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Effect of biocide concentration on electrorotation spectra of yeast cells

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Abstract

The effect of the biocide Cosmocil (polyhexanide) at different concentrations on the electrorotation spectra of yeast cells is investigated over the frequency range from 1 kHz to 10 MHz. The dielectric properties of the yeast, before and after biocide treatment, were deduced from the electrorotation spectra using two-shell ellipsoid modelling methods that have been well tested for other heterogeneous biological systems. The results show a gradual increase in the cytoplasmic membrane conductivity with increasing biocide concentration, rather than an 'all-or-nothing' breakdown of the membrane. The technique gives a quantitative analysis of the toxic damage by chemicals to cells and can be exploited in the development of new pharmacological agents.

Keywords: Electrorotation; Biocide; Yeast; Toxicity; Membrane conductivity

1. Introduction

The investigation of the effects of chemicals and other environmental factors on the electrical properties of cells is increasingly an important area of study. In many of these studies yeast has been the organism of choice [1–10]. Electrorotation of biological cells in microfabricated electrode systems is a useful technique for studying the dielectric properties of individual cells under minimal physiological damage. Huang et al. [8] investigated viable and non-viable yeast cells by means of combined dielectrophoresis (DEP) and electrorotation (ROT) studies and derived the physical parameters of different yeast cell compartments (i.e. cytoplasm, membrane, cell wall etc.). The non-viable yeast cells used in their studies were obtained by physical treatment (heating at 75°C for 5 min). In other studies [4,5,7,11] it has been shown that the rotation spectra of yeast cells are affected by Ag^+ or Hg(II) in the concentration range of 20–100 nM [7,11] and that halophenols (frequently occurring environmental contaminants) have effects at concentrations in the range 0.3–3 μM (approx. 40–400 ppm) [4]. The effect of low concentrations of biocide on the electrorotation of individual cells is not always an 'all-or-none' effect, but intermediate stages can occur in which the cell membrane is affected and the cell contents leaks out [4,7]. However, in these earlier studies, no attempts were made to quantify these effects in terms of changes of the conductivity of the cell membrane and cell interior. We describe here measurements of electrorotation spectra of yeast cells in the presence of the toxic chemical Cosmocil (a polyhexanide oligomer) of concentrations in the range of 10–500 ppm, where intermediate stages of cell lysis are likely to occur. The data are fitted using an ellipsoidal two-shell model, which allows values of the electrical properties of the different cell compartments to be estimated.

2. Theory

The yeast cells were determined by microscopic inspection to be ellipsoids (average dimensions 5.7 ± 0.7 μm $\times$ 4.8 ± 0.7 μm) and because they possess an outer cell wall as well as a cytoplasmic membrane, we approximated them theoretically in terms of an ellipsoidal two-shell cell model suspended in a continuous medium, as shown in Fig. 1. The two-shell model has been well tested for both spherical and ellipsoidal cells possessing a wall and membrane [8,12–14]. The complex AC permittivity of the suspending medium, the cell wall, the cytoplasm membrane and the interior of the yeast cell are represented by ϵ_4^*, ϵ_3^*, ϵ_2^* and ϵ_1^* respectively. These parameters are determined by $\epsilon_1^* = \epsilon_i - j\sigma_i/\omega (i = 1, 2, 3$ and $4)$, where ϵ_i and σ_i are the permittivity and conductivity, respectively, of the ith phase. The surfaces of the shells are

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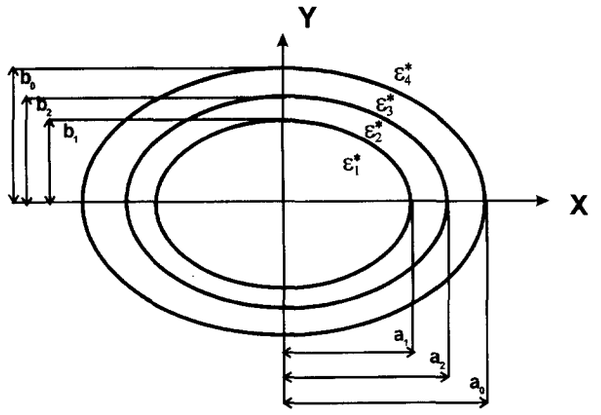

