Development of a Remote Electrode System for Monitoring the Water Content of Materials inside a Glass Vial

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Purpose: This article explores the use of a remote electrode dielectric measurement system to monitor the water content of hydrated ovalbumin inside a glass vial.

Methods: The intrinsic dielectric properties of hydrated ovalbumin were characterized first using conventional parallel plate electrodes. The second stage was to simulate a remote electrode measurement by placing nonconductive, nondispersive polyethylene films between the sample and electrodes. Finally, a study on the dielectric measurement of ovalbumin contained in a 10 ml glass vial was undertaken with the electrodes external to the glass vial.

Results: The dielectric behavior of hydrated ovalbumin was characterized by charge transfer (i.e., protons) in the hydrogen bonded network of water molecules in the bulk sample. The mechanism was identified as an anomalous low-frequency dispersion and a dielectric loss peak (ε_3). The dielectric relaxation time, τ_3, of the ε_3 dispersion was especially sensitive to water content. Moreover, a good correlation ($R^2 = 93\%$) was observed between relaxation times τ_3 obtained from measurements using conventional parallel plate electrodes and the remote electrode system.

Conclusions: Dielectric measurements using remote electrodes attached to a glass vial are therefore applicable for the *in situ* measurement of water content in materials. The application of this technology to the determination of the lyophilization end point is suggested.

KEY WORDS: dielectric relaxation spectroscopy; low-frequency dispersion; remote electrodes; ovalbumin; freeze-drying; lyophilization.

INTRODUCTION

Freeze drying (or lyophilization) is a means by which labile materials (e.g., proteins) can be preserved in a dehydrated form (1,2). The process involves freezing of the bulk water, followed by removal of ice by sublimation under partial vacuum, while gently heating (primary drying). The unfreezable water, remaining from primary drying, is then removed by heating at elevated temperature (secondary drying). The end point of the process of freeze-drying is defined by the sample reaching an optimum water content. Excess moisture may cause instability of the product during storage (3) whereas over drying may result in loss of protein activity due to changes in tertiary molecular structure or the loss of water from the active site (4–6). The analysis and control of the residual moisture content at the end of secondary drying is therefore an essential component of an effective freeze-drying process.

A number of methods (e.g., thermocouples, pressure rise, electronic hygrometer) have been used to determine lyophilization end point, each with varying degrees of success and limitations. The inherent limitations of these techniques highlight the continued requirement for a new method for the *in situ* measurement of residual moisture during lyophilization.

Dielectric spectroscopy is particularly suited to the investigation of partially hydrated proteins (7–9). The wide frequency range of the dielectric spectrum means that it is possible to characterize the material over broad ranges of both time and scale (10,11).

The dielectric response of freeze-dried proteins depends to some degree on the residual concentration of water and the specific interactions between the water of hydration and the protein. One dielectric response of relevance to the characterization of freeze-dried materials is that associated with quasi-dc of charge transport. This process has been observed in a number of diverse systems (12,13) and is particularly prevalent in hydrated granular or porous solids (14). An investigation of this process provides unique information both on the states of binding of water and the porous structure of a material.

Quasi-dc Polarization

A number of studies have shown that most hydrated powders show anomalous dielectric behavior known as a quasi-dc process (12). The quasi-dc process is part of a group of phenomena known as low-frequency dispersions (LFD) and is characterized by a high-rise and parallelism between real and imaginary permittivity at low frequency (12,13). Another characteristic of the quasi-dc process is that it satisfies a "universal" fractional power law dependence on frequency:

$$\varepsilon^*(\omega) \propto (i\omega)^{-p} \quad (1)$$

$$\varepsilon^*(\omega) = A(i\omega)^{-p} \quad (2)$$

where $0<p<1$ *and* A is a pre-exponential factor, involving a cell constant capacitance and characteristic frequency, ω_c, i.e., the frequency representing the intersection point between the real and imaginary permittivities (13). This power law dependence on frequency indicates the presence of a self-similarity of fractal structures in the system, which underpins the percolation of charge in the materials (15,16).

Mechanism of the Quasi-dc Process

Detailed studies have been carried out that ascribe the radio frequency dispersion of hydrated proteins to proton hopping over potential energy barriers (10,17–19). These mobile protons may originate from the ionizable carboxylic groups in the protein structure. In light of the cluster model of Dissado and Hill (13), the origin of the quasi-dc response of hydrated proteins may be explained as follows. The hydrogen-bonded network within the hydration surface of a protein is ordered locally in the form of clusters. These clusters of hydrogen bonds are polarized by a succession of proton transfers, to leave hydrogen bonded OH^- and H_3O^+ groups at each end of the path. At frequencies $\omega>\omega_c$, the dielectric response

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is dominated by proton hopping within the cluster (an intracluster process), and at frequencies $\omega > \omega_c$, the dielectric response is dominated by proton transfer between clusters (an intercluster process). This intercluster process creates a long-range order of proton transport across the bulk material, which can also be described in terms of general percolation theory.

