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PAPER

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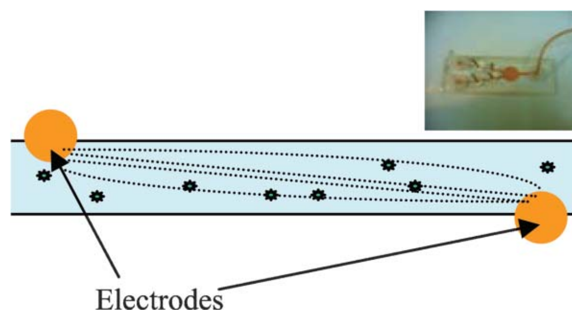
A micro-scale multi-frequency reactance measurement technique to detect bacterial growth at low bio-particle concentrations

Shramik Sengupta, David A. Battigelli and Hsueh-Chia Chang

By increasing the medium resistance (and hence medium *RC* time) in a capillary appropriate microchannel geometry allows the user to detect contributions to the medium capacitance due to bacteria, and hence detect low bacterial loads.



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A micro-scale multi-frequency reactance measurement technique to detect bacterial growth at low bio-particle concentrations

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An on-chip sensor is able to quickly detect the presence of low bacterial counts in fluids (an initial load of ~100 bacteria per ml within three hours) using electrical impedance (reactance) measurements made at easily realizable frequencies of <1 MHz. The technique described enables the user to detect the presence and proliferation of bacteria through an increase in the bulk capacitance (C) of the suspension, which is proportional to the bacteria count, at practical frequencies less than 1 MHz. The geometry of the micro-capillary design employed increases the bulk resistance (R) of the medium, thus increasing its RC time. This makes the measured reactance sensitive to changes in the bulk capacitance, which is usually masked by the much larger surface capacitance. The sensitivity is further enhanced by the existence of a minimum in the value of the reactance at a frequency proportional to the inverse medium RC time. The value of this reactance minimum and the frequency at which the minimum is recorded are dependent on the bacteria count and permit the detection of an initial concentration of ~100 CFU ml^{-1} of *E. coli* within 3 hours of incubation, in comparison with the previous reported values of about 8 hours, with an initial load of 1000 CFU ml^{-1}.

Introduction

A question that is encountered extremely frequently in microbiological practice, whether in clinical, food, environmental, or purely scientific setting, is "How many viable bacteria of a particular kind are present in a given sample of fluid?" Since, in many cases, the answer can vary over several orders of magnitude [from a few or none to millions of colony forming units (CFU) per ml of sample], the standard procedure is to dilute the available sample serially over a few orders of magnitude, and to plate these serially diluted samples out on an agar plate containing growth medium with the desired selectivity. The plates are then kept at a temperature favoring growth (typically 37 °C), and the number of colonies established counted after allowing the bacteria sufficient time to proliferate. Depending on the species and the medium, this time may range from 12 hours (overnight) to weeks.[1]

This method, essentially the same as those first used by Robert Koch in the last three decades of the 19th Century, utilizes a lot of material and is, moreover, slow, tedious and labor intensive. Hence, there has been a lot of interest in, and effort devoted to, developing alternate, more automated and less materially wasteful, methods of enumerating viable bacteria in a given sample. These methods span a variety of techniques, from those based on detecting the activity of a specific enzyme[2] or the release of a specific metabolite like carbon dioxide, radio-labeled[3] or otherwise,[4,5] to those that look for an increase in the electrical conductivity[6] or a change in the pH (color)[7] of the medium as a whole.

Many of the methods are employed in commercial devices such as the Bactec™ that detects the amount of radio-labeled carbon dioxide released, Coli-Check™ swabs that use Bromocresol Purple as an indicator to measure the decrease in pH due to bacterial metabolism, and the Bactometer™ (Bactomatic Ltd.), Malthus 2000™ (Malthus Instruments Ltd.) and RABIT™ (Don Whitley Scientific Ltd.) systems that use electrical impedance.

The common underlying feature of these techniques, including those which use electrical impedance, is that they rely on bacterial metabolism to produce a discernable change in a material property of the medium (such as pH, optical density, amount of carbon dioxide dissolved, electrical conductivity). The amount of metabolite processed by an individual bacterium is extremely small. [Based in our knowledge that the specific oxygen consumption rate for *E. coli* is 20 mmol of oxygen per hour per gram (dry weight) of bacteria[8] and a typical bacterium has a dry weight $\sim 10^{-12}$ g,[9] we estimate that one bacterium consumes only 2×10^{-14} moles of oxygen in one hour]. Hence, there has to be a sufficiently large number of bacteria present (either *a priori* or arising due to proliferation from the smaller number initially present) before the signal generated (change in the material property of the suspension) can be effectively measured. If the bacterial count in the original suspension happens to be small (1000 CFU ml^{-1} or lower) this results in the automated indirect system taking as long as the culture plates to provide the desired result. Thus, while these systems are used frequently in certain niche applications, the primitive plating technique continues to be the most widely used method for estimating bacterial counts.

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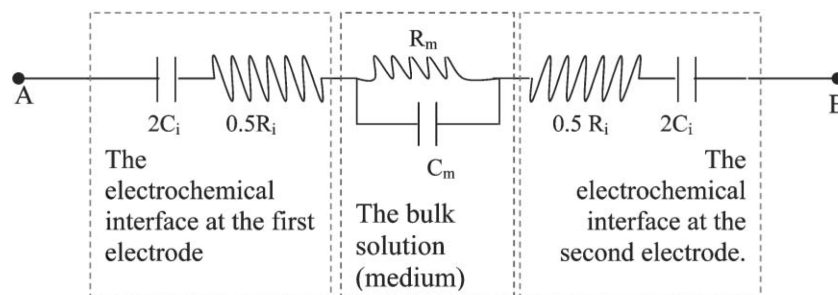
Among all these automated or semi-automated methods, electrical methods are especially attractive because they can potentially be incorporated into small bench-top units or hand-held devices, and/or used in instruments designed to handle a large number of samples simultaneously. However, electrical data generated by solutions containing metabolizing and proliferating bacteria are difficult to interpret. Something regarding the nature of the problems encountered in doing so can be gauged from the electrical circuit representation of the net measured impedance between two electrodes in direct contact with an aqueous solution (as shown in Fig. 1).[10] As is shown by the accompanying equation, the measured impedance is influenced by both the "bulk" and the "surface" quantities (resistance and capacitance). Typically, the bulk quantities reflect the state of the solution/suspension of interest, and the surface circuit represents the electrochemical interface (the "double layer") in equilibrium with the bulk. While the resulting net reading is thus correlated to events in the bulk, asynchronous changes occurring in the surface and the bulk may lead to erroneous conclusions being drawn about the state of the bulk solution.[11]

Under different circumstances, this representation may be modified according to the behavior of individual systems. For instance, when probing highly conductive solutions that lack charged macromolecules with low voltages, one may ignore the bulk capacitance and the surface resistance. This yields the classical result[12] for describing the behavior of metal electrodes in contact with a conductive solution. However, since several equivalent circuits produce similar or identical impedance spectra (especially when the observed range of frequencies is limited), the model must be true to the known (or, at the very least, plausible) physics of the system.

