Depth Profiling of Mammalian Cells by Photoacoustic Spectroscopy: Localization of Ligands

K. Narayanan, S. Chandani, T. Ramakrishna, and Ch. Mohan Rao
Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

ABSTRACT Phase-resolved monitoring of photoacoustic signals can provide information about the depth profile of a sample. We describe an application of this principle to determine the depth profiles of ligands and antitumor agents in mammalian cells. Measurements of the in-phase and quadrature components of the photoacoustic spectra (which yield information from the surface and the interior, respectively) of a tumor cell line, AK-5, treated with the antitumor agent coralyne chloride have been made. They clearly show that the drug accumulates in the cell interior and is not seen on the cell surface, providing in situ evidence for the localization of this drug. Histochemical dyes which stain cells uniformly give identical in-phase and quadrature spectra; spectra of cells incubated with nuclear stains demonstrate a differential staining of the nucleus and the cytoplasm. These results demonstrate the usefulness of phase-resolved photoacoustic spectroscopy in monitoring differential interactions of drugs and other ligands with cells.

INTRODUCTION

Nondisruptive means of monitoring molecular events confer the advantage of presenting a view of molecular interactions in the context of complex milieus such as those found in biological systems. In the visible and UV regions, very few methods are available for investigating cellular events in situ, even though most probes yield results in these regions of the spectrum. A generalized method that is quantitative and nondisruptive and can utilize any probe capable of absorbing in the visible region would offer considerable insights into the working of biological/cellular systems.

Photothermal methods have emerged as useful tools for the optical and thermal characterization of transparent, opaque, or scattering materials (Braslavsky and Heihoff, 1989; Mandelis, 1992). The photothermal methods can be broadly classified into two groups: photoacoustic spectroscopy (PAS) and thermooptical techniques. In PAS, pressure waves resulting from temperature changes are detected; in thermooptical techniques refractive index gradients produced as a result of changes in temperature are measured. Photoacoustic spectroscopy is eminently suited to the study of opaque, weakly absorbing, and highly light scattering materials in the condensed, solution, or gas phases (Balasubramanian et al., 1984; Balasubramanian and Rao, 1986; Rao, 1987; Lachaine et al., 1993). The absorption of intensity-modulated optical radiation by the sample causes intermittent heat generation as a result of nonradiative deexcitations. The heat generated diffuses to the surface and causes pressure fluctuations in the gas surrounding a sample kept in an airtight cell of constant volume, which can be monitored by a pressure-sensitive detector. Scanning the sample around the wavelength of interest yields the photoacoustic (PA) spectrum. The heat liberated in the deeper layers of the sample takes longer to reach the surface; hence signals from the surface and those from deeper layers differ in phase. The magnitude of the phase difference depends on the depth and on the thermal diffusivity of the sample. These signals would thus provide information on the depth profile of the chromophores in the sample. There are a few examples wherein depth profiling of biological samples using PAS has yielded information on in situ structure/organization (Mackenthun et al., 1979; O'Hara et al., 1983; Anjo and Moore, 1984; Nery et al., 1987). The earliest such example was a study of the distribution of astaxanthin-bound proteins in the lobster shell (Mackenthun et al., 1979). These studies exemplify the potential of this technique for examining biological tissues. However, such studies at the cellular level, to the best of our knowledge, have not been reported. The method described here provides a means of localizing the antitumor agent coralyne chloride in situ and demonstrates the use of PAS in monitoring the differential interaction of histological stains and other ligands with normal and cancerous cells.

MATERIALS AND METHODS

Coralyne chloride was obtained from Sigma Chemical Co. (St. Louis, MO); toluidine blue O and methyl green were obtained from Hi Media Co. (Mumbai, India). All other chemicals were of analytical reagent grade.

Cells of the Zajdela ascitic hepatoma (ZAH), a chemically induced tumor of rat hepatocytes (Zajdela, 1964), and AK-5, a histiocytoma (Khar, 1986), were obtained by withdrawing ascitic fluid from the peritoneal cavity of CFY and Wistar rats, respectively. Cells were washed four or five times with and suspended in phosphate-buffered saline (PBS) (pH 7.4). Cell suspensions of normal rat liver were prepared mechanically (Jacob and Bhargava, 1962). The cells were washed and suspended in Earle's balanced salt solution containing 0.06 mM $CaCl_2$, 100 units/ml penicillin G, and 40 mM HEPES (pH 7.4). Cells were fixed by adding cell suspensions dropwise to chilled 70% ethanol with constant, gentle stirring.