The investigation of quasi-dc processes can therefore be used to probe hydrogen-bonded networks at two levels of scale. The application of these studies to freeze-dried systems, in general, should therefore provide information on both the microscopic and mesoscopic structure of a nearly dry freeze-dried material, in particular during the secondary drying stage. This is interesting to both process development and on-line process control of the freeze-drying cycle. However, to make full use of this approach for monitoring the freeze-drying cycle, it is first necessary to develop a system of measurement that is both nondestructive and noninvasive to the process.

In Situ Measurements

Previous attempts to use electrical measurements for process control during freeze-drying were based on the placement of electrodes inside the glass vials, within the freeze-drying chamber (2). These approaches were therefore destructive to the sample and could not be used for routine analysis of an entire batch.

To obtain noninvasive measurements of a material inside a freeze-drying vial, it is necessary to develop a remote-electrode system specifically for this purpose. In the context of this work a remote electrode system is defined as electrodes that are separated from the sample by nonconductive and nondispersive medium, i.e., the glass vial used for the freeze-drying process. With insight into the frequency response of such composite dielectric systems (20) (in this case a sample contained inside a glass container), it should be possible to carefully select a frequency range over which the sample characteristics dominate the dielectric response of the composite system. It will then be possible to undertake measurements of sample properties without introducing probes into the system. Low hydrated ovalbumin (< 0.2 g/g) was chosen as a model material for the end product of freeze-drying proteins.

To establish the validity of the remote electrode approach, it was first necessary to characterize the intrinsic dielectric properties of hydrated ovalbumin, over the frequency range 10^{-1}–10^{6} Hz, using conventional parallel plate electrodes. In this stage, deuterated ovalbumin was also investigated to confirm that proton hopping was the principal mechanism responsible for the dielectric response of hydrated ovalbumin. The second stage was to simulate a remote electrode measurement by placing nonconductive, nondispersive polymer films between the sample and electrodes. Finally, a study on the dielectric measurement of ovalbumin contained in a 10 ml freeze-drying vial was undertaken with the electrodes external to the glass vial. Measurements, in each of the three stages, were taken as a function of the water content of a commercial spray-dried ovalbumin. Spray-dried ovalbumin was used in most of the measurements, as it is readily available and inexpensive. Freeze-dried ovalbumin was used for the final test to investigate whether the use of remote electrodes, attached to a glass vial, could be applied in principle to the determination of water content at the end point of a freeze-drying cycle.

MATERIALS AND METHODS

Materials

Hydrated and Deuterated Spray-Dried Ovalbumin

Spray-dried ovalbumin (crude, dried egg white, grade II, Sigma) was hydrated at 100% relative humidity (25°C) in a desiccator for up to 10 days, to give hydration levels between 0–0.2 g/g. The hydration level was defined as gram water per gram dry ovalbumin (abbreviated to g/g). The basal water content of the as-received sample was determined by drying the sample in a vacuum oven (80°C, ~40 mbar) until no mass reduction was observed. For deuteration, the spray-dried ovalbumin was stored in a desiccator containing D_2O at 25°C for up to 10 days. All values for water content quoted in this article include the basal water content of the received material.

Freeze Dried Ovalbumin

Ovalbumin solution (10% w/v), 2.5 ml aliquots, were placed in 10 ml glass vials and lyophilized in a Secfroid Lyolab G freeze-drier. The following cycle was used: 24 h freezing at –50°C, 24 h primary drying at –20°C, 24 h secondary drying at 0°C, 24 h secondary drying at 20°C.

Methods

Dielectric Measurements

The frequency dependent dielectric response of each sample (at ~20 °C) was measured between 0.1 Hz–10 MHz, using a Solartron 1296 dielectric interface connected to a Solartron 1255 frequency response analyzer. A voltage (3 V_{rms}) was applied to each sample, giving an output of amplitude and phase shift of the resulting current. These output parameters were then converted to dielectric parameters (e.g., impedances, capacitances, permittivities) using Solartron Impedance Measurement Software version 3.1. Curve fitting to complex functions were undertaken using commercial software (Zview™).

Dielectric Measurements Using Parallel Plate Electrodes

Samples of hydrated ovalbumin (thickness 3–3.5 mm) were placed between two circular brass electrodes, each with surface area of 3.14×10^{-4} m^2. For measurement using "remote" parallel plate electrodes, thin sheets of polyethylene film (d = 0.025 mm) were placed between each brass electrode and the sample to create a remote electrode system.

Dielectric Measurements Using Remote Electrodes on a Glass Vial

Samples of hydrated ovalbumin were placed in 10 ml glass vials, each with a rubber bung inserted in the top of the vial (Fig. 1). Each vial was placed in turn on a metal base, sandwiched between custom-made electrodes, and the sample packed to an approximate height of 10 mm. The experimental setup was designed to imitate the real conditions of the freeze-drying process. The brass electrodes were custom-

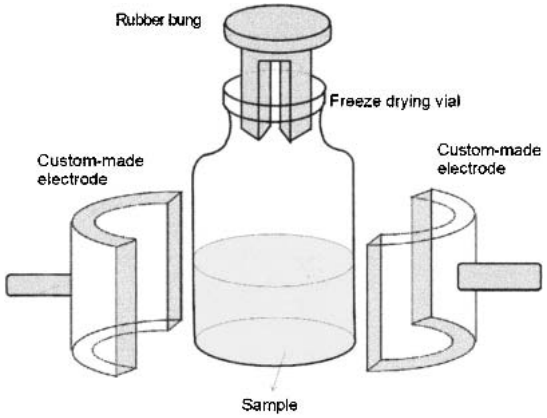

Fig. 1. Custom-made electrodes sandwiching glass vial, creating a remote electrode system for dielectric measurement of a material inside the glass vial.

made to the shape and dimensions of the vials used, with a surface area of 2.5×10^{-4} m^2. The dimensions of the custom-made electrodes were chosen to minimize measurement residuals (e.g., stray capacitances).

