Cells-on-chip based transducer platform for probing toxicity of metal nanoparticles

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In this work, we developed a cells-on-chip based transducer (CoCT) platform for probing toxicity of silver nanoparticles (Ag NPs) using non-Faradaic electrochemical impedance spectroscopy (nFEIS). This transducing platform was consists of arrays of capacitor on chip in which each capacitor was functionalized with living Escherichia coli cells. These cells were capable of responding to exposure of different size and concentration of Ag NPs. The capacitive response of CoCT was dependent on size and concentration of NPs. E. coli cells-on-chip response exhibited dramatic loss of capacitance and showed that maximum toxicity to cells-on-chip occurred with smaller 10 nm sized Ag NPs compared to larger size of 100 nm NPs. The cells tend to resist to the larger 100 nm size of NPs that did not affect the cells-on-chip. Our results demonstrated that whole-cell biosensor chip response at a particular frequency enabled determining the severity of the stress imposed by smaller size of Ag NPs. Further, our results were validated through fourier transform infrared spectroscopy (FTIR) and growth of cell/cellular debris and also determined the NPs stress induced toxicity in cells as a proof-of-concept. The methodology developed in this study potentially be extended to other nanomaterials (NMs) for classifying toxic from non-toxic NMs.

1. Introduction

The nanotechnology industry is rapidly growing with promises of substantial benefits that will have significant economic and scientific impacts. It is also applicable to whole host of areas ranging from aerospace engineering and nano-electronics to environmental remediation and medical healthcare [1]. It is estimated that over 500 consumer goods that are already available consist of variety of nanomaterials (NMs). Nanoparticles (NPs) are present in some sunscreens, cosmetics, toothpastes, sanitary-ware coatings, silicon chips and even in food products. Worldwide investment on nanotechnology is on the rise [2,3] and the trend is expected to continue over the next decade. Unusual physicochemical properties of engineered NMs are attributable to their small size, chemical composition, surface structure, solubility, shape, and aggregation [4]. Yet, concerns have been raised due to the unique properties of nanostructured materials could potentially lead to unforeseen health and environmental hazards. Due to expanding use of NMs and commercialization of nanotechnology products, their exposure in the environment and to humans tend to increase with time. Colloidal silver, including formulations now known to contain silver nanoparticles (Ag NPs), has been used commercially for almost 100 years [5]. However, registration of nanosilver products has increased dramatically over the last decades [5,6], most likely as a result of improved capabilities in nanoscience and engineering that allow Ag NPs to be formulated to confer increased durability and/or sustained antibacterial action, even under harsh environmental conditions [7,8]. As is the case for many other types of nanomaterials, a controversy has arisen about whether the Ag NPs should be subjected to increased regulatory scrutiny compared to macroscale or “bulk” silver. Indeed, Ag NPs have been demonstrated to exhibit toxic effects in plants and bacteria at environmentally relevant concentrations [9]. To date, most traditional biological methods for in vitro and in vivo toxicological studies of Ag NPs and other engineered NMs on microbial cells are based on cellular activity and proliferations. These methods include growth and viability assays [10–12], proteomic assays, reactive oxygen species (ROS) detection tests [13–15] and molecular-level evaluations based on genetic responses [5,16,17]. Recently, size dependent effect of Ag NPs against aerobic bacteria Escherichia coli and anaerobic oral pathogenic bacteria Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Streptococcus mitis, Streptococcus mutans and Streptococcus sanguis have been studied through cell growth
method [18]. Among all of the above methods, in vitro cytotoxicity methods are currently employed, which required labeling with fluorescent molecules for detection. These methods are used as markers for cell-viability and consist of procedures that provide results only at a final time-point [19]. The existing conventional analytical techniques reported in the literature usually requires a lengthy and time-consuming process and often produce false positives, and often cannot be implemented for studying cell behavior without interference from its surrounding environment. Hence, there is a demand for a rapid, sensitive and accurate method for assessing toxicity in cells.

