



# 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 ( $\mu$ CP) 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].  $\mu$ CP 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,  $\mu$ CP offers lower cost, and more experimental speed, simplicity, and flexibility for forming specific and wide-ranging patterns than these other methods. Additionally,  $\mu$ CP is considered one of the most useful fabrication methods for larger area patterning, which demonstrates its scalability [24]. There are several key advantages of  $\mu$ CP 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  $\mu$ CP 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.  $\mu$ CP 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  $\mu$ m VCTP 2.5 in. VCTP02500 filter to form rGO sheets with around 2  $\mu$ m 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  $\mu$ L 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

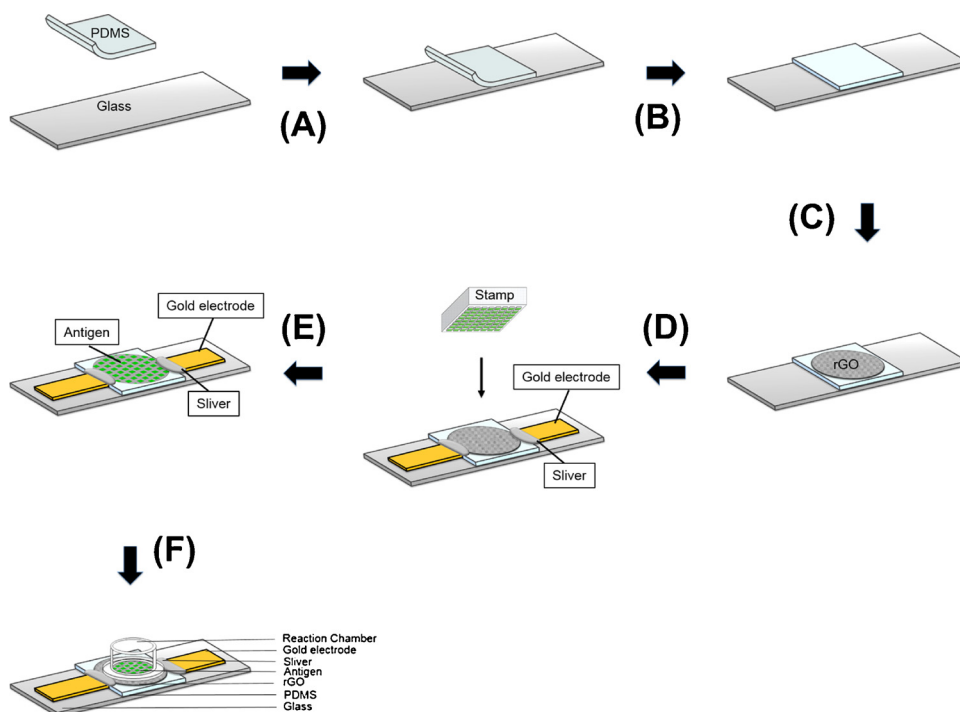
The thickness profiles of the thin rGO films were measured by cross section image of SEM (Zeiss Gemini 500), while the electrical characteristics were measured by Model 600E Series Electrochemical Analyzer/Workstation. The FTIR results were characterized by Cary 630 FTIR Spectrometer. The fluorescent imaging was performed with an inverted Nikon Eclipse TE2000-U fluorescent microscope. Each photo was taken at  $\times 60$  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  $\mu$ m 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  $\Omega$ . However, after annealing at 400 °C for 2 h, the exfoliate rGO sheet resistance achieves around 100  $\Omega$ . 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  $\Omega$ .

High throughput and controllable patterning of the surface modification of cocci antibody can be performed by  $\mu$ CP. 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



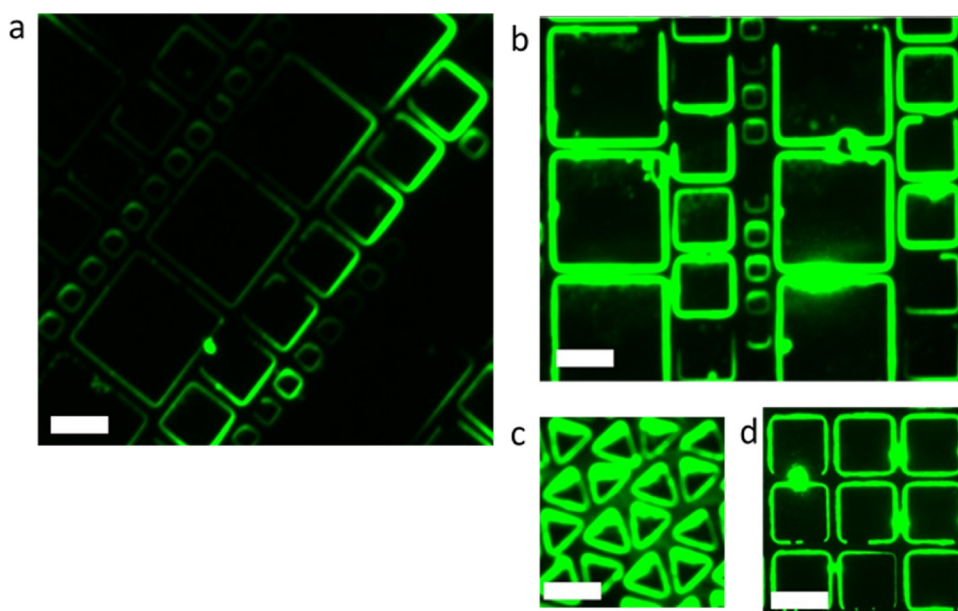
**Fig. 1.** (A)–(F) Schematic illustration that depicts our fabrication process (A) PDMS is spin coated to the glass slide followed by (B) PDMS adhering on top of the glass slide. (C) Placement of rGO sheet pretreated by UV Ozone onto PDMS. (D) The thin gold electrode was sputter coated onto the graphene. (E) PDMS stamps pretreated by UV/Ozone and the subsequently absorbed protein solution is deposited onto the rGO substrate surface (F) The plastic containment chamber was deposited onto the testing surface and adhered PDMS.

