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ORIGINAL PAPER

In situ observation of a cell adhesion and metabolism using surface infrared spectroscopy

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Abstract In this study, we report on an in situ monitoring system of living cultured cells using infrared absorption spectroscopy in the geometry of multiple internal reflections (MIR-IRAS). In order to observe living cultured cells, the temperature in the sample chamber of a FT-IR spectrometer was maintained at 37 °C and a humidified gas mixture containing 5% CO_2 was introduced into the sample chamber. Human breast cell line MCF-7 cultured on Si MIR prisms were placed in the sample chamber and infrared spectra of MCF-7 cells were collected for 5 h. It was found that the adhesion and metabolism of MCF-7 cells could be monitored by the absorption intensity of amide-II protein band (1,545 cm^{-1}) and also by the absorption intensities of CH_x bands (2,700–3,100 cm^{-1}). These results suggest that our system is useful for a nondestructive and non-label monitoring of cell viability. Our method based on infrared absorption spectroscopy has a potential for bioscreening application.

Keywords Human breast cell line MCF-7 · Si prisms · Infrared absorption spectroscopy · Cell metabolism · In situ observation

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Introduction

Bioassay is the search for new chemicals in living matter and an effective method to discover a new drug. Many kinds of new biological active substances have been found from a biological resource. It is necessary to shorten the screening time because most of bioprospecting research activity is a race against time. The bioassay using cultured cell has been developed to screen for kinds of the biological active substances and their influences (Isoda et al. 2002; Han et al. 2002; Talorete et al. 2002).

In our previous report, we have successfully determined genetically modified maize, and distinguished between genotypes of rice varieties using infrared absorption spectroscopy (IRAS) (Emura et al. 2006). And, recently we proposed an in situ monitoring of biomolecular interactions using IRAS (Miyamoto et al. 2005, 2006). High surface

sensitivity was achieved using the multiple internal reflections IRAS (MIR-IRAS) geometry using a small Si prism, with MIR-IRAS particularly, which is well suited for observing interactions or conformational changes of such biomolecules in aqueous environment. Thus MIR-IRAS can be a powerful tool for the primary screening of bioassay. Infrared absorption spectrum provides various information on conformation or structure of biomolecules and it may attend to the needs of multifactorial screening.

On the other hand, there is a large body of literatures about IRAS measurement of biomolecules: DNA, protein, tissue sample, bacteria sample and cultured cells (Mantsch and Chapman 1996; Gremlich and Yan 2001; Hutson et al. 1988; Miyazawa and Blout 1961). Hutson et al. (1988) have reported on a technique for monitoring cell growth of Chinese hamster ovary cells using attenuated total reflection (ATR) FT-IR measurement, however, general ATR method needs expensive ATR crystal made of germanium or zinc selenide and a size of the crystal is much larger than our MIR-IRAS prism. Then, MIR-IRAS prism made of Si has advantages in terms of cost and size. And a surface stability of Si prism covered with chemical oxide layer should be another advantage.

In this study, we report on a method for in situ observation of cell adhesion and metabolism using MIR-IRAS. We maintained the environment of a sample chamber of an infrared spectrometer to be the same as a general cell culture and succeeded in in situ observation of the cell adhesion and metabolism on the Si prism. The result of the MIR-IRAS measurement shows spectral enhancements at amide-I, amide-II and CH_x absorption bands due to the cell adhesion and metabolism.

Materials and methods

Cell and cell culture

The Human Breast Tumour Cells (MCF-7, RCB No.1904) were purchased from the Riken cell bank, Japan. The cells were cultured in RPMI 1640 medium, supplemented with 10% heat inactivated FBS, penicillin (50 IU/mL) and streptomycin (50 μg/mL). The cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in an incubator.

Materials

RPMI 1640 medium (Gibco), FBS (Sigma), streptomycin–penicillin solution (MP Biomedicals Inc.), 25% gultaraldehyde solution (Wako), paraformaldehyde (Wako), 4% osmium tetraoxide solution (Heraeus), 1 M HEPES buffer (pH 7.0–7.6, Sigma).

