In situ real-time monitoring of apoptosis on leukemia cells by surface infrared spectroscopy

doi: 10.1063/1.3068203

In situ real-time monitoring of apoptosis on leukemia cells by surface infrared spectroscopy

Ryo-taro Yamaguchi,[1] Ayumi Hirano-Iwata,[1,a)] Yasuo Kimura,[1] Michio Niwano,[1] Ko-ichiro Miyamoto,[2] Hiroko Isoda,[3] and Hitoshi Miyazaki[3]
[1]*Laboratory for Nanoelectronics and Spintronics, Research Institute of Electrical Communication, Tohoku University, 2-1-1 Katahira, Sendai, Miyagi 980-8577, Japan*
[2]*Department of Electronic Engineering, Graduate School of Engineering, Tohoku University, 6-6-05 Aza-Aoba, Aramaki, Aoba-ku, Sendai, Miyagi 980-8579, Japan*
[3]*Alliance for Research on North Africa, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan*

(Received 8 August 2008; accepted 4 December 2008; published online 22 January 2009)

We have investigated *in situ* real-time monitoring of apoptosis on human promyelocytic leukemia (HL-60) cells using infrared absorption spectroscopy with the multiple internal reflection (MIR-IRAS) geometry. Actinomycin D (Act D)-induced apoptosis on HL-60 cells was monitored for 24 h. Apoptotic cells showed two strong peaks around the protein amide I and amide II bands probably due to the leakage of cytoplasmic proteins, while growing viable cells showed a peak corresponding to the secretion of metabolites and two downward peaks corresponding to uptake of nutrients from culture media. In addition, IR absorption peak intensity of the amide I and amide II bands was proportional to the extracellular concentration of lactate dehydrogenase, a marker protein for cell damage. These results demonstrate that our MIR-IRAS method is useful for discrimination of apoptotic cells from viable ones and cell apoptotic processes can be monitored *in situ* by analyzing the amide I and amide II peak intensity. © *2009 American Institute of Physics*.
[DOI: 10.1063/1.3068203]

I. INTRODUCTION

During recent years cell-based assays are becoming the major alternative methods to animal testing for preclinical pharmaceutical discovery,[1] as researchers directly study the effects of test chemicals upon a wide variety of cells. There are many conventional multistep biochemical assays for evaluating cytotoxic effects of test chemicals such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test,[2] neutral red uptake,[2] ATP measurement,[3] lactate dehydrogenase (LDH) measurement,[3] or growth assay such as colony forming efficiency.[4] However, most of these methods evaluate cellular responses at every scheduled time point, and do not provide kinetic information on the dynamic interactions occurring between living cells and test chemicals. Moreover, these assays are time consuming and labor intensive, since they often need multiple washing/centrifugation, staining, and destruction of the cells.

Recently, several techniques have been reported for real-time monitoring of cytotoxic effects, such as impedance method,[5,6] quartz crystal microbalance,[7] photonic crystal sensor,[8] and infrared absorption spectroscopy (IRAS).[9,10] IRAS presents several advantages, such as rapidity and non-requirement for staining or probe reagents. In addition, IRAS has potential to provide information on multiple analytes without disturbing the biological samples. However, the strong water absorption requires very short path length in the order of several micrometers. Transmission measurements with thin path length are not suitable for cell cultures, because culturing in such a narrow space may have harmful effects on cell viability. On the other hand, IRAS in the multiple internal reflection (MIR) geometry provides an ideal configuration to combine cell culture condition and aqueous-solution phase measurements. We have recently developed a real-time cell monitoring system by using MIR-IRAS.[9] As a MIR prism, we used silicon (Si) substrate whose surface was covered with biocompatible silicon oxide. The cell activities were maintained by controlling the temperature and humidity in a sample room to be 37 °C and >80%, respectively. By using this system, we have *in situ* monitored a cytotoxic effect of Tween20 on HL-60 (human promyelocytic leukemia) cells. Tween20 is a surfactant known to cause necrotic cell death. Since HL-60 is a floating cell that does not adhere to a surface, Tween20-induced cell death caused a leakage of the cytoplasmic proteins to the extracellular media, leading to an increase in the IR absorption in the protein amide I and amide II band regions.

In this study, we have applied our MIR-IRAS system to long-time (~24 h) continuous monitoring of cell apoptosis. Apoptosis is a genetically regulated process that plays an essential role in the development and maintenance of cell homeostasis.[11] Effects of several anticancer agents are strongly related to induction of apoptosis. There have been several IR spectroscopic studies on apoptosis.[12–16] However, with these methods, cells are measured either as a dehydrated biofilm[12,13,15] or as a cell suspension after washing out of the culture medium.[14,16] In contrast to such *ex situ* approaches, our method enables *in situ* real-time monitoring of cell apo-

[a)]Author to whom correspondence should be addressed. Present address: Graduate School of Biomedical Engineering, Tohoku University, 6-6 Aoba, Aramaki, Aoba-ku, Sendai, Miyagi, 980-8579, Japan. Electronic mail: ahirano@bme.tohoku.ac.jp.

