

# Avidin–Biotin Technology in Gold Nanoparticle-Decorated Graphene Field Effect Transistors for Detection of Biotinylated Macromolecules with Ultrahigh Sensitivity and Specificity

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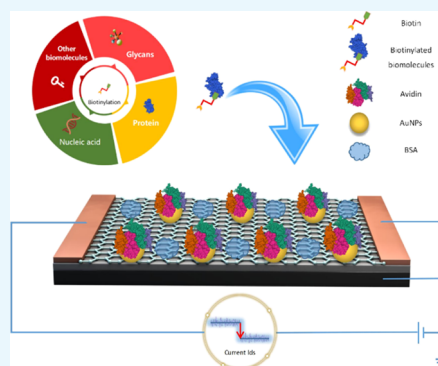


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Supporting Information

**ABSTRACT:** The strong and specific noncovalent interaction between avidin and biotin is widely exploited in different types of enzyme-linked immunosorbent assay kits, labeled immunosensors, and polymer-based sensing devices for the detection of different biomarkers specific to different diseases such as cancer and influenza. Here, we employed the avidin–biotin technology in a novel gold nanoparticle-decorated graphene field-effect transistor (AuNP-GFET) and demonstrated the specific detection of the biotinylated macromolecules such as biotinylated proteins and nucleotides in the sub-picomolar (pM) range. The AuNP-GFET was constructed by fabricating six pairs of interdigital electrodes on graphene transferred on a SiO<sub>2</sub>/Si substrate. The sensing performance of AuNP-GFET was characterized by the real-time two-terminal electrical current measurement upon injection of the analyte solution into a silicone pool preattached onto the electrodes. Avidin, a tetrameric biotin-binding protein with strong affinity and specificity, immobilized on AuNP-decorated single-layer graphene, was used as the sensing platform and transduced the electrical signal upon binding to the analyte macromolecules. The sensing capability of the AuNP-GFET was tested with the biotinylated protein A. Sensitivity of the present biosensor was estimated to be ~0.4 pM. The specificity and applicability of the biosensor were confirmed using both synthetic and real samples. Because the biotin label can retain its binding capability to avidin with strong affinity and specificity even after conjugating with varieties of proteins and nucleotides, the present AuNP-GFET biosensor is expected to promote the research in developing different biosensors.



## 1. INTRODUCTION

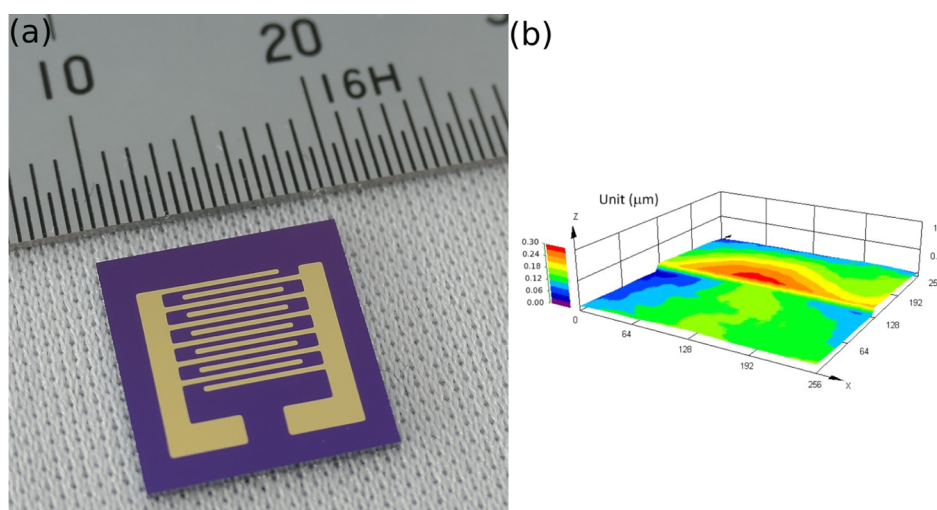
Because of the most specific and strongest noncovalent interaction and labeling capability of biotin, avidin–biotin technology is used in ELISA (enzyme-linked immunosorbent assay) kits, polymer-based detection methods,<sup>1</sup> and labeled immunosensors<sup>2</sup> for the detection of different biomarkers for different diseases including cancer and influenza. The detection limits were reported from 2 pM to 84 nM using different types of analytical techniques such as electrochemical, cyclic voltammetry, competitive immunoassay, and immunoaffinity chromatography. Avidin is a tetrameric protein, which is found in the white part of the eggs of birds, reptiles, and amphibians. Avidin has strong affinity for binding with biotin. Dimeric members of the avidin family are also reported in some bacteria.<sup>3</sup> In the case of chicken egg white, approximately 0.05% of the total protein is avidin (approximately 1800 μg per egg). Avidin contains four identical subunits (homotetramer), and each of these subunits can bind to biotin (vitamin B7, vitamin H) with high specificity.<sup>4</sup> Each biotin can bind to avidin through very strong noncovalent interaction.<sup>4</sup> Naturally, biotin involves in a wide range of metabolic processes with different types of fats, carbohydrates, and amino acids, both in humans and other organisms.<sup>5</sup> Indeed, biotin can work as a

useful label for many proteins and nucleotides, keeping their original properties unaltered because of its small size and tendency of nonbonding of the carboxy-containing side chain with avidin. The chemical process of labeling a biomolecule with biotin is known as biotinylation. Biotin protein ligases can also be attached to specific lysine residues in vitro or in living cells.<sup>6</sup> On the other hand, avidin can be attached to a solid surface or many biomolecules, such as probes.

To date, most of the biomolecule and biomarker detections are based on the ELISA, where quantitative detection is achieved through the measurement of intensity of transmitted light by spectrophotometry. Experimentally, the specific conjugation of biomolecules is realized in terms of optical signals, which is then converted into electrical signals by spectrophotometry for quantitative reading. The direct

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**Figure 1.** (a) Optical image of the six pairs of interdigital electrodes fabricated on a  $\text{SiO}_2/\text{Si}$  substrate. (b) 3D image reflecting the height of electrode above the substrate.

