formation of carbon dopant in nTiO$_2$ (Figure S2a). The O peak at 532 eV is from the formation of the Ti−O bond. The core-level spectra of C 1s were deconvoluted into characteristic peaks using a Shirley baseline with a Gaussian profile (Figure S2b). The nTiO$_2$ peaks at 284.6, 287.0, and 288.9 eV correspond to the C−C neutral bond and oxidized C species such as C−O and −COOH, respectively.49 The presence of these peaks may be due to the formation of carbonate-like species by interstitial carbon in the nTiO$_2$ lattice. The wide-scan spectra of GF showed the C 1s and O 1s peaks, and after coating the GF−nTiO$_2$ composite with anti-ErbB2, the N 1s peak appeared (Figure S3a). The core-level spectra of the GF−nTiO$_2$ composite had a Ni 2p peak at 852−858 eV as a result of
the use of Ni template during the synthesis of GF (Figure S3b).

The peaks at 852.3 and 856.9 eV are due to Ni 2p\(^{1/2}\) and Ni 2p\(^{3/2}\) in the GF−nTiO\(_2\) composite. The Ti 2p peak was deconvoluted into characteristic peaks (Figure S3c). The Ti 2p\(^{1/2}\) and Ti 2p\(^{3/2}\) peaks for nTiO\(_2\) due to spin−orbit splitting photoelectrons were located at 465.4 and 459.4 eV, respectively, indicating the existence of Ti\(^{4+}\) ions (higher oxidation state) in nTiO\(_2\).\(^{49}\)

The XPS C 1s peaks at 284.3 and 285.2 eV for the GF electrode alone, without nTiO\(_2\) and anti-ErbB2, correspond to the core-level spectra of carbon due to the graphitic nature of sp\(^2\) (C−C) and a defect (sp\(^3\), C≡O), respectively (Figure 4a, bottom graph).\(^{50}\) After the GF surface was modified with nTiO\(_2\), an additional peak occurred for C 1s at 288.9 eV, indicating the presence of C≡O groups wherein the atomic ratio of the peak due to C≡O is reduced 6.7% (Table S1). After the functionalization of the antibodies on the oxygen-plasma-treated GF−nTiO\(_2\) by EDC−NHS chemistry, the binding energy of the C−O peak shifted by 1.1 eV, and there were two additional peaks, at 287.1 and 290.0 eV, corresponding to N−C=O and carboxylic groups (O−C=O), respectively. The formation of an amide bond (N−C=O) is the result of the covalent interactions between −COOH groups on GF−nTiO\(_2\) and −NH\(_2\) groups on anti-ErbB2. Table 1 presents the percent relative atomic concentration, the full width at half-maximum (fwhm), and the peak position of the deconvoluted C 1s, O 1s, and N 1s peaks in electrodes of various compositions. The O 1s peaks at 532.6 and 532.1 eV correspond to C−O and C≡O, respectively, indicating the presence of defects in GF. With antibody loading, these peaks shifted to higher binding energies. Figure 4c shows the N 1s core-level spectra for GF−nTiO\(_2\) with and without anti-ErbB2. In addition, there were no peaks for electrons of N 1s in the GF−nTiO\(_2\) composite alone. The three peaks at the binding energies of 398.3, 401.3, and 402.7 eV for the anti-ErbB2/GF−nTiO\(_2\) are assigned to the core-level electron of N 1s, the amide N (CO−NH) present in the antibody/matrix, and the \(\equiv\)N species, respectively.\(^{50}\) This result indicates the successful functionalization of anti-ErbB2 molecules on the surface of the GF−nTiO\(_2\) electrode.

### 3.3. Electrochemical Characterization.

The fabricated electrodes within their corresponding devices underwent EIS as a function of frequency (1.0 to 100 × 10\(^3\) Hz) to investigate properties, such as charge transfer resistance (\(R_{ct}\)) and double-layer capacitance (\(C_{dl}\)). The results are presented in Table 1.

