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On the dielectric method of monitoring cellular viability

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Abstract

It is shown that the dielectric method is a valid way of monitoring cellular viability in situations where the mechanism causing death leads rapidly to gross cell lysis. We also show that the monitoring of capacitance at a single low radio frequency does reflect the actual dielectric properties of the cell suspension as judged from frequency scanning methods. It is tentatively suggested that the initial rise in capacitance of the suspension after the addition of the solvent is probably due to the cells' size/membrane area increasing, rather than a direct effect of the solvent on the membrane permittivity.

INTRODUCTION

The study of the passive electrical (dielectric) properties of suspensions of biological cells has been an area of active interest, and over the years has yielded a lot of information on the structure and physiology of cells (ref. 1 and 2). The real power of the suspension technique is that it is totally non-invasive, since the electrodes used are just dipped into the suspension without the need for the insertion of an electrode into an individual cell. This fact makes such measurements very convenient to make and in recent years they have been exploited for the development of novel bio-instrumentation. One of the major applications has been in the on-line and real-time measurement of cellular biomass in fermentations (ref. 3). This is a variable of very major importance to the fermentation industry and has previously proved to be extremely difficult to measure under the rather hostile conditions found in industrial fermenters. We have developed a dielectric spectrometer (the Biomass Monitor) that has been optimised to work under such conditions; this has proved of great utility in a wide variety of biomass measurement applications (ref. 3). In this work we show that we can indeed measure biomass, as opposed to necromass, by comparison of the results of dielectric studies with those given by the more conventional viability staining methods. We take as our model system the slow lysis of yeast cells by the membrane active agent octan-1-ol (ref. 4). The interaction of such solvents with cells resulting in cell death is of significant interest, since organic solvents are now of some importance as medium components in biotransformation systems (ref. 4 and references therein).

THEORY

The most convenient method to measure the electrical properties of cell suspensions in the frequency range d.c to 100 MHz is by the use of a capacitative probe. Here the probe in essence consists of the plates of a capacitor and the cell suspension is the dielectric. By changing the frequency (in Hz) of the applied (low amplitude) electric field and recording the capacitance (C in Farads) and conductance (G in Siemens) of the cell suspension (in the admittance domain) one can build up a dielectric spectrum. As the frequency is increased one finds that the capacitance falls and the conductance increases in a series of steps called dielectric dispersions. Detailed reviews of the mechanisms underlying the dispersions at frequencies up to 100 MHz, and the precautions that are required to measure them, can be found in references 1, 2, 5 and 6.

The capacitance (C) and conductance (G) of the cell suspension depends on the geometry of the electrodes used to measure them. Thus to model the physical processes going on in the suspension one must convert

them to values independent of electrode geometry. To do this one converts capacitance to relative permittivity (ε', unitless) and conductance to conductivity (σ' in $S.m^{-1}$). The equations used to do this are

$$\varepsilon' = C\,K/\varepsilon_0 \quad (1)$$

$$\sigma' = G\,K \quad (2)$$

where K is the cell constant (in m^{-1}) of the electrode, which reflects the electrode geometry, and ε_0 is the permittivity of free space (8.854×10^{-12} $F.m^{-1}$). For phenomena of present interest, conductivity reflects the concentration of aqueous ions, their mobilities and valencies, whilst relative permittivity (ε') reflects the extent to which localised charge distributions can be distorted by the applied electric field (ref. 7).

The dispersive phenomena of interest in this study is the so called dielectric β-dispersion which is due to the charging up of the plasma-membrane capacitance by ions moving in the applied electric field. For a full discussion of the theory and physical basis of the β-dispersion see references 1 and 2. The frequency dependency of the permittivity of a cell suspension during the β-dispersion is shown diagrammatically on Fig. 1.

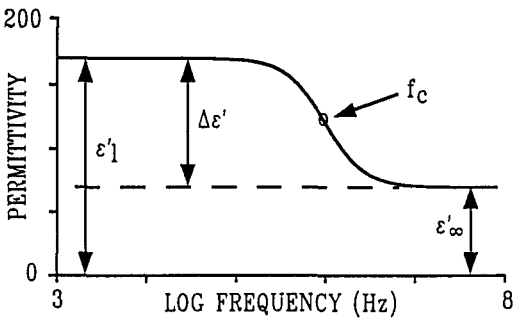

Fig. 1. A diagramatic representation of the β-dispersion. See the text for a full discussion.