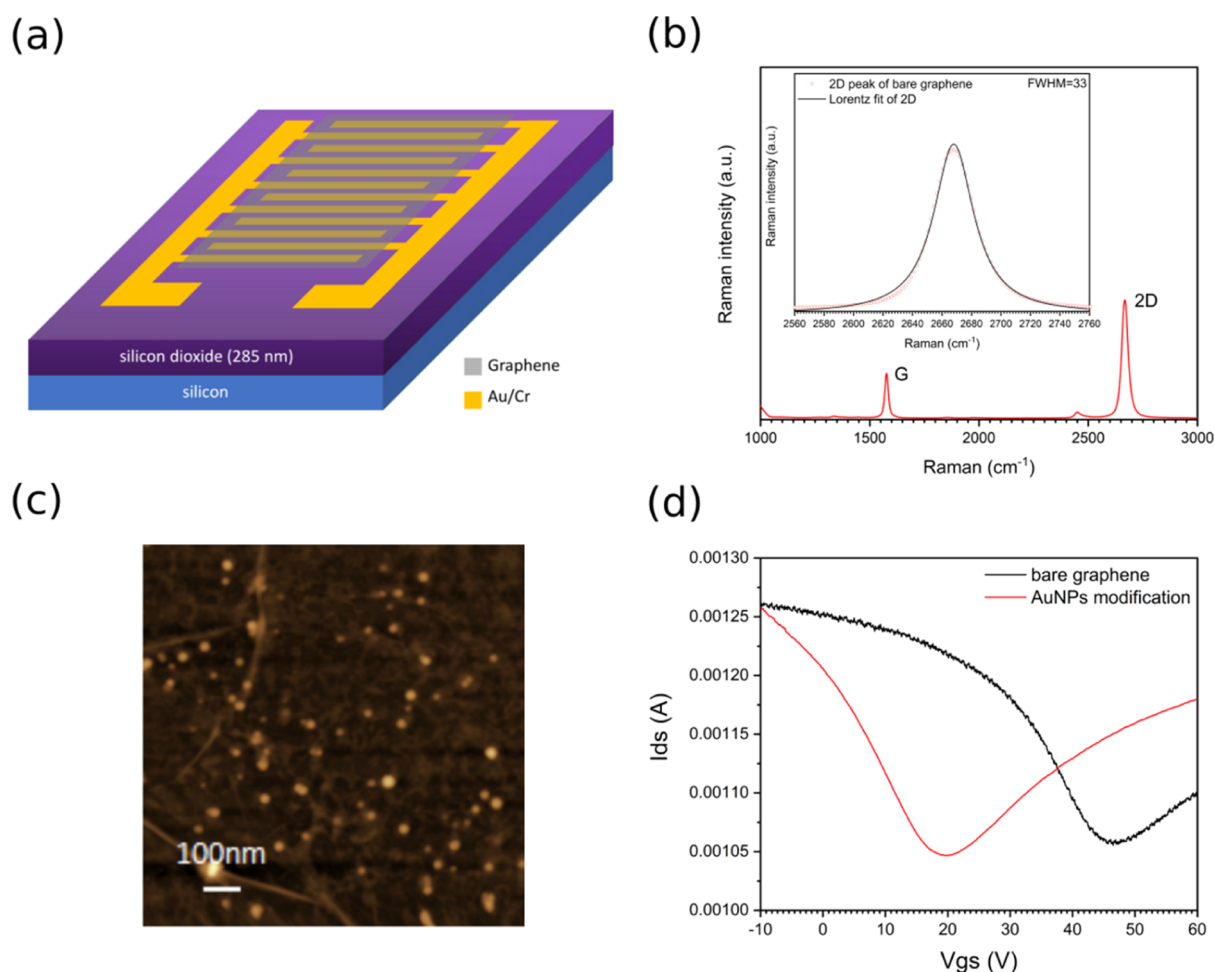
conversion of biological conjugation into electrical signals is of great significance in clinical diagnosis, especially for the development of a simple, easy to use, and low-cost sensing devices. The direct electrochemical detection methods possess not only the advantages of simplicity, fast responses, and ease of use but also are promising for miniaturization of the diagnostic instruments into low-cost microscale dimensions.<sup>7</sup> Of course, the present status of ELISA detection methods in medical diagnosis is unshakeable because of its widespread application and commercialization of the kits. Hence, other methods of biomolecule detection including field-effect transistor (FET)-based sensors might not immediately be the alternative to ELISA but complementary to ELISA as an initial screening method for dealing with large-scale epidemic situations.

Biosensors are composed of mainly two components. A biorecognition molecule or receptor such as an antibody or antigen (or capture molecule) that determines the specificity and a signal transducer such as graphene that determines the sensitivity of the sensor.<sup>8</sup> The extremely high carrier mobility (ranging from 5000 to 12,000  $\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$  with an average mobility of  $\sim 8800 \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$ ),<sup>9</sup> ambipolar transfer characteristics,<sup>10,11</sup> physical flexibility, and robustness under ambient conditions make graphene an ideal material as a sensing platform of ultrasensitive graphene FET (GFET) sensors including biosensors. Because of the compatibility, sensitivity, and the advancement of the state-of-the-art nanofabrication technique, the GFET sensor has drawn much attention as the most promising approach for the rapid, sensitive, specific, and low-cost detection and quantification of biomarkers.<sup>12</sup> Because of its simple design and rapid detection, the GFET sensor can be used as a point-of-care diagnostics tool. Indeed, a number of studies have been reported on the potential applications of the GFET as a biosensing device.<sup>12–25</sup> The detection of bio-macromolecules including cancer markers and RNAs by GFET biosensors has been reported using various types of acceptor/receptor designs.<sup>12–20</sup> The electrolyte-gated GFET has been reported for pH and protein detection.<sup>21</sup> The aptamer-modified GFET has been used for the label-free detection of immunoglobulin (IgE) proteins.<sup>14</sup> The GFET can detect ethanol molecules in the ppb level. The label-free sensing of exosomes using

functionalized graphene-based FET has been reported by Tsang et al.<sup>23</sup> Seo et al. have reported the rapid detection of COVID-19-causative virus (SARS-CoV-2) using GFET.<sup>26</sup> Aerosol-jet-printed graphene-based sensors were used for label-free cytokine monitoring in serum and food safety.<sup>27,28</sup> Because of the easy attachment of avidin on solid surfaces, avidin immobilized on graphene can be utilized to detect biotin and biotinylated protein in the form of electrical signals, allowing real-time point-of-care diagnosis.

