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Detecting exposure to environmental organic toxins in individual cells: towards development of a micro-fabricated device

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ABSTRACT

We are developing a new method for quickly screen for the human exposure potential to polycyclic aromatic hydrocarbons (PAHs) and organochlorines (OCs). The development involves two key elements: identifying suitable signals that represent intracellular changes that are specific to PAH and OC exposure, and constructing a device to guide the biological cell growth so that signals from individual cells are consistent and reproducible. We are completing the identification of suitable signals by using synchrotron radiation-based (SR) Fourier-transform infrared (FTIR) spectromicroscopy in the mid-infrared region (4000-400 cm^{-1}). We have observed distinct changes in the IR spectra after treatment of human cells in culture medium with PAHs and OCs. The potential use of this method for detecting exposure to PAHs and OCs has been tested and compared to a reverse transcription polymerase chain reaction (RT-PCR) assay that quantifies increased expression of the *CYP1A1* gene in response to exposure to PAHs or OCs.

Keywords: Polycyclic aromatic hydrocarbons (PAHs), organochlorines (OCs), dioxin, infrared spectroscopy, *CYP1A1*, RT-PCR

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) and organochlorines (OCs) are ubiquitous environmental toxins that are known rodent carcinogens and suspected human carcinogens. Human exposure to these compounds is of great concern and needs to be assessed. Traditional assessment of human exposure and health risk to these toxins relies primarily on animal experiments. A major uncertainty inherent to this approach is the extrapolation from the high-dose animal experiments to the low-dose and long-term human exposure. To overcome this uncertainty, a common strategy is to complement animal experiments with short-term assays that use human cell culture systems and biomarkers that are indicative of exposure. A global biomarker for PAH and OC exposure is the induction of the *CYP1A1* gene expression and/or increase in the associated enzyme activity. The selection of this biomarker is based at least on the following two widely observed phenomenon. The cytochrome P4501A1, encoded by the gene *CYP1A1*, metabolizes PAHs. *CYP1A1* levels increases as a result of exposure to PAHs or OCs through binding of PAHs or OCs to the Ah receptors [1]. The increase in *CYP1A1* transcript levels represents the levels of the exposure, which can be quantified by reverse transcription-polymerase chain reaction (RT-PCR) [2].

In this study we are developing a faster (than current RT-PCR techniques) and still sensitive method for detecting the exposure of individual human cells to PAHs and OCs. The development involves two key components. First, we identify suitable signals that represent intracellular changes that are specific to PAH and OC exposure. Second, we will construct a device to so guide the biological cell growth that suitable signals from individual cells are consistent and reproducible for a given set of exposure conditions. This paper describes our use of synchrotron- radiation-based (SR) Fourier-transform infrared (FTIR) spectromicroscopy in the mid-IR

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region (4000-400 cm^{-1}) as a tool to identify IR radiation signals (from individual cells) that are specific to the intracellular response after their exposure to PAHs or OCs. SR FTIR spectromicroscopy was used because it has been proven to be a sensitive analytical technique capable of providing molecular information in a biological system quickly (within minutes) at dilute concentrations and a spatial resolution of 10 microns (3-4). The potential use of the new method to screen for the exposure to PAHs and OCs at environmentally relevant concentrations was tested using human cells in an environmental medium for which contaminant types and levels are known. In these tests, HepG2 (human hepatoma derived) cells modeled the human epithelial cells that would activate the test PAHs and OCs; benzo(a)pyrene (BaP) modeled the PAHs, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) modeled the OCs. HepG2 cells metabolize PAHs efficiently. They also show *CYP1A1* induction after their treatment with PAHs and OCs. The potential use of our marker signals for detecting intracellular response to mixtures of PAHs was also demonstrated in an environmental medium with dilute concentrations of coal tar, a complex mixtures of different PAHs.

