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Multifrequency calorimetry

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Abstract

Multifrequency calorimetry is a technique aimed at measuring directly the dynamics of the energetic events that take place during a thermally induced transition by measuring the frequency dispersion of the heat capacity. This is done by modulating the conformational equilibrium using a small oscillatory temperature perturbation (≈0.05–0.1°C) centered at the equilibrium temperature of the system. The information is obtained from the attenuation in the amplitude or phase angle shift of the response of the system to the temperature oscillation. The analysis of the data is performed in terms of the transfer function defined using control systems theory. In this paper we describe new applications of MFC to the study of phase transitions in lipid bilayer systems.

INTRODUCTION

Multifrequency calorimetry is a new technique aimed at measuring simultaneously the energetics and time regime involved in macromolecular transformations. Biological macromolecules (proteins, nucleic acids and lipid components of biological membranes) often exist in equilibrium between different conformations. These conformations can be associated with different functional states, or be present during the process of folding or assembly of macromolecular structures. The goal of multifrequency calorimetry is to determine the magnitude and time scale in which energy changes take place.

Briefly, multifrequency calorimetry allows determination of the frequency spectrum of the heat capacity of a macromolecular system from the response function of the system to a periodic temperature perturbation. Consider for example, a macromolecular solution contained in a

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disc-shaped calorimetric cell of diameter d and thickness x. If the temperature on one side of the calorimeter cell (excitation side) is forced to oscillate in a periodic fashion with a characteristic amplitude and frequency, then the oscillations on the other side (measuring side) will be attenuated and retarded with respect to those at the origin ($x = 0$). It is in the magnitude of the attenuation and phase angle shift of the response wave that the energetic and dynamic information are contained. Typically, the overall amplitude of the temperature oscillation is of the order of 0.1°C or less. The measurements are performed at different system temperatures in order to define a temperature–frequency surface which is then analyzed to obtain thermodynamic and dynamic information.

Multifrequency calorimetry has been used to measure phase transitions in different phospholipid bilayer systems and the folding/unfolding transitions of proteins [1–3]. Also, the multifrequency calorimeter is being used to measure heat capacities of different compounds at specified temperatures.

FUNDAMENTAL IMPLEMENTATION OF MULTIFREQUENCY CALORIMETRY

Multifrequency calorimetry (MFC) is a stationary perturbation technique that allows measurement of the frequency dispersion of the heat capacity of a material. It is closely related to AC calorimetry [4] except for the instrumental design which is dictated by the objective of measuring the response of the system simultaneously at multiple frequencies. The theoretical foundation of multifrequency calorimetry is a consequence of the application of linear response theory to the equation describing the increase in the heat content (or, equivalently, the enthalpy at constant pressure) of a system as its temperature is changed at a finite rate. The relaxation times of the enthalpy fluctuations may be determined from the frequency dependence of the heat capacity.

The theory of multifrequency calorimetry has been presented elsewhere [1–3]. Briefly, the heat capacity at constant pressure C_p is directly proportional to the mean square amplitude of the equilibrium fluctuations in the enthalpy of a system

$$C_p = [\overline{H^2(t)} - \bar{H}^2]/RT^2 \quad (1)$$

where the overbars indicate the long time average, $H(t)$ is the enthalpy at time t, $\bar{H}$ is the long time average of the enthalpy, R is the gas constant and T the absolute temperature. This result can be derived, assuming the system to be ergodic, from Einstein fluctuation theory [5] or directly from the partition function.

Because the thermodynamic heat capacity can be defined as a long time average, it contains no information about the dynamic characteristics of the

enthalpy fluctuations. That information is contained in the normalized autocorrelation function

$$[\overline{H(t+t')H(t)} - \bar{H}^2]/[\overline{H^2(t)} - \bar{H}^2] \quad (2)$$

the measurement of which is a central concern for the spectral resolution of enthalpy fluctuations. Linear response theory and the fluctuation–dissipation theorem indicate that the enthalpy autocorrelation function is accessible, in the frequency domain, from stationary temperature perturbation experiments, the relevant observable being a frequency-dependent heat capacity [1–3, 5, 6].

