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Electrorotation of liposomes: verification of dielectric multi-shell model for cells

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Abstract

The reliability of multi-shell dielectric models, used to describe the ac electrokinetic behaviour of cells, has been tested by performing electrorotation and dielectrophoretic measurements on unilamellar, oligolamellar, and multilamellar liposomes of diameters ranging from 5 to 24 μm . Fluorescence microscopy, flow cytometry and electron spin resonance were used to characterise the morphology and membranes of the liposomes. The dielectric properties of the various types of liposomes, based on appropriate dielectric shell models, were then analysed using a general purpose, recursive, algorithm. Through simulations, the confidence levels that can be assigned to parameters derived through application of simple shell models are estimated. From this, we confirm that electrorotation data enable accurate determinations to be made of the dielectric properties of the outermost membrane of liposomes, and provide good indications of the level of complexity of the shells and internal compartments. We also demonstrate that, used with sufficient additional information, such as that provided by dielectrophoresis, electrorotation data yields unique solutions for the dielectric parameters of liposome-like particles.

Keywords

Electrorotation; Liposomes; Dielectric; Membrane capacitance; Membrane conductance

1. Introduction

ac Electrokinetic phenomena such as dielectrophoresis, electrorotation and travelling field effects are increasingly being investigated as potential tools for the selective manipulation and characterisation of cells and bioparticles (e.g., [1-4] and references cited therein). In such work, the so-called multi-shell model (a system of concentric spheres) is often employed to interpret observed effects in terms of the dielectric properties of sub-cellular components (e.g., cell wall, membrane, cytoplasm, nucleus). This model, originating from the early electrostatic theories of Maxwell (see Ref. [5] for a review), was used by Fricke [6] to provide evidence of the ultrathin, resistive, nature of the red blood cell membrane, and was later refined by Irimajiri et al. [7] to model lymphoma cells. More recently it has been used in dielectrophoresis and electrorotation studies to characterise viable and non-viable yeast cells [8], the morphological changes accompanying induced differentiation of murine erythroleukaemia cells [9,10] and to predict the behaviour of cells in travelling electric fields [11].

Employing the multi-shell model in these ways is analogous to using an equivalent circuit to model a complicated impedance network. There is no guarantee that the equivalent circuit bears any physical correspondence to the real network. Thus, the fact that a shell model fits electrorotation or dielectrophoresis data does not necessarily ensure that the parameters derived from it have a true correspondence with the cellular structure, and the model need not provide a good predictor of electrokinetic behaviour. One way of testing whether good correspondence exists is to make measurements on simple particles, whose physical characteristics can be manipulated and are thoroughly characterised. Liposomes fall into this category and they have analogous structures to cells and to multi-shell dielectric models. Also, Wicher et al. [12,13] have made comparisons between the known physical properties of multi- and oligolamellar liposomes and the parameters derived from fitting one- and two-shell dielectric models to electrorotation and dielectric data.

In the work described here, which significantly extends the studies of Wicher et al. [12,13], unilamellar, oligolamellar, and multilamellar 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes of diameters ranging from 5 to 24 μm were synthesised using the giant vesicle method [14,15] and investigated by electrorotation and dielectrophoresis. Fluorescence microscopy, flow cytometry and electron spin resonance using spin probes were used to characterise the morphology and membranes of the liposomes. The dielectric properties of the various types of liposomes, based on appropriate dielectric shell models, were then analysed using a general purpose, recursive, algorithm. Through simulations, the confidence levels that can be assigned to parameters derived through application of simple shell models were estimated. From this, we confirm that electrorotation allows accurate determinations to be made of the dielectric properties of the outermost membrane of liposomes, and is a good indicator of the level of complexity of the shells and internal compartments. We also demonstrate that, under typical experimental conditions, electrorotation data cannot be analysed to yield unique solutions for the liposome dielectric parameters without additional data such as that provided by dielectrophoresis.

2. Materials and methods

2.1. Synthesis of liposomes

The phospholipid DOPC was chosen because its low phase transition temperature of -14°C [16] ensured that the vesicles remained physically stable over the experimental temperature range from 13 to 40°C . Cholesterol was also used as an additive to decrease the fluidity of the vesicle membranes and ion leakage across the membrane [14,15], and Percoll (density 1.129 g ml^{-1}) was also introduced to the medium to be encapsulated to facilitate vesicle centrifugation and their settling down onto the micro-electrodes for electrokinetic measurements by increasing their effective density with respect to that of the suspending medium used in experimentation. Because Percoll consists of particles having a diameter of about 50 nm, this introduced a constraint on the minimum thickness for aqueous compartments in multilamellar vesicles, but otherwise it should not have affected the liposome preparation techniques employed here. Finally, the conductivity (ca. 1.5 S m^{-1}) of the encapsulated medium was chosen to be about 100-times greater than that of the suspending medium, to ensure [17] that an interfacial dielectric dispersion occurred in the frequency range around 100 kHz to 1 MHz and along with it co-field electrorotation and a dielectrophoresis response that changed polarity.

