Martian Soil Biosensors Based on Dielectric Spectroscopy

by John H. Miller, Jr., and David Warmflash

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ABSTRACT—Researchers are studying the electromagnetic responses of live organisms, and the potential of such measurements to develop biosensors with applications in astrobiology and medicine. For example, dielectric spectroscopy measurements at different temperatures can distinguish live organisms from nonliving complex macromolecules and may eventually be suitable for *in situ* astrobiology studies on the surface of Mars or in the liquid ocean beneath the ice of Europa. More recent studies have involved nonlinear (harmonic generation) responses of biological systems to oscillatory electric fields. Some results suggest that active biological motors and other enzyme complexes generate harmonics over specific frequency ranges. These include complexes in the mitochondrial inner membrane, such as the molecular turbine ATP synthase and pumps in the outer plasma membrane. In addition, the harmonic generation spectra of chloroplasts, responsible for photosynthesis in plants, exhibit light-activated features. This provides evidence that the technique detects physiologically active processes, which could lead to fundamental advances in understanding of biochemical and other complex macromolecular systems.

MARS HAS BEEN A candidate for the possible existence of life, either in the distant past or at present,[1] for many decades. If life were to be discovered on Mars, the scientific implications would be profound for the distribution of life in the cosmos and the evolution of life on Earth. In 1976, the Viking program made an attempt to detect evidence for living or fossilized organisms in Martian soil, which yielded ambiguous, somewhat negative results.[2] The exciting, and more recent, studies[3] of the Martian meteorite Allan Hills 84001 (ALH84001) suggest that microbial life existed on Mars about four billion years ago. Reported evidence includes magnetite (Fe_3O_4) crystals found in carbonate globules and their associated rims in the meteorite.[4] About one fourth of these tens-of-nanometer sized magnetites are nearly identical to those produced by magnetotactic bacteria on Earth and are not expected to be produced by abiotic means. It has thus been argued that such Martian magnetite crystals are magnetofossils, which, if true, would constitute evidence of the oldest life forms known.[5]

Further studies indicate that subsurface Martian life could potentially survive even today.[6] There is geological evidence that ice was once deposited in the regolith, where it may be present above mid-latitudes.[7] This ice, which could extend several kilometers below the surface, might be a source of liquid water near magmatic intrusions.[8] On Earth, the biomass of subterranean organisms may even exceed that at the surface.[9] These organisms can live in highly saline conditions at temperatures from 115°C to –20°C.[10,11] Such conditions might prevail beneath the surface in an aquifer or hydrothermal system. Therefore, there is considerable interest in developing new techniques for detecting subsurface life on Mars. Furthermore, the likelihood that oceans of liquid water exist below the icy surfaces of Europa and other moons make these exciting candidates for possible extraterrestrial life.

This research is further motivated by the hypothesis that an oscillatory field induces proteins and other macromolecules to change conformation. The rate of conformational change depends on frequency and on each enzyme's charge distribution, structure, and state of activity. The resulting motion of charged macromolecules leads to a nonlinear response and to the generation of higher harmonics, providing a powerful functional spectroscopy tool. This hypothesis is supported by the following observations.

First, oscillatory fields induce ac components of transmembrane potentials that add to the intrinsic potentials.[12] A low-frequency electric field polarizes live cells or macromolecules,[13] resulting in enormous dielectric responses, and also modulates the membrane potential of each cell.[14] Second, sinusoidal fields can induce membrane pumps to translocate cations[15,16] and generate harmonics.[17] Membrane proteins exhibit nonlinear behavior[18] since domains with dipole moments interact with the induced transmembrane potential, driving them to change conformation. The combination of conformational changes and ion translocation creates a nonlinear response. For example, cation pumps such as P-type ATPases,[19] have been reported to generate harmonics.[20] We have developed a sensitive method,[21] using superconducting quantum interference devices (SQUIDs) to measure the harmonics produced by such membrane pumps at low frequencies. Further support is provided by our recent harmonic generation spectroscopy measurements, which will be discussed in the section "Results and Discussion."

