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High-throughput label-free microcontact printing graphene-based biosensor for valley fever

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ABSTRACT

The highly prevalent and virulent disease in the Western Hemisphere Coccidioidomycosis, also known as Valley Fever, can cause serious illness such as severe pneumonia with respiratory failure. It can also take on a disseminated form where the infection spreads throughout the body. Thus, a serious impetus exists to develop effective detection of the disease that can also operate in a rapid and high-throughput fashion. Here, we report the assembly of a highly sensitive biosensor using reduced graphene oxide (rGO) with *Coccidioides*(cocci) antibodies as the target analytes. The facile design made possible by the scalable microcontact printing (μCP) surface patterning technique which enables rapid, ultrasensitive detection. It provides a wide linear range and sub picomolar (2.5 pg/ml) detection, while also delivering high selectivity and reproducibility. This work demonstrates an important advancement in the development of a sensitive label-free rGO biosensor for Coccidioidomycosis detection. This result also provides the potential application of direct pathogen diagnosis for the future biosensor development.

1. Introduction

Coccidioides spp. are the root cause for the invasive, pathogenic fungal disease Coccidioidomycosis, commonly known as 'San Joaquin Valley Fever' or 'Valley Fever'. Valley Fever is listed as a nationally notifiable disease by the Center for Disease Control (CDC) [1] Between 1998 and 2011, there were 117,717 cases of Valley Fever reported to the CDC, 97% of which were from Arizona and California [2]. The disease is considered to have high morbidity and potential mortality [3]. Valley Fever is usually indistinguishable from bacterial or other infections without specific laboratory tests, such as coccidioidal serological testing [4]. While Valley Fever cases continue to be endemic in regions, including the southwestern US and parts of Central and South America, the incidence has become more frequent far beyond those regions [3]. The widespread infection has led a tremendous economic burden, often due to delays in diagnosis, extended medical stays, and health care utilization and costs [5]. Treating this disease is limited by the inability to offer reliable, deterministic, and timely diagnoses. This motivates the need for more rapid and sensitive diagnostic tests and earlier diagnoses.

Thus far, diagnostic methods are limited to histopathology, serology, culturing, and ELISA (Enzyme linked Immunosorbent Assay) [6–13]. Among these methods, ELISA is considered the most commonly used method; however, the disease is difficult to diagnose in its preliminary stages of infection [14]. In addition, utilizing ELISA requires a significant amount of sample preparation time specialized training, and is challenging to implement for multiplexed detection [15,16]. The time-to-results in ELISA assays is several hours, while label free biosensors offer real time detection. Recent progression in the field of BioMEMs (Biomedical MicroElectroMechanical Systems) provides prospects to overcome many of the aforementioned limitations of assays, such as ELISA. BioMEMs devices now offer many of the desired features for the next generation of label free biochemical sensors, including higher sensitivity and faster results for medical detection with less human operating involvement and processing [17]. To improve BioMEMs capabilities, there have been recent investigations of new functional materials such as graphene. Graphene is an ideal substrate candidate gathering great scientific interests because of its

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biotechnological potential stemming from its unique physical properties. Graphene derivatives, such as graphene oxide (GO), and reduced graphene oxide (rGO), have emerged as new and viable alternatives because of their biologically compatible, chemically addressable, and electrically conductive basal plane. Their great surface area to volume ratio is optimal for high sensitivity [18]. Furthermore, they can be readily synthesized through a low cost chemical exfoliation and the two-dimensional conformation makes it possible to integrate into diverse platforms for new diagnostic devices [19–21].

In addition to synthetic scalability, there are production challenges needed to be overcome before rGO can be implemented as the substrate for the device stack in the sensing device. The challenges are twofold. The first challenge is to develop a suitable method for the large-scale production of quality rGO. The second challenge is to address the basal plane with the chemically favorable environment for antibody conjugation. Furthermore, to control the device fabrication status, it is of paramount importance to place rGO with the spatial orientation necessary for the subsequent fabrication of placing electrodes to spectroscopically translate biological responses into distinguishable readouts. Even with these challenges in mind, rGO has been desirable for large scale production due to its relative ease of synthesis and quality levels [22].

