Gold nanoparticle-assisted plasmonic enhancement for DNA detection on a graphene-based portable surface plasmon resonance sensor

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Abstract

The impact of different gold nanoparticle (GNP) structures on plasmonic enhancement for DNA detection is investigated on a few-layer graphene (FLG) surface plasmon resonance (SPR) sensor. Two distinct structures of gold nano-urchins (GNu) and gold nanorods (GNr) were used to bind the uniquely designed single-stranded probe DNA (ssDNA) of Mycobacterium tuberculosis complex DNA. The two types of GNP-ssDNA mixture were adsorbed onto the FLG-coated SPR sensor through the π-π stacking force between the ssDNA and the graphene layer. In the presence of complementary single-stranded DNA, the hybridization process took place and gradually removed the probes from the graphene surface. From SPR sensor preparation, the annealing process of the Au layer of the SPR sensor effectively enhanced the FLG coverage leading to a higher load of the probe DNA onto the sensing interface. The FLG was shown to be effective in providing a larger surface area for biomolecular capture due to its roughness. Carried out in the DNA hybridization study with the SPR sensor, GNu, with its rough and spiky structures, significantly reinforced the overall DNA hybridization signal compared with GNr with smooth superficies, especially in capturing the probe DNA. The DNA hybridization detection assisted by GNu reached the femtomolar range limit of detection. An optical simulation validated the extreme plasmonic field enhancement at the tip of the GNu spicles. The overall integrated approach of the graphene-based SPR sensor and GNu-assisted DNA detection provided the proof-of-concept for the possibility of tuberculosis disease screening using a low-cost and
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(Some figures may appear in colour only in the online journal)

1. Introduction

The world-shattering Coronavirus disease (COVID-19) pandemic has been an important reminder of the urgent need for rapid screening and diagnostic methods of respiratory and airborne pathogen infection [1–3]. Another major threat from the respiratory disease class is tuberculosis (TB) infection, which typically spreads in poor and developing countries [4]. The morbidity and mortality rate of TB was predicted to significantly as a consequence of the reallocation of resources during the COVID-19 pandemic worldwide [5, 6]. The Mycobacterium tuberculosis complex (MTBC) is considered to be the pathogenic species causing the TB disease. It has been declared to bring out deadly effects that have infected almost a quarter of the world population, predominantly in the low-to-middle income countries in Africa, Asia, South America, and Eastern Europe [3, 4]. Unfortunately, the human-to-human airborne transmission of this bacterium makes it hard to be tested in a rapid diagnostic or screening protocol. One of the well-known DNA sequence biomarkers of MTBC is the fragment IS6110, which is stable for decades from the mutation genetic part, and extremely specific to the MTBC species. This fragment exists in several copies in different locations of the MTBC genomic profile. This versatility has made this sequence a favorable biomarker for polymerase chain reaction (PCR) and sequence-based screening diagnostics.

PCR, as the golden standard for quantification of the pathogen through specific nucleic acid amplification, is still regarded as indirect, laborious, and expensive. These drawbacks have been the main challenges for low-to-middle income countries to perform routine and extensive PCR tests in society [1, 7]. Additionally, the typical bench-top PCR system is far from being portable, limiting its application for TB cases, which are most likely to occur in remote and harsh sites. Biosensor technology has long been spotlighted to provide an alternative to the time-consuming standard diagnostic methods, and its growth has been momentously accelerated by the current pandemic situation toward the realization of next-generation diagnostic methods. Among the myriads of disease biomarkers, the preference of the nucleic acid biomarker from the antigen is highly advantageous because its base-pairing hybridization is highly specific and robust [8] and easy to be functionalized with nanoparticles (NPs) and other surfaces [9]. On the other hand, the detection of serology biomarkers requires complicated purification–separation protocols due to the complex protein matrices in blood or bodily fluids [10, 11].

