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RESEARCH REPORT

Detection of PAH Carcinogens at the Single Molecule Level

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Introduction:

Polycyclic aromatic hydrocarbons (PAH-s) form an important class of chemical carcinogens in view of their ubiquitous nature and assimilation by human beings through environmental pollution, smoking, and food constituents. Benzo[a]pyrene (BaP), is one of a family of PAH-s which undergo metabolic activation in vivo and bind covalently to DNA as well as proteins, causing mutations and possibly cancer. In our laboratory we have developed (Dasari RR, 1994, Ozbal CC, 1995) a cryogenic fluorescence spectroscopy technique to quantitatively determine sub-femtomole amounts of BaP adducts of DNA and proteins. We have observed that while synthetic adducts invariably gave a single, strong, symmetric band at 377nm, real-life samples sometimes showed subtle differences in their positions as well as shapes, indicating the presence of possible different types of adducts in different samples. If the nature and source of these differences could be identified it might become possible to get information on the different types of *in vivo* metabolic pathways and different PAH exposure patterns. A suitable method to carry out such characterisation would be to separate and study the individual adducts. Since the total amount of adducts available will frequently be in the subfemtomole range, separation and detection present formidable difficulties. We have therefore set up an HPLC- LIF system for studies involving samples in the picogram range. Some of the initial results are presented here.

Experimental:

An HP-1050 pumping module with an HP-1040A detection system and a Waters C18 (3.9X300mm) column were used for the HPLC separation. A Liconix 3230 He-Cd laser operating at 325nm was used for excitation. The output from the column was passed through a capillary (200 m dia.) at flow rates of 0.25-0.5 ml per minute. The laser (10mW) was focused on to the capillary and fluorescence emission was collected by a 40X (NA 0.75) microscope objective. The fluorescence at 377nm was detected using a Spex-1681 model (0.22m) monochromator and R-928 cooled photomultiplier operating at 900-1000 volts. A solution of benzopyrene derivatives was used to test the performance of the system.

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✹ RESEARCH REPORT

The Thermoelastic Basis of Ablation of Biological Tissue

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Laser radiation is currently used in several fields of medicine including ophthalmology, dermatology, urology, and cardiology. However, the interaction between laser light and biological tissue is not completely understood. The fundamental mechanisms of laser ablation are under current debate. In order to optimize laser parameters (wavelength, pulse length, fluence), an understanding of the ablation process is needed. In this article we present our latest results regarding the mechanism of short pulse laser ablation.

A review of experimental results reported in the literature reveals that the energy density required to initiate ablation of biological tissue with nanosecond laser pulses is tenfold less than that required for vaporization. This holds for a wide range of laser wavelengths [Albagli et al, 1994a]. An explanation for this discrepancy is that vaporization (a photothermal process) does not occur at all, and the material ruptures when laser-induced stresses and stress gradients exceed the material's strength. A one dimensional photomechanical model of laser-induced spallation correctly predicts the reduced energy density, but also predicts that damage should occur approximately one absorption depth beneath the surface [Dingus et al, 1991]. In fact, ablation occurs at or near the surface. For lasers and wavelengths used in ablating biological tissue, the optical absorption depth is usually comparable to the transverse laser dimension, and a one dimensional approximation is not appropriate. The discrepancies can be reconciled by including three dimensional effects. We have solved the fully time dependent three dimensional thermoelastic equation of motion, which predicts significant tensile stresses to form on the surface, (precisely where ablation is observed to occur), and also predicts the thermoelastic expansion of the surface as a function of time.

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The Spectroscopy Laboratory houses two laser research resource facilities. The MIT Laser Research Facility, supported by the National Science Foundation, provides shared facilities for core researchers to carry out basic laser research in the physical sciences. The MIT Laser Biomedical Research Center, a National Institutes of Health Biomedical Research Technology Center, is a resource center for laser biomedical studies. The LBRC supports core and collaborative research in technological research and development. In addition, it provides advanced laser instrumentation, along with technical and scientific support, free of charge to university, industrial and medical researchers for publishable research projects. Write or call for further information or to receive our mailings, (617) 253-9774.

