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Microscale electronic detection of bacterial metabolism

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Abstract

In this paper, we present a microscale impedance-based technique for detecting the metabolic activity of a few live bacterial cells. Impedance-based detection relies on measuring changes in the ac impedance of two electrodes in contact with a liquid where the bacteria are cultured, caused by the release of ionic species by metabolizing cells. Rapid detection of a few live cells (1–10) is, in theory, possible if the cells are confined into a volume on the order of nanoliters. A microfluidic biochip prototype has been fabricated to explore this technique, consisting of a network of channels and chambers etched in a crystalline silicon substrate. The complex impedance of bacterial suspensions is measured with interdigitated platinum electrodes in a 5.27 nl chamber in the biochip at frequencies between 100 Hz and 1 MHz. After 2 h of off-chip incubation, the minimum number of live cells suspended in a low conductivity buffer that could be easily distinguished from the same number of heat-killed cells was on the order of 100 *Listeria innocua*, 200 *L. monocytogenes*, and 40 *Escherichia coli* cells, confined into the 5.27 nl chamber. A number on the order of 100 live *L. innocua* cells suspended in Luria–Bertani (LB) broth produced a significantly higher signal than the same number of heat-killed cells, and a difference is evident even down to ∼5–20 cells. To the best of our knowledge, this is the first demonstration of microscale impedance-based detection of bacterial metabolism.
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Keywords: Biochip; Impedance; Bacteria; Detection; *Listeria*

1. Introduction

Detection of a few live pathogenic bacterial cells in food, water, or clinical samples is an important technological problem. For example, in the US, foodborne pathogens cause an estimated 14 million illnesses, 60,000 hospitalizations, and 1800 deaths each year [1]. Despite great efforts to reduce bacterial infections in developed countries, their incidence has tended to remain constant over the last decades [2]. And, with the current threat of bioterrorism, rapid bacterial detection has become a national security issue for many countries. Detecting pathogenic bacteria in food or bodily fluids is a very challenging problem, mainly because these bacteria can be present in very small numbers (their pathogenic dose can be as low as 10 cells). In food samples, these few cells are normally mixed with large numbers of particulates, proteins, carbohydrates, oils, and large quantities of non-pathogenic microorganisms, whose characteristics might be very similar to those of the pathogenic bacteria being searched for. Additionally, in most cases it is very important to detect only live cells. Some pathogens release toxins when alive, which remain in the food, and some could release toxins even after death; but most common pathogens are harmless when dead. Many food products that are likely to contain pathogens are subjected to processing conditions designed to inactivate these bacteria. In these cases, the food products are likely to contain dead cells that were originally pathogenic, and hence the purpose of screening is to ensure that no live ones remain after processing. Any assay that detects both live and dead cells would not be appropriate in this situation. Currently, the official screening procedures established by regulatory agencies (Food and Drug Administration, FDA, and US Department of Agriculture, USDA) are very sensitive, able to identify as few as 3 colony-forming-units per gram of food (cfu/g), but require a long time to complete with the work of highly skilled laboratory personnel [3,4]. The earliest a negative result can be obtained is about 2 days, and the examination of candidate positive results takes more than three days. A full test of samples that show ambiguous results can take up to 7 days. By the time a pathogen is identified in a food sample, the food would probably have been sold and consumed. Few automated methods exist with

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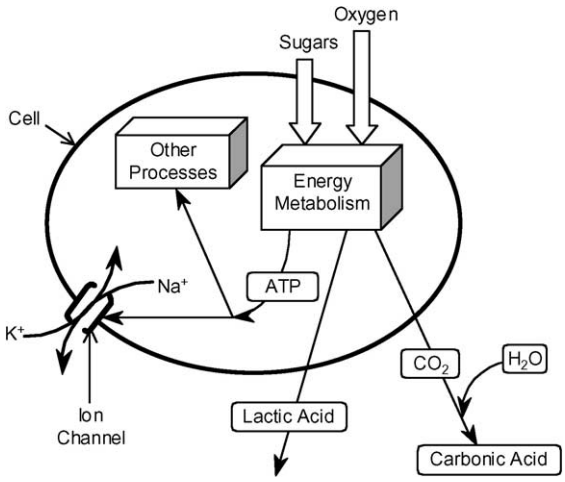

Fig. 1. Schematic representation of the two major mechanisms responsible for the release of ionic species by living cells: energy metabolism (catabolism) and ionic exchange across the membrane [9].

sensitivity and reliability comparable to those of the manual methods; and the most sensitive automated techniques cannot discern between live and dead cells.

One common automated bacterial detection method is based on the changes in the electrical characteristics of a medium where bacteria are cultured. These changes are produced by the release of ionic metabolites from live cells, measured by electrodes in contact with the medium [5]. The most common embodiment of this technique involves monitoring over time the ac impedance of a pair of electrodes immersed in the culture medium. If the impedance changes beyond a certain threshold, a positive detection is indicated. This technique was first identified more than 100 years ago, and has been used for many years in bacterial growth monitoring, bacterial load control on perishable products, and bacterial detection. There are numerous reports in the literature dealing with its application to rapid bacterial detection [6]. And equipment for automated impedance monitoring ranging from laboratory, bench-top size up to industrial scale is commercially available from several vendors. In fact, the method is recognized by the Association of Official Analytical Chemists International (AOAC) as a standard technique for the detection of *Salmonella* in food [7,8]. The ions released by bacteria into their environment have two main origins, as illustrated in Fig. 1: energy metabolism (catabolism) and ion exchange through the membrane. Catabolism, which consumes oxygen and sugars, and produces CO_2 and organic acids such as lactic and acetic, is by far the largest contributor to the release of ionic species. Carbon dioxide adds to the ionic release when it hydrates to produce carbonic acid. A smaller contribution comes from ions, such as K^+ and Na^+, that are actively transported across ion channels embedded in the cell membrane. This exchange serves to regulate the membrane potential, and the osmotic difference between the interior and exterior of the cell.

