

## Real-time monitoring of cell death by surface infrared spectroscopy

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We have developed a method for real-time monitoring of the cell responses to cytotoxicants using Fourier transform infrared spectroscopy with the multiple internal reflection (MIR-FTIR) geometry. To prevent cell damages induced by measurement environments, we have constructed specialized chambers, in which temperature was maintained at  $(37 \pm 0.5)$  °C and humidified air containing 5% CO<sub>2</sub> was supplied. We monitored cell death induced by cytotoxic surfactant Tween20 using MIR-FTIR spectroscopy. It was found that cell death can be monitored by the absorption intensity of amide II band. This result suggests that our method has a potential to be applied for real-time cytotoxicity assay. © 2007 American Institute of Physics. [DOI: 10.1063/1.2813013]

During recent years, cell-based assays have attracted much attention for drug discovery, bioprospecting, and environmental assessment of chemicals. Since cellular responses are highly sensitive to trace amount of toxins, cell viability and toxicity assays are particularly important for screening biological active substances.<sup>1,2</sup> Conventional multistep biochemical assays evaluate cellular responses at every scheduled time point after destruction of the cells. On the other hand, several techniques have been reported for real-time monitoring of cytotoxic effects, such as impedance method,<sup>3,4</sup> quartz crystal microbalance,<sup>5</sup> photonic crystal sensor,<sup>6</sup> and Fourier transform infrared (FTIR) absorption spectroscopy.<sup>7</sup>

FTIR spectroscopy has the potential to provide information on multiple analytes without disturbing the biological samples. There is a large body of literatures on cytotoxicity study using FTIR spectroscopy.<sup>7-14</sup> Commonly, cells are measured either as a dehydrated biofilm or as a cell suspension after washing out of the culture medium. In contrast to such *ex situ* approaches, Hutson *et al.* have reported on a technique for *in situ* monitoring of mammalian cell growth using attenuated total reflectance (ATR) FTIR spectroscopy.<sup>7</sup> Chinese hamster ovary cells were attached on germanium (Ge) ATR prisms and cell growth on the prisms was continuously monitored under physiological conditions. However, recent studies have revealed that Ge-containing compounds are toxic to some kind of cells.<sup>15</sup> Therefore, it is desirable to construct safer systems for the development of cell viability and toxicity assays.

In the present study, we report on a method for *in situ* observation of cell death using FTIR spectroscopy in the multiple internal reflection (MIR) geometry with a Si substrate as a MIR prism. MIR-FTIR provides an ideal optical configuration to combine sensitive detection and aqueous-

solution phase measurements. As a model sample, we used human promyelocytic leukemia (HL-60) which are floating cells and do not adhere to a surface. To maintain cell activities, the temperature and humidity in acrylic chambers were maintained at 37 °C and >80%, respectively. After confirming the growth of HL-60 cells in the acrylic chamber, the cells were exposed to cytotoxic surfactant Tween20, and the process to cell death was monitored with MIR-FTIR spectroscopy. We demonstrated that HL-60 cells grow in our FTIR system and the absorption intensity of amide II band is useful for measuring the cell cytotoxicity.

A Si prism with an optical path length of 15 mm was fabricated and used as a MIR prism. Floating zone Si (100) (5250–7050 Ω cm, double side polished, 450 μm in thickness) wafer was anisotropically etched in tetramethylammonium hydroxide at 90 °C to make the structure as that depicted in Fig. 1(a). Before each experiment, the prism was cleaned in 1:1 H<sub>2</sub>O<sub>2</sub>:H<sub>2</sub>SO<sub>4</sub> solution for 5 min. During this process, the surface of Si prism was covered with a chemical oxide layer. The prism was set on the bottom of an acrylic chamber. Infrared light beam from an interferometer (BOMEM MB-100) was focused at normal incidence onto one of the two anisotropic etch pits of the Si prism and penetrated through the Si prism. The light that exited the Si prism through the other bevel was focused onto a liquid-nitrogen cooled mercury-cadmium-telluride detector. HL-60 cells (Riken cell bank) were cultured in RPMI 1640 medium (Gibco), supplemented with 10% heatinactivated fetal bovine serum (Sigma), penicillin (50 IU/ml), and streptomycin (50 μg/ml) (denoted as culture medium) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere in an incubator.