#### Table 1. Charge Transfer Resistance (\(R_{ct}\)), Double-Layer Capacitance (\(C_{dl}\)), HET Rate Constant (\(k_s\)), Time Constant (\(\tau\)), and Surface Coverage Percentage (\(\theta\)) of the Fabricated Electrodes

<table>
<thead>
<tr>
<th>Electrodes</th>
<th>(R_{ct}) (kΩ)</th>
<th>(C_{dl}) (μF)</th>
<th>(k_s) (cm s(^{-1}))</th>
<th>(\tau) (μs)</th>
<th>(\theta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF</td>
<td>29.8 ± 0.2</td>
<td>21.5 ± 0.07</td>
<td>18.6 ± 0.1 × 10(^{-6})</td>
<td>0.64 ± 0.007</td>
<td>NA</td>
</tr>
<tr>
<td>anti-ErbB2/GF</td>
<td>45.3 ± 0.1</td>
<td>11.03 ± 0.4</td>
<td>12.2 ± 0.01 × 10(^{-6})</td>
<td>0.49 ± 0.008</td>
<td>34.2 ± 0.3%</td>
</tr>
<tr>
<td>GF−nTiO(_2)</td>
<td>86.7 ± 0.2</td>
<td>4.2 ± 0.09</td>
<td>6.4 ± 0.09 × 10(^{-6})</td>
<td>0.36 ± 0.004</td>
<td>NA</td>
</tr>
<tr>
<td>anti-ErbB2/GF−nTiO(_2)</td>
<td>99.9 ± 0.7</td>
<td>3.9 ± 0.03</td>
<td>5.5 ± 0.03 × 10(^{-6})</td>
<td>0.38 ± 0.001</td>
<td>70.1%</td>
</tr>
<tr>
<td>BSA/anti-ErbB2/GF−nTiO(_2)</td>
<td>152.6 ± 1.2</td>
<td>3.2 ± 0.01</td>
<td>3.6 ± 0.05 × 10(^{-6})</td>
<td>0.48 ± 0.003</td>
<td>80.4 ± 0.7%</td>
</tr>
</tbody>
</table>
nTiO2 coating on the GF scaffold, which hinders electron transfer. Interestingly, the carbon-doped electrodes further enhanced the value of \( R_{\text{ct}} \) at high frequencies and implies a charge-transfer-limited behavior. The values of \( R_{\text{ct}} \) and \( C_{\parallel} \) can be obtained from the real \( (Z') \) and imaginary \( (Z'') \) components of complex impedance at different frequencies for a parallel RC circuit using:

\[
Z(\omega) = R_+ + \frac{R_{\text{ct}}}{1 + j\omega R_{\text{ct}} C_{\parallel}} = R_+ + \frac{j\omega R_{\text{ct}}^2 C_{\parallel}}{1 + \omega^2 R_{\text{ct}}^2 C_{\parallel}^2}
\]

The frequency associated with the maximum \( Z'' \), \( f_{\text{max}} \), and the time constant \( \tau \) can be calculated using:

\[
R_{\text{ct}} C_{\parallel} = \frac{1}{2\pi f_{\text{max}}} = \tau
\]

The GF-based device had an \( R_+ \) of 29.8 kΩ. However, after modification of GF with anti-ErbB2, the increase in \( R_+ \) to 45.3 kΩ was due to the formation of functional groups, e.g., -COOH, C=N, and C=O, on the GF via plasma oxidation and EDC–NHS treatment, as evidenced by XPS (Figure 4), as well as to the insulating nature of ErbB2 antibodies at the GF, which hinders electron transfer. Interestingly, the carbon-doped nTiO2 coating on the GF scaffolds enhanced the value of \( R_\parallel \) by nearly 3 orders of magnitude (87.7 kΩ) because of the semiconducting properties of the oxygenated nTiO2 compared to those of the GF alone. The porous GF provides easy access for nTiO2 to reach the interior scaffolds, thus enhancing the loading capacity of the GF for anti-ErbB2. After immobilization of the immunoelectrode with BSA proteins, \( R_\parallel \) increased to 152.6 kΩ because of the insulation provided by the proteins, which obstructed the redox conversion of [Fe(CN)]\(^{3-}/4^-\). The heterogeneous electron transfer (HET) rate constant \( (k_s) \) for all the electrodes is defined as:

\[
k_s = \frac{RT}{n^2 F^2 A R_C C}
\]

where \( R \) is the gas constant (8.314 J/K mol), \( T \) is the temperature (298 K), \( n \) is the electron transfer constant of the redox couple, i.e., 1, \( F \) is Faraday’s constant (96485.3 s A/mol), \( A \) is the area of the electrode (0.096 cm\(^2\)), and \( C \) is the concentration of the redox couple (5 mM) in the surrounding solution. \( k_s \) of the GF electrode was higher (18.6 \( \times 10^{-6} \) cm s\(^{-1}\)) than those of the anti-ErbB2-immobilized GF and the nTiO2-modified GF (Table 1). BSA and anti-ErbB2 loaded onto the GF–nTiO2 electrode further reduced \( k_s \) to 3.6 \( \times 10^{-6} \) cm s\(^{-1}\), which indicates a reduced electron transfer process inside the GF. Interestingly, the time constant \( \tau \), as defined by eq 2, for the GF electrode was higher than that for the GF–nTiO2 electrode (Table 1), indicating a fast reaction for redox conversion. However, the immunoelectrode BSA/anti-ErbB2/GF–nTiO2 had a high \( \tau \) compared to those of the other electrodes (GF, GF–nTiO2, anti-ErbB2/GF, and anti-ErbB2/GF–nTiO2) presumably because of the slow diffusion of [Fe(CN)]\(^{3-}/4^-\) ions at the protein layer/solution interface of the electrode (Table 1). The surface coverage of anti-ErbB2 and BSA can be estimated using the relationship \( \theta = 1 - R_{\text{ct}(\text{ele})}/R_{\text{ct}(\text{ele+bio})} \), where \( \theta \) is the fraction of the occupied binding sites and \( R_{\text{ct}(\text{ele})} \) and \( R_{\text{ct}(\text{ele+bio})} \) are the charge transfer resistances of the electrode before and after immobilizing biomolecules are present, respectively. The \( \theta \) of the GF-based immunoelectrode with and without nTiO2 was 70.1 and 34.2%, respectively. Incorporation of nTiO2 into the GF may be responsible for the higher coverage of anti-ErbB2 on the GF electrode surface. The BSA/anti-ErbB2/GF–nTiO2 immunoelectrode had the maximum \( \theta \) of 80.4% after BSA immobilization. The higher \( k_s \) of the BSA/anti-ErbB2/GF–nTiO2 electrode (3.6 \( \mu \)cm/s) compared to that of other reported electrodes such as BSA/anti-ErbB2/ZnO (1.11 \( \mu \)cm/s)\(^{11}\) and enzyme/TiO2–CH (0.17 \( \mu \)cm/s)\(^{35}\) indicates higher electron transfer kinetics of the immunoelectrode used in this sensor. Figure Sb shows the DPV signals at the sensors integrated with the different fabricated electrodes. Due to the higher overall conductivity of the GF, the peak current of DPV for the GF electrode (77.5 \( \mu \)A) was more than three times that for the GF–nTiO2 electrode (25 \( \mu \)A) and was also higher than that for the anti-ErbB2/GF electrode (63.4 \( \mu \)A). The nTiO2 on the GF

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surface reduced the electron transfer rate, as was evident by the smaller $k_s$ obtained in the EIS studies, thus resulting in the lower electrochemical current. Furthermore, the peak current decreased with the incorporation of anti-EbrB2 (21.7 $\mu$A) and BSA molecules (18.5 $\mu$A) onto the GF$^-n$TiO$_2$ electrode. This is attributed to the presence of functional groups such as $-\text{COOH}$, $\text{C}-\text{OH}$, and $\text{N}-\text{C}=\text{O}$ at the anti-EbrB2- and BSA-immobilized GF$^-n$TiO$_2$ immunoelectrodes. The reduction of the current is indicative of the immobilization caused by proteins on the surface of GF$^-n$TiO$_2$.