2. EXPERIMENTAL

2.1. Cells and Cell Treatment

HepG2 cells were obtained from the American Tissue Culture Collection (Rockville, MD). Cells were maintained in MEM (Minimum Essential Medium, Eagle) with non-essential amino acids and Earle's BSS supplemented with 10% fetal calf serum, 1 mM L-glutamine, 10 mM HEPES and antibiotics. Cells were sub-cultured every 7 days. Cells were treated for 2 - 20 hours with various amounts of BaP, coal tars, and TCDD dissolved in DMSO.

2.2. SR FTIR spectromicroscopy

The infrared spectromicroscope in conjunction with Beamline 1.4.3 at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory was used to monitor intracellular changes in response to TCDD, BaP, and coal tars exposure. Figure 1 depicts the experimental setup for this direct measurement. As shown in the diagram, the infrared microprobe uses a synchrotron source that has much higher brightness than a conventional thermal IR source. The synchrotron light is focused into a Nicolet Magna 760 FTIR bench, then passes through a Nic-Plan IR microscope. As detailed in [3] the spot size of the unmasked synchrotron beam focused through an infrared microscope is 10 μm , nearly diffraction-limited, and significantly smaller than the 100- μm spot size of a conventional thermal IR source. This leads to an improved signal to noise level and finer spatial resolution than is possible for conventional source FTIR spectromicroscopy. Since the synchrotron experimental procedure is non-destructive to the biological materials being studied, SR FTIR spectromicroscopy is extremely useful in detecting subtle intracellular changes as the cells are exposed to environmental stimuli.

All SR FTIR spectra were recorded in the 4000–650 cm^{-1} infrared region. This region was selected because it is the region that contains unique molecular fingerprint-exhibiting absorption features of intact biomolecules. For each IR measurement, 128 spectra were co-added at a spectral resolution of 4 cm^{-1} . All spectra were obtained in the reflection geometry and were ratioed to the reflectance spectrum of a gold-coated slide to produce absorbance values (see Figure 1).

2.3. Semi-quantitative RT-PCR

After the treatment of cells (as described in 2.1), total RNA was isolated using TRI Reagent. Total RNA was reverse transcribed using oligo dT, MMLV reverse transcriptase. *CYP1A1* expression was measured by determining the *CYP1A1* transcript level relative to a constantly expressed internal control gene (2). Primers designed to span an intron were used to generate PCR products. PCR conditions and cycle numbers were optimized for each target sequence to ensure the reaction is in the linear phase of product accumulation. After amplification, the products were separated by electrophoresis on a polyacrylamide gel. The gel was stained with SYBR Gold fluorescent stain (Molecular Probes) and the gels were scanned on a Molecular Dynamics STORM 860 laser scanner. The fluorescent signal for each band was quantified using ImageQuant software.