Recently, due to the advantages of automation of fluids and minimization of human errors, integration of a cells-on-a-chip system is gaining importance for nanotoxicity assessments [20]. In recent studies, chip-based electrochemical approach was used to test the toxic effect of NMs. The measurements were recorded based on differential pulse voltammetry and or electrical impedance methods [21–23]. All the above cells-on-chip approaches for nanotoxicity assessment were based on Faradaic-electrochemical measurements that require a redox mediator/chemical reagent to generate detectable signal which often leads to undesirable quenching effects with NPs. Therefore, the present study aims to develop toxicity assay using electrical sensing platform that provides a new tool to understand the toxicological impacts of Ag NPs as model for other NMs using bacterial cell-functionalized biosensor chip.

In this study, we designed a CoCT using electrical sensing platform that provides a new tool to understand the toxicological impacts of Ag NPs as model for other NMs. This CoCT measures the capacitance from immobilized cells over electrodes as a function of applied AC frequency and its measuring principle is based on non-faradic method. Changes in capacitance can be detected that occurred as a result of changes in the cellular activity after their interaction with NPs. The results obtained from the CoCT studies were confirmed through optical imaging, FTIR as well as probing cellular interactions at the cell-membrane after exposure of NPs by growth assay. The fact that the present study is different from the previous reports in an important consideration that include the developed biochip was tested for metal oxide NPs and it is important to test metal NPs response as well to extend the applicability of developed CoCT to other NMs [24].

2. Material and methods

2.1. Reagents

Silicon wafers of 4 inch size, (100) oriented, p-type with the resistivity of 9–12 Ω cm and thicknesses of 500 ± 25 μm with 1 μm thick SiO2 layer on top were obtained from University Wafers, USA. Wild-type E. coli DH5α strain was used as model living bacterial cells in this study. Luria-Bertani broth (LB-broth) and Luria-Bertani agar (LB-agar) were obtained from Difco (MI, USA). Phosphate-buffered saline (PBS), 3-mercaptopropionic acid (MPA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), Ag NPs (with sizes of 10, 20 and 100 nm) were purchased from Sigma-Aldrich, Germany. All other reagents used in this study were of analytical grade and filtered through 0.22 μm sterile filters.

2.2. Characterization of Ag NPs

The shape and size of Ag NPs used in this study were characterized using transmission electron microscopy (TEM) technique and dynamic light scattering (DLS, Malvern Zetasizer). For TEM characterization sample preparation, 5% solution of each size of Ag NPs were suspended in aqueous medium and then placed on fromvar coated copper grids. The grids were then allowed to dry at room temperature. The images were captured using 2000 SX JEOL-TEM at 160 kV EHT voltage with a magnification of 250Kx.

2.3. Fabrication of capacitor arrays

Gold interdigitated electrode based capacitor sensor arrays were patterned on SiO2 surface using image reversal technique. In this process, the metal layers were patterned using the dual tone photoresists AZ5214E. A 2.5 μm thick AZ5214E photoresist was patterned with the help of a mask for a lift-off process in pure acetone as a solvent. Following this step, a very thin tungsten layer of 50–60 nm size was layered to improve the adhesion of gold on the SiO2 film by DC sputter deposition and about 200–210 nm thick gold layer was deposited. The dimension of each electrode was 800 μm in length, 40 μm in width with a distance between two electrodes of 40 μm. Each capacitor sensor contained 24-interdigitated gold electrodes within a total area of 3 mm2.

2.4. Immobilization of E. coli cells on capacitor array chip and exposure of NPs

The bacterial strain used in this study was E. coli DH5α. Actively growing E. coli cells were inoculated into fresh Luria-Bertani (LB) medium and allowed to grow till mid-logarithmic growth phase. The cells were then harvested by centrifugation at 1000g for 3 min and washed thrice with phosphate buffered saline (PBS) pH 7.2, and resuspended in same buffer. The cell concentration was determined by colony counting after serial dilution followed by plating on LB-agar plates. The chip was first rinsed with sterile distilled water and dried with pure nitrogen followed by immersing the chip in 20 mM of mercaptopropionic acid (MPA) and incubated overnight at room temperature. The chips were then incubated with a mixture of 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide (EDC) and 50 mM of N-hydroxysuccinimide (NHS) for 2 h. The chip was then removed, thoroughly washed with distilled water and each capacitor in an array was incubated with 5 μl of bacterial suspension containing 8 × 107 colony forming units (CFU) in PBS solution for 2 h. This sensing area was incubated with a series of Ag NPs concentrations (0.2–2 μg/ml PBS containing 0.01% Tween 20) in 5 μl volumes containing three different sizes 10, 20 and 100 nm, respectively for 2 h at 25 °C. The chip was quickly washed with PBS and dried using nitrogen gas. The dried chips were then subjected dielectric measurements.

