Capacitance based real time monitoring of three kinds of endocytosis

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ABSTRACT

Endocytosis is a process in which a substance gains entry into a cell without passing through the cell membrane. This process is subdivided into three different types, receptor mediated endocytosis, pinocytosis and phagocytosis. In this study, we developed a capacitance sensor to monitor endocytosis in real time. During the receptor mediated endocytosis, a capacitance peak was observed. In contrast, the capacitance declined without a capacitance peak when nanoparticles or bacteria were taken up via non-specific pinocytosis or phagocytosis. In addition, we have developed a capacitance sensor array to demonstrate capacitance-based high-throughput screening. These results demonstrated that three types of endocytosis are able to be differentiated using our capacitance sensor.

Keywords: Capacitance sensor, Receptor-mediated endocytosis, Pinocytosis, Phagocytosis.

1 INTRODUCTION

All cells, whether prokaryotic or eukaryotic, have a membrane. It separates cell interior from the environment and let some substances pass through freely, some pass through to a limited extent, and blocks some from passing through at all, which is called “Endocytosis”. Viruses and hydrophilic molecules, like nucleic acids, cannot pass through the hydrophobic cell membrane [1]. They are internalized into the cell via receptor-mediated endocytosis, in which a specific receptor on the cell surface recognizes and binds to an extracellular ligand or absorbed by cell membrane unspectifically. Otherwise, pathogens are internalized by cells with phagocytic activity, which is followed by pathogen killing and destruction. All three types of endocytosis are required for general cellular functions. [2] In particular, receptor mediated endocytosis and pinocytosis is essential process for therapeutic gene/drug delivery [3] and phagocytosis is key to the induction of immunity [4]. Therefore, a variety of techniques and reagents have been developed to detect endocytosis but most procedures are invasive, time consuming, and potentially subject to interference.

In this study, we have developed a capacitance sensor composed of two electrodes spaced 10 μm apart, a distance comparable to the size of most cells. We tested the capacitance sensor for monitoring cellular uptake of adenoviruses (Ads), antibodies (Abs), and polystyrene (PS), nanoparticles, and Escherichia coli. Furthermore, we fabricated a capacitance sensor array for high-throughput applications and demonstrated the feasibility of its application to large-scale screening to identify Abs or ligands with high affinity to specific target molecules.

2 EXPERIMENT PART

2.1 Cell lines, adenoviruses and materials

All cell lines were cultured in Dulbecco’s modified essential medium (DMEM) (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and penicillin–streptomycin (100 IU/ml) at 37 °C in a humidified atmosphere of 5% CO2. The hepatoma (Hep1), non-small lung cancer (A549), breast cancer (MDA-MB-435), fibroblast (IMR90), Murine macrophage (RAW 264.7) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Table 1 shows information of adenovirus, nanoparticles and Escherichia coli.

2.2 Fabrication of capacitance sensors

Individual capacitance sensor with gap areas of 10 μm×20 μm was fabricated on a glass substrate (Fig. 1(a)). The 100-nm-thick Au electrodes were patterned by photolithography and lift-off techniques. Next, a 50-nm-thick SiO2 passivation layer was deposited at the top of the Au electrodes to minimize the influence of cells; thus the sensor was regarded as a parallel-plate capacitor. For cell culture, silicon well with fluidic channels was mounted onto the electrodes. The Ads, Abs, or nanoparticles were
diluted in DMEM supplemented with 5% FBS and were introduced into the well through the fluidic channel. The capacitance sensor array, composed of four sensors with gap areas of 50 μm x 4000 μm, was also fabricated on the glass substrate (Fig. 3(a)). An acrylic well with a volume of 200 μl for cell culture was attached to each sensor with a curing agent (polydimethylsiloxane and toluene mixed at 1:10).