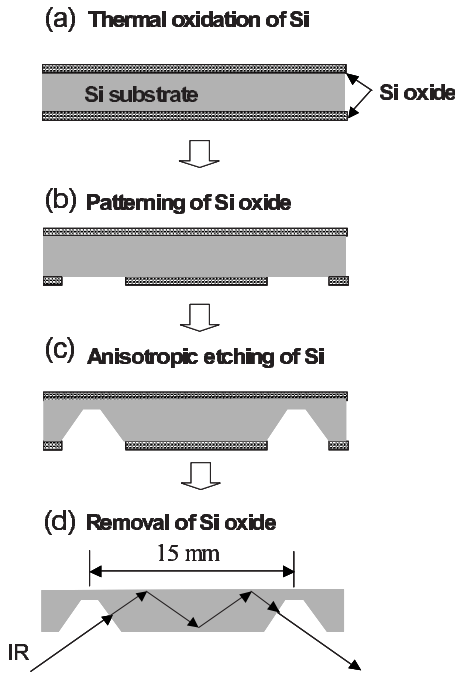

FIG. 1. Procedure for the formation of a short MIR prism. (a) Thermal oxidation of Si wafer, (b) patterning of Si oxide by photolithography, (c) anisotropic etching of Si, and (d) removal of Si oxide. A path for IR light propagation is shown in (d).

ptosis in an aqueous culture medium. We monitored IRAS spectra of HL-60 cells after induction of apoptosis in comparison with viable growing cells. Since MIR-IRAS is intrinsically sensitive to surfaces, which was covered with culture medium rather than the floating HL-60 cells, extracellular changes in relation to cellular process were sensitively detected. Owing to this feature, clear discrimination between the apoptotic and viable cells was achieved by MIR-IRAS. The peak intensity of the amide I and amide II bands was found to be proportionally related to the extracellular concentration of lactate dehydrogenase (LDH), which is known as a cell injury marker. Therefore, it is demonstrated that MIR-IRAS is useful for the *in situ* real-time monitoring of cellular processes including cell growth and apoptotic cell death.

II. MATERIALS AND METHODS

A. Fabrication of short silicon prisms

A Si prism with an optical path length of 15 mm was fabricated and used as a MIR substrate, since longer optical path length results in complete absorption of the IR radiation below 1500 cm^{-1}.[17,18] Figure 1 shows the procedure for the fabrication of the Si prisms. FZ Si (100) (5250–7050 Ω cm, double side polished, 450 μm in thickness) wafers were first thermally oxidized. The thickness of the Si oxide layer was about 1 μm. Then, the Si oxide layer was photolithographically patterned, followed by anisotropic etching of Si surfaces in 25% tetramethylammonium hydroxide solution under constant stirring at 90 °C.[19] It is well known that aqueous bases etch Si (111) face much more slowly than other faces. As a result, etch pits with 54.7° bevels were generated. The Si oxide layer was used as the masking layer

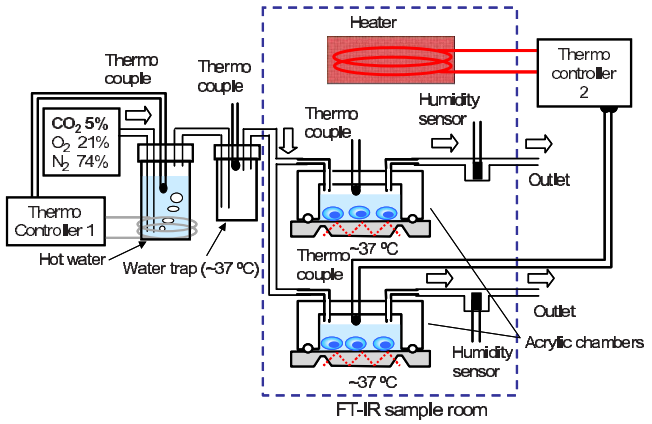

FIG. 2. (Color online) A schematic of the MIR-IRAS measurement system which controls temperature and humidity. A gas mixture containing CO_2 was humidified by passing through a hot water bottle via a bubbler and introduced to the acrylic chambers through silicone tubes. The temperature of the water trap and the chamber was controlled by thermo controller 1 and 2, respectively.

of Si anisotropic etching. After etching, the Si oxide layer was removed by a 5 % hydrofluoric acid solution. The thickness of locally thinned Si layers was about 50 μm. Before each experiment the prism was cleaned in 1:1 mixture of H_2O_2 and H_2SO_4 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. An O-ring made of polydimetylsiloxane (PDMS), which is biocompatible and commonly used for laboratory-on-a-chip devices,[20] was used for sealing between the acrylic chamber and Si prism. To avoid absorption of the IR radiation by PDMS, the O-ring was placed outside the optical path, as shown in Fig. 2.