That the presence of bacteria and their metabolic activity changes the electrical properties of the bulk medium in which they are suspended has been known for a long time. They are known to do so in two ways. Firstly, by breaking down sugars commonly present in growth media to more conductive species like pyruvic, lactic, and carbonic acids they increase the conductivity (decrease the bulk resistance) of the medium.[16] Secondly, by acting as dipoles and possibly even storing charge, the very presence of individual bacteria (along with macromolecules present in the growth medium) contributes to the bulk capacitance of the solutions, thereby affecting the measured reactance (imaginary, or out-of-phase component of the impedance).[17,18] The effect of metabolism alone on the dielectric constant (bulk capacitance) of the solution, decoupled from the contribution due to the physical presence of bacteria alone, seems not to have been studied and is, moreover, expected to be negligible given that metabolism breaks down both molecules that raise the dielectric constant of the solutions (like proteins and peptides) and molecules that lower the dielectric constant (like carbohydrates). The various approaches adopted thus far to developing an impedance based system for bacterial detection and/or enumeration differ from each other with respect to (a) which of the two effects above they seek to monitor, and (b) the electrical circuit models that they use as a framework to better quantitatively explain the behavior of their systems.

Virtually all of the work available in literature, and all the commercially available instruments, depend on monitoring the effect of bacterial metabolism on the electrical properties of the growth medium. The simplest technique,[17] merely tries to relate the absolute value of the measured impedance to the changes in the system. Other methods[6,19,20] ignore the interface (surface) resistance and bulk capacitance (reactance). This approach implies that all the contributions to the resistance (real, or in-phase, component of the impedance) are from the bulk, and that the capacitances that contribute to the measured reactance reside on the electrode–solution interface. Some of these approaches[6] ignore the bulk or medium reactance and try to relate the changes in resistance to the effects of bacterial metabolism. Others[19–21] ascribe the change in the measured reactance to changes in the interfacial capacitance and try to relate that to the effects of bacterial metabolism on the solution. This approach probably works because there is an adsorbed layer of solutes on the electrode that is in equilibrium with the bulk solution,[22] and this affects the interface capacitance.



$$Z_{measured} = \left(R_i + \frac{R_m}{1+\omega^2 R_m^2 C_m^2} \right) - j\left(\frac{1}{\omega C_i} + \frac{\omega R_m^2 C_m}{1+\omega^2 R_m^2 C_m^2} \right)$$

Fig. 1 Electrical representation of the material between the two metal electrodes in direct contact with a solution, showing the contributions from the interface and the bulk (medium) to the measured impedance.

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However, comparatively much less effort has been directed towards detecting the physical presence of bacteria *via* an increase in the bulk capacitance. Prior efforts were made to evaluate the contributions of the surface and bulk quantities to the measured impedance[23] which found that, at the operating frequencies of 1 MHz and lower, the magnitude of measured reactance was inversely proportional to the frequency of measurement. This led to the conclusion that the value of the bulk capacitance was too small to be measured at these frequencies. While this conclusion is a valid one, a more rigorous way of explaining the observations would be to say that the inverse *RC* time of the bulk solution was much larger than 1 MHz, whereas that of the surface was below 1 MHz and this made it impossible to monitor changes in the bulk capacitance at low frequencies using standard impedance instrumentation. In other words, the value of the quantity $R_{bulk}C_{bulk}$ was much lower than that of $R_{surface}C_{surface}$.

This problem of electrode surface "screening" is encountered in many other applications. Standard electrochemical cells and electrodes, such as the ones actually used in the study being referred to, try to decrease the value of $R_{surface}C_{surface}$ relative to that of $R_{bulk}C_{bulk}$ by using a low voltage to minimize electrode reaction and selecting an electrode material with a polished surface to minimize double layer charging. But despite these efforts, in the present case the *RC* value of the surface remains much higher than that of the bulk. As a consequence, at frequencies below 1 MHz, the interface remains the primary contributor to the measured reactance.

The inability to measure the expected increase in bulk capacitance seems to leave one with only the option of looking for the cumulative effects of bacterial metabolism on the medium. As was explained earlier, this approach prevents us from assaying for low bacterial loads within a short interval of time (a few hours). Though there may be other ways to get around this problem, such as by pre-concentrating bacteria through dielectrophoretic (DEP) capture alone,[24] or more efficiently through coupling DEP to electro-osmotic flow field traps,[25] it would be valuable to be able to measure the presence of low loads of viable bacteria directly and effectively in many cases, such as when the total sample volume is limited, or when there are a number of unviable bacteria in the sample.

The work that we present begins on the known hypothesis[17] that the presence of bacteria in a system would increase the bulk capacitance. It shows a way to monitor this change by reducing the inverse *RC* time of the bulk by increasing the value of the bulk resistance (R_{bulk}) without changing the composition of the solution. This is achieved by positioning electrodes along the length of a channel in a microfluidic system, resulting in a higher bulk resistance of the measured sample since the resistance is directly proportional to the path length and inversely proportional to the cross sectional area of the current carrying pathway. This enables us to monitor some of the effects of an increased bulk capacitance at a relatively low frequency (<1 MHz). Moreover, using a multi-frequency measurement technique, we show that the bacteria-induced capacitance increase produces a distinctive signature, *viz*. a minimum in the reactance at a frequency proportional to the inverse *RC* time of the medium that greatly enhances the sensitivity of the detection of a low number of bacteria. The theoretical basis of the technique is explained in the following section.

Theory and numerical simulations

For a solution, the measured impedance (Z) is a vector sum of the real (in-phase) resistance (R) and the imaginary (out-of-phase) reactance (X). In other words

$$Z = R - \mathrm{j}\,X \quad (1)$$

where j is the square root of -1, and the negative sign denotes a phase-lag (as is typically observed for aqueous solutions). The measured reactance (X), in turn, may be assumed to be composed of two parts, the reactance of the interface (X_i) and the reactance of the medium (X_m). Or

$$X = X_i + X_m \quad (2)$$

or

$$X = \frac{1}{\omega C_i} + \frac{\omega C_m}{G_m^2 + \omega^2 C_m^2} \quad (3)$$

where, C_i is the interface (surface, or series) capacitance, C_m is the medium capacitance, and G_m is the inverse of the medium resistance R_m (as depicted in Fig. 1). It may be verified that eqn. (3) is identical to the equation embedded in Fig. 1.