2.5. Optical microscopy imaging of immobilized E. coli cells on chip

Optical images of the sensors after immobilization of E. coli cells on chip were taken using Carl Zeiss Axio Scope A1 MAT.

2.6. Impedance/capacitance measurement

The impedance/capacitance responses were measured sequentially to ensure that chips remain active after every step that includes; (a) bare capacitor sensor, (b) after E. coli immobilization and (c) after NPs exposure. A negative control experiment was conducted using capacitor chip containing heat- killed E. coli cells. For this, capacitor chips containing immobilized E. coli cells (8 × 107) were subjected to heat treatment in an air-tight pre-heated humid chamber at 95 °C for 5 min followed by quickly freezing at ~70 °C for 5 min, and thrice at 25 °C for 15 min. The above treatment process was repeated thrice and finally the capacitive chip was dried under N2 gas. The capacitance response in between the gold interdigitated electrodes of capacitor sensor surface was measured in the frequency range 50 MHz to 1 GHz using a Network Analyzer (Karl-Suss
PM-5 RF Probe Station and Agilent-8720ES). The Network Analyzer was calibrated using SOLT (short-open-load-through) method. The impedance values were exported to MATLAB® software for the analysis. The absolute capacitance values of triplicate experiments were extracted at an effective frequency ($f$) range (150–300 MHz) and the standard deviations were shown as errors. The relative capacitance variations were calculated from the data obtained within 150–300 MHz frequency range under standard assay conditions using the following Eq. (1) as described previously [25].

$$\frac{C - C_0}{C_0} \times 100$$  \hspace{1cm} (1)

where $C$ is the actual capacitance after the interaction of each sizes of NPs with $E. coli$ cells at a particular concentration and $C_0$ is the capacitance before interaction.

2.7. FTIR spectra of whole-cell based biosensor chip after exposure of Ag NPs

The immobilization of $E. coli$ cells on capacitor chip and effect of different sizes of Ag NPs were confirmed by Fourier transform infrared spectroscopy (FTIR) using Thermo scientific Nicolet™ iS™10 FT-IR Spectrometer.

2.8. Determining the growth curves of bacterial cells exposed to different sizes of Ag NPs

To examine the growth curves of bacterial cells exposed to Ag NPs, LB broth with Ag NPs (0, 2 μg/ml) was used, and the bacterial cell concentration was adjusted to $10^7$ CFU/ml. Each culture was incubated in a shaking incubator at 37°C for 24 h. Growth curves of bacterial cell cultures were attained through repeated measures of the optical density (O.D.) at 600 nm by using Synergy HTX microplate reader (Biotek) plate reader.

3. Results and discussion

The goal of this study was to develop a noninvasive, label-free whole-cell based capacitive biochip to test Ag NPs’ toxicity. This aim was accomplished by probing responses of cells as biological reporters under the applied AC electrical frequency. For this, gold interdigitated microelectrodes based capacitor were patterned on SiO₂ surface by photolithography technique. $E. coli$ cells were covalently coupled on sensor platform (Scheme 1). The electrical response of immobilized $E. coli$ cells with and without exposure of NPs on capacitor surfaces was captured based on the change in dielectric properties, conductivity or charge distribution. The response of capacitive chips was further validated through FTIR and growth curves of bacterial cells exposed to different sizes of Ag NPs.

3.1. Characterization of Ag NPs by TEM and DLS

The three different sizes of Ag NPs used in this study were characterized using TEM analysis. The average sizes of NPs were found to be 10, 20 and 100 nm, respectively in TEM images as shown in Fig. 1a–c. The TEM images showed that the NPs were spherical and monodispersed in their distribution patterns that facilitated defined nano-sizes of Ag NPs to study their impact on exposure to $E. coli$ cells-on-chip.