B. Temperature and humidity control system

A schematic of the present MIR-IRAS measurement system is shown in Fig. 2. To maintain cell activities, the cell culture condition inside the acrylic chamber was controlled to be 37 °C in a humidified 5% CO_2 atmosphere. Since the sample room in the Fourier transform infrared (FTIR) spectrometer needs to be purged with air which was freed from water and carbon dioxide vapor, a humidified gas mixture containing CO_2 was introduced to the chamber through silicone tubes. A gas mixture containing 5% CO_2, 21% O_2, and 74% N_2, 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 ~37 °C using a thermocontroller. Another thermocontroller was attached to the chamber. 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 ± 0.5 °C and the humidity in the outlet of the chamber to be more than 80%. We have previously shown that the HL-60 cells kept in this MIR-IRAS measurement system are viable in terms of morphology and proliferation even after 24 h incubation.[9]

Since cell activities and cell cycles vary among different sample batches, it is preferable to compare IR spectra between apoptotic cells and control cells measured at the same time under the identical condition. For this purpose, two sample chambers were mounted on two movable stages, whose positions were 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 IRAS measurement.

C. Cell cultures and FTIR spectroscopy

HL-60 cells were purchased from the Riken cell bank and were cultured in RPMI 1640 medium (Gibco), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma), penicillin (50 IU/ml), and streptomycin (50 μg/ml) (denoted as culture medium) at 37 °C in a humidified 5% CO_2 atmosphere in an incubator. Cells were seeded at 5×10^4 cells/ml in T75 flasks and incubated in a CO_2 incubator for 24 h. The viable cells were counted by using trypan blue (Gibco). Then 1200 μl portions of the HL-60 cell suspension ($\sim1.5\times10^5$ cells/ml) were transferred to the acrylic chambers, which were placed in a FTIR sample room, as depicted in Fig. 2. A reference spectrum of each chamber was collected at 30 min after the addition of the cell suspension in the chamber. Then, apoptosis inducer was added to the cell suspension and infrared spectra of the cell suspension were collected at an interval of 30 min. 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. In the wavelength region of amide I and II bands (~6 μm), the depth of evanescent field penetration,[21] defined as the distance required for electric field amplitude to fall to e^{-1} of its value at the surface, is calculated to be ~0.38 μm when we assume that incident angle is 54.7° and refractive indices of Si and water are 3.5 and 1.3, respectively. The light that exited Si prism through the other bevel was focused onto a liquid-nitrogen cooled mercury-cadmium-telluride detector. All the measurements were performed in a culture medium prepared from H_2O. The water vapor peaks were removed by subtracting water vapor spectra which were obtained in blank experiments, yielding the final spectra for the HL-60 cells.

D. Apoptosis induction

Apoptosis was induced by 17 μM Actinomycin D (Act D, Wako or Sigma). Act D treatment is known to induce apoptosis in HL-60 cells.[22–24] Stock solutions of Act D were prepared at a concentration of 850 μM in ethanol, and a 24 μl portion was added to the acrylic chamber to give a concentration of 17 μM.

E. LDH assay

The cell injury of HL-60 was also quantified by using a LDH-cytotoxic test kit (Wako). LDH is a cytosolic enzyme present within all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid.[25] After the MIR-IRAS measurements, a 100 μl aliquot of the culture supernatant was mixed with a 100 μl aliquot of coloring solution [nitrotetrazolium blue (3.7 mg/vial), diaphorase and reduced nicotinamide adeninde dinucleotide (NADH) dissolved in 50 g/l DL-lithium lactate] and kept for 45 min at room temperature. The reaction was terminated by adding a 200 μl portion of 0.5*M* HCl into the mixture. The absorbance at 560 nm of the reaction mixture was measured with a UV/visible spectrometer (Hitachi U-1800).

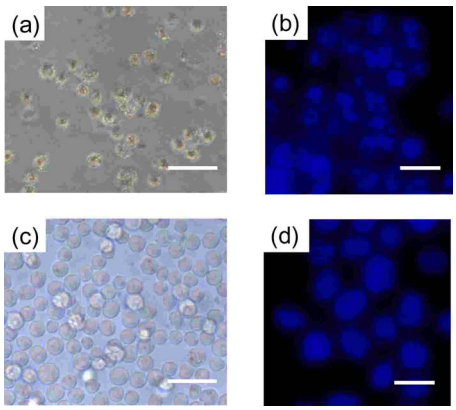

FIG. 3. (Color online) Microscopic images of apoptotic [(a) and (b)] and control [(c) and (d)] HL-60 cells after 24 h incubation in the FTIR sample room. [(a) and (c)] Phase contrast images. [(b) and (d)] Fluorescent images after stained with Hoechst 33342. Scale bars: 50 μm for the phase contrast images and 20 μm for the fluorescent images. Apoptosis was induced by treating with 17 μM Act D.