In the application of DNA on a biosensor, the uniform surface coverage by probe DNA molecules is essential to achieve an overall inherent biorecognition signal after hybridization with the target. The interfacial engineering for biomolecular captures, such as via cross-linking agents [12], electrostatic attraction [13], NP-assisted capture [14], and many other methods has been crucially developed to achieve the best sensing device performance. Gowtham et al reported the mechanism of physical absorption of nucleobases onto graphene and carbon nanotubes. Adenine (A), thymine (T), cytosine (C), and guanine (G) interaction between the graphene layer was dominated by the molecular polarizability, which played a vital role in the attractive dispersion force between these materials [15]. The orientation of the binding was reported to be almost parallel to the graphene layer with a separation distance of around 3.5 Å, and it indicated the interaction characteristic for the π–π stacking mechanism [15, 16]. Aptamer binding on graphene was also studied for application in α-thrombin monitoring. The aptamer probes were functionalized by the amine or sulfide group and adsorbed on the graphene surface by the π–π stacking force mechanism. Subsequently, the presence of the α-thrombins attracted the labeled aptamer from the graphene surface due to a massive covalent bond between the amino or sulfide group and the α-thrombin [17]. In addition, nanomolar and sub-nanomolar range detection of DNA hybridization by the use of graphene for surface-assisted adsorption of the DNA strand were also reported [18, 19].

The utilization of solution-based graphene such as graphene oxide (GO) for capturing the DNA strands via the π–π interaction was highly regarded as a simple and low-cost approach to initialize biomolecular recognition on the sensor’s interface [20–22]. A significant challenge in working with solution-based graphene is transferring it onto another solid substrate, such as a gold (Au) layer, typically used in the surface plasmon resonance (SPR) sensing setup. Song et al recommended the improvement of Au {111} crystallization on the surface to enhance the adsorption energy of the graphene onto the Au layer [23]. Another method emphasized the high possibility for colloidal graphene on a Au SPR chip through metal–carbon bonding with a considerably high uniform coverage rate [20]. The application of deformed multi-layer graphene for effective biomolecular capture was also studied by Hwang et al, who successfully created electronic hotspots from the capture of DNA on the crumpled graphene layer [24].

Among the metallic NP clusters, gold NPs (GNPs) have gained significant attention and have been combined with DNA in a number of sensing platforms, including
electrochemical, transistor-based, and optical sensors, due to their high bio-affinity, chemical and physical stability, surface area-to-volume ratio, and excellent optical features [25–31]. Specifically, the plasmonic enhancement from GNP s has been applied in a variety of optical sensing setups, such as in SPR, photoluminescence, and surface-enhanced Raman spectroscopy biosensors, showing ultrasensitive DNA or aptamer detection [20, 32–35]. The plasmonic coupling is predominantly defined by the nature, size, shape, structure, composition, and aspect ratio of the NPs and the environment dielectric constant and inter-spatial arrangement within the NPs on a solid surface [36]. Some studies have reported a remarkable electric field enhancement of branched GNP s with protruding spikes and sharp tips useful in the biosensing configuration [37, 38].

In this article, we present the proof-of-concept of the MTBC screening method, using few-layer graphene (FLG) on an SPR sensing system. The biorecognition signal from DNA was assisted by the tagging of probe DNA with GNP s with two distinct structures, gold nano-urchins (GNu) or gold nanorods (GNr), to be adsorbed onto the FLG layer via π–π stacking. The hybridization event with the target DNA was checked through the release of the gold nanostructure-tagged probe from the sensing interface. The plasmonic activity from both gold nanostructures was compared, and it was noted that GNu provided the most significant enhancement with an impressive limit of detection (LOD). The proposed work combined both the ease of solution-based graphene integration onto the portable SPR chip and facile detection methods for nucleic acid-based TB detection. Typically, experimental works associated with infectious diseases are carried out in a high biosafety level laboratory which highly restricts the circulation of equipments from and to the laboratory. Therefore, biosensor technology can be a potential approach to eliminate contamination, particularly the modular biosensing technology with the feature of component disposability.

2. Materials and method

The primary platform of the portable SPR biosensor utilizing an organic light source in this experiment was described in our previous reports and is depicted in figure 1 [39]. Modular-based instruments and their portability offer an advantage for the real detection scheme of MTBC, which commonly occurs in remote areas or third-world countries. The disposability of the modular component is beneficial for handling the toxicity of the samples.

