Abstract—The ability to produce indigenous diagnostic tests for neonatal meningitis, that are novel, specific yet cost-effective would now provide a huge impact on public health management in developing countries. Escherichia coli (K1) is the major cause of neonatal meningitis through the interaction with human brain microvascular endothelial cells (HBMEC). Outer membrane protein A (OmpA) is a major protein in the E. coli outer membrane. However, there is not any rapid detections for OmpA. We proposes the construction of a molecular recognition layer composed of thiol molecules and further activation with EDC-NHS complexes on the surface of a quartz crystal microbalance (QCM). Immobilization of antibodies onto the crystal will be allowed to detect OmpA antigens for nanomedicine applications. This study originally developed the aforementioned QCM method to rapidly detect OmpA antigen. The method utilizes the piezoelectric immunosensor onto whose thiol activated crystal surface the affinity-purified antibodies would be immobilized, and the main objective of this study is to optimize the surface monolayer conditions to provide efficient binding of anti-OmpA IgG antibody and to exploit sensing surface for the detection of OmpA antigen, as an alternative diagnostic test. Though the frequency shift was lower for OmpA antibody, still it can able to detect lower than 20 ng /ml of OmpA antigen. The detection of OmpA protein by QCM method is comparatively simple and sensitive than other systems.

Key words—Biosensor; Quartz Crystal Microbalance (QCM); Outer membrane protein A (OmpA); Thiourea; Flow-injection Assay

1. INTRODUCTION

The illnesses caused by many species of Gram-negative bacteria are easily confused with other viral, bacterial, and parasitic illnesses because of the similarity in their clinical manifestations, making clinical diagnosis a difficult challenge. Escherichia coli K1 is the most common Gram-negative organism causing neonatal meningitis. OmpA is an immunodominant protein that is involved in the E. coli-host cell attachment process. Outer membrane proteins (OMPs) are key molecules in the interface between the cell and its environment. Outer membrane proteins (OMPs) are good vaccine candidates, since they are usually abundant and are in direct contact with the host immune system. OmpA is abundant in the cell wall and conserved across different species, which suggests it has an important functional role in bacteria [1].

Earlier studies have shown that OmpA is one of the major factor responsible for Escherichia coli traversal across the blood-brain barrier that constitutes a lining of brain microvascular endothelial cells (BMEC).

Inadequate knowledge of the pathogenesis and pathophysiology of this disease has contributed to this poor outcome [2]. The diagnosis of E. coli infections has been achieved by techniques including serology (enzyme-linked immunosorbent assay [ELISA], and immunofluorescence [IFA]) and molecular detection (polymerase chain reaction [PCR] and real-time PCR). None of these approaches are 100% sensitive for diagnosing the bacterial infection. Limited availability of these methods in many countries and laboratories that lack equipment and training may have resulted in absence of knowledge of the true incidence of neonatal meningitis infections. However, limiting issues that prevent DNA technology from general use in the diagnostic laboratory is that it is expensive to perform and requires sophisticated equipment, which may not be available in the developing countries.

Immunological assays have been the most rapid methods for specific identification of bacterial infections. These tests employ antibody or antigen recognizing only one or a group of bacteria. The highly specific binding of antibody to antigen, plus the simplicity and versatility of this reaction, has facilitated the design of a variety of antibody assays. The antibody based assays comprise the largest group of rapid antibody based assays. The main limitations of ELISA include relatively time-consuming, labor-intensive, cross-reactivity, and efficiency of the signal detection.

Thus, the need for a more rapid, sensitive and portable assay remains as urgent as ever, providing the impetus for the development of next generation technologies. However there is no effective detection method available for the quantification of OmpA antigen. Further, there is no available report on the application of QCM for the detection
of OmpA antigen. Quartz crystal microbalance (QCM) based biosensors are a viable alternative to the previously described methods due to their sensitive mass detection capabilities and their ability to monitor in real time [3]. The study involves the piezoelectric detection of OmpA protein from *E. coli*, where a decrease in resonance frequency is correlated to the mass accumulated on the surface.