Graphene sheets decorated with metal nanoparticles (MNPs) such as gold nanoparticles (AuNPs) are excellent materials for biosensor platforms because of the biocompatibility of AuNPs and the further enhancement of the carrier mobility of the graphene.<sup>29,30</sup> Hence, instead of 1-pyrenebutanoic acid succinimidyl ester (PBASE)-modified graphene, AuNP-modified graphene is expected to possess the advantage of immobilization of avidin in terms of simplicity, stability, and sensitivity. Recently, AuNP-decorated reduced graphene oxide FET has been reported for the label-free detection of miRNA using peptide nucleic acid as the biorecognition molecule.<sup>31,32</sup> These studies reported to date utilize a surface-immobilized recognition probe to selectively interact with a biological analyte in solution, that is, the GFET device is designed for the detection of a specific biological analyte. Indeed, the binding capability of the biorecognition molecule limits the application of the sensor, that is, if the biorecognition molecule can be tailored to bind different macromolecules with high specificity, then, the same sensing platform can be used for different target analytes. It is thus of great importance to develop a universal point-of-care sensing platform that can be used for a wide range of detection purpose simply by labeling or linking the bio-macromolecule to be detected.

Here, we employed the avidin–biotin technology on the AuNP-decorated pristine GFET (AuNP-GFET) biosensor for the detection of biotinylated protein A. The GFET was composed of AuNP-decorated single layer graphene supported on the six pairs of interdigital electrodes prefabricated on the  $\text{SiO}_2/\text{Si}$  substrate. Sensor performance was investigated by real-time current measurements between the source and drain while injecting the analyte solution into the silicone test pool preconstructed on the electrodes. The limit of detection was found to be lower than  $\sim 0.4 \text{ pM}$  for the biotinylated protein A



**Figure 2.** (a) Schematic illustration of the graphene transferred on the SiO<sub>2</sub>/Si substrate covering all six pairs of interdigital electrodes. (b) Typical Raman spectrum of graphene after transferring onto the interdigital electrodes. The prominent G and 2D bands are indicated. (c) Atomic force microscopy (AFM) image of AuNPs decorated on transferred graphene on the SiO<sub>2</sub>/Si substrate. The sphere-shaped bright protrusions randomly distributed over the surface are AuNPs. (d) *I*–*V* curves for clean and AuNP-modified graphene.

in the case of the present AuNP-GFET biosensor. Specificity of the sensor was investigated by both the real and synthetic samples. Because the scope of biotinylation with different proteins, nucleotides and other macromolecules is wide, the present biosensor can be used as a platform for the rapid and low-cost point-of-care detection of different biomolecules and biomarkers specific to different diseases including infectious diseases such as influenza and COVID-19.

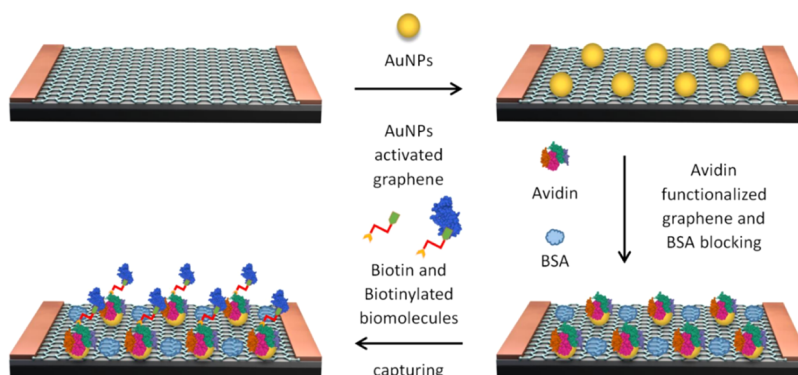
## 2. RESULTS AND DISCUSSION

**2.1. Characterization of the Sensor Device.** The optical image of the six pairs of interdigital electrodes fabricated on the SiO<sub>2</sub>/Si substrate is shown in Figure 1a. Both the width of each electrode and the interelectrode gap are estimated to be 200 μm on average. Thickness of the Cr/Au electrode was obtained from the 3D height profile, as shown in Figure 1b. The estimated thickness was around 65 nm.

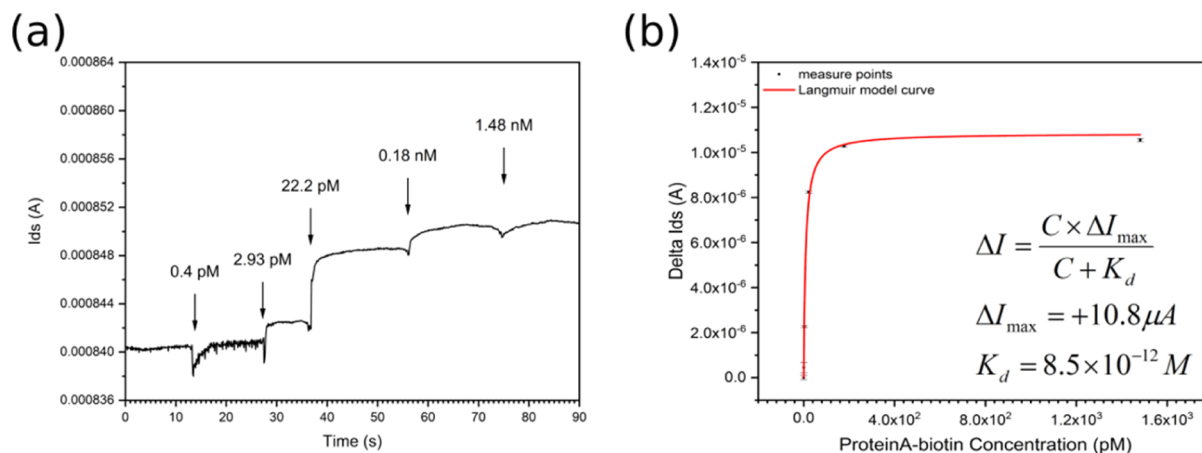
The schematic of the top and side views of the graphene transferred on the interdigital electrodes (i.e., AuNP-GFET) is shown in Figure 2a. The quality of the graphene transferred on the electrodes is ensured by Raman measurements at different places chosen randomly over the entire regions. Figure 2b shows a typical Raman spectrum of the graphene transferred on interdigital electrodes. The two characteristic peaks 2D and

