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Impedimetric cell-based biosensor for real-time monitoring of cytopathic effects induced by dengue viruses

Ming Soon Cheng [a], Suk Hiang Lau [b], Kwai Peng Chan [c], Chee-Seng Toh [a], Vincent T. Chow [b,*]

[a] Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, Singapore 637371, Singapore
[b] Host and Pathogen Interactivity Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, 5 Science Drive 2, National University Health System, National University of Singapore, Singapore 117545, Singapore
[c] Virology Section, Department of Pathology, Singapore General Hospital, Outram Road, Singapore 169608, Singapore

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ABSTRACT

We describe an impedimetric cell-based biosensor constructed from poly-L-lysine (PLL)-modified screen-printed carbon electrode for real-time monitoring of dengue virus (DENV) infection of surface-immobilized baby hamster kidney (BHK-21) fibroblast cells. Cytopathic effects (CPE) induced by DENV-2 New Guinea C strain (including degenerative morphological changes, detachment, membrane degradation and death of host cells), were reflected by drastic decrease in impedance signal response detected as early as ~30 hours post-infection (hpi). In contrast, distinct CPE by conventional microscopy was evident only at ~72 hpi at the corresponding multiplicity of infection (MOI) of 10. A parameter that describes the kinetics of cytopathogenesis, CIT_{50}, which refers to the time taken for 50% reduction in impedance signal response, revealed an inverse linear relationship with virus titer and MOI. CIT_{50} values were also delayed by 31.5 h for each order of magnitude decrease in MOI. Therefore, based on the analysis of CIT_{50}, the virus titer of a given sample can be determined from the measured impedance signal response. Furthermore, consistent impedance results were also obtained with clinical isolates of the four DENV serotypes verified by RT-PCR and cycle sequencing. This impedimetric cell-based biosensor represents a label-free and continuous approach for the dynamic measurement of cellular responses toward DENV infection, and for detecting the presence of infectious viral particles.

1. Introduction

In recent decades, outbreaks of emerging and re-emerging diseases caused by infectious viruses such as dengue virus (DENV), ebola virus, influenza virus, severe acute respiratory syndrome coronavirus and West Nile virus pose ongoing threats to global biosecurity. In confronting these infectious diseases that spread well beyond the initial affected regions, their surveillance and control often create serious challenges for public health organizations. Hence, the development of rapid, effective and safe virus detection tools has become a major priority of the global community. Current clinical laboratory virus detection tests based on real-time and multiplex PCR techniques, provide a promising platform for simultaneous nucleic acid amplification and detection of multiple target sequences in a single test (Caliendo, 2011; Gullett and Nolte, 2015). However, these molecular techniques are incapable of identifying live infectious viral particles since they detect the nucleic acids originating from both infectious and non-infectious viruses. Virus samples usually comprise high viral particle-to-plaque-forming unit or PFU ratio (Cheng et al., 2012), which may indicate that the minority of viruses in a given sample is infectious, or the presence of non-infectious viruses with mutated or damaged genomes, or failure of most of the viruses to successfully infect due to the complexity of the infection cycle (van der Schaar et al., 2007). Proof of infectious viral particles is highly important and can only be accomplished by conventional cell culture assays, which is time-consuming and labor-intensive. Clearly, it is essential to develop an effective and simple virus detection tool for the assessment of virulence and identification of infectious viral particles to aid in the control of an epidemic.

The electrochemical impedance spectroscopic (EIS) technique has recently gained popularity in cell-based assays in view of advantages such as high sensitivity, non-invasive measurement, accessibility of time-dependent and quantitative data. Cell-based assays using adherent mammalian cell cultures such as fibroblast and endothelial cells have been utilized in toxicological and virological studies (Peters et al., 2012; Hofmann et al., 2013). Since the first reported application of EIS in cell culture study (Giaever

* Corresponding author. Fax: +65 67766872.
E-mail address: micctk@nus.edu.sg (V.T. Chow).

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and Keese, 1984), this technique has been widely employed for in vitro monitoring of dynamic responses of cells toward drugs, toxic agents and pathogens (Kiilerich-Pedersen et al., 2011; Asphahani et al., 2012; Xu et al., 2012). Principally, the EIS cell-based analytical system involves quantitative monitoring of the spreading, morphology and viability of adhered cell cultures in real-time, by applying a constant alternating current (AC) electric field. Due to the low conductivity of cellular membrane, formation of a confluent cell monolayer on the electrode surface often constricts current flow. The corresponding change in impedance signal response can be continuously monitored to obtain information on cellular growth or coverage on the electrode surface (Cheung et al., 2005). Ultrasensitive impedimetric methods have recently reported non-invasive cell-based analyses of viral infections including herpes simplex virus infection of Vero cells (Cho et al., 2007), human cytomegalovirus infection of human fibroblasts (Kiilerich-Pedersen et al., 2011), and bluetongue virus infection of bovine endothelial cells (Drew et al., 2010). The direct, real-time investigation of DENV-induced cytopathogenesis in mammalian fibroblast cells using the EIS cell-based analytical system is first reported in this study.