Fig. 1. Cross-section of an ellipsoidal two-shell model representing a yeast cell in the $x-y$ plane. ϵ_4^*, ϵ_3^*, ϵ_2^* and ϵ_1^* represent the complex permittivities of the suspending medium, cell wall, cytoplasmic membrane and cell interior, respectively.

represented by three confocal ellipsoids for convenience of mathematical treatment, and an ellipsoidal co-ordinate system is introduced by assuming the outermost surfaces of the shelled ellipsoid to be a standard ellipsoid with semi-axes a_0, b_0, and c_0 [14]:

$$\frac{x^2}{a_0^2+\xi}+\frac{y^2}{b_0^2+\xi}+\frac{z^2}{c_0^2+\xi}=1 \tag{1}$$

where ξ is a parameter representing a family of surfaces of confocal ellipsoids. When $\xi=0$ and $\xi=-\delta_i$, Eq. (1) represents the outermost and ith surfaces respectively of the shelled ellipsoid [13,14]. The semi-axes a_i, b_i, and c_i of the ith surface are given by:

$$a_i=\left(a_0^2-\delta_i\right)^{1/2}\quad b_i=\left(b_0^2-\delta_i\right)^{1/2}\quad c_i=\left(c_0^2-\delta_i\right)^{1/2} \tag{2}$$

2.1. Electrorotation

Electrorotation (ROT) occurs as the result of the rotational torque exerted on a polarised particle subjected to a rotating electric field [15] and a good theoretical basis for understanding this effect has been established [16–19]. When a particle is placed into a rotating field, the induced dipole moment will rotate with the applied field. The electrostatic interaction between the applied field and the induced dipole moment causes a torque to be exerted on the particle. Co-field rotation occurs when the induced dipole moment lags behind the applied field by phase angles ranging from 0° to 180°, whilst anti-field rotation occurs when the induced dipole moment lags by more than 180°. The resulting rates and directions of rotation of the particle are dependent on the frequency of the applied field and on the effective dielectric properties of the particle and its surrounding medium.

We consider the yeast cell as a homogeneous ellipsoid surrounded by a cytoplasmic membrane and a cell wall. For such a particle in an imposed electric field E of angular frequency ω, the electrorotation torque $\Gamma(\omega)$ is given by [14]:

$$\Gamma(\omega)=0.5V_c\epsilon_m\mathrm{Im}[\chi_\alpha(\omega)]E^2\ (\alpha=x,y) \tag{3}$$

where $V_c(V_c=\frac{4\pi a_0b_0c_0}{3})$ is the volume of the ellipsoid and $\chi_\alpha(\omega)$ $(\alpha=x,y)$ is given by:

$$\chi_\alpha(\omega)=\frac{\epsilon_{effp}^*-\epsilon_4^*}{\left(\epsilon_{effp}^*-\epsilon_4^*\right)A_{0\alpha}+\epsilon_4^*}\ (\alpha=x,y) \tag{4}$$

where ϵ_4^* is the complex permittivity of suspending medium and $A_{i\alpha}(i=0,1,2;\ \alpha=x,\ y)$ is the depolarising factor along the x and y axes where

$$A_{ix}=0.5\,a_ib_ic_i\int_0^\infty\frac{d\xi}{(a_i+\xi)R_i} \tag{5a}$$

$$A_{iy}=0.5\,a_ib_ic_i\int_0^\infty\frac{d\xi}{(b_i+\xi)R_i} \tag{5b}$$

with

$$R_i=\left[\left(a_i^2+\xi\right)\left(b_i^2+\xi\right)\left(c_i^2+\xi\right)\right]^{1/2}\ (i=0,1,2) \tag{5c}$$