G confirm the quality and cleanliness of the graphene. The absence of any peak around 1300 cm<sup>-1</sup> (D band) indicates a defect-free surface. The estimated intensity ratio [*I*(2D)/*I*(G)] 2.6 indicates the defect-free monolayer graphene.<sup>33</sup> Further support for the monolayer graphene comes from the only one Lorentzian peak (*R*<sup>2</sup> is above 0.99) fitting to the 2D band and full width at half-maximum 33 cm<sup>-1</sup> (inset of Figure 2b).<sup>34</sup>

Deposition of AuNPs on the transferred single-layer graphene on the SiO<sub>2</sub>/Si substrate was investigated by AFM, *I*–*V* measurements, and X-ray photoelectron spectroscopy (XPS). A typical AFM image of the AuNP-deposited surface is shown in Figure 2c. The small ball-shaped protrusions distributed all over the surface are the deposited AuNPs. The size of the AuNPs was estimated to be ~25–70 nm. Besides, the wrinkled characteristics of the transferred graphene were also observed in the AFM image. The appearance of AFM image is in agreement with the previously reported image of AuNPs on transferred graphene on the SiO<sub>2</sub>/Si substrate.<sup>32</sup> The presence of AuNPs on the surface is confirmed by the Au 4f and Au 4d peaks at ~85 and 360 eV (shown in Figure S2 in Supporting Information). The two components Au 4f and Au 4d peaks relate to the spin–orbit coupling. The intensity ratio and separation of the two components of the Au 4f peak ensure the purity of the AuNPs on the surface.



**Figure 3.** Schematic of the modification of graphene with AuNPs and subsequent binding of avidin, blocking by bovine serum albumin (BSA), followed by specific capture of biotin or biotinylated biomolecules.



**Figure 4.** (a) Real-time current ( $I_{ds}$ ) upon addition of different concentrations of biotinylated protein A solution at different time intervals. (b) Plot of current change ( $\Delta I$ ) vs concentration extracted from (a) and Langmuir fitting to the plot. The fitting equation and extracted  $\Delta I_{\max}$  and  $K_d$  values are indicated.

$I$ – $V$  measurements are done for the clean and AuNP-modified graphene. A typical  $I$ – $V$  curve for the clean and AuNP-modified graphene is shown in Figure 2d. Compared to the pristine graphene, a large shift of the Dirac point toward lower voltages is observed in the case of AuNP-modified graphene. This shift in the Dirac point toward lower voltage indicates that the decoration of graphene with AuNPs decreases the extent of P-doping characteristics. This phenomenon can be attributed to differences in the work function between graphene and Au. The work function of graphene and Au are around 4.2 and 5.1 eV respectively. When the AuNPs are deposited on the surface of the graphene, the work function difference induces the electronic charge transfer from AuNPs to graphene. Binding of proteins on MNPs has been extensively studied. Accumulation of proteins onto large gold particles (>3 nm) can result in a stable adsorption layer, which is often referred to as the hard protein corona.<sup>35</sup> Avidin, a tetrameric protein, is also expected to bind onto the AuNP on graphene. The binding of avidin on AuNPs decorated on graphene was characterized by XPS measurement, as shown in Figure S3 in Supporting Information. On the avidin-modified surface, the N 1s, Cl 2p, and Na KLL peaks are observed in addition to Au-, graphene-, and substrate-related peaks. The N 1s peak comes from the amino acid of the avidin protein. The Cl 2p and the Na KLL peak come from the residual phosphate-buffered saline (PBS) buffer on the device. Thus, the presence of avidin on the AuNP-decorated GFET device was confirmed.

Note that avidin cannot be immobilized onto the pristine graphene without using any linker molecule such as PBSE.<sup>36</sup>

**2.2. Detection of Biotinylated Protein A.** The immobilized avidin on AuNP-decorated graphene is expected to retain its capability of selectively binding to the biotinylated macromolecules. The schematic of graphene modification and the subsequent binding of avidin and the biotinylated macromolecules is shown in Figure 3. Quantitative detection of the biotinylated macromolecules was performed by injecting the biotinylated macromolecule solutions into the test pool (shown in Figure S4 in Supporting Information).

It is well known that avidin can bind not only to free biotin through noncovalent interaction with high specificity but also to any biotinylated protein or nucleic acid. The most commonly used biotinylation methods are primary amine biotinylation, enzymatic biotinylation, carboxyl biotinylation, sulfhydryl biotinylation, glycoprotein biotinylation, and oligonucleotide biotinylation.<sup>37</sup> Indeed, conjugation of biotin with proteins, nucleic acids and other macromolecules does not affect the original properties of the molecules because of the small size of biotin.<sup>38</sup> Hence, the present AuNP-decorated GFET could be a common platform for various kinds of sensing applications through biotinylation of the target protein or nucleic acids. To demonstrate the wider sensing ability of the present device, we performed a similar experiment with a typical commercially available biotinylated macromolecule, protein A. Protein A is widely used in biochemical research and

clinic diagnosis applications because of its ability to bind different macromolecules such as immunoglobulins, most notably human IgGs.<sup>39</sup> Protein A can also conjugate with other macromolecules such as a fluorescent dye, colloidal gold, enzymes, or radioactive iodine, without affecting the antibody binding site.

