Rapid determination of antibiotic resistance in *E. coli* using dielectrophoresis

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Abstract

In recent years, infections due to antibiotic-resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and ciprofloxacin-resistant *Escherichia coli* are on the rise, and with them the demand for rapid antibiotic testing is also rising. Conventional tests, such as disc diffusion testing, require a primary sample to be tested in the presence of a number of antibiotics to verify which antibiotics suppress growth, which takes approximately 24h to complete and potentially places the patient at severe risk. In this paper we describe the use of *dielectrophoresis* as a rapid marker of cell death, by detecting changes in the electrophysiology of the cell caused by the administration of an antibiotic. In contrast to other markers, the electrophysiology of the cell changes rapidly during cell death allowing live cells to be distinguished from dead (or dying) cells without the need for culturing. Using Polymyxin B as an example antibiotic, our studies indicate that significant changes in cell characteristics can be observed as soon as 1 hour after isolating a culture from nutrient broth.

Keywords

Electrokinetics, bacterium, diagnostic, DEP-Well

PACS Classifications

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Introduction

In recent years, infections due to antibiotic-resistant strains of bacteria such as methillicin-resistant *Staphylococcus aureus* (MRSA) and ciprofloxacin-resistant *Escherichia coli* are on the rise (Wellcome Trust, 2005), and with them the demand for rapid antibiotic testing is also rising. Conventional tests, such as disc diffusion testing, require growth of a primary sample from the patient to obtain a pure culture and to identify the bacterium. This culture is then tested in the presence of a number of antibiotics to verify which antibiotics suppress growth. Since each culture step takes approximately 24h, it takes roughly two days to determine which antibiotic is most adequate. While this is sufficient for many mild infections, it can be too long in the case of severe infections, placing the patient at severe risk. Therefore there is growing demand for more rapid testing methods that give a reliable indication of antibiotic resistance in less time.

In general fast techniques fall in two groups: detecting resistance genes and detecting if a bacterium is actively metabolising. Both have drawbacks that can lead to false positive or negative identification. Genetic tests typically use PCR to amplify a DNA sequence that is known to be a marker for resistance, and detect whether that sequence is present. This method has potential for significant time savings as it is sensitive enough to be applied directly to a sample from a patient, saving nearly two days. However, due to its sensitivity it easily picks up contaminations, or detects resistance in harmless bacteria that are also present in the sample (Borst et al., 2004). Additionally it can only detect known resistance genes, so bacteria that express a previously unknown resistance mechanism, or a new variant of the resistance gene, cannot be detected. Metabolism markers try to detect if an organism is metabolising in the presence of antibiotics. A common way is to detect metabolism by measuring the build-up of CO₂ above the sample; other procedures use radioactively labelled sugars or fluorescent markers to distinguish live and dead bacteria. However natural breakdown of nutrient in the medium can slowly raise the CO₂ content above the sample and thereby give either false positive results or mask slow growing organisms, while florescence markers can be metabolised by bacteria.

In this paper we describe the use of *dielectrophoresis* as a rapid marker of cell death, by detecting changes in the electrophysiology of the cell caused by the administration of an antibiotic. In contrast to other markers, the electrophysiology of

the cell changes rapidly during cell death allowing live cells to be distinguished from dead (or dying) cells. We have used Polymyxin B as an example since its mechanism is well understood. Our studies indicate that significant changes in cell characteristics can be observed as soon as 1 hour after isolating a culture from nutrient broth.

Dielectrophoresis

Dielectrophoresis (DEP) is the motion of dielectric particles in a non-homogeneous electric field, and was first observed by Pohl (1951). In contrast to electrophoresis, which affects only charged particles, DEP affects all particles that have different dielectric properties to the medium they are suspended in. A homogenous electric field induces a dipole in the particle and leads to forces acting on both sides of the particle, however, since the field is homogenous, the forces on both will cancel out so no net force is exerted on the particle. An inhomogeneous field, as used in DEP, means the strength of the force on the two sides of the dipole varies across the particle and thereby a net force is exerted on the particle. Since the dipole is induced by an inhomogeneous electric field, the dipole will be stronger where the field gradient is strongest, independent of the field polarity, which has no influence on the direction of the force (Figure 1). This allows the use of AC fields for DEP, offering the additional advantage that electrophoresis (based on net charge) is eliminated. The use of AC fields also prohibits electrochemical reactions such as electrolysis at the electrodes, and thereby reduces corrosion of the electrodes.

The strength and direction of the force depends on the polarisability of the particle in relation to the surrounding medium at a given frequency. If particle polarisation is greater than the medium at a given frequency, the particle will be attracted to the regions of higher field gradient (normally the electrode edge), while a particle that is less polarisable than the medium will be repelled from regions of high field gradient, phenomena termed positive and negative DEP respectively. Since the complex permittivity of the particle is frequency-dependent, the same particle can experience different magnitudes of positive or negative DEP at different frequencies. By mapping the force spectrum it is possible to determine the dielectric (and hence electrophysiological) properties of particles such as cells by recording the strength and direction of the DEP force against frequency and fitting a mathematical model of the particle against the recorded data.

