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Detection and differentiation of bacteria by electrical bioimpedance spectroscopy

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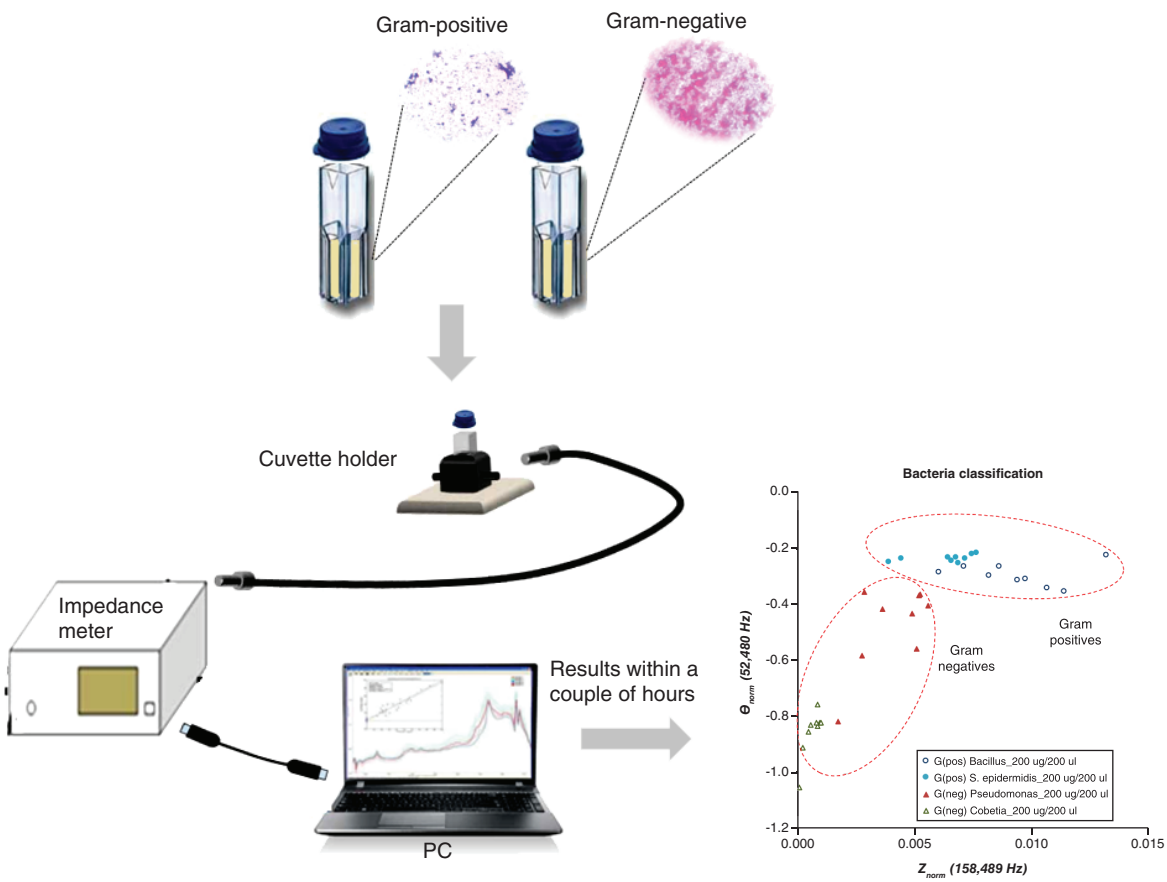
ABSTRACT

Detecting bacteria in samples and differentiating between Gram-negative and Gram-positive species is an important challenge, and the most common method, Gram staining, is very time consuming. The aim of this study was to evaluate the electrical bioimpedance spectroscopy (EBIS) technique as an inexpensive and practical tool for real-time detection of bacteria and differentiation between Gram-positive and Gram-negative species. The relevant sensitivity for differentiating between species was found in the magnitude and phase at frequencies of 158,489 and 5248 Hz, respectively, at a bacterial concentration of 1 μg/μl. Subsequently, the sensitivity was estimated as a function of bacterial concentration. Our results demonstrated that EBIS can potentially distinguish between presence and absence of bacteria as well as between different types of bacteria.

GRAPHICAL ABSTRACT

Methodology infographic. Step-by-step schematic representation of the electrical bioimpedance spectroscopy capable of determining and identifying bacteria type in any biological sample in a couple of h.



METHOD SUMMARY

We have developed a novel nonlabeling (or nonstaining) method to detect bacteria and to differentiate between Gram types based on their electrical properties, cell wall structure and size. This method uses electrical bioimpedance measurements and is a rapid, inexpensive and real-time technique for identification and classification of bacteria.

KEYWORDS:

bacteria ● electrical bioimpedance spectroscopy ● Gram negative ● Gram positive

A fast, simple and reliable tool for differentiating Gram-positive and Gram-negative bacteria has significant value in many applications: in clinical medicine for the diagnosis of diseases caused by harmful microbes [1,2] and in the food industry and water treatment systems for monitoring contamination and maintaining appropriate food and water quality [3–5]. This tool has particular significance in economically underdeveloped regions, where infections spread rapidly and widely into large populations [3].

Gram staining is still the most common method for detecting bacteria and distinguishing between Gram-negative and Gram-positive species. This method, developed by Hans Christian Gram in 1884, relies on the different cell wall staining properties of the two bacterial types [6]. However, the method is time-consuming due to the lengthy process of culturing and staining bacteria (1–3 days). In recent years, researchers have attempted to accelerate the process of detecting bacteria, quantifying their amount and defining their type with simpler methodologies such as fluorescence flow cytometry [7] and imaging analysis [8].

However, fluorescence-based detection needs dye labeling [9], is expensive and also requires professional training for operation. Imaging techniques require the development of identification algorithms and are difficult to perform in real time [10]. Researchers invented a series of label-free detection methods such as quartz crystal microbalance [11] and microfluidic [12] and electrochemical methods [1]. Some of these techniques can detect bacteria rapidly and accurately; however, most of them have to be performed in specialized laboratory environments with the assistance of sophisticated equipment.

