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Aspects of Nonlinear Dielectric Spectroscopy of Biological Cell Suspensions

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***Abstract*— In this paper the technique of nonlinear dielectric spectroscopy is employed to examine the nonlinear response of a suspension of the yeast *S. cerevisiae* to a low frequency perturbating ac electric field. Metabolically active and resting yeast states, as well as the electrolyte medium are considered, and experimental time-course spectral data are presented. Conductivity is found to increase in the active case, resulting in variations in magnitude of the applied field. An empirical model is fitted to the experimental data at discrete points over time, enabling simulation and resulting in a software-based method to compensate for these variations in effective field strength.**

I. Introduction

The nonlinear response of biological cell suspensions to a sinusoidal perturbing field has been of interest as a potential method to probe the status of a biological system. Work appearing in the literature has suggested that this nonlinear response can indicate the metabolic state of a biological system [1, 2]. This type of physiological information would be significant in applications such as the noninvasive determination of blood glucose concentrations, as well as in the evaluation of unknown substances in field-deployable counter bio-terrorism devices. The goal of the work presented in this paper was to evaluate the practical aspects and confounding factors in the application of nonlinear dielectric spectroscopy.

II. Background

Nonlinear dielectric spectroscopy (NLDS) measures the nonlinear response of a substance placed in a perturbating electrical field. Typically a stimulating sinusoidal input signal is applied to a substance in the form of an ac voltage or current through a pair of drive electrodes. The test substance response to this electric field is then measured as a voltage at a pair of recording electrodes. Unfortunately this measured response can also include measurement artifacts, such as those due to electrode polarization impedance. A single pair of electrodes can provide both stimulus and sensing capabilities, but typically a four electrode system is used to reduce electrode polarization artifacts in the recording pair [3]. Recently a high-sensitivity magnetic field detector has been employed in place of the recording electrodes in the NLDS apparatus of [2].

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In NLDS it is the nonlinear relationship between current and voltage that is of interest. In the time domain this nonlinearity appears as a distortion in the quasi-sinusoidal voltage at the recording electrodes, while in the frequency domain this distortion appears as distinct spectral components - the harmonics - occurring at integer multiples of the input frequency. Analysis is typically done in the frequency domain by examining the magnitude of the resulting harmonics.

Woodward and Kell [1] first presented results indicating that NLDS can be used to indicate the metabolic state of a suspension of the yeast *S. cerevisiae*. The resting metabolic state of the yeast suspension occurs when there is no metabolizable source available and is characterized by dominant odd harmonics. The active metabolic state occurs when a metabolizable source, such as glucose, is supplied to the yeast. This active state is characterized by dominant even harmonics. The *a priori* metabolic state of yeast can then be determined by examining the behavior of the second and third harmonic magnitudes.

The nonlinearity of the yeast-electrode system can be placed into two source categories: *A)* nonlinearities due to the biological cells; and *B)* nonlinearities due to the electrode-electrolyte system [4]. These two sources appear in the system as impedances in series, and as such, separating their respective contributions is difficult [5]. Therefore in measuring the response of the cells, confounding nonlinearities that may mask the biological signal of interest are introduced. These two sources are discussed in the following sections.

A. Nonlinear Biological Response

The nonlinear biological response is the signal of interest in this work. This response is caused by dielectric membrane effects occurring in the α-dispersion, a low frequency range spanning milliHertz to kiloHertz [7]. In the considered biological system, the mechanism behind these effects are attributed to the transmembrane protein H^+ATPase [1, 6]. This is an ATP fueled proton pump used to control intracellular pH, and to create an electrical gradient to enable the uptake of nutrient ions. It does this by binding the substrate H^+ on the intracellular side, then changing conformation or shape to physically transport the substrate to the exterior. As this protein is a dipolar molecule fixed in the plasma membrane, in the presence of an electric field it is unable to rotate to align its dipole moment, but it can change its conformation to best align with the field. An applied ac field can cause the resting protein to oscillate between conformations, resulting in the transport of ions. The time delay of these conformation changes due to frictional and

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other forces causes a distortion of the effective field. The theoretical development of a nonlinear response indicative of transmembrane protein state was presented in [6], in which the magnitude of the second harmonic is shown to be sensitive to substrate levels. Active transport of molecules by transmembrane proteins is a common strategy in living cells, making NLDS of interest in sensing applications beyond that of yeast.

B. Nonlinear Electrode-Electrolyte Effects

Electrode-electrolyte systems under low current conditions are typically treated as linear. High resolution measuring equipment can determine nonlinear electrode behavior at extremely low stimulating amplitudes [8]. This nonlinearity increases with current, and the level at which it becomes significant depends on the physical properties of the system such as electrode material and electrolyte concentration [4]. The mechanism giving rise to this nonlinearity is electrode polarization.

