Ultrasonic and rapid detection of malaria using graphene-enhanced surface plasmon resonance

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Abstract

Extraordinary optical, electrical and chemical properties of 2D materials have potential to be useful for quick and sensitive detection of pathological diseases. One important example is malaria disease that can progress rapidly and cause death within days. Therefore, fast, accurate and cost-effective malaria diagnosis available at the point of care is urgently needed to facilitate precise treatment. Here we report rapid and highly sensitive malaria detection with an inexpensive graphene-protected copper surface plasmon resonance biosensor. Using phase sensitive surface plasmon resonance technique and a graphene functionalization protocol for attaching end-tethered DNA probes that were complementary to a malaria specific DNA target, we were able to significantly improve the detection limit of the malarial plasmodium parasite. The phase sensitivity of our graphene-enhanced sensors exceeds by two orders of magnitude the sensitivity of analogous optical biosensors. This enhanced sensitivity could provide means to detect low copy number bacterial infectious agents and to associate dormant bacterial populations with chronic inflammatory diseases using simple label-free optical detection.

1. Introduction

2D materials show great promise for applications in various areas of science and technology [1–3]. One such exciting application emerges in biosensing and healthcare where the large surface-to-volume ratio of 2D materials leads to strong signals produced by interaction of bio-objects [4, 5]. Optical interrogation of bio-interaction with 2D materials appears to be the most promising technique for development of novel 2D biosensors [6, 7]. The sensitivity of optical label-free bio-detection by 2D layered materials can be greatly improved by plasmons [8, 9] and phonons [10].

Recently, we proposed a layered material platform for surface plasmon resonance (SPR) biosensing which was based on an original idea of using graphene not only as a protector of plasmonic properties of reactive metals (Cu, Ag, etc) [11] but also as bio-functionalized surface for detection of low molecular weight bio-objects (e.g. HT-2 toxin important for food safety) [12]. The use of alternatives to Au (with better morphology) in graphene-enhanced SPR biosensing platform allows one to employ phase sensitive methods of optical interrogation which are known to provide unrivalled bio-sensitivity [13–16].

In this work, we used graphene-covered SPR chips based on Cu thin films for the ultrasensitive detection of malaria. Malaria is a life-threatening disease caused by plasmodium parasites that are transmitted to people through adverse contact with
infected female *Anopheles* mosquitoes. It is worth noting that severe malaria can spread rapidly and cause death in a very short time. Additionally, the incorrect prescription of drugs as a result of misdiagnosis leads to malaria drug resistance which increases the difficulty of treating malaria. Thus, there is an urgent need for fast and accurate malaria diagnosis at the low-density of parasite level for early diagnoses and effective therapy. Microscopy and rapid diagnostic tests (RDTs) are the primary choices for diagnosing malaria in the field [17–19] with the results of parasitological confirmation being available in 30 min or less. However, neither of these methods is capable of detecting low density (<20 parasite/µl) parasite infections, which is common in both low and high transmission rate settings. Nucleic acid amplification tests (NAATs) including PCR, LAMP, QT-NASBA, and ELISA [20–24], enable sensitive detection of low density malaria infections (below 1 parasite/µl), but these methods are complex, expensive, time consuming and require highly specialized facilities which cannot be easily used by the non-specialist in remote underdeveloped areas.

Graphene-protected Cu SPR can significantly improve the detection limit of biosensing due to its inherent high phase sensitivity [11, 12]. The suggested graphene-protected SPR chips are inexpensive compared to commercial Au chips (10–100 times cheaper) and could be applied to realise sensitive in-field malaria diagnostics [21, 25]. The possibility of simplifying diagnosis and improving sensitivity by using graphene-protected biosensors is intriguing; however, protocols for graphene functionalization for detection of malaria-related DNA sequences are required to realise selectivity of SPR detection. Here, low-to-high concentrations of malaria DNA sequences were detected using appropriately functionalized graphene-protected copper SPR chips. Since in our SPR chips graphene was used not only for metal protection but also for bio-functionalization we will refer to these chips as graphene-enhanced SPR (GESPR) chips. The phase sensitivity of GESPR to relatively small malaria plasmodium DNA targets was 12 pM which is comparable with the current detection limit based on labelling techniques [26]. As the mass of the studied DNA sequence was 9.75 Da, this yields the mass detection limit of 100 pg ml⁻¹ which is 50 times better than the 5 ng ml⁻¹ optical detection limit quoted in [21].

