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Solvent interconnectedness permits measurement of proximal as well as distant phase transitions in polymer mixtures by fluorescence

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Abstract

We monitored the fluorescence intensity and anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated in bovine serum albumin (BSA) and dimyristoylphosphatidylcholine (DMPC) vesicle membranes, which in turn were embedded in optically clear gelatin solutions, as a function of temperature. DPH in BSA gave unanticipated large changes in fluorescence intensity and anisotropy at the instant of gelatin gel melting. Both steady state anisotropy and fluorescence intensity reported the gel–sol transition point in gelatin unambiguously, which was independently confirmed as physical-pour point of the gel. In the case of DMPC vesicles, fluorescence intensity indicated the gelatin transition, while the anisotropy indicated DMPC phase transition. This fluorescence methodology uniquely offered a common probe for two distinct transitions in two distinct domains interconnected by the solvent, water.

Keywords: Bovine serum albumin (BSA); Dimyristoylphosphatidylcholine (DMPC); Gelatin; Fluorescence anisotropy

1. Introduction

A major reason for the use of fluorescence probes is their sensitivity to variations in the local milieu, dielectric changes, as well as the dynamics [1,2]. Use of such probes in studies on polymers, such as proteins and membranes, focuses mostly on local interactions. The function of biopolymers is well recognized to be dependent on the 'environment' of the polymer, i.e. the 'external' environment, consisting of other proteins, solutes and the bulk solvent, i.e. water [3–5], as well as the interior of the protein and the polymeric motions [6]. There is a need to investigate whether the fluorescent probe yields information simultaneously in its local milieu within a polymer, as well

Abbreviations: BSA, bovine serum albumin; DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, dimyristoylphosphatidylcholine; THF, tetrahydrofuran; T_{gel}, gel–sol transition temperature of gelatin solutions

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as on the environment of the polymer. This should be possible, provided the external environment of the polymer itself exhibits variations in state, as in gels. There are a number of examples of biological interest of interactions within polymers, including transitions and related phenomena (as in membrane-bound proteins and polymeric gels, blood clotting/lysis, gels in which eggs are embedded, etc.).

What properties of a gel are desirable to control the environment external to the polymer, be it a protein or a membrane? Obviously it should be without intrinsic fluorescence, optically clear, and should exhibit a pronounced gel–sol transition dependent on temperature. Gelatin is a unique choice for such a polymer, as it does not contain tryptophan and contains a negligible amount ($\leq 0.5\%$) of tyrosine residues, and hence has little intrinsic fluorescence. It exhibits a temperature-dependent, reversible, gel–sol transition, which is a first-order-like phase transition [7].

Interactions within a gel are of two kinds, with two consequences. Firstly, there are monomer interactions, which largely determine the gel–sol transition and the transition temperature [8,9]. In the case of gelatin, monomers form random coils in sol temperatures, and become aggregated in the form of triple helices bonded by hydrogen bonds as the solution becomes a gel [8–10]. The bonding is also described as junction zones [11] or cross-linking junctions for gelation [12]. As the T_{gel} (i.e. at the sol–gel transition) is approached, the molecular weight of the polymeric gelatin rises sharply and approaches infinity at the gel point [8]. Thus, it forms an interconnected 3-D network, in such a fashion as if a huge single molecule with very high molecular weight were dissolved in water. Secondly, polymer–water (solvent) interactions determine the state (i.e. volume) of the gel, viz. swollen and collapsed [13]. The former may be continuously tuned by the concentration of the monomers, while the latter by the solvent availability (chemical potential, composition, etc.).

Thus, gelatin solutions at concentrations of 2% (w/v) and above in water fulfil all the criteria required of 'gels' [8], and are therefore extensively studied for their melting behaviour, volume changes and other rheological properties. Gelatin is an abundantly available protein polymer of biological origin. It is without tertiary structure and has the least intrinsic (adiabatic) compressibility. In comparison, other abundantly occurring globular proteins, such as albumins, have greater adiabatic compressibility [14]. Among the albumins, BSA has the highest intrinsic compressibility for a monomer protein [14,15]. Here we report unique changes in fluorescence intensity and anisotropy of DPH incorporated in BSA embedded in gelatin gels by employing the temperature-dependent gel–sol transition in gelatin. When other polymer aggregates, such as lipid bilayer vesicles, were substituted for BSA, surprisingly, the fluorescence anisotropy and intensity indicated independent transitions occurring in lipid bilayers and gelatin gel, respectively.

2. Experimental procedures

2.1. Materials

The fatty acid-free BSA, DMPC, gelatin (Type A, acid-cured from porcine skin, approx. 300 bloom) and DPH were purchased from Sigma Chemical Co., St. Louis, USA. Tetrahydrofuran and ethanol were purchased from Fluka, Switzerland. DPH was re-crystallized in ethanol before use. Gelatin was extensively dialysed in the incubation buffer prior to use. The commercially available gelatin varies in background brown colour and fluorescence yield of hydrophobic probes. Specimens of nearly white colour were selected and washed for 1–2 h with freshly distilled diethylether under constant stirring before extensively dialyzing against sodium phosphate buffer, 10 mM, pH 7.4, to yield consistent results. All other chemicals were of analytical grade and Millipore or Laboconco (USA) purified water was used for all purposes.