Since individual bacteria demonstrate the ability to store charge, and thereby act like capacitors,[26] the number of bacteria in solution should have a direct bearing on the value of the bulk (medium, or parallel) capacitance of the system, and this should be reflected in the behavior of the reactance of the system. In fact, if the medium capacitance is primarily due to the bacteria suspended in it, and the capacitance of individual bacteria are not themselves a function of frequency, then

$$C_m \sim C_{bacteria}\,\phi \quad (4)$$

where ϕ is the volume fraction of bacteria in the medium. And in the limit $\omega C_m \ll G_m$ (which typically holds for the medium and should also work for low to moderate volume fractions of bacteria in it),

$$X_m \approx \omega\, R_m^2\, C_{bacteria}\,\phi \quad (5)$$

Thus, higher bacteria counts should produce a clear and rapid increase in the medium reactance. In contrast, the medium resistance R is equal to the volume-fraction weighted sum of the solution resistance and the bacteria-attributed resistance. The small magnitude of ϕ (less than 10^{-6} even at a million cfu ml^{-1}) stipulates R is insensitive to bacteria count. Prior efforts have been reported in literature[23] to gauge this by measuring the reactance of solutions at frequencies realizable by relatively inexpensive equipment (typically, frequencies up to 1 MHz). There it was seen that the interfacial reactance was much greater than the medium reactance. But, while the behavior of measured (total) reactance did deviate from what one would have expected due to the interfacial reactance alone (especially at high frequencies), the deviations were not

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deemed significant enough to lead to any meaningful conclusions about the changes in bulk capacitance (if any) occurring during the observed time period.

To gauge the effect of a change in medium capacitance on the frequency response of the measured reactance of the system, consider, the function

$$f(\omega) = C_{\mathrm{m}}\, X(\omega) \tag{6}$$

or

$$f(\omega) = \frac{C_{\mathrm{m}}}{\omega C_{\mathrm{i}}} + \frac{\omega C_{\mathrm{m}}^2}{G_{\mathrm{m}}^2 + \omega^2 C_{\mathrm{m}}^2} \tag{7}$$

which can be rewritten as

$$f(\omega) = \frac{\alpha}{\omega} + \frac{\omega}{\beta^2 + \omega^2} \tag{8}$$

where $\alpha = C_{\mathrm{m}}/C_{\mathrm{i}}$ is the ratio of the medium and interface capacitances, and $\beta = G_{\mathrm{m}}/C_{\mathrm{m}} = 1/R_{\mathrm{m}}C_{\mathrm{m}}$ is the inverse of the medium (bulk solution) *RC* time.

For low values of ω ($\omega \to 0$)

$$f(\omega) \approx \alpha/\omega \tag{9}$$

and for high values of ω ($\omega \to \infty$)

$$f(\omega) \approx 1/\omega \tag{10}$$

So, as depicted in Fig. 2, one can expect the function given by eqn (8) to either have a single inflexion point, or a pair of local extrema points (one maximum and one minimum) depending on the value of α.

To obtain more a more rigorous condition for the existence of maxima and/or minima for $f(\omega)$, one set its first derivative (with respect to ω) equal to zero. Rearranging to solve algebraically for ω (or rather, ω^2), one obtains the condition

$$\omega^4(1 + \alpha) - \omega^2\beta^2(1 - 2\alpha) + \alpha\beta^4 = 0 \tag{11}$$

For a solution to exist, the discriminant of the above equation needs to be greater than zero. On applying this condition, one obtains

$$\alpha < 1/8 \tag{12}$$

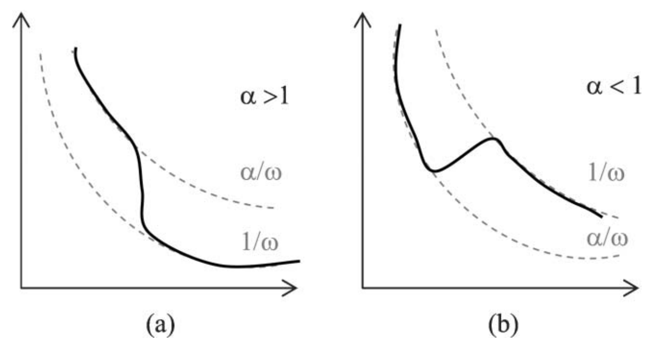

Fig. 2 Expected behavior of the function $f(\omega)$ [$= C_{\mathrm{m}}X(\omega)$] (a) when $\alpha > 1$ (no extrema values), and (b) when $\alpha < 1$ (one minimum and one maximum value).

as the condition that determines whether or not the function $f(\omega)$ [and, as a consequence, the frequency response of the measured reactance, *i.e.*, $X(\omega)$] has local minima and maxima.

Note that $\alpha = C_{\mathrm{m}}/C_{\mathrm{i}}$. This means that a minimum or maximum can be obtained only for values of C_{m} that are substantially lower than C_{i}. The values of C_{m} have been estimated to be lower than C_{i} by a factor of 1000 or larger in more traditional millilitre-scale systems[23,27] (leading to a value of ~ 0.001 for α) and is not expected to change much given the geometry of our microfluidic system. We hence examine the behavior of the system for low values of α [or, mathematically speaking, in the limit $\alpha \ll 1$].

The solution to eqn (11) yields the values of ω at which the maxima and minima are observed. The two roots of eqn (11) are given by

$$\omega_{\mathrm{ext}}^2 = \frac{\beta^2(1-2\alpha) \pm \sqrt{(1-8\alpha)}}{2(1+\alpha)} \tag{13}$$

The first extremum, which, from Fig. 2 we expect to be a minimum, is obtained at the lower value of ω. Thus,

$$\left(\frac{\omega_{\mathrm{min}}}{\beta}\right)^2 = \frac{(1-2\alpha) - \sqrt{(1-8\alpha}}{2(1+\alpha)} \tag{14}$$

which, in the limit $\alpha \ll 1$, reduces to

$$\left(\frac{\omega_{\mathrm{min}}}{\beta}\right)^2 \approx \frac{\alpha}{1+\alpha} \approx \alpha + O(\alpha^2) \tag{15}$$

Hence,

$$\omega_{\mathrm{min}} \approx \sqrt{\alpha\beta^2} \tag{16}$$

which, if one considers the definitions of α and β, can also be expressed as

$$\omega_{\mathrm{min}} \approx \frac{1}{R_{\mathrm{m}}C_{\mathrm{m}}}\sqrt{\frac{C_{\mathrm{m}}}{C_{\mathrm{i}}}} = \frac{1}{R_{\mathrm{m}}(C_{\mathrm{m}})^{0.5}(C_{\mathrm{i}})^{0.5}} \tag{17}$$

Further, the value of the function, $f(\omega)$, obtained at this frequency ω_{min}, is (in the limit $\alpha \ll 1$) given by the expression

$$f(\omega_{\mathrm{min}}) = \frac{2\sqrt{\alpha}}{\beta} = 2R_{\mathrm{m}}C_{\mathrm{m}}\sqrt{\frac{C_{\mathrm{m}}}{C_{\mathrm{i}}}} = 2R_{\mathrm{m}}(C_{\mathrm{m}})^{1.5}(C_{\mathrm{i}})^{-0.5} \tag{18}$$