Electrode interfacial polarization is caused by the accumulation of ions at the electrode-electrolyte boundary, creating a double layer of charge. This contributes to the total impedance Z_t measured between the electrodes. This can be described, when treated as linear, by:
$Z_t = Z_{bulk} + (Z_{p1} + Z_{p2})$, where Z_{bulk} is the electrolyte impedance, and Z_{pn} is the polarization impedance of one electrode [9]. Z_{bulk} varies with current density and frequency and can therefore yield a nonlinear I-V relationship. Z_{pn} will also vary with both current density and frequency, giving rise to interfacial polarization nonlinearity [4, 9].

III. Materials and Methods

A. Medium and Cell Suspension

Biological suspensions were prepared in an electrolyte medium composed of 20 mmol·L^{-1} KH_2PO_4, 30 mmol·L^{-1} KCl, and 20 mmol·L^{-1} $MgCl_2$ in distilled water, as given in [1]. The biological cells were the yeast *S. cerevisiae*, rehydrated in the medium from a freeze-dried powder (Muntons Active Brewing Yeast) at a concentration of 50 mg·mL^{-1}, and allowed to stand for one hour. Resting cases have no metabolizable substrate added to the yeast. In the active cases, glucose at a concentration of 170 mmol·L^{-1} is dissolved in the medium and mixed in with the yeast. Sample sizes of 2 mL were pipetted into open glass cylinders with a diameter of 16 mm.

B. Apparatus

A four electrode system was employed as shown in Fig. 1 under voltage clamp conditions, with the outer two electrodes supplying the perturbating current and the inner two electrodes recording the response. Electrodes were Ag-AgCl, prepared by chloriding 22 gauge (640 μm dia.) silver wire (A-M Systems, 99.99% pure) in sodium hypochlorite. On-center spacing of electrodes was 2.54 mm, with a working length of 14 mm. New electrodes were used for all test cases.

The sinusoidal drive input of 17 Hz at 1.5 V was supplied by a function generator (Agilent 33120A). Drive current was monitored by sampling the voltage across a 470 Ω resistor R_m, in series with the signal source. The response was measured by sampling the voltage across the inner recording electrodes. Amplification, sampling and analog to digital conversion was done using a National Instruments DAQPad 6020E, with 12 bit resolution, operating at a sampling rate of 2048 Hz. Six channels of data were obtained simultaneously from three parallel test cells. This made it possible to obtain data under the same conditions.

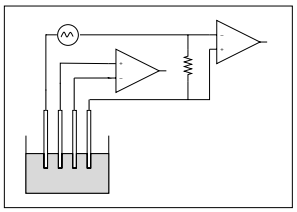

Fig. 1. Nonlinear dielectric spectroscopy experimental setup showing the two outer drive electrodes in series with the resistor R_m, the two inner recording electrodes, and the test cell.

All experiments were performed at approximately 22°C and shielded from direct light to prevent photosensitive artifacts.

C. Signal Processing

Signal processing was performed on a PC running MATLAB. Frequency domain analysis was done by estimating the power spectral density (psd) using Welch's averaged, modified periodogram method. Signals were windowed with a Hamming window of length 1024 samples and overlapped by 50%.

IV. Results

A. Active and Resting Yeast Cases

Data were collected over a twelve hour period to form the time-course plots of harmonic magnitude for the metabolically active and resting yeast cases shown in Fig. 2 and Fig. 3 respectively, as well as for the medium with no yeast (not shown). These results show a possible correlation between harmonic variation and the addition of glucose. The active case is characterized by three phases of relative harmonic magnitude. Phases one (hours 0-1.5) and three (hours 5-12) correspond with periods of lower metabolic activity, and show a third harmonic greater than the second. Phase two (hours 1.5-5) corresponds with the period of greatest metabolic activity, and is characterized by the second harmonic increasing above the third.

The resting case is characterized by only two phases. Phase one (hours 0-1) shows the second harmonic rising to intersect the third. Phase two (hours 1-12) exhibits a second harmonic greater than the third. While the resting case had no added glucose, phase one coincides with the metabolism of endogenous stores of energy within the yeast.

Repeatability of harmonic magnitudes is difficult to achieve with the existing apparatus, but other results typically showed a response that differed between the active and resting cases.