Goal of the Project

The goal of this project is to study dielectric spectroscopy[22] and related methods, such as harmonic generation spectroscopy,[23] for the detection of live organisms. Toward this end, the project aims to identify and explain possible signatures of active macromolecular complexes unique to living biological systems. Possible signatures include unusual behavior, distinct from those of inanimate materials, in the frequency- and temperature-dependent dielectric response, and in harmonic generation spectroscopy, in which generated harmonics are plotted vs. frequency and amplitude.

Methodology

Previous reports focused on variable temperature dielectric spectroscopy of live organisms and Martian soil simulants. Our experiments on linear dielectric response employed a Solartron Impedance Analyzer, which measures complex admittance at frequencies up to 32 MHz. In this report, we report on harmonic generation spectroscopy measurements that suggest a potentially unique method of detecting physiologically active processes.

A four-electrode setup is employed in conjunction with a Stanford Research SR780 signal analyzer, operated as a spectrum analyzer, for measurements at kilohertz frequencies. A function generator applies a sinusoidal signal to the outer electrodes, while the voltage difference between the inner electrodes is fed into Channel 1 of the SR780, which records the induced harmonics. A reference spectrum is acquired using a supernatant, whose conductivity has been adjusted (with distilled water, to compensate for the volume fraction of the cells present in the sample) to be identical to that of the sample at the frequency of the point of interest. The supernatant typically consisted of an aqueous solution of ~1- 10 mM *NaCl*. Two different types of control files are used, depending upon whether the reference is to be logged using the same set of electrodes or a separate matched reference cell. In either case, the logging, windowing, and Fourier Transform routines were identical and provide a power spectrum of the reference cell, which is also recorded as a data file in the computer. Finally, the sample power spectrum obtained from the sample (*e.g.*, cell suspension or soil sample) of interest is divided by the reference power spectrum, and also stored. The entire procedure is automated using LabVIEW data acquisition software. The power of this approach stems from the fact that it allows the researcher to deconvolve the effects of nonlinearities within the electrochemical system from those attributed to the biological cells themselves.

Equipment and Special Technology

Most of our experiments at kilohertz frequencies employ the 4-electrode configuration shown in Fig. 1, where electrodes are immersed into a suspension of cells or organelles. In Fig. 1, the waveform generator applies a sinusoidal voltage of high spectral purity to the two outer electrodes, while the response across the inner electrodes is measured with a Stanford Research SR780, which shows the generated higher harmonics. Typically, the second or third harmonic generated by the suspension is recorded vs. amplitude and frequency, and all measurements are automated with LabVIEW software.