2.1. Material and instruments

Graphene powder and its dispersion solution were purchased from Angstron Materials Inc. (Ohio, USA). GNu with 70 nm size and GNr with an aspect ratio of 2.5 were bought from NanoSeedz (Hong Kong, PRC). DNA sequences were purchased from Purigo Biotechnology Co. (Taipei, Taiwan). The DNA probe and target sequences were derived from the MTBC DNA fragment IS6110. The single-stranded (ssDNA) probe sequence used in this study was SH-CGTGCGGCTATTACGAGGAC TCCACGCTGG (30 mers). The sequence of the complementary ssDNA (cssDNA) target was CCGAT AATGCTCCTG AGGTGCGACC (25 mers). In comparison, the sequence of mismatched single-stranded DNA (mssDNA) utilized in the specificity test was ACAGC ATTGCGCGTT CAGACACCGC (25 mers). The target DNA strand was designed with shorter sequences (5 mers shorter) to
accommodate the spacing activity in the probe DNA. A phosphate-buffered saline (PBS) tablet from Sigma Aldrich (Missouri, USA) was employed to produce the PBS buffer solution. NaCl, K2HPO4, and KH2PO4 for the salt buffer solution were purchased from Sigma Aldrich (Missouri, USA). The RAMaker system from Protrustech, Co, Ltd. (Tainan City, Taiwan) was applied to perform Raman spectroscopy with a charge-coupled device camera system coupled with an Olympus microscope body. The collimation of light excitation was performed by a 100X objective lens (NA 0.5). The laser power source was 100 mW, the exposure time was 2 s, and the accumulation number was 3. The electron microscopy images were obtained from a field-emission scanning electron microscope (FESEM) with the instrument series of JSM-7500F (JEOL Co., Tokyo, Japan), and transmission electron microscopes (TEM) with the instrument type of JEOL JEM-1230 and JEOL JEM-2100 PLUS (JEOL Co., Tokyo, Japan). Finite-difference time-domain (FDTD) simulation was performed by Numerical (Pennsylvania, USA).

2.2. Sensing chip preparation

A gold sensing chip was prepared and produced based on the protocol explained in our previous report [40]. Next, the chip was annealed in a baking chamber at 300 °C for 30 min with a very slow ramp-up of the temperature, followed by an immediate quenching process by exposing it to N2 gas at 20 °C. The quenching ramp-down temperature should be carefully handled to avoid substrate cracks. The annealing–quenching step is critical for improving Au [111] crystalization [23, 41]. The dispersant solution of graphene was diluted in deionized water to reach a 1% concentration. To prepare the FLG solution, graphene powder was then dispersed in 1% of the dispersant solution until reaching a concentration of 0.1 mg ml−1 before ultrasonic treatment for 20 min. Subsequently, the FLG solution was dropped onto the sensing surface and baked at 80 °C (the boiling temperature point of the solution was avoided) until the sensing layer was dry.

2.3. DNA immobilization and detection

In this study, the ssDNA probe was tagged with the GNu before adsorption onto the sensing membrane. First, 70 nM GNu solution was centrifuged and suspended in PBS solution with a rotation speed of 700 x g for 30 min. The GNu solution was then mixed with the ssDNA in PBS until a final concentration of 100 nM ssDNA was reached. This mixed solution was kept at room temperature for 20–24 hours to allow effective covalent binding between the GNu and ssDNA probe. The target DNA was prepared through a serial dilution stage of surface functionalization. This FLG solution drop-casting method holds a potency for a straightforward and brief process for graphene-based SPR biosensor development.