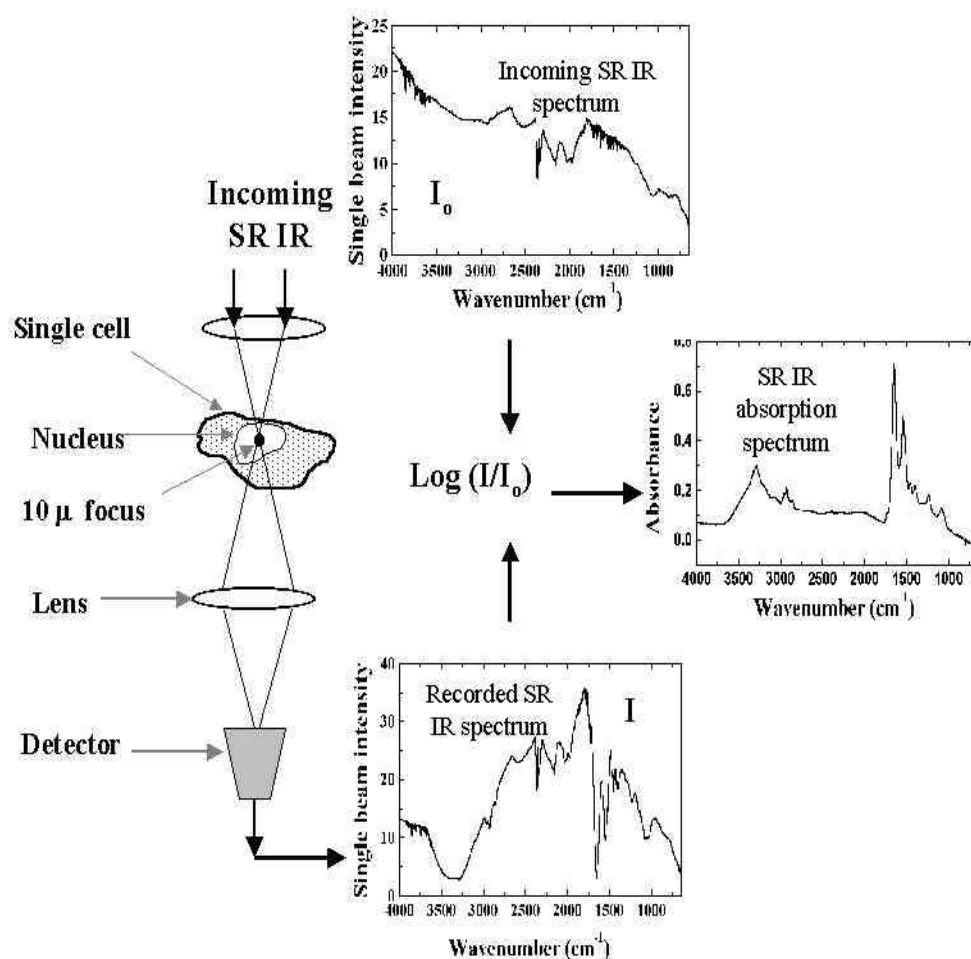


Figure 1. Diagram illustrating experimental setup for monitoring intracellular response to polycyclic aromatic hydrocarbons (PAHs) and organochlorine (OCs) exposure. (Note that cells were placed on treated surfaces on a microscope stage. All measurements were made in reflectance mode.)

3. RESULTS AND DISCUSSION

3.1. SR FTIR absorption spectrum characteristics of the nucleus of a single HepG2 cell

We obtained SR FTIR absorption spectra in the range (1750–1000 cm^{-1}) of interest at different locations of a randomly selected single HepG2 cell prior to its exposure to the test chemicals. Figure 2 shows a typical absorption spectrum recorded at the proximity of a cell nucleus. The overall spectral characteristics are similar to those reported in (6). It shows three main informative spectral regions for the architectural details of nucleic acid helices; each corresponds to a molecular subgroup (6). Absorption bands in Region I (~ 1750–1500 cm^{-1}) arise from in-plane vibrations of the bases (in additions to other proteins), and are sensitive to base-pairing and base-stacking effects. Absorption bands in Region II (~ 1490–1230 cm^{-1}) arise from base deformation motions coupled through the glycosidic linkage to sugar vibrations. Their spectral characteristics are strongly dependent on the glycosidic torsion angle. Absorption bands in Region III (~ 1230–1000 cm^{-1}) arise from phosphate vibrations and sugar vibrations. The absorption band positions and intensities depend strongly on the base-base interactions and the helical geometry in nucleic acids. The architectural structures of nucleic acid helices are extremely sensitive to the surrounding

environments. We anticipated that exposing individual cells to the test chemicals, at the appropriate dose and exposure time, will lead to changes in the SR FTIR spectral characteristics in the three regions.

