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Short communication

Monitoring change in refractive index of cytosol of animal cells on affinity surface under osmotic stimulus for label-free measurement of viability

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ABSTRACT

We demonstrated that a metal-clad waveguide (MCW)-based biosensor can be applied to label-free measurements of viability of adherent animal cells with osmotic stimulation in real time. After Chinese hamster ovary (CHO) and human embryonic kidney cell 293 (HEK293) cells were attached to a Concanavalin A (Con A)-modified sensor surface, the magnitudes of cell responses to non-isotonic stimulation were compared between live and dead cells. The live cells exhibited a change in the refractive index (RI) of the cytosol caused by a redistribution of water through the cell membrane, which was induced by the osmotic stimulus, but the dead cells did not. Moreover, the normalized change in the RI measured via the MCW sensor was linearly proportional to the viability of attached cells and the resolution in monitoring cell viability was about 0.079%. Therefore, the viability of attached animal cells can be measured without labels by observing the relative differences in the RI of cytosol in isotonic and non-isotonic buffers.

1. Introduction

Cell viability is a fundamental physiological characteristic of a cell, and has been of general interest in various kinds of cell-based biological research and applications. In general, cell viability has been determined by staining cells with vital dyes such as trypan blue (TB) and Methylene Blue. However, these dyes have some problems that the number of blue-stained cells increases by lapse of time (Jones and Senft, 1985) and determining viability may be inaccurate when viability is $<95\%$. Alternatively, fluorescent dyes such as Acridine Orange (AO), Alcian Blue (Fukudome et al., 2002), Congo Red (Kovács and Foote, 1992), ethidium bromide (EB) (Maria et al., 1997), and SYTOX Green (Breeuwer and Abee, 2000) have been used to assess cell viability. It is impossible to recover cells for further analysis and to continuously monitor over the entire culture procedure because all of these assays are cell invasive and require multiple steps and end-point detection (Hynes et al., 2003).

The release of specific molecules such as ^{51}Chromium (Vennström et al., 2008), iododeoxyuridine (Schneiderman et al., 1991; Gee et al., 1985), [^{3}H] praline (Vázquez et al., 2003), [^{75}Se] selenomethionine, and [^{3}H] uridine (Hu et al., 2000) from cells can also be utilized to measure cell viability, which may be toxic to the cells and limit sensitivity of the assay. Based on cellular metabolic activity, the bioluminescence-based ATP assay using luciferase (Kanges et al., 1984; Cree, 1998) and the MTT/XTT assay (Carmichael et al., 1987) also suffer from the limitation such as the requirement of cell lysis and lower sensitivity, respectively.

An ideal method of measuring cell viability would not use chemicals that are toxic and hinder measurements of cell viability, but remain sensitive enough to measure viability accurately in real time. A number of assays have been developed that allow cell viability to be assessed label-free and noninvasively such as the

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assay based on monitoring the metabolic activity of living cells via consumption of oxygen has been developed (O'Riordan et al., 2000). However, it has potential problems such as the self-quenching of reagent at high concentrations and phototoxic effects.

The ultimate objective of our research including this work is development of a method for label-free and real-time measurement of cell viability using a surface-refractometric sensor, overcoming the limitations of existing cell viability assays. In case of using a surface-refractometric sensor, however, target cells should be contacted with the sensor surface for measurement. Nevertheless, this method could be applied to most of cell systems since all cells would sink to contact with the sensor surface due to gravity. In this report, as a preliminary result, we assessed the feasibility of label-freely estimating the viability of animal cells adhering to solid surfaces using a metal-clad waveguide (MCW) sensor in real time by monitoring changes in the refractive index (RI) of cytosol due to osmotic stimuli. A recent study showed that surface plasmon resonance (SPR) can be used to monitor volume changes caused by osmotic stimuli in a confluent monolayer of cells (Robelek and Wegener, 2010). Compared to SPR, the MCW sensor with the extensive penetration depth (>1.5 μm) of the optical field is comparatively efficient for monitoring of changes in cell volume (Zourob et al., 2003). Intentional imbalances between osmolarity of the cytosol and ambient media cause the diffusion of water through the cell membrane and consequent alterations in cell volume and the RI of cytosol, which could be observed only in live cells. We investigated the correlation between the changes in RI of cytosol in cells adhering to a surface measured using the MCW sensor and their viability.