Unfortunately, the detection time of the conventional impedance-based method can be quite long when the number of bacterial cells present in the sample is very small. The lower the initial concentration of microorganisms, the longer it takes for the impedance to change by a measurable amount. A simple theoretical analysis, corroborated by experimental observations, shows that the detection time t_d (time required for the impedance to cross an arbitrary threshold) is related to the initial cell concentration C_0 by the following expression [5,10]:

$$\log(C_0) = -\alpha t_d + \beta. \quad (1)$$

In this equation, α ($\alpha > 0$) and β are constants that depend on the particular microorganism, the growth conditions, etc. Eden and Eden [5] provided experimentally-derived values of $\alpha = 0.96$ and $\beta = 7.75$, for t_d in hours and C_0 in cfu/ml of incubation broth. Fig. 2 shows a plot of Eq. (1) with these values of the parameters, indicating that detection times range from 1 h at $C_0 \sim 10^7$ cfu/ml to 8 h at $C_0 \sim 1$ cfu/ml.

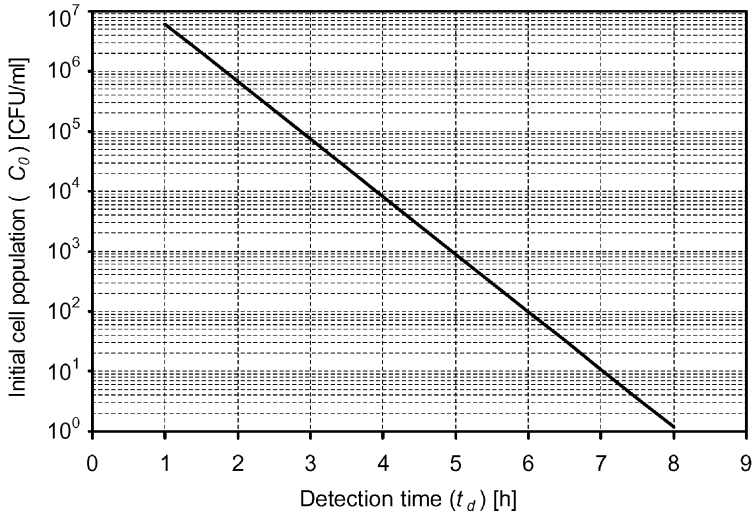

Fig. 2. Plot of Eq. (1) with values of $\alpha = 0.96$ and $\beta = 7.75$ obtained from [5].

Dupont et al. [10] reported values of 1.08 and 11.33 for α and β, respectively, for a particular set of food samples tested for *Escherichia coli* contamination (C_0 in cfu per 100 g of food sample). Corroborating these trends, Edmiston et al. [11] reported detection times ranging from about 1 to about 7 h, for initial bacterial populations of 10^7 and 40 cfu/ml, respectively. Therefore, the conventional impedance-based method is not adequate for detecting bacterial concentrations on the order of 100 cfu/ml or even lower in 2 h or less. This limitation can be overcome if those few cells are confined into a very small volume while the impedance is being measured. In this way, the effective cell concentration is increased without increasing the number of cells, such that a small number of cells can rapidly change the ionic concentration of a very small volume of growth medium. This concept leads to the idea of implementing the impedance-based detection in a microfabricated device (biochip), where volumes on the order of nanoliters can be easily created. One hundred bacterial cells confined into a volume of 10 nl results in a concentration of 10^7 cells/ml. Such application of microfabrication technologies seems especially fitting, since miniaturization of the impedance-based assay has the potential to directly reduce the time for detection by a significant factor.

In this paper, we present results of tests of the microscale impedance-based technique for detecting metabolic activity using a microfabricated device, with three types of bacteria: *Listeria innocua*, a non-pathogenic species of the genus *Listeria*; *L. monocytogenes*, a pathogenic species of the same genus; and a non-pathogenic strain of *E. coli*. *L. monocytogenes* is an ubiquitous rod-shaped, motile, Gram-positive, pathogenic bacterium, that can be found in soil, water, and food. *L. innocua* and *L. monocytogenes* are closely related and are often found together in food samples [12]. The group of illnesses caused by *L. monocytogenes* is referred to as *listeriosis*. This bacterium, in spite of not forming spores, is very resistant to adverse environmental conditions such as high salinity, acidity, and both low and high temperatures. A very important characteristic from the point of view of food safety is its ability to grow at temperatures as low as +3 °C [2]. *L. monocytogenes* enters the body via contaminated foods, most commonly on ready-to-eat meats and soft cheeses. While most healthy persons would not show any symptoms after ingestion of food contaminated with *L. monocytogenes*, individuals with weak immune systems (i.e. the elderly, newborns, and cancer, diabetes, or AIDS patients) can develop serious complications, including septicemia, meningitis (or meningoencephalitis), and encephalitis. The overall fatality rate for *L. monocytogenes* is approximately 20% [13] (20% of those that develop symptoms of listeriosis die). The minimum pathogenic dose of *L. monocytogenes*is unknown, but there is evidence that as few as 1000 viable (live) cells may cause listeriosis [2]. Consequently, any screening method must be able to detect between 100 and 1000 live bacterial cells/ml of sample, with a sample volume of a couple of milliliters.

2. Experimental procedures

2.1. Microfluidic biochip

Since the fabrication of the biochip is discussed in detail somewhere else [14], here we will only briefly describe the most important features of the device. The biochip consists of two groups of chambers connected in series by channels, both etched to a depth of 12 μm onto a crystalline silicon substrate and covered with a 0.45 μm layer of SiO_2 for electrical insulation. The chambers range in size from 80 μm × 80 μm to 530 μm × 850 μm, resulting in volumes between approximately 75 pl and 5.3 nl, while the channels have widths between 20 and 100 μm. Platinum electrodes are patterned at the bottom of the chambers to measure the impedance of liquids injected into them; these electrodes are connected by platinum traces to pads on the periphery of the chip where electrical contacts with measuring equipment are established. The top side of all the channels and chambers is covered by a bonded glass slab that provides perfect hermeticity. Deep trenches are etched at the edge of the chip for insertion of microbore tubes that inject and drain the liquids that are flowed through the chip. The size of the whole biochip, excluding the tubes, is 9.12 mm × 8.9 mm. A very simplified schematic diagram of the chip design is presented in Fig. 3.