Thus, the minimum value of the recorded reactance is given by

$$X_{\mathrm{min}} = \frac{f(\omega_{\mathrm{min}})}{C_{\mathrm{m}}} = 2R_{\mathrm{m}}\sqrt{\frac{C_{\mathrm{m}}}{C_{\mathrm{i}}}} = 2R_{\mathrm{m}}(C_{\mathrm{m}})^{0.5}(C_{\mathrm{i}})^{-0.5} \tag{19}$$

From eqns (4) and (17) we see that the location of the reactance minimum is proportional to the inverse of the square root of the volume fraction ($\boldsymbol{\phi}$) of bacteria in the medium, and is hence expected to be quite sensitive. From eqn (19), we see also the dependence of the minimum amplitude on the bacteria volume fraction, but in this case it is proportional to the square root of $\boldsymbol{\phi}$. We further note that (a) other factors remaining equal, one would expect to observe a minimum in the frequency response of the reactance at a lower frequency when the bulk (medium) resistance is high, and (b) if the bulk capacitance of the solution were to rise (other factors remaining equal), the minimum in the plot of reactance

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against frequency would, again, be observed at lower frequencies. Similarly, eqn (19) predicts that the recorded minimum for the measured reactance would decrease if either the medium resistance or the medium capacitance were to decrease independently, with all other quantities remaining unchanged.

If a certain number of bacteria are inoculated into a growth medium, we would expect from the above arguments that as time progresses and the bacteria metabolize and reproduce, the medium resistance (R_m) would drop but not significantly and the medium capacitance (C_m) would increase proportionally to the bacteria count, as seen in (4), at the low bacteria count and high frequency limits. One might then expect the value of the minimum reactance measured to increase with time and the critical frequency (at which the minimum is recorded) to decrease with time. Thus, there may be cases (for really low initial medium capacitances) for which the critical frequency initially lies beyond the range of frequencies at which measurements are taken, but as time progresses the critical frequency shifts to within the observed range. Then, initially, one would not be able to observe any minimum in $X(\omega)$, but would do so as time progressed. The "appearance" of the minimum within the window of measured frequencies would hence be a "signature" of the presence (and proliferation) of bacteria within the growth medium. Given that, in general, signal sensitivity improves drastically near an extremum of a measured quantity, our system, which examines the location and amplitude of the minimum, should be very sensitive to changes in the medium (bulk) capacitance, and consequently, the bacteria count. As seen in eqn (8), the amplitude and curvature of this minimum is also sensitive to bacteria count. However, its location eqn (15) shows the highest sensitivity.

Prior literature[23] reports only a monotonic decrease in the total measured impedance across frequencies ranging from approximately 10 Hz to 100 KHz when the system had an initial medium conductance of 0.005 mhos (a bulk resistance of 200 Ω). If one takes into account that their interfacial capacitance is of the order of 10^{-6} farads [based on a typical value of 10 μF cm^{-2} for the electrodes[27]] and bulk capacitance is of the order of 10^{-10} farads or lower, it can be seen (using eqn (17)) that for the minimum to lie within their range of observed frequencies, the bulk resistance must lie between 10^3 Ω and 10^8 Ω. The fact that the bulk resistance starts out at a value lower than 1000 Ω (and presumably decreases further as time progresses) prevented the previous investigators from observing this signature.

Thus, to facilitate the observation of the minimum (at frequencies lower than 1 MHz), the initial bulk resistance needs to be increased. The bulk resistance of a particular volume of sample may be increased by confining the sample within a long, narrow geometry—thereby decreasing the effective cross-sectional area of the electrodes and increasing their separation. Another way to look at the effect of the increased bulk resistance R_m (decreased bulk conductance G_m) is through eqn (3) where a higher bulk resistance (lower medium conductance G_m) raises the relative contribution of the bulk reactance to the total measured reactance. As is shown in Fig. 3 (left panel), this minimum is a consequence of a significant contribution to the measured reactance from the medium that, unlike the interface reactance, increases rather than decreases with increasing frequency over the "observed" frequency range of 1 kHz to 1 MHz. The frequencies close to the one at which the minimum is observed are also the region where the bulk and interface contributions are comparable (as illustrated by Fig. 3 (left)), and if one desires to reliably compute the values of both interface and bulk parameters by fitting eqn (3) to the observed data, one has to take measurements in this region of the frequency spectrum.

Another advantage of using a microcapillary with a small cross-sectional area is to increase the contribution of the bacteria capacitance to the medium capacitance. The correlation in eqn (4) is based on a parallel circuit model at low bacteria volume fraction. It implicitly assumes uniform distribution of a small number of bacteria in the capillary. With a small capillary, bacteria may occupy a significant fraction of the cross sectional area and a serial model with a higher bacteria sensitivity may be more appropriate. This is especially true if all the bacteria are concentrated over one region in the capillary. However, we will show from our data that the reactance is indeed proportional to the bacteria count, as suggested by eqn (5), and hence our capillary dimension has not reached a sufficiently small value that the reactance shows a sensitivity to ϕ that is more than linear.

As the bulk (medium) capacitance increases, in a plot of the measured reactance *versus* frequency, the minimum reactance recorded is larger, and the minimum is recorded at a lower frequency—as is illustrated in Fig. 3 (right panel). In a test sample, if these features are observed over time (or a minimum in the reactance is recorded when none existed before), they may be taken to constitute a signature for an increase in the number of bacteria in the medium.

Experimental protocol and results

In order to incubate the bacterial cultures and measure the change in their electrical properties, a microfluidic cassette was constructed using liquid phase photopolymerization of a commercially available UV curable polymer blend, a process that has been described in detail elsewhere.[28,29] A schematic of the cassette and a picture of the final device are shown in Fig. 4. A batch of 10 such cassettes was fabricated, and each cassette was re-used as needed for the experiments described subsequently. Fluidic connectors and electrical contacts were added after the fabrication process. After each experiment, the fluidic and electrical connectors were removed and each cassette was washed thoroughly with soap, water and alcohol. After installing new electrodes and fluidic connectors, the cassettes were sterilized, first by wetting with Clorox solution for 20 min and then flushing with sterile DI water followed by autoclaving at 120 °C for 20 min. They were then loaded with the solution to be studied and impedance measurements were taken using an Agilent 4284A LCR meter. The LCR meter was used in the "List Sweep" mode, which allowed us to measure the resistance and reactance at 10 logarithmically spaced frequencies between 1 kHz and 1 MHz.