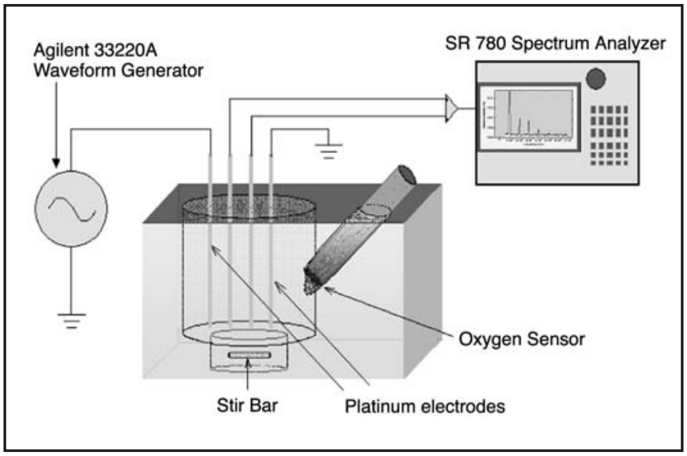

Figure 1. Schematic diagram of 4-electrode setup for *in vitro* measurements, in which platinum electrodes are immersed directly into a suspension (expanded scale) of cells, organelles, or reconstituted vesicles. The spacing between the outer electrodes is about 1 cm and the housing rests on a magnetic stirrer. The Agilent 33220A Waveform Generator produces a sinusoidal voltage of high spectral purity, while the Stanford Research SR780 Dynamic Signal Analyzer is operated as an FFT (fast Fourier transform) spectrum analyzer. Both instruments are interfaced to a computer (not shown) using a GPIB (general purpose interface bus). The experiments are automated with LabVIEW software. The oxygen sensor enables one to monitor the rate of oxygen consumption (or production for the case of chloroplasts), which can be correlated with the generation of harmonics created by enzymatic activity.

Experiments have been carried out on suspensions of whole cells, mitochondria, and chloroplasts, the latter of which provide validation and are of fundamental interest since the photosynthetic enzyme complexes are light activated. Many of these measurements have been carried out in collaboration with the group headed by Prof. William R. Widger in the Department of Biology and Biochemistry at the University of Houston. Additional experiments have been performed on whole organisms, such as plants and earthworms.

Results and Discussion

Some examples of observed harmonic generation spectra in the kHz range are illustrated by Fig. 2, which shows generated harmonics vs. applied frequency for *S. cerevisiae*, which lacks mitochondrial complex I, and uncoupled mammalian mitochondria, in which complexes I, III, and IV have been activated by glutamate malate but ATP synthase (complex V) is inactive due to the lack of a transmembrane potential. (We observed similar, though not identical, behavior by activating complexes II, III, and IV with succinate.)

Several features of the harmonic spectra are noteworthy. First, the spectral features generated by *S. cerevisiae* increase with cell concentration (upper left). We also find that potassium cyanide, which binds to complex IV and completely shuts down ATP production, suppresses the observed spectral features, as seen in the *B. indicas* data shown here. Second, the higher frequency feature (~12 kHz) observed in *S. cerevisiae* and *B. indicas* is not seen in uncoupled mitochondria, in which