A molecule existing in thermodynamic equilibrium between different conformations undergoes dynamic structural fluctuations between the various states that are accessible to it under the externally imposed conditions. According to the fluctuation–dissipation theorem [5], the dynamics of those fluctuations are characterized by the same parameters that characterize the return to equilibrium after a small external perturbation. The natural frequencies of the normal modes of the spontaneous fluctuations are related to the frequencies at which the system can optimally absorb thermal energy (which can be substantial, in view of the excess heat capacity these systems display). The relaxation times for the normal modes are given as the inverses of the natural frequencies, and they characterize the frequency dependence of the apparent excess heat capacity.

In the multifrequency calorimeter, one face of the sample cell (the excitation face) is subject to a steady-state oscillatory temperature perturbation. This temperature disturbance propagates through the sample, creating a temperature wave. The response of the system is measured on the opposite face of the cell (measuring face) and has the form

$$T(x, t) = T_0 + \delta T[\exp(-\Psi^+)\sin(\omega t - \Psi^-)] \quad (3)$$

where

$$\Psi^{\pm} = x(\omega C_p^{\ominus}/2k)^{1/2}\left[1 + (0.5C_p^{\ominus})\sum C_{p,\mathrm{ex,j}}(1 \pm \omega\tau_j)/(1 + (\omega\tau_j)^2\right]$$

In the equation above, T_0 and δT are the equilibrium temperature and perturbation amplitude, respectively, x is the distance from the excitation face, $\omega = 2\pi f$ is the angular frequency of perturbation, k is the thermal conductivity, and $C_p^{\ominus}$ is the non-relaxing heat capacity per unit volume. The non-relaxing heat capacity contains contributions arising from the solvent as well as the intrinsic heat capacity of the sample under study. The above equation has been written for the general case in which the thermodynamic excess heat capacity is composed of contributions from multiple relaxation modes, each characterized by a relaxation time τ_j, and amplitude equal to the equilibrium excess heat capacity $C_{p,\mathrm{ex,j}}$ [1]. As indicated by eqn. (3), the

response signal is attenuated in amplitude and lags behind the excitation signal. The magnitude of these effects is proportional to the excess heat capacity of the system and associated relaxation times as shown in eqn. (3), thus providing a way of estimating its magnitude and relaxation kinetics. Because the magnitude of the amplitude attenuation is proportional to the heat capacity, it is predicted to be maximal at the transition temperature of the system under study. It is clear from eqn. (3) that the relaxation times and amplitudes of the excess heat capacity are available independently either from response amplitude or phase shift measurements. In our studies we have primarily relied on the analysis of the frequency dependence of the response amplitudes because, over the low-frequency bandwidth of these experiments, they can be measured more accurately.

ANALYSIS OF MFC DATA

The relationship between the excitation $X(t)$ and the response $Y(t)$ temperature oscillation functions can be analyzed in terms of the transfer function $G(s)$ defined as the quotient between the Laplace transform of the response function and the Laplace transform of the excitation function

$$G(s) = \frac{\mathrm{L}[Y(t)]}{\mathrm{L}[X(t)]} = \frac{Y(s)}{X(s)} \tag{4}$$

where s is the complex variable $\mathrm{s} \equiv \sigma + \mathrm{j}\omega$, σ is a convergence factor, ω is the angular frequency and $\mathrm{j} = \sqrt{-1}$. In the multifrequency calorimeter, the excitation signal is given by a superposition of sinusoidal functions of characteristic amplitudes and frequencies. The response to an excitation function of the form $X(t) = A \sin(\omega t)$ is given by

$$Y(t) = |G(\mathrm{j}\omega)| \sin(\omega t + \phi)$$

The magnitude $G(\omega) \equiv |G(\mathrm{j}\omega)|$ and the phase angle ϕ constitute the frequency response of the system.

