

Broad-Band Attenuated Total Reflection Spectroscopy of a Hydrated Protein Film on a Single Mode Planar Waveguide

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Received February 7, 1996. In Final Form: April 19, 1996[⊗]

The development of a multichannel spectrometer based on single mode planar integrated optical waveguide and capable of measuring broad-band visible absorbance spectra of a weakly absorbing molecular film at a solid-liquid interface is reported. The system was successfully tested by measuring spectra, over a 100 nm bandwidth, of a hydrated film of cytochrome *c* adsorbed to a glass waveguide surface. The approach should prove useful in spectral characterization of structure and function in thin film molecular assemblies at condensed phase interfaces.

Multimode planar and cylindrical waveguides have been widely employed for characterization of interfacial samples using attenuated total reflection (ATR) spectroscopy.¹ The single mode planar integrated optical waveguide (IOW) is an inherently more sensitive geometry.² At visible wavelengths, a single mode planar IOW supports up to several thousand total internal reflections per centimeter of beam propagation.³ This reflection density is orders of magnitude greater than conventional (i.e., much thicker) multimode waveguides, and yields a concomitantly larger ATR path length.⁴

This sensitivity advantage has been exploited in the application of planar IOW-ATR techniques to research in thin film structure, surface characterization, and chemical sensing.⁴⁻⁶ A major factor that has limited wider use is the difficulty of measuring broad-band spectra. Since the mode propagation angle in an IOW is dependent on wavelength,² the mode coupling angle is also a function of wavelength. A conventional prism or grating coupler does not usually compensate for the waveguide dispersion and is therefore only efficient for a very narrow range of

wavelengths. Consequently, almost all previous IOW-ATR-based studies have utilized a laser as the source.^{4,5}

A few previous attempts have been made to implement broad-band IOW-ATR spectroscopy using an incoherent lamp as the source. Piraud et al.⁶ examined a highly absorbing phthalocyanine film (ca. 20 monolayers) deposited on the surface of a channel IOW. Light was launched into the IOW using a single mode optical fiber butt-coupled to the waveguide endface. Kato et al.⁷ used a multimode optical fiber to deliver light over a 2.5° range of input angles to a prism-coupled planar IOW, on which was deposited a Langmuir-Blodgett film doped with a Cu-porphyrin. Both systems used a scanning monochromator for wavelength selection and a single channel detector, an arrangement that negates the inherent multichannel advantage of a single mode IOW. (Since each spectral component coupled into a single mode IOW propagates at a different reflection angle, the IOW itself functions as a dispersion device. Thus in principle, a monochromator is not needed.) Equally important is that in both systems, the scheme employed for incoupling a broad spectral bandwidth was very inefficient.

Here we report development of a multichannel, planar IOW spectrometer that is capable of measuring broad-band visible absorbance spectra of a weakly absorbing molecular film at a solid-liquid interface. The concept was successfully demonstrated by measuring spectra (100 nm bandwidth) of a hydrated film of cytochrome *c* (cyt *c*) adsorbed to a glass surface.

The heart of our system (Figure 1) is a modified version of a recently reported achromatic planar IOW input coupler.⁸ This device, which consists of a prism and a pair of diffraction gratings, cancels the IOW chromatic dispersion up to the second order, which produces a large incoupling spectral bandwidth. The achromatic input coupler is used to couple a loosely focused beam (NA = 0.04) from a 150 W Xe lamp into a glass planar IOW⁹ that supports a single guided mode across the visible spectrum. After propagation in the waveguide, the beam is simultaneously outcoupled and dispersed at an output diffrac-

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⊗ Abstract published in *Advance ACS Abstracts*, June 15, 1996.

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(3) Reflection densities are computed using a ray optics model, which describes light propagation in an IOW as a process of repeated total reflection at the boundaries between the waveguiding layer and the adjacent media.^{2b}

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(7) Kato, K.; Takatsu, A.; Matsuda, N.; Azumi, R.; Matsumoto, M. *Chem. Lett.* **1995**, 437-438.