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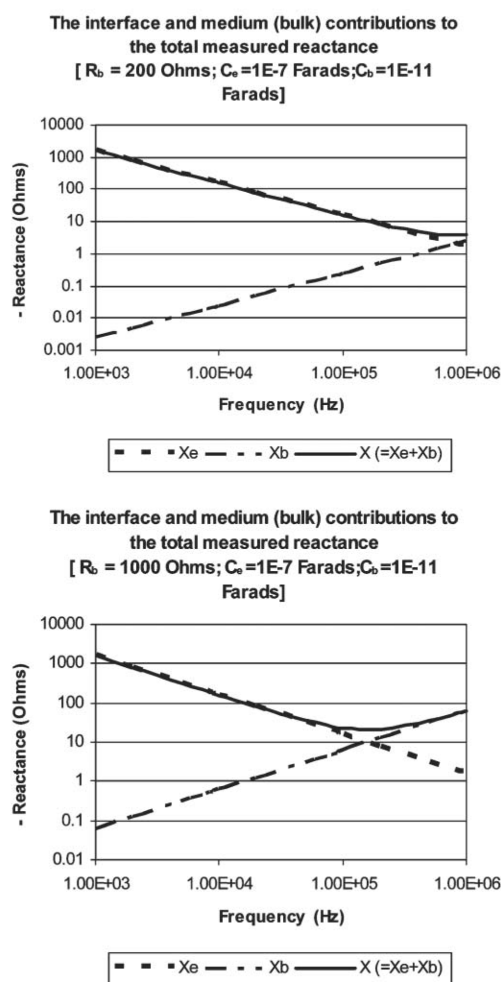

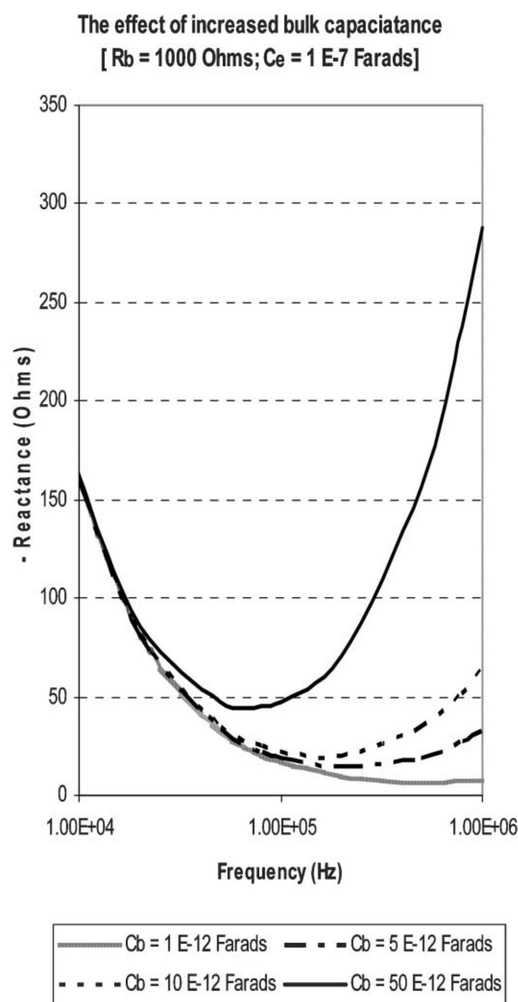

Fig. 3 Pseudo plots illustrating the effect of the various parameters in eqn (3) in enabling the detection of increased bulk (medium) capacitance at low frequencies. Left: An increase in medium resistance increases the value of the medium reactance (X_m) relative to the interface reactance (X_i), thereby causing a minimum in the measured (total) reactance (X). Right: The effect of rising bulk capacitance on the measured impedance, *viz*., the appearance, and subsequent rise in the value and shift to a lower frequency, of a minimum in $X(\omega)$.

The following experiments were performed using these cassettes

Experiment 1 (Overall Control). Filtered and unfiltered samples of 30 mg ml^{-1} sterile (autoclaved) solution of Criterion™ Typtone Soy Broth (TSB) (Hardy Diagnostics, Santa Maria, CA) was loaded into the cassettes. All cassettes were subsequently placed on a slide warmer (Lab-Line Instruments, Model 26020) maintained at 37 °C and allowed time to equilibrate. Impedance measurements were then taken at two subsequent time intervals. Fig. 5 (left) graphically displays one sample data set from each of the two solutions used.

Experiment 2 (Addition of inert carbon nano-tubes). In order to increase the bulk capacitance of the solution, multi-wall carbon nano-tubes (CNTs) [ID 1–3 nm, OD 3–10 nm, length 0.1–10 μm, purity >90% from Aldrich (Lot # 02710EC)], particles with known capacitive properties,[30] were suspended in TSB. Three solutions were prepared, with estimated concentrations of 0.5, 5 × 10^{-3}, and 5 × 10^{-5} mg ml^{-1}, respectively, of CNTs in TSB. In order to do so, first a stock solution of 5 mg ml^{-1} CNTs was prepared by adding 20 mg of CNTs to 1 ml of Nafion® 117 solution (Lot # 119257 from Fluka Chemie GmBH) and 3 ml of 0.1X PBS. 300 μl of this stock solution was added to 2.7 ml of TSB to obtain the first solution. The second and third solutions were obtained by serially diluting this solution further in TSB. Fig. 5 (right) shows how the reactances of the solutions vary as a function of frequency.

Experiment 3. A scoop from a plate culture of F-amp *E.coli* (ampicillin resistant strain of *E.coli* ATCC No. 700891) was added to the 10 ml of TSB laced with 20 μg ml^{-1} ampicillin (GIBCO, Carlsbad, CA) to suppress the growth of other bacteria and incubated overnight with shaking at 37 °C. 1 ml of the resulting cloudy solution was centrifuged (10 000 rpm, 5 min, using a Beckman Microfuge 22R), and after removing the supernatant, the deposited pellet was re-suspended in 0.1X phosphate buffer saline (PBS). It is estimated that the concentration of bacteria in this solution is ~10^{9} cfu ml^{-1}. A set of serial dilutions were performed by adding 100 μl of the solutions with bacteria to 900 μl of 0.1X PBS. Thus, solutions were generated with approximately 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4}, and 10^{3} bacteria per ml. 300 μl each of the solutions, with an

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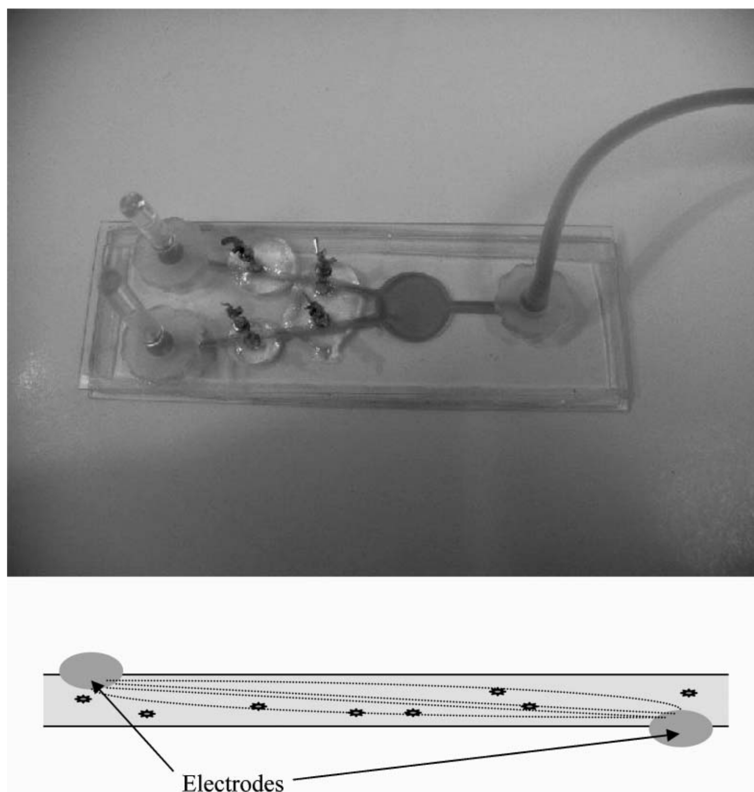


Fig. 4 Picture of the microfluidic cassettes used (top) and a schematic showing the positioning of the electrodes within the channels (bottom). The dotted lines within the channel are the electrical lines of force between the electrodes (ovals), some of which encounter bacteria (small black structures) suspended in the medium.