### 2.3. Capacitance measurements

Cells were cultured for 10-12 hours at 37 °C in a 5% CO₂ incubator prior to the capacitance measurement. Capacitance was measured with an LCR meter (Agilent 4284A) at a frequency of 3 kHz with a 10 mV peak-to-peak AC signal. The single capacitance sensor was placed in a home-made environmental control chamber mounted on an optical microscope (Leica DMI 3000B) for simultaneous electrical and optical measurements. The environmental control chamber was maintained at 37 °C, but the CO₂ concentration and the humidity were not controlled. For the capacitance sensor array, the samples were placed in an incubator and maintained at 37 °C in 5% CO₂ during the capacitance measurements. The electrical connection between the sensor inside the incubator and the LCR meter outside the incubator was made via electrical connectors mounted on the side of the incubator. The capacitance was measured simultaneously from all four connectors mounted on the side of the incubator. The capacitance was measured as a function of time (Fig. 1(c)).

### 3 RESULTS AND DISCUSSION

#### 3.1 Receptor mediated endocytosis

We first studied the real time capacitance of cells placed between the 10 m gap of the capacitance sensor electrodes (Figure 1 (a)). We compared capacitance measurements in Hep1 hepatoma cells infected and not infected (control) with replication-incompetent Ad (Ad-ΔE1/ΔE3; Figure 1(b)). In the cells infected with Ad-ΔE1/ΔE3, a sharp capacitance peak appeared and its height increased with increasing concentrations of Ad-ΔE1/ΔE3 viral particles (VPs). To prove the capacitance peak could be ascribed to the specific endocytosis of Ads, we pre-incubated Hep1 cells with an Ab specific to the coxsackievirus and adenovirus receptor (CAR; 50 μg/ml in serum-free medium) to inhibit the internalization of Ads. Subsequently, 10^5 VPs of Ad-ΔE1/ΔE3 was added and capacitance was measured as a function of time (Fig. 1(c)). In cells treated with CAR-specific Ab, the sharp capacitance peak was not observed. Since the internalization of Ads was inhibited with CAR-specific Abs, this finding confirms that the capacitance peak was specifically caused by the endocytosis of Ads. In order to determine whether the real time capacitance measurements reflected cellular behaviors, we measured the time lapse total internal reflection fluorescence (TIRF) and fluorescence microscope images. The TIRF microscope can image only the fluorescent probes located near the cell membrane on the substrate, while the fluorescence microscope can image the fluorescent probes inside the cells. Cells were transduced with replication-incompetent Ad-ΔE1/IX-GFP, which carried the capsid fusion protein, IX-green fluorescent protein (IX-GFP). Fig. 1(d) and (e) shows the time-lapse TIRF and fluorescence microscope images, respectively. In the TIRF microscope images, the intensity of green florescence from Ad-ΔE1/IX-GFP bound to the cell membrane increased until about 30 min, and then decreased. On the other hand, the time-lapse fluorescence microscope images showed the emergence of green florescence within the cell after about 40 min. The combined time lapse TIRF and fluorescence microscope images suggested that the number of Ad-
ΔE1/IX-GFP bound to CARs on the cell membrane became maximum at about 30 min, and then declined due to internalization of Ad-ΔE1/IX-GFP. Compared with the results in Fig. 1(b), it is clear that the time at which the maximum number of Ad-ΔE1/IX-GFP was bound to the cell membrane coincided with the capacitance peak time. These results suggested that the capacitance increased while Ads were binding to CAR on the cell membrane and then, the capacitance declined while the Ad–CAR complexes budded inward and pinched off inside the cell. Next, we collected the time-lapse TIRF and fluorescence microscope images (Fig. 2(f) and (g), respectively), with CAR-specific Abs and then infected with Ad-ΔE1/IX-GFP.

Unlike the untreated A549 cells, green fluorescence was not observed on the cell surface in the TIRF images until about 30 min. After about 40 min, green emission was only observed from the unbounded Ad-ΔE1/IX-GFP that had collected on the bottom of the culture well. Moreover, the fluorescence images showed that green emission was observed only on the surface of the cell, but not inside the cell even after 1 h. This indicated that endocytosis of Ads was significantly inhibited by the CAR-specific Abs. The above experimental results indicate that the capacitance increased when Ads were bound to the receptors on the cell membrane and the capacitance decreased when the receptor–ligand complexes were internalized, resulting in the capacitance peak.