2.2. Preparation of the bacteria

Unless otherwise noted, bacterial suspensions were obtained by the following "standard" procedure.

1. A certain volume, typically 5 ml, of Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Michigan) was inoculated with bacterial cells extracted from a gel stock culture (in this stock the cells were maintained at room temperature in BHI-agar gel) with a wire loop.

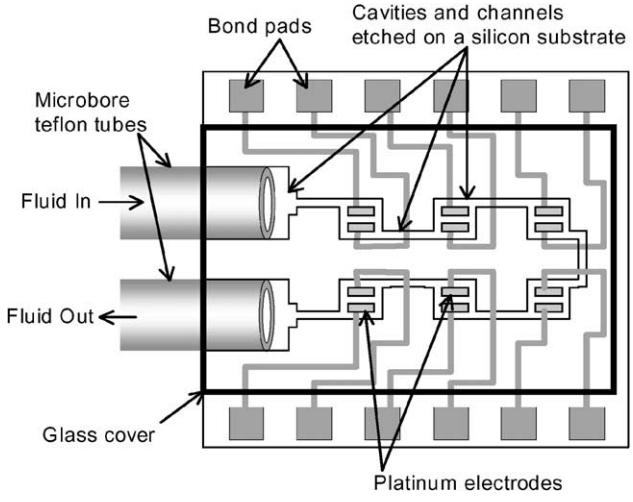

Fig. 3. Simplified schematic representation of the biochip used for microscale, impedance-based detection of bacterial metabolism [14].

2. This inoculated broth was incubated for at least 16 h at 37 °C to create a primary culture.
3. The desired volume of BHI, which depends on the experiment that will be performed, was inoculated with an aliquot from the primary culture. The volume of the aliquot is equal to 1% of the volume being inoculated.
4. This second inoculated broth was incubated for at least 16 h at 37 °C to create a secondary, or ''working,'' culture.
5. If dead bacteria were needed, a fraction of the working culture was placed in a water bath at 80 °C for 20 min to kill all the cells.
6. The whole or an aliquot of the working culture and/or the heat-killed suspension were centrifuged at $2300 \times g$ for 8 min in a microcentrifuge.
7. The supernatant was removed from the centrifuged sample and the pellet was resuspended in the medium where measurements were performed.
8. The previous two steps were repeated a minimum of four times to completely remove all ionic species that were present in the original growth medium.
9. The washed cells were resuspended at the desired concentration (or concentrations) in the chosen medium, at a total volume of 4 ml typically.
10. After washing and resuspending, a sample from one of the suspensions was diluted and plated on BHI-agar plates. The plates were incubated at 37 °C for 24 h, and the number of growing colonies were counted to estimate the number of live cells in the original suspensions.
11. The bacterial suspensions were incubated off-chip at 37 °C for 2 h to allow the bacteria to release ionic species into the medium.
12. From the end of the incubation to the point where they were injected into the biochip, the suspensions were kept at approximately 4 °C to decrease bacterial activity.

For all the tests presented here, the bacteria were incubated off-chip in a macroscale volume before injecting them into the chip for measuring, without performing any further incubations inside the chip. This was done as a first test of the detection concept at the microscale, before proceeding to make more elaborate tests with on-chip incubation. Since the incubation volume was on the order of milliliters, the cell concentrations were on the order of 10^5 to 10^9 cells/ml, which result in a number of cells on the order of 1–10,000 in a 5.27 nl volume. Additionally, at this time, we have not yet perfected the means to selectively capture a few bacterial cells inside the biochip, which in the future would allow us to perform the incubation on-chip.

Two types of suspension media were used for the measurements, one with very low ionic content but none of the nutrients necessary for bacterial growth, and another one with a much higher ionic content and all the nutrients needed by the bacteria to multiply. In a medium with low ionic content, even though bacterial metabolism might be suppressed and thus not releasing large amounts of ionic species, a very small amount of extra ions will produce a large relative change in impedance. On the other hand, in a richer medium, where bacteria metabolize and grow rapidly, a large amount of ions are needed to change the impedance by a measurable amount.

The low ionic content medium is Tris–glycine–dextrose (Tris–Gly–Dext) buffer, which contains 0.18 mM Tris, 47 mM glycine, and 5 mg/ml of dextrose as a metabolic substrate. The Tris concentration was modified around the nominal value to adjust the pH to 7.5. The conductivity of this medium is 17 μS/cm. This medium has very low ionic content and does not include any of the minerals, proteins, vitamins, and other nutrients that bacteria require for long-term survival and multiplication. Additionally, its low osmolarity subjects the cells to a large osmotic pressure, which would tend to make them swell and could eventually cause them to burst. Monitoring of *L. innocua* cells suspended in Tris–Gly–Dext indicate that cell survival in this medium is fairly unpredictable. For this monitoring, cells were washed and suspended in Tris–Gly–Dext at a concentration of approximately 10^7 cfu/ml and incubated at 37 °C for up to 4 h. Samples from the suspension were taken at three points in time: immediately before incubation, after 2 and 4 h of incubation. The samples were diluted 10,000-fold and 100 μl or 200 μl aliquots from this dilution were placed on each of three BHI-agar plates. These plates were then incubated for 24 h at 37 °C, after which the number of colonies growing on them were counted. Fig. 4 shows the number of colonies after each incubation step, relative to the number immediately before incubation, from three separate experiments. In two cases, a large fraction of *L. innocua* cells died after 2 h, while in another case all cells survived. After 4 h, the cell population increased in one case, while it decreased in the other two.

