Live cell adhesion assay with attenuated total reflection infrared spectroscopy

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Living confluent fish fibroblast cells RTG-P1 from rainbow trout adherent on diamond were examined by attenuated total reflection (ATR) infrared (IR) spectroscopy. In particular, IR spectra were recorded dynamically during the adsorption of the cells onto the diamond and during their biochemically induced structural responses to the subsequent addition of trypsin and cytochalasin D. It is shown that changes in the IR spectra result from changes in cell morphology and surface coverage. The results demonstrate the potential and the applicability of ATR IR spectroscopy for live cell adhesion assays. © *2007 American Vacuum Society.* [DOI: 10.1116/1.2710336]

I. INTRODUCTION

The explicit study of cell structure and function ultimately requires living cells. *In vitro* experiments are most commonly based on biochemical assays and optical light microscopic methods. However, spectroscopic techniques can provide complementary or additional information. IR spectroscopy probes molecular vibrations and is a powerful diagnostic tool for investigations of chemical composition and molecular structure. As a noninvasive, nondestructive, noncytotoxic as well as a label- and stain-free method it is very well suited for the study of living cells.[1–3] It offers the possibility of time-resolved *in situ* monitoring of biochemical or physical cellular changes which may be triggered by internal or external events. Application of the attenuated total reflection (ATR) geometry gives enhanced surface sensitivity and decouples the path length, i.e., the thickness of the sample penetrated by the evanescent field, from the actual extent of aqueous medium during observation. The latter provides ease of sampling in view of the high IR absorptivity of water. In contrast to relevant optical microscopy techniques (e.g., phase contrast, interference reflection, and total internal reflection fluorescence) ATR IR spectroscopy thus has the combined virtues of being surface sensitive and label-free and of providing molecular-level spectral information. It was applied to investigations of bacteria[4] and to living mammalian cells in studies with regard to cell growth and inhibition [5] as well as infection and activation.[6]

As was alluded to in the literature,[5] ATR IR spectroscopy seems to be suitable also for studies of cell adhesion due to its distinctive nature as a surface probe. In this article, we addressed this potential and explored the applicability of ATR IR spectroscopy for live cell adhesion assays by studying fish fibroblast cells. Specifically, adherent cells on diamond were exposed to trypsin and cytochalasin D, and the cellular responses were monitored *in situ* by ATR IR spectroscopy.

II. EXPERIMENT

Cells were obtained from ATCC (Manassas, VA) and cultured according to the following protocol. The cells were grown in 10 cm petri dishes with Leibovitz L-15 medium (Gibco, Carlsbad, CA), supplemented with 10% FBS (Cambrex, Walkersville, MD) and 1% Pen/Strep (Gibco). Cells were incubated in the dark at 23 °C in 100% air atmosphere. Cells were used for experiments from P4 to P8.

For the ATR IR experiments, a Bruker VERTEX 70 Fourier transform IR spectrometer equipped with a PIKE MIRacle ATR sampling accessory with a 45° single reflection diamond/ZnSe horizontal crystal plate was used. A

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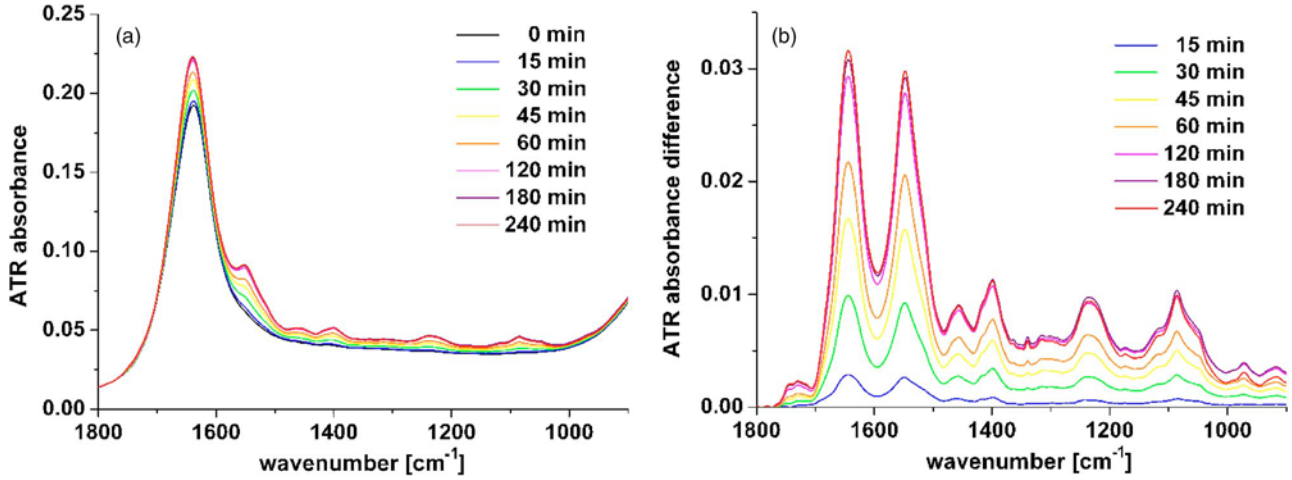

