Non-linear dielectric monitoring of biological suspensions

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Abstract. Non-linear dielectric spectroscopy as a tool for in situ monitoring of enzyme assumes a non-linear behavior of the sample when a sinusoidal voltage is applied to it. Even many attempts have been made to improve the original experiments, all of them had limited success. In this paper we present upgrades made to a non-linear dielectric spectrometer developed and the results obtained when using different cells. We emphasized on the electrode surface, characterizing the grinding and polishing procedure. We found that the biological medium does not behave as expected, and the non-linear response is generated in the electrode-electrolyte interface. The electrochemistry of this interface can bias unpredictably the measured non-linear response.

1. Introduction
Non-linear dielectric spectroscopy is a promissory technique for in situ monitoring of membrane-bounded proteins in microorganism suspensions [1-6]. Theoretical models suggest that a sinusoidal electric field applied to a biological suspension interacts with the membrane-bounded proteins (mainly H⁺–ATPase in Saccharomyces cerevisiae). The cyclic change in the charge distribution of the protein behaves as a non-linear system, and harmonic components can be observed in the polarization current [7].

The measurements are performed in a typical tetrapolar cell [1], with two outer electrodes used to inject the signal, and two inner electrodes used to register the signal. The current distortion is typical of a non-linear system and can be characterized with Fourier series analysis. However, the non-linear interfaces established between the outer electrodes and the medium behaves also as non-linear components and their response encumber the biological expected response.

Some attempts were made to overcome the interface-related errors with polymeric coating and advanced signal analysis [8-10], without much success. Other researchers evaluated alternative technologies and equipment, facing the same limitation than Woodward’s [11-14]. In these publications, some controversy was build around the source of the non-linear behavior, and some authors attributed it to the interfaces, rather than to the medium [15; 16]. Even the controversy was never clarified, our results suggest that the medium itself behaves linearly, and there is no observable, if any, non-linear response. We have already supported that the non-linear phenomenon is originated in the EEI, and can be affected by the presence and metabolic state of some microorganism [13].
this paper we present some modifications to the non-linear spectrometer used and add new results to the previously published.

2. Materials and Methods

2.1. Equipment

The setup has been briefly described somewhere else [13], and we will comment some updates of the hardware used. The equipment, depicted in figure 1(a), is built around a central PC which manages the experiments, sets up an electrochemical analyzer Solartron SI1287 (Solartron, New Hampshire, England), generates polarization signals, digitizes the signals applied to the sample and turns on/off the stirrer and peristaltic pump.

The user sets frequency and voltage ranges in the PC, and these signals must be applied to the sample. The PC generates for each frequency-voltage combination a digital signal that is communicated to the SI1287 via a digital-analog converter. The SI1287 receives the signal and acts a driver unit, amplifying this voltage and applying it to the cell through the outer electrodes. A high input impedance electrometer connected to the inner electrodes measures the voltage in the medium, and a secondary electrometer measures the current through the cell using a standard resistance. The voltage of the power driver is adjusted as much as necessary to match the input voltage received from the PC to the voltage measured in the medium. This is done automatically by the SI1287 unit fast enough to maintain a sinusoidal waveform, regardless the non-linear interfaces. If the sample connected between the inner electrodes is non-linear, harmonics will be observed in the current that circulates through the sample. Both current and voltage waveforms are communicated to the PC for further digitizing.

Emphasis must be given to the digitizing process to obtain utmost resolution and SNR of the acquired signals. Four mechanisms are controlled by the PC to achieve this requirement: (i) the

![Figure 1. The non-linear spectrometer and measurement cells: (a) Measurement equipment, (b) tripolar cell, (c) low interface impedance tetrapolar cell, and (d) flat parallel electrodes tetrapolar cell. Numbers 1 to 4 indicates how each cell is connected to the equipment.](image-url)
resistance used to measure current is variable, ranging from 0.1Ω to 10MΩ. (ii) there is a controlled amplification stage (×1 or ×10) for each signal previous to acquisition, (iii) the input of the SI1287 has a variable attenuator (×1 or ×0.01) to obtain maximal SNR in the input signal and (iv) the digitizing stage includes a selectable gain (×1, ×2, ×4 or ×8).