Herein, we present a high-throughput and controllable process to fabricate a reduced graphene oxide-based device for rapidly and sensitively diagnosing Valley Fever. The chemical and thermal reduction process can obtain high conductivity of the rGO and can retain the carboxyl group for surface modification. In addition, we utilized Microcontact printing (μCP) techniques by patterning self-assembled monolayers (SAMs) onto the graphene surface, combining a highly precise design to the unique material properties of the rGO [19]. μCP is a surface patterning technique that offers an ability to manipulate SAMs with molecular-level detail on a surface with the properties of materials [23]. Other patterning processes such as photolithography, electron beam (e-beam) lithography, and direct writing can be used for fabricating graphene sheet patterns. However, μCP offers lower cost, and more experimental speed, simplicity, and flexibility for forming specific and wide-ranging patterns than these other methods. Additionally, μCP is considered one of the most useful fabrication methods for larger area patterning, which demonstrates its scalability [24]. There are several key advantages of μCP that allow it to meet the high- throughput screening demand for high fidelity, convenience, and low cost. One aspect is the efficiency and efficacy with which μCP easily manages an extremely high level of precision that greatly reduces the amounts of expensive chemical or biological agents required for functionalization of the surface material. μCP techniques also can construct multiple stamps from a single master, which allows easily reproducible and reliable results with exceptional control over the pattern's featured size, area, and position on the micro-fabrication design [23]. We performed the testing by having the antigen surface-immobilized and the cocci antibody as the target analyte, which is the usual method for diagnosing infections [25]. The analysis of the properties and characterization of the rGO was performed by Fourier-transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). We used electrochemical testing and fluorescent imaging to validate our technology, and to anticipate and eliminate false positives. This developed biosensor achieved ultrasensitive detection as low as sub picogram/milliliter (2.5 pg/ml) levels for cocci antibody, which can provide advantageous new strategies for sensing the disease.

2. Methods

2.1. Materials

The rGO dispersion was synthesized according to the published methods [26]. GO colloids (0.5 mg/ml, 40 ml) made from the modified Hummers' approach were mixed in a flask with 0.1 ml hydrazine (35 wt % in water) and 0.56 ml ammonia (28 wt% in water) to adjust the pH to 11 and then stirred in an oil bath at 95 °C for 1 h. IDCF Cocci antigen and antibody were kindly provided by IMMY (IMMY, OK, USA). Polydimethylsiloxane (PDMS, Sylgard 184) is purchased from Dow Corning.

2.2. Fabrication of rGO biosensors

The dispersion of rGO (0.5 mg/ml, 8 ml) was filtered with a Millipore, Isopore filter with a pore size of 0.1 μm VTCP 2.5 in. VCTP02500 filter to form rGO sheets with around 2 μm thickness. The sheets were then immersed into acetone for 30 min to lift off and separate the rGO sheet from the filter. The sheet was then allowed to anneal for 2 h at 400 °C in a furnace for exfoliation [27]. The rGO sheet was then adhered to a spin coated PDMS layer on a glass slide. Sputter coating deposition was then used to layer the thin gold electrode of thin gold onto the rGO sheet. An acrylic ring-shaped holder was cut by a laser cutter (0.25 in., 1 in. in diameter) and was used to hold the plastic chamber. PDMS was then used to adhere the chamber to the rGO to provide insulation and sealing.

2.3. Surface functionalization

The rGO sheet was pretreated with UVO cleaner to enhance the carboxy-group exposure on the surface. Next, the rGO sensors were incubated in 100 μL of a 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide (EDC, 40 mM, Thermo Sci, USA) and *N*-succinimide (NHS, 20 mM, Thermo Sci, USA) plus IDCF Cocci antigen in PBS (0.1 mg/ml, IMMY, Inc.) solution diluted 10 times in 0.1 M MES Buffered Saline (pH = 6.0) for 1 h at room temperature.

2.4. Measurement and characterization

The thickness profiles of the thin rGO films were measured by cross section image of SEM (Zeiss Gemini 500), while the electrical characteristics were measured by Model 600E Series Electrochemical Analyzer/Workstation. The FTIR results were characterized by Cary 630 FTIR Spectrometer. The fluorescent imaging was performed with an inverted Nikon Eclipse TE2000-U fluorescent microscope. Each photo was taken at ×60 magnification.

3. Results and discussion

3.1. Characterization of the Cocci antigen on rGO surface

rGO operated as the substrate for the construction of the Cocci biosensor as detailed in Fig. 1. In order to sustain a transferable rGO sheet, maintaining the proper thickness of the rGO sheet is critical, 8 ml of 0.5 mg/ml rGO solution after passing through the filter can achieve the thickness of 2.5 μm rGO sheets. The thickness was confirmed by characterizing the graphene sheets using scanning electron microscopy (SEM). The major morphology observation in the SEM image is the layered structure, which demonstrates the success of the rGO synthesis (Fig. S1). Due to the organic solvent and the presence of the oxygen containing functional groups attached on carbon, the resistance of the rGO sheet after being peeled off from the filter was around 1000 Ω. However, after annealing at 400 ° C for 2 h, the exfoliate rGO sheet resistance achieves around 100 Ω. Longer and higher temperatures for the annealing process will remove the chemical modifiable carboxyl group in the rGO sheet, which will affect the conjugation amount and functional ability of rGO. With the antigens modified onto the surface, the resistance will increase to around 140 Ω.

High throughput and controllable patterning of the surface modification of cocci antibody can be performed by μCP. Fig. 2 shows the successful initial attempts to print patterns of cocci antigen on rGO surfaces with PDMS stamps. We can observe the performance of the fluorescent-labeled rGO sheet by observing strong fluorescence