Infrared spectroscopy

Figure 1a illustrates the equipment made of Teflon® we used in this study for MIR-IRAS measurements. This equipment was also used in our past studies (Miyamoto et al. 2005, 2006). The volume of the sample solution was 100–200 μL. A homemade Si prism was 0.5 × 10 × 30 mm^3 with 45° bevels on each of the short edges, and contacts with the sample solution. An infrared light beam from an interferometer (BOMEM MB-100) was focused at normal incidence onto one of the two bevels of the Si prism, and penetrated through the Si prism, internally reflecting about 60 times. The light that propagated

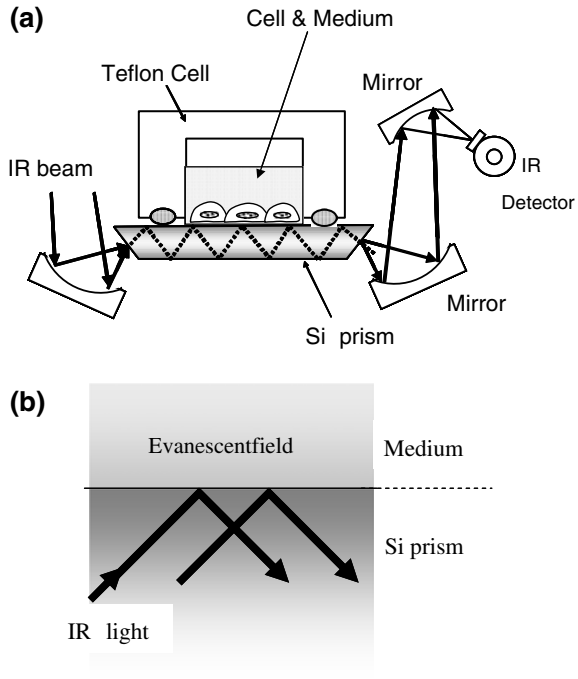

Fig. 1 (**a**) Experimental setup of MIR-IRAS measurement and (**b**) schematic illustration of evanescent field on the Si prism surface. Evanescent field of infrared light penetrates about 500 nm deep into the medium solution

the Si prism through the other bevel was focused onto a liquid-nitrogen cooled Mercury–Cadmium–Telluride (MCT) detector. The internal reflections excited an evanescent field on the Si prism surface (Fig. 1b), and the chemical species of the biomolecules inside the evanescent field influenced the propagated light.

To observe the living cultured cell, we constructed an environment control system. A schematic view of the environment control system is shown in Fig. 2a. First, we mounted a homemade temperature controller (a heater, a small fan and thermo controller 1) to the infrared spectrometer described above. Second, a mixed gas containing 5% of CO_2 gas was introduced into MIR-IRAS equipment via a bubbler bottle. To humidify the mixed gas, the bubbler bottle was maintained at about 40 °C using thermal controller 2 and an attached heater. The temperature of the sample chamber of the spectrometer is maintained at 37 °C and the MCF-7 cell sample can be cultured under enough humidity and CO_2, 5% as same as a typical cell culture environment. A time course of the temperature monitored by thermo couple 1 is shown in Fig. 2b. The temperature of the sample chamber is well maintained at 37 ± 0.3 °C for 30 min.

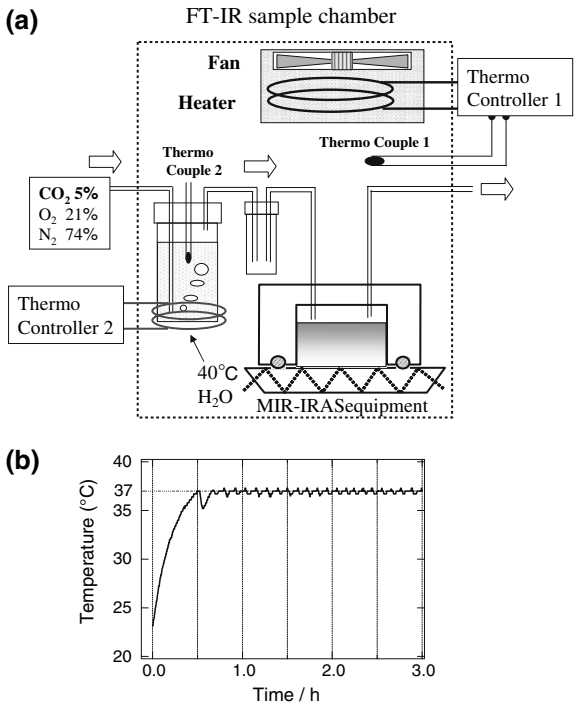

Fig. 2 (**a**) Schematic view of a FT-IR sample chamber. A mixed gas containing 5% of CO_2 gas was introduced into MIR-IRAS equipment via a bubbler bottle, and temperature of the sample chamber was maintained at 37 ± 0.2 °C. (**b**) A Temperature versus time plot obtained for the FT-IR sample chamber