Electrical bioimpedance spectroscopy (EBIS) [13–21] offers many advantages over the conventional techniques and has been increasingly applied in biosensing because of its sensitivity, simplicity and cost–effectiveness.

The electrical impedance of a biological sample reflects the electronic and physical properties of its cellular membranes as well as the molecular shape, structure and composition, the concentration and the interaction of molecules in solution that undergo a water dipole effect [22].

Clausen *et al.* recently reported the development of a microfluidic sensor to assess the levels of bacteria in water using multifrequency electrical impedance. They focused on differentiating *Escherichia coli* from solid particles, estimating bacterial concentration and distinguishing between a Gram-positive and a Gram-negative species (*Staphylococcus aureus* vs *E. coli*). The microfluidic sensor displayed an overlap of bacterial differentiation in a Gaussian distribution but could successfully identify up to 84% of the bacteria in a mixed sample [23].

Most of the methodologies that can distinguish between Gram-negative and Gram-positive bacteria take advantage of the differences in the bacterial cell walls. A much thicker peptidoglycan layer is found in Gram-positive than in Gram-negative bacteria, which have a cell wall/membrane composed of a lipid bilayer and a thin peptidoglycan layer [24]. Because each bacterium has a distinct shape, size and composition, EBIS could be used as a marker of bacterial types, properties or even species by establishing clusters.

In this study, real-time EBIS measurements were performed on label-free samples of bacteria in suspension with the aim of detecting the microorganisms as well as differentiating between the Gram-positive (*Staphylococcus epidermidis* and *Bacillus* sp.) and Gram-negative (*Pseudomonas aeruginosa* and *Cobetia* sp.) species. The appropriate frequencies were determined and the sensitivity of the technique was investigated as a function of bacterial concentration (by weight). The EBIS values for the four selected bacteria were plotted as magnitude versus phase to examine possible differentiation clusters.

Materials & methods

Experimental design

Two Gram-positive (*S. epidermidis* and *Bacillus* sp.) and two Gram-negative (*P. aeruginosa* and *Cobetia* sp.) bacteria were cultured by standard techniques [25]. Three procedures were then carried out with the selected bacteria: Gram staining to identify Gram-positive and Gram-negative bacteria; generation of standard calibration curves of bacterial weight as a function of optical density (OD); and resuspension of bacteria in phosphate-buffered saline for EBIS measurements in a frequency range of 100 Hz–1 MHz, with samples of each organism prepared in triplicate. The experimental design and setup are illustrated in Figure 1.

Stain characterization

All four species were characterized by Gram staining [25]. Microscopic slides of bacterial samples were prepared by carefully adding one drop of each bacterial solution. The cells were stained with crystal violet dye for 1 min and then rinsed with a gentle stream of water for a maximum of 5 s to remove the unbound stain. Iodine solution was added to form a complex with crystal violet, and 1 min later the

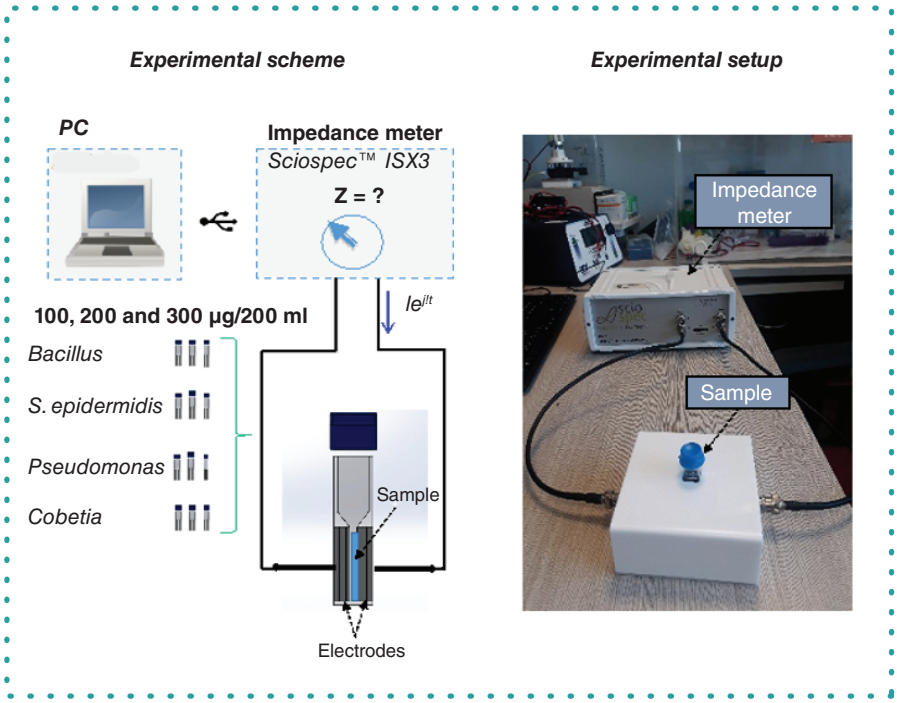


Figure 1. Scheme of the experimental design and setup.