As one can see the relative permittivity (ε') goes from a high low-frequency plateau (ε'_1) to a low high-frequency one (ε'_∞), as the frequency is increased. The change in the permittivity between the two plateau values is called the dielectric increment (Δε'). The frequency at which the *change* in permittivity is half complete (i.e when $\varepsilon' = (\Delta\varepsilon'/2) + \varepsilon'_\infty$) is called the critical frequency (f_c in Hz). The f_c values typical for the β-dispersion range from 100 kHz to 10 MHz. The equation that models the change in permittivity (ε') as function of frequency that is classically used for the β-dispersion is that of Cole and Cole (ref. 1, 2, 8).

$$\varepsilon'_\omega = \Delta\varepsilon'((1 + (f/f_c)^{1-\alpha} \sin(0.5\alpha\pi))/(1 + 2(f/f_c)^{1-\alpha} \sin(0.5\alpha\pi) + (f/f_c)^{2-2\alpha}) + \varepsilon'_\infty \quad (3)$$

The terms in this equation are as in Fig 1. In addition f is the frequency of the applied electric field and the α value is the so called Cole-Cole α, which nominally reflects the magnitude in the distribution of relaxation times underlying a dispersion (although it is difficult to reconcile this interpretation with typical β-dispersion data for biological cells (ref. 9)).

A number of equations in the literature relate the macroscopic features of the β-dispersion (as seen in Fig. 1) to the structure of the cells in the suspension (ref. 1 and 2). The two equations of importance in this study are given below.

$$\Delta\varepsilon' = (9\,P\,r\,Cm)/4\,\varepsilon_0 \quad (4)$$

$$Cm = (\varepsilon'_m\,\varepsilon_0)/d \quad (5)$$

Δε' and ε_0 are as described earlier. P is the volume fraction (unitless) of biological cells in the suspension with *intact* plasma-membranes and r is cellular radius (in m) to the outside of the plasma-membrane. Cm is the capacitance per unit area of plasma-membrane (in $F.m^{-2}$) and typically takes values close to 0.01 $F.m^{-2}$ (ref. 1 and 2). Cm is related to the structure of the plasma-membrane by equation (5). In this equation ε'_m is the permittivity of the membrane bilayer and d is the membrane thickness (in m). The conductance of the membrane does not appear in equation 4 because it is very small compared to that of the cytoplasmic and suspending medium conductances and so is regarded as being a non-conductor (ref. 2 and 10). In fact to decrease the size of Δε' significantly the conductivity of the plasma-membrane has to be increased enormously, to values as high as 1 $S.m^{-2}$ (ref. 10).

One can see that equation (4) predicts that the magnitude of the $\Delta\varepsilon'$ of the β-dispersion is directly proportional to the volume fraction (P) of cells with *intact* plasma membranes present, provided r .Cm is constant. It is this relationship between $\Delta\varepsilon'$ and P which forms the basis of the measurement of biomass in fermentors. The aim of this study is to see if $\Delta\varepsilon'$ really does measure viable cells (defined dielectrically as having *intact* plasma-membranes) as opposed non-viable (lysed) cells, by deliberately killing cells slowly, measuring the changes in $\Delta\varepsilon'$ and correlating them with the cell viability as measured by viability staining. The most convenient way to kill cells slowly is to add to the suspension an organic solvent that is relatively insoluble in water. Under these conditions the poor solubility of the solvent in the aqueous phase controls the rate of partitioning of the solvent into the membranes. The solvent slowly binds into the membrane, expanding it and eventually causing lysis (ref. 4).

MATERIALS AND METHODS

Saccharomyces cerevisiae was obtained locally in the form of a paste and washed and suspended in a sorbitol/HEPES buffer exactly as in ref 4. The viability of the yeast during slow killing by octanol was determined using methylene blue (MB), ethidium bromide and fluorescein-diacetate (FDA) stains. The methylene blue stain was prepared and used as in ref. 4. The ethidium bromide/FDA stain was made up immediately before use, using the methods of Kemp (ref. 11). A couple of crystals of ethidium bromide were added to 5ml of the yeast suspension medium. A few crystals of FDA were added to 1ml of acetone and 4 drops added to the ethidium bromide solution using a Pasteur pipette. For the assessment of viability, 10 µl of cells were added to 1ml of suspension medium and 50 µl of the mixed stain. The ethidium bromide stained the dead cells a bright fluorescent red and FDA stained the living cells a bright fluorescent green. The fluorescence was observed immediately after the addition of the stain using an epifluorescence microscope. A stain containing just ethidium bromide was also used. This was prepared and used exactly as above, except that the FDA in acetone was omitted.