DOPC (Avanti Polar Lipids) and cholesterol (Sigma) were each stored in chloroform; the fluorescent dye *N*-(3-sulfopropyl)-4-(*p*-dodecylaminostyryl) pyridinium inner salt (Di1OASP-PS) obtained from Molecular Probes and the spin label 5-doxyl-stearate (5-DS) (Sigma) were each stored in ethanol. The so-called giant vesicle method [14,15] was used to produce liposomes of radii ranging from 2 to 15 μm . This involved mixing 2 ml of 20 mg ml^{-1} DOPC, 1 ml of 4 mg ml^{-1} cholesterol, 5.0 μl of 2.5 mg ml^{-1} Di1OASP-PS and 30 μl of 0.39 mg ml^{-1}

5-DS in a scintillation vial. The solvents were removed from the lipid mixture by rotary evaporation in a 40°C water bath under a gentle stream of nitrogen gas, which eliminated atmospheric oxidation effects. After 30 min, films of phospholipid that were free of chloroform odour formed on the sides and bottom of the scintillation vial. 10 ml of Percoll (Pharmacia BioProcess Technology) containing 150 mM sodium chloride, 20 mM HEPES and 5 mM EDTA (Sigma) was gently layered onto the film. After 24 h undisturbed incubation at 37°C, lamellae of the phospholipids became detached from the glass surface to form free-floating lipid globules. Giant vesicles were formed by gently drawing the suspension in and out of a Pasteur pipette several times over the course of a few s.

The liposomes produced by this technique thus contained an aqueous medium that was relatively conductive (1.47 S m^{-1} at 21°C), contained EDTA to capture divalent ions such as Ca^{2+} and Mg^{2+} ions that can cause aggregation effects amongst liposomes [15] as well as transition metals that can catalyse lipid oxidation, and contained Percoll to increase its density to 1.14 g ml^{-1} . This stock suspension of liposomes could be stored at 4°C for up to 20 days without degradation.

2.2. Fluorescence microscopy

The lipophilic fluorescent dye Di1OASP-PS ($\lambda_{\text{ex}} = 496 \text{ nm}$ and $\lambda_{\text{em}} = 614 \text{ nm}$) was used as a fluorescent probe to visualise the conformation and concentration of lipids in each vesicle. Since this dye became evenly distributed throughout the phospholipid layers of all liposomes, the intensity of emission under fluorescence microscopy was used to investigate the uniformity of membrane layers in the liposomes, to distinguish between unilamellar and multiply-stacked lamellae [15], and to discriminate between unilamellar liposomes and those containing multiple compartments. For gross characterisation of samples and the determination of particle size and lipid content distributions, stock vesicle suspensions were investigated on an Analyser (Becton-Dickinson) flow cytometer to provide scatter plots of lipid content versus vesicle volume, following a ten fold dilution with physiological saline.

2.3. Electron spin resonance (ESR)

Intact liposomes were harvested and rendered largely free of lipid droplets by centrifugation. To accomplish this, the stock liposome preparation was first diluted 1:10 with 5% mannitol to lower the suspension density with respect to the liposome interiors without causing the liposome membranes osmotic stress. The diluted mixtures were then centrifuged at $4500 \times g$ for several min at 4°C and the pelleted liposome samples were transferred to 50 μl capillary tubes and investigated in a Varian E-109 ESR spectrometer over the temperature range 5 to 45°C at 5°C increments. Spectra of the 5-DS incorporated in the lipid phase were acquired and the rigidity parameter S [18] of each spectrum was analysed by computer.

2.4. Electrorotation (ROT) and dielectrophoresis (DEP) measurements

Just prior to ROT and DEP measurements, the stock liposome preparation was diluted 100-fold with a 5% mannitol solution to produce a suspension containing ca. 10^6 liposomes ml^{-1} of radius larger than 2.5 μm dispersed in an isotonic medium of conductivity 13 mS m^{-1} and density 1.016 g ml^{-1} . In this way, the liposomes were introduced into an environment that was significantly less dense, and had a much lower conductivity, than their internal medium and in which they suffered no osmotic stress to their membranes.

ROT measurements were conducted in a flowthrough chamber whose lower surface comprised a polynomial electrode array [19] of 400 μm tip-to-tip spacing of gold-on-glass construction. The four electrodes of the array were energised with sinusoidal signals in phase quadrature that produced an essentially homogeneous rotating electric field in the central region of the array [20]. Following injection of a new sample, liposomes settled almost immediately because

of their high density value. Rotation measurements were then taken over the frequency range 500 Hz to 150 MHz at four points per decade for liposomes that lay within 65 μm of the geometrical centre of the array and that were at least three diameters from any neighbour. Experimental data points were taken at frequencies chosen in random order. These procedures ensured that measurements were taken on liposomes in the most homogeneous region of the applied field, that they were not subjected to particle-particle interactions, and that systematic errors caused by time-dependent changes [21] in the liposome were avoided. The temperature of the measurement chamber was maintained at $21 \pm 0.1^\circ\text{C}$ by a massive platform (1.5 cm thick $\times$ 7 cm $\times$ 10 cm brass block) through which fluid from a thermostatted heat exchanger was circulated. The apparatus was mounted on a Diaphot (Nikon) inverted microscope, and liposome rotation rates were timed by stopwatch with the aid of video-enhanced microscopy. Each liposome measured was classified according to its structure by switching briefly to epifluorescent observation of the lipids as described above.