F. Hoechst assay

HL-60 cells after the MIR-IRAS measurement were fixed in 4% paraformaldehyde overnight. The treated cells were washed twice with PBS and stained with 12.5 μg/ml Hoechst 33342 (Wako) in PBS for 10 min. The sample cells were examined with a fluorescent microscope (Leica DM IRBE M2FL III) with an ultraviolet filter.

III. RESULTS AND DISCUSSIONS

A. Apoptosis induction

Figure 3 shows the phase contrast and fluorescent micrographs obtained from HL-60 cells treated and untreated (control) with Act D for 24 h in the acrylic chambers in the FTIR sample room. Nearly all of the control HL-60 cells [Figs. 3(c) and 3(d)] showed normal morphology similar to that of the viable HL-60 cells in a CO_2 incubator (data not shown). As described in our previous paper, HL-60 cells in the FTIR sample room showed almost same growth rate as those in a CO_2 incubator,[9] confirming the viability of the HL-60 cells kept in our sample room. On the other hand, most of the HL-60 cells treated with Act D [Figs. 3(a) and 3(b)] showed cytoplasmic shrinkage, membrane blebbing, and chromatin condensation, which are characteristic of apoptotic cells. More than 95% of the cells were dead 24 h after incubation with Act D, indicating apoptotic cell death.

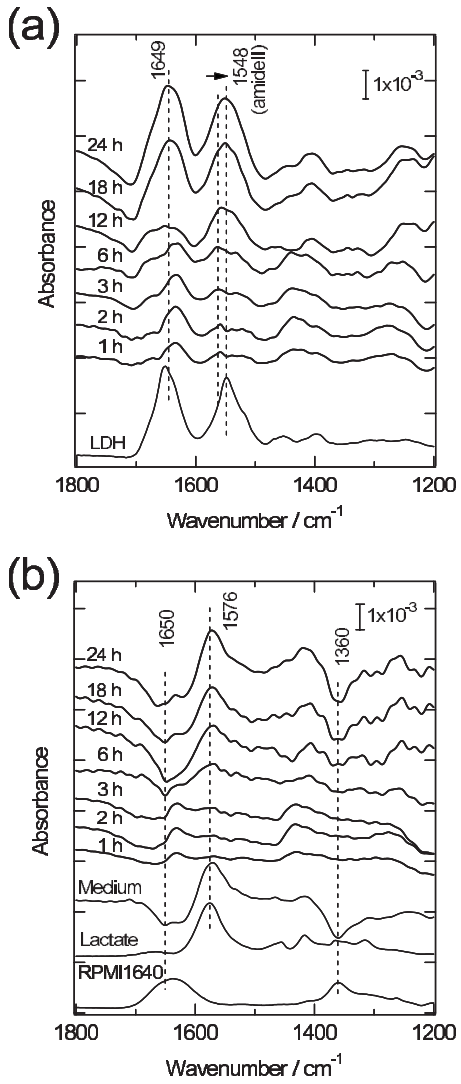

FIG. 4. IR spectral changes of (a) apoptotic and (b) control HL-60 cells during 24 h in the FTIR sample chamber. The reference was the spectrum recorded 30 min after the cells were placed in the FTIR sample room. IR spectral changes for the culture medium during 24 h incubation were subtracted. IRAS spectra of (a) 4000 U/ml LDH and (b) 50 mg/ml DL-lithium lactate and RPMI1640 are also shown. The reference for these spectra was the spectrum of pure water. (b) also shows a difference spectrum of the culture medium containing RPMI1640, FBS, penicillin, and streptomycin before and after incubated with HL-60 cells for 48 h in a CO_2 incubator. IR spectral changes for the medium during 48 h incubation were subtracted from the raw spectrum.