All dielectric measurements were made using a 4-terminal dielectric spectrometer, the Biomass Monitor obtained from Aber Instruments, Aberystwyth Science Park, Cefn Llan, Aberystwyth SY23 3AH, Dyfed, U.K. The electrode was a standard 25 mm diameter fermentation-type probe ending with 4 solid gold electrode pins. The cell constant of the electrode was 0.63 cm^{-1} as judged by the methods in ref. 4. Baseline scans of capacitance versus frequency indicated that the 4 gold pin electrode arrangement was essentially non-polarisable under the conditions used. The electrode was mounted horizontally in the side of a plastic beaker, in which all measurements were made. The beaker had a single thin plastic baffle opposite the electrode and this arrangement allowed vigorous mixing with the production of a fine homogeneous suspension of octanol, while preventing the formation of a vortex in front of the electrode (see ref. 4).

Before the start of any work on yeast suspensions the capacitance of the suspending buffer was set to zero at 0.4 MHz, this has the effect of setting ε'_{∞} on Fig. 1 to near zero. Two forms of monitoring the yeast suspension during treatment with octanol were used. In the first the capacitance and conductance was recorded only at 0.4 MHz. A low pass filter of time constant 1s was used to filter out any stirrer noise. 0.4 MHz was used as this was well below the f_c of the β-dispersion of the yeast in this medium and so the capacitance measured at this frequency reflected the magnitude of $\Delta\varepsilon'$ the β-dispersion (see Fig 1). The yeast suspensions used in these studies always had a capacitance at 0.4 MHz, prior to the addition of solvents, of 25 pF as judged by this method (see ref. 4 for details).

The second method involved doing frequency scans on the suspension and its equivalent polarisation controls as described in detail in references 6 and 8. The resulting capacitance spectrum of the cell suspension after the polarisation control data has been subtracted from it was then converted to the permittivity spectrum using equation 1. Equation 3 was then fitted to the data using the Levenberg-Marquardt non-linear least squares method incorporating the robust weighting algorithm of Mosteller and Tukey *via* the computer program GraFit (ref. 8). This enabled the actual $\Delta\varepsilon'$, f_c and α of the dispersion to be followed as a function of time after the addition of solvent.

In all experiments where octanol was added to the yeast suspension, a control was also done to monitor the effect of the addition of that compound to the medium alone. In no instance was there a significant change in capacitance or conductance at 0.4 MHz, or at any other frequency. A control experiment showed that increasing the conductance of a 25 pF yeast suspension by 0.34 mS with KCl, gave no measurable change in capacitance at 0.4 MHz.

RESULTS AND DISCUSSION

It has been known for some time that aliphatic alcohols can partition into erythrocyte membranes and increase their area (ref. 12). If this occurs, then one might expect this to be reflected in the size of the $\Delta\varepsilon'$ of the β-dispersion, which should increase as the cell size and/or membrane area increases (see equation 4).

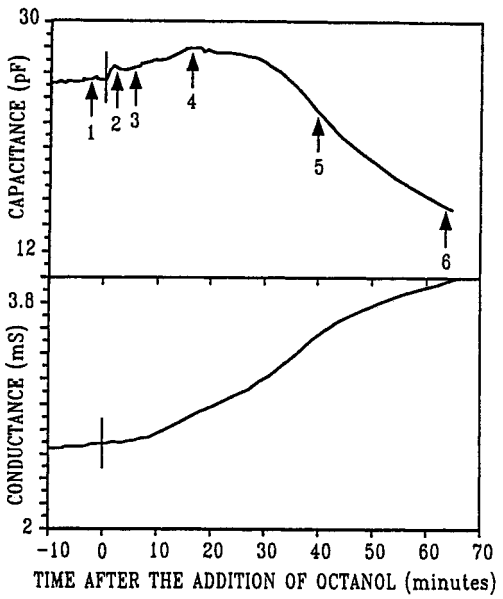

Fig. 2. Effect of octanol on the electrical properties of yeast at 0.4 MHz. The numbered arrows indicate frequency scans. The fitted $\Delta\varepsilon'$ to these scans (as described in the methods section) along with the standard error of the fitted parameter (in brackets) were 1: 191 (1.6), 2 and 3: 202 (2.3), 4: 213 (1.9), 5: 180 (2.2) and 6: 132 (2.0).

However as was pointed out in reference 13, black lipid membrane (BLM) studies have shown that the incorporation of solvent into membranes increases its thickness and therefore *decreases* the membrane capacitance (see equation 5). This effect would be reflected by a fall in the capacitance of the suspension on solvent addition (see equations 4 and 5). Whatever the dominant effect of the solvent on the cell membranes one would expect the permittivity (capacitance) at a frequency low with respect to the f_c of the β-dispersion to be affected by changes in the membrane/cell properties . In a previous paper (ref. 4) we showed that a range of solvents known to partition into membranes indeed changed the capacitance of the cell suspension as they partition into the membranes. In addition, after a threshold level of partitioning has occurred cell lysis is seen as a fall in capacitance and a rise in conductance of the suspension, which correlated with viability as judged by methylene blue staining. Solvents that did not enter the membranes in significant concentrations did not affect the capacitance or viability of the suspension. In this paper we aimed to study these effects in more detail.