Staining of the cells with toluidine blue O was performed following the method of Scragg and Ferreira (1991). Fixed cells were washed with a

Received for publication 2 December 1996 and in final form 11 February 1997.

Address reprint requests to Dr. Ch. Mohan Rao, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India. Tel.: +91-40-672241; Fax: +91-40-671195; E-mail: mohan@ccmb.globemail.com.

0006-3495/97/05/2365/04 $2.00

solution of 1% sodium tetraborate, suspended in a minimal volume of the same solution, and stained with 1% toluidine blue O. Cells were stained with methyl green as described by Gurr (1962). Excess stain was washed off with PBS. AK-5 cells (10×10^6) were incubated with coralyne chloride (100 $\mu g/10^6$ cells) and then washed with and suspended in a minimal volume of PBS. The cells were layered on a 20×3 mm strip of Hybond N+ membrane (Schleicher and Schuell, Inc.) previously soaked with PBS, and excess buffer was absorbed by filter papers placed below the strip. The strip was then transferred to a PAS sample holder.

PA spectra were recorded on an extensively modified OAS-400 PA spectrometer (EDT Research, London). The light beam from a 300-W xenon lamp was intensity-modulated using a continuously variable mechanical chopper (HMS 222; Ithaco, Ithaca, NY) operating at a chopping frequency of 30 Hz. The beam was monochromated and passed on to a sample cell (15 mm length, 5 mm width, 1.2 mm depth, machined in an aluminum block) with provisions for attaching a microphone. The microphone and the sample compartment were connected through a small opening (5 mm length, 1 mm diameter). The air column above the sample was 2 mm. The signals were recorded using a B and K 4165 microphone (Bruel and Kjaer, Naerum, Denmark) coupled to a power supply (B and K 2804). The sensitivity of the microphone was 50 mV/Pascal. The signal was passed through a preamplifier (EG and G 113; Princeton Applied Research, Princeton, NJ) and a lock-in analyzer (EG and G 5206), the phase resolution of which was $\pm 0.1°$ with a phase increment of 0.025°. Data were collected on an IBM PC. Normalization of the spectra to constant input light intensity was achieved by using the PA spectrum of carbon black.

RESULTS AND DISCUSSION

Depth profiling of samples using PAS can be carried out using two methods. Varying chopping frequency yields information at varying depths relative to the sample surface. The photoacoustic response is only sensitive to heat generated within the thermal diffusion length (μs) of the sample:

$$\mu s = (2\alpha/\pi f)^{1/2},$$

where α is the thermal diffusivity in cm^2/s, and f is the chopping frequency in Hertz. This length gives an approximate depth to which a sample can be probed. To achieve resolution on the order of a micron, assuming thermal properties of an aqueous environment, the chopping frequency required would be very high. Such high frequencies generate undesirable signal-to-noise ratios. An alternative method for obtaining information about the surface and interior of the samples is to monitor phase-resolved signals. This is done by recording in-phase (surface) and quadrature (interior) components of the photoacoustic signal. The magnitude of the PA signal can be expressed as

$$S(\phi)\lambda = S_0[\lambda]\cos\theta + S_{90}[\lambda]\sin\theta,$$

where $S_0[\lambda]$ and $S_{90}[\lambda]$ are the in-phase and quadrature components of the photoacoustic signal from the sample. It is thus possible to estimate contributions from various locations by varying the phase angle (ϕ) of the demodulator. In the present study we have used this approach to obtain information on the surface and interior properties of cells treated with dyes and antitumor agents. Fig. 1 shows the PA spectra of ZAH cells stained using the dye methyl green. Microscopic examination of the cells shows that they are uniformly stained. The in-phase and quadrature spectra of

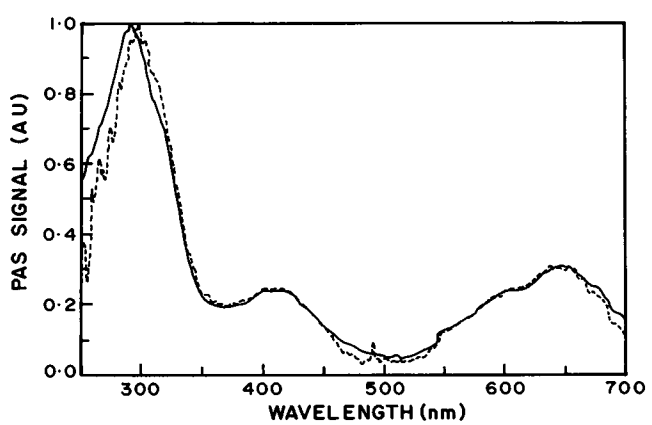

FIGURE 1 The in-phase (——) and quadrature (– – –) spectra of ZAH cells stained with methyl green. The spectra were intensity-normalized at 280 nm. Cells were processed as described in Materials and Methods.