The surface expansion of tissue immediately after short-pulse laser irradiation is related to the thermal gradient which causes internal stresses and mechanical properties of the tissue. Measuring the surface expansion for fluences below ablation threshold reveals information on these parameters. An interferometric technique, with spatial resolution $\approx$ 3 nm and temporal resolution $\approx$3 ns, has been developed to monitor the surface of tissue [Schaffer et al, 1995]. Laser induced thermoelastic expansion is measured by a Michelson interferometer which uses a He-Ne probe laser, the sample is the end mirror in one arm, and a rotating corner cube prism is included in the reference arm. By causing the reference arm length to vary at a constant velocity, the system is given a built-in bias which allows the direction as well as the speed to be measured; thus, the surface position as a function of time can be determined. The thermal input pulse is a 7.5 ns, frequency tripled, Q-switched Nd:YAG laser operating at a wavelength of 355 nm.

Using a fully time dependent numerical solution of the thermoelastic wave equation based on the Adams-Bashforth time stepping method [Albagli, 1994], we can predict the surface expansion of a material given the incident laser profile and material constants. In figure 1 this numerically predicted surface expansion is shown as a function of time for a rounded top-hat laser profile and some typical material parameters. From features of this curve, some unknown material parameters, can be inferred using the known beam radius, w [Itzkan et al, 1995]. These parameters are longitudinal and transverse speeds of sound, C_l and C_t, Poisson's ratio, s, optical penetration depth, D, and the ratio of the expansion coefficient to the heat capacity, β/C_V. Finally, the stresses can be calculated from the now known material properties and the quasi-steady state displacement, F_4.

The interferometer was first calibrated using glass and acrylic as samples. Experimental results compared well with theoretical predictions. The measured quasi- steady state values are plotted in figure 2 versus fluence for acrylic and glass. The relation between fluence and displacement is linear as expected. Also in this figure is the theoretically predicted value of this linear relationship as

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The Thermoelastic Basis …

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Figure 1. The time evolution of the thermoelastic expansion, predicted by the three-dimensional model allows the determination of mechanical, optical, and thermal properties from features in the surface movement. These features also allow determination of the magnitude of the induced stresses. Quasi steady-state displacement is normalized to 100.

Figure 2. Quasi steady-state displacements of acrylic and glass as a function of fluence.

calculated from the manufacturer's data for these materials, with no free parameters. For glass, the agreement is excellent, even to ablation threshold. In acrylic, there is initial agreement at low fluences, but an as yet undetermined non-linear effect causes the displacements to be lower than expected at high fluences. Also, for acrylic there is a larger spread in the data with each subsequent shot at the same location, each one producing less thermoelastic expansion. Small permanent defects, observed with a microscope, are created in acrylic by sub-threshold laser pulses.

The acrylic samples were studied microscopically, and a large number of defects were observed. Under high magnification the defects appear to be fracture patterns. They are concentrated near the surface of the sample, and the number of defects increases with subsequent laser pulses. Defect formation also correlates with the non-linear fall-off of quasi-steady state displacement as a function of fluence shown in figure 2. These "microcracks" weaken the overall strength of the acrylic, and their accumulation may play a significant role in the ablation process, called the "incubation

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effect," where a material does not seem to be affected by n pulses at a given sub-threshold fluence, but then ablates on the n+1 pulse.

An analogous effect to the microcrack in hard tissue is cavitation in soft tissue. The thermoelastic surface expansion of meniscus, a soft biological tissue, showed an unexpected feature; and additional expansion lasting 1-4 μs. These results are described in reference 3 in detail. Laser fluences used were very low, corresponding to a temperature of $<10^{o}$ C. Water doped with $FeCl_3$ was also used to determine whether the anomalous behavior in meniscus could ascribed to water which comprised 70% of meniscal tissue. The same feature which appeared in mensicus was observed in water and can be explained by the creation of cavitation bubbles. The observed times of growth and decay are consistent with the times for formation and collapse of cavitation bubbles. Cavitation is created in water under tension. Since the collapse of cavitation bubbles is known to be destructive to adjacent solid material, the presence of cavitation at such low fluences may have serious consequences for medical laser procedures.