Our work not only provides a fast and easy way to detect malaria with high sensitivity using graphene-enhanced copper SPR biosensors, but also provides general recipes for detection other DNA-based biomolecules, important in many applications, such as human health, food and environmental safety where sensitive, specific, simple, and rapid detection is required.

2. Materials and methods

2.1. Fabrication of graphene-protected copper SPR sensor chips

SPR chips were produced by depositing 1.5 nm Cr and 43.5 nm Cu films onto a glass substrate using electron beam evaporation (MiniLab060, Moorfield Nanotechnology). The depositions were performed at a base pressure of about 10⁻⁷ mbar and growth rate of 0.3 nm s⁻¹ while the film thickness was measured by calibrated quartz microbalance. 99.99% Cu beads from Sigma-Aldrich were used as an electron-beam target. Microscope slides of sizes 25 mm by 25 mm and thickness of 1 mm were used as substrates for all the studied samples. The substrates were ultrasonically cleaned in heated acetone and isopropanol before depositions. The depositions were followed by an immediate transfer of a chemical vapour deposited (CVD) graphene flake on the top of the copper film.

Single layer graphene films were grown on copper using the CVD method. In short, a 25 micro-metre thick Cu foil was placed inside a quartz tube and then heated to 1000 °C with a H₂ flow at rate of 20 cm³ min⁻¹ and a pressure of 200 mTorr. The foil was annealed and then a gas mixture of H₂ and CH₄ was introduced into the chamber where the CVD growth of graphene was performed at a pressure of 600 mTorr for 30 min. Finally, the CVD chamber was rapidly cooled to room temperature in a H₂ atmosphere. CVD-grown graphene was checked with Raman (WITec, DV401-A-BV-352, CCD-11889) (which confirmed graphene single layer nature of the CVD grown films over the used area of around 1 x 1 cm²) and then transferred onto the target sample by using the procedure described in detail in [11]. The procedure included covering of CVD graphene on copper foil with spin coated poly(methyl methacrylate) (PMMA). Then, the PMMA film attached to the graphene layer was isolated by etching away the Cu foil. The resultant PMMA-graphene film was transferred onto the target copper films. To promote adhesion between the graphene and copper films samples were annealed in a H₂/Ar atmosphere at 150 °C for 3 h. The PMMA layer was removed in acetone and the graphene surface was cleaned by rinsing in IPA.

2.2. Graphene functionalization for malaria detection

To the best of our knowledge, there exist no previous studies that use graphene-protected copper SPR chips for the detection of a plasmodium malaria membrane DNA target. Here, we developed a new protocol for functionalization of graphene in GESPR chips designed to achieve high selectivity of malaria DNA sequence detection (see Supplementary Information). This protocol includes two generic steps: i) electrochemical graphene grafting with the COOH
Figure 1. Schematic illustration of the functionalization of graphene-protected copper SPR sensor chip for detection of malaria DNA sequences.