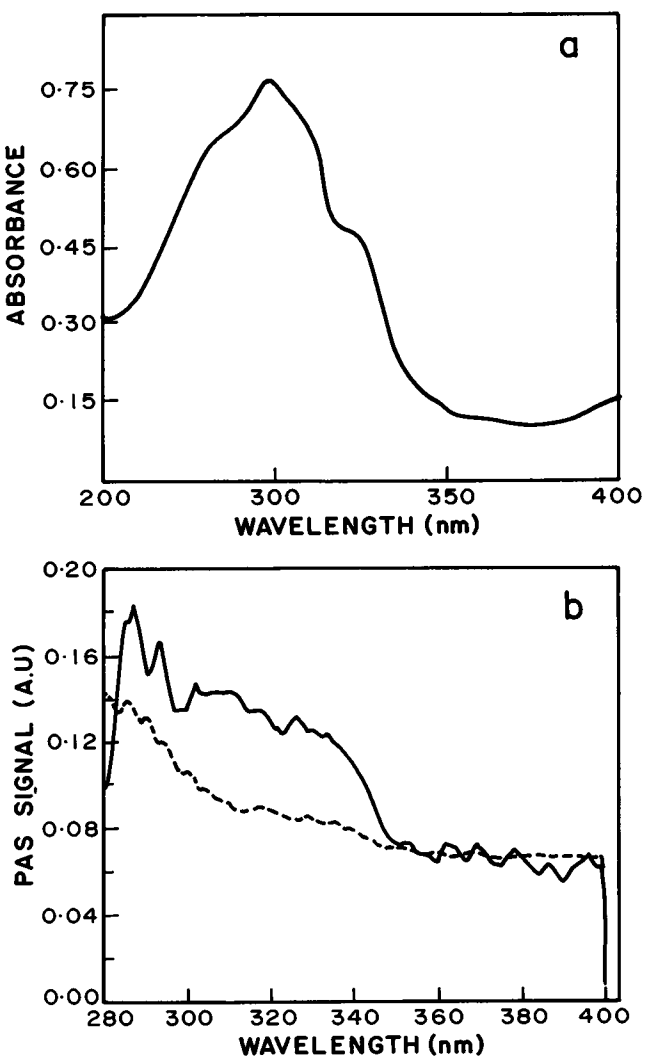

FIGURE 2 (*a*) The absorbance spectrum of coralyne chloride. (*b*) The in-phase (– – –) and quadrature (——) spectra of AK-5 cells incubated with coralyne chloride for 120 min.

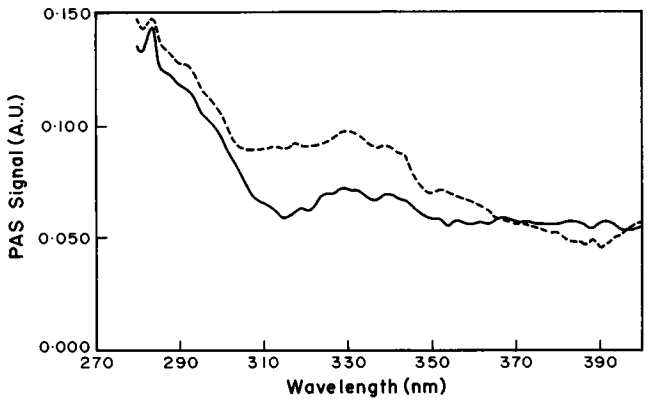

FIGURE 3 Difference quadrature spectra of AK-5 cells incubated with coralyne chloride for 40 min (——) and 120 min (– – –). Difference spectra were obtained as described in the text.