intensity with fluorescence microscope, which independently confirms that the  $\mu$ CP facilitated surface modification process worked on the rGO sheets. The resulting patterned arrays in the square stamps were  $100\ \mu\text{m} \times 100\ \mu\text{m}$ ,  $50\ \mu\text{m} \times 50\ \mu\text{m}$  and  $10\ \mu\text{m} \times 10\ \mu\text{m}$  (2a, 2b), the triangle pattern were  $50\ \mu\text{m}$  each side (2c) and Fig. 2d was  $50\ \mu\text{m} \times 50\ \mu\text{m}$  squares. After testing the different patterns, we obtained our results by using  $50\ \mu\text{m} \times 50\ \mu\text{m}$  patterned arrays. The cocci antibody on the surface of rGO sheet was investigated using FTIR (Fig. S2). By comparing the antigen conjugated rGO and unconjugated rGO, the presence of amine groups on the surface was determined. Sensor performance was measured utilizing an electrochemical analyzer, Fig. 3a shows the current-voltage characterizations of the bare graphene surface and the surface modified graphene. The conductance of

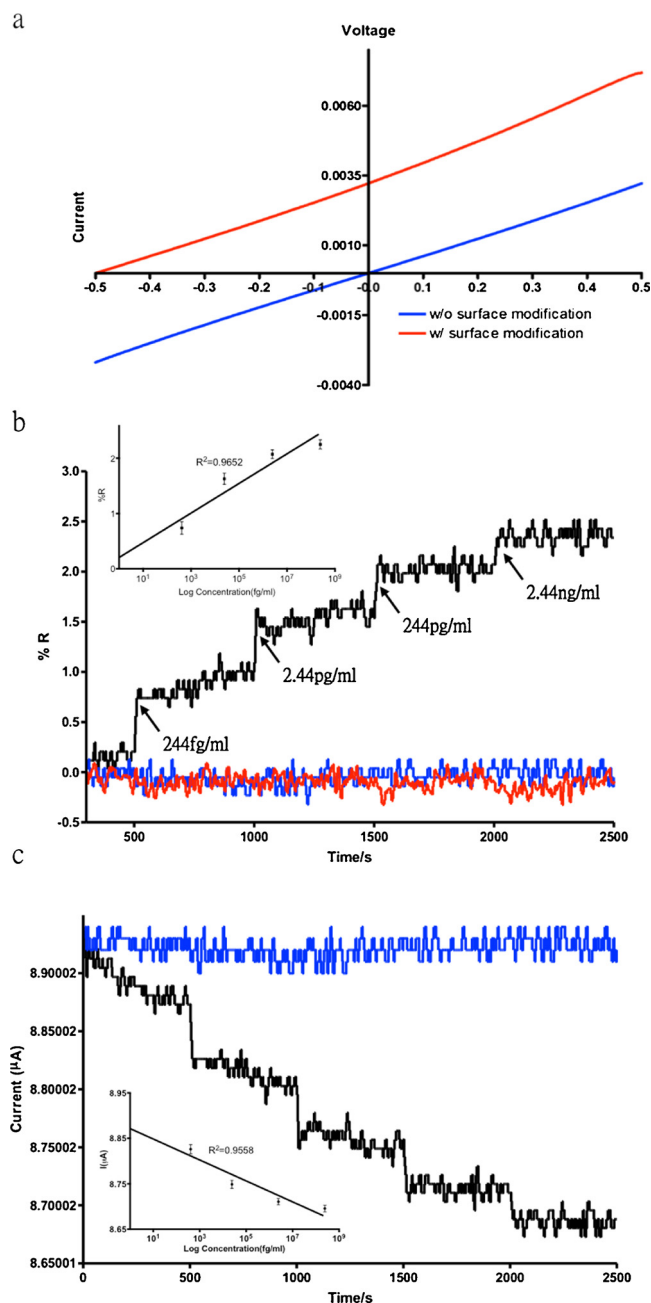
the rGO sheet before surface modification by cocci antibody is  $81.3 \pm 3.85\ \text{S/cm}$  and after cocci antibody conjugated rGO conductance increase to  $119.3 \pm 7.25\ \text{S/cm}$  (Fig. S3). We can observe that the modified graphene surface has resistivity changes, which can be attributed to the binding of the antigen.