II. MATERIALS AND METHODS

A. Reagents and materials

All Escherichia coli strains were kindly provided by Dr. K.S. Kim (Division of Pediatric Infectious Diseases, School of Medicine, Johns Hopkins University, Baltimore, MD); they are summarized in Table 1. E44 is K1 strain RS218 (O18.K1.H7) isolated from the cerebrospinal fluid of a neonate with meningitis (17). E91 is an E44 mutant lacking the entire OmpA gene. E105 is an OmpA complemented mutant of E91 that carries PRD87 (which contains the ompA gene on pUC9). E111 is E91 with pUC9 vector control. E109 was obtained by transformation of E91 with a pRD87 derivative that lacks the C-terminal 53 amino acids of OmpA. MG1655 is a nonpathogenic strain that is noninvasive for the BBB. Bacteria were grown in brain heart infusion broth with appropriate antibiotics (all from Difco Laboratories, Detroit, MI). For infection experiments, overnight cultures were expanded in brain heart infusion broth and incubation at 37°C for 2 to 3 hours to mid-log phase. Bacteria were centrifuged and resuspended in cell culture medium without antibiotics. [4]

For chemical modification on the quartz crystal, thiosalicylic acid, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Chemical Co. Recombinant Protein G was also purchased from Sigma Chemical Co., which retains its affinity for IgG, but lacks albumin and Fab-binding sites and membrane-binding regions. Bovine serum albumin (BSA, A2153) and Human serum albumin (HSA, A9511) was procured from Sigma Chemical Co. All the other chemicals and reagents used in this experiment were purchased from Sigma (USA) and of analytical grade. All the solutions used in the analytical procedures except thiosalicylic acid were prepared with water purified by Milli-Q Millipore system (Bedford, MA, USA).

B. Quartz Crystal Microbalance

The quartz crystal used as a sensor in the QCM technique is not only sensitive to changes in mass of the working electrode. It is also highly sensitive to changes in temperature, pressure and viscous loading. Temperature and pressure can be controlled, but the principle of a switch-flow cell experiment is to change the electrolyte. Consequently, the changes in viscosity have to be compensated for. This can be done by simultaneous recording of the quartz crystal resonance frequency and resistance [5]. In the QCM, the equation of the resonant frequency change for mass change has been presented by Sauerbrey equation [6]:

\[ \Delta f = -2f_0^2 \frac{\Delta m}{A} \left( \frac{\rho_s \mu_o}{2} \right)^{1/2} \]

In the commonly used case of AT-cut quartz crystals, thickness-shear vibrations in a thin quartz plate are induced by means of the piezoelectric effect when an alternating electric field at the resonant frequency is applied to the exciting electrodes attached to either side of the plate.

C. Antibody immobilization

The gold-coated quartz crystals (8 MHz, AT-cut, CH Instruments) were first cleaned by immersion into piranha solution (1:3 (30% v/v) H₂O₂–concentrated H₂SO₄) for 1 min at room temperature, followed by subsequent rinses with ethanol and distilled water and dried in a stream of nitrogen. After rinsing with DI water and dried with nitrogen gas, the crystals were mounted inside the QCM flow cell chamber. Degassed phosphate buffer saline (PBS, pH 7.4) was then injected into the flow cell via peristaltic pump at flow rate of 20 µL/min until the stable baseline was obtained. Then the gold surfaces were exposed to 10 mM thiosalicylic acid for 3 h. After rinsing with ethanol and water, the thiol-modified crystals were treated with 100 mM EDC–NHS until the frequency response have been tending stable (around in 1 hr) to convert the terminal carboxylic group to an active NHS ester. Followed by rinsing with PBS for 10 min, an anti-OmpA IgG antibody (10 µg/mL) was injected into the flow cell and the flow was stopped for 60 min to allow the antibody immobilization on the carboxylated crystal surface. After the antibody immobilization, PBS was injected to remove un-bound antibody and the unreacted NHS-esters remaining on the sensor surface were deactivated by the adding 60 µL of 0.1 M ethanolamine and the flow was stopped for 60 min before a final wash with PBS.