For dielectric measurements of freeze-dried ovalbumin, the glass vial containing the ovalbumin "cake" obtained from the lyophilization process was placed directly between the custom-made electrodes.

RESULTS

Dielectric Measurement Using Parallel Plate Electrodes

Figure 2 shows a typical dielectric spectrum of hydrated spray-dried ovalbumin (0.073 g/g) from a measurement using conventional parallel plate electrodes. The spectrum was characterized by a low-frequency response ($\omega<\omega_c$) and high-frequency response ($\omega>\omega_c$), where ω_c is the frequency at which the real and imaginary permittivities cross over. The low-frequency response ($\omega<\omega_c$) is characterized by a significant increase in both real and imaginary permittivities, whereas the high-frequency part ($\omega>\omega_c$) is characterized by a dielectric loss peak, which was designated as ε_3. (The symbol

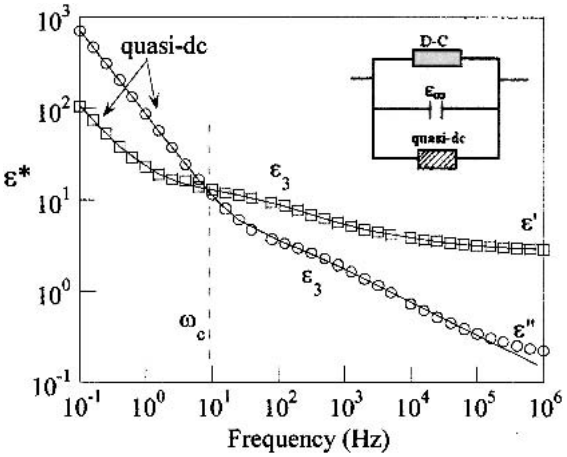

Fig. 2. Real and imaginary permittivities of hydrated ovalbumin (0.073 g/g), showing the quasi-dc part and ε_3 dispersion. Solid lines represent the curve fitting, symbols represent experimental data. The inset shows the circuit used to fit the graph.

ε_3 is used in the article for high-frequency dispersion. Some publications may refer to different symbols, such as α, β, or γ dispersion. In other circumstances, discussed in the next section of this article, there will be ε_2 dispersion. ε_1 dispersions are found at frequencies lower than those used in this study.)

The parallelism of the real and imaginary permittivities at low frequency (Fig. 2) and the fulfillment of the universal fractional power law of frequency (Eq. [2]) confirmed that the low-frequency response of the sample ($\omega<\omega_c$) was associated with a quasi-dc process.

The high-frequency ε_3 dispersion was a weak relaxation and only observed at low hydration levels (< 0.12 g/g). With increasing hydration, the ε_3 dispersion was either obscured, due to overlap with the low-frequency dispersion, or shifted to frequencies above the experimental frequency window. Both the quasi-dc response and the ε_3 dispersion shifted to higher frequency as the hydration level was increased.

Dielectric spectra of hydrated ovalbumin were fitted to a parallel circuit comprising a quasi-dc element, a Davidson-Cole element, and an instantaneous permittivity ε_∞ (see Eq. [3] and inset in Fig. 2):

$$\varepsilon^*(\omega) = A(i\omega)^{-p} + \frac{\Delta\varepsilon_3}{(1 + i\omega\tau_3)^{\beta_3}} + \varepsilon_\infty \qquad (3)$$

The quasi-dc element ($\varepsilon^*(\omega) = A(i\omega)^{-p}$) models the universal fractional power law response of the low-frequency part ($\omega < \omega_c$), the instantaneous permittivity, ε_∞, represents the permittivity at $\omega\to\infty$, and the remainder (the Davidson-Cole element) models the high-frequency part ($\omega > \omega_c$). For the Davidson-Cole element, $\Delta\varepsilon_3$ is dielectric relaxation strength, τ_3 is relaxation time, and β_3 reflects the distribution of relaxation time ($0<\beta<1$).

The use of the Davidson-Cole model gave the smallest error in all dielectric parameters, and gave better fitting results compared with the Havrilliak-Negami model. This is not surprising, because the low-frequency arm of the ε_3 dispersion is largely hidden below the quasi-dc response. It follows that compensation between the parameters in the quasi-dc response and those in the Havrilliak-Negami expression will give rise to large uncertainties in the fit parameters.

The pre-exponential factor A and exponent parameter p, obtained from the quasi-dc element (Eq. [2]) are shown in Fig. 3a and 3b, respectively, as a function of water content. A transition is observed at hydration level at ~0.08 g/g for both the pre-exponential factor A and the exponent parameter p.

