Chapter 8

Graphene Oxide Based Surface Plasmon Resonance Biosensors

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Additional information is available at the end of the chapter

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1. Introduction

Graphene oxide (GO), an amorphous insulatormaterial, has consists of a hexagonal ring based carbon network having both sp^2-hybridized carbon atoms and sp^3-hybridizedcarbons bearing hydroxyl andepoxide functional groups on either side of the sheet, whereas the sheet edges are mostly decorated by carboxyl and carbonyl groups [1-6]. These unique properties hold great promise for potential applications in many technological aspects such as nanoelectronics [7-10], nanophotonics [11-16], and bio-sensors [9, 17-21], and nanocomposites [22, 23].

This chapter is intended to demonstrate a facile and effective method to construct GO sheets onto the self-assembled monolayer (SAM) at Au films based surface plasmon resonance (SPR) technique for proteins immunization and deoxyribonucleic acid (DNA) detection. The amine functionalized grapheme oxide has demonstrated various applications. Given the high number of functional groups in the GO, such as -OH, -COOH, and epoxides, compared to those in Au electrode. The oxygen functional groups on graphene oxide surfaces, including carboxylic acid groups at the edge and epoxy/hydroxyl groups on the basal plane can be utilized to change the surface functionality of graphene oxide.

We introduced a novel and simple methods for fabricating high-sensitivity, high-resolution GO based SPR biosensors that provide high accuracy and precision over relevant ranges of analyte measurement. We used SPR technique todetect the binding phenomenonbetween proteins and GO films. SPR is a surface-sensitive optical technique that very suitable for monitoring of bio-molecular interactions occurring in very close vicinity to sensor surfaces. It allows real-time and label-free detection of analysis by exploiting the interfacial refractive index changes associated with any affinity binding interaction between a biomolecule immobilized on a sensor surface and its biospecific partner in solution. Rapid and real-time analysis without labeling, highly specific detection with extremely low detection limits

creating the unique potential for the application of SPR in biosensor. Owing to these powerful advantages of GO-based SPR technique, many applications of GO-based biosensors in such areas as molecular engineering, biomedical diagnostics, drug discovery, environmental monitoring and food analysis.In this chapter, we report progresses in the following areas:

- Characterization of functionalized graphene oxide
- Characterization optical of graphene oxide sheets
- Graphene oxide based SPR biosensorstechnological
- Graphene oxide layer effect
- Biomolecular detection

This chapter addresses the preparation, characterization and potential biosensing applications of graphene oxide-based SPR, which have beenparticularly relevant in our research group at bio-plasmonic. Special attention will be dedicated to GO precursor as a building block for the preparation of these nanocomposites. Interesting and promising applications for these materials are also discussed.

2. Characterization of functionalized graphene oxide

The oxidized counterpart of graphene, functionalized graphene sheets, which is also called graphene oxide (GO) sheets as shown at Fig. 1(a), usually has abundant functional groups which are advantageous for biosensor applications [9, 17-21]. It has been used as a platform for the detection of proteins [24] and DNA [25] by utilizing its good water dispensability, and versatile surface modification. There have many method to evidencing the presence of –COOH, –OH, and C=O groups at the edge of the GO sheet, like infrared spectroscopic, extensive nuclear magnetic resonance, and electron diffraction.The basal plane of GO is covered with mostly epoxide and –COOH groups as shown at Fig. 1(b). Semi-quantitative study of the EDC/NHS activation of acid terminal groups at modified graphene oxide surfaces. Thus, we use GO as a medium for the covalent binding reaction with proteins. It is expected that forming the novel GO sheets will taking advantages of the striking properties of GO [26-28]. Therefore, we can obtain hybrid material manipulation and new electrochemical properties.

2.1. Characterization optical of graphene oxidesheets

The GO is synthesized by the oxidation of graphene, and its unique atomic and electronic structure has been revealed toshow small sp^2 carbon clusters that are isolated by the sp^3 matrix [29, 30]. GOsheets bearing carboxyl groups (-COOH) located atthe sheet edges andhydroxyl (-OH) located at the sheet surface, these functional groups makes GO sheets have strongly hydrophilic, which allows GOto readily swell and disperse in water[31].Unlike other conventional materials, the valence band and conduction band in graphene are smooth-sided cones that meet at a point, called the Dirac point, and graphene has no energy band-gap and readily

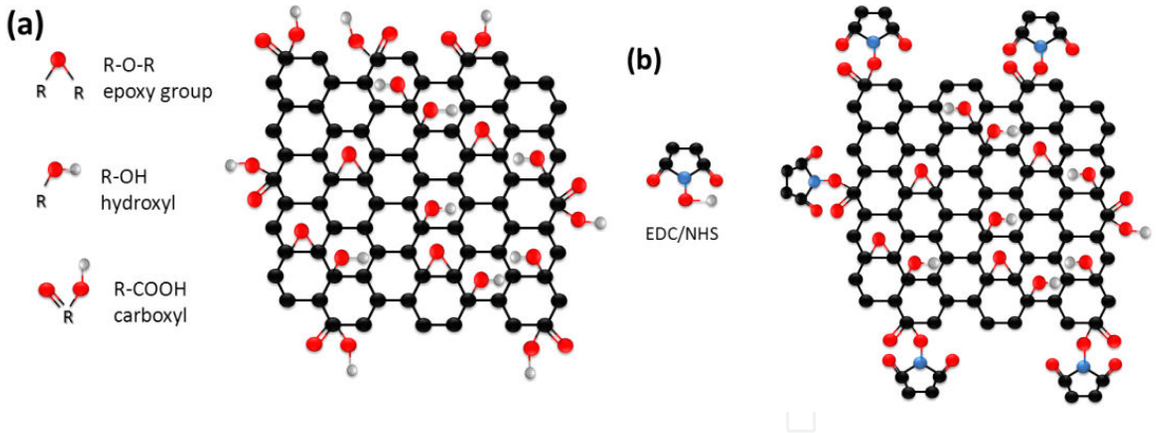

Figure 1. (a) graphene oxide structure, (b) chemical modificationamine-reactive EDC/NHS esters can be created on any carboxyl-containing molecule.

absorbs allphotons at any wavelength[32].Due to the adsorption of oxygen inthe form of epoxy, carboxyl, hydroxyl, andether groups on graphemeoxide surface not only opens up the band gap, but also provides anoption to tune the electronic, optical, andmechanical properties by means of controlled oxidation [15].

The nonlinear optical properties andultrafast relaxation dynamics shows that the unique atomic and electronic structure of GO leads to the emergence of variable absorption processes under different input intensities, and the different absorption processes are distributed between the sp^2 clusters and the sp^3 domains of GO [33]. Consequently, the nonlinear optical properties of GO are determined by the combined action of the sp^2 and the sp^3domains. Owingto theunique and large two-dimensional (2D) π-electron conjugation systems of graphene, much like that in zero-dimensional (0D) fullerene and one-dimensional (1D) carbon nanotubes (CNTs), we expect that GO would exhibit good nonlinear optical properties. Finally, Zhibo Liu et al. [34] had demonstrated that graphene oxide has a large two photon absorption coefficient.

Thus, we used ITO as substrate, attaching with graphene oxide by APTES, forming GO sheets (0.275 mg/ml), and get multilayer GO sheets through layer by layer (LBL) technique [35]. In Fig. 2., we observed that the relative transmittance fall down when GO layers rises. The result here was corresponded to Liu's [34] theory. The GO sheets that we used hasmany advantages for optical sensing of biomolecules detection which compared with conventional immune interactions.These advantages arise from the unique physical and chemical structure of GO.