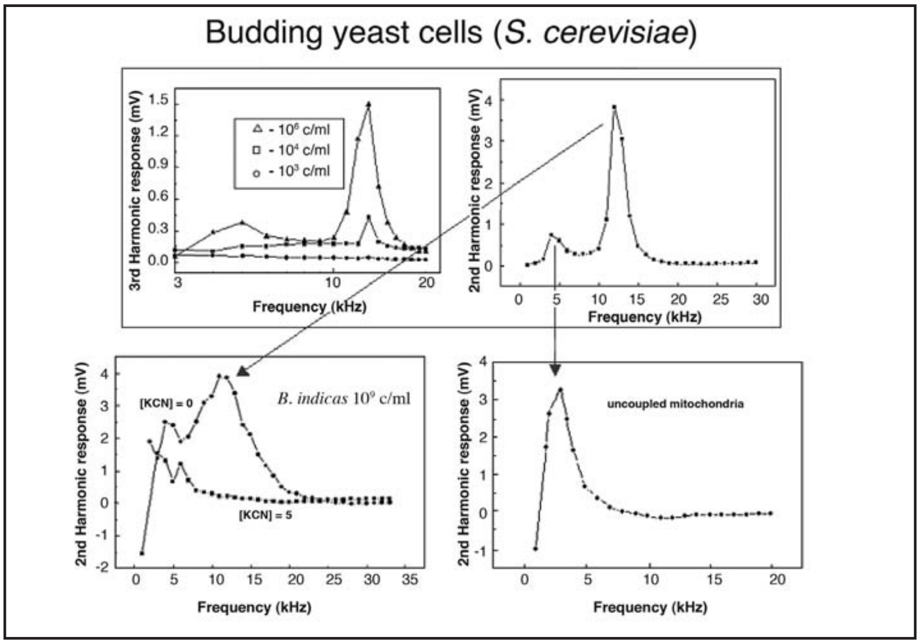

Figure 2. Harmonic generation spectra (applied field amplitude = 5 V/cm) of suspensions of budding yeast (*S. cerevisiae*, top), a relative of the mitochondrial ancestor (*B. indicas*, lower left, and uncoupled mammalian mitochondria, in which ETC complexes I, III, and IV have been activated by adding glutamate malate (lower right). The data were obtained with the 4-electrode setup shown in Fig. 2, in which a sinusoidal voltage is applied across the outer two electrodes and the induced harmonics measured across the two inner electrodes with a spectrum analyzer. The spectral features generated by *S. cerevisiae* are observed to increase with cell concentration (upper left).

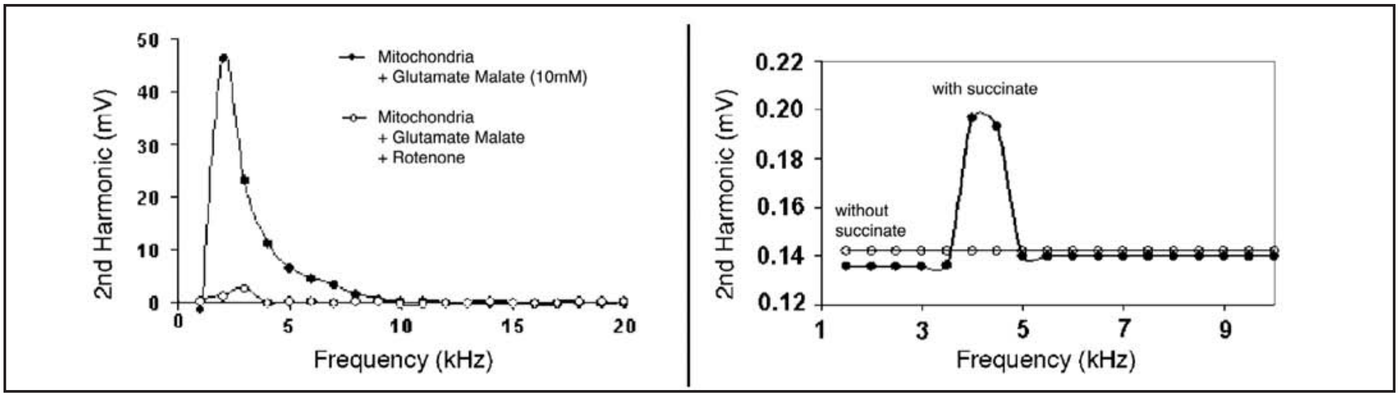

Figure 3. (*Left*) Generated second harmonic vs. fundamental frequency (applied field amplitude = 5 V/cm) of a suspension of uncoupled mammalian mitochondria, in which ETC complexes I, III, and IV are activated by adding glutamate malate (closed circles) and subsequently inhibited (open circles) by adding rotenone, which binds to complex I. Here the second harmonic was by far the largest of the higher harmonics, and the magnitude of the second harmonic response (2-40 kHz) was measured for applied frequencies ranging from 1 to 20 kHz. (*Right*) Generated second harmonic vs. fundamental frequency (field amplitude = 1 V/cm, 4-electrode method) of a suspension of uncoupled mammalian mitochondria before (open circles) and after (closed circles) activating ETC complexes II, III, and IV with succinate.

ATP synthase (complex V) is inactive. We hypothesize that the higher frequency feature is generated during ATP production by this molecular turbine. Finally, note that, although a lower frequency (~3 kHz) peak in harmonic generation spectra is present in *S. cerevisiae*, *B. indicas*, and uncoupled mitochondria, this feature is significantly smaller in *S. cerevisiae*. There could be at least two possible reasons for this. One is that complex I is missing in *S. cerevisiae*, and it may contribute to the more pronounced feature seen in *B. indicas* and mitochondria.