The particle sizes of Ag NPs used in this study was determined using dynamic light scattering measurement technique. In this technique, the hydrodynamic radius of the NPs was measured based on the principle of Brownian motion. The size distributions of the used NPs were depicted in Fig. 1d–f. As seen in case of 10 nm NPs, the particles were not seen monodispersed, but the average hydrodynamic radius of NPs was found to be around 16 nm (Fig. 1d). The estimated size in the DLS technique is based on the hydrodynamic diameter of the theoretical sphere that diffuses with identical size of particles and therefore, the measured size could be influential due to electrical double layer moving along with the particle. Thus, the measured size of 10 nm of NPs by TEM found differs than its measured hydrodynamic radius by DLS. The hydrodynamic radius for other two sizes of NPs were found to be around 20 and 100 nm (Fig. 1e and f), respectively.
whole-cell chip exhibited size dependent toxicity of silver NPs. It was observed that the capacitive response decreased with smaller size of Ag NPs (10 nm), which is attributed to the change in the potential of cell membrane upon the NPs’s interaction with cells at the interface of the microelectrodes (Fig. 3a). However, the sensor response with larger 100 nm size of Ag NPs did not impose significant damage to the living cells-on-chip when compared with the control (no NPs, only cells-on-chip) (Fig. 3c). These results suggested that E. coli cells were sensitive to smaller NP sizes and severely affected by 10 nm NP sizes as evidenced by the dramatic loss of capacitance responses probably due to damage or collapse of living cell structure on sensor. This type of response was not seen with E. coli cells exposed with larger 100 nm sizes of Ag NPs which was probably because of their ability to resist against the larger sized Ag NPs (Fig. 4a and b). Fig. 4b shows the relative change in chip response of control (only cells), tests upon exposure of 10, 20 and 100 nm sizes of Ag NPs at 200 MHz applied AC frequency. The chip response against the heat-killed cells consistently showed significant drop in relative capacitance. This result suggested the inability of heat-killed cells to respond, which is an indication that the sensor response is attributed to the living activity of cells. This type of signal was correlated to the impact of NPs imposed on the living cells. It is clear from Fig. 4b that after heat-killing cells, the relative capacitive response dramatically dropped to a maximum extent and a similar response were also seen with normal cells exposed to 10–20 nm Ag NPs. This result can be compared to the sensor response obtained with heat-killed cells that show similar responses and suggest the smaller Ag NPs are lethal to cells. The capacitive response of chip upon exposure of 10–20 nm size at 0.2–0.8 μg/ml concentration was found to decline

Fig. 2. Optical microscopic images of E. coli cells immobilized on: (a) capacitor chip at 5× and (b) a magnified portion of interdigitated electrodes of capacitor chip at 10× magnification.

3.2. Optical micrographs of E. coli immobilized chip

The optical images of E. coli cells immobilized on capacitor chip are shown in Fig. 2a and b. The optical microscopic images clearly indicated the density and distribution of cells indicating the dense immobilization of cells on electrodes of chip which is most suitable for probing NPs’ toxicity in living E. coli cells-on-chip.

3.3. Capacitance response of whole-cell based biosensor chip after exposure of Ag NPs

The capacitance responses of whole-cell chip after exposure of Ag NPs were measured as a function of AC frequency (150–300 MHz). The capacitive response of Ag NPs on whole-cell biochip with three different sizes 10, 20 and 100 nm was shown in Fig. 3a–c, respectively. The cellular responses obtained through the

Fig. 3. Change in capacitance from whole-cell chip as a function of applied frequency (100–300 MHz) when exposed to different concentration (0.2–2 μg/ml) of (a) 10 nm (b) 20 nm and (c) 100 nm size of Ag NPs.
significantly (Fig. 4a). The results suggest that the increase in concentration of NPs (ranging 0.2–0.8 μg/ml) also affected the cells that increased the severely of cellular toxicity of NPs. Such effects normally occur due to accumulation of NPs or transport activity of NPs across the cell membrane, and this combined with impaired cell membrane function making the reduced cell’s growth potential [15,26]. The interaction of bacterial cells with NPs probably yielded a reduction of net surface charges and this may have resulted in a decrease in relative change in capacitance response. However, relative capacitive response with exposure of 100 nm sizes of NPs did not show a considerably lethal effect which was closer to control responses. This result suggested that the cells did not affected severely by the 100 nm size of Ag NPs compared to smaller size (10–20 nm).