ϵ_{effp}^* is the effective complex permittivity of the two-shelled ellipsoid along the α $(\alpha=x,\ y)$ axes.

$$\epsilon_{effp}^*=\epsilon_3^*\frac{\epsilon_3^*+\left(\epsilon_{ieff}^*-\epsilon_3^*\right)\left[A_{1\alpha}+\nu_1(1-A_{0\alpha})\right]}{\epsilon_3^*+\left(\epsilon_{ieff}^*-\epsilon_3^*\right)\left(A_{1\alpha}-\nu_1A_{0\alpha}\right)} \tag{6}$$

where ϵ_{ieff}^* represents the effective complex permittivity of the inner-most ellipsoids after they have been smeared together:

$$\epsilon_{ieff}^*=\epsilon_2^*\frac{\epsilon_2^*+\left(\epsilon_1^*-\epsilon_2^*\right)\left[A_{2\alpha}+\nu_2(1-A_{1\alpha})\right]}{\epsilon_2^*+\left(\epsilon_1^*-\epsilon_2^*\right)\left(A_{2\alpha}-\nu_1A_{1\alpha}\right)} \tag{7}$$

with

$$\nu_j=\frac{a_jb_jc_j}{a_{j-1}b_{j-1}c_{j-1}}\ (j=1,2)$$

where the subscripts 1, 2 and 3 represent the internal phase, cytoplasmic membrane and wall of a yeast cell, respectively.

Furthermore, we assume the ellipsoidal particle is prolate, and $a_i>b_i=c_i>0$, so that the depolarising factor in Eq. 5a and Eq. 5b can be derived as:

$$A_{ix}=\frac{a_ib_i^2}{2\left(a_i^2-b_i^2\right)^{3/2}}\ln\left(\frac{2a_i^2-b_i^2+2a_i\sqrt{a_i^2-b_i^2}}{b_i^2}\right)-\frac{b_i^2}{a_i^2-b_i^2} \tag{8a}$$

$$A_{iy}=\frac{a_i^2}{2\left(a_i^2-b_i^2\right)}-\frac{a_ib_i^2}{4\left(a_i^2-b_i^2\right)^{3/2}}\times\ln\left(\frac{2a_i^2-b_i^2+2a_i\sqrt{a_i^2-b_i^2}}{b_i^2}\right)=\frac{1}{2}-\frac{1}{2}A_{ix} \tag{8b}$$

According to Edwardes' theory [20], the viscous frictional torque $\Gamma(\omega)$ for rotation of a rigid ellipsoid about the z axis at small Reynolds numbers is given by:

$$\Gamma(\omega) = -R_f \omega_c \quad (9)$$

where R_f is a parameter characterizing the hydrodynamic resistance to rotation of the body.

$$R_f = 2V_c\eta\left[\left(a_0^2 + b_0^2\right)/\left(a_0^2 A_{ox} + b_0^2 A_{0y}\right)\right] \quad (10)$$

Here η is the viscosity of the external medium, A_{0x} and A_{0y} are defined by Eq. 5a and Eq. 5b, respectively, and ω_c is the angular velocity of rotation about the z axis. We thus obtain the following expression for the electrorotation velocity:

$$\omega_c = 0.5\epsilon_m \frac{V_c}{R_f} \mathrm{Im}\left[\chi_x(\omega) + \chi_y(\omega)\right] E_0^2 \quad (11)$$

Over the frequency range investigated here (1 kHz to 10 MHz) the aqueous suspending medium does not exhibit a dielectric dispersion and the value of ϵ_m in Eq. (11) remains fixed at around $78\epsilon_0$, where ϵ_0 is the permittivity of free space ($8.854 \cdot 10^{-12}$ F m^{-1}).