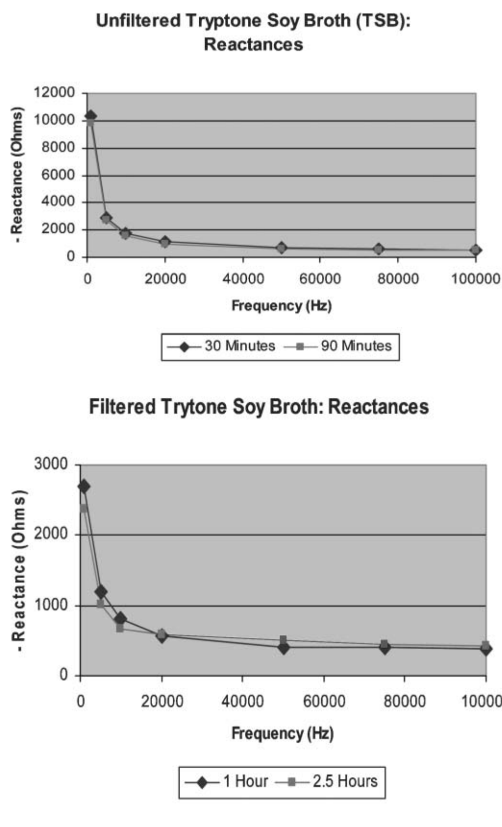
estimated bacterial count $\sim 10^4$ and 10^3 per ml, were added to 2.7 ml volumes of TSB (laced with 20 μg ml^{-1} ampicillin) to yield two solutions—one with approximately 1000 and the other with approximately 100 bacteria per ml. These solutions were loaded into sterile cassettes, which were then placed on a slide warmer maintained at 37 °C. The cassettes and their contents were allowed to sit for about half an hour to equilibrate before impedance measurements were taken. Three impedance measurements were taken again at 2 hour intervals after the first reading. The reactances measured (along with the resistances) are displayed in Figs. 6 and 7 for the solutions with initial loads of ~ 100 cfu ml^{-1} and 1000 cfu ml^{-1}, respectively. Four control cassettes were also used corresponding to each experimental condition (both values of the initial bacterial load). The contents of one cassette were extracted at roughly the same time as the impedance measurements were taken and (after diluting if deemed necessary) spread on agar plates to obtain plate counts to estimate the concentration of bacteria at that particular instance in the samples whose impedance is being measured.

Discussion and analysis of the results

Tryptone Soy Broth (TSB) is a commonly used liquid medium for culturing bacteria. This broth contains casein digests of soy protein in addition to glucose and sodium chloride. When its reactance is recorded at frequencies ranging from 1 kHz to 1 MHz (Fig. 5 left), it is seen that the reactance decreases monotonically with increasing frequency for both filtered and unfiltered samples. This indicates that the minimum in $X(\omega)$ occurs at a frequency higher than 1 MHz. Further, the measured reactances do not change with time, in keeping with the expected lack of metabolic activity within the solution.

However, when carbon nano-tubes (CNTs), particles that are known to possess significant capacitive properties, are suspended in the TSB, we see a change in the manner in which the reactance behaves as a function of frequency (Fig. 5 right). Although the $X(\omega)$ for the solution with the lowest concentration of suspended CNTs looks qualitatively similar to that for TSB, the two more concentrated solutions display minima within the observed range of frequencies (with the minimum value being larger and occurring at a lower frequency for the most concentrated solution). This is in qualitative agreement with eqn (17), which predicts that an increase in medium capacitance (C_m) will cause the minimum in $X(\omega)$ to occur at lower frequencies. This observation also suggests that an increase in the number of capacitive particles suspended in the medium (brought about by means physical or biological) could lead to the transformation of the plot of $X(\omega)$ from one that is monotonically decreasing over the observed frequency range to one that displays a minimum.

Figs. 6 and 7 show how the ability to detect the increase in medium capacitance provided by suspended material with capacitive properties can be utilized to monitor the growth of bacteria in solutions. When the first measurement is taken for the solution inoculated with ~ 100 cfu ml^{-1} of *E. coli* (Fig. 6),



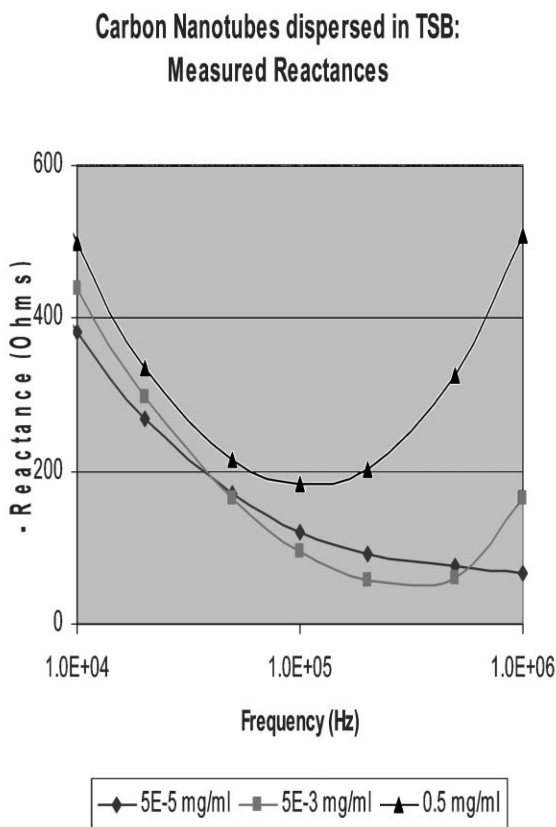


Fig. 5 Plots showing the electrical behavior of sterile suspensions. Left: unfiltered (top) and filtered (bottom) Tryptone Soy Broth (TSB), showing a stable (over time) monotonic decrease of measured reactance with frequency. Right: TSB solutions with carbon nano-tubes (CNTs) suspended in them. Higher concentrations of suspended CNTs lead to higher medium capacitance, and the effects are similar to those predicted in Fig. 3.