Microscopic observation

Optical images of the MCF-7 cells on the Si prism were observed using Digital microscope (VHX-200, Keyence Co., Japan). And a cross-sectional image of MCF-7 on a silicon wafer was observed using scanning electron microscope (VE-7800, Keyence Co., Japan). To obtain the cross-sectional image, the MCF-7 cells on the silicon were pre-fixed over night. The pre-fix solution was composed of 2% paraformaldehyde and 2.5% glataraldehyde in 0.1 M HEPES buffer. The sample was post-fixed for 1 h in 1% osmium tetraoxide in 0.1 M HEPES buffer. The sample was then dehydrated through graded ethanol and a thin gold layer was deposited. The fixed sample and the Si wafer were cleaved before the observation.

Result and discussion

As a preliminary experiment, we have estimated MIR-prism dependence on the cell adhesion of MCF-7 cells. Two kinds of MIR prisms, Si and GaAs, were compared. We found that MCF-7 did not adhere on the GaAs surface as shown in Fig. 3a. It may be due to a toxicity of arsenide. On the other hand, when MCF-7 cells (2×10^5 cells/mL, 200 μL) were introduced into the MIR-IRAS equipment with a Si prism in Fig. 1a, and incubated for 24 h in the CO_2 incubator, the MCF-7 cells adhered on the Si prism surface, and its surface area coverage was about 70%, indicating a stable cell adhesion on the Si prism (Fig. 3b). Considering that less toxic SiO_2 layer was formed on the surface of the Si prism during washing with Piranha solution (H_2SO_4:H_2O_2 = 1:1), the SiO_2 layer enabled cell growth of MCF-7 cells on the Si prism. Thus we used the prisms made of Si in this study.

We next examined the cell adhesion of MCF-7 cells using MIR-IRAS. The MIR-IRAS equipment was mounted in the FT-IR sample chamber and infrared spectra of the cultured MCF-7 cells on the Si prism were collected for 300 min at an interval of 30 min. A reference spectrum was collected at 60 min after the

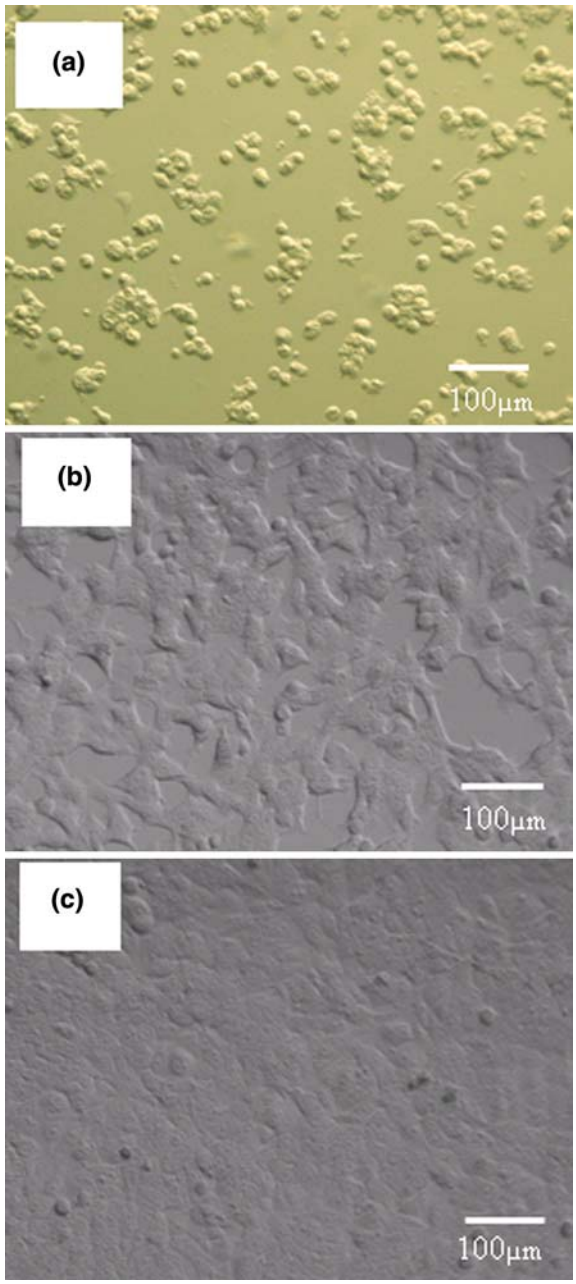

Fig. 3 The micrographs of the MCF-7 cell on (**a**) GaAs substrate and (**b**), (**c**) on a Si prism surface. (**b**) The cells were added in the MIR-IRAS equipment and incubated for 24 h in a CO_2 incubator. (**c**) The cells after (**b**) were placed in the FT-IR sample chamber for 300 min of MIR-IRAS measurement