For a typical experimental condition in which the sample cell is loaded with a macromolecular solution existing in equilibrium between different states, the frequency response of the multifrequency calorimeter is given by the equation

$$G(f, T) = G_1(f)\{G_2(f) + K_0 \exp[-x(\pi f/\alpha(T))^{1/2}]\} \tag{5}$$

where $G_1(f)$ and $G_2(f)$ are characteristic instrument response functions independent of the sample under study. The sample contribution is contained in the exponential term through its thermal diffusivity $\alpha(T)$, K_0 is the value of the unattenuated signal, x is the width of the MFC cell,

which can be set anywhere between 0.2 and 1.4 mm, and $f = \omega/2\pi$ is the frequency in Hz. The thermal diffusivity of the sample $\alpha(t)$ is given by the formula

$$\alpha(T) = k(T)/\rho(T)C_p(T) \tag{6}$$

where $k(T)$ is the thermal conductivity, $\rho(T)$ the density of the solution and $C_p(T)$ the heat capacity at constant pressure.

The instrument response constants $G_1(f)$ and $G_2(f)$ are independent of the nature of the sample under study and essentially independent of temperature within the operational temperature interval of 0–100°C. Therefore, the temperature dependence of the measured response function $G(f, T)$ for a pure liquid or a macromolecular solution originates completely in the temperature dependence of the thermal diffusivity. In the absence of a phase transition, the thermal diffusivity is a slowly varying function of temperature. A phase transition or a macromolecular conformational transition, however, will induce significant changes in thermal diffusivity within the transition region. These changes contain thermodynamic and kinetic transition parameters.

For dilute macromolecular solutions, $k(T)$ and $\rho(T)$ are not significantly affected by the existence of a conformational transition. The heat capacity, however, can be written as

$$C_p(T) = C_p^{\ominus}(T) + C_{p,ex}(T) \tag{7}$$

where $C_p^{\ominus}(T)$ represents the heat capacity of the sample in the absence of a transition and $C_{p,ex}(T)$ the excess heat capacity associated with the presence of a temperature-induced transition.

For a sample exhibiting a phase transition, the excess heat capacity function is maximal at the transition temperature. For this reason, the thermal diffusivity (eqn. (6)) will have a minimum and the exponential term in the response function (eqn. (5)) will exhibit a maximal attenuation. This situation is illustrated in Fig. 1 for the solid–liquid transition of benzene. In this figure, we have plotted the surface defined by the temperature and frequency dependence of the response function. For this experiment, the frequency response to a temperature oscillation of ≈0.1°C was measured as a function of temperature in the frequency range 0–0.5 Hz. In general the signal is exponentially attenuated as a function of the square root of the excitation frequency (see eqns. (3) and (5)). However, in the presence of a transition an additional attenuation due to the excess heat capacity function will be visible within the temperature range of the transition. It is clear in the figure that the response function has a pronounced valley at 5.2°C, the phase transition temperature of benzene. At this temperature the heat capacity of the sample is maximal and therefore the attenutation of the signal is also maximal.