The digitally generated sinusoidal signal has a sampling frequency 500 times greater than the signal frequency itself, while the input signals are sampled at a frequency 100 greater than signal’s. The length of each acquisition is slightly longer than 4 full cycles to achieve a resolution of 0.25Hz in the frequency spectrum. The current waveform acquired is divided into 100 overlapping sections, each section windowed with a Blackman function and Fourier transformed. With all the spectra, an average frequency spectrum is obtained. The amplitude of the first five harmonics (expressed in dB) is extracted and plotted as function of frequency and amplitude of the applied signal. This process generates five surfaces, each one representing a different harmonic. We use a frequency range of 1Hz to 100Hz, with eleven points (logarithmic scale), and the voltage range is variable depending on the cell used, but also includes eleven points.

The system is completed with a reservoir with fresh solution, a magnetic stirrer and a peristaltic pump to avoid settling of the suspension. These devices are controlled by the PC to avoid stirring during acquisition. After 90 seconds of measurement, acquisition is paused and suspension is agitated during 10 seconds. Inhibitors or substrate of the H\(^+\)-ATPase are added to the reservoir and, the suspension is agitated for 1~2 minutes.

2.2. Tripolar cells

The tripolar cell (figure 1(b)) has been described somewhere else [13; 17]. It consists of a high-area low-impedance hemispheric counter electrode, a low area working electrode and a thin reference electrode. In this experiments, the cell was provided with two different working electrodes, made of stainless steel AISI304 (ϕ = 8mm, grinded with sandpaper grit 600, Buheler) and 18-carat gold (ϕ = 8mm, polished with diamond past and 1μm alumina powder). These electrodes are made with a piece of metal embedded in an acrylic disc, and an additional stainless steel coil (Dentaurum ϕ = 1mm) enclosed in a thin acrylic center-hollowed disc located between working and counter electrode serves as reference. This removable disc has an impermeable seal close to the working electrode and is placed after the working electrode is polished.

Initially two tripolar systems were used (reference and test cell respectively [17]) to remove the interfaces response, as proposed by Woodward [1]. However the impedance of the working electrodes is hardly matched (even made of the same material and equally polished), and the response of each system is not necessarily the same. Consequently, we decided to measure continuously in a single interface, and analyzed changes in the non-linear response when some biological perturbation was added.

2.3. Tetrapolar cells

Two different tetrapolar cells are used, both shown in figure 1(c) and (d). The former is a high volume (~400cc) cell, composed by two large-area outer electrodes separated by a thin acrylic layer. The two chambers are connected together through a central hollow in the acrylic layer, and two bounded steel coil (Dentaurum ϕ = 0.75mm) serve as reference electrodes.

The geometry of the hollow (length l and diameter ϕ) determines the impedance of the sample, typically much higher than the interface impedance between the medium and the outer electrodes. We used two different acrylic pieces (ϕ1 = 1mm, l1 = 1mm y ϕ2 = 1mm, l2 = 10mm). Each chamber included a magnetic bar and the suspension was stirred along with the reservoir.

The latter tetrapolar cell is an acrylic low-volume cylindrical arrange. It has two flat stainless steel AISI 304 outer electrodes (ϕ = 10mm) and two inner stainless steel coil electrodes (Dentaurum ϕ = 1mm). The outer electrodes and the acrylic are threaded for mounting and un-mounting the electrodes before and after each experiment. These electrodes are grinded with sandpaper (grit 600, Buheler).
before each experiment. The cell is also provided with tubing to allow circulation of fresh suspension when the pump is turned on.

2.4. Measurement performed
We measured the non-linear response of the samples, added a biological perturbation and thereafter measured again. We used an stimulator of the H$^+$/ATPase (glucose 100mM) and an inhibitor (sodium metavanadate 1mM). For each single measurement the entire range of voltage and frequency described previously were tested. The time and amount of measurements performed was variable, and will be detailed along with the results. Whenever a variation compatible with biological response was observed, additional test were performed to confirm the origin of such response.