3. Materials and methods

3.1. Yeast cells

Yeast cells investigated were those of *Saccharomyces cerevisiae* strain R12, a gift from Dr. R. Hölzel, Free University, Berlin. The cells were grown in an aqueous medium consisting of 0.5% yeast extract, 0.5% peptone and 5% sucrose at 30°C in an incubator-shaker (Jencons Scientific) at 200 rpm. The cells were harvested at 24 h after inoculation and washed three times in deionized water following centrifugation at $100 \times g$ for 15 min. The biocide used was Cosmocil (polyhexanide oligomer), and has a chemical structure of $[-(CH_2)_3\text{-NH-CNH-NH-CNH-NH-CNH-NH-CH}_2)_3 -]_n \cdot HCl$, where n averages 11 [21]. It was supplied by Dr. I.M. Eastwood of Zeneca Biocides. Biocide treated cells were prepared by first diluting the suspension 100-fold using deionized water. 20 μl yeast suspension was introduced into a 3 ml glass tube, and then 20 μl of different concentrations of the biocide ranging from 10 ppm to 500 ppm were pipetted into these yeast suspensions. The final yeast concentration was the same in each experiment. The suspensions were washed three times following 15 min of exposure of the yeast cells to the biocide at room temperature, and results were obtained for five such samples at each biocide concentration. The conductivity of the incubation suspension was adjusted to conductivities ranging from 0.5 mS m^{-1} to 1.5 mS m^{-1} using small amounts of a concentrated NaCl solution. At these conductivity values the effect of the medium conductivity on the yeast cell wall conductivity is likely to be small [22]. The conductivities of the suspending media were determined using a Hewlett Packard (model 4192A) impedance analyser, and to minimise electrode polarisation effects measurements were made at a frequency of 100 kHz, using platinum-black electrodes of cell constant 1.536 cm^{-1} determined by calibration against KCl solutions of known concentration. This procedure to adjust and measure the conductivities of the media took of the order 4 ± 1 min, after which the electrorotation measurements were commenced within 2 min.

3.2. Electrorotation measurements

For the ROT measurement the same experimental procedure was used as described elsewhere [21]. Polynomial electrodes were used that were deposited onto glass microscope slides by photolithography. The electrode material was gold and the distance between opposing electrodes ranged from 400 μm to 2 mm. The electrodes were cleaned thoroughly, and soaked in deionized water for 12 h to ensure minimal adherence of cells to the electrode substrate. Phase-shifted sinusoidal voltages were applied to the electrodes in the frequency range 1 kHz to 10 MHz to induce electrorotation. The applied voltages had peak-to-peak values in the range from 4 V to 10 V (equivalent to electric fields of the order 18 to 45 V cm^{-1}) and an electrorotation spectrum was usually completed within 20 to 30 min. The rotation behaviour of yeast was monitored using a Nikon microscope coupled to video camera equipment so records of the electrorotation could be re-analysed after the experiment. A total of 80 untreated yeast cells and 300 biocide treated cells were investigated, and electrorotation rates were determined manually using a stop-watch. The rotation spectra obtained, for 15 untreated cells and for at least 5 cells per biocide concentration, were then analysed theoretically. Because of cell capture by the electrodes or cells becoming stuck on the glass substrate, good rotation spectra extending over the full frequency range 1 kHz to 10 MHz were obtained for only about 20% of cells investigated.

4. Results and discussion

Single viable yeast cells were measured between frequencies of 1 kHz and 10 MHz at 4 points per decade and a typical electrorotation spectrum is shown in Fig. 2, together with a theoretical curve fitted according to the two-shell ellipsoidal model of Eqs. 3, 4, 6 and 7. In agreement with theory, there are two relaxation peaks of nearly the same size. The cells exhibited anti-field rotation at frequencies below 100 kHz, due to the presence of a non-conducting cytoplasmic membrane, and co-field rotation above 500 kHz, which results from the difference in the dielectric properties of the cell interior and the suspending medium.