Figure 1: In electrophoresis affected charged particles experience a force, dielectric particles polarise and distort the electric field but charges cancel out so there is no net force. In dielectrophoresis the electric field is highly inhomogeneous, so particles (and the surrounding medium) polarise to a different extent on opposite sites of the particle and the particle experiences a net force. If the particle is more polarisable than the medium it will move toward the field gradient (positive DEP) if it polarises less than the medium the particle is repelled from the field gradient (negative DEP)

The DEP-Well system

DEP spectrum data was recorded using the DEP-Well system (Fatoyinbo et al., 2005; Hoettges et al., 2005; Hübner et al., 2005). The DEP-Well system consists of chip devices with electrodes along the wall of a micro-well with a transparent base, allowing determination of cell behaviour in a similar manner to the observation of cells in conventional well-plate assays. The devices consist of a laminate of metal and polymer layers; consecutive metal layers are connected to opposite phases of a signal generator. Wells are defined through the laminate. The resulting structures have thin rings of electrodes evenly spaced around the wall of the well.

Compared to conventional flat electrode structures, DEP-Well devices have several advantages. First, the well structures energise a larger volume than conventional flat electrode of the same footprint. They also contain the sample, allowing multiple samples to be analysed in different wells on the same chip simultaneously. Data extraction is simpler as the cells are either pulled towards the wall or pushed towards the centre of the well, giving radial symmetry. For evaluation, simple absorption measurements can be used since positive DEP pulls the cell to the wall and removes them from the light path while negative DEP pushes cells into the light path increasing light absorption. For small cells, the field may be insufficiently

strong to affect all the cells in the well, but even if only the outer 30% of the radius are affected this makes up more than 60% of the enclosed volume. If the cells within the well are observed using a microscope, image processing algorithms can be used to evaluate the volume closest to the wall of the well and thereby increase the signal quality.

To interpret data gained from experiments the cells' response at different frequencies can be matched to mathematical models. By matching a computer model to experimental data the properties of the particles can be determined. The model used here is based on an advancement of the work of Irimajiri (1979) by Broche (2005) and considers a cell to be a homogenous sphere (the cytoplasm) suspended in a second sphere (the cell wall) which is in turn surrounded by the suspending medium. Other models for non-spherical geometry such as ellipsoids or rods are reported in the literature and can be used to model particles such as red blood cells (Jones 1995), elongated bacteria (Sanchis et al., 2004; Castellarnau et al., 2006) or carbon nanotubes (Mureau et al., 2006). Various algorithms and programs for computer models are published as well (Hughes 2002; Morgan and Green 2003). The model can be curve fitted to the data by adjusting the conductivity, permittivity and layer thickness for each layer. This allows extracting the electrophysiology of the particle. In the case of cells, the properties of the cytoplasm and membrane can give valuable information. Since factors such as ion flux or even water efflux change the conductivity of the cytoplasm, while damage to the membrane or membrane folding change membrane parameters, this allows label free investigation of ion channel activity, cell viability or interactions with drugs.

The bacteria used in the experiments are shaped like a short rod with rounded ends (sometimes described as “pill-shaped”), and no model for this shape has been presented in the literature so far. In the past, several models for *E. coli* have been used (Sanchis et al., 2004; Castellarnau et al., 2006); however these models consider the orientation of the particles in the field as well as the geometric shape of *E. coli* and therefore have difficulties in extracting accurate quantitative data from the experiments. In this study, we have not focussed on accurately quantifying changes in the cell, but have instead focussed on rapidly detecting the change in the cell that indicates viability. This can be achieved using a much simplified spherical model for the particle. The model assumes just one outer shell that consists of the cell membrane and cell wall, while the inner sphere consists of the cytoplasm. The results allow

examining broadly if changes occur at the surface or inside the cell. In general such model consists of two dispersions (changes in the relative dielectric properties, indicated by curves between plateaux on a DEP spectrum). Assuming the geometrical parameters of the cells such as cell radius and, cell wall and membrane thickness stay constant, the initial value of the spectrum at low frequencies relates to the conductivity of the outer shell, the first dispersion is determined by the permittivity of the outer shell, the second dispersion relates to the cytoplasm conductivity and the high frequency level indicated the cytoplasm permittivity, however this final level is only rarely observed with the technology used in this experiment (Broche et al., 2005). A reduction in cytoplasm conductivity should manifest itself as a shift of the higher frequency dispersion to lower frequencies, while a more conductive membrane should raise the DEP spectrum at before the first dispersion (2).