### 3.2. Sensing performances for the Cocci antibody detection

The commercial enzyme immunoassay (EIA) that detects IDCF and IDTP antigens is the current method used in Coccidioidomycosis diagnoses (CDC, 2017, Information for Healthcare Professionals about Valley Fever (Coccidioidomycosis)). It has reliable results and follows a standard procedure, therefore the IDCF antigen was chosen for the



**Fig. 2.** Fluorescent image of antibody-conjugated rGO. 2a, 2b) patterned square array  $100\ \mu\text{m} \times 100\ \mu\text{m}$ ,  $50\ \mu\text{m} \times 50\ \mu\text{m}$  and  $10\ \mu\text{m} \times 10\ \mu\text{m}$  respectively. 2c) patterned equilateral triangle array  $50\ \mu\text{m}$  each side. 2d) patterned square array  $50\ \mu\text{m} \times 50\ \mu\text{m}$ .



**Fig. 3.** Sensing performances. 3a) Current-Voltage (I–V) diagram for the device with (red) and without (blue) cocci antigen functionalization to the rGO. (3b) Time dependent impedance response (inset: Log values for  $\Delta \%R$ ) and (3c) amperometric response (inset: Log values for  $\Delta I$ ) to the addition of Valley Fever antibody at different concentrations demonstrating high dependence of Valley Fever antibody concentrations. (Valley Fever antibody: black; PBS baseline: blue; and NADPH negative control: red) (Valley Fever antibody concentrations: 244 fg mL<sup>-1</sup>, 2.44 pg mL<sup>-1</sup>, 244 pg mL<sup>-1</sup>, 2.44 ng mL<sup>-1</sup> respectively) (n = 6) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

device to detect the antibody in solution. The device was tuned by first running the test with 100  $\mu$ L PBS for 500 s to establish an equilibrated resistance. Next, we examined the sensor response in the presence of NADPH as our negative control. As seen in Fig. 3b, the sensor showed minimal response to the non-target agents, demonstrating the suppression of nonspecific binding by non-target proteins and high selectivity.

Successive doses were sequentially increased on the sensor (Fig. 3b

and c) and tested to determine the sensitivity of the measurements. Fig. 3b is a resistance increasing ratio vs time plot, which shows the increasing steps of the resistance every 500 s after the addition of the next concentration of antibodies. The cocci antibody can be detected on each addition for four different concentrations (244 fg/mL, 2.44 pg/mL, 244 pg/mL, 2.44 ng/mL) with a few seconds of response time observed. The resulting calibration plot displayed a strong linear relationship between the resistance response and the logarithm of the concentration. With a correlation coefficient of 0.9652, it clearly demonstrates the dependence on the concentration of the cocci antibody. An amperometric analysis showed similar results (Fig. 3c). These results were confirmed through multiple tests (n = 6) of our devices, and shows that the biosensors were able to reliably detect cocci antibody concentrations as low as 244 fg/mL, which is much lower than current methods (0.03 ng/mL) [13,14,28–30]. The electrochemical analysis technique has been applied in biosensors to measure the change of the electrode interface based on biological and chemical molecule recognition. This method of evaluating the performances and capability for our device offers a more rapid, sensitive, and reliable tool compared to the current ELISA method used for diagnosing Coccidioidomycosis infections.

#### 4. Conclusion

The development of biosensor is growing rapidly due to new materials and improved fabrication techniques [31]. rGO can undergo versatile surface modifications, and has unique properties while maintaining superb electroconductivity [32]. The advantages of rGO applied towards the various applications has been reported across different scientific disciplines [33]. We have developed an effective biosensor that is easily and expeditiously assembled for high-throughput Valley Fever detection, by combining  $\mu$ CP patterning with the widely applicable sensing material rGO. By detecting the measured conductance shift due to specific antibody binding, the device is compatible with the most commonly used diagnosis methods, yet it can provide more reliable, low cost, and rapid screening for early disease diagnosis. The fabrication method we used in this study can provide reliable, low cost, and on the spot screening for disease diagnostic purposes, and it suitable to the large scale production. These characteristics demonstrate the potential to detect many other target analytes. Future approaches to optimizing the combination of precise surface modifications, synthesis and fabrication of high quality rGO sheets might extend the performance of the biosensor to have multi-functionality and a significant impact on large-scale production clinical testing.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.colsurfb.2018.06.011>.

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