temperature in the FT-IR sample chamber became stable. The collected spectra of MCF-7 on the Si prism are shown in Fig. 4. Figure 4a, b shows different region in 1,450–1,750 and 2,700–3,100 cm^{-1}, respectively. In Fig. 4a, it is clearly visible that two absorbance bands are centered around 1,640 and 1,545 cm^{-1}. The absorption band around 1,640 cm^{-1} corresponds to amide-I protein absorption band and is mainly attributed to C=O stretching mode, and the other absorption band around 1,545 cm^{-1} corresponds to amide-II protein absorption band mainly attributed to N–H bending mode and C–N stretching mode (Socrates 2001). In Fig. 4a, these amide-I and II absorption bands were enhanced as incubation time is increased.

In another region in 2,700–3,100 cm^{-1}, some CH_x absorption peaks are observed. In Fig. 4b, three absorption peaks at 2,854, 2,925 and 2,960 cm^{-1} increased with elapsed time. These absorption peaks are known as stretching modes of C–H stretching modes. According to Socrates (2001), the peak at 2,854 and 2,925 cm^{-1} can be assigned to a symmetry stretching mode and an asymmetry stretching mode of CH_2 group, respectively. Another peak at 2,960 cm^{-1} can be assigned to an asymmetry stretching mode of CH_3 group. These absorption peaks are also enhanced as incubation time is increased as in the case of amide-I and II absorption bands. These spectral changes shown in Fig. 4 may be due to the metabolic activity of the MCF-7 on the Si prism.

On the other hand, the surface coverage of the MCF-7 cells, which was estimated through the microscopic observation, was about 70% before MIR-IRAS measurements and almost 100% after 300 min as shown in Fig. 3b and c. It suggests that the environment of the spectrometer was maintained appropriately for cell culture and the spectral changes described above are due to not only the metabolism but also the adhesion of the MCF-7 cells. The surface coverage on the Si prism surface should directly affect on the infrared absorption spectrum.

We also performed a control experiment without MCF-7 cells. Absorption spectra obtained by the control experiment are shown in Fig. 5. In Fig. 5a, any significant spectral changes are not observed in amide-II absorption band. On the other hand, small spectral changes were observed in amide-I absorption band, which is probably due to a strong absorption of water overlapping with the amide-I absorption band. In the higher wavenumber region, enhancements of the absorption peaks of CH_x groups were observed, as shown in Fig. 5b. However, the

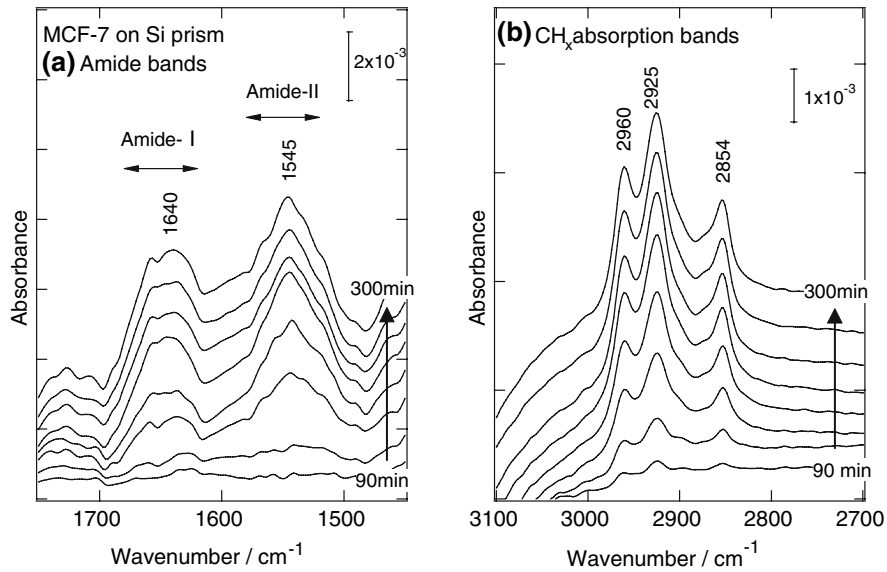

Fig. 4 MIR-IRAS spectra of MCF-7 in the wavenumber region of (**a**) 1,450–1,750 cm^{-1} and (**b**) 2,700–3,100 cm^{-1}