the medium capacitance is lower than the value needed for the minimum in the measured reactance to be observed at a frequency below 1 Mz. Two hours after the first measurement is taken, a minimum in the measured reactance is recorded at 100 kHz. This noticeably different behavior of the system, brought about by an increase in the medium capacitance of the system due to bacterial proliferation, can be taken as a signature for bacterial presence in applications such as detecting the presence of bacteria in food products, where the requirement is merely to detect the presence of bacteria (typically present at low concentrations). As can be seen, this signature is recorded less than 3 hours post inoculation giving us a "Time to Detection" (TTD) of less than three hours for an organism whose generation time is expected to be about 25 min. This compares extremely favorably to the technique for detecting bacteria based on monitoring resistance[19] which has a TTD of 6.7 hours for a sample having an initial load of 1000 cfu ml^{-1} of bacteria with a generation time of 30 min. (The initial load needed is roughly ten times ours and the TTD is more than twice as long.) The aforementioned technique forms the basis of a commercial system called the RABIT (Rapid Automated Bacterial Impedance Technique) developed by Don Whiltley Scientific, UK. Moreover, while the RABIT uses a medium whose composition is specially designed to maximize the sensitivity of the measured resistance change to the effects of bacterial metabolism, our system uses a generic growth medium. The relative efficacies of the techniques based on resistance and reactance can be gauged by looking at the plots showing the evolution of the measured resistances with time accompanying the plots for reactances in Figs. 6 and 7. By the time the unambiguous signature for bacterial growth is recorded using the reactance (in Fig. 6), the measured resistances change by less than 2%.

When a minimum in $X(\omega)$ is recorded at the first reading, the evolution of this feature over time may be used to monitor bacterial growth. For instance, the reactance of the sample with an initial estimated load of $\sim$1000 cfu ml^{-1} displays a minimum when the first measurement is taken (at t = 45 min). But then the minimum evolves over time. As can be seen in Fig. 8, between 4.75 hours and 6.75 hours the minimum value of the reactance and the frequency at which the minimum is recorded (functions of the medium capacitance) change significantly, the former increasing by over 85% and the latter decreasing by about 100 kHz. Again in contrast, the values of the measured resistances change by only about 2.5% during this time.

One can also obtain a value of the medium capacitance of the solution by using standard curve fitting techniques to fit the parameters in eqn (3) to the recorded values of the reactance. A Matlab® program was used to perform a least-squares fit to the data recorded. The fits typically resulted in R values over 0.9 and 95% confidence intervals of $\sim \pm 10\%$ of the value of the predicted parameters. The values obtained (as seen in Fig. 8) seem to change over time like the bacterial load in the

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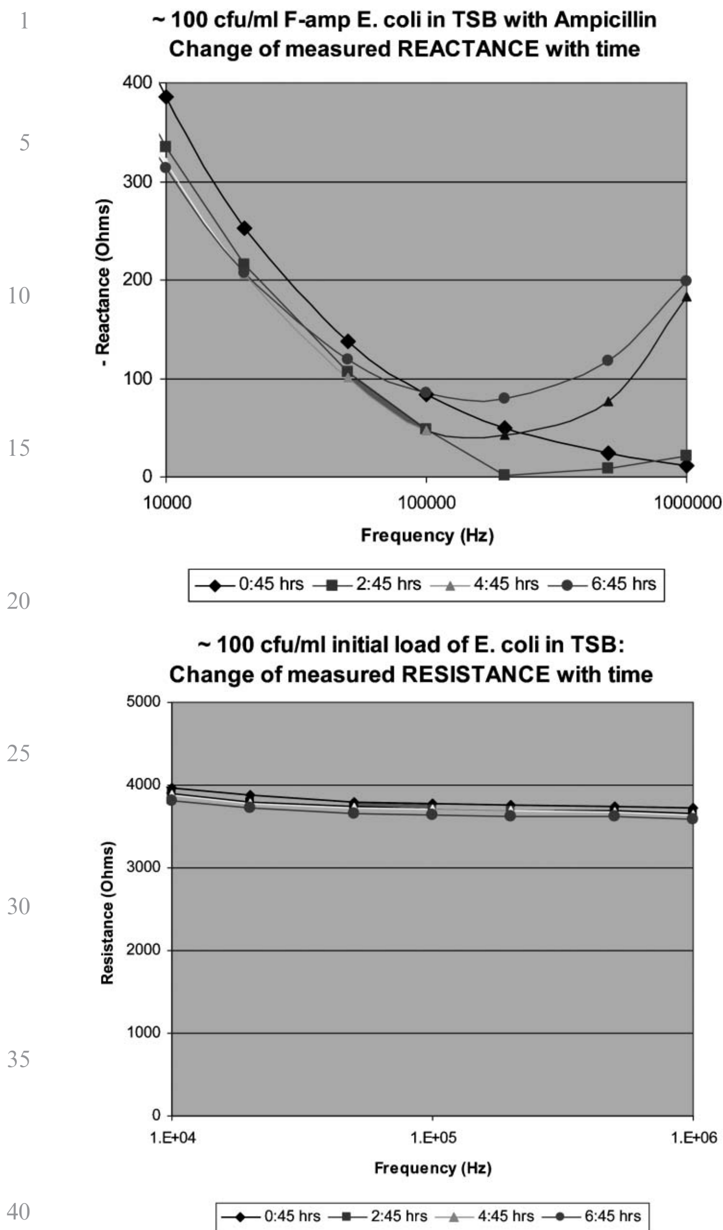


Fig. 6 Plots showing the change with time of measured reactance (top) and resistance (bottom) for a bacterial culture initially loaded with $\sim$100 cfu ml^{-1}; the former is caused by bacterial proliferation and the latter by metabolic activity. While the change in reactance is significant within less than 3 hours [plot begins to display a minimum in $X(\omega)$], the change in resistance values is less than 2% by this time. Even after 6.75 hours, it is still about 3.5%.