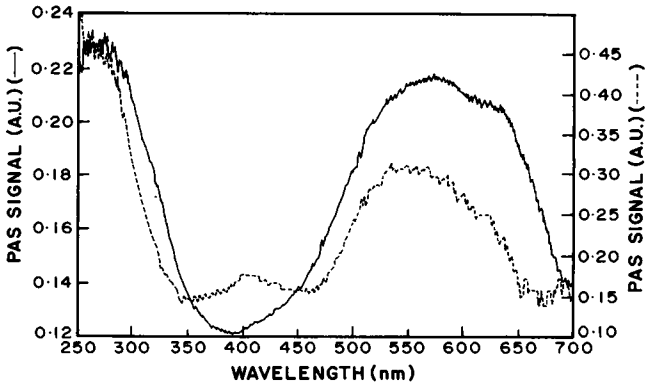

FIGURE 5 PA spectra of rat liver cells stained with toluidine blue O. ——, In-phase spectrum; – – –, quadrature spectrum.

the methyl-green-stained cells, intensity-normalized at 280 nm, overlap almost completely, showing that the dye is present not only on the surface, but also in the interior of the cell.

We next studied the effect of coralyne chloride, an alkaloid with antitumor properties (Zee-Cheng and Cheng, 1976), on AK-5 cells. Coralyne chloride has been shown to bind to DNA in vitro and to inhibit RNA polymerase activity (Wilson et al., 1976). It has been suggested previously that the antitumor activity of this alkaloid stems from its interactions with DNA (Wilson et al., 1976). The absorption spectrum of coralyne chloride shows a peak at 295 nm and a shoulder at 325 nm (Fig. 2 *a*). The in-phase and quadrature spectra of AK-5 cells treated with coralyne chloride for 2 h are presented in Fig. 2 *b*. Whereas the in-phase spectrum shows a weak signal in the 320–340-nm region, the quadrature spectrum shows a very strong signal in this region, suggesting that coralyne chloride accumulates in the interior of the cell. Quadrature spectra of AK-5 cells treated with coralyne chloride for 20, 40, and 120 min were recorded. The spectrum recorded at 20 min did not show any signal in the 320–340-nm region. There was a distinct signal in this region after 40 min of treatment with coralyne chloride, which increased upon incubation for 120 min. To observe these changes more clearly, we subtracted the quadrature spectrum recorded at 20 min from those recorded at 40 and 120 min. Fig. 3 shows an increase in the PA signal in the 320–340-nm range upon incubation with coralyne chloride. Other cellular chromophores, such as NADH, NADPH, and $FADH_2$, also absorb in the same region. However, PA spectra of untreated cells as well as those of cells incubated with coralyne chloride for short periods of time (up to 20 min) do not show a detectable signal in this range. The increase in PA signal in the 320–340-nm region observed by us after extended incubation with coralyne chloride can thus be attributed to arise from the alkaloid moiety. Measurements of the amount of coralyne chloride remaining in the supernatant and subsequent buffer washes suggest that approximately 2×10^{10} molecules of coralyne chloride per tumor cell could be detected.

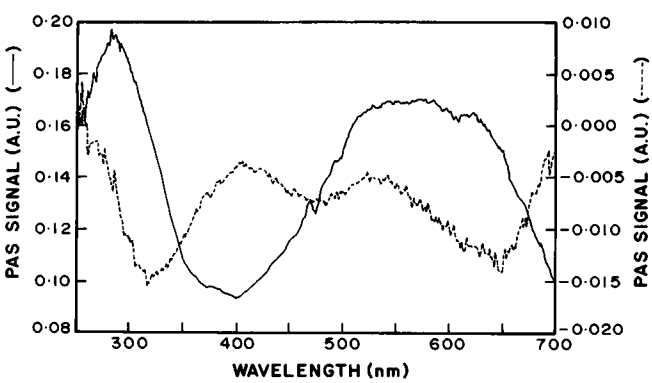

FIGURE 4 PA spectra of AK-5 cells stained with toluidine blue O. ——, In-phase spectrum; – – –, quadrature spectrum.

To further examine the usefulness of PA spectroscopy in depth profiling of cells, we studied the toluidine blue O staining of some normal and tumor cells. This dye stains the cytoplasm blue and the nucleus red. The in-phase spectrum of AK-5 cells stained with toluidine blue O shows a broad peak around 550 nm and a peak at about 280 nm (Fig. 4). The quadrature spectrum shows peaks around 550 nm and 280 nm and, in addition, a peak between 400 and 450 nm, due to the differential staining of the nucleus. We have also examined liver cells with the same stain (Fig. 5). Here, too, we see a similar trend, with minor differences in relative intensities. Whereas the quadrature spectra in Figs. 4 and 5 show the peak in the 400-nm region distinctly, there is a significant difference in the relative intensities of the 550-nm and 400-nm peaks. The nuclei of the rapidly dividing tumor cells are known to be large because of differences in ploidy, which could account for the large 400-nm signal seen in AK-5 cells (Fig. 4).