Dielectric Measurement Using "Remote" Parallel Plate Electrodes (with Polyethylene Films)

The insertion of thin sheets of polyethylene between the sample and the circular brass electrodes, to create a remote electrode system, gave rise to a Maxwell-Wagner like process, designated as the ε_2 dispersion, while the ε_3 dispersion was still observed (Fig. 4). With increasing hydration, both the ε_2 and ε_3 dispersions shifted to higher frequency.

To confirm the origin of the proposed Maxwell-Wagner process, the dielectric responses from the remote electrode measurement were fitted to the following circuit: a quasi-dc element, a Davidson-Cole element, and an instantaneous permittivity in parallel, in turn connected in series to a permittivity representing the electrical capacitance of the polyethylene spacers (see Eq. [4] and inset in Fig. 4).

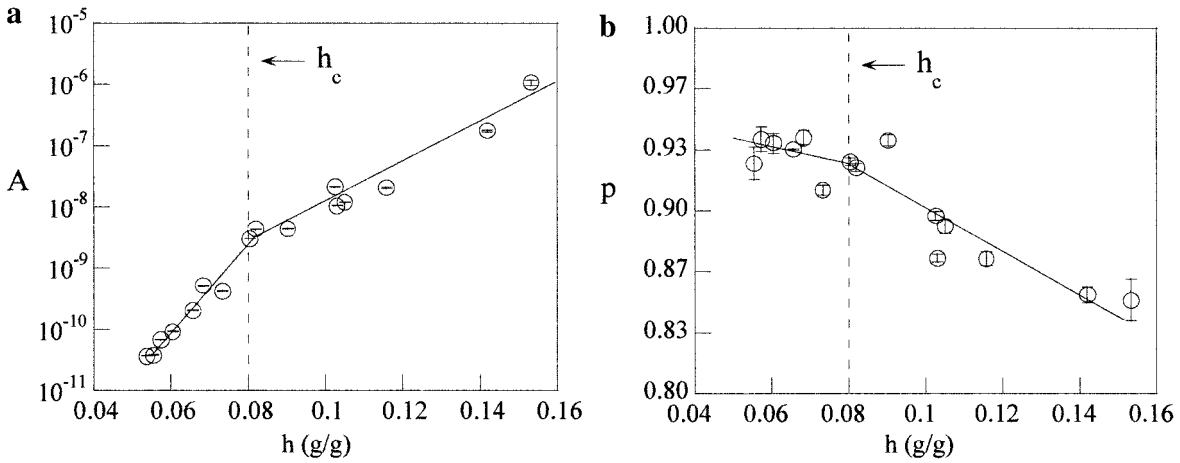

Fig. 3. (a) Pre-exponential factor A and (b) exponent parameter p from fitting of the quasi-dc element. Solid lines represent the tendency of the data. h_c = transition hydration.

$$[\varepsilon^*(\omega)]^{-1} = \left[A(i\omega)^{-p} + \frac{\Delta\varepsilon_3}{(1+i\omega\tau_3)^{\beta_3}} + \varepsilon_\infty \right]^{-1} + (\varepsilon_b)^{-1} \quad (4)$$

The ε_2 dispersion also fits a simple Davidson-Cole model. Therefore, the dielectric response from measurements using remote electrodes can also be modeled by a parallel circuit of two Davidson-Cole elements (representing the ε_2 and ε_3 dispersions) according to:

$$\varepsilon^*(\omega) = \frac{\Delta\varepsilon_3}{(1+i\omega\tau_3)^{\beta_3}} + \frac{\Delta\varepsilon_2}{(1+i\omega\tau_2)^{\beta_2}} + \varepsilon_\infty \quad (5)$$

The subscripts "2" and "3" in Eq. (5) denote ε_2 and ε_3 dispersions, respectively.

Both Eqs. (4) and (5) were shown to fit the data adequately, and the relaxation times, τ_3, obtained from both equations are comparable. Ultimately, Eq. (5) was chosen as the final fitting model for all measurements using polyethylene spacers. The reason for this choice was that the relaxation time τ_2 could be used to confirm that proton hopping was responsible for the observed quasi-dc process.

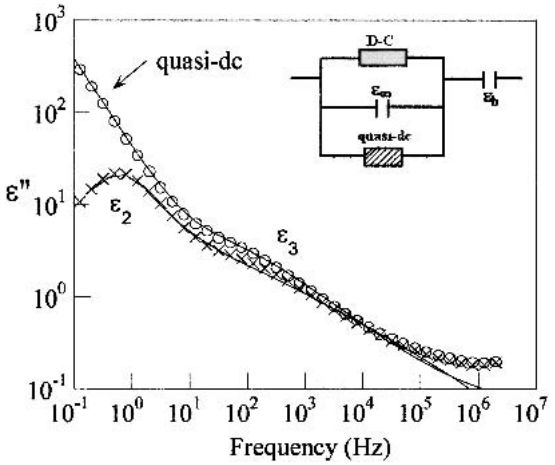

Fig. 4. Imaginary permittivity of hydrated ovalbumin (0.066 g/g) measured using conventional parallel plate electrodes without and with polyethylene film. With remote electrodes (i.e., with the use of polyethylene films), the quasi-dc part was eliminated by ε_2 dispersion, whereas the ε_3 dispersion was not affected by the use of remote electrodes. Symbols 'o' and 'x' represent measurement without and with remote electrodes, respectively. Solid lines represent the curve fitting, symbols represent experimental data. Inset shows the circuit used to fit the graph.