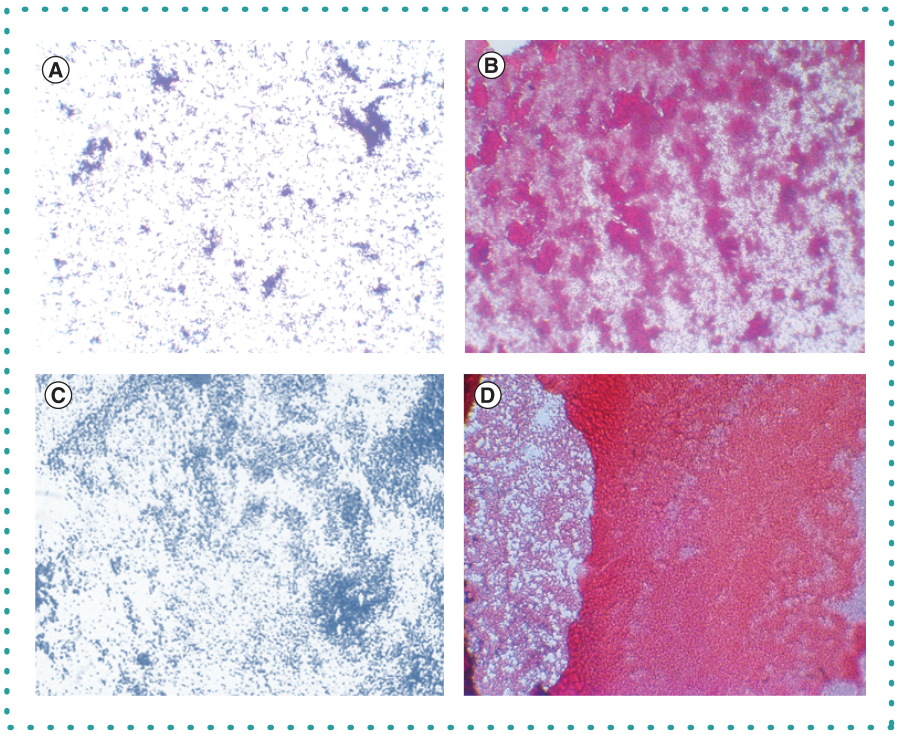


Figure 2. Gram staining of the four selected bacteria. (A) *Bacillus* sp. (Gram positive), **(B)** *Cobetia* sp. (Gram negative), **(C)** *Staphylococcus epidermidis* (Gram positive) and **(D)** *Pseudomonas aeruginosa* (Gram negative) viewed under a light microscope at 20× magnification.

slides were rinsed with acetone for decolorization. Subsequently, safranin was added to the slides to generate a red stain. The stained bacteria were observed under light microscope and identified (Figure 2).

Table 1. Number of bacterial colonies (CFU/ml).

Bacteria type	CFU/ml
Bacillus sp.	1.945×10^{11}
Cobetia sp.	1.150×10^{11}
S. epidermidis	7.650×10^{10}
P. aeruginosa	2.125×10^{11}

Bacterial culture & resuspension

Bacterial cultures of all organisms were prepared from a single colony selected from a plate containing 10 g/l of tryptone, 5 g/l of yeast extract, 10 g/l of NaCl and 20 g/l of agar, and freshly streaked with 10 ml LB broth inoculum (Difco, USA). The samples were incubated at 37°C for 18 h. The inoculums then were added to 1 l liquid LB medium for incubation in a shaking incubator (MRC Labs, Israel) at 37°C with a rotational speed of 150 rpm. The bacterial growth was examined every 4 h for 3 days by measuring the OD at 600 nm. The resulting bacterial biomass was collected by centrifuging 100 ml of each sample at 4000 $\times g$ for 30 min, rinsing twice with 0.9% saline solution, centrifuging for another 15 min and oven drying at 60°C for 24 h until a constant weight was obtained. Standard calibration curves of OD versus dry weight were then generated. Electrical impedance is affected by cell membrane and surface area; the selected bacteria have different sizes and membrane shapes, so using the dry weight concentration will generate an equal total membrane surface area for all the selected bacteria. For impedance measurements, three different biomass concentrations of each bacteria type were prepared in phosphate-buffered saline to achieve a final volume of 200 µl (100, 200 and 300 µg to give concentrations of 0.5, 1.0 and 1.5 µg/µl, respectively). For each concentration, three samples were prepared and for each sample, three impedance measurements were performed. Every impedance spectrum was normalized with respect to its highest value which was used as control procedure; in this sense, every experiment represents its own control.

Counting method

Serial dilutions, plating and counting were performed to determine the number of bacteria in a given population. Different serial dilutions (at least four) of a solution containing an unknown number of the selected bacteria (*Bacillus* sp., *Cobetia* sp., *S. epidermidis*, *P. aeruginosa*) were obtained. The bacterial samples were plated and the total number of bacteria in the original solution determined by counting the number of colony-forming units (CFU), each CFU representing a bacterium that was present in the diluted sample. The number of CFU was divided by the product of the dilution factor and the volume of the plated diluted suspension to determine the number of bacteria per milliliter in the original solution (Table 1).

Impedance measurements

The system for measuring multifrequency electrical impedance in bacteria was created by connecting an electroporation disposable cuvette (model 610, 2-mm gap size, BTX/Harvard Apparatus, Inc, MA, USA) to a bioimpedance meter (ISX-3, ScioSpec, Germany), followed by injecting a 100-mV peak potential difference in the frequency range of 100 Hz–10 MHz. The current was monitored to estimate the impedance of the system. To store the output data, ScioSpec was programmed on a PC.

Statistical analysis

Bioimpedance data were analyzed by two nonparametric statistical tests. The Kruskal–Wallis test [26], a rank-based test used to explore statistically significant differences between two or more groups of an independent variable, was used to determine differences between the four groups of bacterial species. The Mann–Whitney U test [27] allowed us to determine differences between two species groups at every frequency. Differences were considered significant at $p < 0.05$.

Results & discussion

As shown in Figure 2, *S. epidermidis* and *Bacillus* sp. are stained in purple, indicating that they are Gram-positive. *S. epidermidis* grows in grape-like clusters, forming raised, cohesive colonies about 1–2 µm in diameter; *Bacillus* sp. is rod-shaped and typically forms small clumps, short chains or single cells with a size of 0.5–1.0 µm by 4–10.0 µm. Conversely, *P. aeruginosa* and *Cobetia* sp. are stained red, indicating that they are Gram-negative. *P. aeruginosa* is asporogenous with a size of about 1–5 µm by 0.5–1.0 µm, and 0.8–0.9 µm for *Cobetia* sp. [28–32]. The standard calibration curves (Figure 3) of weight versus OD for the four species showed a coefficient of correlation (R^2) greater than 0.93 in all cases.