Figure 2. Third harmonic amplitude measured in:
(a) tripolar cell with AISI 304 electrode: difference between before and after SMV adding, with a single polishing at the beginning.
(b) tripolar cell with AISI 304 electrode: temporal evolution measured at 10Hz and ~400mV: (—) hydrated yeast, (—- ) boiled yeast, and (—- ) hydrated yeast pre-incubated with SMV. A 1mM injection of SMV was added after two hours of measurement, indicated by the arrow.
(c) tripolar cell with golden electrode: temporal evolution measured at 10Hz and 900mV: (— ) hydrated yeast and (— ) boiled yeast. 100mM of glucose was added after two hours of measurement, as indicates the arrow.
(d) tetrapolar cell with low interface impedance: difference between averaged measurements before (9) and after (7) a single injection of SMV.
(e) tetrapolar cell with low flat electrodes: same proceedings as (d), but 11 and 13 measurements were performed before and after SMV injection.
Figures (a), (d) and (e) amplitude’s are color-coded using the colorbar shown in figure (e).
The suspension was made with commercial dry yeast (50mg dry weight x ml\(^{-1}\) in the same solution used by Woodward [1]), with distilled water with conductivity minor to 5\(\mu\)S/cm. All measurements were made on fresh suspension, with no more than four hours of being prepared. It was mainly observed the third harmonic, because it is the most referenced in the literature [1-6; 11; 12; 17].

3. Results

3.1. Tripolar cells

The difference in the third harmonic for a single measurement on stainless steel electrode before and after adding SMV is shown in figure 2(a). There is a decrease close to 30dB in the harmonic content measured for voltages between 0.45V and 1V, and a frequency range between 2.5Hz and 10Hz.

Repeated measurements in steel electrodes usually showed a biasing of 15dB in the first two hours of experiments (close to 10 measurements), without addition of glucose or SMV. Experiments that did not show such bias are shown in figure 2(b). In these three records SMV was added close to two hours of measurement and response of –30dB was observed only in hydrated yeast (—). The remaining experiments, which did not show any response, were performed on boiled and inhibited yeast (pre-incubated with SMV).

The biasing observed when using the golden electrode was less significant, and the time evolution of measurements performed on hydrated yeast is shown in fig. 2c. When glucose was added a decrease close to –30dB was observed for the third harmonic (- -). This response was reestablished approximately one hour later. The same experiment but with boiled yeast did not show any change (—).

3.2. Tetrapolar cells

The tetrapolar cells did not show a priori linear behavior for single measurement, regardless of adding SMV or glucose. Therefore we averaged repeated measurements and also examined the temporal evolution of the response.

We first evaluated the cell presented in figure 1(c) (central disk: \(\phi = 1\)mm, \(l = 1\)mm). Averaged measurements before and after SMV addition were subtracted, and the result is shown in figure 2(d). There is no appreciable change due to the SMV in the entire range evaluated (electric field up to 5000mV/cm). Experiments with other geometries of the central hollow of the acrylic piece show similar behavior, with surfaces compassed between –5dB and 5dB and no response to SMV or glucose.

The flat parallel-electrodes tetrapolar cell showed similar response with fields applied up to 1300mV/cm, apparently linear and no change due to SMV or glucose (figure 2(e)).

4. Discussion

The experiments performed in tripolar cell have shown response attributable to biological source, however this results are hardly repeatable under the same conditions (same voltage and frequency of the applied signal). The main source of variability was due to the polishing process. Even using the same electrode, abrasives and procedure, the impedance of the interfaces was hardly reproduced after each new polishing. This lead to new experimental conditions for every new test performed.

Repeated measurements over the same electrode reduced the polishing-related problems, but introduced a new one. The electrochemical corrosion of the interface, after continuous measurement, alters its impedance. There is, therefore, an intra-experiment variation that cannot be controlled. There were cases with minimal biasing of the impedance, being those cases reported here. Further biochemical test were done to prove the biological origin of the non-linear response observed.

With steel electrodes (figure 2(b)) we found a decrease in the third harmonic when SMV was added, and such decrease was not observed in boiled yeast or in yeast initially hydrated with SMV. This additional measurements confirmed the biological source of the response, an also invalidated a
possible electrochemical interaction of SMV. The value of all three traces after 2 hours of experiments correspond to non-metabolizing H\(^+\)–ATPase (whether inhibited or boiled). However, the traces do not show all the same value, suggesting that magnitudes between experiments are still incomparable and temporal evolution must be analyzed within each experiment.

No explicit change could be attributed to glucose in steel electrodes. However, we did found response to glucose in gold electrodes (figure 2(e)). The biological source was also tested with boiled yeast under the same conditions, and the temporal response is compatible with the previous experiments of glucose uptake [9].