A schematic view of the present FTIR system is shown in Fig. 1(a). To maintain cell activities, the cell culture condition inside the acrylic chamber was controlled to be 37 °C in a humidified 5% CO<sub>2</sub> atmosphere, as in a CO<sub>2</sub> incubator. Since the FTIR sample room needs to be purged with air which was freed from water and carbon dioxide vapor, a

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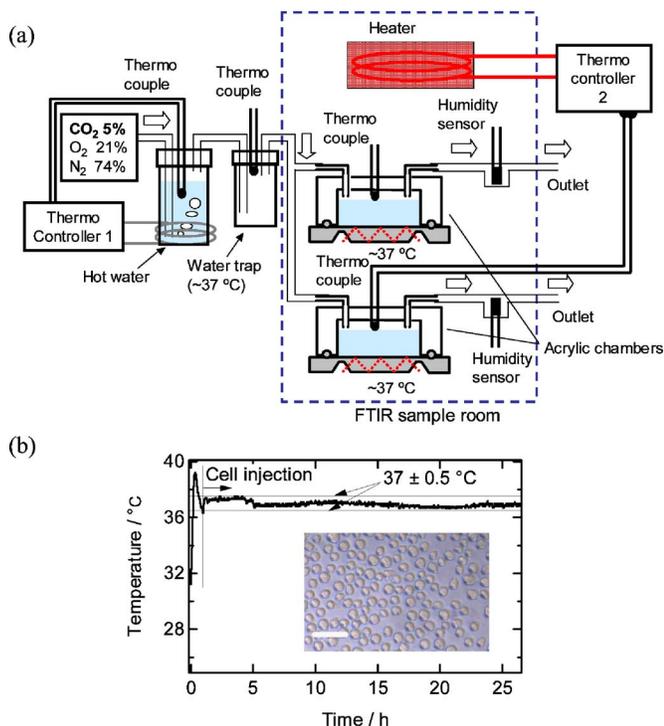


FIG. 1. (Color online) (a) A schematic view of the FTIR sample room which controls temperature and humidity. A gas mixture containing CO<sub>2</sub> was humidified by passing through a hot water bottle via a bubbler and introduced to the acrylic chambers through silicone tubes. The temperature of the water trap and the acrylic chamber was controlled by thermocontroller 1 and 2, respectively. (b) Time course of temperature in the acrylic chamber. The inset figure shows a photomicrograph of HL-60 cells incubated for 24 h in the acrylic chamber placed in the FTIR sample room. Scale bar: 50  $\mu\text{m}$ .

humidified gas mixture containing CO<sub>2</sub> was introduced to the chamber through silicone tubes. A gas mixture containing 5% CO<sub>2</sub>, 21% O<sub>2</sub>, and 74% N<sub>2</sub> was humidified by passing through a hot water bottle via a bubbler. The humidified gas passes through a water trap prior to entry into the FTIR sample room to minimize condensation dropping down into the chamber. The temperature of the water trap was maintained at  $\sim 37^\circ\text{C}$  using a thermocontroller. Another thermocontroller was attached to the chamber. As shown in Fig. 1(b), the temperature in the chamber reached at  $\sim 37^\circ\text{C}$  after  $\sim 50$  min and then kept constant within the error of  $\pm 0.5^\circ\text{C}$  over the whole time range examined. The humidity of the gas mixture in the outlet of the chamber was also monitored with the capacitive humidity sensor (Toplas Engineering Co., Ltd). Prior to introduction of the HL-60 cells into the chamber, the temperature in the chamber was controlled to be  $(37 \pm 0.5)^\circ\text{C}$  and the humidity in the outlet of the chamber to be more than 80%.

Since cell activities and cell cycles vary among different sample batches, for cytotoxic assays, it is preferable to compare IR spectra between drug-treated and control cells measured at the same time under identical conditions. For this purpose, two acrylic chambers were mounted on a movable stage, whose position was controlled automatically by using four-axis stage controller (Sigma Koki, Mark-204) and a computer. Each chamber was driven into the optical path 3 min before each FTIR spectroscopic measurement. All the measurements were performed in a culture medium prepared from H<sub>2</sub>O.