The second medium used, with a much larger ionic content than Tris–Gly–Dext, is Luria–Bertani (LB) broth, which is composed of yeast extract (5 g/l), tryptone–peptone (10 g/l), and dextrose (3.3 g/l). These ingredients provide all the nutrients necessary for fast growth of *L. innocua* and *E. coli*. The original formulation of this medium includes 5 g/l of NaCl, but we eliminated this component to reduce the ionic content. This medium has a conductivity of 2 mS/cm, compared to 17 μS/cm for Tris–Gly–Dext. To test the effect of various concentrations of NaCl in the medium, growth curves of *L. innocua* in LB broth were obtained by the following procedure: LB medium with 0.625 g/l NaCl was inoculated at a 5% (v/v) level with *L. innocua* (previously grown in BHI) and incubated for 24 h at 37 °C. This culture was then used to inoculate three samples of LB at a 1% (v/v) level, with the following NaCl concentrations: 5, 0.5, and 0.00625 g/l (this last concentration, 6.25 mg/l, results from the salt carried into the sample by the inoculum). The inoculated samples were incubated at 37 °C while their absorbance at 595 nm was measured every 10 min (the absorbance was corrected based on the absorbance

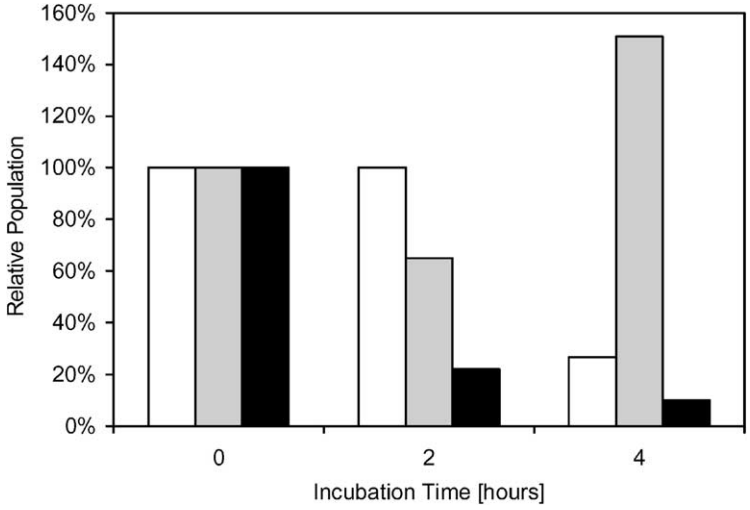

Fig. 4. Survival of *L. innocua* cells suspended in Tris–Gly–Dext buffer at a concentration of approximately 10^7 cfu/ml, estimated from plate-count experiments, after 2 and 4 h of incubation at 37 °C. The bars correspond to the number of colonies obtained after each period of incubation, relative to the number of colonies obtained before incubation. Each shade of gray represents a separate experiment.

of a sample without bacteria) using a Beckman Coulter DU-640 spectrophotometer (Beckman Coulter Inc., Fullerton, CA). Fig. 5 shows the time course of the absorbance over a period of 24 h, indicating that the growth rates of *L. innocua* in LB with and without NaCl are very similar. With 5 g/l NaCl the generation time during exponential growth is 55 min, while with 6.25 mg/l NaCl the generation time is 50 min.

2.3. Viability detection

After creating bacterial suspensions using the standard procedure mentioned earlier, the suspensions were injected into the chip sequentially, using a simple pressurized nitrogen-driven injection system, and were allowed to flow through the chip for 15 min before measuring to properly flush the whole fluidic path. Fig. 6 shows the general arrangement of the measurement system. Impedance measurements were performed in a chamber that is 530 μm wide by 850 μm long by 12 μm deep, for a total volume of 5.27 nl (taking into account that the walls of the chamber have a 54.74° angle). This chamber has two interdigitated platinum electrodes with five fingers each. The exposed area of each finger is 450 μm by 50 μm, and the distance between finger centers is 80 μm. An HP4284A LCR meter (Hewlett-Packard, now Agilent Technologies, Palo Alto, CA) measured the impedance of the interdigitated electrodes at 52 frequencies, logarithmically spaced between 100 Hz and 1 MHz, with a 50 mV (amplitude) voltage excitation. The impedance of the wiring and probes was automatically subtracted from the measurements by the instrument, so that only the impedance of the elements in the chip was recorded. Identical

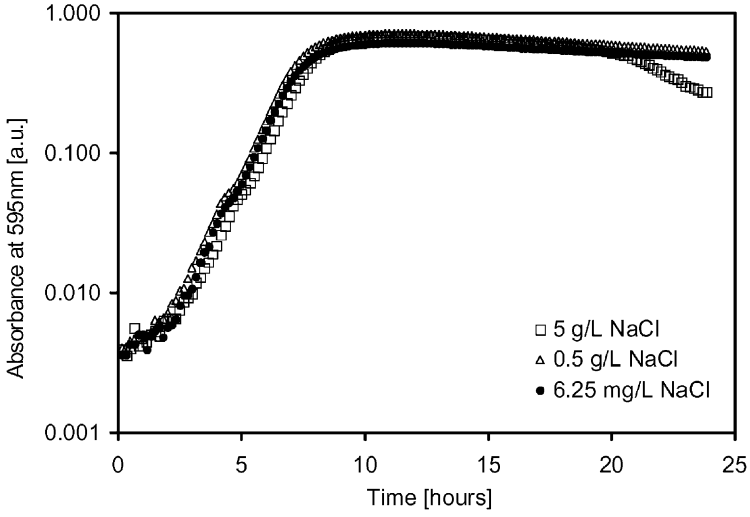

Fig. 5. Growth of *L. innocua* cells incubated at 37 °C in LB broth with different NaCl concentrations, measured as the absorbance of the suspension at 595 nm. Generation times, extracted from the exponential growth phase are: 55, 52, and 50 min, for 5 g/l, 0.5 g/l, and 6.25 mg/l NaCl, respectively.