The analysis of the response function in Fig. 1 can be performed by

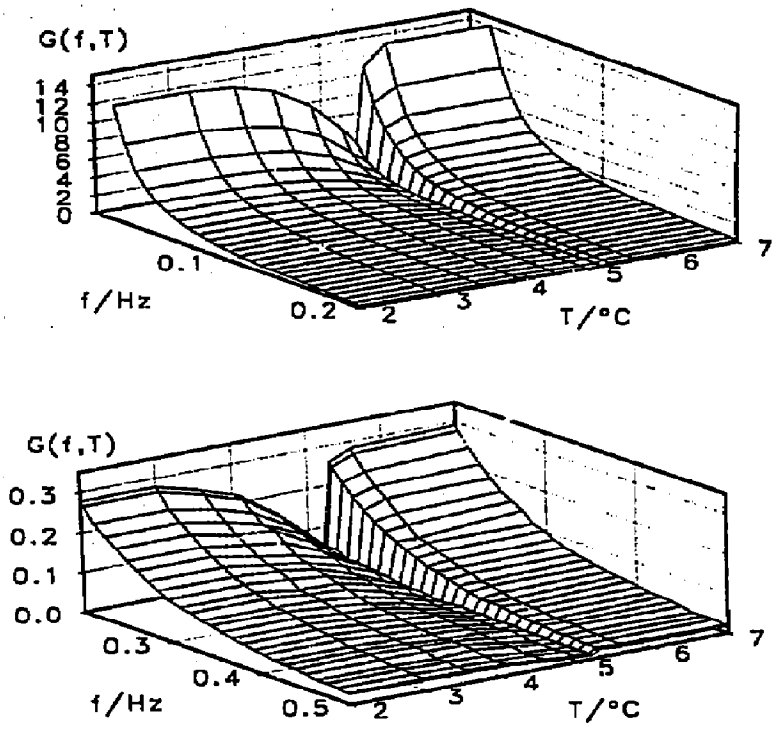

Fig. 1. Temperature dependence of the MFC frequency response associated with the fusion of benzene. For clarity of presentation the graph has been divided in two parts. The top panel covers the frequency range 0–0.2 Hz and the bottom panel the frequency range 0.2–0.5 Hz.

subtracting the intrinsic contributions of the sample to the response function from those arising from the existence of a phase transition. This is somewhat analogous to the baseline subtraction in conventional differential scanning calorimetry (DSC) except that in this case the entire surface is subtracted. The *basesurface* is also given by eqn. (5), except that the thermal diffusivity does not contain the $C_{p,ex}(T)$ term. The differential surface is given by the equation

$$\Delta(f, t) = G(f, T) - G^*(f, T) \tag{8a}$$

$$= G_1(f)K_0\{\exp(-x[\pi f/\alpha(T)]^{1/2}) - \exp(-x[\pi f/\alpha^*(T)]^{1/2})\} \tag{8b}$$

where $\alpha(T)$ and $\alpha^*(T)$ can be easily obtained by a multiexponential analysis of the data.

The thermal relaxation time of the sample in the absence of a phase transition is given by

$$\tau(T) = x^2/\alpha(T) \tag{9}$$

and that observed in the presence of the transition is

$$\tau^*(T) = x^2/\alpha^*(T) \tag{10}$$

Thus, the difference between both relaxation times can be expressed as

$$\tau^{*}(T) - \tau(T) = x^2 \rho(T) C_{p,ex}(T)/k(T) \quad (11a)$$

$$= x^2/\alpha_{ex}(T) \quad (11b)$$

This difference is equal to the transition relaxation time $\tau^{exc}(T)$, and can be measured as a function of temperature. Finally, the excess heat capacity function associated with the phase transition can be obtained from the formula

$$C_{p,ex}(T)/C_p^{\ominus}(T) = (\alpha(T)/\alpha^{*}(T)) - 1 \quad (12)$$

ANALYSIS OF THE GEL–LIQUID CRYSTALLINE PHASE TRANSITION OF DPPC BILAYERS

Figure 2 shows a conventional DSC scan of an aqueous suspension of DPPC multilamellar vesicles. The heat capacity function is characterized by a very large and sharp peak centered at 41.6°C. This peak corresponds to the gel–liquid crystalline transition which is a highly cooperative process