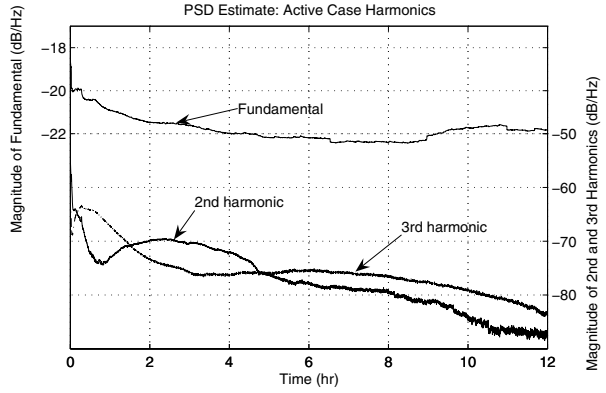

Fig. 2. The magnitudes of the spectral harmonics were calculated every six seconds to examine the nonlinear behavior over time.The period of greatest metabolic activity occurs between hours two and five.

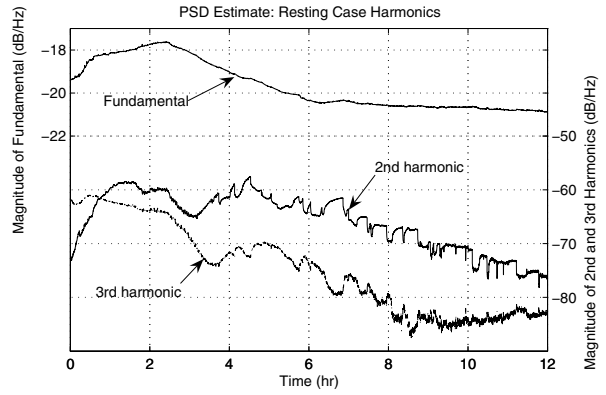

Fig. 3. The resting case shows a third harmonic lower than the second after an initial rise in the second harmonic in the first hour.

In a nonlinear system power is spread across the spectrum rather than being contained at the fundamental frequency. Variations in the psd can be caused by system nonlinearities redistributing power elsewhere in the spectrum, or by variations in the system input amplitude. The power distribution can be shown by modelling the yeast-electrode system as a Taylor series expansion about $v_{in} = 0$:

$$\begin{aligned} v_o &= k_o + k_1 v_{in} + k_2 v_{in}^2 + k_3 v_{in}^3 + \dots \\ &= \sum_{n=0}^{\infty} k_n v_{in}^n. \end{aligned} \quad (1)$$

Applying a sinusoidal input $v_{in}(t) = A\sin(2\pi F_{in} t)$ to (1), expanding, and employing trigonometric identities, will yield the parametric k_n contribution to the spectral components.

To understand the degree to which variability in the signals shown in Fig. 2 and Fig. 3 is the result of changes in system nonlinearity, or to changes in effective input amplitude, the current effectively supplied to the system was analyzed. Spectral analysis of the signal recorded at the drive series resistor R_m for the active, resting and medium cases is shown at the fundamental frequency in Fig. 4, normalized to remove slight differences in initial conductivity. Voltages in the drive circuit, assuming linear behavior over the small range of interest, are described by $v_s = v_{eff} + v_m$, where v_s is the source voltage, v_{eff} is the effective voltage across the drive electrodes, and v_m is the voltage across the resistor R_m. As v_m increases, indicating increased current flow, v_{eff} decreases. From Fig. 4 it can be seen that conductivity increases the most in the active yeast case, increases a small amount in the resting yeast case, and decreases a small amount in the medium alone. Insertion of R_m enables monitoring current, but causes v_{eff} to vary with the observed current changes. A working theory for the change in conductivity can be found in the Discussion.

B. A Method to Compensate for Input Variability

As discussed earlier, the insertion of the drive series resistance R_m results in variability of v_{eff} as current changes. Similarly, other series impedances such as drive electrode polarization will also affect v_{eff}. This variation may serve to mask the nonlinear response of the yeast. To compensate for variation in v_{eff} the Taylor series expansion given in (1) is employed as a transfer function to model the system input-output relationship at a time point. Equation (1) was truncated after the seventh order term and fit to the experimental data sampled at the record electrodes, to obtain the k_n parameters at each six second time point. Thus a time-course of parameters describing the system for a given v_{eff} was obtained. Assuming that the changes in v_{eff} that are to be compensated for are small enough that the the model is reasonably accurate, the effect of a constant v_{eff} can be simulated. Equation (1), with the time-course of experimentally determined parameters, was then solved numerically for $v_{eff} = v_{in}(t) = B\sin(2\pi F_{in} t)$ to simulate the effect of constant B on harmonic behavior. The results of this analysis for the active and resting cases are shown in Fig. 5.

The active case of Fig. 5(a) is approximately characterized by four phases of relative harmonic magnitude. Phase one (hours 0-1.5) and phase three (hours 4-8) show a second harmonic greater than the third. Phases two (hours 1.5-4) and four (hours 8-12) show a third harmonic greater than the second. These results appear quite different from the uncompensated active data which are characterized by only three phases. The relative magnitude of the harmonics is also reversed from the uncompensated results up until hour eight.