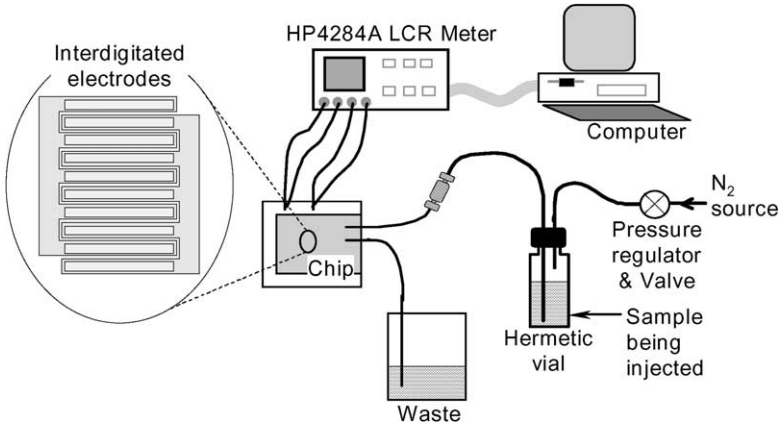

Fig. 6. General arrangement of the measurement system.

measurements were performed on suspensions of *L. innocua*, *L. monocytogenes* and *E. coli*, with very similar results.

Fig. 7 shows the complex impedance (magnitude and angle) as a function of frequency of eight different samples injected into the biochip: de-ionized (DI) water, Tris–Gly–Dext buffer, and six *L. innocua* suspensions in Tris–Gly–Dext. The six suspensions correspond to live and heat-killed cells at three different concentrations, approximately 10^5, 10^7, and 10^9 cells/ml, resulting in 1–4, 50–200, and 5000–20,000 cells in the 5.27 nl chamber, respectively. These ranges are due to relatively large uncertainties in the actual concentration of the suspensions for this particular set of samples. It can be seen in the figure that the plateau in the impedance (between approximately 1 and 20 KHz for four samples) decreases as the number of cells increases, and the hump in the phase (in the same frequency range as the magnitude plateau) moves towards higher frequencies. Impedance curves obtained from *L. monocytogenes* and *E. coli* display the same behavior. This behavior can be understood by looking at an electrical model of two electrodes in contact with the bacterial suspension, which is presented in Section 3.

3. Data analysis and results

A fairly simple circuit model of a pair of electrodes immersed in an electrolytic solution is shown in Fig. 8 [15], where C_{di} is the dielectric capacitance (it contains dielectric contributions from all the materials surrounding the electrodes, including the solution), R_s is the bulk solution resistance (charge transport across the bulk solution), and Z_w is the interfacial impedance (the so-called Warburg impedance), which accounts for the changes in the ionic gradient

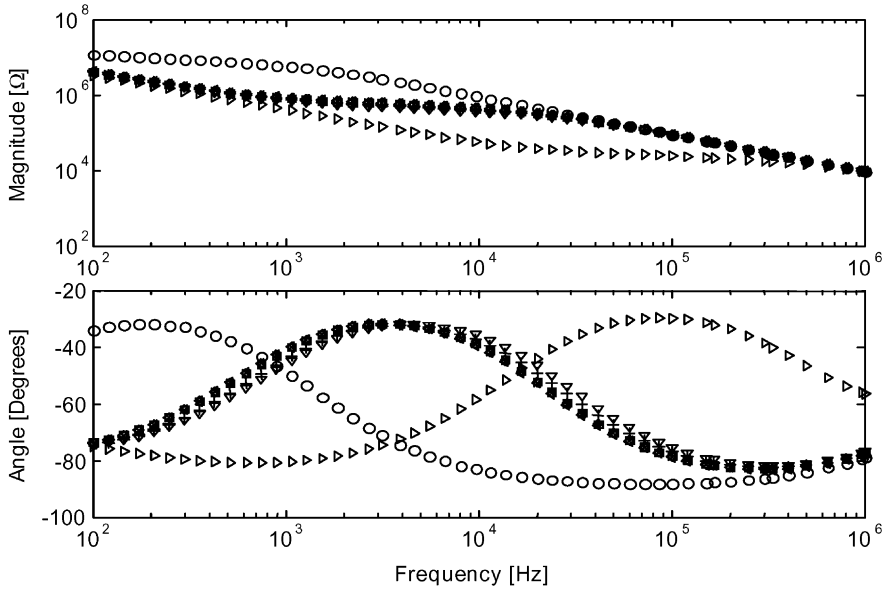

Fig. 7. Complex impedance (magnitude and angle) of DI water (◯), Tris–Gly–Dext (◁), and six different samples of *L. innocua*: 1–4 dead (×) and live (◇) cells, 50–200 dead (□) and live (▽) cells, 5000–20,000 dead (+) and live (▷) cells. These numbers correspond to the number of cells in the 5.27 nl chamber in the chip, where impedance is measured.

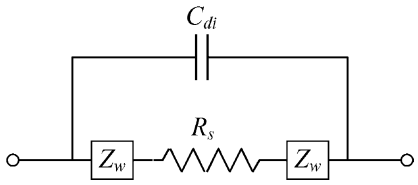

Fig. 8. Equivalent circuit of two identical metallic electrodes in contact with an electrolyte (C_{di} = dielectric capacitance; R_s = bulk electrolyte resistance; Z_w = interfacial impedance) [15].

at the interface [16,17]. The simplest model of the interfacial response to ac signals yields the following expression for Z_w:

$$Z_w = \frac{\sigma(1-j)}{\omega^{1/2}}, \tag{2}$$

where $j = \sqrt{-1}$, ω is the angular frequency of the electrical signal, and σ is a parameter that depends on the diffusive properties of the electrolytes, and the area and characteristics of the electrodes. From this expression, we can see that the phase difference between the applied voltage and the resulting current will be 45° at all frequencies. However, actual systems show a phase difference that can be anywhere between 0° and 90°. Thus, a better model for the interfacial impedance is [15]:

$$Z_w = \frac{1}{(j\omega)^n B}, \tag{3}$$

where n and B are parameters that depend on the properties of the electrolytes and of the electrodes. Eq. (3) assumes that the phase between voltage and current is constant at $n\pi/2$ rad. Experimental data indicate that the value of n is typically close to 1, in which case, the interfacial impedance Z_w is mostly capacitive. The hump in the phase observed in Fig. 7 occurs in a frequency range where R_s is larger than Z_w, but much smaller than C_{di}, so that R_s dominates the total impedance of the circuit. For lower frequencies Z_w dominates, while C_{di} dominates at higher frequencies. As the ionic concentration of the solution increases, the bulk solution resistance decreases, shifting the range where it dominates to higher frequencies, and decreasing the overall impedance in that same range.