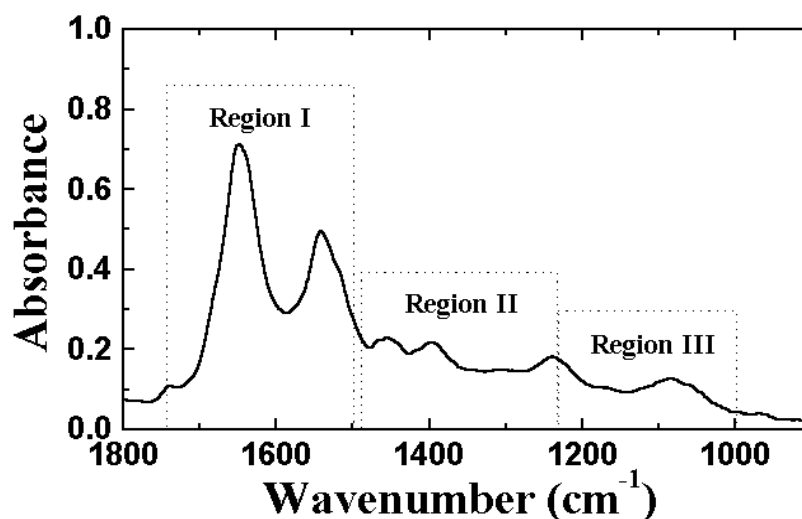


Figure 2. A typical SR FTIR absorption spectrum recorded near/at the nucleus of a single HepG2 cell prior to its exposure to the test chemicals. The spectrum shows three main informative spectral regions. Regions I-III are discussed in text.

3.2. Identifying marker signals specific to the intracellular response

To identify spectral signals that were specific to the induction of *CYP1A1*, cells were exposed to TCDD known to interact specifically with the Ah receptor that regulates *CYP1A1* expression. HepG2 cells were treated with different concentrations of TCDD (0, 0.01, 0.1, 0.5, and 1.0 nM) for 20 hours. SR FTIR spectra were measured for randomly selected individual cells, and examined for significant changes in the spectral characteristics as the dose increased. Figure 3 shows the normalized SR FTIR absorption spectra recorded. There

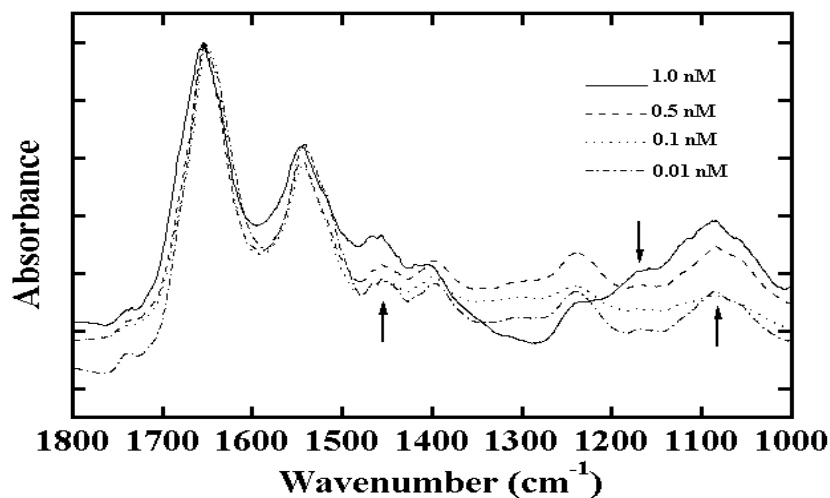


Figure 3. Normalized absorbance spectra of individual HepG2 cells after 20-hour exposure to different TCDD concentrations.

are considerable differences in the SR FTIR spectra, with one difference being the increased absorption of the vibration band 1180 - 1160 cm^{-1} , centered at $\sim 1170 \text{ cm}^{-1}$. This systematic spectral change might be related to the alteration in the DNA base structure (7). This will be the subject of future investigation. For our experimental system, the normalized absorbance intensity for individual cells increased from 0.007 to 0.21 when the TCDD concentration increased from 10^{-11} to 10^{-9} M (Figure 4). The normalized absorbance intensity at $\sim 1170 \text{ cm}^{-1}$ for individual control cells was 0.005. This is a 42-fold increase in the absorbance intensity, which is extremely significant considering the definition of absorbance (see Figure 1).