Figure 4a shows the real-time  $I_{ds}$  values upon injection of different concentrations of biotinylated protein A. Before injection of protein A solution, a blank test was done using 20  $\mu$ L of PBS solution. No change in stable  $I_{ds}$  values was observed. Then, 20  $\mu$ L of biotinylated protein A solution of different concentrations in PBS (0.8 pM, 8 pM, 80 pM, 0.8 nM, and 8 nM) was introduced to the test pool subsequently. Considering the dilution of the injected solution by the subsequent addition, the corrected concentrations are estimated to be 0.4 pM, 2.9 pM, 22.2 pM, 0.18 nM, and 1.48 nM. The  $I_{ds}$  value reaches to the saturation value at 1.4 nM. Thus, the detection limit of biotinylated protein A was estimated to be 0.4 pM to 1.4 nM. From the magnitude of the real-time current changes shown in Figure 4a,  $\Delta I_{max}$  (maximum current change) and  $K_d$  (the dissociation constant) can be estimated from the Langmuir adsorption model  $\Delta I = (C \times \Delta I_{max}) / (C + K_d)$ , where  $C$  represents the concentration of the biotinylated protein A. The change in  $I_{ds}$  values with different concentrations are shown in Figure 4b. The  $\Delta I_{max}$  and  $K_d$  of +10.8  $\mu$ A and  $8.5 \times 10^{-12}$  M, respectively, are extracted by fitting to the  $\Delta I$  versus concentration curve shown in Figure 4b. The fitting correlation coefficient ( $R^2$ ) is above 0.98. Note that injection of biotinylated protein A in the device without avidin but passivated with BSA does not show any significant change of the  $I_{ds}$  value. Hence, the possibility of binding of biotinylated protein A to AuNPs can be ruled out.

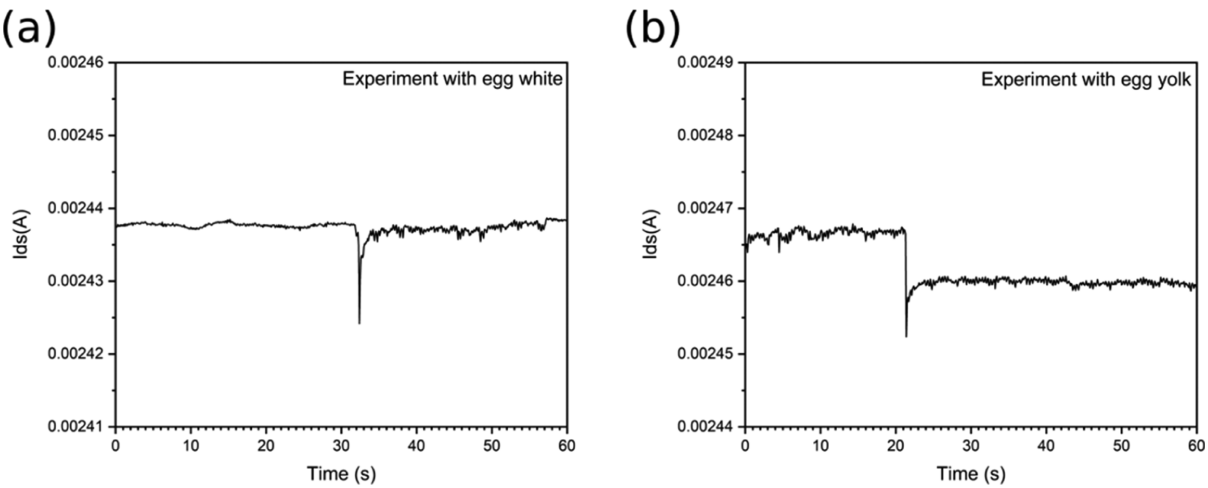
Note that the spikes in  $I_{ds}$  versus the time curve upon addition of the solutions are the signature of an interfacial capacitance that is disrupted by the fresh solution added and then recovers with time. For the large bio-macromolecule adsorption, the doping characteristics of the graphene is expected to be different from the AuNPs and other linkers such as PBASE. It is mediated by the interface capacitance (of the electrical double layer), not through direct charge injection into the channel. Most of the bio-macromolecules such as proteins contain carboxyl, amino groups, and other complex moieties, which make them hold different degrees of charge in the PBS solution. The type and strength of the charges are related to the pH value of the solution and the isoelectric point (pI) of the bio-macromolecules.<sup>21</sup> Because these bio-macromolecules themselves are not conductive and large in size compared with the linkers such as AuNPs, the charge of these bio-macromolecules cannot directly regulate the conductance of graphene by doping (direct charge injection into the channel of the graphene) in the PBS solution, but the charges of the bio-macromolecules could affect the charge distribution at the interface double layer between the graphene and the PBS solution to achieve the effect of regulating the conductivity of the graphene. To our knowledge, the pI of protein A is around 5, and the pH of our PBS solution is 7.4; thus, protein A maintains the negative charge in the PBS solution. The  $I$ - $V$  measurement before the real-time test (Figure S5) shows the successive functionalization steps also preserve the transistor behavior, and the GFET is polarized in the hole branch. It is remarkable that the stable  $I_{ds}$  values gradually increase by the subsequent injection of the biotinylated protein A from lower to higher concentrations.

Because there is an interface capacitance in the graphene and PBS solution, the binding of biotinylated protein A with the immobilized avidin induces a positive charge or a change in the net charge to a positive value on the graphene channel, resulting in the positive response in current change upon injection of the solution in the test pool.

**2.3. Specificity of the Sensor.** One of the key challenges in the practical applicability of a biosensor is the specific detection of the target molecule or marker. Avidin is a glycoprotein and contains four identical subunits of 16,400 Da each, giving an intact molecular weight of approximately 66,000.<sup>40</sup> Each subunit contains one binding site for the biotinylated macromolecule. The tetrameric protein is highly basic, having a pI of about 10. Tryptophan and lysine residues in each subunit are known to be involved in forming the binding pocket.<sup>41</sup> Once the biotinylated macromolecule is bound to avidin, the avidin-biotin complex becomes much robust against breakdown. A minimum of 6 to 8 M guanidine at pH 1.5 is required for inducing complete dissociation of the avidin-biotin complex.<sup>42,43</sup> Such robust nature of the avidin-biotin complex against any denaturing agent makes the complex very useful in bioconjugate chemistry. Even biotinylated molecules and avidin can also bind together and make the complex under the extreme conditions. It can be said that the specificity of the avidin-biotin interaction is similar to that of antibody-antigen or receptor-ligand recognition but with much higher affinity. Indeed, the specific binding of biotinylated molecules with avidin cannot be prevented by variations in the buffer salt, pH, the presence of denaturants or detergents, and extreme temperatures.<sup>44</sup> Because of the high pI and carbohydrate content, natural avidin has tendency to bind nonspecifically with components other than biotin or biotinylated macromolecules; hence, it shows some disadvantages to use for sensing purpose. However, this disadvantage of tendency to nonspecific binding has been eliminated in the chemically modified avidin, such as NeutrAvidin used in the present study, through deglycosylation and reducing its pI through covalent modification of charged residues. Hence, the present avidin-immobilized AuNP-GFET is expected to exhibit high specificity.