The circuit model shown in Fig. 8 was fitted (using least-squares criteria) to the curves in Fig. 7, with Z_w given by Eq. (3), to obtain values for C_{di}, R_s, B, and n. An additional resistor R_{tr} was added in series to the circuit to account for the resistance of the metal traces on the biochip and the contacts to them. Values of $R_{tr} = 1909\,\Omega$ and $C_{di} = 17.76$ pF were extracted from fitting to the Tris–Gly–Dext buffer data and held fixed when fitting the model to all other samples. Fig. 9 shows an example of how well the circuit model fits the measured impedances. The following normalized differences for the various cell concentrations are calculated from the parameters extracted from the fit, and will be referred to as the "signals" obtained from the impedance measurement:

$$\overline{\Delta Z} = \frac{|Z_{\text{buffer}} - Z_{\text{bacteria}}|}{|Z_{\text{buffer}}|}, \quad \text{at } f = 11.43\,\text{kHz} \tag{4}$$

$$\overline{\Delta R_s} = \frac{|R_{s-\text{buffer}} - R_{s-\text{bacteria}}|}{R_{s-\text{buffer}}} \tag{5}$$

$$\overline{\Delta B} = \frac{|B_{\text{buffer}} - B_{\text{bacteria}}|}{B_{\text{buffer}}}. \tag{6}$$

In Eq. (4), Z_{buffer} and Z_{bacteria} refer to the as-measured complex impedances of the Tris–Gly–Dext buffer and of the bacterial suspension, respectively, at a frequency of

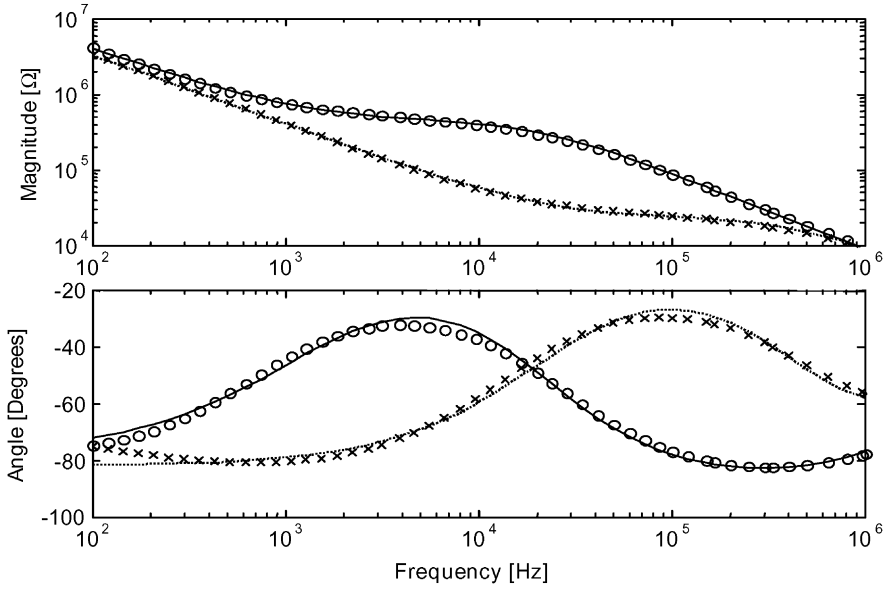

Fig. 9. Fits of the circuit in Fig. 8 to the complex impedance of the *L. innocua* samples at a concentration corresponding to 5000–20,000 cells in the 5.27 nl measurement chamber (from the same data as in Fig. 7): dead cells measured (◯); dead cells fitted (—); live cells measured (×); live cells fitted (···).

11.43 KHz. Both $\overline{\Delta Z}$ and $\overline{\Delta R_s}$ should all increase as the ionic concentration in the suspension increases. As the ionic concentration increases, more ions are available for transporting charge inside the liquid and the bulk resistance of the suspension R_s decreases; this in turn causes the total impedance Z to decrease, especially in the frequency range in which R_s dominates. The behavior of B is much more complicated and unpredictable because this parameter is