Figure 5 shows the dielectric parameters $\Delta\varepsilon_3$ and β_3, obtained from fitting the Davidson-Cole model to the ε_3 dispersion. The dielectric parameters $\Delta\varepsilon_3$ and β_3 were obtained only up to a hydration level of about 0.12 g/g. Above this hydration level, the ε_3 dispersion became indistinguishable. The general agreement between β_3 values obtained from measurements with and without polyethylene films showed that the mechanism of the ε_3 dispersion is independent of the use of remote electrodes. The independence of the ε_3 dispersion on the use of remote electrodes is also shown in the correlation of relaxation times, τ_3, between measurements using parallel plate electrodes, with and without the insertion of the polyethylene film (Fig. 5c). A good correlation (i.e., R^2 = 99%) was obtained from the linear fitting to the data, with gradient 47.5° and digression from the origin of 0.004.

There is some evidence from the dependence of the dielectric parameters (A, p, $\Delta\varepsilon_3$, and β_3) on hydration that indicates a transition at a hydration level of ~0.08 g/g. The relaxation strength $\Delta\varepsilon_3$ was almost independent of hydration below ~0.08 g/g but increased with hydration above ~0.08 g/g (Fig. 5a). The parameter β_3 decreased from 0.05 to 0.08 g/g and was then almost independent of hydration above 0.08 g/g. A water content of 0.08 g/g therefore appeared to reflect some critical hydration level in ovalbumin.

Figure 6 shows the comparison of relaxation times τ_2 and τ_3 of hydrated and deuterated ovalbumin based on the degree of hydration level, in which the deuteration level was corrected to the hydration level by normalizing to the molecular weight. The data was fitted well using a power function. Both relaxation times τ_2 and τ_3 from deuterated ovalbumin are higher than hydrated ovalbumin by a factor of ~1.3.

Dielectric Measurements Using Remote Electrodes on Glass Vial

Figure 7a shows the imaginary permittivity of hydrated spray-dried ovalbumin (0.08 g/g), measured by a number of approaches: (i) using conventional parallel plate electrodes, (ii) using parallel plate electrodes with polyethylene spacers

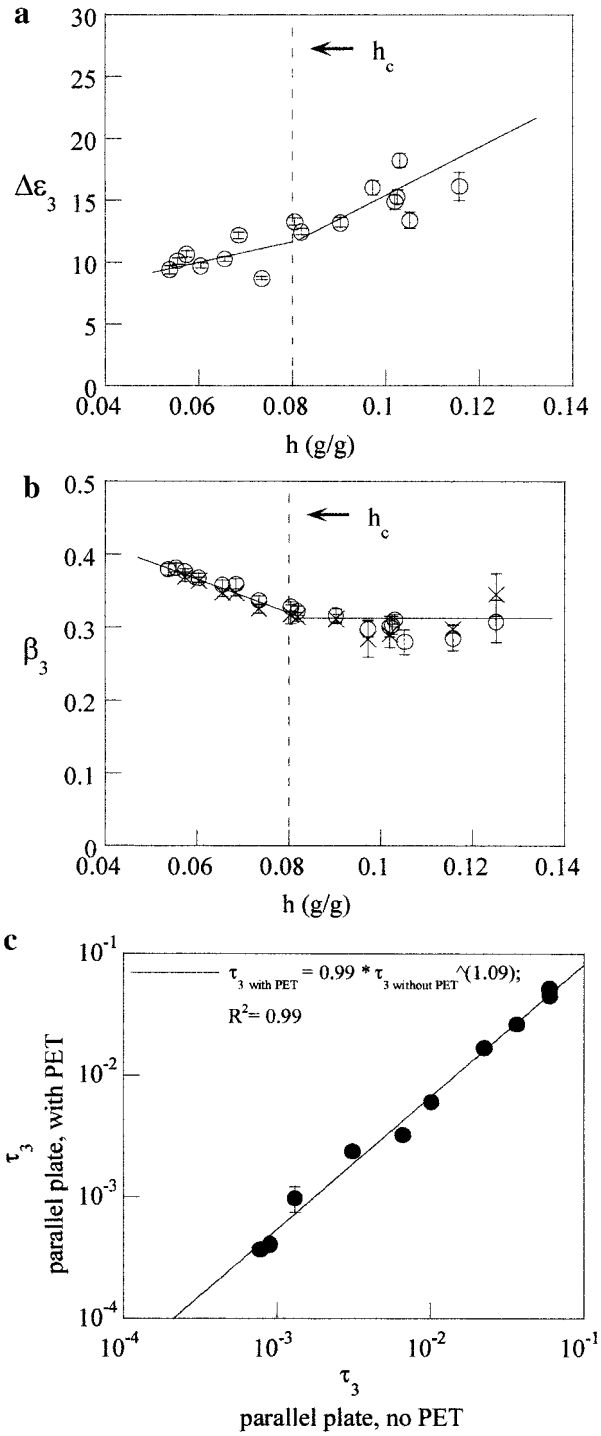

Fig. 5. (a) Relaxation strength ($\Delta\varepsilon_3$) and (b) distribution parameter (β_3) of hydrated ovalbumin from the ε_3 dispersion measured using conventional parallel plate electrodes. The overlapped symbols for β_3 originate from the measurement data with and without remote electrodes. Symbols 'x' and 'o' represent measurement with and without remote electrodes, respectively. h_c = transition hydration. (c) Relaxation times τ_3 of various hydrated ovalbumin, from measurement using parallel plate electrodes with and without polyethylene (PET) films.