2.2. Graphene oxide based SPR biosensors technological

The surface plasmon resonance (SPR) behavior of free electrons or plasma at the interface of a metal-dielectric material has been widely studied [36–43]. Prism coupling between Kretschmann and Otto configurations has been used extensively to study the optical properties of metallic thin films, including refractive index (n), extinction coefficient (k), thickness (d), and roughness [44, 45]. Conventionally, SPR biosensors are used in biochemistry and biology to detect molecular concentration, thickness, and specific chemistry analytes. In biochemistry,

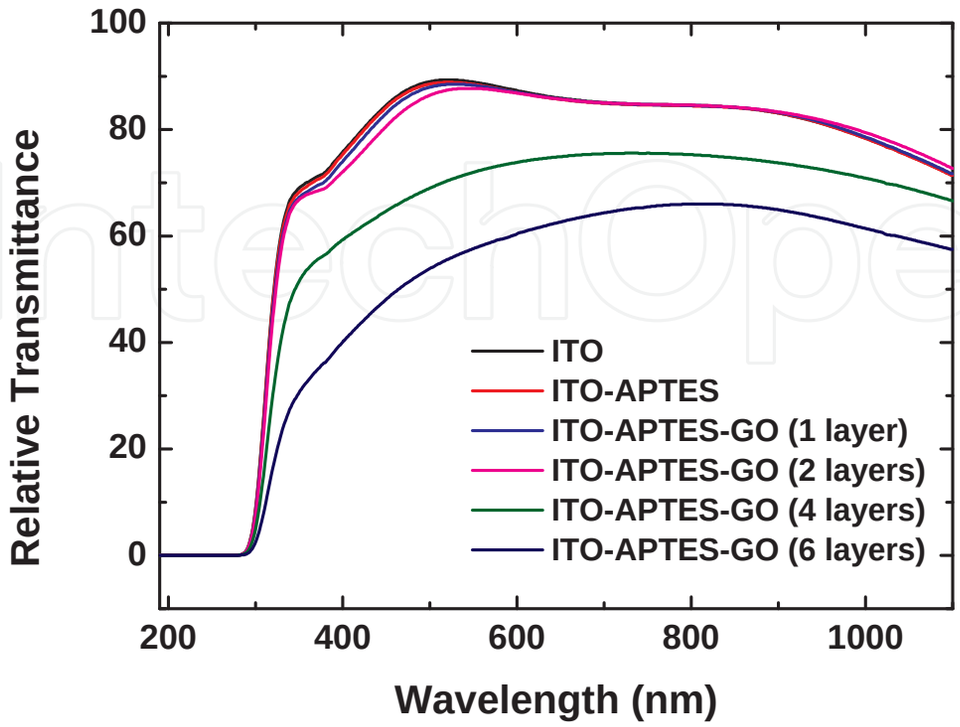

Figure 2. Relative Transmittance (%T) of ITO film, ITO-APTES film, ITO-APTES-GO (0.275 mg/ml) 1 layer, 2 layers, 4 layers, and 6 layers, respectively.

analyte concentration is determined from the SPR angle shift by a biosensor operating in the angular interrogation mode. The shift or difference between the initial and final values of the SPR angles provides a quantitative measurement of the analyte concentration.

The SPR is another interesting field of research for rising applications of GO. SPR occurs when radiant energy is coupled or transferred to electrons in a metal film. The wavelength of light at which coupled occurs depends on the characteristics of the metal that is illuminated and the optical properties of the surrounding environment. When there is a match or resonance between the energy and wavenumber of the light photons and the electrons at the metal surface, a transfer of energy occurs.

The electrons charge on a metal boundary can perform coherent fluctuations which are called surface plasmon oscillations. Their existence has been demonstrated in plasma losses by fast electrons at thin films experiments.The volume and surface plasma losses ofmaterial can be identified in reflection experiments by varying the primary electron beamenergy and observing the intensity variation of the energy loss peaks at a fixed scattering angle. The frequency $\omega$of these longitudinal oscillations is tied to its wave vector k_x by a dispersion relation $e(k_x)$. These charge fluctuations, which can be localized in the Zdirection within the Thomas-Fermi screening length of about 1Å, are accompanied by a mixed transversal and longitudinal electromagnetic field which disappears at $|Z| \to \infty$, Figure 3(a), and has its maximum in the

surface Z=0, typical for surface waves. This explains their sensitivity to surface properties. Maxwell's equations yield the retarded dispersion relation for the plane surface of a semi-infinite metal $\left(\varepsilon_m = \varepsilon_m^{'} + i\varepsilon_m^{''}\right)$, adjacent to a medium dielectric , Fig. 3(b), has an interface metal $\left(\varepsilon_m = \varepsilon_d^{'} + i\varepsilon_d^{''}\right)$/dielectric (ε_m), on which electromagnetic fields propagates in the x direction.The coupling of light into a metal surface produces a plasmon. The plasmon, in turn, generates an electro-magnetic field that typically extends on the order of about 100 nm above and below the metal surface and oscillates with optical frequencies [36, 46-48].

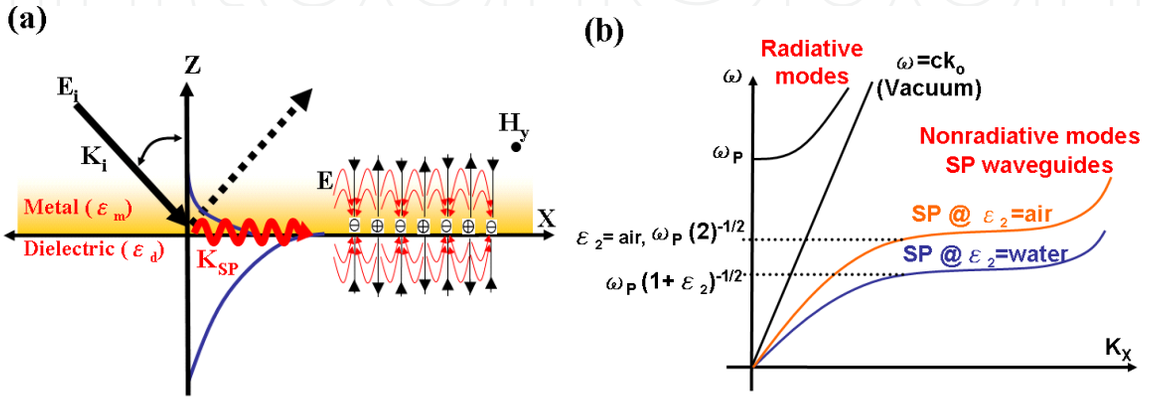

Figure 3. Schematic presentation of surface plasmons is constituted of resonantly oscillating surface charges at a metal/dielectric interface and of the electromagnetic surface wave that originates from these surface charges. (a) Electric field lines of a surface plasmon on a smooth surface and the electromagnetic field of SPs propagating on a surface in the x direction. (b) The dispersion relation of non-radiative SP modes and radiative modes.

The most common approach to excitation of surface plasmons is by means of a prism coupler and the attenuated total reflection method (ATR). There are two configurations of the ATR method-Otto geometry [36, 37] and Kretschmann geometry [36, 38]. If light wave is reflected at a metal surface covered with a dielectric medium (ε_0>1) e.g., with a quartz prism, its momentum becomes (ε_d) instead of $(\hbar\omega/\mathbf{c})\sqrt{\varepsilon_p}$ and its projection on the surface as shown in Fig. 4(a). The plasmonic dispersion relation for SPs propagating on the interface $(\hbar\omega/\mathbf{c})$ can thus be satisfied between the lines c, beyond which limit total reflection at an interface $\mathbf{E_d}/\mathbf{E_m}$ takes place.

The grapheme oxide can provide new perspectives in SPR ways: (1) as a surface which directly supports surface plasmons at visible range, (2) as a tunable propagation and excitation platform whose optical properties can be tuned by an external electromagnetic field, and (3) as an oxygen-functional groups coating for the existing plasmonic devices. These oxygen-containing functional groups on graphene oxide not only opens up the band gap, but also provides an option to tune the electronic, optical, and mechanical properties by means of controlled oxidation [15, 16, 49-51].

The formation scheme of gold surface multilayer modification system for site-oriented immobilization of GO sheets, GO is assembled on a gold film by a layer-by-layer technique using a self-assembled linker monolayer. The GO modified electrode is represented as Au/

linker/GO. Protein contains both amine group (–NH2) and carboxylic acid group (–COOH). It is evident that GO possess a number of carboxylic acid group (–COOH) that can easily bind with the free –NH2 terminals of the protein to result in a strong amide covalent linkage. Figure 4(b) shown schematic representation of the sensing configuration for the GO based SPR immunoassay.