We studied the onset of ablation in beef cortical bone, a tissue whose behavior is similar to that of acrylic. Figure 3 shows typical surface displacement of bone. The quasi-steady state displacement was normalized to 100 for several fluences, and time dependent behavior is identical throughout the fluence range. The theoretically predicted movement is compared to the measured movement in figure 3. However, the predicted alternating contractions and expansion were "washed out." For a turbid media such as bone, scattering within the target will change the temperature distribution and blur the sharp temperature gradient at the radial edge of the laser beam. The temperature distribution was calculated numerically using the absorption coefficient and the effective scattering coefficient for in a Monte Carlo calculation [Itzkan et al, 1995]. The predicted thermoelastic surface motion of the bone, using this new temperature distribution, is also plotted in figure 3. The agreement between theory and experiment considerably improved when scattering effects are taken into account, lending credence to the hypothesis that scattering is responsible for the "washing out" of the small expansions and contractions.

In the apparatus, the He-Ne probe beam can be translated across the sample's surface relative to the pump beam, so that it can be positioned at different radii, including radii outside the irradiated area. Even though the material receives no laser irradiation here, it shows displacement since it is pulled up by the "hot" adjacent material. Off-center monitoring allows us to observe the behavior of the rim during ablation. Although the surface is destroyed once ablation occurs and material is ejected, we can monitor the surface outside the irradiated area (r>w) to determine when ablation occurs. Figure 4 shows the motion of bone just

Figure 3. Surface movement of cortical bone compared to theoretical predictions which include a correction due to optical scattering. Four different fluences are displayed with quasi steady-state values normalized to 100.

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outside the irradiated area when irradiated with sufficient energy to cause ablation, compared to the sub-threshold theoretical curve. Ablation occurs at a time about 350 ns after the laser pulse. There is an abrupt departure from the theoretical curve, the surface at the rim undergoes a large contraction to a position 200 nm below the original surface position, followed by a slow recoil expansion for several microseconds to the limit to which we monitored time in this measurement. This experiment was repeated for acrylic and surface damage was observed under a microscope. The appearance of surface damage always corresponded with the large negative surface motion of the rim.

Interferometric surface monitoring is an important tool for studying the fundamental mechanisms of ablation of biological tissue. A three dimensional theoretical model for thermoelastic surface expansion after sub-ablation threshold laser irradiation has been developed which establishes the relationship of this movement to important mechanical and optical properties of the tissue. The surface movement of bone, glass, and acrylic were measured after irradiation with a 7.5 ns, 355 nm pulse. The results for glass and acrylic were quantitatively consistent with the theoretical model presented; although, acrylic did show a deviation from theory as defects were formed in the material. The results for bone were in good agreement with the theory after the effects of scattering were taken into account. Finally, the interferometric surface monitoring technique has the ability to monitor the ablation event by placing the probe beam outside of the pump beam area. Currently, further studies are underway to measure the optical and mechanical properties of bone, and to determine the threshold for cavitation and its effects in soft tissue ablation.

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Figure 4. Ablation of bone is monitored by placing the He-Ne probe beam outside of laser irradiated area (w=400 mm, r=700 mm). The fluence used, 35.4 mJ/mm^2, is the threshold value for causing ablation. At about 350 ns after the laser pulse, the cold rim recoils to a depth of -250 nm, and then expands. These motions are very large compared to the sub-threshold values and appear only when ablation occurs.