between the electrodes and sample, and (iii) inside a 10 ml glass vial using remote electrodes attached to the outside of the vial. Two dispersions (i.e., ε_2 and ε_3) were observed with both remote electrode systems (i.e., the measurement with

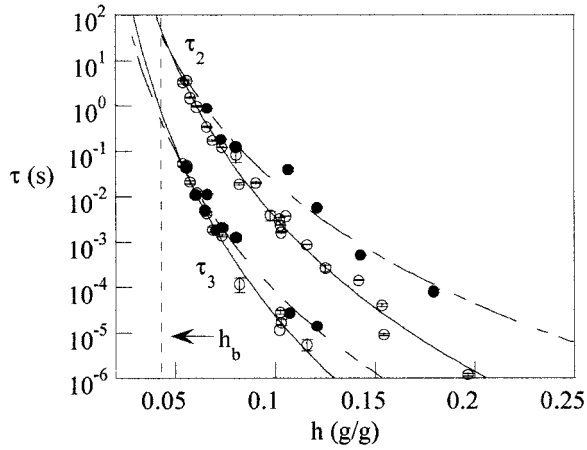

Fig. 6. Relaxation times τ_2 and τ_3 of various hydrated and deuterated ovalbumin, measured using parallel plate electrodes with polyethylene films. Solid lines and broken lines represent the curve fitting for hydrated and deuterated samples, respectively. Symbols represent experimental results. Open symbols represent hydrated samples, closed symbols represent deuterated samples.

the polyethylene spacers between the sample and parallel plate electrodes, and the glass vial measurement). The ε_2 dispersion is due to the Maxwell-Wagner effect associated with a composite dielectric, comprising the glass vial and hydrated ovalbumin, or the polyethylene spacer and hydrated ovalbumin. The relaxation time τ_3 for the ε_3 dispersion from measurements inside the glass vial was comparable with the results obtained from measurements using conventional parallel plate electrodes. Figure 7b shows the comparison of relaxation times τ_3 from measurements inside glass vial and measurements using conventional parallel plate electrodes, for various hydration levels. The linear curve fit characterized by a gradient of 45.15° and an intercept of 0.12 shows a good correlation (R^2 = 93%) of relaxation times τ_3 between measurement inside glass vial and conventional parallel plate electrodes.

Dielectric Measurement of Freeze-Dried Ovalbumin

Figure 8 shows the imaginary permittivity of a sample of freeze-dried ovalbumin inside a glass vial, as measured by remote electrodes. This freeze-died ovalbumin was obtained directly from the lyophilization process. As shown in Fig. 8, no relaxations were observed when the ovalbumin cake from the freeze-drying process had a trace amount of moisture (~0.01 g/g). When the sample contained 0.04 g/g moisture, the ε_3 dispersion was clearly observed, whereas the ε_2 dispersion was not, as it was below the experimental frequency window. However, when the sample contained ~0.07 g/g moisture, both the ε_2 and ε_3 dispersions were clearly observed.

DISCUSSION

Dielectric Measurements Using Parallel Plate Electrodes

Jonscher, Dissado, and Hill (12,13) described the origin of the quasi-dc process as a "many body" interaction within a material. If the exponent parameter p characterizing the response was between $0.5 < p < 1$, then the precise mechanism was said to be due to charge carrier hopping. The exponent