The specificity of the sensor device was confirmed in the case of biotinylated protein A using both synthetic and real samples. The set of synthetic samples consisted of vitamin C, lactoferrin, BSA, and biotinylated protein A. Figure S6 in Supporting Information represents the  $I_{ds}$  value upon injection of 20  $\mu$ L of 0.01 mg/mL of vitamin C, lactoferrin, BSA, and biotinylated protein A solution in PBS into the test pool sequentially. These results indicate that the immobilized avidin on AuNP-decorated graphene does not bind to vitamin C, lactoferrin, and BSA but readily binds to the biotinylated protein A, that is, the present AuNP-GFET is very specific to the biotinylated molecules.

Chicken egg white was taken as a real sample to test the specificity of the sensor. It is well known that chicken egg whites and yolk contain avidin and biotin, respectively, in addition to many other components. The major components of chicken egg white are ovalbumin (phosphoglycoprotein), conalbumin, ovomucoid (glycoprotein), ovomucin, lysozyme, avidin (0.05% of egg white protein), ovoglobulin, and ovomucoid.<sup>45</sup> The egg yolk contains lipoproteins, which include lipovitellins and lipovitellinin, carbohydrates, different fatty acids, and vitamins including biotin (13–25  $\mu$ g).<sup>46</sup> Biotin in egg yolk remains in conjugation with a protein, which is

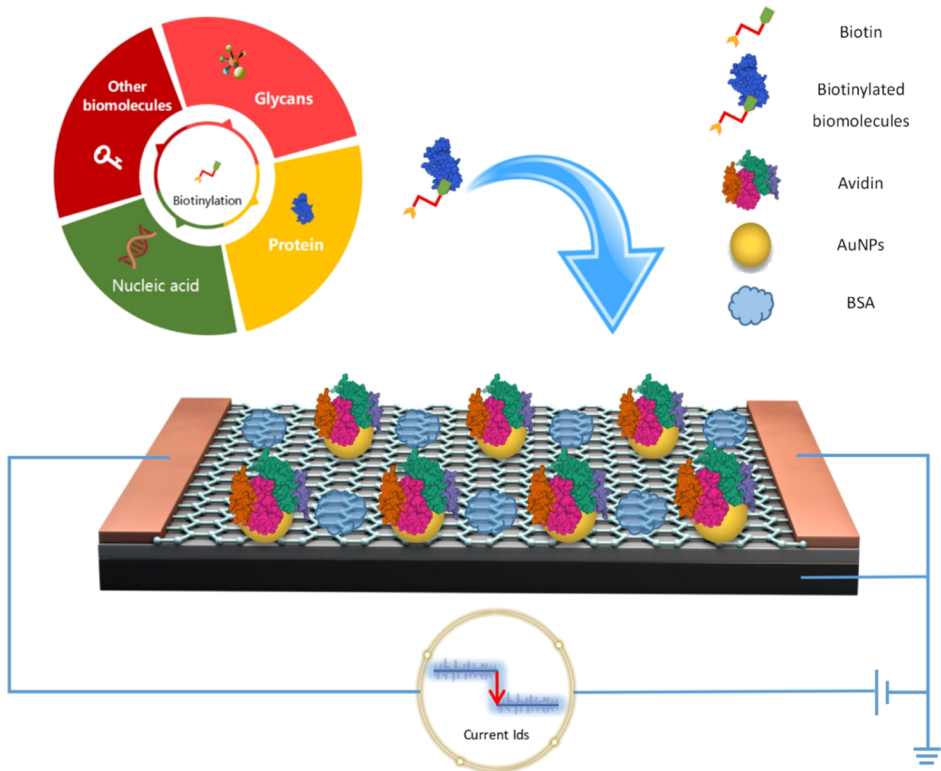


**Figure 5.** (a) Real-time current ( $I_{ds}$ ) vs time upon the addition of 50  $\mu$ L of egg white and (b) egg yolk diluted with PBS (1:100). Before injecting the sample solution, 50  $\mu$ L of PBS was added.

**Table 1. Avidin–Biotin Technology Used in Different Biosensors<sup>a</sup>**

| platform                          | material                   | method                             | limit of detection | time (min) | reference |
|-----------------------------------|----------------------------|------------------------------------|--------------------|------------|-----------|
| electrochemical magneto biosensor | streptavidin               | cyclic voltammetry                 | 84 nM              | 40         | 55        |
| boron-doped diamond electrode     | streptavidin               | electrochemical sensing            | 5 nM               | 10         | 50        |
| sepharose beads                   | antibiotin antibody        | immunoaffinity chromatographic     | 41 pM              | 30         | 56        |
| beads and AuNPs                   | antibiotin antibody        | competitive immunoassay            | 2 pM               | 15         | 49        |
| boron-doped diamond electrode     | captavidin                 | electrochemical sensing            | 1 nM               | 10         | 57        |
| GFET biosensor                    | streptavidin (NeutrAvidin) | current $I_{ds}$ real-time monitor | 0.4 pM             | 1          | this work |

<sup>a</sup>Limit of detection and time required for data acquisition compared to the present study are indicated.