B. IR spectral changes due to apoptosis induction

Figure 4 shows IR spectra of HL-60 cells treated with Act D, together with those of control cells kept in the FTIR sample room. Reference was the spectrum collected 30 min after the cells were placed in the FTIR sample room. IR spectral changes in the culture medium containing FBS during incubation were subtracted from the raw spectra. It is expected that the absorption of serum proteins in FBS to the Si surface reaches the steady state during the 30 min waiting, since rapid adsorption of serum proteins to solid surfaces has been reported.[26,27] Furthermore, by subtracting the spectral change of the culture medium during the incubation period, the effect of serum protein adsorption on the IRAS peak height was minimized. 1 h after addition of Act D, the IR spectrum of the cells treated with Act D showed two peaks around the protein amide I (1630–1650 cm^{-1}) and amide II (1550–1570 cm^{-1}) bands. The intensity of both bands increased with time, as shown in Fig. 4(a). These peaks were assigned to the C=O stretching (amide I) and N–H in-plane bending and C–N stretching modes (amide II). The time-dependent increase in the peak intensity is considered as the gradual leakage of cytoplasmic proteins from dead cells. Cytosolic enzyme LDH is a most probable candidate for such proteins, because LDH is known as a marker for cell damage and is released into extracellular space when the cell membranes are damaged.[25] The IR spectrum of LDH [Fig. 4(a), the bottom spectrum] showed similar profiles to those of the late apoptotic cells (18 and 24 h). The release of LDH in the extracellular medium was confirmed by detection with LDH-cytotoxic test kit. It is suggested that cytosolic proteins including LDH are released from the apoptotic dead cells, leading to IRAS signals of the amide I and amide II bands.

In contrast to the apoptotic cells, control cells exhibited a peak at 1576 cm^{-1} and two downward peaks at 1650 and 1360 cm^{-1}, which were more pronounced for late incubation period (12–24 h), as shown in Fig. 4(b). These spectral features are clearly different from those obtained with apoptotic dead cells. To interpret the spectra, we measured IR spectral change for extracellular culture medium after incubation with HL-60 cells [Fig. 4(b)]. The spectrum thus obtained showed similar profiles to those of the control cells for the late incubation period, indicating that the IR spectrum observed with the control cells reflects composition changes in extracellular medium during the cell growth. We then measured IR spectra of culture medium and lactate, which is a major cellular metabolite. Lactate showed a strong absorption peak at 1576 cm^{-1} [Fig. 4(b)], which corresponds to the carboxyl group. The culture medium (RPMI1640), before addition of FBS and penicillin, showed strong absorption bands at 1640 and 1360 cm^{-1} [Fig. 4(b)], possibly due to nutrients, such as amino acids and glucose. When the spectra of the control cells for the late incubation period were compared to those of lactate and the culture medium, the positive band with the control cells was similar to the absorption band of lactate, and the positions of two downward bands were close to the absorption bands of the culture medium (RPMI1640). These results suggest that the IR spectral changes observed with the control cells reflect the compositional changes in extracellular medium due to the excretion of lactate and consumption of nutrients. Such spectral changes were clearly different from those for apoptotic dead cells which reflect leakage of cytoplasmic proteins including LDH.

In the case of the cells treated with Act D, the amide II band shifted from 1564 to 1548 cm^{-1} during apoptosis induction. Similar peak shift was also observed with the amide I band, however, the shift was not further discussed due to strong water absorption around the amide I band. The red-shift in the amide II band was probably due to the gradual process of the apoptotic cell death. In the early incubation period, most of the cells are alive, excreting metabolites such as lactate. Then the number of apoptotic dead cells gradually increased due to the effect of Act D, releasing cytoplasmic

protein, such as LDH. On the other hand, secretion of lactate was decreasing. Therefore, the observed redshift probably reflects the gradual decrease in secretion of lactate together with the gradual increase in LDH release. These results demonstrate that dynamic processes during apoptosis can be also analyzed with MIR-IRAS through monitoring of compositional changes in the extracellular media.

There have been many reports on the measurements of apoptosis by IR spectroscopy.[12–16] However, since they measured IR spectra for intracellular components of the target cells, it was difficult to discriminate the subtle difference between apoptotic and control cells. On the other hand, our MIR-IRAS method can detect the compositional changes in extracellular medium rather than those inside the cells, leading to clear discrimination between the apoptotic and control cells: the apoptotic cells exhibit amide I and amide II bands due to the leakage of cytoplasmic proteins, while the viable cells exhibit IR signals corresponding to secretion of cell metabolites and uptake of nutrients. Furthermore, MIR-IRAS does not require any pretreatments, such as drying, resuspension,[12,13,15,16] and fractionation with apoptosis-detecting reagents,[14] which were commonly required for the other IRAS approaches. This feature is advantageous especially for *in situ* and real-time monitoring of cellular processes.