We have carried out the experiments to investigate DPV responses of our immunosensor under different conditions, including temperature, pH of PBS solution, and concentration of antibody for immobilization. First, to optimize temperature, the microfluidic sensor was tested under different temperatures using a hot plate. Figure S4a presents the DPV responses of the sensor to 0.1 $\mu$M concentration of ErbB2 antigen at different temperatures from 20 to 45 °C. This sensor provided a maximum peak current at 30 °C. Therefore, all sensing measurements were conducted at 30 °C. Second, Figure S4b shows the influence of pH values for the PBS solution on DPV responses of the sensor. Similarly, 0.1 $\mu$M concentration of ErbB2 antigen was used in this experiment. The maximum peak current was found at pH 7.4 of PBS solution, so we performed all sensing measurements at pH 7.4. In addition, we optimized the concentration of anti-ErbB2 used for immobilization. Figure S4c shows the DVP responses of the sensor functionalized with different anti-ErbB2 concentrations when responding to 0.1 $\mu$M concentration of ErbB2. The result shows that the DPV peak current decreased with increasing concentration of antibody molecules. This may perhaps be a consequence of loading more antibody molecules on the sensor surface that allowed capturing more ErbB2 antigen molecules, resulting in a decreased current. When the concentration of antibody solution went beyond 0.24 $\mu$M, the peak current was found to saturate. We thus chose 0.24 $\mu$M concentration of antibody for immobilization.

To investigate the redox property of the sensor with the BSA/anti-ErbB2/GF$^-n$TiO$_2$ electrode, we conducted cyclic voltammetry (CV) studies at different scan rates (Figure S5a). This sensor exhibited well-defined oxidation and reduction properties of the ferro/ferri cyanide mediator used. By increasing the scanning rate from 20 to 500 mV/s, the oxidation and reduction currents increased and decreased, respectively (Figure S5b), and the oxidation and reduction peak potentials shifted linearly in the positive and negative potential directions, respectively (Figure S5c). This indicates that the diffusion is surface-controlled, and the process is quasi-reversible. The linear equations, slope, regression coefficients, and intercept values for the anodic and cathodic peak currents and the anodic and cathodic potentials are given in the inset of Figure S5a,b, respectively. The diffusion coefficient ($D$) of redox species $[\text{Fe(CN)}_6]^{3-/-4-}$ for the fabricated electrodes can be obtained using the Randles-Ševčik equation:\textsuperscript{52} $i_p$ (peak current) = $269 \times 10^3 n^{3/2} A D^{1/2} C_{\text{red}}^{1/2}$, where $n$ is the number of electrons transferred in the redox reaction (here, $n = 1$), $A$ is...
the area of the electrode (cm²), $D$ is the diffusion coefficient (cm²/s), $C$ is the surface concentration (mol/cm²), and $v$ is the scanning rate (mV/s). The value of $D$ was found to be 15.6, 1.14, 0.636, and 0.207 μ cm²/s for the GF, GF–nTiO₂, anti-ErbB2/GF–nTiO₂, and BSA/anti-ErbB2/GF–nTiO₂ electrodes, respectively. The incorporation of nTiO₂ into the GF scaffolds resulted in a lower diffusion coefficient compared to the GF electrode alone because of the opposite charges between nTiO₂ and [Fe(CN)₆]³⁻/⁴⁻. The BSA/anti-ErbB2/GF–nTiO₂ electrode had a $D$ value comparable to those of electrodes of other materials, such as ZnS–rGO (6.3 μ cm²/s)⁵³ and CysCdS–Au (7.45 μ cm²/s).⁵⁴