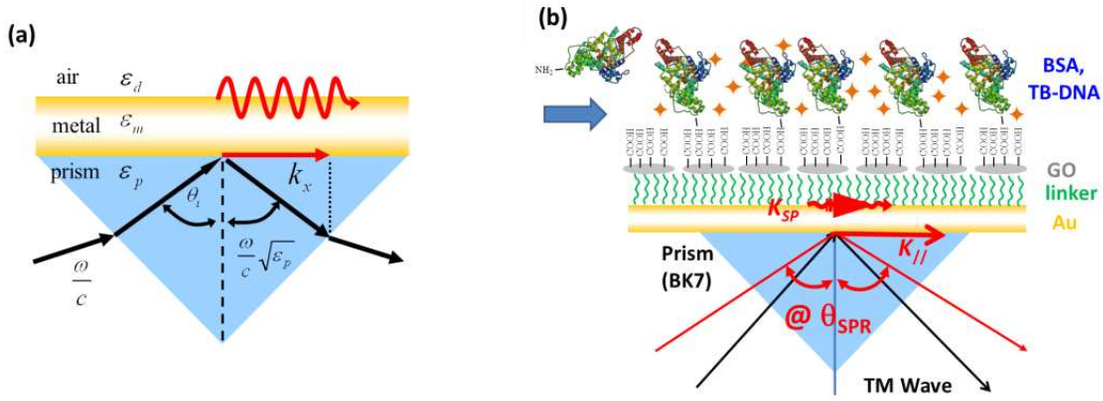

Figure 4. Excitation of surface plasmon in the (a) Kretschmann geometry and (b) functionalized graphene oxide based SPR biosensing mechanisms.

When a light wave propagating in the prism is made incident on the metal film a part of the light is reflected back into the prism and a part propagates in the metal in the form of an inhomogeneous electromagnetic wave [36-38]. This inhomogeneous wave decays exponentially in the direction perpendicular to the prism-metal interface and is therefore referred as to an evanescent wave. If the metal film is sufficiently thin (less than ~50nm), the evanescent wave penetrates through the metal film and couples with a surface plasmon at the outer boundary of the film [36]. In order for the coupling between the evanescent wave and the surface plasmon to occur, the propagation constant of the evanescent wave and that of the surface plasmon have to be equal (1) and (2):

$$\mathbf{E_p / E_m} \tag{1}$$

$$k_x = \sqrt{\varepsilon_p}\frac{\omega}{c} sin\theta_i \tag{2}$$

The dispersion relation of free photos in a dielectric $k_{sp} = \frac{\omega}{c}\sqrt{\frac{\varepsilon_d \varepsilon_m}{\varepsilon_d + \varepsilon_m}}$, and in a coupling prism $c / sin\theta_i$, compared to the dispersion relation for non-radiative surface plasmons at the metal/dielectric interface as shown in Fig. 5.

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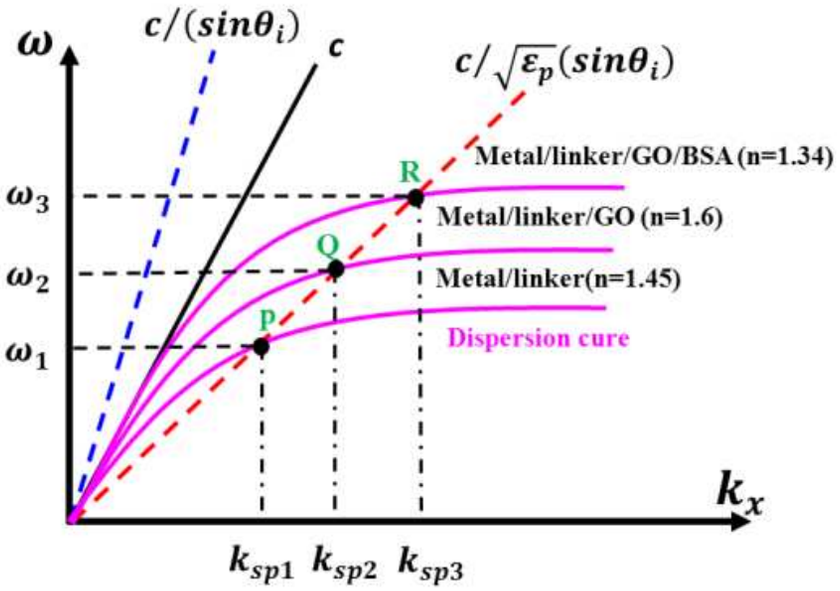

Figure 5. The ATR method: Dispersion relation of SPs for a prism/metal/air system $c/\sqrt{\varepsilon_p}\, sin\theta_i$, c is light line in vacuum, $\varepsilon_d = 1$ is light line in the medium $c/\sqrt{\varepsilon_p}$. Since the light line ε_p lies to the right of the dispersion relation up to a certain $c/\sqrt{\varepsilon_p}$, light can excite SPs of frequencies k_x below the crossing point P, Q and R on the metal and dielectric interface.

To excite the SPR, the layer needs to be thicker than this, but still thin enough for the field to sample the dielectric half-space. For the appropriate metal film thickness the SPR can be excited and the characteristic dip in the reflectance angle scan appears. This phenomenon is illustrated in Fig. 6, which shows reflectance dips produced by the excitation of surface plasmons at the different angle.

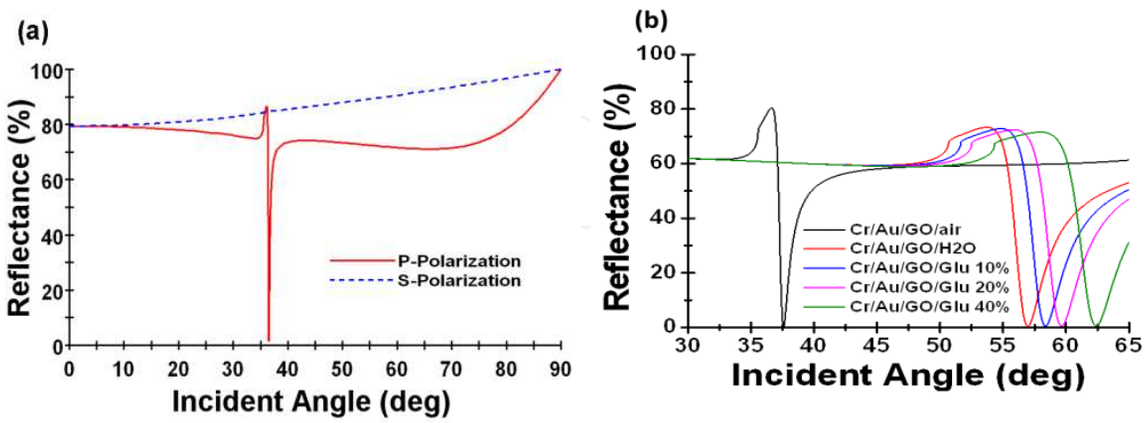

Figure 6. SPR curves with prism coupling in the Kretschmann configuration. (a) It demonstrates that the p-polarization illumination has higher sensitivity than s-polarization. (b) TM reflectance as a function of angle of incidence using the rigorous Fresnal reflection theory. Configuration: SF10 glass/Cr/Au/GO and five different dielectric layers air, Water, glucose 10%, glucose 20% and glucose 40%, respectively.

2.3. Graphene oxide layer effect

It is a novel Au-SAM-GO sheets and taking advantages of the striking properties of both grapheme oxide and Au film, fundamental understanding in hybrid material manipulation and new biosensors properties can be obtained.The sensitivity of proteins immunization detection was determined by GO sheets and compared with that of a conventional Au films. We used bovine serum albumin (BSA) to binding with GO sheets and conventional Au films, respectively. As the meanwhile, we also real-time detect the binding phenomenon by SPR technique. The results shows that GO sheets for SPR biosensor offers a potentially powerful assay, with a highly sensitive analysis, which can reach to 100 of pg/ml of BSA. However, the conventional Au films can just reach to 10μg/ml of BSA. Therefore, the results described herein that GO sheets are a promising approach towards highly sensitive for proteins immunization detection.The sensitivity of TB detection in the DNA-based assay for the amplification of the Insertion Sequence 6110 (IS6110) samples was determined by a GO/Au thin film andcompared with that of a conventional Au/Cr chips. The results show that a GO/Au SPR offers a potentially powerful assay, with a highly sensitive analysis, that may be applicable as an important tool for bio-marker detection.

The GO solution was purchased from GRAPHENE SUPERMARKET. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride(EDC), bovine serum albumin(BSA) and 8-Mercaptooctanoic acid($C_8H_{16}O_2S$; MOA)were purchased from Sigma-Aldrich (USA). Cystaminedihydrochloride (Cys, 97+%), 1-Octadecanethiol ($C_{18}H_{37}SH$; ODT) and N-hydroxysuccinimide (NHS)were purchased from Alfa Aesar (USA). 10X Phosphate-buffered saline(PBS) solution was purchased from UniRegion Bio-Tech (Taiwan).

a. Preparation of conventional Au film

Standard glass microscope slides (18 × 18 mm; High Precision Cover Glass, NO. 1.5H) purchased from Superior Marienfeld (Germany), were used as base substrates.To remove any contaminants, the glass substrates were subjected to ultrasonic cleaningby acetone, IPA, anddeionized water for 5 min before deposition, respectively. The slides were finally dried with nitrogen gas. The thin film grown on the glass substrates was shown in Fig.7. Cr and Au films were deposited by an electron beam evaporator at a vacuum level of about 3×10^{-6} Torr.