III. RESULTS AND DISCUSSION

A. Frequency shift as a function of time during the formation of thiol monolayer

The observed decrease in resonance frequency attributed the formation of TSA monolayer on the quartz crystal. Adsorption of organosulfuric compounds on the gold surfaces by chemisorption via thiol groups and leaving the carboxyl groups. The response of the QCM sensor to thiosalicylic acid of varying time scale was depicted in Fig. 1. Accordingly, Fig. 1. represents the formation of thiol monolayer on the gold surface. Following the self-assembled thiol monolayer, an activation of the TSA-modified crystal surface was constructed by the addition of equi-molar ratio of EDC-NHS, which involves a step-wise formation and replacement of terminal EDC and NHS in sequence to form an NHS ester.
Figure 1. Frequency shift as a function of time during the formation of thiol monolayer.

B. Immobilization of antibodies onto the monolayer modified crystal

The immobilization time was optimized with 180 min and the optimum immobilization concentration was 60 μg/mL, respectively. The surface modification and protein immobilization on the quartz crystals were confirmed by frequency shift variation. Fig. 2 show the OmpA antibodies onto the monolayer modified crystal, its decrease in frequency shift 261.55 Hz.

Figure 2. Immobilization of antibodies onto the monolayer modified crystal.

C. Estimation of the OmpA antigen by flow cell analysis

A flow-injection assay for the detection of the OmpA antigens using antibody-based QCM biosensor has been proposed. Fig. 3 step (a) show the thiolated crystals were activated with EDC-NHS and the first decrease in frequency was noted at 25 min which indicated the formation of active NHS ester on the crystal surface, then wash out by PBS (step (b)). When OmpA antibody was injected into the flow cell, substantial decrease in frequency shift (198.96 Hz) was observed (step (c)). After antibody binding step, any unbound and/or nonspecifically bounded antibody was removed from the flow cell by rinsing with buffer (PBS), showed in step (d). The non-reacted binding sites were occupied by the addition of 10 mM ethanolamine which resulted in slight frequency change (23 Hz). Followed by OmpA antigen binding (decrease in frequency shift 117.84 Hz, step (e)), any unbound and/or nonspecifically bounded antibody was removed from the flow cell by rinsing with buffer and stabilization frequency was achieved, and the results were presented in Fig. 3.

Figure 3. Estimation of the OmpA antigen by flow cell analysis.

D. Estimation of the detection limitation

QCM frequency variation monitored during the addition of different concentration of OmpA antigen to a chemically modified quartz crystal. The data represent the mean of three independent measurements. Calibration curve of antigen binding on site-specifically immobilized antibody, which mean the sensor chip was incubated with various concentrations of OmpA to the antibodies immobilized on the modified crystals. The concentrations were taken into account when plotting the linear relationship of frequency shift vs antigen concentrations. (Fig. 4)

Figure 4. The detection limitation of OmpA antigen by frequency shift.

IV. CONCLUSION

The identification of OmpA protein is remaining important for those countries with higher infection rate. Hence it is necessary to identify the etiologic agent to
provide the correct diagnosis and treatment. For that reason, accurate and easy-to-use methods are required for diagnosis of the most common infections. A rapid, simple and reliable diagnostic test is highly desired. The detection method employed in the present work has the potential to detect the OmpA antigen with accurate, specific, and simple. A flow-injection assay for the detection of the OmpA antigen using ab-based QCM biosensor has been proposed. Satisfactory results were recorded for OmpA ag-ab complex. A linear relationship that existed between frequency shift and the OmpA antigen concentration from 0.02 to 1.20 μg/ml was established. Besides, it can able to detect a way far from lower than 20 ng of OmpA antigen. At any rate, the detection of OmpA protein by quartz crystal microbalance method was comparatively simple and sensitive than other system.

REFERENCES