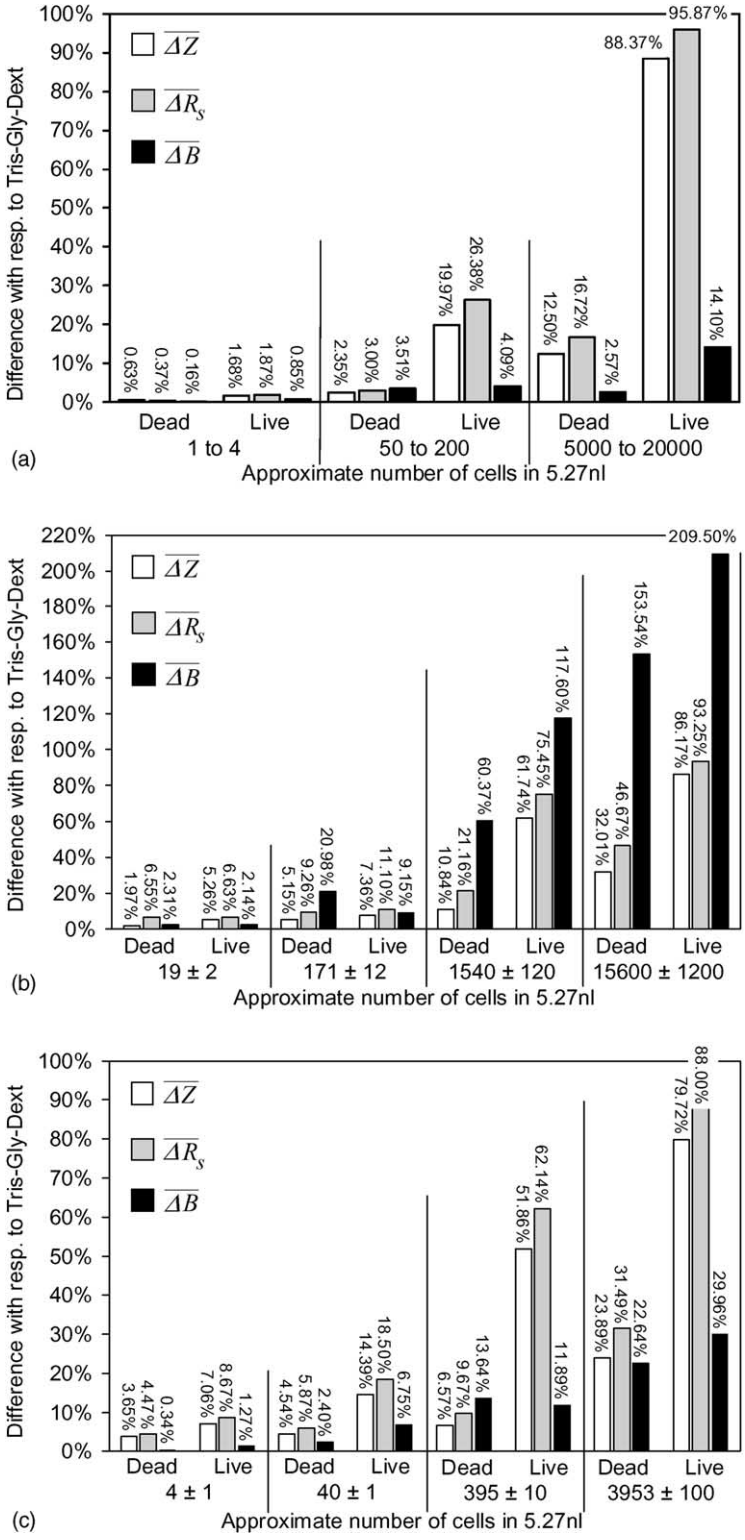

Fig. 10. Normalized differences $\overline{\Delta Z}$, $\overline{\Delta R_s}$, and $\overline{\Delta B}$, as defined in Eqs. (4)–(6), for three species of bacteria suspended in Tris–Gly–Dext buffer: (a) *L. innocua*; (b) *L. monocytogenes*; and (c) *E. coli*.

extremely sensitive to the characteristics of the electrode surfaces. In these experiments, we did not characterize the electrode–liquid interfaces, beyond extracting B and n, nor did we ensure that the interfaces were in a standard state before performing the measurements (by standard cleaning procedures, followed by cyclic voltammetry, for example).

Fig. 10 shows the signals, defined in Eqs. (4)–(6), obtained from three sets of bacterial samples: *L. innocua*, *L. monocytogenes*, and *E. coli* suspended in Tris–Gly–Dext. Each set contained samples of live and heat-killed cells, at three or four different concentrations. It is evident from the figure that both $\overline{\Delta Z}$ and $\overline{\Delta R_s}$ correlate very well with the number of cells. More importantly, the magnitude of these differences is much larger for live cells than for heat-killed ones, which is a clear indication that the change in impedance is caused by the metabolic activity of the cells. The signals observed from suspensions with dead cells are most likely caused by ionic species leaking out of the cells, and by cells that burst due to osmotic pressure. Cellular membranes have very low permeability to ions [18], but leakage is possible through injuries in the cell membrane produced by the heat-killing process and by the natural degradation of the membrane that occurs after death. As was mentioned earlier, Tris–Gly–Dext buffer can induce a large osmotic pressure due to its low osmolarity, which could lead to cell lysis. Live cells have various mechanisms to control osmotic pressure, such as the release or uptake of K^+ ions to reduce or increase the osmolarity of the cytoplasm, but these mechanisms are obviously not active in dead cells, making them more prone to osmotic lysis [19]. The values of $\overline{\Delta B}$ also increase with increasing cell numbers, but their behavior is much less consistent than that of $\overline{\Delta Z}$ and $\overline{\Delta R_s}$. As was mentioned earlier, this is likely caused by the high sensitivity of B to the properties of the electrode surfaces, which were not controlled during the experiments.

The technique was also tested on cells suspended in LB broth, whose composition is described in Section 2.2. The cells were grown, harvested, washed, and incubated following the standard procedure, using LB broth instead of Tris–Gly–Dext buffer. Impedance measurements were also carried out in the biochip using the same procedure as before. Fig. 11 shows the value of $\overline{\Delta Z}$ for four different concentrations of *L. innocua* cells in LB medium, calculated from the impedances at 1.07 and 11.43 kHz. The impedance curves from these samples could not be properly fitted by the circuit model in Fig. 8, making it impossible to extract values for R_s and B. Nevertheless, $\overline{\Delta Z}$ provides a good indication of metabolic activity, with a trend similar to that shown in Fig. 10, except for the small difference in the signals obtained from 500 and 5000 cells. Such small difference could be explained by the fact that 5000 cells in 5.27 nl correspond to a concentration of approximately 10^9 cells/ml, which is higher than the highest concentration these cells attain when growing in LB broth. Once the concentration reaches approximately 10^8 cells/ml, the acidic species released by bacterial metabolism lower the medium's pH below the tolerance limit of the cells (LB broth does not contain any buffering agent). Consequently, further metabolic activity and growth are inhibited by the low pH. In this case, when the concentration is forced to be 10^9 cells/ml (5000 cells in the chamber), the bacteria will only release metabolites up to the level equivalent to 10^8 cells/ml, at which point their metabolism is inhibited by the low pH. This is not necessarily a problem, since we are interested in detecting small numbers of cells. In Tris–Gly–Dext medium, there is a large difference between the signals produced by the two highest cell concentrations because it is buffered by Tris. With this buffering agent, a larger amount of acidic metabolites can be released without the medium's pH falling below the tolerance limit of the cells.