Au	47 nm
Cr	2 nm
Glass (n=1.52)	170 μm

Figure 7. Schematic diagram of the thin film structure (not drawn to scale) in Au/Cr

b. Surface functionalization of conventional Au film (Au-MOA)

Prior tosurface functionalization, the gold-coated glass slide was washed with acetone, IPA, anddeionized water for 3 min, respectively. After that,the gold-coated glass was rinsed with deionized water with sonication. The assay developed was designed as a bio-affinity immobilization assay. Finally, the gold-coated slide was immersed into 1mM MOA solution at room temperature for 24 h to form a self-assembled monolayer on the surface as shown in Fig. 8(a), we called conventional Au film.

c. Surface functionalization of GO sheets (Au-ODT-GO)

Prior tosurface functionalization, the gold-coated glass slide was washed with acetone, IPA, and deionized water for 3 min, respectively. After that,the gold-coated glass was rinsed with deionized water with sonication. The assay developed was designed as a bioaffinity immobilization assay. Finally, the gold-coated slide was immersed into an10mM 1-Octadecanethiol (ODT) solution at room temperature for 24 h to form a self-assembled monolayer on the surface. During the process, organosilane SAMs was formed on Au, we called Au-ODT film. After the Au-ODT substrate was rinsed with ethanol and dried with N_2, the substrate was immersed into a GO solution for 5 h in order to adsorbing GO on the Au-ODT surface, forming GO sheets as shown in Fig. 8(b).

d. Preparation of GO sheets

It is well known that $C_{18}H_{37}SH$ (ODT) can form a stable SAM on Au substrate through the formation of Au-S bond. As shown in Fig. 8(c), Au films modified with SAM of ODT were prepared by immersing the films into ethanol solution of ODT (10 mM) for 24 h at room temperature. The SAM films were then thoroughly rinsed with ethanol and dried with pure N_2 [52], forming GO sheets.

For adsorption of the GO onto the SAM films, the SAM films were immersed into GO solution, 0.275 mg/ml *vs.* 2 mg/ml for 5 h. The concentration of GO was adjusted to achieve films with different surface coverage of GO. The films (denoted as GO sheets) were thoroughly rinsed with deionized water to remove GO which unstable adsorbed onto the Au film surface and then dried with pure N_2 before use.

e. Optical characterization of GO sheets

Figure 9 shows digital microscopy image of bare Au film, Au-ODT film and different concentration of GO sheets. In Fig. 9(a) and 9(b), we cannot observe anything at the surface of the Au film. However, in Fig. 9(c) and 9(d), we observed that GO sheet (2 mg/ml) has higher surface density compared with lower concentration of GO sheet (0.275 mg/ml).

Fig. 10 shows relative transmittance spectra of bare Au film, Au-ODT film and different concentration of GO sheets. The relative transmittance at 670 nm decreased following the formation of Au-ODT film, the relative transmittance of GO sheets also lower than Au-ODT film. Fig. 10(a) shows relative transmittance spectra of bare Au film, Au-ODT film and different concentration of GO sheets. The value of transmittance decreased following the formation of Au-ODT film, the relative transmittance of GO sheets also lower than Au-ODT film. It shows

that higher concentration of GO sheet (2 mg/ml) have more opportunities to covalent binding with the following biomolecules and improved the detection limit.

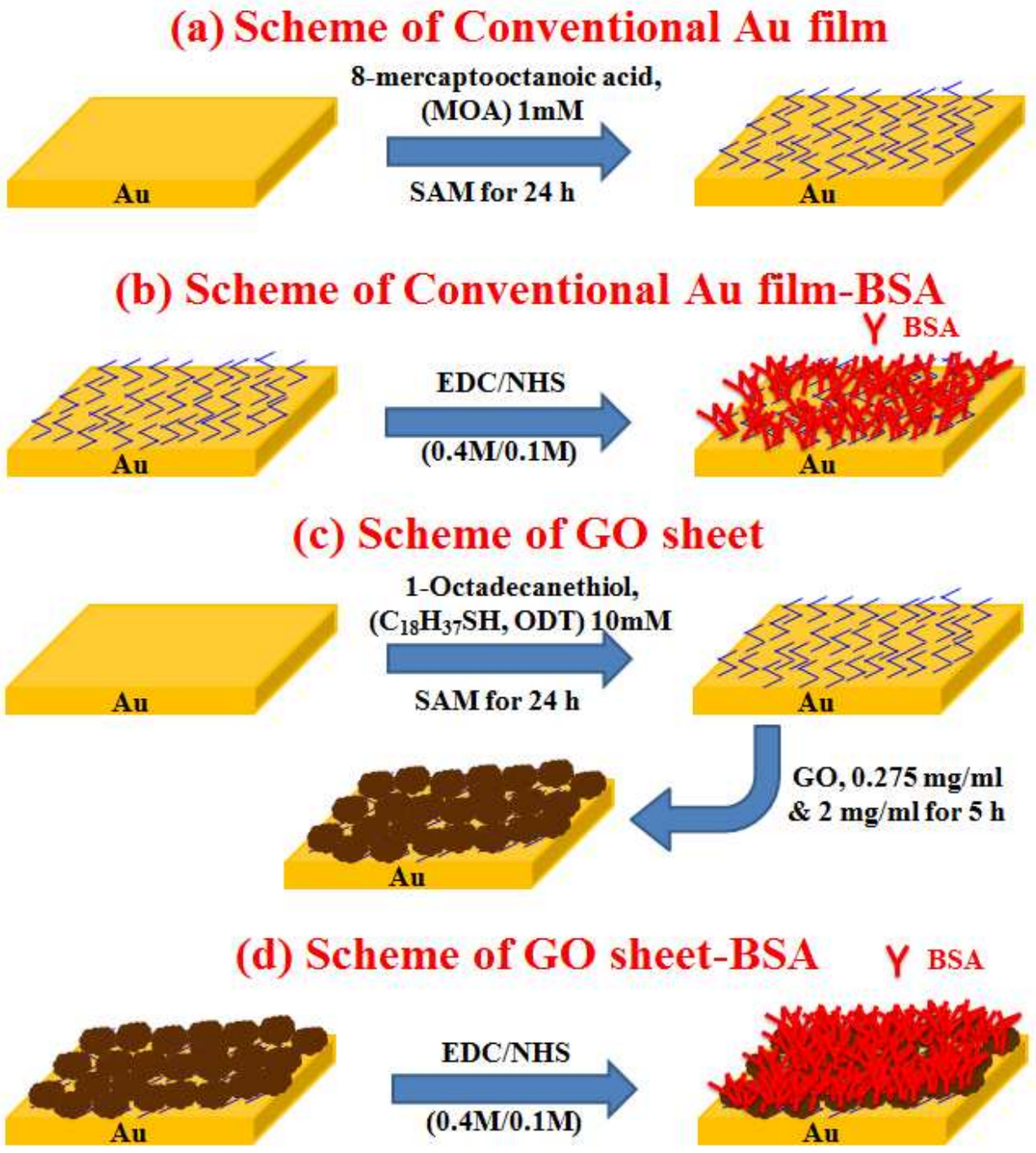

Figure 8. Scheme of (a) conventional Au film, (b) conventional Au film-BSA, (c) GO sheets (0.275 mg/ml & 2 mg/ml) and (d) the GO sheets with BSA for the preparation of GO sheets-BSA conjugate through the activated functional groups at the surface of GO sheets.