### 3.4. Detection of Cancer Biomarker

The microfluidic sensor was tested with regard to how well it detected the breast cancer antigen ErbB2 using the DPV and EIS methods. The concentration of ErbB2 was varied from 1 × 10⁻¹⁵ to 1 × 10⁻⁷ M or 1.0 fM to 0.1 μM. Figure 6a shows the DPV for different ErbB2 concentrations from 1.0 fM to 0.1 μM in the voltage range of 0.3–0.9 V. The sensing potential in the DPV experiments was 0.42 V, at which the maximum peak current occurred. The sensor calibration plot in Figure 6b shows that the DPV peak current was inversely proportional to the logarithmic concentration of ErbB2. The insulating property of the immunocomplex layer formed on the sensor surface barricaded the generated electrons, resulting in a lower current. As the number of ErbB2 molecules bound to the sensor surface increased, the thickness of the insulating layer increased, thus reducing the voltammetric output current. Figure 6b shows that the sensitivity of 0.585 μA μM⁻¹ was obtained when the sensor was exposed to a wide detection range (1.0 fM to 0.1 μM). Thus, the sensor, in conjunction with the DPV measurement method, offered higher sensitivity (0.585 μA μM⁻¹) in the detection of ErbB2 molecules than that of another sensor that also uses DPV but has ZnO nanowire as electrode material.⁵⁵ (Table 2). However, the sensitivity of our sensor was lower than those of other sensors that use gold nanoparticles⁶¹ and graphene nanosheets.⁵⁷

A control experiment for the sensor was carried out with (5 mM) and without Fe(CN)₆³⁻/⁴⁻. Without Fe(CN)₆³⁻/⁴⁻, the impedance response exhibited a nearly straight line with a high $R_{ct}$ value of 1.5 MΩ. However, after adding 5 mM concentration of Fe(CN)₆³⁻/⁴⁻, the sensor provided a semicircle Nyquist plot with a reduced $R_{ct}$ value of 202 kΩ owing to the presence of Fe(CN)₆³⁻/⁴⁻ mediator. Figure 6c shows the Nyquist plots obtained from the EIS measurements at different concentrations of ErbB2 antigen. Table S2 shows the EIS parameters of $R_{ct}$, $R$, and $C_{dl}$ obtained at different concentrations of target ErbB2 antigen. These parameters were extracted from the simulated EIS curves, and one example of how the parameters were extracted from the Nyquist plot is given in Figure S7. Figure 6d shows that $R_0$ increased with increasing concentration of ErbB2. This occurred essentially because of the interaction between anti-ErbB2 and ErbB2 at the binding sites, i.e., paratope for antibody and epitope for antigen, on the sensor surface, which formed the immunocomplex seen in Figure 2. The higher $R_0$ indicates that the presence of the insulating layer of ErbB2 on the anti-ErbB2/GF–nTiO₂ electrode impeded the penetration of the redox species, as indicated by the larger diameter of the semicircle of the Nyquist plot (Figure 6c) and the lower $k$ value (Table 1). The negatively charged ErbB2 on the sensor surface repelled the generated electrons, resulting in a higher impedance signal.⁵⁸

The response of $R_{ct}$ for this sensor showed two linear regions of concentration of ErbB2, i.e., from 1 × 10⁻¹⁵ to 1 × 10⁻¹² M (or 1.0 fM to 1.0 pM) and from 1 × 10⁻¹¹ to 1 × 10⁻⁷ M (or 10 pM to 0.1 μM). Table 2 compares our sensor with other sensors reported in literature with respect to the performance of detecting target cancer biomarkers. Our sensor exhibited competitive sensitivity (123.5 kΩ pM⁻¹ for 1.0 fM to 0.1 μM and 43.4 kΩ μM⁻¹ for 0.1 pM to 0.1 μM) in the detection of ErbB2 compared to that of other sensors with cysteamine–gold nanoparticles (3.83 kΩ pM⁻¹ for 7.4 fM to 7.4 nM)⁵⁹ and functionalized ZnO nanofibers (7.76 kΩ μM⁻¹ for 1.0 fM to 0.1 μM).¹² The higher sensitivity of this microfluidic device is due to the porous and large surface feature of nTiO₂-coated 3D GF electrode that allows more loading of anti-ErbB2 receptors for their interactions with specific ErbB2 antigens. Furthermore, the EIS and DPV measurement results indicate that our sensor, in terms of sensitivity, worked better with the EIS method than the DPV method for the detection of ErbB2.