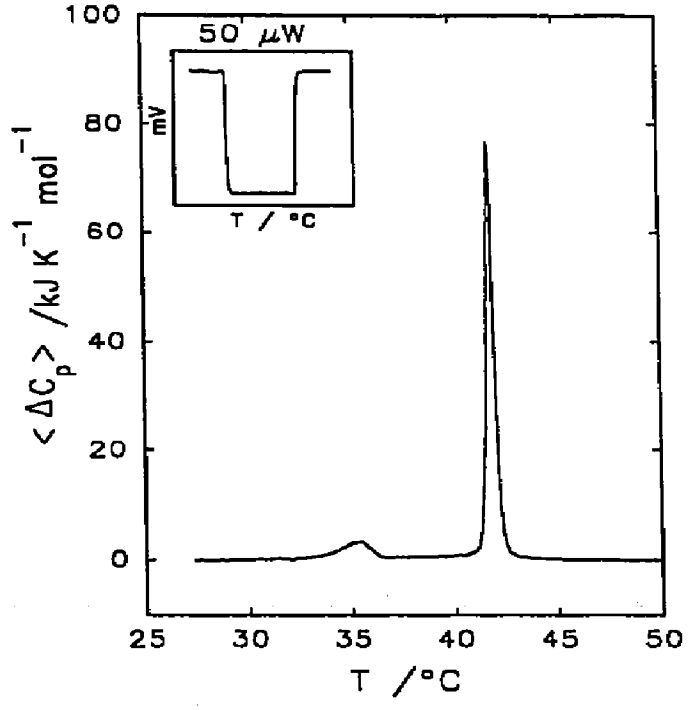

Fig. 2. Excess heat capacity function measured by conventional DSC (DASM4 differential scanning microcalorimeter) for the gel–liquid crystalline transition of an aqueous suspension of DPPC multilamellar vesicles (dipalmitoyl phosphatidylcholine). The inside panel shows a calibration pulse of 50 μW.

triggered by the melting of the phospholipid acyl chains. Below the transition temperature T_m, the hydrocarbon chains of the lipid molecules are in an *all trans* rigid configuration, while above T_m the chains melt and undergo substantial rotameric disorder. The enthalpy change for this transition, the area under the excess heat capacity curve, is equal to 37 kJ mol^{-1}. Also shown in the scan is the small peak centered around 35°C often referred to as the pre-transition.

Figure 3 shows the response function of the multifrequency calorimeter as a function of temperature and frequency for the same DPPC sample. As in the example shown in Fig. 1, a sharp valley is observed at the phase transition temperature of 41.6°C. Subtraction from the *basesurface* as