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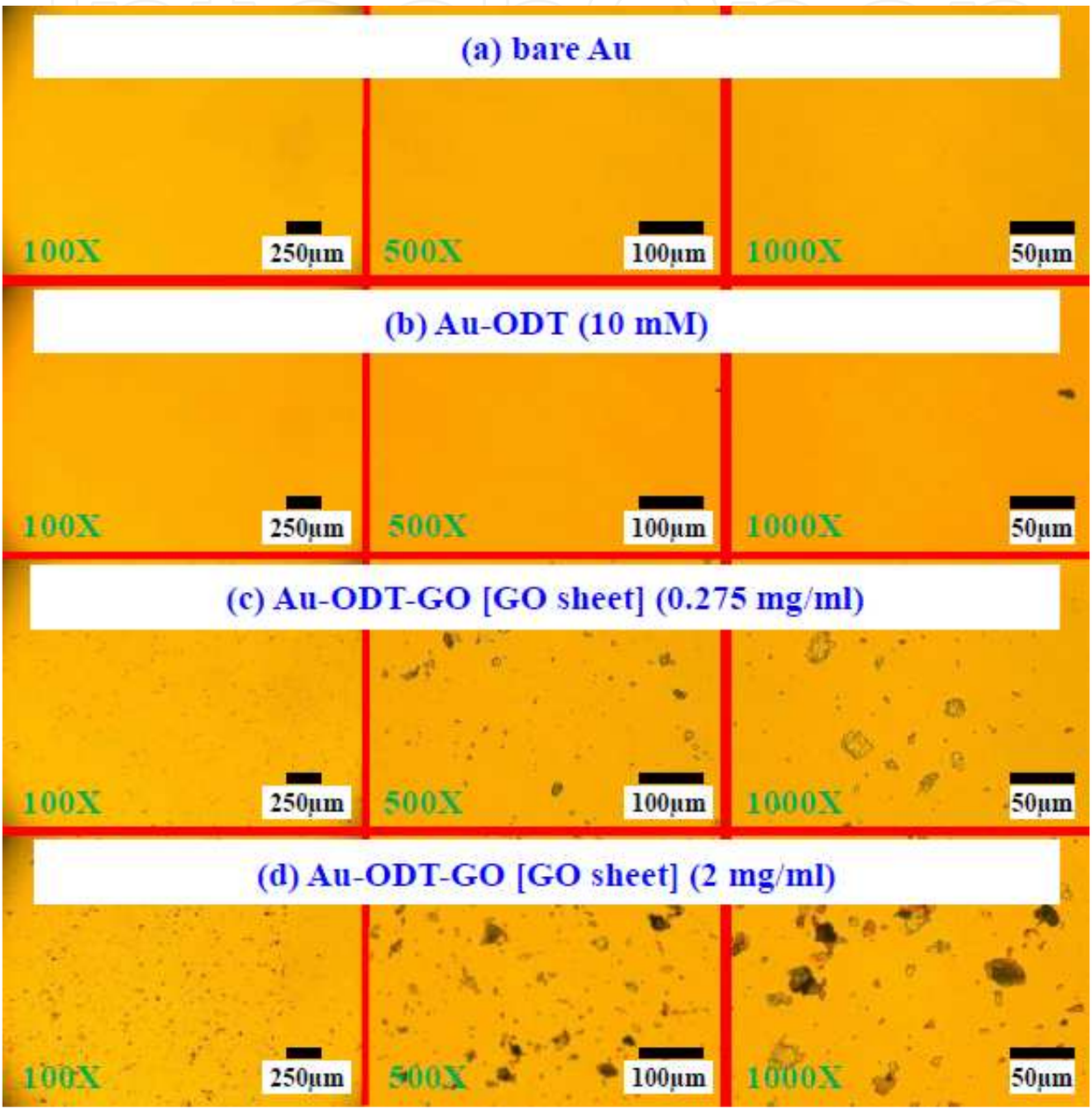

Figure 9. Digital microscopy image of (a) bare Au film, (b) Au-ODT (10 mM) film, (c) GO sheet (0.275 mg/ml) and (d) GO sheet (2 mg/ml).

As shown in Fig. 10(b), we used different films to observe the normalized reflectance intensity and SPR angle. The results show that when the concentration of GO rise, both intensity and SPR angle were risen, too.It demonstrated that GO solutions were successfullyadsorbed on Au-ODT films surface.

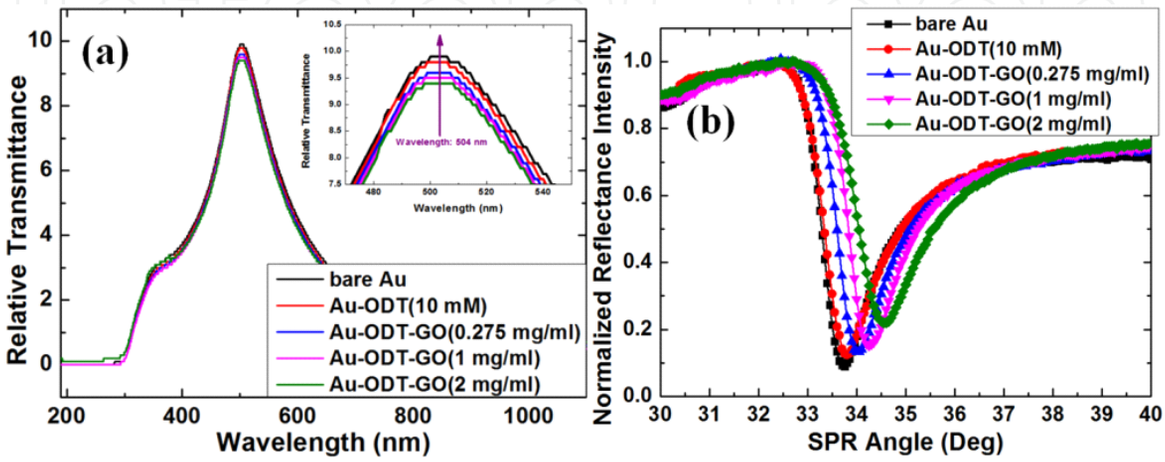

Figure 10. (a) Relative transmittance spectra of bare Au film, Au-ODT film and GO sheets (0.275 mg/ml, 1 mg/ml, and 2 mg/ml) from 190 nm to 1100 nm(Inset: The relative transmittance at 504 nm); (b) Normalized reflectance intensity of bare Au film, Au-ODT film and GO sheets (0.275 mg/ml, 1 mg/ml and 2 mg/ml).

f. Instruments and measurement

The transmission spectra were recorded using a Hitachi U-2900 Ultraviolet-vis (UV-vis) spectrophotometer (Hitachi High-Tech worldwide, Japan) with wavelength in range of 190-1100 nm. The surface plasmon resonance (SPR) measurements were carried out using BI-3000 (Biosensing Instrument, Tempe, AZ) with Kretschmann prism-coupling. These sample devices were prepared to measure the intensities of reflected light at a fixed angle. We are able to provide a real-time detection of immune interaction. The change in reflective intensity wasrecorded as the difference in the lowest point of the SPR reflectivity curve.

2.4. Biomoleculardetection

2.4.1.Detection of proteins immunization using a graphene oxide sheets

Here, we report the GO sheets with BSA for the preparation of GO sheets-BSA conjugate through the activated functional groups at the surface of GO sheets, and real-time observing the binding phenomenon between GO sheets and BSA by SPR technique as shown in Fig. 8(d). When we analysis various type of proteins, serum albumins are the major soluble protein constituents of the circulatory system and have many physiological functions, moreover, it can

also serving as transporters for a variety of compounds. Especially, bovine serum albumin (BSA) has been one of the most extensively studied proteins in this group of proteins, because of its structural homology with human serum albumin (HSA) [53].

The whole preparation process contains two steps: firstly, carboxylic acid groups on GO sheets are activated by N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC). Stable active ester is formed in the presence of N-hydroxysuccinimide (NHS). Secondly, the activation surface of GO sheets reacted with the amine groups of BSA, forming a strongly covalent bond. This preparation process will cause no denaturing of the protein species and guarantee the uniform attachment of BSA on GO sheets. In addition, the present method is promising to be used for bridging other biological systems to GO sheets-based materials.

a. Principle of proteinbinding with GO sheets

It is know that there were abounding epoxy groups at the edge of the GO surface, like carboxylic acid groups and hydroxyl groups [26, 27]. BSA proteins used in this study contains 60 amino moieties in lysine residues and 26 arginine moieties in guanidine side chains and 17 disulfide bonds with one free thiol in cysteine residues [54]. One of the universal methods for connecting proteins to GO sheets surface is covalent binding. Activating agents as EDC/NHS was usually used. After that, the amine groups ($-NH_2$) of BSA and the carboxylic acid groups (-COOH) of GO sheets on their surface will forming a strongly covalent bond.

b. GO sheets for protein biosensing by SPR technique

Figure 11 shows the SPR angle of conventional Au film and GO sheets (0.275 mg/ml *vs.* 2 mg/ml) towards different concentration of BSA. Compared with conventional Au film, GO sheets had higher sensitivity and lower detection limit.