This is consistent with data obtained from uncoupled mitochondria, Fig. 3, in which either complexes I, III, and IV or complexes II, III, and IV are activated. In addition, the plasma membrane of a eukarote, such as *S. cerevisiae*, acts as a high-pass filter due to its finite capacitance. The data in Fig. 3 was also obtained with the 4-electrode method, in which a sinusoidal voltage is applied across the outer two electrodes and the induced harmonics measured across the two inner electrodes with a spectrum analyzer.

We find that potassium cyanide, which binds to complex IV and completely shuts down ATP production, suppresses the observed spectral features, as seen in the B. indicas data shown here. The higher frequency feature (~12 kHz) observed in whole cells is not seen in uncoupled mitochondria, where ATP synthase (complex V) is inactive. We hypothesize that the higher frequency feature is generated during ATP production by this molecular turbine.

Photosynthetic organisms have also proven extremely useful for validation of the technique, since photosynthesis and ATP production are activated by light. Figure 4 shows examples of the second harmonic generation spectra of a whole leaf and a suspension of spinach chloroplasts, which show dramatic differences between the spectra with and without light activation.

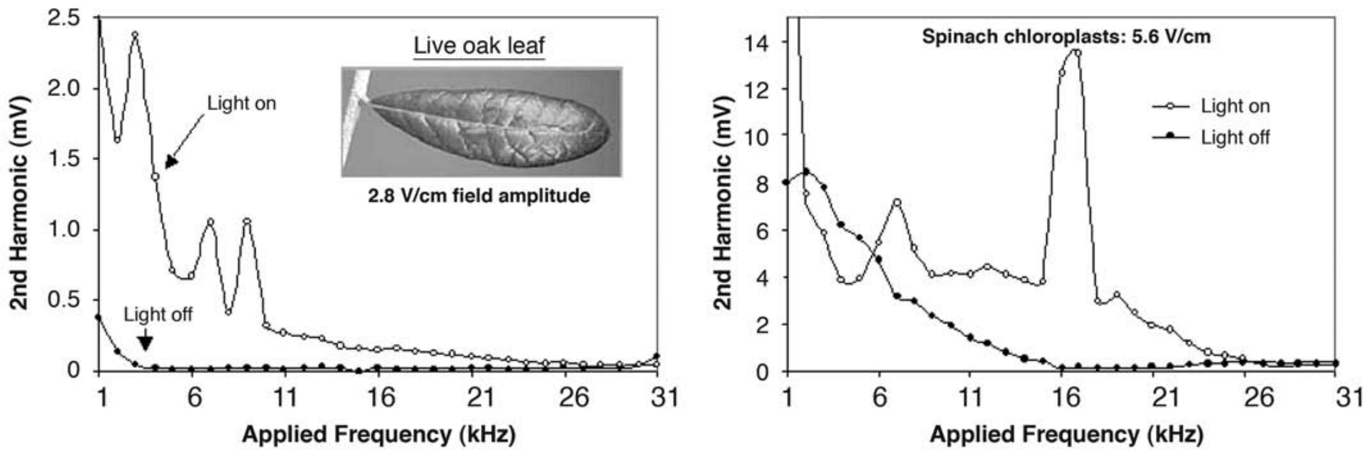

Figure 4. Light activated responses in the 2nd harmonic generation spectra of a whole leaf (left) and a suspension of spinach chloroplasts (right) in which the photosynthetic ETC has been activated by an electron acceptor (ferricyanide).

Conclusions

Our studies thus far have encompassed both linear (impedance, dielectric) and nonlinear (harmonic generation, mixing) responses of biological systems to oscillatory electric fields. Some results suggest that active biological motors and other enzyme complexes generate harmonics over specific frequency ranges. These include complexes in the mitochondrial inner membrane, such as the molecular turbine ATP synthase, and pumps in the outer plasma membrane. In addition, the harmonic generation spectra of chloroplasts, responsible for photosynthesis in plants, exhibit light-activated features. This provides evidence that the technique detects physiologically active processes, which could lead to fundamental advances in understanding of biochemical and other complex macromolecular systems. Moreover, the method could eventually play an important role in the search for extant life elsewhere in the solar system.