The impedance sensitivity results were evaluated using the nonparametric Kruskal–Wallis test and are shown in Figure 4. The bacterial concentration of 1 µg/µl for *S. epidermidis* and *P. aeruginosa* exhibited the largest differences between the two species in terms of magnitude and phase values, compared with the other tested concentrations. The concentrations of 0.5 and 1.5 µg/µl showed smaller differences in magnitude and phase values. This indicates that the bioimpedance measurements are highly sensitive at a concentration of 1 µg/µl; that is, they have a greater ability to distinguish between the two types of bacteria at this concentration. Impedance values for bacterial concentrations at 1 and 1.5 µg/µl are in a relatively close dynamic range compared with those at 0.5 µg/µl; bacterial con-

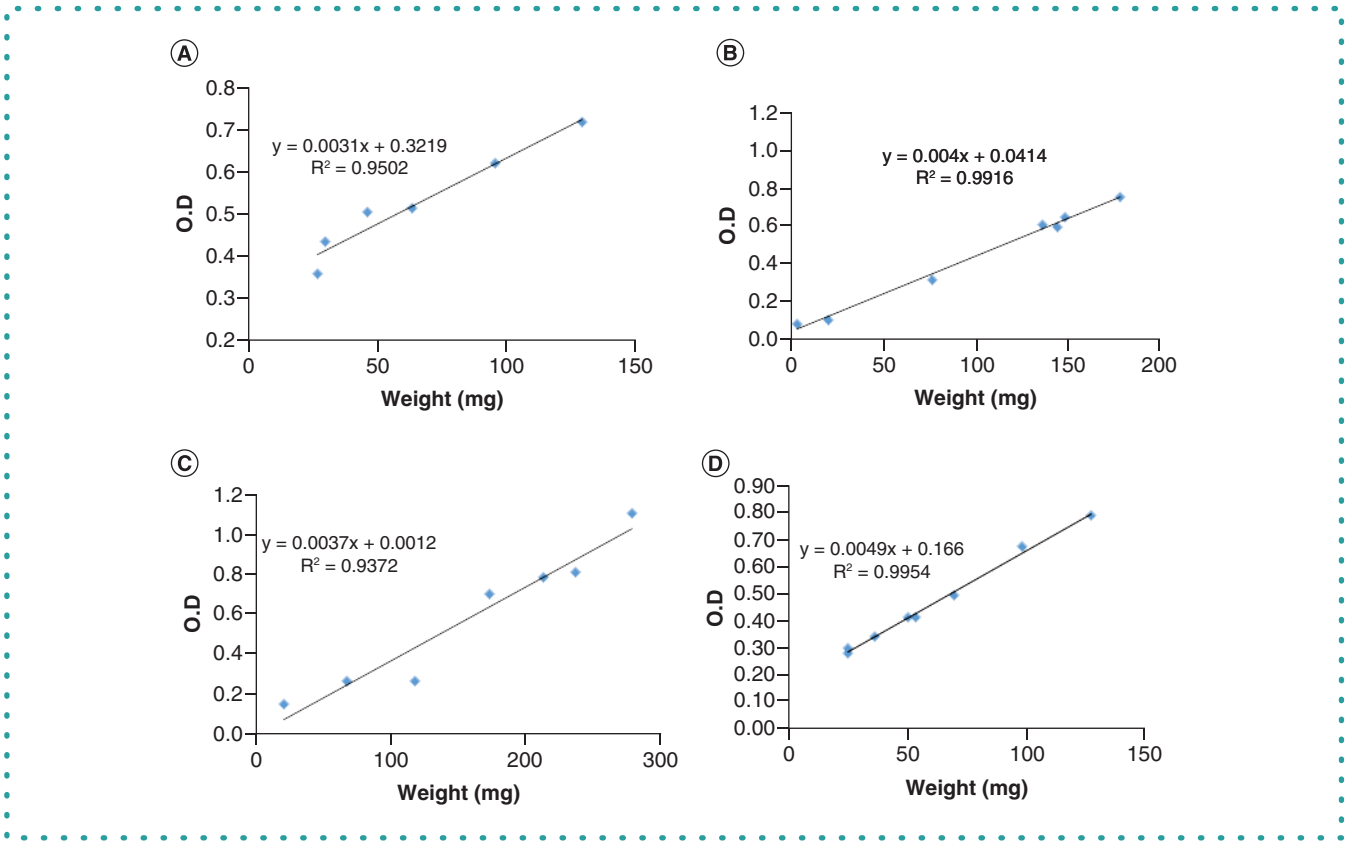


Figure 3. Standard calibration curves of weight and optical density for Gram-positive (A & B) and Gram-negative (C & D) bacteria. (A) *Bacillus* **sp., (B)** *Staphylococcus epidermidis*, **(C)** *Pseudomonas aeruginosa* **and (D)** *Cobetia* **sp.**
OD: Optical density.

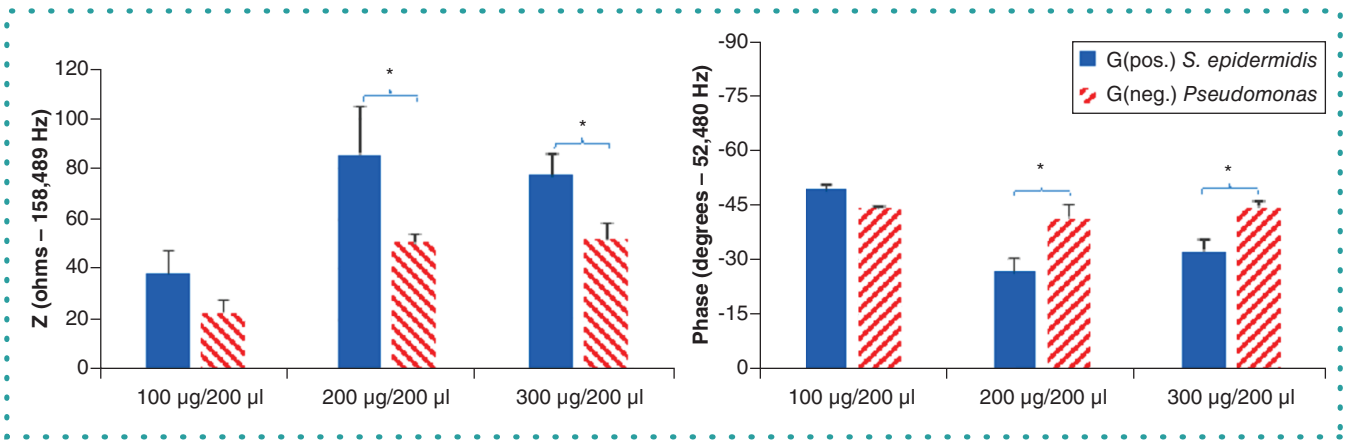


Figure 4. Sensitivity effect based on the concentration (by weight) of Gram-positive and Gram-negative bacteria (*Staphylococcus epidermidis* and *Pseudomonas aeruginosa*) on the magnitude and phase of electrical bioimpedance measurements at specific frequencies.
***Significant differences between groups ($p < 0.05$), as per the Mann–Whitney U test.**