FIG. 1. (a) ATR absorbance spectra of a cell suspension placed on diamond in the time course between 0 and 240 min. (b) Difference spectra calculated with respect to the ATR absorbance spectrum at 0 min.

100 μl aliquot of a cell suspension of 10^6 cells/ml in complete medium was pipetted on the uncoated diamond internal reflection element (diameter=1.8 mm) and covered to prevent evaporation. Mid-IR spectra with a spectral resolution of 4 cm^{-1} were recorded at room temperature using the globar source, a KBr beam splitter, a LN_2-cooled MCT detector, and coadding 256 scans each. As a reference, a single beam spectrum of the bare diamond in air was collected before each experiment. Atmospheric and ATR penetration depth compensations as well as a base line offset with respect to 1800 cm^{-1} were applied using the OPUS software. For each experiment, collection of spectra was repeated with duplicate samples to ensure reproducible results.

III. RESULTS AND DISCUSSION

Figure 1(a) shows the spectra in the range between 1800 and 900 cm^{-1} for a cell suspension placed on the diamond ATR crystal recorded at time intervals between 0 and 240 min. The ATR absorbance spectrum corresponding to 0 min is characterized by a strong absorption band around 1640 cm^{-1} which is assigned to the bending mode of liquid water.[7] In addition to this band, the successive spectra display several other spectral features that increase in intensity with time until a steady plateau is reached after about 240 min. These absorption bands can be seen more clearly in the difference spectra [see Fig. 1(b)] calculated with respect to the spectrum at 0 min. The bands are attributable to the biochemical and structural profile of the cells and can be assigned according to the literature (see Refs. 8 and 9, as well as the supplementary material in Ref. 10). It should be noted that due to the limited penetration depth, the IR radiation interrogates only those cellular fractions which are in close proximity (within an average IR penetration depth of about 1.2 μm) to the surface. The spectra thus reflect the settling and the spreading of the cells on the diamond surface. Maximum coverage or confluence after 240 min is inferred considering the plateau reached. This conclusion was corroborated by visual inspection of the cells on diamond using a low-resolution microscopic external reflection geometry. The persistence of the IR bands of cellular origin after exchange of culture medium and after rinsing with a phosphate buffered saline (PBS, Cambrex, Walkersville, MD) solution (100% and 98% retentions of integrated cell signal intensity, respectively, spectra not shown) indicated good cellular adhesion on diamond which is known to be a biocompatible material for living cells.[11] As a control, cell spreading and adhesion were found to be compromised when the diamond was coated with heat-denatured bovine serum albumin (Sigma, St. Louis, MO) (67% of integrated cell signal intensity with respect to cells on uncoated diamond, dropping to 51% after rinsing with PBS, spectra not shown).

For the cell assays the cells were cultured on diamond for 20 h prior to experiments with an exchange of medium after 10 h. Figure 2(a) shows the ATR absorbance spectra of cells in PBS to which a trypsin/EDTA solution (2.5 g/l of trypsin and 0.38 g/l of EDTA) (Gibco, Carlsbad, CA) was added at the time point denoted as 0 min. Trypsins belong to the serine endopeptidases and are enzymes that cleave peptide bonds in proteins. They can therefore be used to resuspend adherent cells by breaking extracellular attachments to the surface. As can be seen the IR bands of cellular origin decrease over time and vanish within 45 min. For clarity, the difference spectra with respect to the spectrum recorded after 45 min are shown in Fig. 2(b). This experiment demonstrates that ATR IR spectroscopy can be used for *in situ* monitoring of the effect of a stimulus or agent on adherent cells. Also, the observed response to trypsin serves as a further proof for the presence of living adherent cells in our experiments.