The high BSA loading is attributed to the large surface area of GO and oxygen rich functional groups on its surface. Thus, due to the higher surface density, GO sheet (2 mg/ml) is much sensitive than GO sheet (0.275 mg/ml). The BSA detection limit of conventional Au film is 10 μg/ml of BSA. However, the GO sheet (2 mg/ml) can reach to 100 pg/ml of BSA.

Figure 12 shows SPR Angle of conventional Au film and GO sheets (0.275 mg/ml, 1 mg/ml and2 mg/ml) towards different concentration of BSA. Compared with conventional Au film, GO sheets have higher sensitivity and lower detection limit.The detection limit of conventional Au film is 1μg/ml.However, the GO sheet (2 mg/ml) can reach to 100 pg/ml of BSA.The high BSA loading is attributed to the large surface area of GO and functional groups on its surface. Furthermore, owing to the high surface density, the sensitivity of GO sheet (2 mg/ml) was superior to GO sheet (0.275 mg/ml).

c. Kinetic Analysis

Figure13 shows kinetic analysis for conventional Au film and GO sheets. Compared with conventional Au film, GO sheets have higher association constant (K_A) and lower dissociation constant (K_D), it demonstrated thatGO sheets weremuch more sensitive than conventional Au films [55]. The value of K_A also related to the concentration of GO sheets.

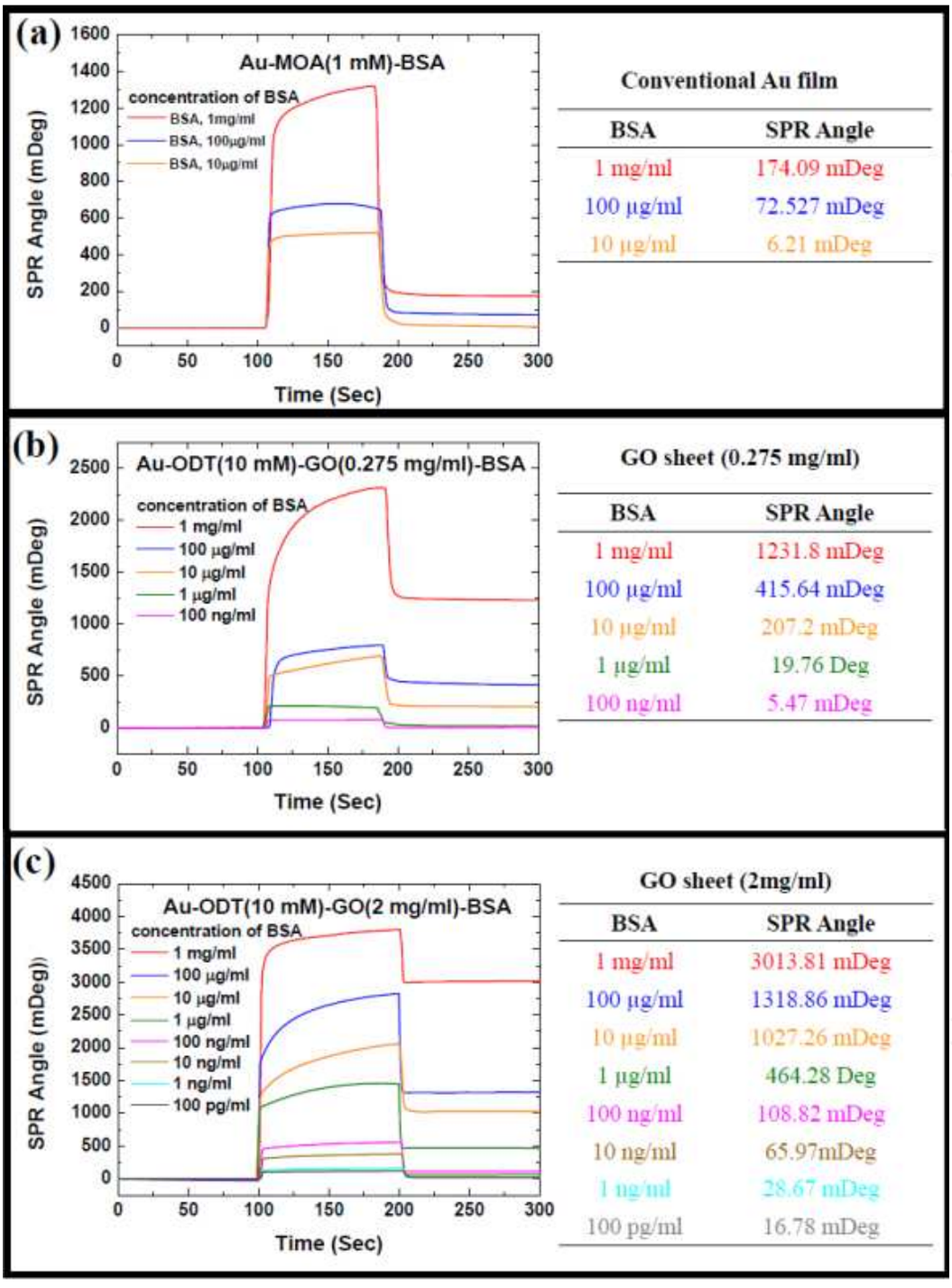

Figure 11. SPR Angle of (a)conventional Au film, (b) GO sheet (0.275 mg/ml) and (c) GO sheet (2 mg/ml) with various concentration of BSA. It shows high sensitivity of GO sheets SPR biosensor.

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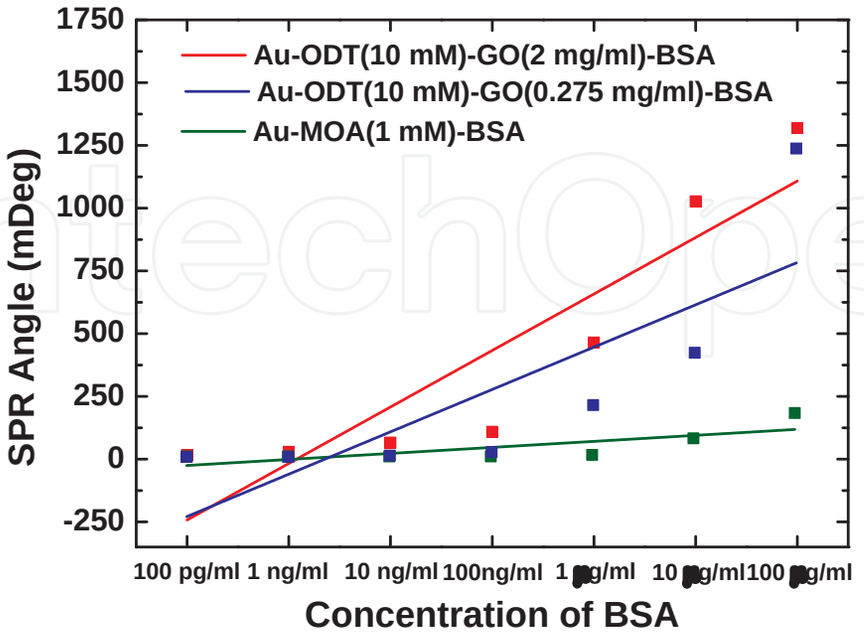

Figure 12. SPR Angle of conventional Au film (Au-MOA) and GO sheets with different concentration of BSA.