centration above 1 µg/µl did not improve sensitivity, probably due to a media saturation effect. Thus this concentration was chosen for further analysis. These differences were obtained at frequencies of 158,489 Hz for magnitude and 52,489 Hz for phase (Figure 5).

The frequencies found to have the highest sensitivity to impedance measurements correspond to the beta dispersion bandwidth [33]. This is congruent with the dispersion region observed in the magnitude of the spectra, starting at approximately 100 kHz. A resonance effect developed in the impedance meter system above 400 kHz, therefore the results from higher frequencies were excluded from the dataset.

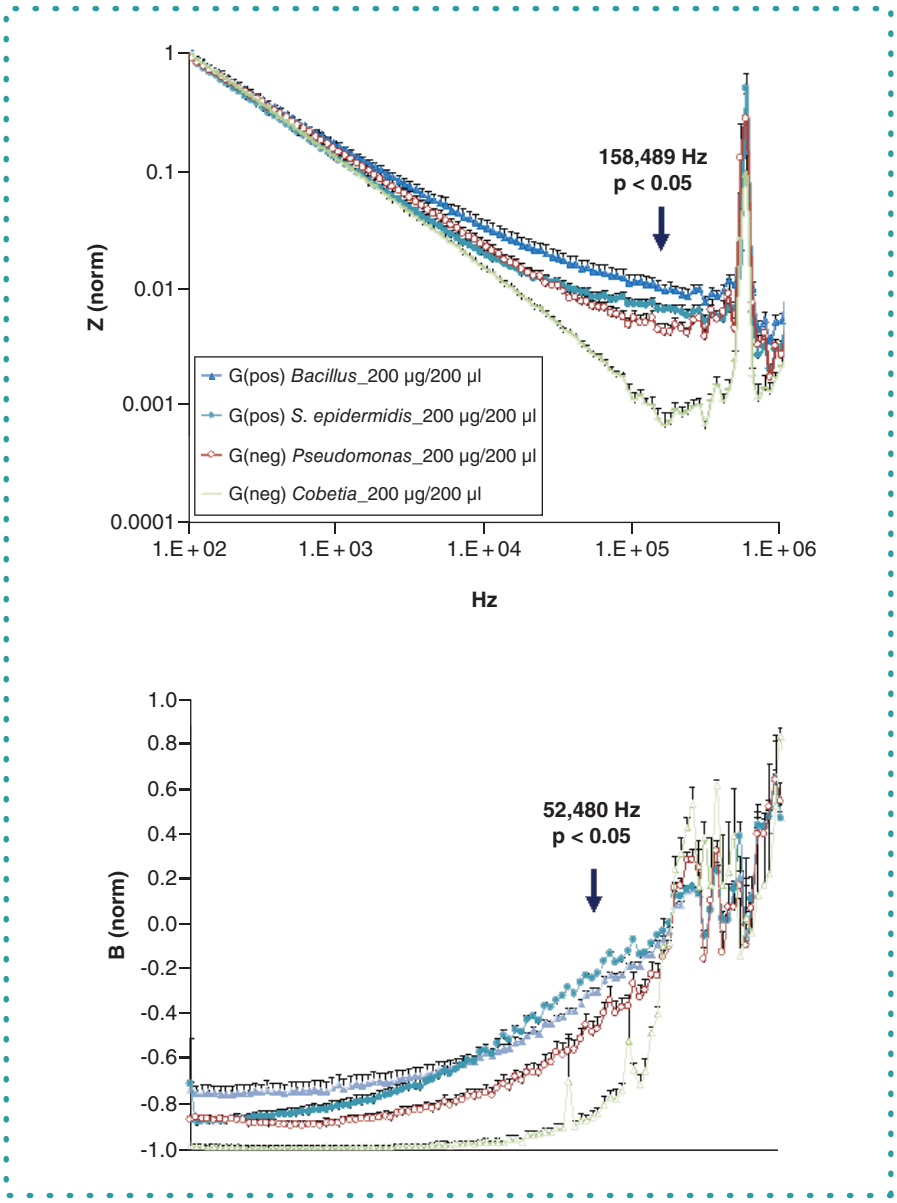


Figure 5. The magnitude and phase of bioimpedance spectra obtained from two Gram-positive (*Staphylococcus epidermidis* and *Bacillus* sp.) and two Gram-negative (*Pseudomonas aeruginosa* and *Cobetia* sp.) bacteria at a concentration of 1 µg/µl. The results are normalized with respect to the corresponding highest value in every spectrum. Data are expressed as the mean ± standard error. The nonparametric Kruskal–Wallis test showed statistical differences between species in the entire bandwidth. The Mann–Whitney U test was used to select the specific frequencies (158,489 Hz and 52,489 Hz for magnitude and phase, respectively) with the greatest significant differences between groups at a minimal p-value.

Figure 6 shows the dispersion of magnitude versus phase of the bioimpedance measurements for two Gram-positive and two Gram-negative bacteria at a concentration of 1 µg/µl. Two cluster regions can be clearly differentiated for the Gram-positive and Gram-negative species at significant frequencies. The cluster analysis also showed that *S. epidermidis*, *Bacillus* sp. and *P. aeruginosa* could be differentiated from *Cobetia* sp. This can be explained by the fact that electrical impedance is influenced by bacterial size and *Cobetia* sp. is the smallest member in the group.