To verify that the rate of ion leakage through the liposome membranes did not seriously affect the conductivity of the supporting or encapsulated phases, the frequency at which the DEP response changed polarity was determined for each individual liposome before and after measurement of the ROT spectrum. In most cases in which cholesterol was present in the lipid, there was little or no change in this DEP crossover frequency (see Appendix A). This can be used as confirmation [22] that the rate of ion leakage through the liposome membrane was insufficient to affect the physical experimental conditions or compromise the accuracy and reliability of the ROT data.

2.5. Analysis of electrorotation data

A dielectric shell model that closely resembled the physical appearance of each liposome in terms of physical dimensions and the number of compartments was used to analyse each ROT spectrum [7,8] by applying a curve fitting method based on the Nelder-Mead simplex optimisation procedure [22,23] provided by MATLAB (The MathWorks). This procedure minimised the error function

$$\text{Min} \left\{ \sum_i^N [R_{\text{sim}}(\omega_i) - R_{\text{exp}}(\omega_i)]^2 + W * Re^2(f_{\text{CMfitted}}(\omega_c)) \right\} \quad (1)$$

for the N frequency points ω_i in the experimental ROT spectrum R_{exp} . A general purpose, recursive algorithm was developed to generate the ROT spectrum R_{sim} based on dielectric parameter estimates for the appropriate multi-shell model (e.g., [8]). W represents the weight given in the minimisation procedure to the real part of the Clausius-Mossotti factor ($f_{\text{CMfitted}}(\omega_c)$) calculated from the dielectric parameters used to simulate R_{sim} . As described in Appendix A, the real part of the Clausius-Mossotti factor has value zero when the DEP force vanishes at the crossover frequency ω_c . We investigated how accurately this curve fitting procedure was able to recover dielectric parameters from idealised ROT spectra. This was accomplished by using the computer to generate multiple sets of 100 spectra having Gaussian noise of up to 10% RMS added. The simulated spectra were then fitted by the method described above, and the recovered dielectric parameter sets were analysed statically in terms of the original data sets used to generate the spectra. In each case this was done both with and without the DEP crossover frequency constraint ($W > 0$ and $W = 0$, respectively) in order to assess the importance of this analysis approach. In the analysis of experimental data for liposomes, the value $W = 1$ was used.

When analysing experimental data for giant liposomes, the measured value of the suspending medium conductivity was provided to the fitting algorithm while the relative permittivity

values for the internal and external aqueous media were fixed at the value for normal bulk water at the measurement temperature [24]. Radii of all shells, as determined from fluorescence microscopy, were also provided explicitly to the algorithm. The optimisation procedure was devised to provide a value for the specific membrane capacitance C_{mem} as well as an estimate of the membrane relative permittivity ϵ_{mem} that was based upon an assumed thickness of each DOPC membrane of 3.8 nm [16]. C_{mem} is related to the membrane properties by $\epsilon_{\text{mem}} \epsilon_0 / \delta$ (where $\epsilon_0 = 8.854 \times 10^{-12} \text{ F m}^{-1}$ is the permittivity of free space, and is expected to have the value 5.1 mF m^{-2} for a membrane of thickness 3.8 nm if the literature [25] value of 2.2 is taken for the relative permittivity of the lipid membrane phases. As shown in Table 1, the experimental values obtained for C_{mem} for unilamellar liposomes were around 6.7 mF m^{-2} , possibly reflecting the facts that the liposomes may not have had perfectly smooth surfaces (e.g., see folded membrane case in Table 1), or may have had effective membrane thickness and permittivity values that differed from those assumed above.

For the single shell model the iterated parameters in the minimisations were thus: the membrane dielectric properties σ_{mem} and ϵ_{mem} ; the internal conductivity σ_{int} ; and the scaling factor k . This parameter took into account the combined influences of the experimental environment on the liposome rotation rate including the square of the local electrical field strength, the effective suspension viscosity in close proximity to the bottom wall of the electrorotation chamber, and the friction between the liposome and the substrate. Under our conditions, k assumed values between 3×10^{11} and 3×10^{12} , with this order of magnitude range largely reflecting an approximately 3-fold variation in the local field strengths encountered by vesicles measured in different regions of the electrode chamber. Because the dielectric parameters for the vesicles determined the shape of the electrorotation spectrum, a lack of knowledge of these parameters, which acted to scale in a frequency-independent fashion the overall spectral response, did not confound the determination of the vesicle dielectric properties. It should be noted that for each new membrane and the resulting new compartment that was added to the dielectric model, three additional parameters were iterated, namely the two new membrane dielectric properties and the conductivity of the new aqueous compartment.

3. Results

3.1. Liposome properties

The liposome populations produced by the giant liposome method were heterogeneous, but typically 30–40% of the liposomes present had radii in excess of $2.5 \mu\text{m}$ and were characterisable by our ROT procedure. Radii of unilamellar liposomes ranged as high as about $10 \mu\text{m}$ whilst those for multilamellar liposomes with multiple compartments ranged to $12 \mu\text{m}$.