3.4. FTIR spectra of whole-cell based biosensor chip after exposure of Ag NPs

E. coli’s outer wall is composed of an asymmetric lipid bilayer. The inner leaflet is mainly composed of L-phosphatidyl ethanolamine (PE) and the outer leaflet is composed of lipopolysaccharide (LPS) biomolecules [27]. The ATR-FTIR peaks of an E. coli before exposure of Ag NPs were assigned to bands at 1634 cm⁻¹ (amide I) arising principally from ν(C=O) stretching vibrations; bands at 1554 cm⁻¹ (amide II) due primarily to N–H bending with contributions from the C–N stretching vibrations of the peptide group as shown in Fig. 5. The bands around 1280–1200 cm⁻¹ arise from the nucleic acid bands overlapping with the asymmetric stretching mode νas of the phospholipid phospho-diester (PO₂⁻) and non-stoichiometric lipopolysaccharides PO₂⁻ (LPS). The peaks between 1200 and 950 cm⁻¹ are due to the vibrations of the sugar rings of LPS and also with the sugar rings from other cell moieties, such as peptidoglycan and exopolysaccharides (EPS) or capsular polysaccharides (CPS) [28].

The changes in the FTIR spectra for the E. coli cells treated with 10 and 100 nm Ag NPs is shown in Fig. 5. The intensity of the spectra was found to decrease with smaller size of Ag NPs. LPS spectra contain a relatively broad peak in the carbohydrate (O-antigen) region [ν(CO, COC); 1055 cm⁻¹, 1168 cm⁻¹] in E. coli spectra. When 100 nm Ag NPs interacted, LPS peak 1055 and 1168 cm⁻¹ were shifted to lower wavenumber of 1045 and 1169 cm⁻¹, respectively. However, these peaks disappeared with cells treated with 10 nm size of NPs. This was probably due to the weakening of C=O, C–C, and POH bond caused by an increase in the degree or strength of hydrogen bonding with NPs [27]. The above results showed that the outer membrane of E. coli was indeed damaged, which was triggered by the toxic nature of smaller 10 nm sized Ag NPs. These results are in accordance with capacitive chip response results when different sizes of Ag NPs exposed to E. coli cells.

3.5. Growth curves of E. coli cells exposed to different sizes of Ag NPs

Growth of E. coli cells exposed to different sizes of Ag NPs was conducted to validate the E. coli CoCT response and all the toxicity parameter such as sizes (0, 10, 20 and 100 nm) and maximum concentration (2 μg/ml) of Ag NPs were kept same as in E. coli CoCT experiment.

The growth curves of bacterial cells treated with different sizes of Ag NPs indicated that Ag NPs could inhibit the growth of bacterial cells. The growth curves of Ag NPs treated E. coli cells are shown in Fig. 6. The bacterial growths of cells treated with 10 and 20 nm Ag-NPs were inhibited in comparison with 100 nm. The bacterial growth of the cells treated with 100 nm of Ag-NPs was also slightly lower than that of cells in the control group. The above result indicated that reactive oxygen species may be formed by lower size of Ag NPs inhibit in cellular respiration. As a result, lower size of Ag NPs cause inhibition of bacterial growth and reproduction, which is in good agreement with a previous study, which showed that the respiratory chain activity in E. coli was inhibited by Ag.
4. Conclusions

In this study, capacitor arrays immobilized with E. coli cells were employed as a whole-cell biosensor chip to probe the cytotoxicity of Ag NPs. Here, E. coli cells were utilized as biological reporters and probed for electrical behavior of cells when interacted with Ag NPs using nEIS. Our results suggested that E. coli cells were sensitively and severely affected by smaller 10 nm sizes of NPs, because cells exhibited dramatic loss of capacitance responses mainly because of the severe cellular damage. Contrastingly, E. coli cells were not severely affected with the larger 100 nm size of Ag NPs which was probably because cells-on-chip were capable of resisting to perturbations induced larger NP sizes. Further, the whole-cell-chip responses were also validated by FTIR analysis and growth of cell/cellular debris before and after the NPs exposure. The biosensor developed in this study can potentially be applied for the rapid detection of nanomaterials and chemicals that induce cytotoxicity in food, pharmaceutical preparations and environmental samples.

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References


Biographies

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