**Figure 6.** Schematic of the AuNP-decorated GFET biosensor that can be used as a common platform for detection of proteins, nucleic acids, glycans, and other biomolecules. Any biotinylated proteins, nucleic acids, glycans, or other biomolecules are expected to selectively bind to the immobilized avidin attached on AuNPs decorated on the graphene channel and induce a current change.

different from egg-white avidin. Upon injection of 50  $\mu$ L of egg yolk solution in PBS into the test pool of the present sensor, a large drop in stable  $I_{ds}$  current is observed in real-time measurements. This drop of  $I_{ds}$  current indicates the presence of biotinylated proteins in egg yolk (Figure 5a). In contrast to egg yolk, no significant change in stable  $I_{ds}$  current (Figure 5b) is observed upon injection of egg white solution in PBS. This implies the absence of not only biotin but also any other chemical components in egg white, which can nonspecifically bind to the immobilized avidin on the present AuNP-GFET. These observations with chicken egg white and yolk further support the specificity and sensing capability of the present AuNP-GFET sensor in the case of unprocessed real samples.

**2.4. Practical Applicability and Comparative Sensitivity.** A number of studies have been reported on the potential utilization of avidin–biotin interaction in sensing technology.<sup>47–51</sup> Using various detection methods such as competitive immunoassay, electrochemical, cyclic voltammetry, and immunoaffinity chromatography, detection limits ranging from 2 pM to 84 nM have been reported (Table 1). It seems that the present AuNP-GFET biosensor based on the avidin–biotin technology shows the highest sensitivity and quick detection time.

Practically, biotin–avidin systems are being utilized in different conventional ELISA kits.<sup>51–53</sup> Similarly, the present AuNP-GFET biosensor is expected to be utilized for the detection of antigen–antibody, nucleic acid systems in body fluids, hormone receptors, tissues and cells, and other bioactive macromolecules through the judicious choice of biotinylation. The potential application of the present AuNP-GFET biosensor as a common platform for detection of various biomolecules is schematically shown in Figure 6. Avidin attached onto the AuNPs deposited on the single layer graphene supported on SiO<sub>2</sub>/Si substrate will function as the common sensing platform. The real-time current response can be monitored by the two-terminal feed through attachment to the Au/Cr electrode fabricated on the graphene. Because of avidin's strong affinity for biotinylated macromolecules with high specificity, different biotinylated proteins, nucleotides, and so forth can be quantitatively detected by monitoring the current ( $I_{ds}$ ) response upon injection on to the device.

### 3. CONCLUSIONS

Extreme electrical and optoelectrical properties of graphene and its compatibility to different organic macromolecules make graphene an ideal platform for holding the receptor molecule and a transducer of a biosensor. With the advancement of the state-of-the-art nanofabrication facility, the GFET biosensors appeared as the most promising point-of-care sensing devices for the rapid, specific, low-cost detection, and quantification of bio-macromolecules with ultrahigh sensitivity. Deposition of AuNPs on graphene further enhanced its compatibility toward varieties of biomolecules. Here, we employed the avidin–biotin technology in AuNP-GFET consisting of six pairs of interdigital electrodes for the detection of biotinylated macromolecules with ultrahigh sensitivity and specificity. The sensing performance of the AuNP-GFET biosensor was detected by real-time two-terminal electrical current measurement while injecting the analyte solution into a silicone test pool. As the receptor of the sensor, avidin, a tetrameric protein which can bind to biotinylated macromolecule with strong affinity and specificity, was immobilized on AuNPs decorated on single-layer graphene transferred on the SiO<sub>2</sub>/Si substrate.

By measuring the real-time current upon injection of different concentrations of biotinylated protein A, sensitivity of the present AuNP-GFET biosensor was estimated to be  $\sim 0.4$  pM. The dissociation constants of avidin–biotinylated protein A complexes were estimated to be  $8.5 \times 10^{-12}$  M from the Langmuir fitting to the  $\Delta I_{ds}$  versus concentration curve. Specificity of the biosensor was confirmed using vitamin C, lactoferrin, BSA, and chicken egg white and yolk. In the specificity test, no significant changes of  $I_{ds}$  value were observed upon injection of vitamin C, lactoferrin, BSA, and chicken egg white solution, but sharp drops of  $I_{ds}$  values were observed upon injection of biotinylated protein A and chicken yolk solution. Note that chicken egg whites contain ovalbumin, conalbumin, ovomucoid, ovomucin, lysozyme, avidin, ovoglobulin, and ovinhibitor, but egg yolk contains lipoproteins, which include lipovitellins and lipovitellin, carbohydrates, different fatty acids, and vitamins including biotin (13–25  $\mu$ g). The results with chicken egg white and yolk further confirm the specificity and practical applicability of the present AuNP-GFET biosensor for various biotinylated molecules. Because the scope of biotinylation is wide and biotin label can still hold its binding capability to avidin, the present AuNP-GFET biosensor can be used as a common sensing platform for detecting different proteins, nucleotides, and macromolecules by labeling or tagging the desired macromolecules to be detected.

### 4. MATERIALS AND EXPERIMENTAL PROCEDURE

**4.1. Materials and Instruments.** The SiO<sub>2</sub>/Si wafer was purchased from ALLIANCE Biosystems (Osaka, Japan). The thickness of SiO<sub>2</sub> layer on a 4 in. wafer was 285 nm. The single-layer chemical vapor deposition graphene on both sides of copper foil was purchased from Chongqing Graphene Technology Co., Ltd. (Chongqing, China). Protein A–biotin was purchased from Sigma-Aldrich (United States). BSA, vitamin C, and lactoferrin were purchased from Wako Pure Chemical Corporation (Osaka, Japan). NeutrAvidin protein and PBS were supplied by Thermo Fisher (Massachusetts, USA). Poly(methyl methacrylate) (PMMA) and ammonium peroxydisulfate were purchased from Sigma-Aldrich (Tokyo, Japan) and Kanto Chemical Co., Inc. (Tokyo, Japan), respectively. Ultrapure deionized water produced by the water purifier WL220 (Tokyo, Japan) was used. All of these chemicals were used as obtained, that is, no further purification was done.