C. Time course

To further explore the relationship between IR spectral changes and LDH release into the extracellular media, the time courses of peak intensity at 1548 cm^{-1} (amide II) and 1650 cm^{-1} (amide I) were compared to that of LDH concentration in the extracellular media. The time course of LDH concentration was measured in separate experiments in which apoptosis was induced to HL-60 cells in multiwell plates placed in a CO_2 incubator. The IR peak intensity and LDH concentration was normalized to those observed 24 h after apoptosis induction. When the HL-60 cells were treated with Act D, the peak intensity at 1548 cm^{-1} continuously increased with time, while only slight increase in the peak intensity was observed with control cells [Fig. 5(a)]. These time courses are similar to those of LDH concentration for both the Act D-treated and control cells [Fig. 5(c)], indicating that the observed time course of the peak intensity at 1548 cm^{-1} reflects the released amount of LDH, a marker protein for cell injury. Similar time-dependent increase in the peak intensity at 1650 cm^{-1} was also observed for the Act D-treated cells, while a gradual decrease in the peak intensity was observed for the control cells [Fig. 5(b)]. The time-dependent decrease in the peak intensity at 1650 cm^{-1} with the control cells was probably due to the consumption of nutrients during cell growth. These results suggest that the peak intensity at both the amide I and amide II bands can be used as a measure for monitoring cell damage and/or cell death. Although the amide I signals were slightly noisier than the amide II ones, possibly due to the water absorption around 1650 cm^{-1}, the opposite spectral changes between the apoptotic and control cells is useful for discriminating them.

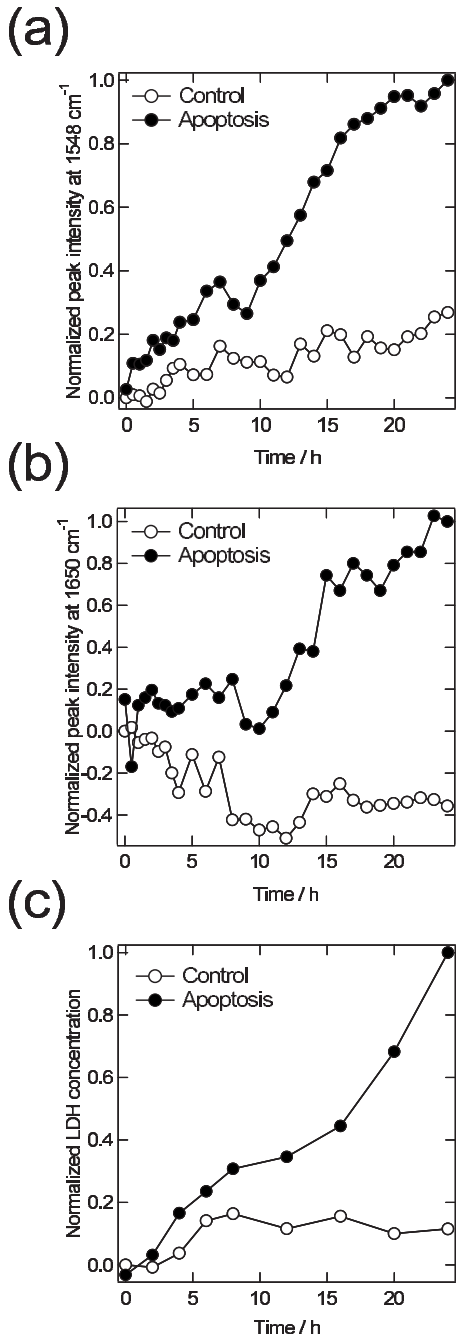

FIG. 5. Time courses of the peak intensity at (a) 1548 cm^{-1} (amide II) and (b) 1650 cm^{-1} (amide I). Typical time courses obtained from one experimental pair of apoptotic and control cells are shown. (c) Time course of LDH concentration in extracellular media. The peak intensity and LDH concentration was normalized to those observed 24 h after apoptosis induction. In the LDH assay experiment, cell suspension was prepared in a same manner as the MIR-IRAS experiment and transferred to a multiwell cell culture plate in a CO_2 incubator. Cell apoptosis was induced by 17 μM Act D and extracellular LDH concentration at given time points was determined using LDH-cytotoxic test kit. Each data point represents average of three measurements.

D. Relationship between the peak height at the amide bands and cell death

Next, we examined a relationship between the peak height of amide bands and extracellular concentration of cell injury/death marker LDH by incubating HL-60 cells at various conditions inducing cell death. LDH assay was per-