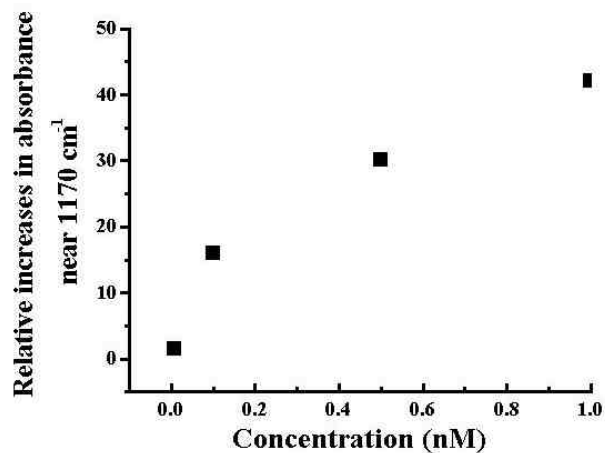


Figure 4. Dose response in absorption intensity at about 1170 cm^{-1} .

We compared the performance of SR FTIR spectromicroscopy with our current RT-PCR technique. The purpose was to evaluate if SR FTIR spectromicroscopy could be a valid and sensitive technique to detect the subtle intracellular changes during the induction of *CYP1A1*. *CYP1A1* expression in HepG2 cells, as measured by RT-PCR, increased 212-fold over the same range of TCDD concentrations used in the SR FTIR study. Relative changes in the absorption intensity at $\sim 1170 \text{ cm}^{-1}$ (detected by SR FTIR spectromicroscopy) as compared to the *CYP1A1* expression detected by the RT-PCR techniques are shown in Figure 4b.

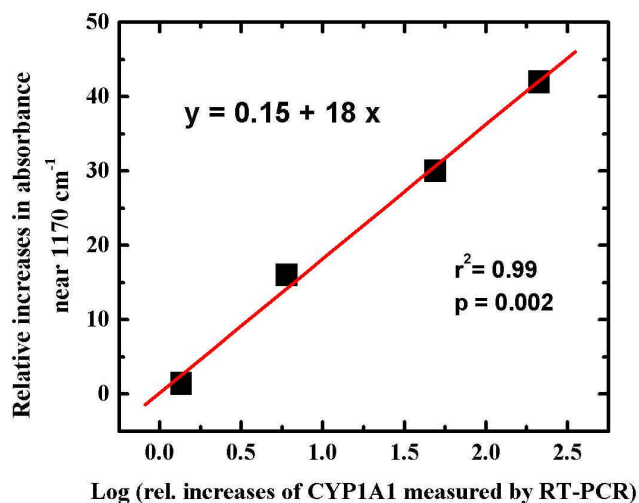


Figure 4b. SR FTIR spectromicroscopy versus RT-PCR techniques.

The solid line in Figure 4b is the least-squares fit to the data. The excellent agreement (with $r^2 = 0.99$) for measurements from the two methods indicates that the fast SR FTIR spectromicroscopy technique is indeed comparable to the RT-PCR technique that specifically measures increases in the *CY1A1* expression. This demonstrates that SR FTIR spectromicroscopy can be used as a substitute for direct measurements of *CY1A1* levels. This observation also implies that SR FTIR spectromicroscopy measures similar intracellular changes.

3.3. Applications of the SR FTIR Biomarkers

3.3.1. Dose response to BaP

When HepG2 cells were treated with different concentrations of BaP (0.5, 2.0, 5.0, and 10.0 μM) for 20 hours, their response was monitored by SR FTIR spectromicroscopy. Similarly, there were considerable changes in the SR FTIR spectra (not shown here). Among these changes some were similar to those observed in the TCDD study. We focussed on the peak centered at $\sim 1170\text{ cm}^{-1}$ because its changes were consistent with those for the TCDD exposure. The normalized absorbance intensity at $\sim 1170\text{ cm}^{-1}$ for individual cells increased 4-fold, from 0.005 to 0.02 when BaP concentration increased from 0.5 to 10 μM (Figure 5b). The normalized absorbance intensity at $\sim 1170\text{ cm}^{-1}$ for individual control cells was 0.005, which is the same as when the cells were exposed to 0.125 mg/mL of BaP for 20 hours.