2. Experiments

2.1. Materials

The materials used in this study are as follows: phosphate-buffered saline (PBS), sucrose, poly-L-lysine (PLL), glutaraldehyde, Concanavalin A (Con A), polyvinylpyrrolidone, monobasic sodium phosphate, dibasic sodium phosphate, sodium hydroxide (NaOH), trypan blue (TB), HEDS (hydroxyethyl disulfide) assay kit, the detail information of which is described in Section S-1 of the Supplementary information.

2.2. Cell preparation

The CHO and HEK293 cells were grown in shake flasks (polycarbonate Erlenmeyer flask, Corning, USA) using HyClone SFM4CHO Cell Culture Media and HyClone SFM4HEK293 Cell Culture Media (Thermo Scientific Rockford, USA) with L-glutamine and 2.2 g/L sodium bicarbonate, respectively. The cultures were performed in an ordinary humidified cell incubator at 37 °C and 5% CO_2 with shaking.

2.3. Preparation of sensor chip and cell attachment

The MCW sensor chips were fabricated with the procedure described in the previous studies (Im et al., 2012; Kim et al., 2014), which is depicted in Section S-2 of the Supplementary information in detail. The surface of the fabricated MCW sensor was modified as illustrated in Fig. 1. The sensor chips were immersed in 5 N NaOH for 10 min with sonication and washed with DW. The sensor chips were soaked in 0.01% PLL solution overnight to produce an amine surface on the chip, followed by washing with DW. To create an aldehyde functionalized surface on the sensor chip, the chips were treated with 2.5% glutaraldehyde for 2 h and then washed with DW. The sensor chips were treated with Con A solution (1 mg/mL) for 2 h at room temperature and washed with DW to adhere cells on the sensor surface.

The CHO and HEK293 cell suspensions were diluted to a concentration of 1×10^6 cells/mL with culture medium, and 1 mL of the diluted cell suspensions was dropped on the Con A-modified sensor surfaces and reacted for 10 min. To remove unattached cells, the chip was washed via mild shaking with sterilized PBS three times and kept in the same buffer until measurement. To obtain attached cells with various viabilities, the attached cells were left alone in isotonic buffer at room temperature to starve.

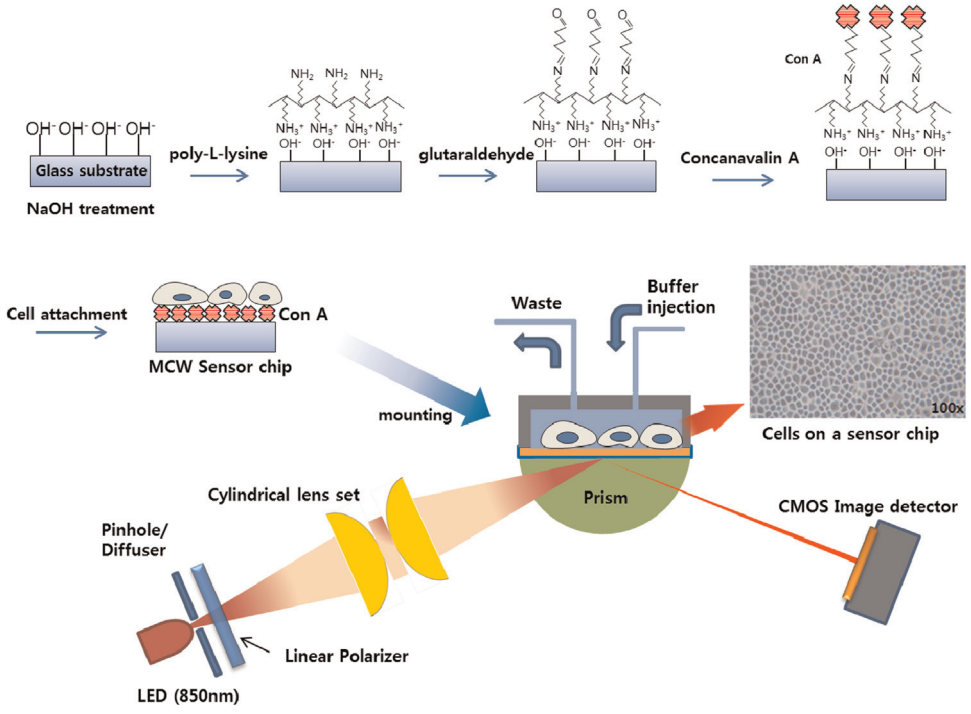

Fig. 1. Procedure of the modification of the sensor surface for attachment of cells and the schematic of the metal-clad waveguide (MCW) sensor system used to measure the change in the refractive index of the cytosol of CHO cells.