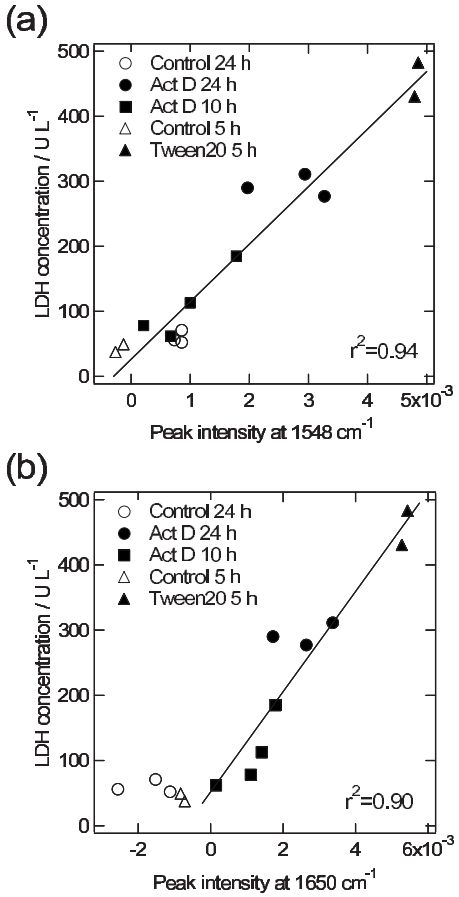

FIG. 6. Statistical correlation between the IR peak intensity at (a) 1548 cm^{-1} and (b) 1650 cm^{-1} and the extracellular LDH concentration. Cell death was induced either by 17 μM Act D or 0.2% (v/v) Tween20. HL-60 cells were treated with Act D for 10 h (■, $n=4$) or 24 h (●, $n=3$), or Tween20 for 5 h (▲, $n=2$). Control cells were kept for 5 h (△, $n=2$) or 24 h (●, $n=3$) without adding any reagents.

formed for the extracellular medium taken from the cell samples after MIR-IRAS experiments and the LDH concentration was plotted against the peak height of the amide I and amide II bands (Fig. 6). Data obtained from necrotic cells treated with 0.2 % (v/v) Tween20 were also included. As shown in Fig. 6(a), a proportional relationship was observed ($R^2=0.94$) between the peak height of the amide II band and the extracellular LDH concentration for all the cells examined, including apoptotic, necrotic, and control cells. This result suggests that the peak height at 1548 cm^{-1} can be a measure for evaluating cell death as well as the amount of LDH release. However, owing to the overlap of the amide II band with the absorption due to metabolites, it was difficult for the amide II band alone to discriminate between the apoptotic cells and control cells incubated for long hours.

We then examined a relationship between the peak height at 1650 cm^{-1} (amide I) and the extracellular LDH concentration [Fig. 6(b)]. A proportional relationship was also observed ($R^2=0.90$) between the peak height of the amide I band and LDH concentration for the apoptotic and necrotic cells. Of note is that plots for the control cells deviated from the proportional line due to the downward peak around 1650 cm^{-1}. These results indicate that the amide I peak can be used as a measure for discriminating the apoptotic and viable ones.

In IR spectroscopic analysis of biological substances in aqueous-solution samples, the peak height of the amide II band rather than that of the amide I band has been commonly used due to the strong water absorption in the amide I region.[10,28] On the other hand, the present results showed that both the peak height of the amide I and amide II bands exhibited a good linearity to the extracellular concentration of LDH, suggesting that both peaks can be a measure for cell damage/death, if the correction for the presence of water was carried out appropriately. This discussion is also supported by our previous MIR-IRAS study on protein-protein interactions in aqueous solutions, in which we demonstrated that the amide I signal was as reliable as the amide II signal for the quantitative analysis of proteins.[29]

Apoptosis has been detected by several assays that analyze distinct biochemical events during apoptosis, such as fragmentation of genomic DNA,[30] externalization of phosphatidylserine,[31] and release of LDH in the extracellular medium.[32] In contrast to these methods, which need multiple washing/centrifugation, staining, and destruction of the cells, MIR-IRAS does not require any pretreatments and is suitable for real-time and *in situ* monitoring of cell apoptosis. Although our method is not feasible for detecting distinct biochemical compounds, IRAS spectra can provide global insight into apoptotic processes. Therefore, MIR-IRAS is a label-free monitoring method for apoptosis, which is complementary to the conventional biochemical assays.

IV. CONCLUSION

We have investigated cell apoptosis on HL-60 cells using MIR-IRAS. It was revealed that apoptotic cell death caused release of cytoplasmic proteins such as LDH in the extracellular medium, leading to an increase in the peak intensity at the amide I and amide II regions. On the other hand, control viable cells showed completely different spectral features from the apoptotic cells. The peak intensity of both the amide I and amide II bands was positively correlated with the extracellular LDH concentrations, demonstrating the usefulness of these bands as a measure for the cell damage/death. Although we investigated floating HL-60 cells as an illustrating example, MIR-IRAS can be applicable to monitoring other cells including adherent cells. Investigation on IRAS monitoring of apoptosis in adherent cells is now in progress. Thus, the MIR-IRAS is useful as a real-time monitoring method for cellular processes without using any external reagents which may cause a potential perturbation to the cells.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Basic Scientific Research (Grant no. 20246008) from the Ministry of Education, Science, Sports, and Culture of Japan.