The tetrapolar cells were aimed with two different but defined objectives: (i) reduce electrode impedance while attaining high medium impedance, or (ii) ensure homogeneity of the electric field regardless of the interface impedance, two design factors that had not been accomplished in the previous publications. In our experiments with tetrapolar cells we did apply a sinusoidal wave to the medium, and obtained a reasonable linear response (figure 2(d) and (e)). Neither SMV nor glucose induced a non-linear response or non-linear change, comparable to the previously reported [1].

All results that account for biological response (figure 2(a), (b) and (c)) were obtained in electrode-electrolyte interfaces, as proposed somewhere else [14; 15]. For every case reported in the previous literature, the third harmonic (or any other) was present in the signal applied to the system, but it was never generated upon a pure sinusoidal, as should be for non-linear systems.

All these experiments suggest that the medium is quite, almost completely, linear. However it can interact with signals with high harmonic content already present in the input signal. The energy coupling between electric field and enzyme appears to be affected by the waveform of the field, particularly its harmonic content. As suggested, the microorganism affects the interfacial behavior and reflects the biological component in the non-linear analysis.

Consistently, the most productive literature [1-6; 8-10; 16] was obtained with a spectrometer that never accounted for a true sinusoidal field to the medium. The real system was a three-component series circuit; two of them were certainly non-linear (the interfaces) and the remaining system was expected to be non-linear (the medium). The controlled sinusoidal voltage applied to the outer electrodes actually resulted in an uncontrolled multi-frequency voltage applied to the medium. The current distortion suffered in the interfaces became visible in the linear medium, and was erroneously considered a non-linear system. This can be easily corroborated and has also been discussed [14; 15].

Our equipment feedbacks the voltage measured between the inner electrodes and adjust the waveform applied to satisfy a sinusoidal field in the medium. For any non-linear behavior, harmonics should have been observed in the current, but none was found.

5. Conclusions
We were unable to obtain the same response that had been published, in spite of many cell geometries, electrodes and polishing treatment to the metal surfaces. All these parameters had not been tested previously. We did found response in electrolytic interfaces, were the microorganism is expected to modify the interface natural response. In these configurations, the electrochemistry of the interface can vary during each experiment and obscure the true phenomenon.

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References
[1] Woodward AM and Kell DB 1990 Bioelectrochemistry and Bioenergetics 24 83-100
[2] Woodward AM and Kell DB 1991 J. Electroanal. Chem. 321 423-39
[3] Woodward AM and Kell DB 1991 Bioelectrochemistry and Bioenergetics 26 423-39
[4] Woodward AM and Kell DB 1991 FEMS Microbiology Letters 84 91-5
[5] Woodward AM and Kell DB 1991 *J. Electroanal. Chem.* **320** 395-413
[6] McShea A, Woodward AM, and Kell DB 1992 *Bioelectrochemistry and Bioenergetics* **29** 205-14
[7] Astumian RD and Robertson B 1989 *The Journal of Chemical Physics* **91** 4891-901
[8] Woodward AM, Davies EA, Denyer S, Olliff C, and Kell DB 2000 *Bioelectrochemistry* **51** 13-20
[9] Woodward AM, Jones A, Zhang XZ, Rowland J, and Kell DB 1996 *Bioelectrochemistry and Bioenergetics* **40** 99-132
[10] Woodward AM, Gilbert RJ, and Kell DB 1999 *Bioelectrochemistry and Bioenergetics* **48** 389-96
[11] Nawarathna D, Miller J, Claycomb JR, Cardenas G, and Warmflash D 2005 *Physical Review Letters* **95** 158103-4
[12] Nawarathna D, Claycomb JR, Miller J, and Benedik MJ 2005 *Applied Physics Letters* **86** 23902-3
[13] Treo EF, Felice CJ, and Madrid RE 2005 *Proceedings of 27th Annual International Conference of the Engineering in Medicine and Biology Society, IEEE-EMBS* p 4588
[14] Blake-Coleman BC, Hutchings MJ, and Silley P 1994 *Biosensors and Bioelectronics* **9** 231-42
[15] Hutchings MJ, Blake-Coleman BC, and Silley P 1994 *Biosensors and Bioelectronics* **9** 91-103
[16] Woodward AM and Kell DB 1995 *Biosensors and Bioelectronics* **10** 639-41
[17] Treo EF, Felice CJ, and Madrid RE 2005 *Revista Argentina de Bioingeniería* **11** 29-34