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(9) A Corning 7059 waveguiding film, 400 nm thick, was fabricated by rf sputtering deposition on a 1 mm thick fused silica substrate.⁸

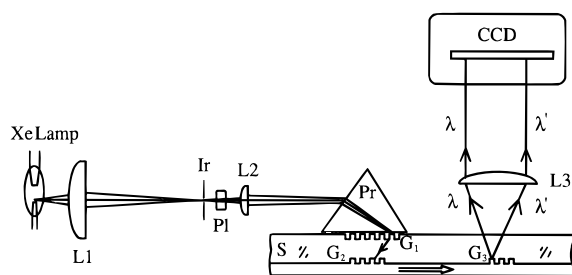


Figure 1. Schematic of a broad-band IOW-ATR spectrometer. L1 = 10 cm focal length lens, Ir = iris, P1 = polarizer, L2 = 17 cm focal length lens, Pr = prism, G1–G3 = diffraction gratings, S = IOW on substrate, CCD = TE-cooled, charge-coupled array detector, L3 = 25.4 mm focal length cylindrical lens. Pr, G3, and S are described in ref 8. Grating parameters: G1, period = 1.921 μm , depth = 0.34 μm ; G2, period = 2.252 μm , depth = 0.12 μm . The distance between the input and output grating couplers is 8 mm.

tion grating, and then collected by a cylindrical lens. A CCD chip is placed at the lens back focal plane, where the spectral components are spatially separated and simultaneously detected. A liquid flow cell (not shown) clamped to the waveguide-coupler apparatus allows the solution in contact with the waveguide surface to be exchanged. The evanescent field that penetrates into the liquid phase is used to probe molecular films at the waveguide–solution interface.

We developed the broad-band IOW-ATR spectrometer specifically to enable spectral studies of hydrated heme protein films. It is well recognized that several biotechnologically important areas, such as transduction in biosensors, affinity-based separations, and materials biocompatibility, are governed by the structural and functional properties of proteins accumulated in a thin film at a solid–liquid interface.¹⁰ These properties (i.e., conformation, orientation, bioactivity) are in turn known to be dependent on the physical and chemical properties of the interface, but these relationships are not well understood, primarily because *in situ* study of protein films is a technically difficult challenge.¹⁰ Elucidating these relationships is a prerequisite to the projected use of such films in molecular device technologies.^{10a}

Heme-containing proteins have been frequently utilized as models for studying structure and function in immobilized protein films.^{11–14} Visible absorbance spectroscopy is one of several experimental techniques that have been employed¹¹ and is a potentially powerful

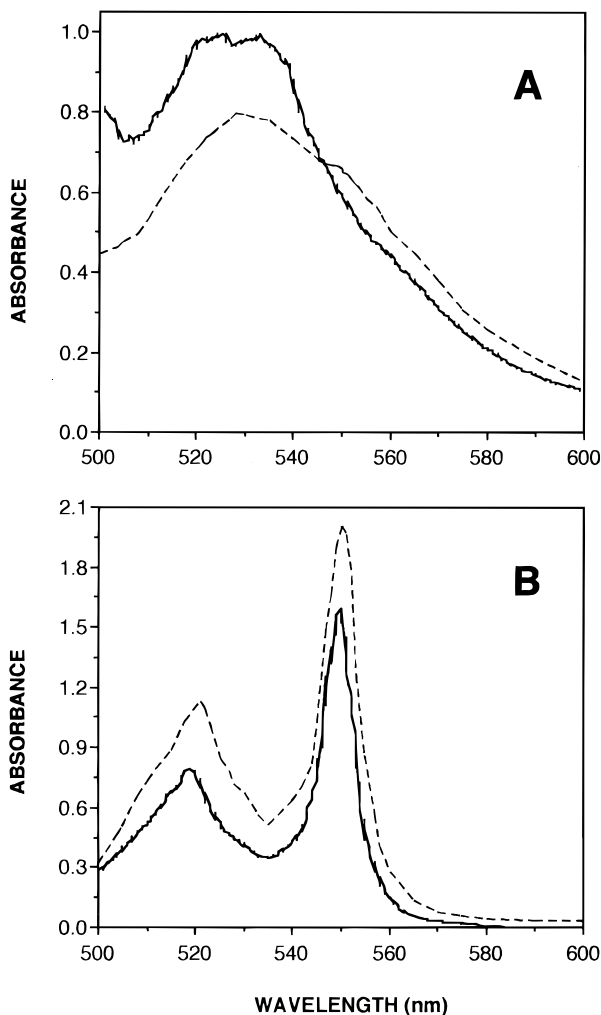


Figure 2. Spectra acquired with the broad-band IOW-ATR device (plotted as solid lines), of cyt *c* adsorbed to glass before (A) and after (B) injection of sodium dithionite into the flow cell, which contained pH 7 phosphate buffer. Also shown are the spectra of dissolved ferricyt *c* (A) and ferrocyanocytocrome *c* (B) (plotted as dashed lines). Dissolved spectra were calculated from published molar absorptivity data²¹ (i) assuming a protein surface coverage of one monolayer and a geometrically random molecular orientation, (ii) using a ray optics model³ for light propagating in the lowest order, transverse electric (TE) waveguide mode, and (iii) applying a correction for wavelength-dependent differences in evanescent electric field strength.^{4,10b}