The 3D height profile of the interdigital electrodes and substrate was generated by 3D laser microscopy using OLS-4000, where a 405 nm laser source was used. The pristine and modified graphene were investigated by Raman spectroscopy using a Nicolet Almega XR Raman microscope. The excitation of the graphene was done with a 532 nm laser. The Raman spectra were acquired with a 100 $\times$  objective, and the spatial resolution on the surface was  $\sim 1$   $\mu$ m. XPS measurements were performed using an AXIS-NOVA XPS system where an Al K $\alpha$  X-ray source was used. The incident and emission angles for X-ray and electrons were 60 and 0 $^\circ$  to the surface normal. For the wide- and narrow-range measurements, the analyzer pass energies were set at 160 and 20 meV, respectively. The analyzer slit was set at 110  $\mu$ m for both wide- and narrow-range measurements. The real-time current measurements of GFET were done by a Keysight 4155B semiconductor parameter analyzer.

**4.2. Device Fabrication.** The commercially available SiO<sub>2</sub>/Si wafer was cut into the desired size (typically 1 cm × 1 cm) by a dicing saw (DISCO Corporation, Japan). Interdigital electrodes are fabricated on the 1 cm × 1 cm SiO<sub>2</sub>/Si substrate. For the fabrication of the interdigital electrodes, the commercially available shadow mask was carefully placed on the 1 cm × 1 cm SiO<sub>2</sub>/Si surface. Chromium (Cr) and gold (Au) were used as the materials for electrodes. Deposition of Cr and, then, Au with desired thickness was achieved by the electron beam vacuum evaporation deposition (EIKO Engineering, Japan) technique. The expected thickness of the Cr and Au electrodes was around 15 and 50 nm, respectively.

Transfer of graphene onto the interdigital electrodes is a crucial step for the fabrication of the sensor device. For transferring the graphene on the interdigital electrodes, a thin layer of PMMA was deposited on the graphene on a copper foil using PMMA in acetone solution. The deposition of PMMA in acetone solution was done using a spin coater (Mikasa Corporation, Japan). Evaporation of acetone by 5 min-heating left the thick layer of PMMA attached onto the graphene. Then, this PMMA-attached graphene on the copper foil was cut into 5 mm × 5 mm size so that the interdigital electrodes can be fully covered. For etching out the copper from the graphene, the PMMA-attached graphene on the copper foil was placed on ammonium peroxodisulfate aqueous solution until the copper was fully dissolved. This etching process left the PMMA-attached graphene film floating on the surface of the solution. The residual ammonium peroxodisulfate adhering to the film was removed by carefully transferring the film in ultrapure water for 30 min. Then, the clean PMMA-attached graphene film was carefully placed onto the interdigitated electrode fabricated on the SiO<sub>2</sub>/Si substrate. After drying in air for 30 min at room temperature, the transferred PMMA-attached graphene film on the interdigital electrodes was heated at 60 °C for 30 min in an oven. Then, the PMMA was removed from the graphene using boiling acetone and isopropyl alcohol, and thereby, the clean graphene attached to the interdigital electrodes was obtained.<sup>54</sup> Indeed, the single and bilayer graphene on the SiO<sub>2</sub> substrate are clearly visualized by an optical microscope. Under the optical microscope, no significant amount of bilayer graphene is observed; hence, the cleanliness of the transferred graphene was confirmed (Figure S1 in Supporting Information). Some small patches of PMMA might exist undetected on the graphene. However, it is unlikely that these trace amounts of PMMA invalidate the results obtained from the present AuNP-GFET biosensor.

**4.3. Graphene Modification.** The single-layer graphene transferred on the SiO<sub>2</sub>/Si substrate was decorated with ligand-free AuNPs using our recently developed technique.<sup>32</sup> The transferred graphene on the SiO<sub>2</sub>/Si substrate was directly immersed into the as-prepared AuNP solution during the AuNP synthesis at room temperature. The AuNPs were synthesized by the reduction of 1 mM HAuCl<sub>4</sub> solution by slow addition of sodium borohydride (NaBH<sub>4</sub>). Thus, homogeneous decoration of AuNPs (25–70 nm) on graphene transferred on the SiO<sub>2</sub>/Si substrate was obtained.<sup>32</sup> To facilitate the real-time measurement upon addition of liquid analytes, a liquid pool made with silicone sheet was fixed on the AuNP-decorated graphene in such a way that all the six pairs of interdigital electrodes are covered.

Avidin was immobilized on AuNP-modified graphene by injecting 100 μL of 1 mg/mL avidin–PBS solution onto the AuNP-decorated graphene enclosed by the silicone pool and holds for 1 h at room temperature. After reaction for 1 h, the device was rinsed with 100 μL of PBS three times. It is necessary to block the residual surface regions not modified by avidin for the prevention of the nonspecific binding of other molecules because this nonspecific binding of other molecules is likely to affect the signal during actual measurements of the sensor device. Hence, the residual unmodified surface regions of the graphene were blocked against any unwanted reaction by reacting with 100 μL of 0.01 mg/mL BSA–PBS solution for 1 h at room temperature. The device was again rinsed with 100 μL PBS three times.

**4.4. Real-Time *I*–*V* Measurements with Liquid Analytes.** Buffer solutions of biotinylated protein A with different concentrations were injected into the liquid pool sequentially. During the injections of solutions, the real-time current was continuously recorded. From the magnitude of current changes, the quantitative detection of biotinylated protein A in PBS solution at pH 7.4 was achieved. During the two-terminal measurement, the source–drain voltage was fixed at 0.1 V, and the gate (Si/SiO<sub>2</sub>) was grounded. The measurements were done by a Keysight 4155B semiconductor parameter analyzer. Two sets of experiments with synthetic samples and a real sample from chicken egg were used to check the specificity of the biosensor. In the first set of the experiment, fixed amounts of vitamin C, lactoferrin, BSA, and protein A–biotin were injected into the testing pool separately while monitoring the current. For the experiment with chicken egg sample, a chicken egg of regular size (50 g) was purchased from the supermarket. About 0.5 mL of egg white and yolk was carefully extracted from the raw egg. Then, both the egg white and yolk were diluted in PBS (1:100), and 50 μL of each solution was injected into the test solution.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c04429>.

Optical image of graphene, XPS spectra of AuNP- and avidin-modified graphene, optical image of the device, *I*–*V* curve after avidin functionalization, and *I*<sub>ds</sub> versus time plot for the specificity test using the synthetic sample (PDF)

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## Notes

The authors declare no competing financial interest.

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