3. Results and discussion

3.1. FLG deposition on Au SPR chip

The deposition of graphene was notably pivotal in interfacing DNA oligonucleotides onto the SPR sensing system. We observed that the drop-casting deposition of the FLG solution onto the Au sensing chip of the portable SPR sensor favorably resulted in graphene with a few-layer structure. In figure S1(a), available online at stacks.iop.org/NANO/32/095503/mmedia, the optical microscopy figure shows uniform coverage of FLG on the Au layer, indicated by the homogeneous transparent color spread onto the surface with tiny clumped particles over it. The surface morphologies visualized by FESEM in figure S2 demonstrate the FLG on top of the Au chip, indicated by some visible creases on the surface in contrast to the Au layer without graphene on top. The FLG structure is typically found in the drop-casting method as a result of the graphene film wrinkling due to the surface tension of water when evaporation takes place during the drying protocol [42]. It is noteworthy that the wrinkled structure of graphene is ascribed to the defects and holes in the lattice of tetrahedral sp3 hybridized carbon atoms, providing extra bonds that finally form atomically rough graphene sheets as compared to pristine graphene [43, 44]. Raman detection of the FLG film in figure S2 presents a substantial uniformity of graphene coverage with very similar spectral features taken at three different sites. Graphene fingerprint peaks were observed, such as visible 2D (∼2680 cm−1), considerably sharp G (∼1571 cm−1), and intense D (∼1340 cm−1) peaks, ascribed to the phonons coupling with two opposite wave vectors, sp2 carbon and sp3 carbon, respectively [45]. The lower intensity of the 2D compared with the G peak indicates FLG formation [46, 47], and the highly pronounced D peak (defects), as well as the small 2D peak, is attributed to the defects due to the removal of oxygen in the basal plane [45, 48]. The rough graphene sheet would be beneficial in trapping the probe due to the high surface area in the next stage of surface functionalization. This FLG solution drop-casting method holds a potency for a straightforward and brief process for graphene-based SPR biosensor development.

3.2. Absorption profiles of GNu and GNr

The architectural building block of gold nanostructures greatly determines its optical properties. The TEM image in figure 2 inset shows that the GNu consists of poly-branch tips with an approximate tip-to-tip diameter of around 70–80 nm. The absorption spectra of GNu and GNr in the PBS medium are demonstrated in figure 2. GNu absorption appears optimally at around 601 nm wavelength. In the presence of ssDNA on the GNu following the preparation of the probe, the absorption peak was shifted to ∼619 nm wavelength. This finding is in good accordance with that of Rotz et al 2015, who reported a similar range of wavelength shifts in a gold nanostar spectrum after DNA conjugation with good maintenance of colloidal stability [49]. In contrast, as seen in the TEM image in figure 2 inset, the dimension of GNr has a
length of ~40 nm and a rod thickness of about 10 nm. GNr absorption showed two major peaks, with the maximum peak at 724 nm wavelength and the minimum one at 522 nm. These peaks emerged as a consequence of the dual orientations of the GNr, which has longitudinal and transverse peaks [50, 51]. In the presence of ssDNA, the minor and major peaks were blue-shifted to 531 nm and 734 nm wavelength, respectively, indicating a successful formation of the ssDNA and GNr nanoprobe complex.

3.3. Annealing–quenching protocol of Au sensing chip and GNP effects on SPR signal enhancement

The annealing and quenching step of the Au SPR sensing chip before graphene solution deposition was an attempt to enhance the Au \{111\} orientation on the surface [41]. Higher-temperature annealing was suggested to obtain highly ordered Au \{111\}. However, considering that the melting point of the BK7 substrate is around 350 °C, the annealing point in our experiment was set to 300 °C. Moreover, the quenching step with the exposure of the substrate to the cold N2 gas was
conducted to avoid substrate cracks, which easily occur in quenching techniques with cool liquid immersion. Song et al reported that Au {111} film exhibited better binding absorption for graphene solution [23]. For the comparison purpose, we measured the probe immobilization on the FLG-covered Au SPR chip in the presence and absence of the annealing–quenching protocol, as displayed in figure 3(a). The strong absorption and homogeneous coverage of the FLG film on the Au SPR sensing chip were assisted by the annealing–quenching protocols, which, in comparison to the non-treated Au chip, dramatically amplified the SPR signal after the immobilization of the GNP-DNA matrix via the π–π stacking force between graphene and ssDNA. The π–π stacking force exists in the mechanism of ssDNA immobilization since both graphene and ssDNA share identical characteristics as electron-rich materials [16, 23]. It is also essential to note the topological impact of the uniform FLG coverage on the Au sensing chip for probe capture. Pertaining to the convection–diffusion reaction during the FLG drop-casting on the Au chip, the convection-triggered evaporation of liquid and surface roughness of the graphene layer could enable molecular absorption, facilitating DNA transfer to the graphene interface, which in turn cuts off the diffusion time and yields high-sensitivity detection [24].