Figure 7 represents a scheme of bacterial size, type and complexity for the four selected species and the effect of these parameters on magnitude and phase of bioimpedance. Surprisingly, we found that *S. epidermidis* and *Bacillus* sp. resulted in an increase in the impedance magnitude compared with *P. aeruginosa* and *Cobetia* sp. This might be explained by the presence of large size bacteria (*Bacillus* sp., *S. epidermidis, P. aeruginosa* and *Cobetia* sp., respectively). In addition, the phase angle describes the contribution of the resistance and capacitance elements to the impedance value [34]. As shown in Figure 7, the presence of the two Gram-negative bacteria (*P. aeruginosa* and *Cobetia* sp.) resulted in an increase in the impedance phase. This clearly demonstrates the bacterial cell wall differ-

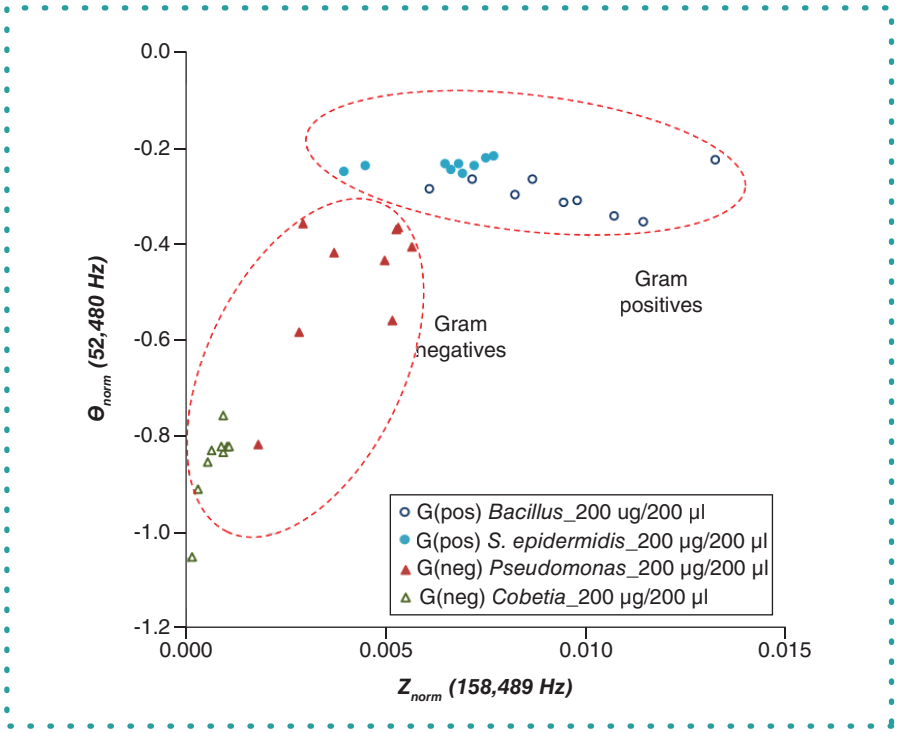


Figure 6. Dispersion of magnitude versus phase of the bioimpedance measurements for two Gram-positive (*Staphylococcus epidermidis* and *Bacillus* sp.) and two Gram-negative (*Pseudomonas aeruginosa* and *Cobetia* sp.) bacteria at a concentration of 1 µg/µl. Data are expressed as the mean value at 158,489 Hz and 52,489 Hz (for magnitude and phase, respectively). Two cluster regions can be clearly differentiated for the Gram-positive and Gram-negative species.