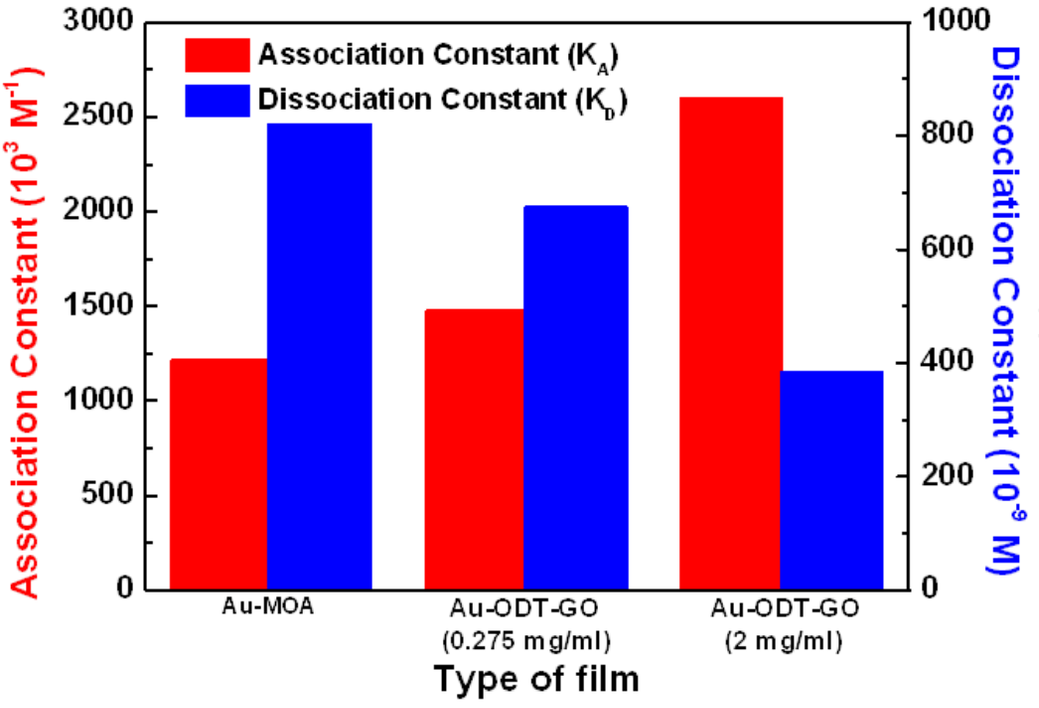

Figure 13. Kinetic analysis of conventional Au film and GO sheets.

2.4.2. Detection of Deoxyribonucleic Acid (DNA) using a graphene oxide sheets

Human tuberculosis bacillus (TB) is a highly contagious bacterial infection that is passed from person to person through droplets in the air. Infection is caused by the bacteria multiplying inside the body, causing tissue and organ damage. The infection of TB-virus often is more difficult to diagnose since the patient does not have the normal signs and symptoms associated with pulmonary TB. Therefore, development of a rapid diagnosis of the viral agent will be important for prevention of TB-viral infection and spreading.According to the estimation of WHO [56], in 2010, there were 8.8 million (range, 8.5–9.2 million)incident cases of TB, 1.1 million (range, 0.9–1.2 million)deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB.Between 1990 and2010, prevalence rates were halved, mortality rates fell by almost 80% and TB incidence rates fell by 3.4% per year.

Development the rapid and efficient diagnosis of infectious pulmonary TB is one of the crucial issues in the global fight to control this disease [57, 58]. TB is one of the most important chronic infectious diseases that cause millions of deaths annually.Traditional TB diagnostic techniques include smear microscopy and Mycobacterial culture and nucleic acid diagnosis. However these techniques are not suitablefor mass screening for some disadvantages: smear microscopyis low sensitivity (105 bacteria/ml), Mycobacterial culture need 30day bacteria culture, and nucleic acid diagnosis is too expensive [53, 57-60]. In addition, the PCR-based [56, 57] assays are among the most promising and attractive methods compared to the routine methods. However, the long reaction time of traditional PCRamplification and the high costof thermal cycler are still major issues for such a screening application.Therefore, it is important to develop a rapid and accurate diagnostic device.

In 2000, Notormi et al. [57] reported a technique for the amplification of nucleic acid has been described, named loop-mediated isothermal amplification (LAMP). The LAMP is advantages as compared to the conventional PCR-based methods.In the LAMP methodare labeling the fluorescent primers method, the so called "Fluo-LAMP" method. It needs using UV irradiation requirements for Loop-amp fluorescent detection reagent. Due to the advantage of Fluo-LAMP reaction are efficient amplication of the target DNA and proceeding at constant temperature, we want integrated SPR-LAMP (surface plasmon resonance) technique to develop a without fluorescent primers and high-sensitivitydetection devices.

We demonstrate the interfacing of graphene with biological molecules to build a novel SPR-LAMP device and a TB DNA-hybridization device with excellent sensitivity. It is evident that graphene possess a number of reactive functional groups that can easily bind with the free-NH2 terminals of the enzyme or protein to result in a strong amide covalent linkage [61-65]. As a result, in this work, we employ graphene as an electrode material for glucose biosensing. SPR biosensors based on immobilize biomolecules are suitablefor a highly specific, sensitive, and rapid analysis of various biological species in vivo and in vitro.

The synthesis and investigation of the graphene oxide (GO) material properties of these stiff materials have been undertaken, although little has been done to explore GO and reduction

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graphene oxide (rGO) in the analysis ofbiomolecules. Herein us studies the ability of water-soluble GO and rGO as a biochip for the sensitiveand high selective detection of TB-DNA.A new SPR-LAMP for TBdevice based on a GO material, which allowed immobilizing gold (Au) film onto the TB DNA antigens detection, was constructed. Figure 14 shows a schematic of a SPR-LAMP chips using graphene sheets with assay DNA format. Most important of all, we combined with the surface plasmon resonance to real-time monitoring the thickness of single layer GO.Surface roughness and specificity of the SPR sensor systems were also evaluated.This dispersion also facilitated the intimate mixing of the graphene oxide sheets with matrix polymers, providing a novel synthesis route to make GO-DNA nanocomposites. In the development of GO-DNA based biosensors, self-assembled monolayers (SAMs) or ordered structures of thiolated DNA on a gold surface can be achieved via use of spacer molecules between DNA and the thiol moiety, since DNA is known to form a disordered formless globular structure.

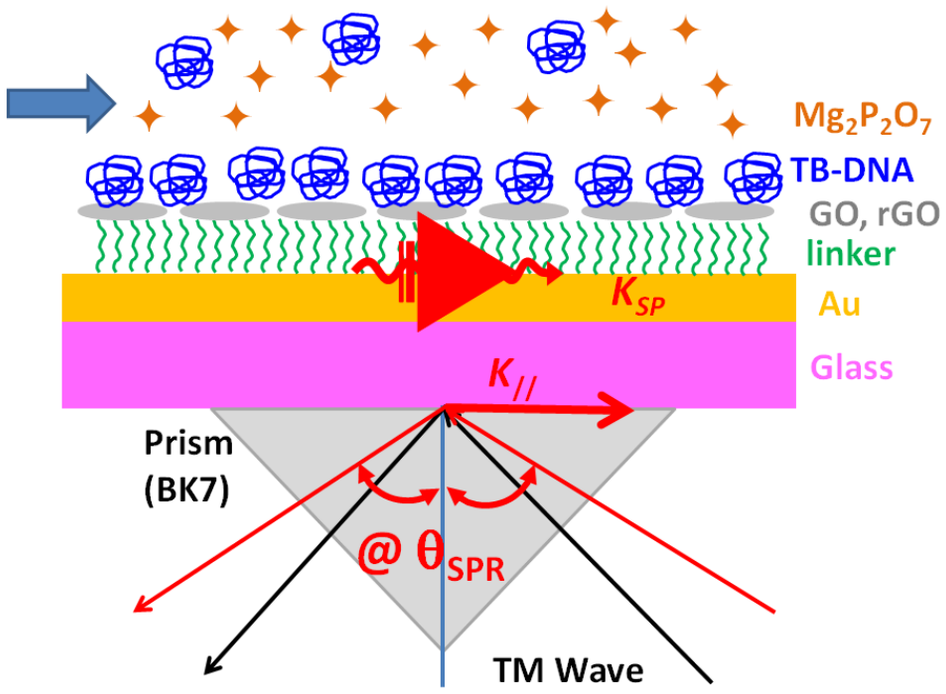

Figure 14. Schematic illustration of the SPR-LAMP detection chips for angle change of the TB DNA-GO binding. The experiments were compared with GO-linked immunosorbent assay, a conventional immunological method. GO-based SPR showed more advantages in providing label-free and real-time detection. Additionally, the high sensitivity and specificity for the detection of TBinfection showed its potential for future development of biosensor for TB DNA diagnosis.