As demonstrated by Gascoyne et al. [23], only four dielectric parameters of a multi-shell cell model can be uniquely and accurately derived from an electrorotation spectrum consisting of two peaks. The parameters we are interested in determining are the conductivities of the cell wall and cytoplasmic membrane, and an effective overall conductivity for the cell interior. We chose the cell wall thickness as the fourth parameter to be determined, because the value obtained for this can be used as a check of the reasonableness of the computer-aided modelling. For the purpose of this work we therefore employed literature values for the relative permittivity of the aqueous suspending medium, cell wall, membrane and cell interior, as well as the thickness of the membrane. The dimensions of the cells were determined by microscopic inspection. The electrorotation spectra were curve-fitted to the theory using a minimisation routine in MATLAB (The Math Works). Thus, the solid line in Fig. 2 representing the best fitted curve according to the two-shell ellipsoidal model (Eqs. 3, 4, 6, 7 and 11) was obtained for a measured medium conductivity of 1.1 mS m^{-1}, and for values of the relative permittivity of the medium (78), cell wall (60), cytoplasm membrane (6), cell interior (60) and membrane thickness (8 nm) were taken from the literature [24]. A starting value of 15 mS m^{-1} for the conductivity of the cell interior, and a cell wall thickness in the range 0.1–0.3 μm, were chosen in accordance with the yeast cell data derived by Huang et al. [8]. For this cell, with major axis 5 μm and minor axis 4.2 μm, the best-fit gives the values for the conductivities of the cell wall, membrane and cell interior as 14 ± 1 mS m^{-1}, 0.25 ± 0.1 μS m^{-1}, 0.2 ± 0.05 S m^{-1}, respectively, as well as a value of 0.2 ± 0.01 μm for the cell wall thickness. These derived parameters of the yeast cell compartments from the best-fitting curves are in good agreement with the work by Huang et al. [8].

Fig. 3 shows the electrorotation behaviour of a single yeast cell after treatment with 500 ppm biocide, together with the best fitted curve using the two-shell ellipsoidal model. It shows that the magnitude of the anti-field rotation is decreased in parallel with the co-field rotation. This can be interpreted as resulting from the cytoplasmic membrane being permeabilized and its conductivity increasing due to the integrity of this cell membrane being violated by the biocide, whilst at the same time the internal conductivity decreases due to leakage of the ionic contents of the cell into the surrounding medium. The measured medium conductivity and relative permittivity were 0.5 mS m^{-1} and 78, respectively. The relative permittivity of the cell wall, cytoplasm membrane and cell interior as well as the thickness of membrane and cell wall were taken to be the same as in Fig. 2. The best-fit gives the values for the conductivities of the membrane and cell interior as 5.5 ± 0.3 μS m^{-1} and 10 ± 2 mS m^{-1}, respectively, whilst maintaining the conductivity of the cell wall fixed at 14 ± 1 mS m^{-1}.

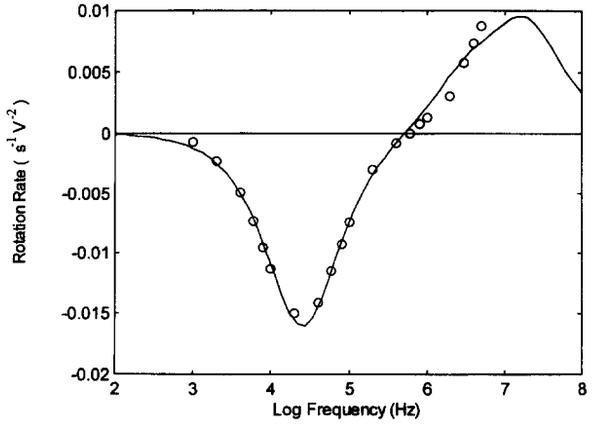

Fig. 2. Electrorotation spectra of a single intact untreated yeast cell.