3.2 Pinocytosis

In contrast to Ads and Abs, nanoparticles are taken up by the cells via non-specific pinocytosis [5, 6]. To investigate whether non-specific pinocytosis can be distinguished from receptor-mediated endocytosis by measuring the capacitance, we measured the capacitance for Hep1 cells treated with PS nanoparticles with a diameter of 100 nm, which is comparable to the size of an Ad. As shown in Fig. 2(a), a dip in capacitance was observed rather than the capacitance peak observed when the Hep1 cells were treated with Ads or Abs. The capacitance decreased until about 120, 400, and 420 min post-treatment for PS nanoparticle concentrations of 5, 10, and 20 μg/ml, respectively. The capacitance then increased, resulting in the observed dip in capacitance. To visualize the behavior of PS nanoparticles, we recorded time-lapse TIRF microscope images of cells treated with rhodamine-loaded PS nanoparticles (Fig. 2(b)). Unlike the Hep1 cells transduced with Ad-ΔE1/IX-GFP, we clearly observed red emission inside the cell from PS nanoparticles even at 12 min, and the region of red emission broadened with time. This implied that PS nanoparticles were internalized immediately after introducing PS nanoparticles to the medium.
from cells that contained no molecules; thus, this served as a negative control. As shown in Fig. 3(b), capacitance peaks were observed for Ad-ΔE1/ΔE3 but not for Herceptin. This indicated that Hep1 cells expressed CAR receptors for Ad-ΔE1/ΔE3, but were not susceptible to Herceptin which specifically binds Her-2/neu receptors. Then, capacitance dip was observed for PS nanoparticles. These results demonstrated that the capacitance sensor array could be used to screen binding molecules to identify specificity to a particular membrane receptor and unspecific pinocytosis.

![Figure 3](image)

Figure 3. (a) Schematic diagram of a capacitance sensor array. Time dependence of C/Co recorded with the sensor array (b) Hep1 cells treated with Herceptin (5 mg/ml), Ad-ΔE1B19 (10MOI), PS nanoparticles (40 μg/ml), and untreated Hep1 cells. Data are shifted only for clarity.

3.3 Phagocytosis

Phagocytosis is the process of internalisation of pathogens by cells with phagocytic activity, which is followed by pathogen killing and destruction. To optimize the conditions of an in vitro phagocytosis assay as realistic situations, we decided to use viable pathogens for infection- Escherichia coli- basis of the Murine macrophage cell line (RAW264.7). 5x10⁴ cells of RAW264.7 were seeded in the wells attached to the array sensor, then three concentrations of E-coli were applied.

![Figure 4](image)

Figure 4. (a) Capacitance result of phagocytosis (b) confocal images of phagocytosis process (RAW264.7: green, E-coli: red).

The capacitance sensor shows a peak during the engulfing of bacterium, precisely; capacitance is abruptly increases due to the competition between engulfing the bacterium and bacteria dividing, then capacitance decreases for internalization of E-coli (Figure 4(a)). The capacitance decreasing time is proportional to E-coli concentrations (5, 50, 500 MOI). The phagocytosis process proves by real time confocal imaging; the E-coli (red) engulfing to RAW264.7cell (green) then engulfing E-coli observed as yellow. Then the internalization time is same as capacitance peak observing time (Figure 4(b)).

4. Conclusion

In summary, we have demonstrated that the capacitance sensors can be used for discriminate three kinds of endocytosis process. The capacitance peaks were observed when Ads or Abs were internalized into the cells via receptor-mediated endocytosis. Based on the comparison of the combined time-lapse TIRF and fluorescence microscope images with the capacitance recordings, we propose that the capacitance increased when Ads or Abs were bound to the receptors on the cell membrane, and the capacitance decreased when the receptor–ligand complexes were internalized. This rise and decline resulted in the observed capacitance peak. Indeed, when PS nanoparticle or E-coli were taken up by the cells via non-specific pinocytosis and phagocytosis, the capacitance decreased without a capacitance peak. To our knowledge, this is the first study to demonstrate that cellular activities like endocytosis could be monitored and distinguished in real time with a capacitance sensor.

We also developed the capacitance sensor array for high throughput applications and showed that the capacitance sensor array could measure several bio-molecules at once to detect whether they were internalized into a specific cell type. Furthermore, we can easily increase the number of sensors in an array. Thus, we expect that, in the future, the capacitance sensor array will be a valuable tool for high-throughput screening to identify antibodies or ligands with high affinities to specific cell types.

REFERENCES