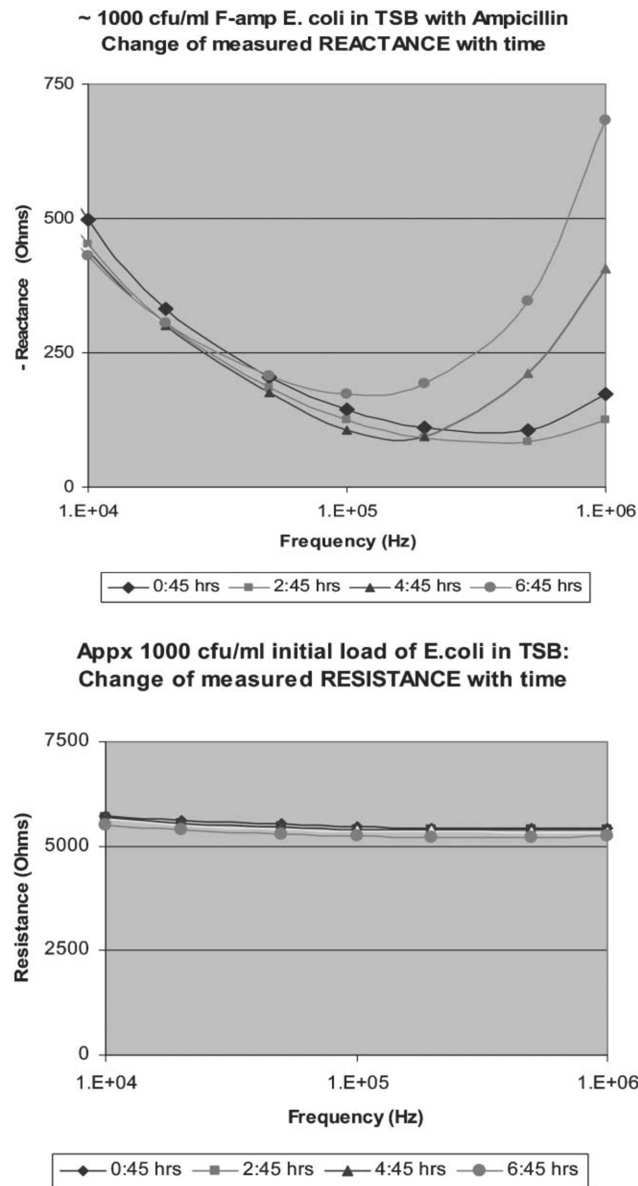
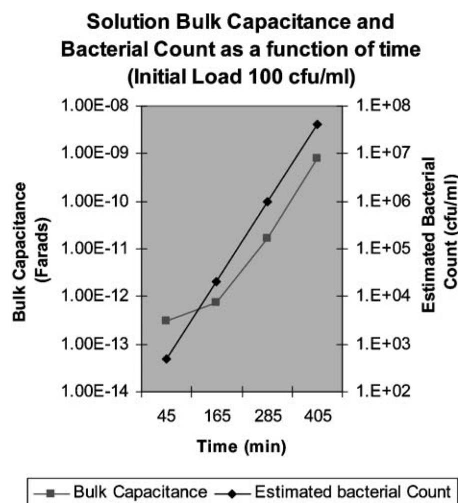


Fig. 7 Plots showing the variation of the measured reactance (top) and the measured resistance (bottom) for a bacterial culture initially loaded with $\sim$1000 cfu ml^{-1} at various points in time. Changes in the behavior of the reactance [$X(\omega)$] are significant—the minimum value of reactance ($X_{\min}$) rising by $\sim$85% between 4.75 and 6.75 hours. In contrast, changes in resistance are less pronounced (by about 2.5% in the same period).

solutions, and hence correlate with the latter. The calculated values of medium capacitance (with its confidence interval) could hence be used as a more rigorous method of monitoring bacterial proliferation. The linear correlation between bacteria count and the medium capacitance is consistent with eqn (4).

There are many sources of variability in the systems described by Fig. 8. Firstly, different independent aliquots from the same solution were introduced into different cassettes. Separate aliquots from the solution that was introduced and more concentrated versions of it in the dilution series were used to establish the bacterial load *via* a plate count. The plate count, by itself, is just a good order-of-magnitude estimate of bacterial load. Besides, given the low number of bacteria in the solution, it is extremely unlikely that all samples started off with exactly the same number of bacteria (although that is the implicit assumption of Fig. 8). With exponential growth, even small differences in initial load can be magnified over time. Moreover, the bacteria, like all living creatures, are subject to small variability among individuals and differences in their metabolic state may be reflected in differing contributions to the medium capacitance. Non-uniform distribution of bacteria within the narrow region



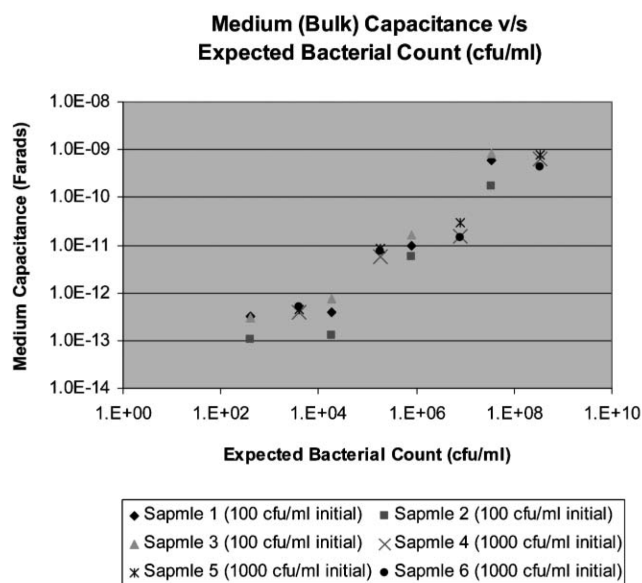


Fig. 8 Evolution of bacterial count and calculated medium capacitance with time (top), and variation of calculated medium capacitance with estimated bacterial count (bottom).

between the electrodes could also contribute to the observed variability, although its effect would be mitigated by the motility of live *E. coli*. However, the most important source of variability could be small differences in temperature between the different cassettes being incubated on the same slide warmer. Not only does temperature affect the conductivity and dielectric constant of the solution, but may change the doubling time of the bacteria by a few minutes. Over 3–7 hours, this could lead to substantial differences in bacterial numbers. Given all these sources of variability, the observed scatter is not unexpected.

Concluding remarks

The effort was motivated by a desire to be able use electrical methods to directly establish bacterial counts in solution instead on relying on monitoring the cumulative effects of bacterial metabolism. It has long been intuitively obvious that medium capacitance (and consequently medium reactance) is a strong function of bacterial count. However, the surface reactance of electrode–solution interface masked the changes in the bulk reactance at frequencies conducive to inexpensive instrumentation (1 MHz and below). We were able to utilize the geometry of microfluidic channels to increase the bulk resistance of the medium, and thereby decrease the inverse RC time of the bulk. This enabled us to notice the effect of increased contribution from the bulk reactance to the total measured reactance in the form of a minimum in the latter quantity. The strong sensitivity of the frequency at minimum to bacteria count allows us to produce a sensitive bacteria detection method for low bacteria counts.

Utilizing our technique, we are able to pick up signatures of bacterial proliferation (and hence presence) in about half the time as an established commercial technique starting with an initial load ten times smaller. Moreover, the electrode characteristics and their location within the channels have not been optimized, and the growth medium used was a popular generic one, not chemically modified in any way. Further advances in the design of the cassette, incorporating modifications in the design of electrodes and their positioning within the channels to optimize the electrical behavior, in conjunction with a specially designed medium with low amounts of macromolecules, would be expected to make the technique even more sensitive by further reducing times to detection. Reducing the capillary dimensions and concentrating the bacteria at one location along the capillary are also attractive strategies. However, smaller chamber dimensions may increase the sensitivity to debris corruption of the signal and an optimum dimension may exist.

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