The fluorescent probe Di1OASP-PS allowed different types of liposomes to be distinguished and the amount of phospholipid present in the membranes of each to be visualised. As shown in Figs. 1 and 2, the liposomes exhibited a range of structural forms including those with a single compartment and an unilamellar membrane (Fig. 1), a single compartment and a multilamellar membrane (Fig. 2a), oligo- and multi-lamellar liposomes having two or more compartments (Fig. 2b and c), and multivesicular liposomes having complex combinations of these characteristics (Fig. 2d). There was no dominant form, and in addition to liposomes many residual globules (containing little enclosed aqueous medium) from the initial suspension of the lipid film were present.

Fig. 3 shows a typical fluorescent plot of lipid content (as gauged by Di1OASP-PS fluorescence) versus liposome volume (determined by both light scattering and Coulter methods) as measured by flow cytometry. From this plot, it is clear that the lipid content of the liposome populations was directly proportional to liposome volume, as evidenced by the linear relationship with slope = 1 between these parameters. (A linear relationship with slope = 2/3

would be expected if the lipid content mirrored the surface area of the liposomes). Also, two distinct classes of liposomes appear to have been present, as evidenced in Fig. 3 by the two parallel bands separated by an order of magnitude in lipid fluorescence. It is likely that the band having the more intense fluorescence represented the residual lipid globules that could be observed under fluorescence microscopy, whilst the less intensely fluorescent band represented liposomes with significant aqueous compartments.

The relationship between the rigidity of the liposome membranes, the temperature, and the cholesterol content of the lipid phase is shown in Fig. 4a. The *S*-parameter derived from the ESR spectra of 5-DS was found [26] to increase with cholesterol content, showing that membrane rigidity became higher. Also, water accessibility to the hydrophobic region of the membrane decreased as the amount of cholesterol present in the membrane increased [26] leading to a reduction of ion leakage through the membrane as judged by measuring the rate of change of suspending medium conductivity for liposomes suspended in a sucrose solution of low conductivity, in agreement with earlier reports [14,15]. In practice, this was an important consideration because ROT spectra provide most information when the internal conductivity of the liposome compartment is much higher than that of the suspending medium. Maintaining a high ion concentration gradient across the liposome membrane for a sufficiently long period of time to complete ROT spectral measurements proved to be difficult in the absence of cholesterol. For this reason, all the ROT experiments reported here were undertaken on liposomes whose lipid contained 10% cholesterol. In this way, ion leakage was minimised and ROT spectra for liposomes were usually found to be stable and reproducible over a period of about 1 h. After 5 or more h, however, changes in the ROT spectrum suggested that significant ion leakage and possible alterations in the liposome outer membrane structure had occurred, however. Therefore, ROT studies reported in this work were undertaken as quickly as possible once the liposomes were suspended in sucrose and DEP crossover-frequency measurements, in which the measurement speed was about 11 times quicker, were utilized to support the estimates obtained for the internal conductivity values.

As well as using 5-doxyl-stearate (5-DS) as a spin label to examine the thermal motions of the membrane lipid chains at carbon position 5, 12-doxyl stearate and 16-doxyl stearate were also employed to investigate such dynamics at positions 12 and 16, respectively. The results shown in Fig. 4b indicate that with increasing distance into the bulk of the membrane, the effective fluidity progressively increases from position 5, through 12 to position 16. With increasing temperature it can also be seen from Fig. 4b that the inner part of the lipid membrane becomes increasingly more fluid than the outer part.

3.2. Electrorotation spectra

The ROT behaviour of more than 150 liposomes were examined, but of these only the simplest morphological classes that most closely resembled the forms shown in Figs. 1 and 2 were analysed in detail. In addition, studies were made of the ROT characteristics of some representative classes of more complex liposomes whose physical appearance was unlike the shell models. These observations helped to establish the degree to which ROT spectra could be used to identify liposomes of a given physical form.

3.2.1. Single compartment liposomes—Single compartment liposomes having membranes containing different numbers of lamellae were identified from the intensity of fluorescence emitted by their lipid phases. The single compartment liposome form is most appropriately described by the single shell dielectric model, and in applying that model for analysis it was sought to determine whether the expected correlation was observed between the thickness of the membrane and its capacitance. Typical ROT spectra obtained for such liposomes, together with the best fit of the single shell model, are shown in Fig. 5a and b. DEP

crossover frequencies and ROT peak frequencies varied by more than an order of magnitude between the liposomes having the thinnest and thickest membranes examined. As discussed in Section 2.5, the optimisation procedure applied for the analysis of the ROT spectra allowed for the estimation of the specific membrane capacitance C_{mem} rather than ϵ_{mem} directly. The membrane capacitance of single compartment liposomes could also be estimated from measurement of the DEP crossover frequency using the relationship