intensities of the observed peaks are smaller than those in the presence of the MCF-7 cells (Fig. 4b). In addition, the relative intensities of the peaks are different with and without the cells. The strongest peak was at 2,925 cm^{-1} with the MCF-7 cells (Fig. 4b), while the strongest peak was observed at 2,960 cm^{-1} in the control experiment (Fig. 5b). The result of control experiment, therefore, suggests that the background signal from medium is relatively small and the spectral changes in Fig. 5 are attributed to other species different from the case of the experiment with MCF-7 cells.

The time course of the peak intensities show another evidences. In Fig. 6a, the intensities of the strongest absorption peak at 1,545 cm^{-1} in Figs. 4a and 5a as a function of time. In the case of MCF-7, the intensity of the peak increased and saturated after 210 min. And there is no absorption peak as shown in Fig. 5a in the control experiment. In Fig. 6b, the three CH_x absorption peaks in Fig. 4b and 5b are plotted against the time. These intensities in the absorption spectra of MCF-7 (Fig. 4b) also saturated after 210 min as same as the peak intensity at 1,545 cm^{-1} as shown in Fig. 6a. And the intensities

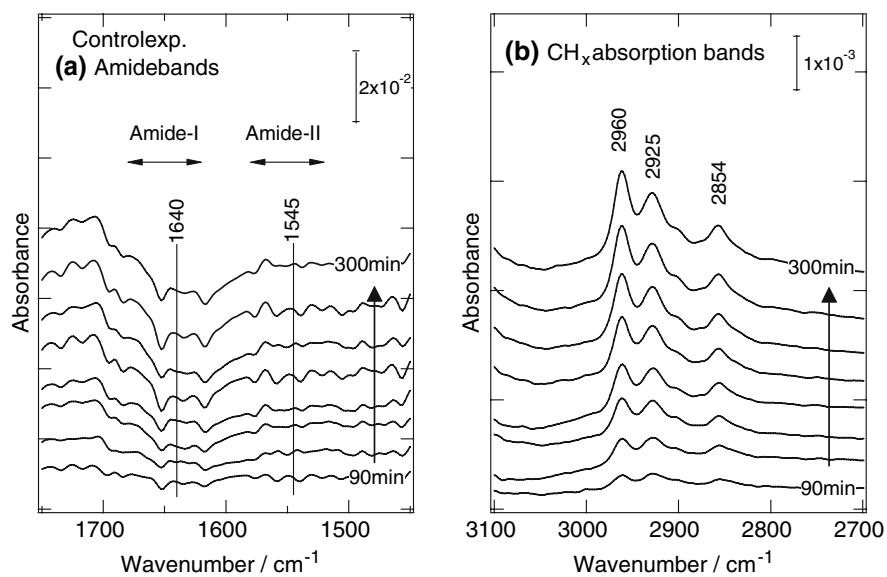

Fig. 5 MIR-IRAS spectra obtained from a control experiment without MCF-7 cells in the wavenumber region of (**a**) 1,450–1,750 cm^{-1} and (**b**) 2,700–3,100 cm^{-1}. The spectra were measured at every 30 min