The compensated resting case shown in Fig. 5(b) more closely resembles the uncompensated data. This is expected as the conductivity increase in the resting case is small.

V. Discussion

The input variability compensation method presented earlier provides a software-based approach to counter the conductivity changes observed. There are a number of factors that can affect conductivity in the system. Conductivity is directly proportional to the surface area of the electrodes, and is inversely proportional to both the electrode spacing and the thickness of the chloride layer. Conductivity is also directly proportional to ion concentration, ion mobility, and temperature. These test cases were all run in parallel so

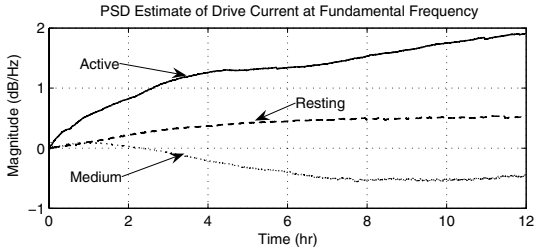

Fig. 4. The voltage across the series resistor R_m was sampled to obtain a signal proportional to current. The psd was estimated and normalized to account for small variations in initial conductivity. The results show that conductivity over time differs between the active yeast, resting yeast and electrolyte medium.

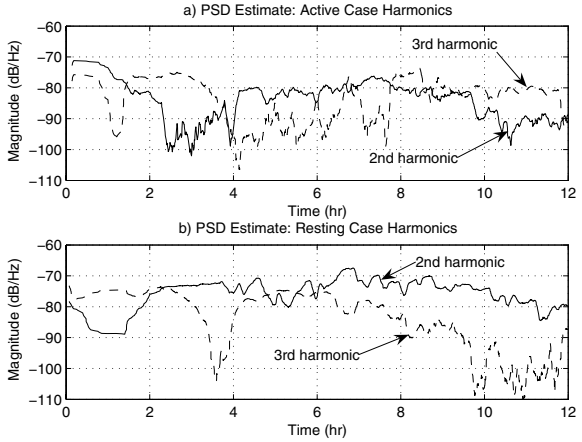

Fig. 5. The system response was simulated for a constant input amplitude for both the active case (Fig. 5a) and the resting case (Fig. 5b).

changes in temperature and electrode properties exhibit the same approximate change in conductivity. The factor that is likely to change only under metabolically active conditions is ion concentration.

S. cerevisiae is a facultatively aerobic organism. Depending on environmental conditions, primarily concentration of glucose and oxygen, metabolism can occur through either aerobic oxidation or anaerobic fermentation, with both reactions producing CO_2. Aerobic respiration is the complete breakdown of glucose through oxidation:

$C_6H_{12}O_6$ + $6O_2$ $\rightarrow$ $6CO_2$ + $6H_2O$ + 38 ATP molecules.

Anaerobic fermentation requires no oxygen:

$C_6H_{12}O_6$ $\rightarrow$ $2CO_2$ + $2C_2H_5OH$ + 2 ATP molecules.

In this experiment approximately 0.3 mmol of glucose was consumed over 12 hours. Assuming aerobic respiration, the consumed glucose produces 1.8 mmol of CO_2. From the ideal-gas law, $PV = nRT$, approximately 44 mL of CO_2 gas is bubbled through the suspension. A portion of this CO_2 will dissolve yielding the reaction :

$CO_2(aq)$ + H_2O $\rightleftarrows$ $H_2CO_3(aq)$ $\rightleftarrows$ $H^+(aq)$ + $HCO_3^-(aq)$.

This results in an increase in highly mobile H^+ ions, and a corresponding increase in conductivity. An electrode-based sensor to measure the conductivity change caused by dissolving CO_2 in water leverages this effect [10].

Electrode polarization effects are a significant hindrance in this work. Schwan has modelled polarization effects using a Taylor series, and [11] developed this further. Hardware approaches to reduce electrode polarization effects have also been employed. Polishing electrode surfaces is often done and a polymer coating has shown promise [12]. The sensor used in [2] avoids polarization effects that can appear in the record electrodes due to current leakage or electrode fouling.

VI. Conclusion

The technique of NLDS was employed to examine the nonlinear response of a suspension of the yeast *S. cerevisiae* to a low frequency perturbating ac field. A possible correlation between the resulting harmonic signature and the addition of glucose was observed, but obtaining repeatable harmonic magnitudes was difficult to achieve. Conductivity was also observed to have the greatest increase with the metabolically active yeast, possibly due to an increase in H^+ ions. A method to compensate for this conductivity change was effectively applied. While this technique might prove useful in compensating for changes in series impedances in the system, the nonlinear nature of electrode polarization can affect its accuracy, and is a significant obstacle that is still not fully understood.

Acknowledgments

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