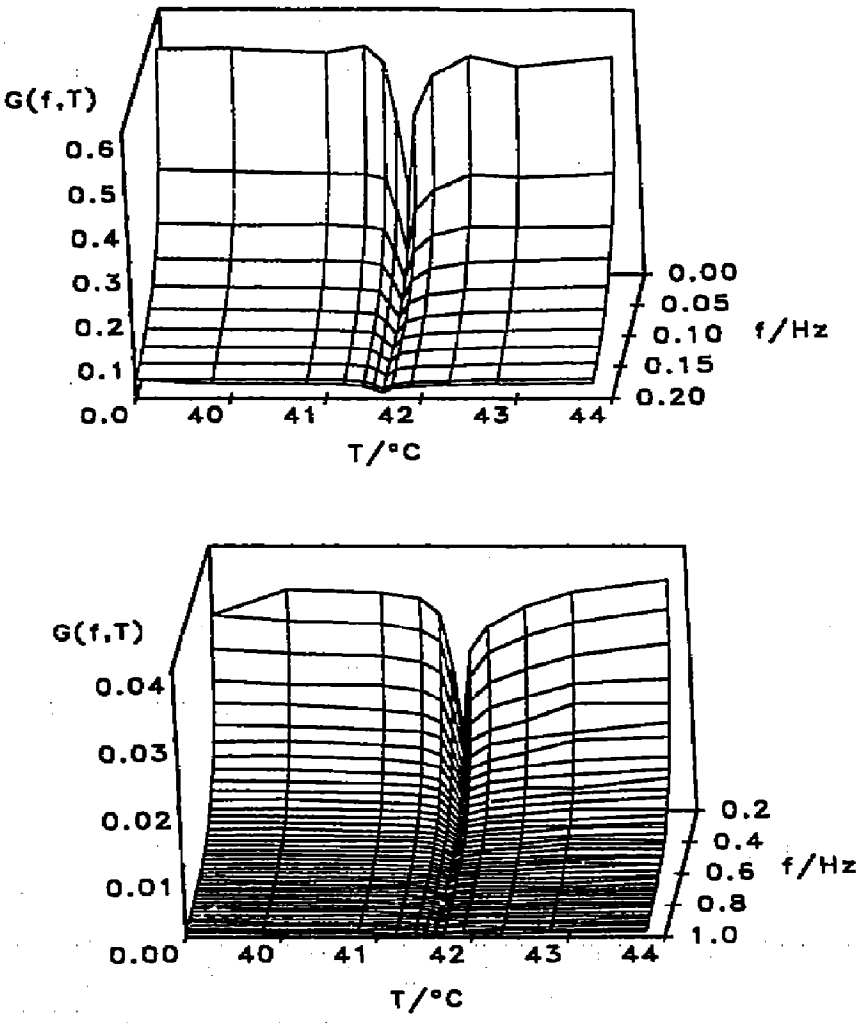

Fig. 3. Temperature dependence of the MFC frequency response associated with the gel–liquid crystalline phase transition of an aqueous suspension of DPPC multilamellar vesicles. For clarity of presentation the graph has been divided in two parts. The top panel covers the frequency range 0–0.2 Hz and the bottom panel the frequency range 0.2–1 Hz.

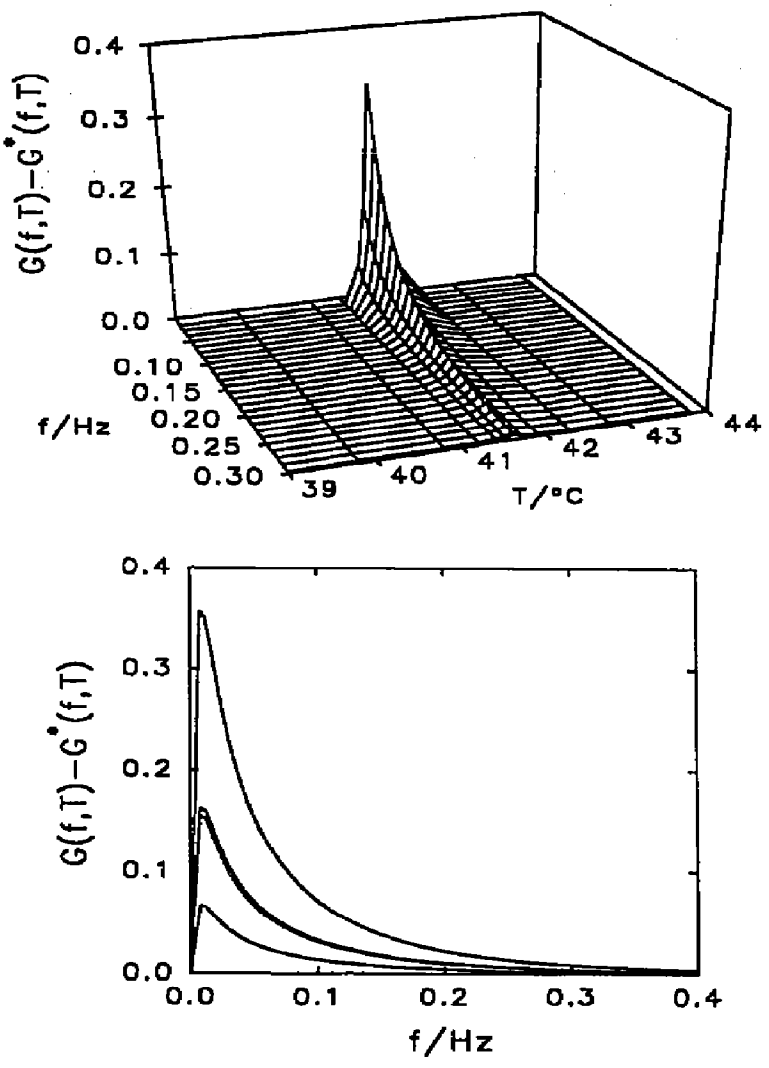

Fig. 4. Temperature dependence of the excess MFC frequency response associated with the gel–liquid crystalline phase transition of an aqueous suspension of DPPC multilamellar vesicles. This function is obtained by subtracting the frequency response from the *basesurface* and originates entirely from the effects due to the existence of the phase transition (see text for details). The bottom panel shows a different view angle of the same surface to illustrate the presence of a maximum in the frequency axis as well.