Cytochalasin D is a cell permeable fungal metabolite that causes the inhibition of actin polymerization and the disruption of actin filaments. This disruption of the supramolecular organization of the cytoskeletal actin filaments causes cells to round up.[12] Figure 3(a) shows the ATR absorbance spectra of cells exposed to a 2 μM solution of cytochalasin D (Sigma) from the time point denoted as 0 min. The IR bands of cellular origin decrease over time. After about 300 min,

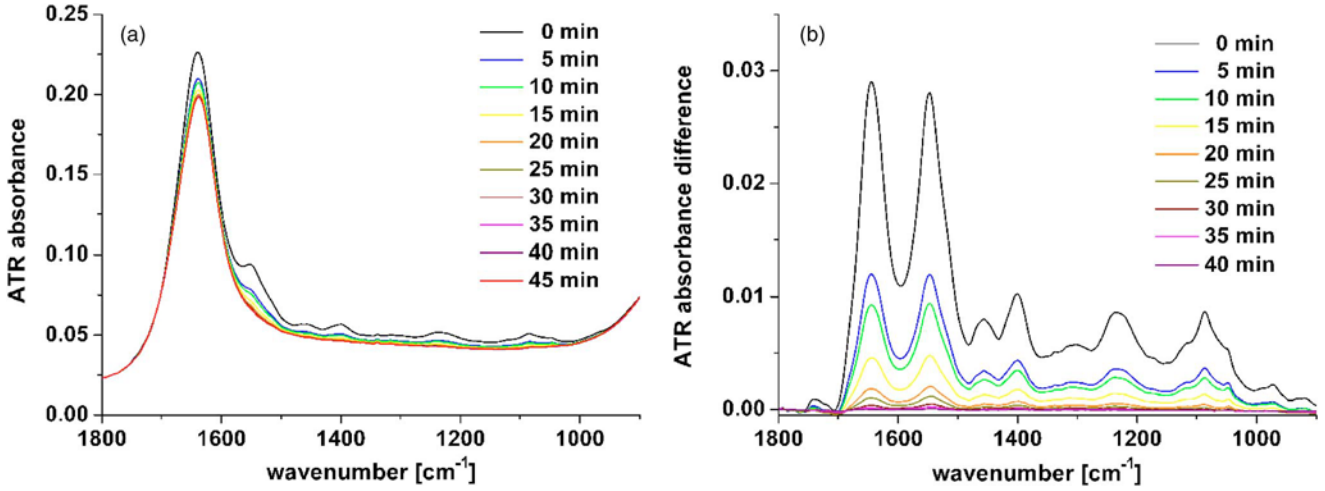

FIG. 2. (a) ATR absorbance spectra of cells initially adherent on diamond in the time course between 0 and 45 min after adding a solution of trypsin/EDTA. (b) Difference spectra calculated with respect to the ATR absorbance spectrum at 45 min.

no significant change in the spectra is detected. The residual integrated signal intensity of the IR bands of cellular origin after 300 min corresponds to approximately 27% of the initial intensity at 0 min [for reference, see the dotted line in Fig. 3(a) which corresponds to the initial spectrum (0 min) shown in Fig. 1(a)]. This means that the cells do not completely detach from the diamond surface. Looking in a conventional culture dish by light microscopy at cells that were treated with cytochalasin D it was found that the confluent cells changed from a spread or flattened morphology into a