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Seminar on

MODERN OPTICS AND SPECTROSCOPY

FALL SEMESTER, 1994

February 21	Curt Wittig, University of Southern California **Ultraviolet Photochemistry of HI Dimers**
February 28	Mara Prentiss, Harvard University **Neutral Atom Photography**
March 14	Edward A. Hinds, Yale University **Testing Fundamental Symmetries with Molecules**
March 21	Ronald R. Parenti, Lincoln Laboratory, MIT **Adaptive Optics for Astronomy**
April 11	Richard Gottscho, AT&T **Getting into the Display Business (Again)**
April 18	Herbert Walther, Max-Planck-Institut Fur Quantenoptik **Single Atoms in Cavities**
April 25	Lev Perelman, Spectroscopy Laboratory **Photon Migration for Spectroscopy and Imaging**
May 2	David Wineland, National Institute of Standards **Entangled States for Spectroscopy and Computations**

May 9 Special Event

Fourth Annual Richard C. Lord Lecture

Richard Zare, Stanford University

"Structure" of the Nitric Oxide Ionization Continuum

TUESDAYS, 11:00-12:00, Marlar Lounge (37-252), Ronald E. McNair Building

Refreshments Served Following the Seminar

Sponsored by George R. Harrison Spectroscopy Laboratory,
Research Laboratory of Electronics, Schools of Science and Engineering,
Plasma Fusion Center and Industrial Liaison Program,
Massachusetts Institute of Technology
Rowland Institute for Science

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Lester Wolfe Workshops in Laser Biomedicine

Optical Probes in Biology and Medicine

Tuesday, May 2, 1995, 4:00-7:00 PM

Can Two-Photon Excitation Illuminate Medical Applications of Laser Microscopy
Watt W. Webb, Cornell University

Photon Migration in Turbid Media With Early Arriving Photons: Towards Optical Imaging Through the Human Body
Jun Wu, MIT Spectroscopy Laboratory

Resonance Raman and Gd Vibronic Sideband Spectroscopy as Probes of Protein Structure and Dynamics
Joel Friedman, Albert Einstein College of Medicine

Applications of Flash-Photolysis to Biological Photochemistry
Robert Redmond, MGH-Wellman Laboratories

HST Auditorium (E25-111), Whitaker College Building, MIT
45 Carlton Street, Cambridge

Refreshments at 3:30 P.M.

Sponsored by MIT Laser Biomedical Research Center,
MGH Wellman Laboratories, MIT Industrial Liaison Program, &
Harvard-MIT Division of Health Sciences and Technology

✸ PERSONALITIES

Dr. Steven R. Tannenbaum was born in New York City and began his lifetime association with MIT in 1954, receiving the S.B. degree in 1958 and the Ph.D. degree in 1962. This was followed by appointment to the faculty of the Department of Nutrition and Food Science (later to be renamed the Department of Applied Biological Sciences). Promotions ensued to Associate Professor and then Professor in 1974. He has been Registration and Admissions Officer of the both the Department of Applied Biological Sciences (1982-1988) and more recently of the Division of Toxicology (1988-present). He is also an Associate Director of the MIT Center for Environmental Health Sciences. Other MIT activities have included the Committees on Educational Policy, Academic Performance, and the committee that founded the Independent Activities Period.

Dr. Tannenbaum's research has spanned a variety of fields ranging from food chemistry to nutritional biochemistry to toxicology. Since the mid-1970's his main interests have centered on carcinogen chemistry and biochemistry, particularly compounds that are in air, food, and water. Research on the relationships between environmental exposure and human disease then led to studies on the endogenous synthesis of carcinogens. In particular, the investigation of the endogenous formation of N-nitroso compounds led to studies on the pharmacokinetics of nitrate and nitrite. A major discovery followed from these experiments: nitrate and nitrite were themselves synthesized in mammals de novo from reduced nitrogen species (NH_4^+ (NO_3^-), and the biosynthesis was stimulated by infection and/or inflammation. These findings contributed to the subsequent discovery by other investigators that the source of nitrate was in fact nitric oxide synthesized by oxidation of arginine.