10 X buffer	5ul
10X DIG labeling Mix (0.35mM)	x
dNTP(2mM)	5ul
F2(1uM)	5ul
B2(1uM)	5ul
Enzyme Mix (3.5U/ul)	0.75ul
ddH2O	24.25ul
LAMP DNA product	5 ul
Total	50ul

Table 1. Detection and confirmation of TB-LAMP reaction assay

a. Preparation of primers for TB-LAMP reaction

In the experiments, LAMP reactions are performed at a fixed temperature(60-65 °C) heatdenaturation and rapid cooling were applied to aDNA sample containing the target sequence (IS6110)and the four specific primers [57, 58]. We use sensitive LAMP-based assay for the detection of *Mycobacterium tuberculosis complex* (MTBC), based on the amplification of the *Insertion sequence 6110* (IS6110). IS 6110 is specific for the members, and first report on a LAMP assay for detection of MTBC in which IS6110 is used as a target sequence [57, 58]. The IS6110-specific LAMP primers were designed according to the general criteria described by Notomi et al. [57] and Aryan et al. [58]. Total volume of sample concentrations of LAMP DNA product assay was 50 μl, as shown in table 1.

b. GO sheets forTB-DNAbiosensing by SPR technique

For the SPR-LAMP analyses, we prepared threedevicesin 10X dilution of LAMP DNA productas shownin Fig.15. The purpose of combination SPR-LAMP technology is that it has excellent ability for surface analysis, high sensitivity for oxygen refractive coefficient, and the ability for real-time detection, so it is a new kind of electrode materials with potential applications in electrochemical sensing and SPR biosensing.We can get the best structure for –COOH groups of GO which have great biocompatible and binding properties. The surface functionalization of GO is covalent functionalization with TB DNA. After NaOH injected, the baseline didn't decrease. It shows the strongly covalent bonding between Au-Cys-GO surface and TB DNA. However, GO is compatible with DNA molecule. Table 2.shows the performance comparisons of the resolution for the three devices.

This measurement results shown, the SPR angle shift of TB DNA was measured for evidence of the adsorption of TB-DNA on GO. We demonstrated the GO sheets for the detection of TB DNA.

Comparison of the detection limits at the SPR signal and agarose gel electrophoresis of the LAMP product assays. We analyzed three different concentrations of LAMP products for 1X, 25X and 100X dilution. In the Au-Cys-Go device, the SPR real-time resonance angle was measured. The experimental results shown in Fig. 16(a) illustrate the performance of Au-Cys-

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Go devices in TB DNA solutions of dilution percentages(deionized water), 1X, 25X and 100X for 1102.499, 85.335 and 23.564 mDeg of SPR angle shift. Following the validation of LAMP reaction, the amplified products elongated to a length of several kbp and when generated they showed complex cauliflower-like structures. We demonstrated that the positive sample reveals many bands of different sizes afteragarose gel electrophoresis (Fig. 16b). Comparison of the detection limits, the SPR-LAMP has high-performance and simple, rapid an advantageous position.

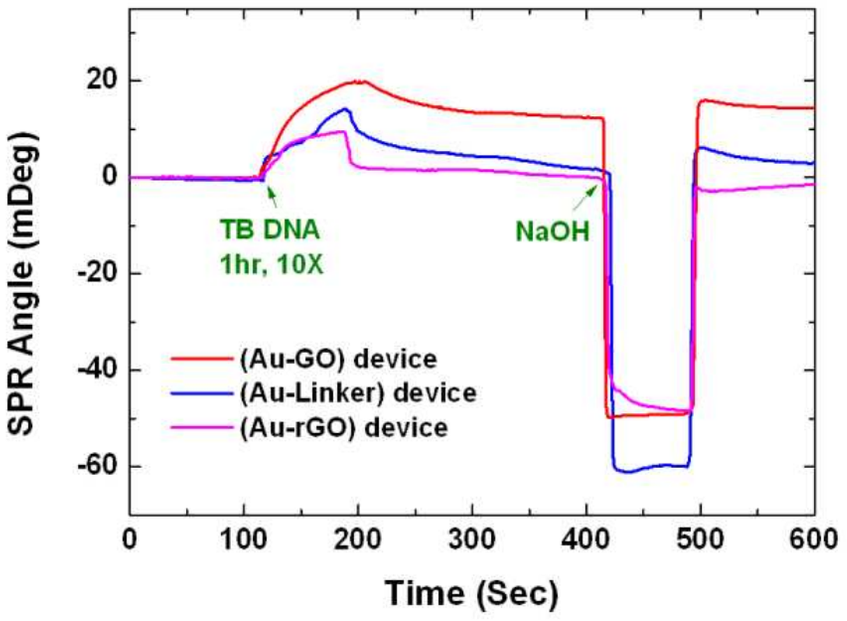

Figure 15. SPR real-time monitoring of the response of the three devices in LAMP product of TB DNA solutions.

Type of chips	SPR shift angle for 10X TB DNA
Au-GO	15.646 mDeg
Au-linker	5.418 mDeg
Au-rGO	2.312 mDeg

Table 2. Resonance angle shift at different SPR devices

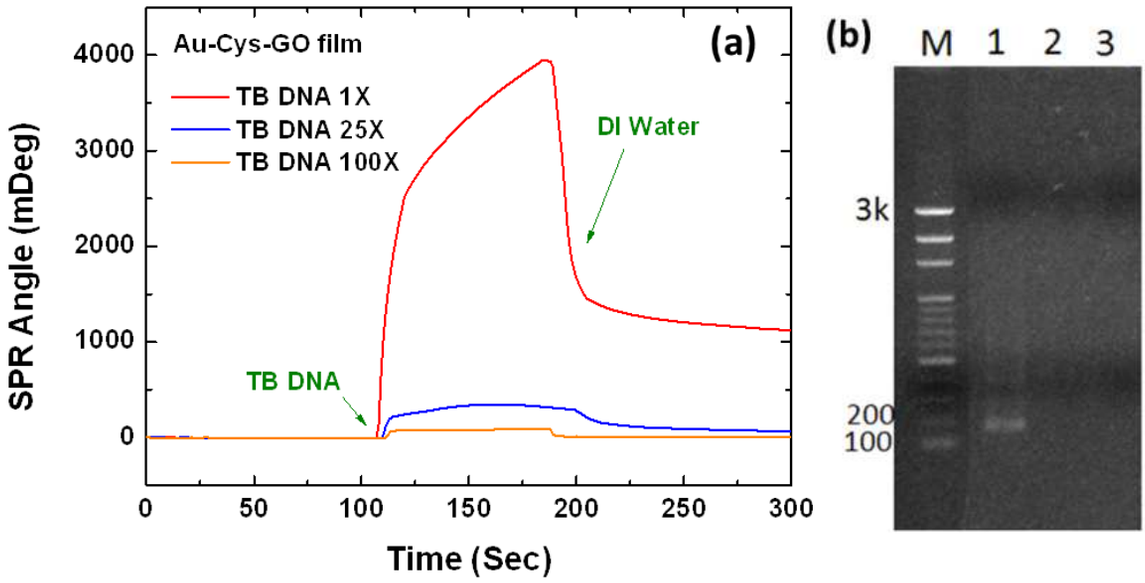

Figure 16. Comparison of the detection limits at the (a) SPR signal and (b) agarose gel electrophoresis of the LAMP product assays. Restriction enzyme analysis of the MTBC-LAMP products. Aliquots of 2.5ul LAMP products were electrophoresed in 2% agarose gels (1×TBE) and then stained with SYBR Green I dye for verification by fluorescent imager. M: 100 bp DNA ladder; 1: TB LAMP reaction (positive control) for 1X; 2: B LAMP reaction (positive control) for 25X; 2: B LAMP reaction (positive control) for 100X.

3. Conclusions

A SPR biosensor of high sensitivity and good detection limit with the employment of a GO functionalized Au-ODT composite (GO sheets) has been successfully demonstrated in this work. The linear range was dramatically improved. We observed a detection limit of pg/ml using the proposed GO sheets, whereas the conventional Au film can just reach to μg/ml. Although this work only reported the detection of BSA in vitro, it is likely that the method based on the GO sheets will ultimately apply in vivo and on-line detection. Therefore, the results described herein that GO sheets are a promising approach towards highly sensitive for diseases with immunization detection. The covalent functionalization of –COOH group utilizes the strong interactions between the graphene oxide and DNA molecules. We demonstrated the GO sheets for the detection of TB DNA. However, the techniques have a high sensitivity. For the practical measurement and mass production in the future, we designed a low-cost, simple, rapid and convenient SPR-LAMP for TB chip. This work can reduce human operation errors and make measurement more easily. Moreover, the SPR-LAMP sensing technique is useful for preventive medicine and personalized medicine. Therefore, another mission to attempt here is to commercialize SPR-LAMP chip and detection platform. The optimization of GO capture layer has been done by using protein BSA and TB DNA with measurable results.

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