$$C_{\text{mem}} \approx \frac{\sigma_s}{R\pi\sqrt{2}f_c} \quad (2)$$

Eq. (2) is valid providing the membrane conductivity is sufficiently low that its influence on the crossover frequency f_c can be ignored (see Appendix A). Here, σ_s is the conductivity of the supporting medium and R is the radius of the liposome. Table 1 summarises the capacitance values determined for single-compartment liposomes having membranes of different thicknesses as determined both from parameter optimisation of the entire ROT spectra and the DEP crossover frequency values. Membrane conductivity values derived from the optimisation analysis were very low, typically below 10^{-6} S m^{-1} , and the confidence level for these values was small because the error function was comparatively insensitive in this range of membrane conductivity. Furthermore, it was difficult to separate the effects in the error function of small changes in membrane conductivity from small changes in the scaling factor k . Thus, we believe that the conductivity values obtained should be taken as no more than a guide. The conductivity of the encapsulated medium in the unilamellar liposome (Fig. 1) was determined to be 1.07 S m^{-1} , compared to the value of 1.47 S m^{-1} originally encapsulated during its formation. This suggests that ions had leaked across the membrane into the surrounding medium. By contrast, the internal conductivity derived for the eleven-times thicker multilamellar liposome (Fig. 2a) was determined to be 1.38 S m^{-1} , around 94% of the known initial value and also within the accuracy level expected of the optimisation procedure used in the single shell model (see Fig. 8).

3.2.2. Two compartment liposomes—For oligolamellar liposomes having two separate medium compartments, and where the innermost compartment was larger than about 30% of the liposome outer radius, the ROT spectra were characterised by a relatively broad anti-field rotation peak. Such spectra could not be fitted using the single shell model, but a three shell (two membrane) model provided a good description, as shown in Fig. 6. Experimenting with this model revealed that the shape of the low frequency shoulder of the antifield ROT peak was determined by the properties of the outermost membrane, whilst the innermost membrane influenced the width and shape of the high frequency region of the antifield peak and the position of the ROT crossover frequency. Computer simulations also revealed that the accuracy of the derived dielectric parameters from the optimisation procedure were in general poor and fell markedly with increasing distance into the liposome (see Fig. 9).

3.2.3. More complex liposomes—The addition of each new concentric membrane compartment adds three new parameters to the dielectric model, namely the new membrane permittivity and conductivity and the conductivity of the new aqueous compartment (which is assumed to have a relative permittivity of 78). The computer simulations showed that the accuracy with which each new parameter set could be fitted fell off rapidly with increasing numbers of shells. Nevertheless, Fig. 7 shows a ROT spectrum for a multilamellar liposome with multiple compartments that can be clearly distinguished in its form from the ROT spectra of more simple unilamellar and oligolamellar liposomes (see Figs. 5 and 6). A model having five shells (3 membranes) was the minimum complexity that was able to give a reasonable fit to this data, showing that while accurate parameters could not be derived for the inner dielectric

properties, such complex liposomes could nevertheless be distinguished from simpler ones on the basis of the complexity of their electrorotation spectra.

It was also possible to detect folding of the outermost membrane in both simple and complex liposomes. Such membrane folding resulted in much higher capacitance values than could be accounted for by a single smooth bilayer. For example, the ROT spectrum of the folded liposome listed in Table 1 indicated a mean membrane capacitance of ca. 14.4 mF m^{-2} because of a high degree of membrane folding that was readily apparent by microscopy. Similar effects were seen in multilamellar liposomes whose outermost membrane capacitance values were as high as 33.1 mF m^{-2} .

3.3. Accuracy of shell models

Confidence levels for the derived dielectric parameters (e.g., membrane and compartmental conductivities and permittivities) were investigated as a function of simulated noise added to the ideal ROT data. Noise was created using the MATLAB 'randn' function, which produces normally-distributed random numbers centred about zero and with a variance of unity. Such noise was first scaled to have a set variance with respect to each rotation value in the theoretically-derived ROT spectra and then added to the ideal values to produce noisy spectra. This approximated the finding that, in the absence of stiction, the variance of an observed rotation rate in electrorotation experiments is proportional to that rotation rate. Using this approach, the effects of noise variance levels of 0%, 3%, 5% and 10% were tested in different simulations. A summary of noise effects, applicable to the single shell model, is shown in Fig. 8. To obtain these results, ROT simulations were limited to the same 500 Hz to 150 MHz frequency range as our experiments on liposomes, in order to derive conclusions applicable to our experimental measurements. For the case of no noise the confidence levels for membrane permittivity, internal medium conductivity and the scaling factor k were all 94% whilst that for membrane conductivity was 89%, within a confidence limit of 90% to 110%. With increasing noise level, the accuracy of the internal conductivity and membrane permittivity fell to 49% and 52%, respectively, whilst that for the membrane conductivity fell rapidly to a value of 22% for the same confidence limits. For noise of 10% variance, the confidence level of the scaling factor was greater than 95% for confidence limits of 90% to 110%.