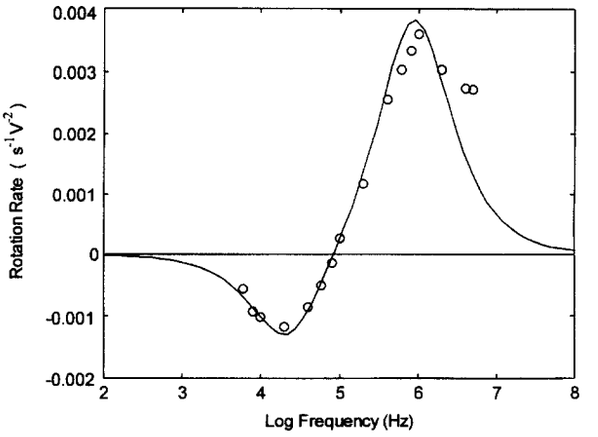

Fig. 3. Electrorotation spectra of a single yeast cell after treatment with 500 ppm biocide (Cosmocil).

To what extent do the dielectric properties of a membrane change upon exposure to toxic chemicals? To answer this, electrorotation spectra were measured for different concentrations of biocide and curve-fitted to the theory described above using the MATLAB minimisation routine. The corresponding values obtained for the conductivity of the membrane and cell interior, based on the averaged results obtained for at least five cells at each biocide concentration, are show in Fig. 4. Although electrorotation spectra could be obtained for biocide concentrations above 500 ppm, because the anti-field rotation response in the frequency range from 1 kHz to 100 kHz became very small with an ill-defined peak value, it was not possible to accurately fit the model to these data. At the lowest concentrations (< 50 ppm) the biocide appears to have no effect, but from then on the membrane appears to become more conductive in a *gradual* rather than in the 'all-or-none' fashion observed for yeast cells exposed to halophenols [4]. When the conductivity of the membrane increases by a factor of about ten times that of the untreated value of

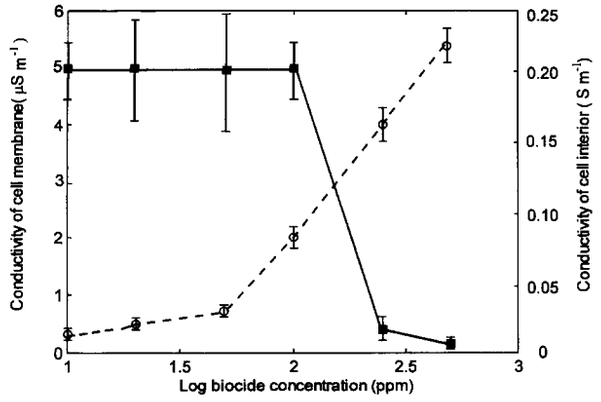

Fig. 4. Variation of cell membrane (○) and cell interior (■) conductivity as a function of the concentration of biocide. Electrorotation of 300 biocide treated cells were studied, and the data shown here are based on those (at least 5 per biocide concentration) for which complete spectra were obtained from 1 kHz to 10 MHz.

around 0.2 μS m^{-1}, the internal conductivity of the cell decreases as a result of ion leakage across the membrane. Similar effects have been observed for biocide treatments of bacteria [25]. When considering these changes, it should be taken into account that the apparent membrane conductivity is composed of a transverse and tangential surface conductance component. No definite values are available for the surface membrane conductance of yeast cells and bacteria, but if it is of the order of that estimated for mammalian cells it can provide a major contribution to the overall effective membrane conductivity [26]. In this way the transverse membrane conductivity, which controls ion leakage, may be more significantly affected by the biocide treatment than is suggested by the results shown in Fig. 4.

5. Conclusions

The effects of various doses of biocide on yeast cell suspensions have been investigated using electrorotation. It is shown that changes in the conductive properties of the cytoplasmic membrane and interior of the yeast cells after biocide treatment can be determined from the electrorotation spectra using two-shell ellipsoid modelling. It was observed that above a threshold concentration the membrane conductivity increases on administering higher doses of the compound Cosmocil (polyhexanide), whilst the internal conductivity only changes when the membrane conductivity has increased to relatively high values. The results indicate that the method of rotating cells in a rotating electrical field may be useful for the quantitative analysis of toxic chemicals effects, in terms of kinetics of the development of cell damage. The work also suggests further potential biotechnological and biomedical applications, as for example in the monitoring of cell viability after exposure to pharmacological agents and toxic chemicals.

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