Fig 2 shows the capacitance and conductance changes that occurred when 0.375 ml of octan-1-ol was added to a yeast suspension and monitored at 0.4 MHz only. The arrows mark the times when frequency scans of the suspension were done (the low pass filter being turned off for these). Several trends are clear: at first there is a rise in the low-frequency capacitance of the cell suspension, suggesting that solvent is partitioning into the membrane. For a given batch of yeast paste the shapes of the peaks in capacitance, on addition of octanol, were very reproducible. These features of the plot must reflect transitions in the shape and size or membrane properties of the cells resulting from the partitioning of the solvent into the membranes. Also seen on this Fig. is the fall in capacitance due to lysis of the plasma membranes, which is accompanied by a significant rise in conductance. These effects result from the lysis of the cells in the suspension, with the release of cytoplasmic ions into the suspending medium. At this frequency (0.4 MHz) the ions inside intact cells are not seen, due to the screening effect of the plasma membrane. These results are in line with those in ref. 4.

Also given in Fig. 2 are the best fit $\Delta\varepsilon'$ values of the β-dispersion derived from the fitting of equation 3 to the frequency scans as described in the Methods. The results demonstrate that the increases in capacitance values measured at 0.4 MHz do reflect the magnitude of the dielectric increment ($\Delta\varepsilon'$) of the β-dispersion. The rise in $\Delta\varepsilon'$ immediately after the addition of solvent does suggest that partitioning into the membrane is being detected dielectrically. From equation 4 the rise could be due an increase in the effective cell radius (or membrane area) and/or an increase in Cm. Octanol is known to bind into cell plasma-membranes and increase their area (ref. 12) and if this resulted in an increase in cell radius then a substantial increase in $\Delta\varepsilon'$ could result. This follows from the fact that $\Delta\varepsilon'$ is proportional to r^4 . This relationship is seen when P in equation 4 is replaced by $(4/3)\,\pi\, r^3\, N$, where N is the number of cells per unit volume. If the cell wall prevents the protoplast expanding radially, thus causing the membrane to become folded, then this too can be expected to increase the apparent Cm of the cells, as was seen in the work of Davey (ref. 14).

From equation 5, an increase in Cm could result from a decrease in the membrane thickness (d) on solvent binding or an increase in the plasma membrane permittivity (ε'_m). It has already been mentioned that BLM studies suggest that octanol will increase the membrane thickness rather than decrease it. As the

permittivity of octanol is about 10, and that of the membrane (ε'_m) is less than 3, one can see that the binding of octanol into the membrane may result in a significant rise in ε'_m and thus Cm , although the concomitant increase in membrane thickness will offset this effect somewhat. As octanol is known to bind into membranes and to increase their area, and as $\Delta\varepsilon'$ is potentially very sensitive to this, it is probably the membrane area/cell radius effects that give rise to the increases seen on the addition of octanol.

To check that the fall in capacitance and rise in conductance on addition of solvent was indeed due to cell death, an octan-1-ol experiment was monitored with three viability stains. It was found that the cell suspension (25 pF) prior to the addition of octanol had methylene blue and ethidium bromide percent viabilities of 98.8 and 95.6 respectively, while FDA gave much less than 5%. As the FDA staining relies on the possession of esterases by living cells (ref. 15), it appears that the strain of yeast used lacked sufficient of these enzymes when in the physiological state found in the yeast pastes. Thus for the vitality staining experiments only methylene blue and ethidium bromide (no FDA added) were used. Fig. 3 compares the viabilities of the yeast after the addition of 0.5 ml of octan-1-ol as judged by capacitance and the two stains.

From Fig. 3 it is evident that there is no straightforward relationship between the viabilities measured using the three methods, but there is good qualitative agreement. The problem is that each method uses a different criterion to judge whether a cell is alive or dead (ref. 16). For a cell not to contribute to the β-dispersion its plasma membrane has to be very badly ruptured. For ethidium bromide to make a cell fluorescent it must first penetrate the plasma membrane and then intercalate in DNA. Thus it is an exclusion dye. Methylene blue is also an exclusion dye which on entry to a living cell is reduced to the colourless leuco-form (ref. 17). Thus viability, as judged by failure to stain, is a function of both the rate of dye penetration and its rate of reduction inside the cell.