group followed by ii) graphene surface tethering to the 5′ end of the DNA probe via use of a polythymi-dillic acid spacer and formation of an amide peptide bond. Figure 1 shows a schematic illustration of the graphene functionalization steps of the graphene-enhanced sensor chip. Firstly, the COOH group was grafted on the surface of graphene by an electrochemical method [27, 28]. The original procedure (grafting from 50 mM HCl solution) was complicated by significant etching of the copper layer. In an optimized protocol we used imidazole and orthophosphoric acid which are well-known copper corrosion inhibitors [29]. The protocol included the following steps. First, to 25 ml of an aqueous solution containing 0.052 mmol of 4-amino-3,5-difluorobenzoic acid (95%, Fluorochem), 0.52 mmol of H₃PO₄ (85 wt% in H₂O, Sigma-Aldrich) and 12.8 mmol of imidazole (99%, Sigma-Aldrich) 0.5 ml of 0.1 M aqueous solution of NaNO₂ (99%, Sigma-Aldrich) were added and the mixture was stirred vigorously for 3 min. Then, the solution was transferred into a single-compartment electrochemical cell consisting of the graphene/Cu sample (working electrode), 2 cm × 2 cm platinum foil (counter electrode) and
Figure 2. A schematic diagram of a flow cell for graphene-protected copper SPR biosensing of malaria DNA oligonucleotide sequences. The sensor chip was pre-functionalized with COOH groups and the NH$_2$-DNA malaria probe sequence shown below in the buffer solution of N’N-dimethylformamide (DMF):5’-(NH$_2$)-TTTTTTGGGTTTAGTTTGACCAAACCGAAT-(CY3)-3’ was attached.

2.3. SPR measurements for malaria detection

Figure 2 shows a schematic diagram of the flow cell for graphene-protected copper SPR biosensing of plasmodium malaria membrane DNA sequences. Graphene-protected Cu films (43.5 nm) were used as sensor chips capable of ultrahigh phase sensitivity in SPR biosensing reported before [13]. SPRs were observed using the Attenuated Total Reflection (ATR) method [31]. The graphene-enhanced sensor chips were pre-functionalized with COOH groups followed by the binding of NH$_2$-DNA probe which contained the susceptibility gene of malaria (see figure 1). During measurements, a SPR sensor chip was fixed in the flow channel and different concentrations of DNA analyte solutions with plasmodium malaria membrane encoding DNA sequence 5’-ATTCCGGTTTGGTGCAAACCGAAT-(CY3)-3’ (derived from the malaria parasite Plasmodium falciparum, clone 3D7, the gene sequence located on chromosome 13 that was found to be unique for Plasmodium falciparum with the GeneBank identification number LR131493.1) in N’N-dimethylformamide...
Figure 3. Graphene-enhanced SPR sensing of malaria DNA detection. (a–b), Ellipsometric parameters $\Psi$ (amplitude) and $\Delta$ (phase) of the SPR curve of functionalized sensor chip after reacting with different concentrations of malaria DNA oligonucleotide target sequence. (c–d), The shift of resonant wavelength ($\delta \lambda$) for $\Psi_{\min}$ and the change of phase ($\delta \Delta$) is shown as a function of concentration of the in-solution target DNA. The dark dashed line shows the sigmoidal fit of $\delta \lambda$ as a function of concentration, and the red dashed line is the same for $\delta \Delta$. The error bars show the standard errors of $\delta \lambda$ and $\delta \Delta$ from data collected after reacting with different concentrations of malaria DNA analyte. The errors on all amplitude $\Psi$ measurements were below 0.1° and on phase $\Delta$ measurements were below 0.05°.
r16S sequence to demonstrate specificity. DNA with single base pair mismatch could easily be a mutated malaria DNA.