Figure 2: Example of modelling bacterial properties. A spherical single shell particle with the properties models to an *E.coli* control (Cytoplasm $r = 0.5 \mu\text{m}$, $\sigma = 0.35 \text{ S/m}$, $\epsilon = 65$, outer shell thickness 8 nm , $\sigma = 30 \mu\text{S/m}$, $\epsilon = 12$, surrounding medium $\sigma = 0.5 \text{ mS/m}$, $\epsilon = 78$). When the cytoplasm conductivity is halved (to 0.175 S/m) the high frequency dispersion shifts downwards in frequency. When the membrane conductivity is doubled (to $60 \mu\text{S/m}$) the low frequency plateau of the curve is shifted upwards.

Experimental

E.coli, (laboratory strain no. JD131) was grown on nutrient agar #3 (Sigma-Aldrich, UK) and stored at +4°C. One colony was taken using a wire loop and incubated at 37°C for 15 h in nutrient broth #2 (Sigma-Aldrich, UK). 3 mL of the broth were taken as a the control sample; the remaining culture was treated with 20 µg/mL Polymyxin B (Sigma-Aldrich, UK) and incubated at 37°C. Further 4mL aliquots were taken of this solution 1h, 2h and 4h after treatment.

Before each experiment the relevant sample was transferred in an iso-osmotic low conductivity medium for DEP experiments, consisting of 280mM mannitol (Sigma-Aldrich, UK) adjusted to a conductivity of 5mS/m by adding a small amount of phosphate buffered saline (Sigma-Aldrich, UK).

3 mL of sample was centrifuged at 6000g for 3 min, the supernatant was discarded and replaced with 1mL of low conductivity medium. The sample was centrifuged and the supernatant replaced a further 2 times. The sample was filled into the well of a DEP-well spectra chip and a frequency was applied for 90sec. A video system monitored the outer 30% of the well and measured the average change in light intensity transmitted through the well against time. After one frequency was applied the sample inside the well was replaced with fresh sample and another frequency was applied. Frequencies between 10 kHz and 20 MHz were spaced in a logarithmic scale with approximately 5 data points per decade (extra points were introduced above 10 MHz since a greater change was expected in this frequency range. The four highest points were repeated. A single-shell model was fitted to the recorded data by manually adjusting parameters until a high correlation coefficient was reached.

To achieve rapid results with a sample grown from a culture plate a similar experiment was conducted whereby the sample was grown on a culture plate for 18 h. A single culture was removed and dispersed in 25 mL pre-warmed nutrient broth #2, incubated for 3h at 37°C to 'acclimatise' the culture to the liquid medium and then treated and analysed as above.

DEP-Well Spectra chips were constructed from a laminate of 17 µm copper interleaved with 75 µm polyimide. 12 copper layers were used and wells of 800 µm were drilled through the laminate. All exposed metal surfaces were plated with 1 µm

gold to ensure bio-compatibility. Devices were manufactured using flex-PCB technology by Tru-Lon (Royston, UK). A glass base was attached to the base of the laminate to seal the base of the well. Signals were applied using a Digimess FG100 signal generator (Farnell, Leeds, UK); signals were monitored using an IDS710 digital oscilloscope (RS electronics, UK). The experiment was performed under a Nikon 50i Microscope (Nikon, UK) with an AVT dolphin 145b camera attached (firstsightvison, Farnham, UK). The Camera, signal generator and oscilloscope were controlled by a Matlab (release 2006b Mathworks, Natick, USA) script. Image processing, signal processing and data analysis were also performed by various Matlab scripts.

To confirm the effectiveness of the antibiotics serial dilutions of the cultures used in the experiments were plated on nutrient agar. This was also used to confirm that the bacteria stayed viable throughout the sample preparation and when stored in the conductivity medium for up to 4 h.

Results and discussion

Figure 3 shows the DEP spectra collected after treating *E. coli* with polymyxin B. As can be seen, there are clear changes in the DEP spectrum even one hour after exposure, where the high-frequency dispersion (the descending part of the curve) is observed to shift down in frequency, whilst the low-frequency portion of the curve rises and changes sign for the earlier time points whilst dropping to a value nearer the initial state after four hours.

To better understand these changes, a dielectric model was manually fitted onto the experimental data. Since only a single-shell spherical model was used, the data must be considered as qualitative rather than representing absolute values for the respective part of the cell. However, the values do give an understanding of the changes occurring in the bacteria relative to the control experiments (Table 1).

Even 1h after drug exposure, the cytoplasm conductivity was observed to drop by nearly two thirds from 0.35 S/m to 0.13 S/m; confirming the dilution of the cytoplasm. After 4h the cytoplasm conductivity drops further to 0.05 mS/m, indicating that most mobile ions in the cytoplasm have diffused out of the cell and the remaining charges are likely part of proteins or bound to the internal cell structures.