prescribed by eqn. (8) yields the excess response function shown in Fig. 4. In this figure a peak is clearly observed as a function of temperature and as a function of frequency (bottom panel). Figure 5 shows the data collected at the transition midpoint and the fit to a double exponential function according to eqn. (8b). Finally, Fig. 6 shows the estimated transition relaxation times as a function of temperature. As expected for a phase transition, the relaxation time is maximal at the transition temperature, reaching a value close to 650 ms. This relaxation time is consistent with values measured by other techniques [7].

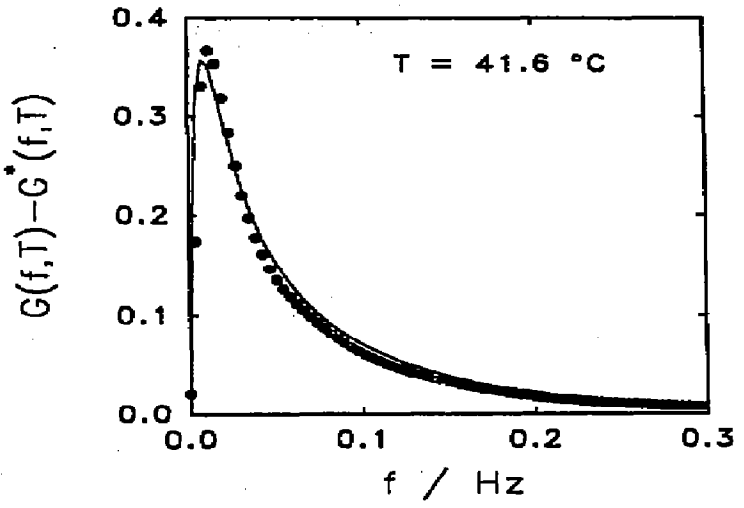

Fig. 5. Excess MFC frequency response isotherm (41.6°C) associated with the gel–liquid crystalline phase transition of an aqueous suspension of DPPC multilamellar vesicles. The solid line represents the fit of the data to a double exponential function as indicated by eqn. (8).

The relaxation times determined by MFC provide a direct measurement of the time scale in which the energy changes take place. This unique feature distinguishes this technique from other techniques in which optical or other non-thermodynamic observables are used to monitor a transtion or phase change. If a transition involves more than two states, non-thermodynamic observables will not generally reflect changes in energetic parameters. Thus, MFC is unique in that it allows a direct measurement of the kinetics of the energetics of a process.

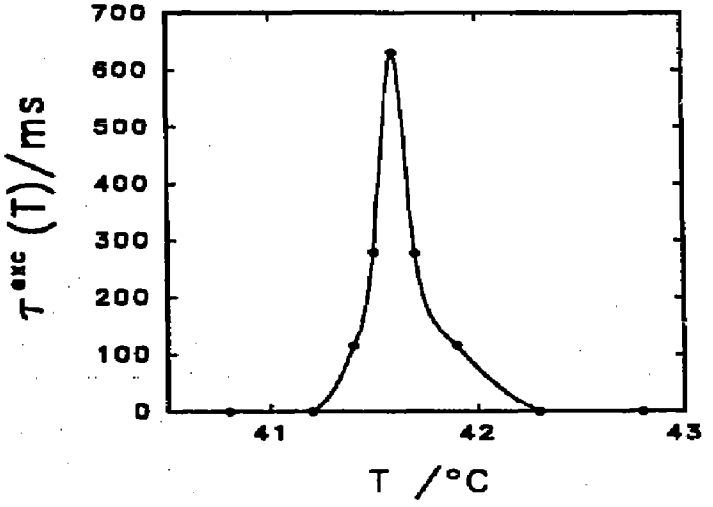

Fig. 6. Temperature dependence of the transition relaxation time for the gel–liquid crystalline phase transition of an aqueous suspension of DPPC multilamellar vesicles.

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