Photoacoustic spectroscopy has been employed in the depth profiling of biological tissues. Frequency modulation has been more frequently used in depth profiling. Mackenthun et al. (1979) studied the organization of lobster shell

carotenoproteins in situ and demonstrated the anisotropic distribution of the pigment astaxanthin bound to specific proteins within the endocuticle. Anjo and Moore (1984) used this method to distinguish signals from β-carotene, hemoglobin, and melanin at different depths from the skin surface. The segregation of distinct pigment layers in the lichen *Acarospora schleicheri*, symbiotically associated with a green alga, was also demonstrated by frequency modulation (O'Hara et al., 1983). On the other hand, Nery et al. (1987) used phase-resolved photoacoustic spectroscopy in their studies to locate herbicides in leaves. Our study is the first attempt to use PA spectroscopy to investigate the effects of drugs such as coralyne chloride on tumor cells and demonstrates the usefulness of phase-resolved PA spectroscopy in locating drugs within or on the surface of cells. It also points to the application of this technique in monitoring differential interactions of other ligands with cells.

REFERENCES

Anjo, D. M., and T. A. Moore. 1984. A photoacoustic depth profile of β-carotene in skin. *Photochem. Photobiol.* 39:635–640.

Balasubramanian, D., and Ch. M. Rao. 1986. Application of photoacoustics to biology—some specific systems and methods. *Can. J. Phys.* 64: 1132–1135.

Balasubramanian, D., Ch. M. Rao, and B. Panijpan. 1984. The malaria parasite monitored by photoacoustic spectroscopy. *Science.* 223: 828–830.

Braslavsky, S. E., and K. Heihoff. 1989. Photothermal methods. *In* Handbook of Organic Photochemistry, Vol. 1. J. C. Scaiano, editor. CRC Press, Boca Raton, FL. 327–355.

Gurr, E. 1962. Staining Animal Tissues. Leonard Hill, London.

Jacob, S. T., and P. M. Bhargava. 1962. A new method for the preparation of liver cell suspensions. *Exp. Cell Res.* 27:453–467.

Khar, A. 1986. Development and characterization of a rat histiocytoma-macrophage tumor line. *J. Natl. Cancer Inst.* 76:871–878.

Lachaine, A., R. Pottier, and D. A. Russell. 1993. Photoacoustic and fluorescence spectroscopy for biological systems. *Spectrochim. Acta Rev.* 15:125–151.

Mackenthun, M. L., R. D. Tom, and T. A. Moore. 1979. Lobster shell carotenoprotein organisation in situ studied by photoacoustic spectroscopy. *Nature.* 279:265–266.

Mandelis, A. 1992. Principles and Perspectives of Photothermal and Photoacoustic Phenomena. Elsevier Scientific, New York.

Nery, J. W., O. Pessoa, Jr., H. Vargas, F. A. M. Reis, A. C. Gabrielli, L. C. M. Miranda, and C. A. Vinha. 1987. Photoacoustic spectroscopy for depth profile analysis and herbicide monitoring in leaves. *Analyst.* 112:1487–1490.

O'Hara, E. P., R. D. Tom, and T. A. Moore. 1983. Determination of the in vivo absorption and photosynthetic properties of the lichen *Acarospora schleicheri* using photoacoustic spectroscopy. *Photochem. Photobiol.* 38:709–715.

Rao, Ch. M. 1987. Photoacoustic spectroscopy—instrumentation. *J. Instrum. Soc. Ind.* 17:219–227.

Scragg, M. A., and L. Ferreira. 1991. Evaluation of different staining procedures for the quantification of fibroblasts cultured in 96-well plates. *Anal. Biochem.* 198:80–85.

Wilson, W. D., A. N. Gough, J. J. Doyle, and M. W. Davidson. 1976. Coralyne. Intercalation with DNA as a possible mechanism of antileukemic action. *J. Med. Chem.* 19:1261–1263.

Zajdela, F. 1964. L'emploi d'hépatomes ascitiques en cytologie du cancer. *In* Colloque Franco-Soviétique: quelques problèmes posé par la cellule cancéreuse. Gauthiers-Villars, Paris. 914–920.

Zee-Cheng, K. Y., and C. C. Cheng. 1976. Tetramethoxydibenzoquinolizinium salts. Preparation and antileukemic activity of some positional and structural isomers of coralyne. *J. Med. Chem.* 19:882–886.