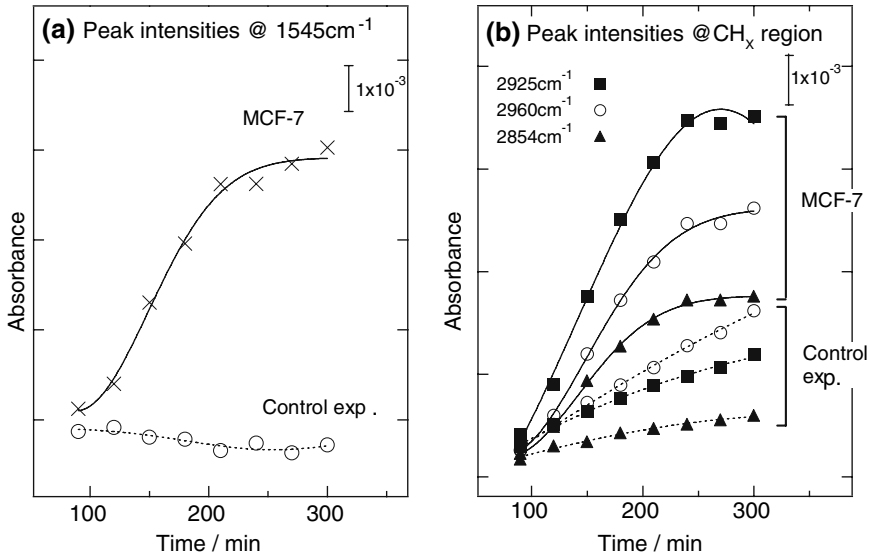

Fig. 6 Time course of the peak intensities at (**a**) 1,545 cm^{-1}, (**b**) 2,925 cm^{-1} (■), 2,960 cm^{-1} (◯) and 2,854 cm^{-1} (▲). Solid lines indicate the peak intensities of MCF-7 cells (Fig. 4), and dashed lines indicate the peak intensities of control experiment (Fig. 5)

in the control experiment (Fig. 5b) increased continuously as incubation time increased. A clear difference can be seen between the time courses of two experiments. The continuous increasing of CH_x peaks in the control experiment may be due to the adsorption of medium components. These intensities of CH_x peaks in the control experiment are smaller than those in the experiment with MCF-7. And besides, MCF-7 cells adhered on the Si prism and covered the Si prism surface, as shown in Fig. 3b, c, suppressing the effect of the background signal from the medium. Thus the obtained signal should be mainly due to the adhesion and the metabolism of MCF-7 cells.

Fig. 7 The cross-sectional SEM image of MCF-7 cells on the Si prism

Figure 7 is a cross-sectional scanning electron microscopy (SEM) image of the MCF-7 cells on a Si wafer. We estimated that the thickness of the MCF-7 cells was about 1–2 μm, and the thickness of the excited evanescent field on the Si prism was about 500 nm from the surface as shown in Fig. 1b. The thickness of the evanescent field covers 25–50% of that of the MCF-7 cells, thus the obtained absorption spectra should contain many absorption bands of lipid membranes, membrane proteins, part of nuclear and also organelle proteins of the MCF-7 cells. Miyazawa and Blout (1961) have assigned absorption bands of typical secondary structures of protein; α-helix, β-sheet and random coil. If these absorption bands in Fig. 4a were mainly attributed to the kinds of protein, the absorption band at 1,545 cm^{-1} can be assigned to the α-helix structure. However, it is probable that the amide-II absorption band contains other absorption bands attributed to the β-sheet and random coil structure at 1,530 and 1,535 cm^{-1}, respectively. According to their study, although the α-helix structure also has another strong absorption band at 1,650 cm^{-1} in amide-I band, it is difficult to identify the peak from Fig. 4a. This is because of an overlapping of H_2O strong absorption band at about 1,640 cm^{-1} to amide-I band. And it is also difficult to identify the bands attributed to the β-sheet and the random coil structured proteins for the same reason.

On the other hand, it is most likely that the absorption bands of other cell components such as the

nucleic acid (Miyamoto et al. 2005 and 2006) or the lipids (Socrates 2001) complicate the obtained spectra. For example, the absorption bands attributed to the asymmetric stretching mode of COO^- group overlaps to the amide-II region, the bending vibrational mode of the N–H group also overlaps to the amide-I and amide-II region, and the base residues of the nucleic acid also have kinds of absorption bands in the amide-I region. And it is also difficult to assign the CH_x absorption peaks in 2,700–3,100 cm^{-1}. There must be large number of CH_x groups on the Si prism surface. As described above, although the assignment of the absorption peaks is very complicated, these absorption bands should be good index to monitor the cell adhesion and metabolism by MIR-IRAS measurement.

Conclusion

We developed the monitoring system of living cultured cell using infrared spectroscopy and demonstrated that MIR-IRAS is capable of in situ observation of living culture cell in this study, specially the cell adhesion and metabolism without any reagents. The sample chamber of the spectrometer was maintained to optimum the condition of the cell culture, and the adhesion and metabolism of MCF-7 can be monitored by the absorption intensity at 1,545 cm^{-1} of amide-II absorption band and also by the absorption intensities of CH_x absorption bands in 2,700–3,100 cm^{-1}. To screen various biological active substances, one of the important information is cell viability or toxicity, which affects the cell metabolism. Thus, this novel method can be used for the screening of many biological active substances. We have tried to observe the other cell types and other cellular activities such as apoptosis or necrosis using MIR-IRAS and these results will be discussed in the next article.

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