Cells such as yeast or bacteria having an outer cell wall can be analysed using the two shell model [7,8] but these cannot be imitated using liposomes. For the case of ROT spectra of the form of Fig. 6 obtained for oligolamellar liposomes and for which the three shell model is appropriate, then it can be seen in Fig. 9 that the confidence levels of the derived dielectric parameters are significantly degraded. This is particularly so for the permittivity and conductivity of the inner-most membrane and the conductivity of the core medium. In the analyses used for Fig. 9, the relative permittivities of the encapsulated inner media and external suspending medium (ϵ_1 , ϵ_3 and ϵ_s , respectively) were given the fixed values of 78.7 applicable for water at 21°C [24] containing an NaCl concentration of 0.15 M with a conductivity 1.4 S m^{-1} . Adopting a fixed value for the relative permittivity of each aqueous compartment overlooked the fact that this parameter was slightly dependent on the conductivity of each compartment. Nevertheless, this approximation greatly increased the speed of iteration and the stability of convergence of the parameter fitting procedure and introduced errors into the estimate of the conductivity parameters that were small compared to the expected fitting precision for real data. The other seven parameters (ϵ_2 , ϵ_4 , σ_1 , σ_2 , σ_3 , σ_4 , k) were free to iterate. The decreasing confidence levels found for the parameters with increasing model complexity appear to reflect a steady deterioration of the conditioning of the solution system. This can be directly attributed to the complex, recursive nature of the dielectric equations describing multishelled dielectric systems. Increases in the complexity of models beyond 4 or 5 layers can be expected to further decrease the accuracy level for derived parameters and possibly lead

even to complete degeneracy among parameters in the solution set. Thus, the electrorotation method appears to suffer certain fundamental limitations in its ability to determine unique dielectric parameters for very complicated multishelled systems if applied alone. However, degeneracy in a solution system can always be eliminated by the provision of a sufficient number of boundary conditions. Therefore, the electrorotation method may still prove to be useful for characterizing even particles of complex structure if a sufficient number of experimentally-determined parametric constraints can be provided.

3.4. Temperature effects

For some of the liposomes of Table 1, ROT spectra and measurements of the DEP cross-over frequency were obtained for the temperature range 10 to 40°C. In the MATLAB minimisation procedure the permittivity and conductivity of the suspending and encapsulated media were corrected for temperature change, and values for the membrane capacitance were obtained as a function of temperature. As shown in Fig. 10, this capacitance decreased with increasing temperature, at a rate similar to that observed by White [26] for glycerol monooleate black lipid membranes. Shown in Fig. 10 are results obtained for unilamellar liposomes, as well as oligolamellar ones possessing two bilayer membranes. Three of the oligolamellar types exhibit membrane capacitance values intermediate to those expected for single and double bilayer membranes. These liposomes possessed a single compartment, and it is possible that aqueous media was trapped between the two bilayers during their formation, giving rise to a high pressure region that stretched the membranes, reducing their thickness and increasing their effective capacitance. An effect such as this has been reported by Lis et al. [27].

The decrease in capacitance with increasing temperature can arise from a combination of increasing membrane thickness, a decrease in the effective permittivity of the lipid membrane material, as well as a reduction of the effective roughness of the membrane surface. Crawford and Earnshaw [28], using quasielastic light scattering, determined that the thickness of glycerol monooleate black lipid membranes increased with increasing temperature. The temperature variation of the effective thickness of our DOPC liposomes (derived by maintaining membrane permittivity and surface roughness constant) is in close agreement with these earlier results of Crawford and Earnshaw. This indicates that the results of Fig. 10 predominantly reflect temperature changes in the membrane thickness, and are in line with effects to be expected from the fluidity changes shown in Fig. 4b.

For some of the liposomes a small hysteresis effect was observed in the capacitance values as the temperature was reduced from 40°C down to 10°C and then reheated to 40°C. Similar hysteresis effects were reported by White [29] for black lipid membranes. These various results, obtained as a function of temperature, provide additional confidence in the application of the single-shell dielectric model for analysing electrorotation and dielectrophoresis data for cell-like particles.

4. Conclusions

We have been able to confirm the earlier findings of Wicher and Gündel [13] that the electrorotational behaviour of liposomes reflect their structure as well as the electrical properties of their interior and exterior. A conclusion of these earlier studies [13] was that liposomes, especially multilamellar ones, could not be described exactly by the single-shell dielectric model. Our own conclusions, from the work presented here, are rather different in several respects, most probably because we have been able to more fully characterise the morphology of the liposome membranes and compartments using fluorescence microscopy and flow cytometry.

As shown in Fig. 5a and b the single-shell model provides close agreement between experiment and theory for unilamellar liposomes as well as for multilamellar ones composed of up to ten or more lipid bilayer membranes. As the number of stacked bilayers increases, the characteristic peak frequencies of the electrorotation spectra advance to higher frequencies in increments that are predictable and consistent with the single-shell model. Furthermore, as shown from the statistical modelling experiment in Fig. 8, if the electrorotation data is coupled with a measurement of the dielectrophoresis cross-over frequency, very accurate values can be derived for the membrane relative permittivity and the liposome internal conductivity. An estimate for the membrane conductivity can also be obtained providing it is not negligible compared with the liposome suspending medium conductivity. For liposomes having thick multilamellar membranes that are resistant to ion leakage, there is excellent agreement between the derived internal conductivity and the known value for the medium entrapped during synthesis. The single-shell model also provides a good analysis for unilamellar liposomes having highly convoluted outer membranes (see Table 1), and the derived temperature dependence of the membrane capacitance of unilamellar liposomes also agrees well with work on lipid membranes (see Fig. 10).

As expected for oligolamellar liposomes having two distinct compartments, their electrorotation spectra cannot be modelled using the single-shell model. However, as shown by Fig. 6 the appropriate three-shell model does provide a good theoretical description.