On the other hand, in the Au SPR chip without the annealing–quenching step, the probe immobilization stage yielded a negligible SPR signal. This is indicative of the poor FLG sheet coverage on the Au chip. The FLG degradation was plausibly caused by buffer streaming in the reaction chamber, creating the lack of Au {111} orientation with higher energy absorption of the graphene layer. This outcome is in a linear agreement with the report of Song et al [23]. The morphology of Au film in the presence and absence of annealing is depicted in figure S3.

### 3.4. Probe immobilization

GNRs with a length of ~40–60 nm and aspect ratio 2.5 were applied to compare the SPR signal enhancement in the GNu-assisted probe immobilization stage. The SPR signal levels are presented in figure 3(b). The GNu-ssDNA achieves a three times higher SPR signal enhancement than the GNr-ssDNA probe’s signal. This phenomenon is closely linked with the plasmonic absorption behavior of GNu and GNr, which likely occurs in different wavelength regions. The plasmonic absorption of GNu is observed to be in the range of 600–620 nm, which is correlated to the intensity peak of the OLED light source in this experiment. Contrarily, the maximum plasmonic absorption of GNr was observed in the wavelength range of 720–750 nm, where the intensity of the OLED light source had dropped below the full-width at half maximum [39]. As displayed in figure 3(b), the GNu in this study successfully provided around three times higher enhancement in the SPR signal than the GNr.

### 3.5. Detection of cssDNA target

Probe DNA immobilization is pivotal in the overall study. In GNr-assisted DNA detection (figures S4(a) and 3(b)), the SPR signal from the GNr was indicative of probe DNA immobilization. However, compared to the GNu-assisted one shown in figures 3(a) and (b), the GNr signal is extremely low since the saturation level was achieved very rapidly within the low range level of the SPR signal (figure S4(a)). In the detection of a series of cssDNA concentrations, the SPR sensor detected the degradation of the signal level after the washing step by PBS solution in both GNr- and GNu-assisted DNA detection (figures S4(b) and 4(a)), respectively. It is also notable that in both approaches, the SPR signal dramatically leaped as the cssDNA target was injected into the reaction chamber due to the immediate change in the refractive index value resulting from the salt buffer from the target dilution. The DNA binding affinity is greatly affected by the ionic strength [52], and salt mainly shows interactions with the negatively charged phosphate molecules [53]. The contrast was seen in the SPR signals after the PBS wash in GNu-assisted DNA detection, which shows more pronounced signal reduction trends as a higher cssDNA concentration was injected into the sensing chamber (figure 4(a)). The drop of
### Table 1. Performance comparisons of our work with other SPR-based DNA detection studies.

| No | Sensing platform | Remarkable technique | Detection target | LOD | References |
|----|------------------|----------------------|------------------|-----|------------|
| 1  | Fiber optic SPR  | SAM-PEG surface chemistry binds the streptavidin for signal enhancement | DNA-protein | 2 nM | [59] |
| 2  | SPR              | DNA-templated polyaniline deposition | DNA-PNA | 5 nM | [60] |
| 3  | SPR imaging      | Signal enhancement by colloidal GNP | DNA hybridization | 10 pM | [61] |
| 4  | SPR imaging      | Signal enhancement by GNP | MicroRNAs | 10 fM | [62] |
| 5  | Laboratory SPR   | GO, signal enhancement by GNP | DNA hybridization | 10 fM | [20] |
| 6  | Commercial SPR Sensia | Graphene, signal enhancement by Au nanostar | DNA hybridization | 0.5 fM | [32] |
| 7  | Localized SPR    | Au nanoisland, signal enhancement by Au nanostar | DNA hybridization | 0.2–40 nM | [63] |
| 8  | Localized SPR    | Doxorubicin-modified GNP for signal enhancement | DNA hybridization | 600 pM | [64] |
| 9  | Commercial SPR Bia-core X | Signal enhancement by streptavidin | MicroRNA | 17 pM | [65] |
| 10 | Commercial SPR Bia-core X | Signal enhancement by super-sandwich assembly and streptavidin | MicroRNA | 9 pM | [66] |
| 11 | Laboratory SPR   | Surface-anchored rolling circle and GNP amplification | TB DNA | 5 pM | [67] |
| 12 | Modular and portable SPR | Few graphene layers, signal enhancement by GNu | TB DNA | 24.5 fM | This work |