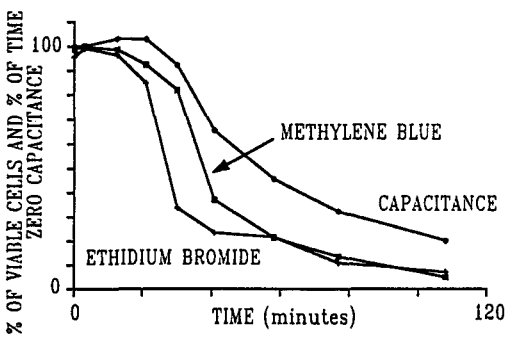

Fig 3. The viability of a yeast suspension as measured by capacitance (at 0.4 MHz) and two viability stains as a function of the time after the addition of 0.5 ml octanol.

A feature often seen in dielectric monitoring of viability is the fact that the capacitance of the suspension never falls to zero even when vitality staining suggests all the cells are dead (see Fig. 3). This is probably due to the fact that the cells have membranes damaged enough to allow the stains to indicate that the cells are dead but not so damaged that they do not contribute to the β-dispersion $\Delta\varepsilon'$ at all. Thus one may find that the suspension contains grossly lysed cells not contributing anything to the $\Delta\varepsilon'$, along with cells with high membrane conductances which still contribute a little. A set of frequency scans would need to be done to settle this point. If the yeast in the paste are left to die naturally before making suspensions one can get a situation where the methylene blue stain viability is zero whilst the capacitance at 0.4 MHz indicates 100% viability (as judged by comparison to fresh cells with 100% MB viability). This reiterates the dielectric method's emphasis on *intact* plasma-membranes for viability, as opposed to an intact metabolic apparatus.

CONCLUSIONS

In this paper we have shown that the dielectric method is a valid way of monitoring cellular viability in situations where the mechanism causing death rapidly leads to gross cell lysis. This is true in the case of the acute toxicity of solvents to cells resulting in lysis, and in practice also seems true of cells dying in many fermentation situations. We have also shown that the monitoring of capacitance at a single low frequency does accurately reflect the actual dielectric properties of the cell suspension as judged from frequency scanning. We can also tentatively suggest that the rise in capacitance of the suspension after the addition of the solvent is probably due to the cells size/membrane area increasing, rather than a direct effect of the solvent on the membrane permittivity.

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REFERENCES

1. C.L. Davey and D.B. Kell, In Treatise on Bioelectrochemistry, Vol. VI, Birkäuser (1992), in press.
2. K.R. Foster and H.P. Schwan, CRC Crit. Rev. Biomed. Eng. 17, 25-102 (1989).
3. D.B. Kell, C.L. Davey and R.W. Todd, Biotechnology International, 2nd Edition (R.N. Greenshields, ed.), Grosvenor Press, London, pp. 294-297.
N.G. Stoicheva, C.L. Davey, G.H. Markx and D.B. Kell, Biocatalysis. 2, 245-255 (1989).
5. H.P. Schwan, In Physical Techniques in Biological Research, Vol. VIB, Academic Press, New York (1963).
6. C.L. Davey, G.H. Markx and D.B. Kell, Eur. Biophys. J. 18, 255-265 (1990).
7. R. Pethig, Dielectric and Electronic Properties of Biological Materials, Chichester, Wiley (1979).
8. C.L. Davey, H.M. Davey and D.B. Kell, Bioelectrochem. Bioenerg. (1992), in press.
9. G.H. Markx, C.L. Davey and D.B. Kell, Bioelectrochem. Bioenerg. 25,195-211 (1991).
10. C.M. Harris and D.B. Kell, Eur. Biophys. J. 13,11-24 (1985).
11. R.B. Kemp, R.W.J. Meredith and S.H. Gamble, Food Chem. Toxicol. 23, 267-270 (1985).
12. P. Seeman, Pharmacol. Rev. 24, 583-655 (1972).
13. L.E. Ferris, C.D. Davey and D.B. Kell, Eur. Biophys. J. 18, 267-276 (1990).
14. C.L. Davey, D.B. Kell, R.B. Kemp and R.W.J. Meredith, Bioelectrochem. Bioenerg. 20, 83-98 (1988).
15. R.P. Jones, Process Biochem. 22, 118-134 (1987).
16. A.S. Kaprelyants and D.B. Kell, J. Appl. Bacteriol. 72, 410-422 (1992).
17. L.F. Hewitt, Oxidation-Reduction Potentials in Bacteriology and Biochemistry, London County Council, London (1948).