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Funding and Proposals

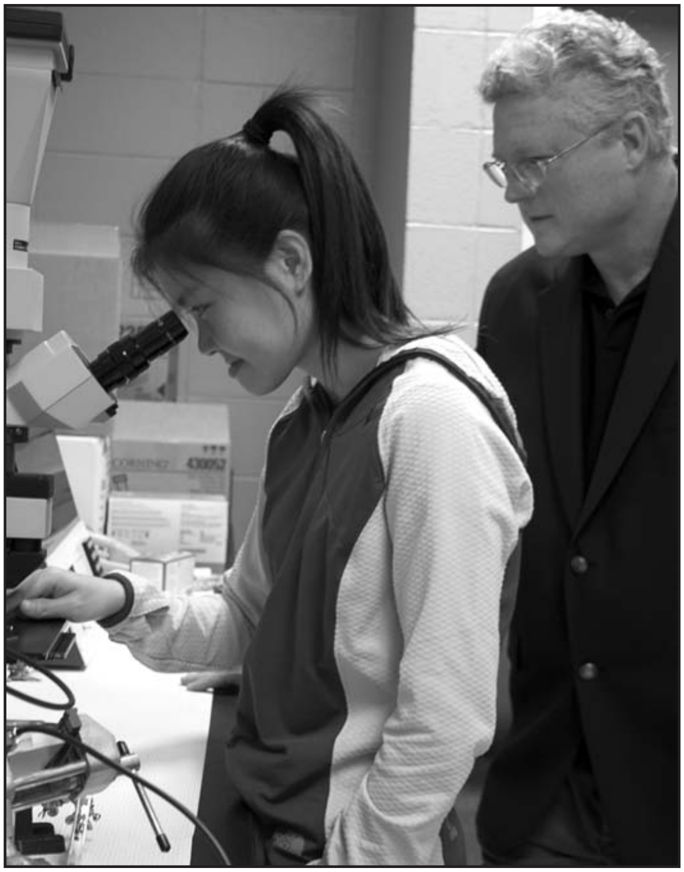
Miller, John H., Jr. “Dielectric Spectroscopy of Chemical and Biological Systems,” Robert A. Welch Foundation, June 1, 2004–May 31, 2007. $165,000.

Miller, John H. Miller, Jr., PI, William R. Widger, Co-I, “Noninvasive Sensors of Metabolic Activity,” NIH, RFA-HL-07-007, Bioengineering Approaches to Energy Balance and Obesity (R21), $150K/yr of direct costs requested for 3 years. Additional co-investigators/collaborators include Dale J. Hamilton, MD, and Richard J. Robbins, MD, of Methodist Hospital.

Miller, John H., Jr., PI, “Nonlinear Impedance Spectroscopy of Chemical and Biological Systems,” Robert A. Welch Foundation, renewal of E-1221, $60,000/year direct costs requested for three years.

Funding Initiative

NIH (R01), United Mitochondrial Disease Foundation and American Diabetes Assoc.: Marin Laughlin, an NIDDK program director in NIH, and others in NIBIB and NHLBI have expressed considerable interest in UH ideas and methodology for detecting mitochondrial function.



SPECTROSCOPY—Dr. John H. Miller Jr. supervises Shih-Ying Hsu, a Taiwanese doctoral student in biophysics, in a study of electromagnetic responses of living cell suspensions.

SCIENCE AND ENGINEERING—The Science and Engineering Research and Classroom Complex on the UH main campus is a 200,000-sq. ft. facility offering five floors of laboratory space capable of supporting an estimated 40 research laboratories.