2.4. Measurement of changes in RI of the cytosol using the MCW sensor and confirmation of cell viability

The schematic diagram of the MCW sensor system is illustrated in Fig. 1, which was also described in a previous study (Im et al., 2012), and the detail of the system is represented in Section S-3 of the Supplementary information.

The sensor chips covered with CHO and HEK293 cells with various viabilities were installed on the prism of the MCW measurement system. Isotonic or non-isotonic buffer solutions were irrigated on the surface of the chip using a two-channel fluidic system and a syringe pump with a flow rate of 10 μL/min. After measuring the changes in the RI of the cytosol using the MCW sensor, cell viability was estimated by averaging the counted values obtained from three different regions on the sensor surface using TB staining and an automatic cell imaging counter (CYT-100; ECI Inc., Tokyo, Japan). The TB dye exclusion method was used after the cells were stained with 0.2% TB solution for 1 min and washed with sterilized PBS. For HEDS assay as another method to estimate cell viability, HEDS and 5.5-dithiobis 2-nitrobenzoic acid (DTNB) were prepared as the manufacturers' instructions in the glucose-free growth medium and glutathione buffer, respectively, and stored at 4 °C. After measuring using the MCW sensor, a pre-casted poly-dimethylsiloxane (PDMS) well was covered on the region of the MCW chip where the cells were adhering, which plays the role of a reservoir for the HEDS reagent and the medium. 5 μL HEDS reagent was dropped in the well and incubated in a humidified CO_2 incubator at 37 °C for 2 h. The amount of mercaptoethanol (ME) produced by bio-reduction of HEDS was estimated via measuring optical density (OD) at 412 nm using a spectrometer (HR4000, Ocean Optics, USA) with a micro-cuvette.

3. Results and discussion

3.1. Monitoring the change in refractive index of cytosol against osmotic stress

We hypothesized that the MCW signals of attached cells caused by osmotic stimulation are directly proportional to their viabilities.