This work using liposomes, as realistic models for relatively simple cells, serves to confirm the general value of the dielectric multishell model. The results obtained for the folded membranes (Table 1) indicate that morphological changes of the outer membrane, such as those accompanying induced differentiation of cells [9,10] can be analysed with confidence. The behaviour of cells in travelling fields can be predicted if both the electrorotation and dielectrophoretic spectra are known for a particle type [11]. The fact that even complicated cell structures are amenable to modelling using three- or five-shells is thus important, because as shown in Figs. 5-7 the dielectrophoretic response can be derived from the electrorotation data (and vice versa).

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Appendix

Appendix A. Dielectrophoresis DEP cross-over frequency

The dielectrophoretic force acting on a particle of radius R in a suspending medium of relative permittivity ϵ_s is given by:

$$F = 2\pi\epsilon_s R^3 \operatorname{Re} \left(\frac{\xi_p - \xi_s}{\xi_p + 2\xi_s} \right) \nabla E^2 \quad (\text{A1})$$

where E is the RMS electric field strength, ∇ is the del vector operator that defines the field gradient, and each $\xi = \epsilon - j\sigma/\omega$ is a complex permittivity with subscripts 'p' and 's' denoting the particle and suspending medium, respectively. At the DEP cross-over frequency $f_c = \omega_c/2\pi$ no net dielectrophoretic force is exerted on the particle, which means that

$$Re\left(\frac{\xi_p - \xi_s}{\xi_p + 2\xi_s}\right) = 0 \quad (\text{A2})$$

where Re denotes that the real part of this complex function is to be taken. From this we obtain the following expression for the cross-over frequency:

$$f_c = \frac{1}{2\pi} \left\{ \frac{(\sigma_s - \sigma_p)(\sigma_p + 2\sigma_s)}{(\epsilon_p - \epsilon_s)(\epsilon_p + 2\epsilon_s)} \right\}^{1/2} \quad (\text{A3})$$

The dielectric multishell model [7,8] considers a cell to consist of a set of concentric homogeneous spherical shells, with the number of shells increasing as the structural components of the cell increases. The number of interfacial dielectric dispersions also increases with the number of shell interfaces [7]. For the single shell model we have an inner sphere of radius r surrounded by a thin membrane of thickness d . If this membrane has a conductivity that is much lower than the surrounding medium, charges build up at the membrane-medium interface with a characteristic relaxation time τ and thus at a characteristic dispersion frequency $f_d (=2\pi/\tau)$. The effective permittivity of the cell can then be written [7] as

$$\xi_p = \xi_{\text{mem}} \frac{\left(\frac{R}{r}\right)^3 + 2\left(\frac{\xi_{\text{in}} - \xi_{\text{mem}}}{\xi_{\text{in}} + 2\xi_{\text{mem}}}\right)}{\left(\frac{R}{r}\right)^3 - \left(\frac{\xi_{\text{in}} - \xi_{\text{mem}}}{\xi_{\text{in}} + 2\xi_{\text{mem}}}\right)} \quad (\text{A4})$$

where $R=r+d$ (with $R \gg d$. and the subscripts 'in' and 'mem' refer to the internal compartment and the membrane, respectively.

For frequencies well below the dispersion at f_d (i.e., typically well below 1 MHz) we have that [17]

$$\frac{\xi_{\text{in}} - \xi_{\text{mem}}}{\xi_{\text{in}} + 2\xi_{\text{mem}}} \cong 1 \quad (\text{A5})$$

$$\begin{aligned} \xi_p &= \xi_{\text{mem}} \left\{ \frac{\left(\frac{1}{1-d/R}\right)^3 + 2}{\left(\frac{1}{1-d/R}\right)^3 - 1} \right\} \\ &\cong \xi_{\text{mem}} \left(\frac{R+d}{d}\right) \cong \xi_{\text{mem}} \left(\frac{R}{d}\right) \end{aligned} \quad (\text{A6})$$

We can define the specific membrane capacitance as $C_{\text{mem}} = Re(\xi_{\text{mem}})/d$, which from Eq. (A6) gives:

$$C_{\text{mem}} = \frac{\epsilon_p}{R} \quad \text{in } F \text{ } m^{-2} \quad (\text{A7})$$

For frequencies well below the interfacial dispersion frequency f_d , where the low conductivity of the membrane dominates the effective conductivity of the cell, the effective permittivity ϵ_p greatly exceeds that for the surrounding aqueous medium ϵ_s and the effective conductivity

σ_p is much less than the conductivity σ_s of the surrounding medium [17]. In this situation, Eq. (A3) can be approximated as:

$$f_c \approx \sqrt{2} \frac{\sigma_s}{\epsilon_p} \quad (\text{A8})$$

From Eq. (A7) and Eq. (A8) we obtain the result given in Eq. (2) of the main paper:

$$C_{\text{mem}} \approx \frac{\sigma_s}{r\pi\sqrt{2}f_c} \quad (\text{A9})$$

This result, although not previously explicitly derived, has been verified in earlier work on mammalian cells [30]. In Table 1, the mean values for the membrane capacitance obtained from the DEP cross-over frequency were derived from the slopes of plots such as those of Fig. 11 for liposomes of different radii.

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Fig. 1.
Liposome having a unilamellar membrane and single compartment, which can be analysed using the single-shell dielectric model.