The outer shell conductivity rises significantly in the first two hours, confirming the that Polymyxin B disrupts the membrane integrity, opening more and more holes that let ions equilibrate with the surrounding medium. However, an interesting effect is the downward shift of the first dispersion frequency (the upwards curve beginning at approximately 100kHz). This indicates that the outer shell becomes more capacitive than before, possibly caused by the membrane shrivelling up and thereby increasing the membrane-area-to-cell-volume ratio. The drop of shell conductivity after 4 h might be caused by re-stabilising of the membrane. The near doubling of the membrane capacitance per unit area from 13.3 mF/m² to 24.3 mF/m² on the other hand stays constant even after 4 h confirming that the outer shell of the cell does not recover from the interaction by the polymyxin.

The practical application of the DEP as a tool in determining rapid antibiotic resistance is limited by the fact that most current assays grow cultures on plates rather than broth. Since DEP-Well chips need evenly suspended cells it is difficult to use a culture directly from a plate for analysis, as they rarely disperse evenly when put into a liquid medium. To overcome this, as well as demonstrating the rapidity of the DEP assay, a culture was taken directly from a plate and dispersed in fresh culture medium (at 37°C) and incubated for 3 h before treatment (Figure 4). The results are similar to cultures grown in liquid medium only. Again, clear changes in the DEP-Spectrum are visible after 1 hour. To analyse the changes, curves were again manually fitted with a single shell model (Table 2). The trends shown by the model are similar than the previous experiment. The different starting values were most likely caused by the different culture conditions as well as the limitations of the spherical model. This experiment shows clear potential in DEP for antibiotic studies, providing clear results just four hours after taking a culture from a plate. This gives a very significant time saving compared to conventional disk diffusion tests that need at least 16 h to give a result. By optimising the procedures, further savings may be envisaged allowing even faster detection of antibiotic resistance.

Figure 3: *E.coli* after treated with 20 $\mu\text{g/mL}$ polymyxin B after 15h culture in broth. The graphs show the experimental data (points) and best-fit lines (parameters in table 1) for *E. coli* before treatment with polymyxin B and after 1, 2 and 4 hours of treatment.

Time	σ_{cyto} [S/m]	C_{spec} [mF/m ²]	G_{spec} [kS/m ²]	Correlation coefficient R
0 h (control)	0.35	13.3	7.75	0.9805
1 h	0.13 (-63%)	24.3 (+82%)	12.8 (+65%)	0.9369
2 h	0.12 (-65%)	24.3 (+82%)	17.8 (+130%)	0.9008
4 h	0.05 (-86%)	24.3 (+82%)	10.3 (+33%)	0.9552

Table 1: modelling parameters for *E.coli* exposed to polymyxin B. Best-fit values for the conductivity of the cytoplasm (σ_{cyto}) and the capacitance per unit area and conductance per unit area of the shell (C_{spec} and G_{spec} respectively). Values in brackets indicate change relative to control. After exposure to Polymyxin B the cytoplasm conductivity drops first to more then half and after 4 ha even to a 7th of the original value, while the shell conductivity first rises strongly but stabilises later, also the shell capacity approximately doubles.

Figure 4: *E.coli* taken from plate incubated for 3h in broth before treatment with 40 μ g/mL polymyxin B. The graphs show the experimental data (points) and best-fit lines (parameters in table 2) for *E. Coli* before treatment with polymyxin B and after 1, 2 and 4 hours of treatment

Time	σ_{cyto} [S/m]	C_{spec} [mF/m ²]	G_{spec} [kS/m ²]	Correlation coefficient R
0 h (control)	0.21	19.9	8.38	0.9744
1 h	0.10 (-52%)	44.3 (+120%)	19.0 (+127%)	0.7502
2 h	0.08 (-62%)	44.3 (+120%)	25.3 (+205%)	0.8621

Table 2: modelling results for *E.coli* taken from plate. Best-fit values for the conductivity of the cytoplasm (σ_{cyto}) and the capacitance per unit area and conductance per unit area of the shell (C_{spec} and G_{spec} respectively). Values in brackets indicate change relative to control. With the culture only briefly cultured in liquid medium similar results to the only liquid cultures appear. The cytoplasm conductivity drops dramatically; the outer shell becomes more conductive and capacitive.

Conclusion

This study provides a starting point in the assessment of DEP-based drug resistance assays for bacteria. When *E. coli* was exposed to polymyxin B, significant time savings were achieved compared to conventional disk diffusion. However, to fully assess the potential of the technique, a broader study is needed. Polymyxin B affects the membrane, and so leads to an easily identifiable change in the DEP spectrum. For antibiotics which have different mechanisms of action, these results (both in extent

and change of properties) may vary according to the drug and also on the bacterium, particularly whether the bacterium is gram-positive or gram-negative.

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