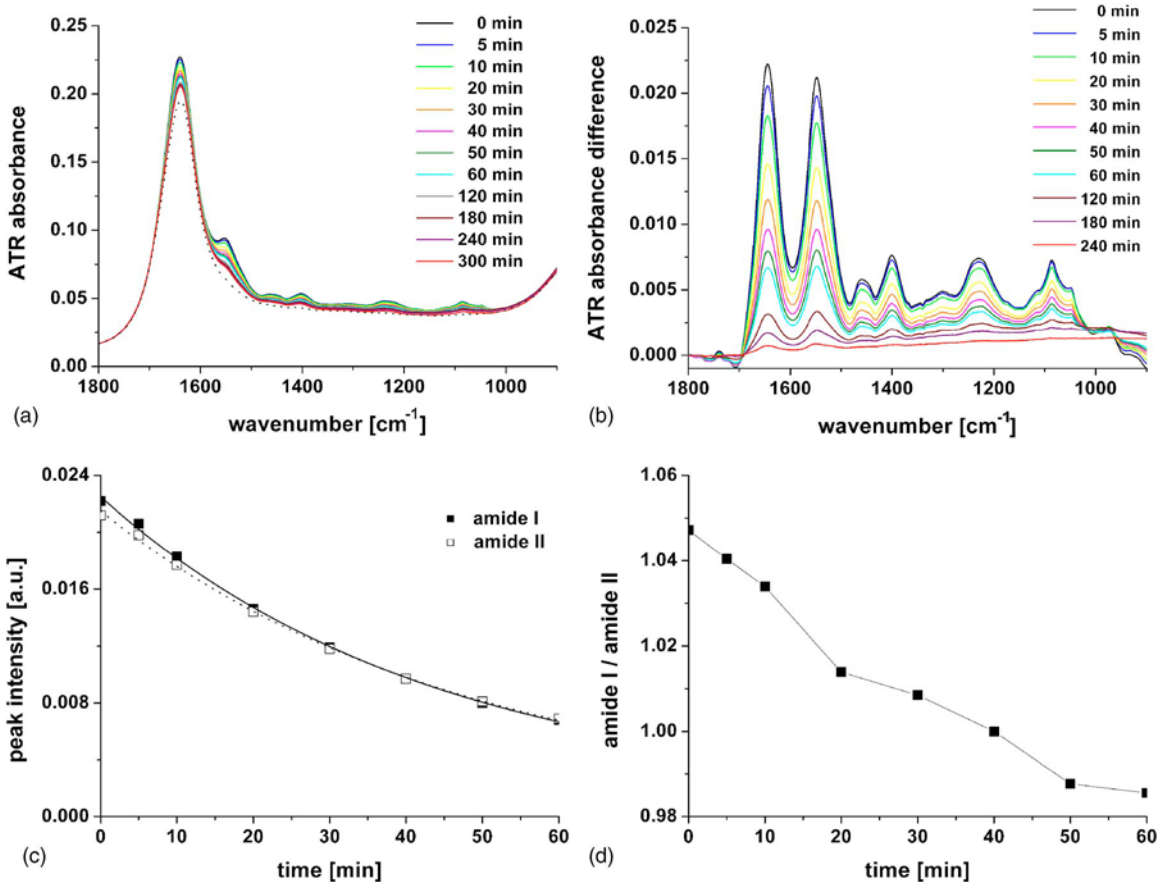

FIG. 3. (a) ATR absorbance spectra of cells on diamond after adding a solution of cytochalasin D. For reference, the initial spectrum (0 min) shown in Fig. 1(a) is added (see dotted line). (b) Difference spectra calculated with respect to the ATR absorbance spectrum at 300 min. (c) The peak maxima and (d) the ratio of the peak maxima of the amide I and amide II bands during the treatment of cells with cytochalasin D.

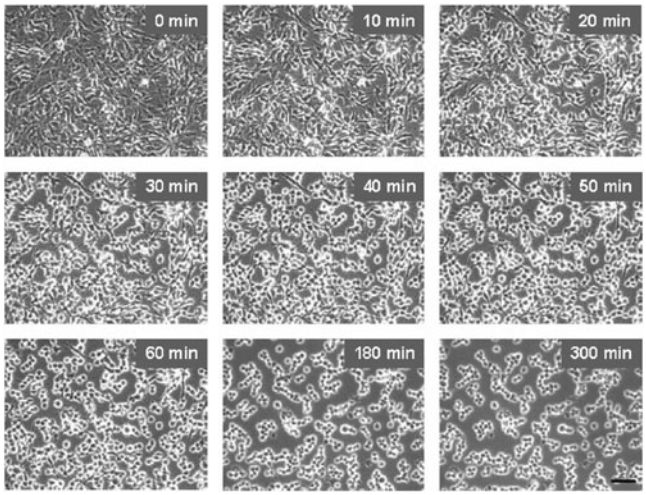

FIG. 4. Light microscopy images of cells in a culture dish that were treated with a 2 μM solution of cytochalasin D. The scale bar in the bottom right-hand corner marks 50 μm.

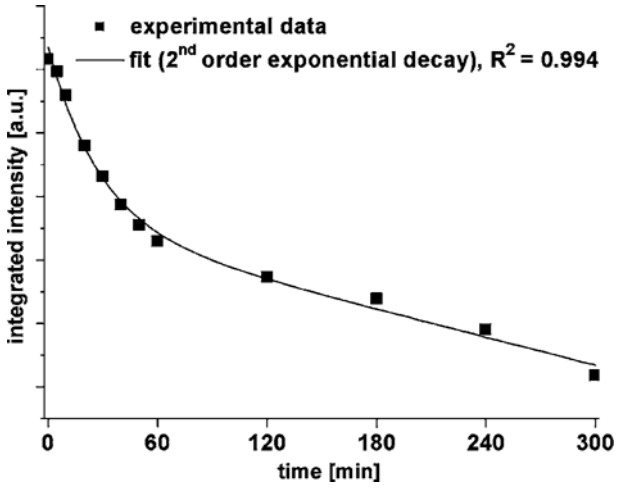

FIG. 5. Integrated intensity for the ATR absorbance spectra acquired during the treatment with cytochalasin D [cf. Fig. 3(a)] as a function of time.