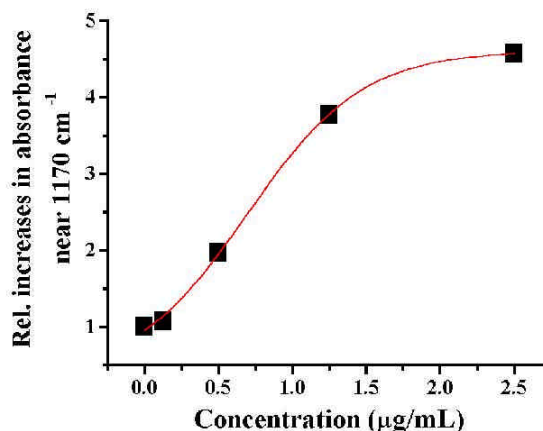


Figure 5. Increasing absorption intensity at about 1170 cm^{-1} with increasing BaP concentrations (The curve-fit is for visualization purpose).

3.3.2 Time response to a mixture of PAHs - coal tars

Humans usually are exposed to mixtures of PAHs rather than to individual compounds. Coal tars represent complex mixtures of different PAHs. In this demonstration, we used hexane extracted coal tar to model the PAH mixtures. All cells were treated at a concentration of $0.5\text{ }\mu\text{g/mL}$ for 2, 4, and 8 hours. Spectral changes were monitored by SR FTIR spectromicroscopy. Changes in spectral intensities (Figure 6) at the marker peak ($\sim 1170\text{ cm}^{-1}$) were significantly stronger than those detected for BaP response. This observation was consistent with quantitative results from the RT-PCR technique. The normalized absorbance intensity at $\sim 1170\text{ cm}^{-1}$ for individual cells increased almost 9-fold, from 0.07 to 0.61 when exposure time increased from 2 to 8 hours. The normalized absorbance intensity at $\sim 1170\text{ cm}^{-1}$ for individual control cells was 0.005.

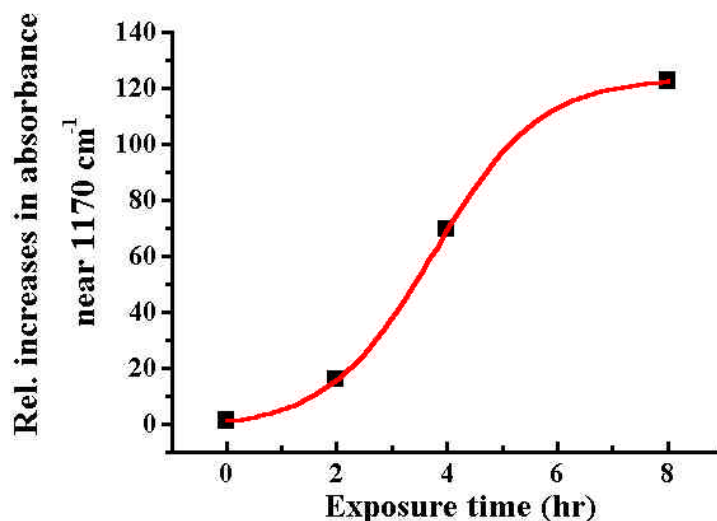


Figure 6. Increasing absorption intensity at about 1170 cm^{-1} with increasing exposure time to coal tar (The curve-fit is for visualization purpose).

4. CONCLUSIONS

We have succeeded in using SR FTIR spectromicroscopy to identify specific signals (in the $1250\text{-}1000\text{ cm}^{-1}$ infrared region) that represent the intracellular response to PAH and OC exposure. Further development will allow the technique to distinguish biological responses to other different environmental toxins. Our next step is to construct a micro-fabricated device that can guide the biological cell growth so that the IR signal at $\sim 1170\text{ cm}^{-1}$ from individual cells is consistent and reproducible for a given set of exposure conditions. This could lead to an anticipated saving of time and money especially when a large number of samples are analyzed as a component of a large-scale study of potential exposure to PAHs and OCs.

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