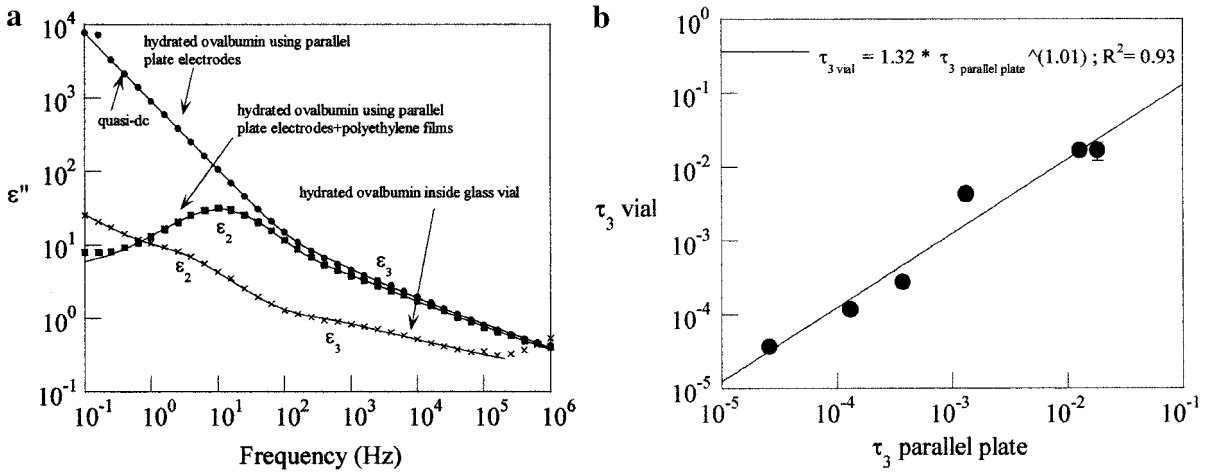

Fig. 7. (a) Imaginary permittivity of hydrated ovalbumin (0.08 g/g), showing the comparison of measurement using conventional parallel plate electrodes (with and without polyethylene films) and custom-made electrodes (with a glass vial). Solid lines represent the curve fitting, symbols represent experimental data. (b) Relaxation times τ_3 of ε_3 dispersion for various hydration, from measurement using conventional parallel plate electrodes (with polyethylene films) and custom-made electrodes (with glass vial).

parameter p, obtained from fitting a universal power law element to dielectric spectra of ovalbumin, measured using conventional parallel plate electrodes, was in the range of $0.8 < p < 0.98$ (Fig. 3b). Therefore, it can be said that the quasi-dc process observed on hydrated ovalbumin was probably due to charge hopping. Because this quasi-dc process is greatly affected by water content, it is probable that the exact mechanisms of charge hopping corresponds to proton transfer within the hydrogen-bonded water-protein system. The mechanism of proton transfer is confirmed from the deuteration study (Fig. 6) and is explained further in the next section.

The good fit of the spectra to the model consisting of quasi-dc and Davidson-Cole elements in parallel showed that the quasi-dc response observed for hydrated ovalbumin is a bulk property of the sample. If the quasi-dc observed was due to an interfacial or Maxwell-Wagner effect associated with the sample-electrode interface, then the dielectric response would be modeled by the quasi-dc element in series with the Davidson-Cole circuit. The independence of the quasi-dc dispersion on the compaction and thickness variation of the sample also confirmed that the quasi-dc process reflected a volume/bulk property of the sample.

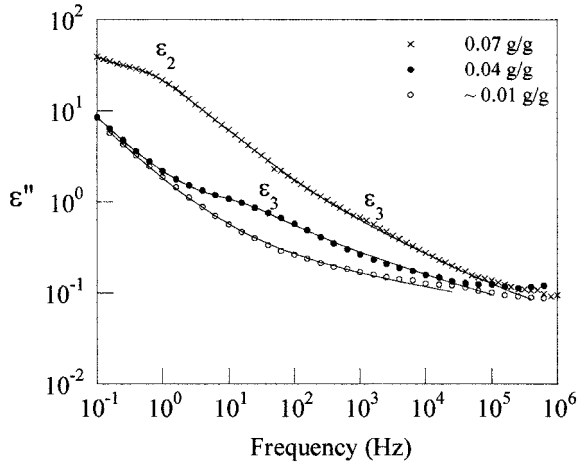

Fig. 8. Imaginary permittivity of freeze dried ovalbumin cake inside glass vial, comparing different hydration level. Solid lines represent the curve fitting, symbols represent experimental data.

Dielectric Measurements Using "Remote" Parallel Plate Electrodes (with Polyethylene Films)

Two dispersions (ε_2 and ε_3) were observed when taking dielectric measurements of the composite dielectric material, comprising thin sheets of polyethylene sandwiching the sample between the circular brass electrodes.

ε_2 Dispersion

The ε_2 dispersion was attributed to a Maxwell Wagner process associated with the heterogeneous dielectric mixture at the boundary of the analyzed system, i.e., polyethylene/sample interface. Considering the nondispersive and inert nature of the polyethylene spacers, it is possible to infer that changes in the ε_2 dispersion also will depend primarily on changes to the hydrogen-bonded network within the protein water system. This is supported by the observation that the relaxation time for the Maxwell Wagner process shows a strong dependence of water content (Figs. 5 and 6)

A comparison of the dielectric response of ovalbumin, hydrated using water and deuterated water, was undertaken to confirm that the process of proton hopping was responsible for the quasi-dc process, and hence the dependence of ε_2 on water content.

As seen in Fig. 6, the relaxation times τ_2 for deuterated ovalbumin are approximately 1.3 times higher than hydrated ovalbumin. The total mass of the proton in D_2O is twice than in H_2O, so the relaxation times for deuterated ovalbumin should be approximately $\sqrt{2}$ times higher than for hydrated ovalbumin; that is, if mobile protons are the reason for the dielectric relaxation. It can be concluded, therefore, that proton transport is responsible for the low-frequency process ($\omega < \omega_c$) in hydrated ovalbumin.

The intersection of relaxation times, τ_2, of hydrated and deuterated ovalbumin at a hydration level of about 0.046 g/g,

corresponds to the basal hydration (i.e., the hydration level of the as-received sample).