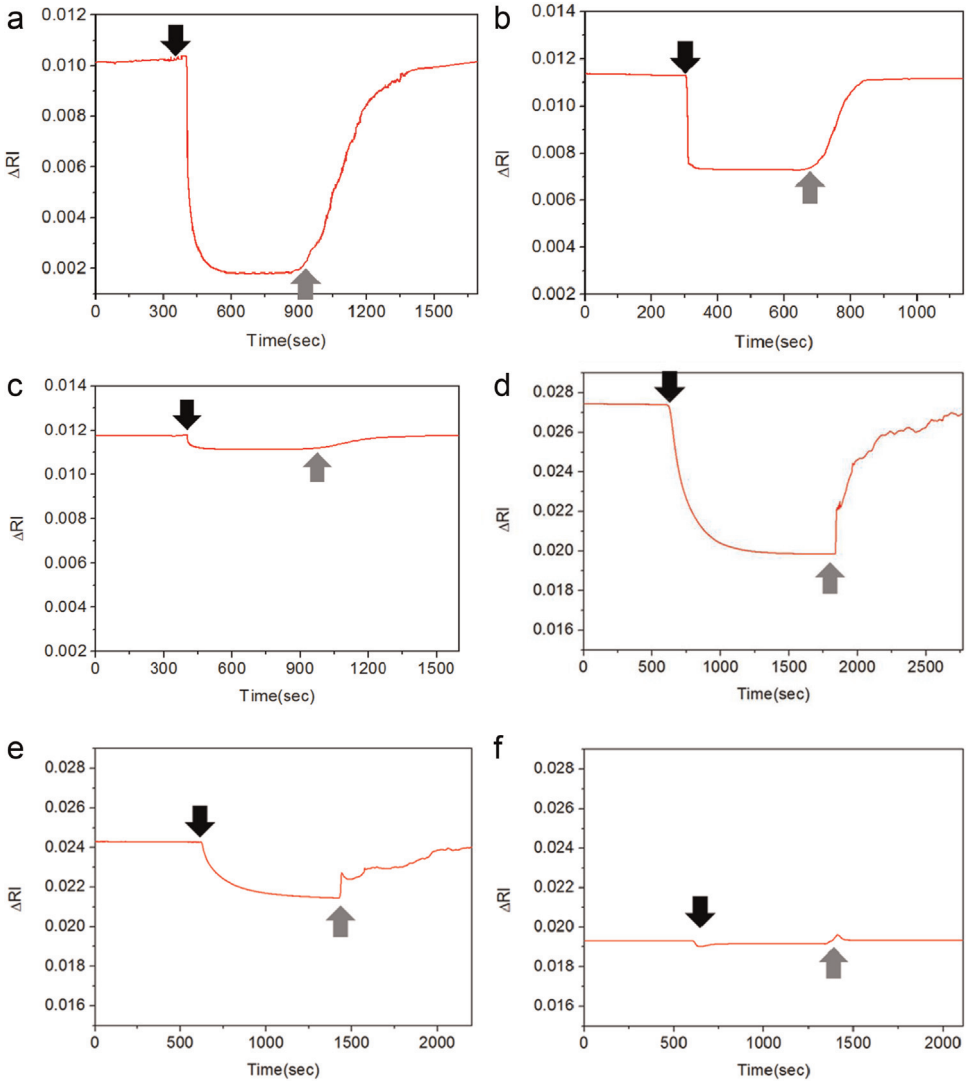

Fig. 2. (a–f) Sensorgrams obtained using the metal-clad waveguide (MCW) sensor curve under hypotonic stimulation to attached cells on sensor chips as the time for which the cells are left alone increases; (a) CHO cells after 6 h on the chip, (b) CHO cells after 15 h, (c) CHO cells after 25 h and (d) HEK293 cells after 6 h, (e) HEK293 cells after 18 h, (f) HEK293 cells after 30 h of being left alone. Black arrow indicates the buffer change from isotonic running buffer (sterilized PBS) to hypotonic buffer ($\Delta = -140$ mOsm/L). Grey arrow indicates the change back to the isotonic buffer; cell images obtained after staining with trypan blue (g) CHO cells after 6 h on the chip, (h) CHO cells after 15 h, (i) CHO cells after 25 h, (j) HEK293 cells after 6 h, (k) HEK293 cells after 18 h, (l) HEK293 cells after 30 h of being left alone in isotonic buffer. The length of scale bar is 100 μm. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