approach, since the position, shape, and intensity of the heme absorption bands in the 350–600 nm range are markers of ligand binding, the oxidation state of the central metal ion, and protein conformation.¹⁵ However, in the transmission geometry commonly used for protein films supported on planar substrates, low sensitivity is an inherent and significant limitation.¹⁶ The initial results we have obtained for cyt *c* adsorbed to a hydrophilic glass waveguide surface¹⁷ show that our IOW-ATR system circumvents this limitation. The experiment was performed as follows: After the flow cell was filled with

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(16) The surface coverage of a close packed monolayer of a small, globular protein is $\leq 10^{-11}$ mol/cm².^{10b} In a transmission experiment on a planar substrate (both sides coated), the pathlength is only 2 monolayers thick, which is less than 100 \AA .^{11a–c}

(17) Before each experiment, the IOW surface was cleaned with PCC-54 surfactant (Pierce), rinsed in deionized water, and dried under N₂.

phosphate buffer and a reference spectrum collected, a ferricyt *c* solution¹⁸ was injected into the cell and allowed to incubate for 30 min. The cell was then flushed with fresh buffer and a spectrum of the protein adsorbed to the waveguide surface was acquired.¹⁹ An 8 mM solution of sodium dithionite in buffer was then injected to reduce the adsorbed protein, and another spectrum was acquired immediately.

The adsorbed protein spectra shown in Figure 2 were obtained following application of a stray light correction and subtraction of the reference. A broad, featureless absorption band is observed for adsorbed ferricyt *c*; two narrower bands centered at 520 and 550 nm are present in the spectrum of ferrocyc *c*. Spectra calculated for a film of the native (dissolved) protein are also plotted in Figure 2.²⁰ The ratio of absorbances at the peak of the band near 550 nm in the adsorbed spectra (ferro/ferri) is 2.8. Although this ratio agrees reasonably well with the

corresponding ratio of 3.1 in the calculated spectra, it suggests that not all of the adsorbed protein can be reduced. Furthermore, the major band in the adsorbed spectrum of ferricyt *c* appears shifted relative to the native protein spectrum. This shift may be evidence of conformational changes induced by adsorption to the waveguide surface.^{10a-e} Although their origin is unknown at this time, these spectral differences illustrate one of our major motivations for developing the broad-band IOW-ATR method, to enable studies of adsorbed protein conformation, and will be a subject of continued investigation in our laboratories.

In conclusion, the adsorbed spectra in Figure 2 demonstrate that the very high sensitivity of the IOW-ATR geometry can be extended to the broad-band regime. The ability to measure visible ATR spectra of weakly absorbing molecular films in contact with a gas or a liquid should have a significant impact in several areas, including: (i) spectral characterization of structure-property relationships in thin film molecular assemblies and (ii) chemical and biochemical sensing for multiple analytes that can be spectrally resolved and thus detected simultaneously.

Acknowledgment. S.B.M. gratefully acknowledges support from CAPES (Brazilian Agency Supporting Graduate Programs). This work was supported by the National Science Foundation (Grant CHE-9403896 to S.S.S. and L.L.) and the National Institutes of Health (Grant R29 GM50299 to S.S.S.).

LA9601162

(18) Ferricyt *c* from horse heart (Sigma) was dissolved in sodium phosphate buffer (50 mM, pH 7.2), purified on a Sephadex G-25 gel filtration column, and diluted to a final concentration of 50 μ M in the same buffer.

(19) Under these conditions, a protein surface coverage of approximately 1 monolayer is obtained. Lee, J. E.; Saavedra, S. S. Manuscript submitted for review.

(20) We investigated the influence of the imaginary part of the refractive index of the protein film, assuming a surface coverage of 1 monolayer. The absorbances at 550 nm, where the molar absorptivity of ferrocyc *c* is greatest in the spectral region of interest, were calculated using a rigorous wave model (Li, L. *J. Opt. Soc. Am. A* **1994**, *11*, 984-991) and the ray optics model. Good agreement was found (a difference of ca. 10%), which is expected because of the extremely weak absorbance of the monolayer film.

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