3. Results and discussion

3.1. Detection of malaria with graphene-enhanced copper SPR biosensor

GESPR chips were used to detect malaria DNA short sequences in positive reaction and to check non-specific binding with non-malaria DNA sequences in negative reaction. Three different concentrations of the complementary DNA analyte solutions in DMF buffer (8.3 nM, 50 nM and 500 nM) were applied to measure the SPR sensor response to malarial DNA sequences. After introducing each concentration of malaria analyte solutions in DMF buffer, distilled water was pumped into the flow channel and the SPR response of the sensor chip was measured. When the binding of DNA analyser to DNA probe took place in the detection area, Ψ and Δ of the graphene-protected copper SPR curve changed as shown in figures 3(a) and (b). Figure 3(a) depicts the red-shift of resonant wavelength due to the local refractive index changes induced by the binding of the target DNA sequence. In addition, the change of effective refractive index around the copper thin film modifies the optimum angle of SPR excitation which causes the increase of Ψmin. In figure 3(c), we can see that the shift of resonant wavelength (from lowest to highest concentration) is about 8.5 nm. For lowest concentration, the value of δλ was around 2.8 nm. Since the recorded spectra were acquired with the spectral of 1 nm, this yields the limit of detection (LOD) for amplitude measurements as 3 nM. (This LOD value can be improved by performing spectrum fitting near the resonance dip which allows deduction of the resonance position more accurately by using a larger amount of spectral points). Figure 3(b) plots changes in the ellipsometric phase produced by the binding with increasing concentration of malaria DNA analyte. We observe an extremely large phase jump (∼180 degrees) near the resonant wavelength which confirms high phase sensitivity of SPR in graphene-protected copper films [11]. The relationships between phase change (δΔ) and the concentration of malaria DNA analyte is shown in figure 3(d). The phase change (∼34.6 degrees) after pumping 8.3 nM of DNA analyte is quite large. This allows calculation of the phase LOD by taking into account...
the experimental phase resolution of our ellipsometric system $\sim 0.05^\circ$. This yields phase LOD as 12 pM which is two orders of magnitude greater than the detection limit of amplitude measurements, see the discussion of LOD in amplitude and phase measurements in [11, 12]. The improvement over conventional amplitude sensitivity comes about from the darkness of graphene-protected copper resonances and enhanced stability of phase measurements [14]. Taking into account the mass of the studied DNA sequences (9.75 Da), we get the mass detection limit of 100 fg $\mu$l$^{-1}$. These numbers obtained by our label-free phase GESPR technique are approaching those achieved with complex labelling methods [26]. (It is worth noting that amplitude surface plasmon resonance was used to detect only reasonably large antibodies of *plasmodium falciparum* [32] with four orders of magnitude lower mass LOD than that obtained in our work.) As has been reported in previous SPR studies [33], the change of measured variables ($\delta$ and $\Delta\delta$ in our experiments) with increasing target malaria DNA sequences is described well by a sigmoidal function [34]. The sigmoidal fits are shown in figures 3(c) and (d) and yield values of cooperativities $nH = 2.6$ and 1.4, and ligand concentrations $K_H = 1.4$ and 0.87, respectively. The positive cooperativity implies that the binding of a ligand increases the binding affinity of the neighbouring sites.

### 3.2. Negative control of graphene-protected copper SPR biosensor using 16S ribosomal DNA

To confirm the selectivity of the GESPR sensor, we performed negative control hybridisation-based studies on the pre-functionalized GESPR chips. Three different concentrations (500 pm, 5 nM, and 100 nM) of 16S ribosomal DNA probe sequence (not connected to malaria) were employed to check the non-specific binding as shown in figure 4. Experimental conditions are the same as in figure 3. The change of $\Psi$ and $\Delta$ of graphene-protected copper SPR curves in distilled water is shown in figures 4(a) and (b). The corresponding shift of resonant wavelength ($\Delta\lambda$) for $\Psi_{\text{min}}$ and phase change ($\delta\Delta$) is depicted in figures 4(c) and (d). The shift of resonant wavelength in case of non-specific 16S ribosomal DNA binding was 4 times smaller than that for the complementary malaria sequence, while the phase shift was an order of magnitude smaller. Thus, we conclude that non-specific binding has a little effect on the process of specific malaria detection.

### 4. Conclusions

In summary, we demonstrate a real-time, label-free, highly sensitive and inexpensive graphene-enhanced SPR sensor for malaria diagnosis based on malaria specific oligodeoxyribonucleotides. The phase SPR detection limit based on 2D materials (graphene) was 12 pM. More importantly, the GESPR sensor required simple functionalization procedure and label-free optical malaria detection can be performed in minutes. Our approach has great potential to be used for point-of-care testing and is suited to be used by non-specialists. Given the maturing of graphene functionalization technology and advanced phase detection systems with higher phase resolution, our technique paves the way to realize the broad applications of graphene-protected copper SPR sensors for ultrahigh sensitive specific molecule detection in the near future.

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