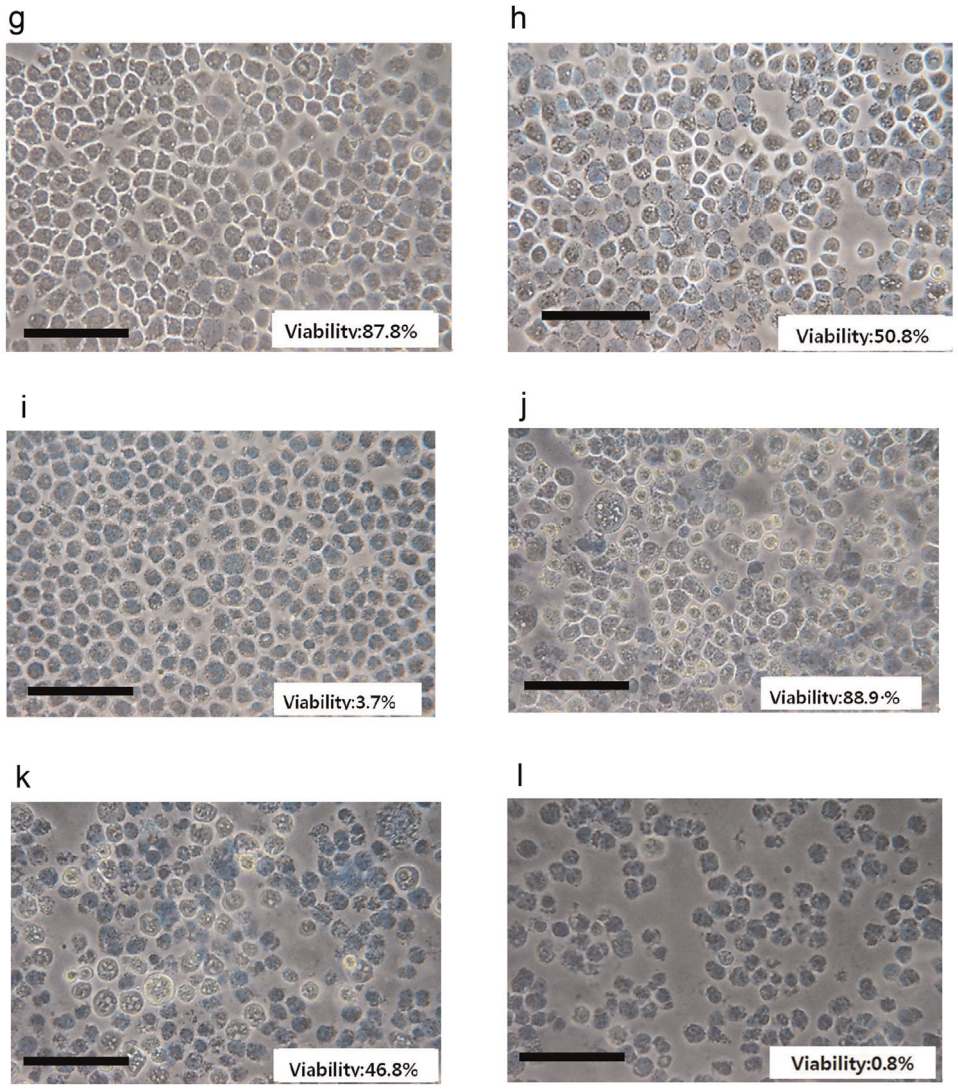

Fig. 2. (*continued*)

To verify our hypothesis, first, it should be confirmed that the recorded MCW signal originate from only a cellular response against an extracellular non-isotonic stimulation, not a simple change in the bulk RI of the buffer. Therefore, we precluded the influence of the bulk RI of buffer via equalizing the RI of the non-isotonic buffer with that of the isotonic one as described in Section S-4 of the Supplementary information.

Before measuring the change in RI of the cytoplasm under osmotic stimulus for the various viabilities of the attached cells, we first investigated the responses of CHO cells to osmotic stress in two extreme cases; all cells are alive and dead as positive and negative controls, respectively, which is described in Section S-5 of the Supplementary information. To quantitatively investigate a correlation between the measured the RI change of cytosol and cell viability, the attached CHO and HEK293 cells with various viabilities were obtained by leaving the cells alone for various amounts of time. Attached cells in a sample channel were exposed to the RI-equalized hypotonic buffers so that the changes in RI of the intracellular fluid were induced by an imbalance in the osmolarity between the cytosol and the environment across the cell membrane. The responses of the cells to low osmotic pressure of the hypotonic buffer were monitored using the MCW sensor system.

Fig. 2(a–f) shows the sensorgrams measured while applying the hypotonic stimulus to the attached cells that were left alone for various amounts of time. The measurements were performed three times for identical durations of leaving the cells alone using different MCW sensor chips with confluent cell layers. As a result, the sensorgrams for the same conditions showed similar aspects, and the representative sensorgrams of each triplicate measurement are illustrated in Fig. 2. For all cells attached to the chips, the RI measured by the MCW sensor dropped abruptly after the beginning of hypotonic stimulation (black arrow) and reached a stable equilibrium after 7–10 min, as anticipated. After changing the buffer back to isotonic PBS (grey arrow), the MCW signal begins to rise and finally reached a value close to the initial level after 5–7 min. Fig. 2(a–c) shows the sensorgrams for the CHO cells and (d–f) shows those for HEK293 cells after leaving the cells for different times. In Fig. 2(a–c) for CHO cells, the relative differences in RI values between the isotonic and hypotonic levels were 8.41×10^{-3} RIU, 4.16×10^{-3} RIU, and 0.62×10^{-3} RIU when the signal was compensated with the response signal from the reference channel. The relative differences in RI values were 7.54×10^{-3} RIU, 3.13×10^{-3} RIU and 0.13×10^{-3} RIU in the plot for the HEK293 cells, respectively (Fig. 2(d–f)). Regardless of cell species, as the time of leaving the cells alone increased, the

magnitudes of the responses decreased, which was probably due to a reduction in the number of cells responding to osmotic stress.

3.2. Correlation between RI changes of cytosol and cell viability

After we observed changes in the RI of the cytoplasm in cells using the MCW sensor, the chips were removed from the prism and the adherent cells were stained by TB to assess the viability of cells. Fig. 2(g–i) and (j–l) show images of the stained CHO cells and HEK293 cells, respectively, on the MCW sensor chips captured using an cell imaging counter. The images were obtained from the same chips used in the measurements of the sensorgrams of Fig. 2 (a–f) in the previous section. The cells stained with blue were nonviable, whereas intact cells were viable. Fig. 2(g) shows an image of CHO cells with the 87.8%, and (h) and (i) show images of CHO cells exhibiting steady drops in viability to 50.8%, and 3.7%, respectively, as time passed. Fig. 2(j–l) shows images of HEK293 cells with viabilities of 88.9%, 46.8%, and 0.8%, respectively, which also represent decreased viability.