Table 2 also shows that other breast cancer biomarker sensors with CdTe–CdS quantum dots,⁶⁰ optofluorescent ring resonators,⁷ Au nanoparticles,⁶¹ and GO–SiO₂,²³ had detection ranges of 25 fM–25 nM, 189.5 pM–1.46 nM, 1.4 pM–145.7 pM, and 1 pM–1 μM, respectively. The available functional groups of the carbon-doped nTiO₂ and the large surface area of the nTiO₂–GF allowed more antibody molecules to be loaded, resulting in the detection of femtomoles of antibodies and over a wider biomarker concentration range from 1.0 fM to 0.1 μM. The antibody–antibody binding kinetics of the nTiO₂–GF composite-based electrode were studied using the Hill plot.⁶¹ The association and dissociation behavior of an antibody–antigen complex on the surface of an immunoelectrode can be expressed by

### Table 2. Comparison of the Performance of Our Device with That of Other Electrochemical Sensors

<table>
<thead>
<tr>
<th>bioelectrodes</th>
<th>measurement methods</th>
<th>biomarkers</th>
<th>sensitivity</th>
<th>detection range</th>
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<tr>
<td>opto-fluidic ring resonator²</td>
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<td>HER2</td>
<td>30 nm/RIU</td>
<td>189.5 pM–1.46 nM</td>
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<td>fluorescence</td>
<td>carcinoma embryonic antigen</td>
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<td>25 fM–25 nM</td>
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<td>zinc oxide nanowires⁵⁵</td>
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<td>BRCA1</td>
<td>6.36 nA μM⁻¹</td>
<td>10.0–100.0 μM</td>
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<td>graphene nanosheets⁷⁷</td>
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<td>carcinoembryonic antigen</td>
<td>0.1 μA pM⁻¹</td>
<td>2.7–333.3 pM</td>
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<tr>
<td>nTiO₂ (this work)</td>
<td>pulse voltammetry</td>
<td>ErbB2</td>
<td>0.076 μA pM⁻¹</td>
<td>1.4–145.7 pM</td>
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<td>reduced graphene oxide–SiO₂ nanoparticles²³</td>
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<td>functionalized ZnO nanofibers¹¹</td>
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<td>cysteamine–AuNPs⁵⁶</td>
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where $K_a$ and $K_d$ are the association and dissociation constants, respectively, $\text{Ag}'$ represents ErbB2 antigen in solution, $\text{Ab}$ denotes the immobilized anti-ErbB2 on the sensor surface, $\text{Ag}'_n\text{Ab}$ is the antigen–antibody complex, and $n$ represents the Hill coefficient or the degree of binding. The dissociation constant for the interaction between ErbB2 and anti-ErbB2 is $K_d = 1/K_a = \left[ \text{Ag}' \right]^n[\text{Ab}]/\left[ \text{Ag}'_n\text{Ab} \right]$. A low $K_d$ value indicates strong binding between ErbB2 antigen and anti-ErbB2. The Hill coefficient is equivalent to the slope of the Hill plot. Therefore, $K_a$ can be derived from the ordinate intercept of the Hill plot, based on eq 5, and its reciprocal is $K_d$.

$$\log \frac{Y}{1-Y} = \log \frac{1}{K_d} + n \log[\text{ErbB2}]$$

where $Y = \Delta R_{ct}/\Delta R_{ct\text{max}}$ and $\Delta R_{ct}$ and $\Delta R_{ct\text{max}}$ denote the change and the maximum change in charge transfer resistance, respectively. On the basis of the Hill plot for ErbB2 (Figure S8), the values of $K_d$, $K_a$, and $n$ for our sensor were $2.32 \times 10^{-3}$ M, $0.43 \times 10^3$ M, and 0.24, respectively. A value of $n < 1$ indicates negative cooperativity of the immunocomplex between ErbB2 and anti-ErbB2 on the sensor surface and a
decrease in the affinity of anti-ErbB2/GF–nTiO2 with other molecules. The low $K_d$ of this sensor may be responsible for the higher affinity of the anti-ErbB2 toward the ErbB2 antigen. 