ε_3 Dispersion

The lack of dependence of the ε_3 dispersion on the use of remote electrodes shows that the ε_3 dispersion is not due simply to the effect of the sample-electrode interface or the Maxwell-Wagner effect. This ε_3 dispersion is rather due to the internal structure of the bulk sample itself. However, the nature of the ε_3 dispersion is still in question. Dissado and Hill (13) treated the ε_3 dispersion, observed in bovine serum albumin, as a supplementary loss peak that may occur in the hydrogen-bonded system besides the quasi-dc process. The ε_3 dispersion found in this study was assumed to occur because of proton transfer within the cluster itself (intracluster), and the scale of the cluster is probably commensurate with the scale of the individual macromolecules (as reported by Careri *et al.* [17]). The evidence suggesting that proton transport is responsible for the ε_3 dispersion is shown in Fig. 6. In a similar way to the relaxation times for ε_2 dispersion, the relaxation times, τ_3, of the deuterated samples were also approximately 1.3 times higher than hydrated sample, thereby supporting the explanation that proton hopping was responsible for the ε_3 dispersion.

The curves of the relaxation times τ_2 and τ_3 against hydration were not parallel (Fig. 6), thereby supporting the suggestion of an independent process between ε_2 and ε_3 dispersions. The ε_2 dispersion was due to the long-range proton hopping between the clusters (intercluster), while the ε_3 dispersion was due to proton hopping within the cluster (intracluster).

Transition Hydration

The transition observed in the dielectric parameters characterizing the response of ovalbumin (Fig. 3 and 5) may be due to the formation of first hydration stage for ovalbumin. Other authors state the stages of protein hydration, in which the first hydration stage is observed at a hydration level of ~0.07 g/g (21,22). The surface sites on the protein, therefore, are assumed to be fully hydrated at about a hydration level of 0.08 g/g. At higher concentration of water (> 0.08 g/g), the two-dimensional clusters of water molecules grow into a three-dimensional plane. This change leads to a discontinuous increase in $\Delta\varepsilon_3$, beyond 0.08 g/g added water.

Dielectric Measurements Using Remote Electrodes on Glass Vial

The relaxation times (τ_2) of the ε_2 dispersion, derived from measurement inside glass vial, were considerably different from those obtained from measurements using the "remote" parallel plate electrodes. The ε_2 dispersion, as mentioned before, was due to the composite capacitor characteristics of spacer and sample. The glass vial has significantly different permittivity and thickness to the polyethylene spacer ($\varepsilon_{PET} \approx 2$, $\varepsilon_{glass} \approx 5$, $d_{PET} \approx 0.1$ mm, $d_{glass} \approx 3$ mm). Therefore, it would be expected that the relaxation times (τ_2) of the ε_2 dispersion, using glass vial measurements, would be considerably different from those determined using parallel plate and polyethylene as remote electrodes.

The main intention of this study was to show that *in situ* measurement of hydrated ovalbumin inside a glass vial, expressed through the ε_3 dispersion, was equivalent to measurement using parallel plate electrodes and sensitive to water content (Figs. 7 and 8). The ε_3 dispersion is related to the hydration level of the sample, so the measurement of the ε_3 dispersion using this method can be used to monitor the water content in a freeze-dried product, close to the end point of the freeze-drying process.

However, at very low hydration levels (± 0.01 g/g), the ε_3 dispersion is not observed (Fig. 8), because it occurs at frequencies below the experimental frequency window. An alternative indicator therefore is required as the sample approaches low water contents. In this case, it may be possible to use the crossover frequency, ω_c. The cross-over frequency in this study was also outside of the experimental frequency window, but could be obtained by the extrapolation of the real and imaginary permittivities to low frequency. Estimates for ω_c of 0.078 Hz, 0.11 Hz, and 3.2 Hz were obtained for moisture contents of ~0.01 g/g, 0.04 g/g, and 0.07 g/g, respectively.

Another novel strategy could be employed, which involves a method called Eigen-coordinates procedure, to reveal the hidden ε_3 dispersion at very low hydration level (23). This technique is not discussed in this article but will be the subject of continued study.

CONCLUSIONS

This investigation of the dielectric behavior of hydrated ovalbumin shows two independent mechanisms of proton hoppings in the bulk sample, which are distinctly identified as an anomalous low-frequency dispersion and a dielectric loss peak (ε_3). The dielectric behavior also showed a transition at ~0.08 g/g, which was probably related to the first hydration layer in the protein.

Using the technique of dielectric measurement with remote electrodes, it was also possible to measure the ε_3 dispersion. The study therefore suggests that remote dielectric measurements may be employed as a convenient method for analyzing pharmaceutical materials, when contact of an electrode with a sample is either undesirable or impractical. The high sensitivity of the ε_3 dispersion to water content also suggests that the technique could be used for monitoring water content close to the end point of the lyophilization process.

NOTATION

ε^*	complex permittivity
ε'	real permittivity
ε''	imaginary permittivity
ε_3	dispersion at high frequency ($\omega > \omega_c$)
ε_2	dispersion at low frequency ($\omega < \omega_c$)
τ	relaxation time
τ_2	relaxation time corresponding to ε_2 dispersion
τ_3	relaxation time corresponding to ε_3 dispersion
$\Delta\varepsilon$	relaxation strength
$\Delta\varepsilon_3$	relaxation strength corresponding to ε_3 dispersion
β	distribution parameter
β_3	distribution parameter corresponding to ε_3 dispersion
A	pre-exponential factor
p	exponential parameter

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