The SPR signal below the reference signal level (negative ΔSPR signal) implies the release of ssDNA probes from the graphene surface by a stronger hydrogen bond force during DNA hybridization [54] than the π-π stacking force between graphene and probe DNA [55–57]. The removal of the probe is related to the different binding energies, where the DNA hybridization binding energy was estimated at around 65.7 kcal mol⁻¹ (equivalent to 2.85 eV) [35], while the π-π stacking force between the ssDNA and the graphene surface is only approximately 0.49 to 0.61 eV [7, 36]. The removal of the ssDNA and the GNPs away from the sensing area resulted in a significant drop in the plasmonic field, represented by the negative ΔSPR signal. This, by the nature, structure, and morphology of the GNPs, was much stronger in GNu than GNr in providing plasmonic effects. This phenomenon leads to a susceptible sensing method with negative signals.

Figure 4(b) demonstrates the calibration plots from the level signal after PBS washing protocols in the real-time dynamic measurement from GNu- and GNr-assisted DNA detection (figures 4(a) and S4(b)). GNu-assisted DNA detection yields a trendline with a correlation coefficient of 99% and an estimated LOD based on the International Union of Pure and Applied Chemistry (IUPAC) definition using confidence level 3. It means the LOD was calculated by the estimation of the concentration (the x-axis), when the signal level reaches three times the standard deviation (SD) of the reference measurement, in correlation to the dose–response standard curve. The SD of the reference measurement was 0.23, and consequently a calculated LOD of ~24.5 fM cssDNA was achieved, significantly lower than that estimated in the GNr SPR signal shown by 8.2 pM of cssDNA.

The calibration plot from the specificity test performed using non-complementary mssDNA target hybridization with the GNu-cssDNA probe is displayed in figure 4(b). In this test, the hydrogen bond of the DNA hybridization was assumed not to exist, and hence the mssDNA strands were accumulated in the graphene layer due to the π-π stacking force. This mechanism produced a slight shifting of the delta SPR signals to the positive signal [58], which opposed the results from the specific binding of hybridized DNA. The combined calibration plots clearly distinguished the GNu paramount characteristics in providing SPR signal enhancement for both highly sensitive and specific detection of DNA hybridization. The plasmonic field enhancement characteristics of GNu and GNr simulated in FDTD are presented in figure 5. GNu showed prominent hotspots in every tip of its spicules, while the GNr hotspots were likely distributed at the edge of its diameter. The robust plasmonic profile of GNu in the simulation affirms the findings on its UV–vis absorption behavior shown in figure 2. It is important to note two crucial contributions of GNu in DNA detection using our constructed SPR sensor. One, the surface roughness effects on the GNu facilitate higher probe DNA binding sites, as proven by the larger shift in the UV–vis absorption outcomes than in the GNr bonded-probe DNA. Two, the sharp spicules and rough morphology of the GNu exhibit a drastic improvement of the SPR signals compared with the smooth-surfaced GNr, as shown in the FDTD simulation.

Comparing with reported studies in related fields in table 1, it is indicated that our modular and portable sensor using a graphene-based SPR sensor combined with GNu-assisted DNA detection shows excellent advantages in terms of simplicity, portability, and specificity. The detection limit in our proposed study is highly comparable to that of the commercial SPR and laboratory SPR, as well as the SPR imaging system, indicating a promising technique for DNA detection at a low concentration.

### 4. Conclusion

A proof-of-concept of the DNA hybridization of MTBC, assisted by the plasmonic field from GNu, has been presented in a portable graphene-based SPR sensor. The drop-casting of...
an FLG solution on the Au chip of the SPR sensor has paved the way for simple, time-saving, and low-cost sensor production. The high accuracy of detection performed in this study, reflected by the low detection limit and high specificity in DNA detection, offers new insights for early screening methods of bacterial infections such as TB in a rapid a low-cost manner with portable sensors, with the possibility of application in remote areas or third-world countries as well as in pandemic scenarios.

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