Figure 7a,b shows the transient response of the sensor in the presence of ErbB2 using the EIS method. When the sensor was exposed to a [ErbB2] of 1 nM, the real-axis impedance was saturated after 198 s in the EIS method. The longer detection time of the EIS method may be due to the need to measure the impedance at multiple frequencies until the impedance signal is saturated. We also monitored the transient amperometric current for the detection of 1 nM of ErbB2. The current was saturated within about 1 s at 0.3 and −0.3 V.

To test the selectivity of the sensor, we exposed it to two interfering nonspecific antigens, i.e., ErbB3 and ErbB4, both in the ErbB receptor tyrosine kinase family. The DPV method was used to measure the response of the sensor to the presence of the interfering antigens (Figure 8a,b). ErbB2, ErbB3, and ErbB4 each had a concentration of 1 nM. Without the ErbB2 antigen, the microfluidic sensor had a low relative standard deviation (RSD) of ±2.5% from the immunoelectrode (BSA/anti-ErbB2/GF–nTiO2) signal (Figure 8b). When 1 nM specific ErbB2 antigen was added to the solution containing nonspecific ErbB3, ErbB4, and ErbB3 + ErbB4 antigens, the peak current of the sensor did not significantly change, as evidenced by the low RSD (±0.5%). This result demonstrates the good selectivity of the sensor for detecting ErbB2 overexpressed by breast cancer cells. The high selectivity is due to the specific anti-ErbB2 binding sites on the sensor surface and the strong interactions with ErbB2. Within 42 days of putting the immunosensor through a stability test, we observed a low RSD of ±1.0% when the sensor was stored in 4°C (Figure 8c) because of the strong covalent bond of anti-ErbB2 to the GF–nTiO2 matrix. Figure 8d shows the reproducibility of the fabricated device in which five immunoelectrodes were tested, keeping the area of the electrode constant (0.096 cm²) using both DPV and EIS methods. The DPV measurement result shows that the deviation of these sensors was ±2.5%, indicating reasonable reproducibility. In addition, the EIS measurement result shows a deviation of ±5% for the value of $R_a$ as shown in Figure S9a. To explore the extreme case of detecting 10¹⁰ M (1.0 fM), we also conducted four repeated measurements using one of the sensors. The sensor provided a low deviation of ±2.95% for the value of $R_a$ from the initial sensor response. This result indicates that the sensor was reproducible even at 1.0 fM of ErbB2.

4. CONCLUSIONS

We presented a selective, reproducible, and stable microfluidic immunosensor for the detection of breast cancer molecules via antigen–antibody interactions. The sensor utilized a new immunoelectrode made of hierarchical GF modified with electrosynopsis carbon-doped nTiO2 as the electrochemical working electrode. The excellent electrochemical properties of the GF–nTiO2 matrix of the device allow for sensitive detection of the ErbB2 antigen, at minute (1.0 fM) to higher (0.1 μM) concentrations, overexpressed in breast cancer cells. The sensor interfaced with two electrochemical measurement methods, impedance, and pulse voltammetry, used to detect the ErbB2 antigen. With the impedance method (EIS), the higher sensitivity of the device was responsible for the impedimetric property of the nTiO2 coated on the GF scaffolds. The functional groups at the GF–nTiO2 composite interface with anti-ErbB2 and their shape and size were confirmed by XPS, SEM, and TEM. The DPV method also found that the sensor had reasonable sensitivity to ErbB2 within a wide concentration range. Our sensor is highly selective for ErbB2 in the presence of interfering ErbB3 and ErbB4 antigens. Our sensor can be modified flexibly for the addition of a multifunctional chip to realize multiplexed detection of multiple biomarkers such as ErbB1, ErbB3, and ErbB4. Efforts will be made to investigate using modified devices to test serum or cell lysate samples.

ASSOCIATED CONTENT

 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b05648.

Raman and XPS spectra, a table summarizing binding energies, fswhm, and atomic ratio; temperature, pH, and optimization of antibody concentration using DPV; scan rate studies in cyclic voltammetry (CV) measurements; control studies using EIS; simulation of Nyquist plot; table summarizing EIS parameters; Hill plot for kinetic binding studies; and reproducibility studies (PDF)

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Notes

The authors declare no competing financial interest.

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