spherical shape covering only a fraction of the surface (see Fig. 4). The difference spectra with respect to the spectrum recorded after 300 min are presented in Fig. 3(b). The projected area seen in the microscope to be covered by cells after 300 min is about 50% (see Fig. 4). From this it is estimated that only approximately 11% of the actual volume that would be probed in the ATR IR experiment is filled by cells (assuming perfectly spherical cells with a diameter of 10 μm, and an IR penetration depth of 1.2 μm). There is a discrepancy between this estimate and the larger ($\sim$27%) measured residual IR cell signal intensity. The most plausible explanation for this is that on diamond the cells after 300 min have a *spherical-like* geometry and actually a larger contact area with the surface than that expected for cells with a perfectly spherical shape.

These experiments with trypsin and cytochalasin D show the capabilities of ATR IR spectroscopy for live cell assays. The spectra give access to qualitative and quantitative information. For both trypsin and cytochalasin D, the IR bands of cellular origin were found to decrease with time. This is consistent with a decrease of mass or concentration of the cells and their constituent biochemical molecules in the sample volume probed by the IR radiation. This is due to the changes of cellular shape and geometry from a spread and more flat morphology when adherent to a more spherical shape towards cellular detachment. Kinetic data can be derived from the time-resolved spectral changes which are associated with the cellular responses. For the fungal toxin cytochalasin D the cell retraction from the surface occurs slower. Cytochalasin D and trypsin act at different sites and in different ways to interrupt cell adhesion and spreading. The difference in the temporal responses is directly related to the mechanistic differences.

Figure 5 shows a plot of the integrated intensity for the ATR absorbance spectra acquired during the treatment with cytochalasin D [cf. Fig. 3(a)] as a function of time. Assuming a second order exponential decay a good fit (R^2=0.994) to the experimental data was obtained. A simple lift-off of cells during detachment from the surface would be adequately described by a single exponential term. Cytochalasin D, which was found to induce the retraction of cellular protrusions and the rounding up of cells (cf. Fig. 4), however, causes the more complicated kinetic behavior observed in Fig. 5.

The spectra contain specific chemical information on the molecular level. The two most prominent bands in the difference spectra around 1644 and 1547 cm^{-1} can be assigned to the amide I and amide II absorption modes of proteins. Figure 3(c) shows how the peak maxima for these two bands change during the treatment of cells with cytochalasin D. It is noted that the responses are slightly different. Indeed, the ratio of the peak maxima of the amide I and amide II bands decreases with time [see Fig. 3(d)]. The interpretation of this observation seems complicated for two reasons. First, the amide I band is superimposed on the bending mode of liquid water, and during the experiments there is a varying exposure of the ATR crystal to aqueous medium. Second, there is a complex and heterogeneous distribution of proteins in the cells. It was, however, reported that the amide I/II intensity ratios of adsorbed protein films on a Ge ATR crystal were significantly different from those of proteins in solution, suggesting that the difference was associated with changes in the secondary structure.[13] Hence, the amide I/II intensity ratio may be useful to detect changes in the secondary structure of proteins that occur upon adsorption to or desorption from a solid surface. Most recently, a change of the amide I/II intensity ratio was observed upon adhesion of bacteria to an α-Fe_2O_3-coated ZnSe ATR crystal.[9] Therefore, the change of the amide I/II intensity ratio during the treatment of cells with cytochalasin D may implicate changes in protein conformation. Further experiments are required to get detailed insights into the possible origins (e.g., cell-surface proteins) of the variation in the ratio of the amide I and amide II peaks. Further spectral changes, most notably those indicative of cell death,[14,15] were not detected in the measurements.

It seems desirable to extend these cell adhesion studies to mammalian cells. Indeed, it is possible to investigate such cells by ATR IR spectroscopy under the required physiological conditions by controlling *p*H and temperature.[5] Also, the adhesion studies are not restricted to diamond surfaces. The use of different ATR substrates and surface functionalization (e.g., spatially defined decoration with adhesive cues, or surface passivation) can give a versatile approach for the probing of cell adhesion by controlling and tuning surface properties.

IV. CONCLUSION

The biochemically induced structural responses of adherent cells to trypsin and cytochalasin D were investigated by ATR IR spectroscopy. Changes in the IR spectra were correlated with changes in cell morphology and surface coverage. This study demonstrates the potential and the applicability of ATR IR spectroscopy for live cell adhesion assays. As an *in situ* surface probe this spectroscopic technique is more widely applicable to the investigation of cellular adhesion, other cell-surface phenomena, and cellular function in general.

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