Mosquito-borne dengue infections are caused by single-stranded RNA-containing DENV which is transmitted by *Aedes* mosquitoes. DENV exists as four antigenically distinct serotypes designated DENV-1, -2, -3 and -4 (Teles, 2011). Among the serotypes, secondary infection with DENV-2 has proven to be responsible for the more severe symptoms of dengue fever (Vaughn et al., 2000). Invasion of host cells by lytic viruses such as DENV will ultimately result in degenerative changes and damage to host cells, known as cytopathic or cytopathogenic effects (CPE). Such effects are generally characterized by degenerative morphological changes, detachment, membrane degradation and eventual death of cells. In general, decreased tight junctions between cells, and increased separation between cells and electrode arising from CPE may lead to decreased impedance signal response. In comparison to conventional inspection methods such as microscopy and plaque assay that rely on observable changes in the morphology and surface coverage of cells, the EIS technique offers a more promising platform for studying virus–host interactions based on the real-time measurement of CPE-induced impedance response (Cho et al., 2007).

Here we report an impedimetric cell-based biosensor constructed from poly-L-lysine (PLL)-modified screen-printed carbon electrode (SPCE) for real-time monitoring of DENV infection of surface-immobilized baby hamster kidney (BHK-21) fibroblast cells. This in vitro method in which the cell-based biosensor is immersed in the culture medium, detects viral-induced CPE by gradual decrease in the impedance response. The sensitivity of the biosensing system is enhanced using SPCE, which constricts the current to flow within a small area of working electrode (Campbell et al., 2007; Alonso-Lomillo et al., 2010). Furthermore, adherence of a cationic polymeric film (PLL) onto SPCE significantly improves the attachment and spreading of cells (Mazia et al., 1975; Sanders et al., 1975; Frey and Corn, 1996; Carrier and Pézolet, 1984; Khademhosseini et al., 2004; Sitterley, 2008). The excellent performance in terms of rapid detection time, automated analysis, high sensitivity and capability of indirectly identifying infectious viral particles are some desirable features of this impedimetric cell-based approach in real-time biosensing.

2. Materials and methods

2.1. Reagents and materials

SPCE, carbon working electrode, silver pseudo-reference electrode, and platinum auxiliary electrode on ceramic substrate were purchased from DropSens. PLL (MW 70,000–150,000 at 0.01% w/v) and potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$, ~99%) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS), and Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium were obtained from Biowest. Trypsin–EDTA (10 ×) and penicillin–streptomycin (100 ×) were obtained from PAA Laboratories. RPMI-1640 medium was purchased from Thermo Scientific Hyclone. Avicel RC-591 (1.2% solid) was purchased from FMC BioPolymer.

2.2. Fabrication of PLL-modified SPCE

10 μL of 0.01% (w/v) sterile-filtered aqueous solution of PLL was applied onto the carbon electrode surface (with an area of 12.6 mm^2) and the tip of pipette was used to spread the solution evenly over the entire electrode surface. The electrode was inverted and placed in a drying oven at 80 °C for at least 5 min to ensure the transformation of PLL film into the stable β-sheet configuration, and adherence of PLL (at a thickness of 1.05 nm) to the electrode surface (Jordan et al., 1994). Excess PLL solution was aspirated, and the electrode surface was thoroughly rinsed with sterile ultrapure water. The PLL-modified SPCE was characterized using cyclic voltammetry (CV) in the presence of 1.0 mM $Fe(CN)_6^{3-/4-}$ in 1 × PBS, pH 7.4 at a scan rate of 100 mV s^{-1} and potential range from −0.8 to 0.8 V.

2.3. Culturing and maintenance of BHK-21 cells

BHK-21 cells (American Type Culture Collection) were cultured in a T25 tissue culture flask containing 3 mL of growth medium (RPMI-1640 medium supplemented with 10% FBS and 1 × penicillin–streptomycin solution). Cells were grown in a humidified incubator at 37 °C with 5% CO_2. When the cells were 80–90% confluent (after 1–2 days), growth medium was removed from the flask. Cells were washed once with 5 mL of 1 × DPBS, 3 mL of 1 × trypsin–EDTA was added, and incubated at 37 °C with 5% CO_2 for 5 min until cells detached. The detached cells were flushed, trypsin–EDTA was removed, the cells were subsequently resuspended in 2 mL of growth medium, and centrifuged at 6000 rpm for 1 min. The supernatant was discarded, and the cell pellet was resuspended in 3 mL of growth medium. BHK-21 cells were propagated at a sub-cultivation ratio of 1:2.

2.4. Preparation of DENV stocks

DENV-2 New Guinea C (NGC) strain and four clinical isolates each of DENV serotypes 1, 2, 3 and 4 were propagated in BHK-21 cells supplemented with maintenance medium (RPMI-1640 supplemented with 5% FBS, 1 × penicillin–streptomycin). Virus at multiplicity of infection (MOI) of 0.1 was inoculated, and the tissue culture infectious fluid (TCIF) was harvested 4–5 days upon the observation of CPE. MOI represents the ratio of the number of infectious agents (viruses) to the number of infection targets (cells) adsorbed on the electrode surface. Uninfected cells served as the control. TCIF was subsequently centrifuged at 6000 rpm for 1 min. The supernatant containing viruses was collected and diluted with RPMI-1640 to achieve the desired MOI for viral infection monitoring experiments. Plaque assay using Avicel (1.2%) and crystal violet staining was performed to determine the virus titer expressed as PFU mL^{-1}.

2.5. Construction of cell-based biosensor and monitoring of DENV-2 infection

The experimental set-up consists of a CHI 750D potentiostat-