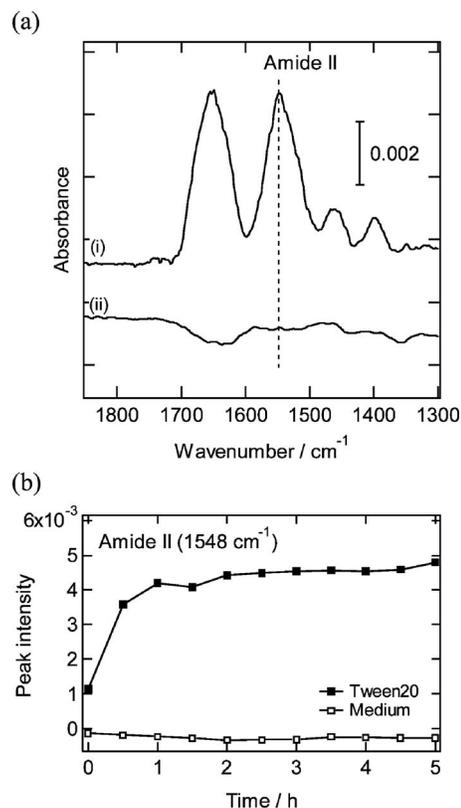


FIG. 2. (a) MIR-FTIR spectra of HL-60 cells obtained 5 h after (i) incubation with 0.2% Tween20 and (ii) incubation with cultured medium as a control experiment. (b) Time courses of peak intensity at  $1548\text{ cm}^{-1}$  (amide II band).

An inset in Fig. 1(b) shows a micrograph of HL-60 cells after 24 h incubation in the acrylic chamber. Nearly all of the cells showed normal morphology with a circular smooth edge and bright shine. Their morphology was similar to that of the viable HL-60 cells in a CO<sub>2</sub> incubator (data not shown). To confirm the cell proliferation, we also counted viable cells by using trypan blue. The number of HL-60 cells increased 2.8-fold [number of measurements ( $n$ ) was 2] during 24 h incubation in the chamber. This growth rate was almost same as that (2.6-fold,  $n=2$ ) of HL-60 cells incubated in a CO<sub>2</sub> incubator. These results suggest that the cells kept in the chamber are viable in terms of morphology and proliferation even after 24 h.

Next, the cytotoxic effect of Tween20 on HL-60 cells was monitored with MIR-FTIR spectroscopy. Tween20 is a surfactant which is often used in cytotoxic test assay for killing all the sample cells.<sup>16</sup> The suspension of HL-60 cell ( $\sim 1.3 \times 10^5$  cells/ml) was transferred to the two acrylic chambers. Then, Tween20 was added to one of the chambers, while the culture medium was added to the other chamber for a control experiment. As shown in Fig. 2(a), the MIR-FTIR spectrum of the cells treated with Tween20 (line i) showed intense peaks centered at  $1655$  and  $1548\text{ cm}^{-1}$ . On the other hand, control cells did not exhibit such peaks [line ii]. Tween20 itself exhibited no absorption peaks in this region (data not shown). The peaks at  $1655$  and  $1548\text{ cm}^{-1}$  were due to the C=O stretching (amide I band) and N—H in-plane bending and C—N stretching (amide II band), respectively.<sup>7,17</sup> It is well known that Tween20 induces destruction of cell membranes to induce cell lysis. Therefore, it is suggested that the observed increase in the peak intensity

of the amide I and amide II bands is due to the leakage of cytoplasmic proteins. Since the strong liquid water absorption overlap the amide I band, variations of liquid water absorption sometimes appeared in the region of amide I band, as shown in the line (ii) of Fig. 2(a). Therefore, the amide II band reflects more accurately the total protein contents of the cells.<sup>7</sup>

Figure 2(b) shows the time course of the peak intensity of the amide II band. When the cells were treated with Tween20, the peak intensity of the amide II band rapidly increased and reached plateau within 1 h. No viable cells were counted after 1 h exposure to Tween20, which is consistent with the results obtained with MIR-FTIR spectroscopic measurements. These results indicate that cell cytotoxicity can be monitored by the absorption intensity of amide II and our method has the potential for application to a real-time cell cytotoxic assay.

In summary, we have developed a real-time method for monitoring cell death by using MIR-FTIR spectroscopy. To maintain cell activities, the temperature and humidity in the acrylic chambers in the FTIR sample room were maintained at 37 °C and >80%, respectively. We have confirmed the cell viability and proliferation of HL-60 cells after 24 h incubation in the chambers. The cytotoxic effect by Tween20 was *in situ* monitored with MIR-FTIR spectroscopy. It was demonstrated that the cell death can be monitored *in situ* by analyzing the amide II peak intensity. Thus, our method can be applied for *in situ* observation of various cellular activities, such as cell proliferation, apoptosis, necrosis, and differentiation.

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