It was the research on the very low levels of carcinogens that are found in people that led to his current program to develop biomarkers of carcinogen exposure at the sub-femtomole level. Early efforts with mass spectrometry gave excellent results for a number of different types of molecules, but another dimension was required. This was provided by the current collaboration with Dr. Ramchandra Dasari and his co-workers in the area of LIF. A number of approaches have yielded impressive results in the quantitative analysis of pyrene-containing structures at altomolar levels, and qualitative results on molecular structure using cryogenic laser-induced fluorescence (LIF) line-narrowing spectroscopy. A current project involves construction of a molecular and/or cell separation system that will push the envelope down to the single molecule level of detection. Support for all of this research has come from the National Cancer Institute, the National Institute of Environmental Health Sciences, and the Superfund.

STEVEN R. TANNENBAUM

In addition to his activities at MIT, Dr. Tannenbaum is very active at the national and international level of professional societies. He is currently on the editorial boards of four journals and has served on numerous committees of the American Association for Cancer Research. He is also currently a member of the Board of Scientific Counselors of the Division of Cancer Etiology of the National Cancer Institute, and tries to find some time for his two grandchildren, Bailey (5) and Seth (0.5) Hanselman. When not in the vicinity of MIT he can sometimes be found wandering the beaches of Cape Cod.

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Results and Discussion:

Figure 1 shows the HPLC detector output and corresponding absorption spectrum for a 2.75 nmole (total) sample. The absorption spectra of all the fractions were identical indicating that the sample contained four different pyrene derivatives. The fluorescence signal from 110 picomole (total) of sample is shown in Figure 2. The inset in Fig.2 corresponds to 11 attomole (total) of the same sample. Figure 2 gives a realistic estimate of the minimum detectable quantity with the present setup, which will be in the zeptomole range. But a more clear picture of the capability of the system can be obtained when we consider the signal output at any

Figure 1. HPLC peaks (bottom) and absorption spectrum (top) of a 2.75 nanomole (total) solution of benzopyrene derivatives. All the four HPLC peaks gave same spectra indicating that the sample contains four different pyrene derivatives only.

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instant of time for the 11 attomole sample. The laser beam is focussed to about 10 microns . With a capillary radius of 100 microns the volume of solution observed by the system at any time is about $3*10^{-7}$ml. Only about 4 attomoles contribute to the most intense peak, and this amount comes out of the system in about 0.25ml. (A flow rate of 0.5ml/minute, with peak half width of 30 seconds). Hence the quantity detected at any time is about $4*4*3*10^{-7}*6*10^{5} = 3$ molecules. This is not surprising, since, in one minute the sample travels about 17 meters and the time spent in the laser beam is about 35 microseconds. With life times of the order of 100 nseconds, each molecule thus gets excited several hundred times and emits a large number of photons. It is thus evident that the present system will be capable of detecting single molecules of BP with very little further effort.

An important consequence of the high sensitivity achieved in the present system is the possibility that it can be used to sort out intact cells containing PAH adducts. Cells from smokers are estimated to contain on an average about 1000 PAH molecules per cell, while non-smokers may have less than a few hundred molecules of PAH per cell. It will not be very difficult with improvements in the present system to carry out such sorting, enabling a preconcentration of adducted cells with any desired degree of adduction. The cells can then be examined for the variations in spectral properties, type and nature of adducts etc. as was mentioned earlier.

It should be noted that many single molecule detection experiments (Nie S, 1994, Fan F-RF, 1995) use relatively high concentrations of samples (nM-mM) and look at an extremely small volume (pico-zepto litres). That is, one has a large number of molecules at his disposal but wants to look at only one at a time. While this is necessary for study of dynamics of single molecules, in applications of detection and quantitation, one faces the reverse problem. That is one has only a very small number of molecules available and wants to look at all of them simultaneously. The two areas merge when a specific molecule can be localised at a given point in space and one can study it as long as necessary.

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Figure 2. Fluorescence signal from 110 picomole (total) of sample of Figure 1. Excitation He-Cd laser, 12 mW. Emission 377 nm. Inset shows the fluorescence signal from 11 attomole (total) of the same sample.

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