Fig. 3 shows the correlation between the decrease in RI of the cytosol due to water diffusion into the cytoplasm and the number of live cells (or cell viability) measured using TB staining and HEDS assay. The net changes in RI for the reference signal as the cell response against hypotonic stimulation exhibited a linearly proportional dependence on the number of live cells attached to the sensor chip (Fig. 3(a)). Because only live cells among attached cells would respond to osmotic stimulus, we were able to expect that the decrease in RI would be linearly proportional to the number of live cells on the chip. However, it was quite interesting that the dependence of the cell response (ΔRI) to the number of live cells was simultaneously represented by an identical linear function for both cells. This probably resulted from that both cells exhibited similar responses to osmotic stress because both of them are mammalian cells, but this should be investigated in depth in the future.

Fig. 3(b) shows the correlation between cell viability, which is the ratio of the number of live cells to the total number of cells on the sensor chip, and changes in the RI under osmotic stress of the hypotonic buffer. As the result of the quantitative analysis with Fig. 3(b), the sensitivity in our detection method of cell viability is 1.0×10^{-4} RIU/% (change in refractive index vs. change in cell viability). The variation of RI without change in osmotic pressure in each channel was below 7.9×10^{-6} RIU (see Section S-4 in the Supplementary information) when the buffer was changed. Therefore, it is expected that the change in cell viability can be discriminated by about 0.079% using the MCW sensor. Fig. 3 (c) shows the result of normalizing the changed values of RI measured using the MCW sensor with the total number of cells attached to the sensor chip, which shows that the normalized changes in the RI represent a dependence with higher linearity than the unnormalized data in Fig. 3(b).

In this study, the pertinence of the MCW sensor as a measuring tool of cell viability was validated once more through investigating the correlation with the RI changes obtained from the MCW sensor and the cell viability estimated using HEDS assay. Viable cells reduce HEDS to mercaptoethanol (ME) in the absence of any toxic effect on cells by intracellular pathways. ME produced inside the cells as a result of HEDS reduction by oxidative pentose phosphate cycle (OPPC) is extruded to the extracellular medium (Ayene et al., 2008; Biaglow et al., 2006). Therefore, live cells can be quantified