[1]F. Zucco, I. De Angelis, E. Testai, and A. Stammati, Toxicol. In Vitro **18**, 153 (2004).
[2]E. Borenfreund, H. Babich, and N. Martin-Alguacil, Toxicol. In Vitro **2**, 1 (1988).
[3]T. Riss, M. O'Brien, and R. Moravec, Cell notes **6**, 6 (2003).
[4]M. D. Shah, A. G. Ramchandani, M. B. Mahimkar, P. D. Potdar, A. N.

Bhisey, and R. A. Bhisey, Toxicol. Lett. **119**, 1 (2001).
[5]J. Z. Xing, L. Zhu, J. A. Jackson, S. Gabos, X.-J. Sun, X. Wang, and X. Xu, Chem. Res. Toxicol. **18**, 154 (2005).
[6]J. H. Yeon and J.-K. Park, Anal. Biochem. **341**, 308 (2005).
[7]S. J. Braunhut, D. McIntosh, E. Vorotnikova, T. Zhou, and K. A. Marx, Assay Drug Dev. Technol. **3**, 77 (2005).
[8]L. L. Chan, S. L. Gosangari, K. L. Watkin, and B. T. Cunningham, Apoptosis **12**, 1061 (2007).
[9]R. Yamaguchi, A. Hirano-Iwata, Y. Kimura, M. Niwano, K. Miyamoto, H. Isoda, and H. Miyazaki, Appl. Phys. Lett. **91**, 203902 (2007).
[10]T. B. Hutson, M. L. Mitchell, J. T. Keller, D. J. Long, and M. J. W. Chang, Anal. Biochem. **174**, 415 (1988).
[11]M. D. Jacobson, M. Weil, and M. C. Raff, Cell **88**, 347 (1997).
[12]J. Zhou, Z. Wang, S. Sun, M. Liu, and H. Zhang, Biotechnol. Appl. Biochem. **33**, 127 (2001).
[13]K.-Z. Liu, L. Jia, S. M. Kelsey, A. C. Newland, and H. H. Mantsch, Apoptosis **6**, 269 (2001).
[14]N. Jamin, L. Miller, J. Moncuit, W.-H. Fridman, P. Dumas, and J.-L. Teillaud, Biopolymers **72**, 366 (2003).
[15]F. Gasparri and M. Muzio, Biochem. J. **369**, 239 (2003).
[16]S. Gaudenzi, D. Pozzi, P. Toro, I. Silvestri, S. Morrone, and A. C. Castellano, Spectroscopy (Amsterdam) **18**, 415 (2004).
[17]M. K. Weldon, B. B. Stefanov, K. Raghavachari, and Y. J. Chabal, Phys. Rev. Lett. **79**, 2851 (1997).
[18]R. Herzig-Marx, K. T. Queeney, R. J. Jackman, M. A. Schmidt, and K. F. Jensen, Anal. Chem. **76**, 6476 (2004).
[19]O. Tabata, R. Asahi, H. Funabashi, K. Shimaoka, and S. Sugiyama, Sens. Actuators, A **34**, 51 (1992).
[20]F. Wolbers, P. Braak, S. L. Gac, R. Luttge, H. Andersson, I. Vermes, and A. Berg, Electrophoresis **27**, 5073 (2006).
[21]N. J. Harrick, *Internal Reflection Spectroscopy* (Harrick Scientific Corporation, New York, 1987).
[22]D. Shiokawa, H. Maruta, and S. Tanuma, FEBS Lett. **413**, 99 (1997).
[23]S. Verhaegen, A. J. Mcgowan, A. R. Brophy, R. S. Fernandes, and T. G. Cotter, Biochem. Pharmacol. **50**, 1021 (1995).
[24]H.-S. Huang and E. Y. C. Lee, J. Biol. Chem. **283**, 18135 (2008).
[25]T. Rae, J. Biomed. Mater. Res. **11**, 839 (1977).
[26]S. J. McClellan and E. I. Franses, Colloids Surf., A **260**, 265 (2005).
[27]Y. Arima and H. Iwata, J. Mater. Chem. **17**, 4079 (2007).
[28]G. Reiter, N. Hassler, V. Weber, D. Falkenhagen, and U. P. Fringeli, Biochim. Biophys. Acta **1699**, 253 (2004).
[29]K. Onodera, A. Hirano-Iwata, K. Miyamoto, Y. Kimura, M. Kataoka, Y. Shinohara, and M. Niwano, Langmuir **23**, 12287 (2007).
[30]P. Huang and W. Plunkett, Anal. Biochem. **207**, 163 (1992).
[31]I. Vermes, C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger, J. Immunol. Methods **184**, 39 (1995).
[32]M. Verghese, D. R. Rao, C. B. Chawan, L. T. Walker, and L. Shackelford, LWT-Food Science and Technology **39**, 1093 (2006).