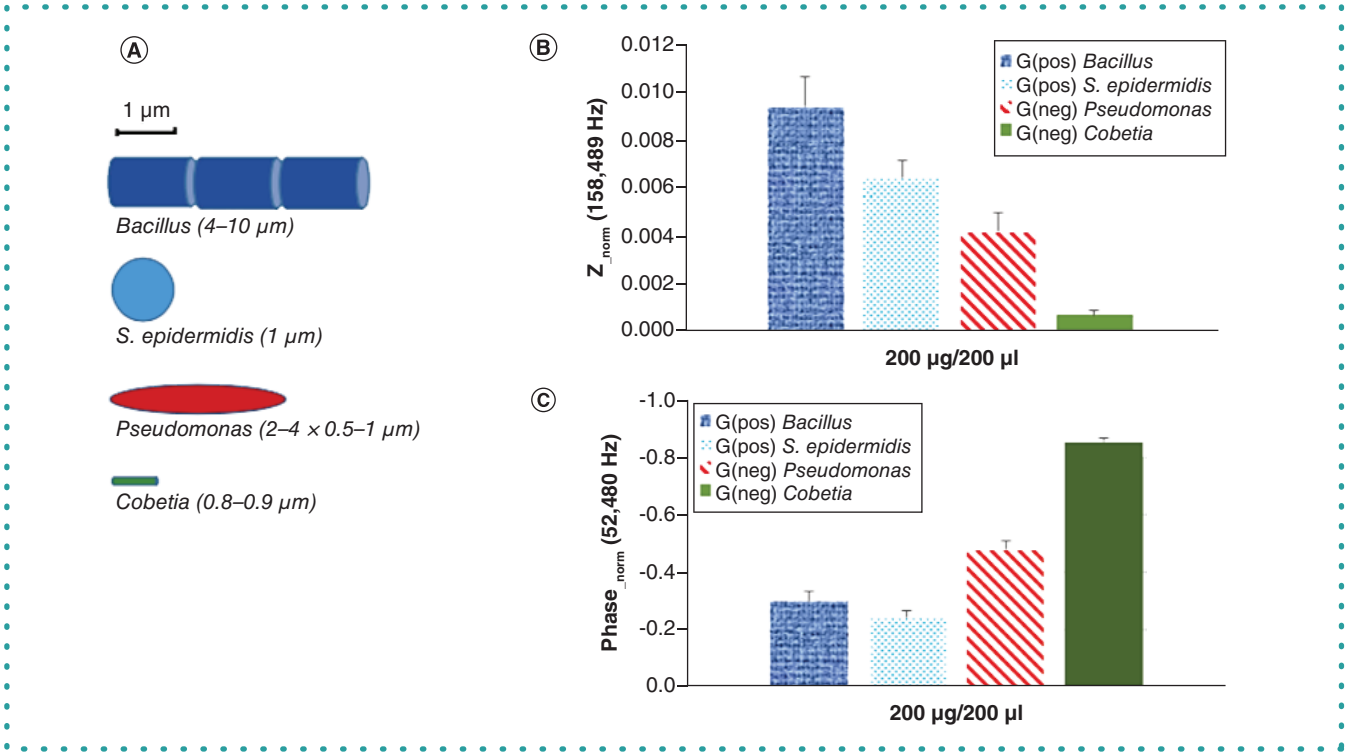


Figure 7. A scheme of bacterial size, type and complexity of *Bacillus* sp., *Cobetia* sp., *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. (A) Scheme of the size and complexity of the bacterial cells as well as the components of the cell wall/membrane in Gram-positive (*S. epidermidis* and *Bacillus* sp.) and Gram-negative (*P. aeruginosa* and *Cobetia* sp.) bacteria. These factors most likely generated the observed differences in resistance and capacitance. **(B)** Mean experimental values found for magnitude and phase of bioimpedance at 158,489 Hz and 52,489 Hz, respectively.

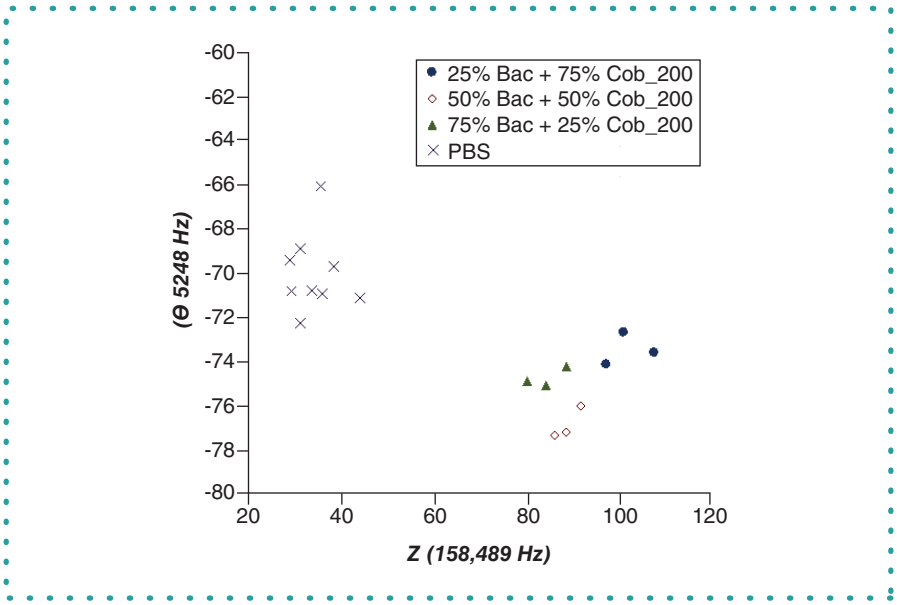


Figure 8. Dispersion of magnitude versus phase of the bioimpedance measurements for two Gram-positive and Gram-negative bacteria (*Bacillus* sp. and *Cobetia* sp.) in different proportions (25/75, 50/50 and 75/25%). Data are expressed as the mean value at 158,489 Hz and 52,489 Hz for magnitude and phase, respectively. Three cluster regions can be clearly differentiated for each proportion.
PBS: Phosphate-buffered saline.

ences effect; the additional outer membrane in Gram-negative bacteria is composed of lipopolysaccharides which conceivably produce greater capacitance. This likely explains the negative impedance phase shift observed. Given the selected frequency range as well as the electric potential used for impedance analysis (tens kHz and mV), it is very unlikely that the observed differences correspond to an effect of the charge of the cell wall.

Figure 8 illustrates the feasibility of this technique for detecting and differentiating a mixture of Gram-positive and Gram-negative cells in a real application. Mixed samples of *Bacillus* sp. and *Cobetia* sp. (Gram positive and Gram negative, respectively) were prepared in three ratios (25/75, 50/50 and 75/25%) at the same overall concentration. Bioimpedance data were obtained at the optimum sensitive frequencies determined above (158,489 and 5248 Hz for magnitude and phase, respectively) and each sample was measured in triplicate. The results show clusters for every tested ratio; the mixed properties associated with size and membrane structure emerge at specific regions in a magnitude versus phase dispersion map. This method has the potential to distinguish between different types of bacteria in a mixed sample using techniques for feature extraction and pattern recognition, and further analyses are warranted; additional experiments with different cell type proportions and concentrations are underway. In addition, it should be noted that Table 1 presents the CFU data for each species at a concentration of 1 μg/μl; this concentration is high compared with real applications and this factor is a limitation of our method. However, lower bacterial concentrations that emulate real conditions are yet to be examined.

A numerical estimation of the impedance frequency response for Gram-positive and negative bacteria was calculated by *LTspice IV*. The equivalent circuits were designed based on a typical model for bacterial cells in suspension; resistive and capacitive elements were added with proposed values to reflect the interactions between the peptidoglycan and lipopolysaccharide layers. Peptidoglycan resistive elements are different in one order value and lipopolysaccharide capacitance applies only in the case of Gram-negative bacteria. The bacteria were modeled by R_i (1.47 KΩ), R_m (1.38 KΩ), C_m (0.5 nF) and R_b (745 Ω) as the intracellular resistance, membrane resistance, membrane capacitance and extracellular resistance, respectively. R_p (1 MΩ) and C_{lyp} (0.01 pF) represent the resistance and the capacitance for a composite interaction between the peptidoglycan and lipopolysaccharide layers. The impedance is the total current-limiting effect in the circuit. The specific impedance contribution of R_p and C_{lyp} is a combination of resistance and capacitive reactance connected in series, thus their ohmic values must be added. However, these two elements are 90° out of phase with each other, which means that changes in R_p and C_{lyp} must be observed in magnitude differences as well as phase shifts. Figure 9 shows the numerical bioimpedance estimation in magnitude and phase; the effect of different peptidoglycan resistive element values is evident in the magnitude results, and the presence of lipopolysaccharide capacitance element promotes differences in phase.