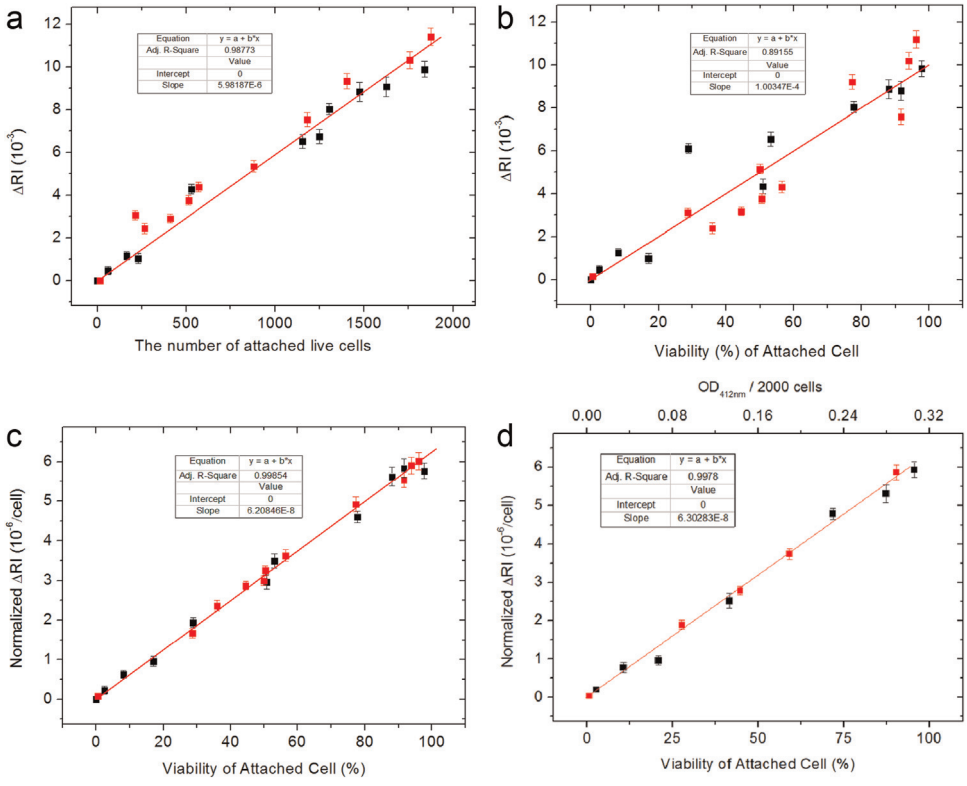

Fig. 3. Plot of dependence of the changes in the relative refractive index measured by the metal-clad waveguide (MCW) sensor (a) on the number of the live cells on the chip and (b) cell viability evaluated using TB staining. (c) Plot of the correlation between cell viability evaluated using TB staining and the normalized change in the refractive index between isotonic level and hypotonic level. (d) Correlation between the cell viability estimated using HEDS and the normalized RI change of the cytosol in the cells under osmotic stress. Upper *x*-axis indicates the optical density at 412 nm, which was calculated in terms of assumption that two thousands of cells were adherent on the detected surface of the sensor chip. Actually, the total number of cells attached to the detected area in the PDMS well was within the range of 1530–2050. Black and red squares represent data for CHO and HEK293 cells, respectively. The measurements were achieved 3-times per one sample in this study and the linear curve fit was iterated up to 50 times in order to minimize error. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

via observing the amount of ME in the extracellular medium (Li et al., 2012). The HEDS reagent was applied to the attached cells in the PDMS well containing the medium (100 μL) after measurement of the changes in the RI of the cytosol in cells using the MCW sensor. After the incubation, the amount of ME in the mixed medium was estimated from the normalized optical density (OD_{norm}) and the cell viability was derived as represented in Section S-6 of the Supplementary information.

Fig. 3(d) compares the normalized change in RI of the cytosol in the cells recorded by the MCW sensor and the cell viability obtained using HEDS assay for the identical cells measured using the MCW sensor. In common with the aspect shown in Fig. 3(c), the normalized RI change of the cytosol under osmotic stress was also linearly proportional to the cell viability measured using HEDS assay.

3.3. *Limitations of monitoring cell viability by cell attachment on the affinity surface*

In this study, we utilized Con A as an affinity surface to ensure contact of cells to the sensor surface. As shown in Fig. S-3 of the supplementary information and a previous report (Ahn et al., 2014), the total number of cells attached to the affinity surface decreased gradually as the viability of cells in the culture medium decreased, which indicates that live cells attach to affinity surfaces more efficiently than dead cells. Therefore, the viability of cells adhering to an affinity surface should be higher than that of cells suspended in original media (Ahn et al., 2014). Because of this reason, the opportunity for fixation of cells on the sensor surface should be equally provided to both live and dead cells via a physical contact due to sedimentation instead of affinity binding if one uses the MCW sensor to measure cell viability. In addition, another limitation of this preliminary study was the need to know the initial total number of cells attached on the sensor surface, which would provide exact estimation of cell viability from changes in the RI of the cytosol. To overcome these limitations, we are currently trying to measure the response of each individual cell to osmotic stimulation after cells physically contact the bottom surface of micro-well array fabricated on the MCW sensor chip via spontaneous sedimentation due to gravity and trapping into micro-wells.

4. Conclusions

We investigated the potential of an MCW sensor for label-free measurement of the viability of animal cells attached to the surface, assisted by osmotic stimulation. It was confirmed that the only live cells attached to the surface exhibited the change in the RI of cytosol caused by the redistribution of water through the cell membrane as a response to osmotic imbalance. The normalized change in the RI of cytosol measured using the MCW sensor was linearly proportional to the viability of the attached cells. Therefore, the viability of the attached cells can be exactly estimated without any labels through monitoring the RI of the cytosol using the MCW sensor after the cells were placed in contact with the sensor surface.

We are currently measuring the response of individual cells after the cells are physically contacted with the bottom surface of micro-well array fabricated on the MCW sensor chip, which will exclude inconsistency in the viability measurements between suspended and attached cells due to the affinity contact.

Acknowledgments

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.007.

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