The most important observation derived from these measurements is that it is possible to discern the metabolic activity of a few live bacterial cells from the signal produced by the same number of heat-killed cells. The minimum

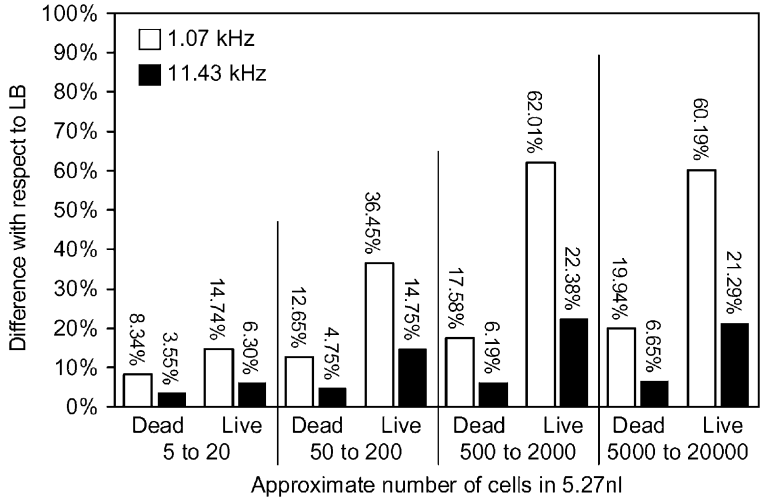

Fig. 11. Normalized difference $\overline{\Delta Z}$ calculated at two different frequencies (1.07 and 11.43 kHz) for *L. innocua* cells suspended in LB broth.

number of live cells suspended in Tris–Gly–Dext buffer that could be easily distinguished from the same number of heat-killed cells was on the order of 100 *L. innocua*, 200 *L. monocytogenes*, and 40 *E. coli* cells, confined into a 5.27 nl volume. For *L. monocytogenes*, there were small differences in $\overline{\Delta Z}$ between live and dead cells for 19–171 cells, but they might not be statistically significant. Similarly, for *L. innocua* and *E. coli*, a small difference between live and dead cells could be observed down to about 4 cells. A number on the order of 100 live *L. innocua* cells suspended in LB broth produced a significantly higher signal than the same number of heat-killed cells, and a difference is evident even down to ∼5–20 cells. Additional experiments using *L. innocua* suspended in Tris–Gly–Dext (data not shown) showed that the value of $\overline{\Delta Z}$ for 1–4 live cells was consistently larger than the value for dead cells by factors between 1.14 and 2.7. The large range of these factors is due to the fact that each of these additional experiments was performed with slightly different compositions of the buffer: adding 0.05% (v/v) of Tween-20 (a detergent) and/or 5% (w/w) of glycerol (an osmoprotectant).

4. Conclusions

We have shown the use of a microfabricated biochip for impedance-based detection of the metabolic activity of bacterial cells suspended in two different kinds of media, one with very low ionic content (Tris–Gly–Dext) and the other with high ionic content (LB broth). The electrical impedance of bacterial suspensions was measured at several frequencies inside a 5.27 nl microfabricated chamber in a biochip. Ionic species released by the metabolic activity of live cells increase the ionic concentration of the suspension, and thus modify its electrical characteristics. After 2 h of off-chip incubation of *L. innocua* and *E. coli* suspended in Tris–Gly–Dext buffer, and *L. innocua* suspended in LB broth, a small difference in impedance could be observed between live and heat-killed cells at concentrations resulting in a number of cells on the order of 5 in the 5.27 nl chamber. A very significant difference in impedance was observed for *L. innocua* and *E. coli* suspended in Tris–Gly–Dext buffer, and *L. innocua* suspended in LB broth for a number of cells on the order of 100. The minimum detection level for *L. monocytogenes* in Tris–Gly–Dext buffer was on the order of 200 cells. These observations seem to indicate that it would be possible to use the impedance-based technique for a microscale system for rapid detection of the viability of a few bacterial cells.

No significant differences were observed in the sensitivity obtained with Tris–Gly–Dext buffer and LB broth. Given that sensitivity is the same, LB broth is more attractive than Tris–Gly–Dext because LB broth does not stress the cells as Tris–Gly–Dext does due to its low osmolarity and lack of nutrients. This is a very important factor when trying to detect bacteria in food samples. Bacteria present in food are very likely to be injured or stressed by low pH, high salt concentrations, and heat treatments, and are likely to die very quickly if they are stressed further by suspending them in Tris–Gly–Dext buffer. This can result in a very low metabolic signal from the impedance-based assay. On the other hand, injured and stressed cells can recover and grow if they are put in LB broth, thus generating a larger impedance change. We are currently working on a microscale detection system where the target bacteria can be selectively captured and incubated inside the biochip. In this system, the metabolic signal used for detection will be a monotonic change in impedance over time, of a sample with the captured bacteria.

A significant factor that would limit the sensitivity of the assay is the fact that heat-killed cells do generate a small change in impedance. Consequently, a large number of dead cells can produce the same as or a larger signal than a small number of live ones. For example, in Fig. 10c, it can be seen that 395 dead cells make R_s change by a larger percentage than 4 live cells. Based on the data collected so far, it seems that it would be difficult to detect live cells mixed with dead ones in ratios larger than 10 to 1 (dead to live). However, the sensitivity to live cells could possibly be increased by examining the shape of the impedance change over time, and not only the final value. It is conceivable that the shape of the change would be different for live and dead cells. Additionally, there is evidence that the antibodies that will be used for bacterial capture exhibit a higher affinity to live cells than to heat-killed ones [20] (the antigens on the cell surface targetted by the antibody are degraded by high temperatures, making them less likely to bind to the antibody). This difference in affinity would improve the ratio of live to dead cells captured by the chip. More experimental data are necessary to fully address this issue.

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