The simulation was designed based on ideal conditions and represents a first approach to better understand the mechanism involved in the differences observed experimentally, thus additional numerical studies considering electrode polarization effect, total suspension volume and biomass weight are warranted.

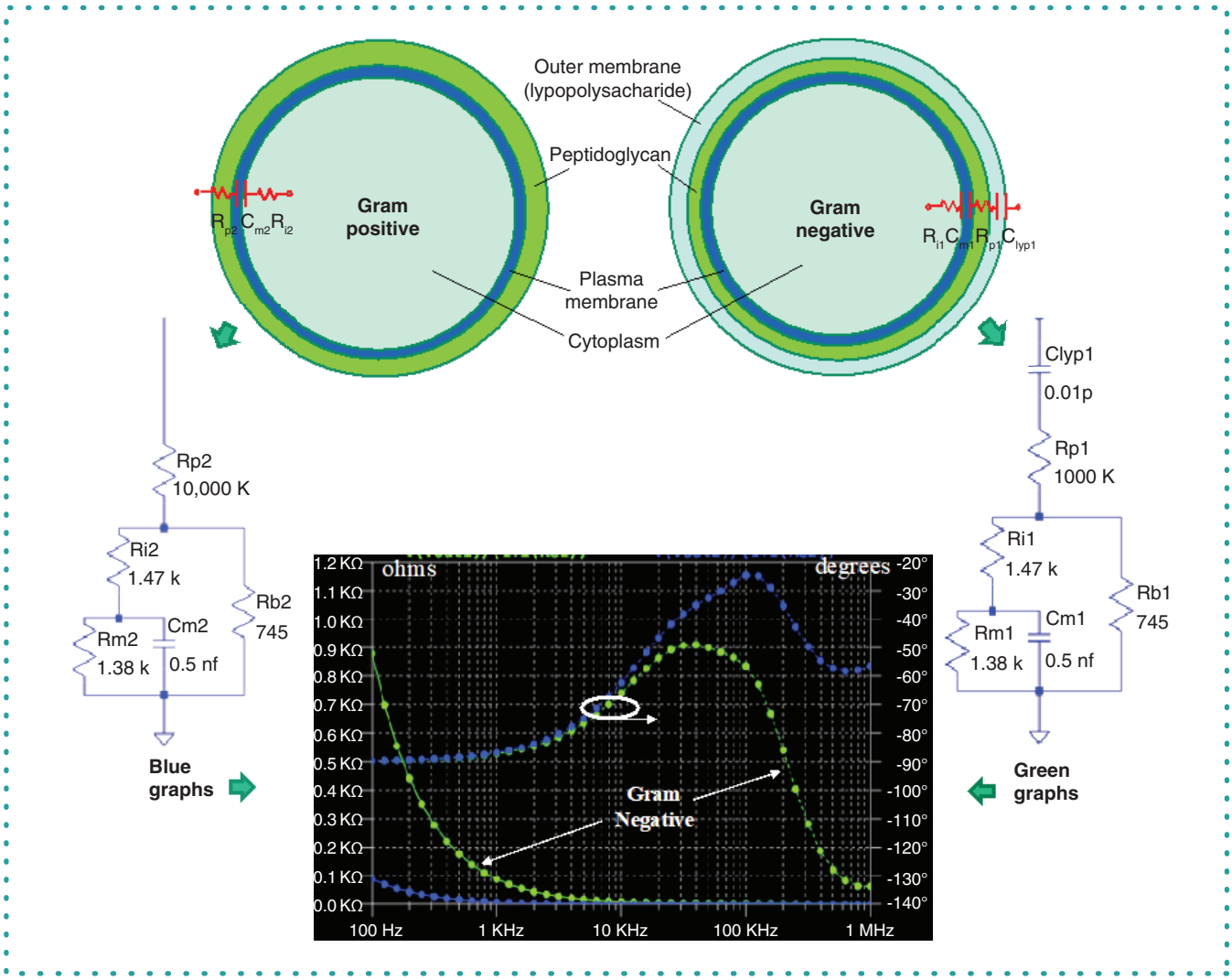


Figure 9. Numerical estimation of the impedance frequency response for Gram-positive and Gram-negative bacteria. Bacteria are modeled by R_i, R_m, C_m and R_b as the intracellular resistance, membrane resistance, membrane capacitance and extracellular resistance, respectively. R_p and C_{lyp} denote the resistance and capacitance for a composite interaction between the peptidoglycan and lipopolysaccharide layers.

This study was designed to evaluate the viability of impedance measurements as an inexpensive and simple technique for real-time detection of bacteria and differentiation between Gram-positive and Gram-negative species. Our results demonstrate that EBIS can potentially distinguish between presence and absence of bacteria as well as between different types of bacteria.

The methodology in this paper offers a simple and cost-effective means of detection and differentiating bacteria in label-free samples. However, appropriate miniaturization, optimization and clinical trials need to be done before any product is launched into market. The widespread use of bacterial cell sensors not only will be a milestone in the sensor industry but will have a profound impact on food, medical, environmental and clinical diagnostics.

Future perspective

Further studies are needed regarding sensitivity, specificity and utility for the bacterial concentrations found in clinical and/or industry applications. To meet the increasing demand for bacterial differentiation, innovative sensor devices like EBIS which provide a satisfactory combination of precision, sensitivity, specificity, speed and usability need to be developed. An excellent integration of biochemistry, biology, nanotechnology, physics and electronics would facilitate the development of high-performance sensors for bacterial detection and differentiation.

Author contributions

R Gnaim, C González and B Rubinsky conceived the idea for the study. C González and A Golberg designed the research protocol. R Gnaim performed the experiments with the help of J Sheviryov, while C González made the measurements and analyzed the data. C González and R Gnaim wrote the manuscript.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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