Fig. 2.

(a) Oligolamellar liposome with two compartments, which in principle can be analysed using the three-shell model. (b,c) Multilamellar liposome having two or more compartments. (d) Multivesicular liposome.

Fig. 3.
A typical fluorescent plot of liposome fluorescence (reflecting fluorescently-labelled lipid) versus volume obtained cytometrically for a suspension of liposomes.

Fig. 4.

(a) The rigidity (S -parameter) derived by electron spin resonance (ESR) for the DOPC liposome membranes as a function of temperature and cholesterol content: Δ , no cholesterol, $\bullet$, 10 wt% cholesterol, $\blacksquare$, 20 wt% cholesterol. (b) The rigidity of DOPC-10% cholesterol membranes at carbon positions 5($\square$); 12 (Δ) and 16 ($\circ$).

Fig. 5. Typical electrorotation (ROT) spectrum obtained at 21°C for liposomes of the form of Fig. 1. ○, Experimental ROT data; ×, experimental DEP cross-over frequency. The lines drawn through the experimental data are the best fits derived theoretically using the single shell model. (a) Unilamellar liposome: radius 5.25 μm; suspending medium conductivity 12.45 mS m⁻¹. The derived values for the membrane thickness, encapsulated medium conductivity, and scaling factor k are 3.8 nm, 1.1 S m⁻¹, and 7.5×10^{11} , respectively. (b) multilamellar liposome: radius 5.64 μm; suspending medium conductivity 13.55 mS m⁻¹. The derived values for the membrane thickness, encapsulated medium conductivity and scaling factor k are 41 nm, 1.4 S

m^{-1} , and 2.85×10^{12} , respectively. For both liposomes the membrane conductivity was estimated to be less than 1 nS m^{-1} .

Fig. 6. Electrorotation spectrum obtained at 30°C for an oligolamellar liposome of the form of Fig. 2a, having inner and outer liposome radii of 3.8 and 5.5 μm , respectively. The conductivity of the suspending medium was 17.3 mS m^{-1} . $\circ$, Experimental ROT data; $\times$, experimental DEP cross-over frequency. The solid line drawn through the ROT data is the best fit using the three-shell model, whilst the dotted line is the corresponding derived dielectrophoresis spectrum. The value derived for the scaling factor k was 7.07×10^{11} .

Fig. 7.

Electrorotation spectrum obtained at 21°C for a complex multilamellar liposome of outer radius 11.8 μm of the form of Fig. 2d. The solid line drawn through the ROT data is the best fit using a five-shell model, whilst the dotted line passing through the experimental DEP cross-over frequency ($\times$) is the corresponding derived dielectrophoresis spectrum. See text for details of dielectric parameters derived from the five-shell analysis.

Fig. 8.

Accuracy (confidence) levels, within a confidence limit of 90% to 110%, of dielectric parameters derived using the single-shell model as a function of noise in the ROT spectrum. ∇ , scaling factor k ; $\square$, membrane permittivity ϵ_2 ; $\circ$, internal conductivity σ_1 ; Δ , membrane conductivity σ_2 .

Fig. 9.

Accuracy (confidence) levels, within a confidence limit of 90% to 110%, of dielectric parameters derived using the three-shell model as a function of noise in the ROT spectrum. ▼, Scaling factor k ; ○, outer-most membrane permittivity ϵ_4 ; ●, outer-most membrane conductivity σ_4 ; ▽, intermediate medium conductivity σ_3 ; □, inner membrane permittivity ϵ_2 ; ◆, **inner membrane conductivity σ_2 ; ▲, core medium conductivity σ_1 .

Fig. 10. Temperature variation of liposome membrane capacitance. Unilamellar membrane ($\blacksquare$); Oligo- (two) lamellar membrane ($\blacksquare$ and $\blacklozenge$), glycerol monooleate black lipid membrane [29].

Fig. 11.

The mean specific membrane capacitance C_{mem} for the 12 unilamellar liposomes of Table 1 was derived, according to Eq. (A9), from the inverse slope of this plot of the DEP cross-over frequency (f_c) versus the ratio (σ_s/R).

Table 1

Values of the mean membrane capacitance C_{mem} obtained from electrorotation (ROT) and dielectrophoresis (DEP) measurements (Eq. (2)) using the single shell model for the various forms of liposome studied

Liposome type	No. of liposomes measured	C_{mem} (ROT) (mF m^{-2})	C_{mem} (DEP) (mF m^{-2})	No. of bilayers
Uni- (Fig. 1)	12	6.48±0.8	6.86±1.3	1
Multi- (Fig. 2a)	9	3.66±0.5	3.82±0.6	2
Oligo-(Fig. 2b)	2	1.98±0.02	2.13±0.07	3
Multi-(Fig. 2c)	1	1.60	1.71	4
Multi-(Fig. 2d)	1	0.62	0.61	11
Folded	3	13.6±2.5	15.3±1.7	1

The type described as folded refers to unilamellar liposomes having a folded outer membrane. In determining C_{mem} the thickness of each DOPC membrane was assigned the value of 3.8 nm [16], and the values given for the closest integral number of bilayers are based on comparison with the mean C_{mem} for the unilamellar liposomes.