2.2. Preparation of large unilamellar vesicles of DMPC

Large unilamellar vesicles (LUVs) of approximately 100 nm in diameter were prepared by

reverse-phase evaporation [16]. The entrapment medium consisted of 0.22 M sucrose, 0.01 M NaCl and 0.01 M NaH_2PO_4 buffer, pH 7.4. In this system, the major osmolyte was the non-electrolyte, sucrose. Secondly, electrolyte was present in a minimal concentration to ensure sufficient ionic strength for LUV formation and screening of head-group charges. DPH was incorporated by pre-incubating aliquots of vesicles (~500 μg of lipid) with 12.5 μM DPH for 30 min in the dark at 30°C.

2.3. Osmotic pressure measurements

Various concentrations of gelatin solutions (%, w/v) containing 10 mM sodium phosphate buffer pH 7.4 and 10 mM NaCl were prepared and the osmolality was measured in a Wescor 5500 vapour pressure osmometer. In the case of gelatin solutions, the very low osmolality values encountered fell in the low non-linear range for the osmometer (20–100 mOs/kg). Therefore, these values were assessed with great care: (i) in triplicates; (ii) in the presence of precalibrated NaCl standards (in parallel using a conductivity meter) in the required range; and (iii) by prior cleaning of the thermocouple to reduce the reading for water alone (purified by reverse osmosis, Labconco, USA) to as low as 16–18 mOs/kg. The NaCl and buffers used were of comparable low osmolality.

2.4. Fluorescence measurements in BSA and DMPC vesicles

Intrinsic fluorescence of BSA was measured with excitation at 290 nm and emission monitored at 340 nm. The assay medium contained 10 mM sodium phosphate buffer, pH 7.4, and 250 μg/ml of BSA. The fluorescent dye DPH was added to the protein at a final concentration of 12.5 μM. A stock solution of DPH was prepared in tetrahydrofuran (THF). Additions were made such that the final concentration of THF did not exceed 0.25%. Requisite checks were made such that THF itself had no contribution to the emission signal of either intrinsic fluorescence or that of DPH. Excitation and emission wavelengths for DPH were 355 and 440 nm, respectively.

Fluorescence measurements were carried out in a Kontron SFM 25 spectrofluorometer with continuous stirring. Fluorescence anisotropy was calculated from fluorescence polarization, P, [2] as:

$$P = (I_{vv} - G \cdot I_{vh})/(I_{vv} + G \cdot I_{vh}) \quad (1)$$

where G is given by (I_{hh}/I_{hv}) and anisotropy, $r = (2 \cdot P)/(3 - P)$. I is the fluorescence intensity, v and h stand for the polariser placed vertically and horizontally, respectively, and the double subscripts stand for the excitation and emission polarisers, respectively. The correction factor 'G' was calculated at every polarization value using the horizontally polarized light (i.e. for each polarization value, a set of four fluorescence measurements was used, with the excitation and emission polarisers oriented in all four combinations). In all measurements reported here, the G value was found to be close to 1.0 (range 1.0–1.25), thereby ensuring that the emission monochromator did not contribute significantly to polarization effects at the wavelengths used [2]. The spectrofluorometer was interfaced via the RS232C port to a 286 IBM-compatible computer and polarization measurements were carried out in the automated mode. Protein was incubated in the dark with DPH for 30 min prior to measurements. Thermal transitions were measured as changes in the fluorescence intensity and anisotropy of the incorporated DPH [17,18]. Temperature-induced transitions were measured while heating at a rate of 0.5°C/min, using a Haake water bath and F3 thermoregulator controlled by a PG 20 programmer (Haake). The actual temperature in the cuvette was independently monitored using a probe connected to a Hewlett-Packard 89100A temperature controller and recorded on a Hewlett-Packard 85B microprocessor.

2.5. Fluorescence lifetime measurements

The fluorescence lifetime of DPH was measured using the 358-nm pulse (FWHM ~ 1.2 ns) from a nitrogen flash lamp (IBH Consultants, UK). The fluorescence was collected using a 420-

nm cut-off filter. An epifluorescence microscope (Nikon Diaphot 300) with a dichroic mirror at 400 nm was used in these experiments. A time-correlated single-photon counting set-up was used for the measurement of fluorescence lifetimes.

Fluorescence decay curves of DPH were analysed by deconvoluting the decay curves with the instrument response function (IRF), to obtain the fluorescence intensity decay function as:

$$I(t) = \Sigma\alpha_i \exp(-t/\tau_i) \quad (2)$$

where $I(t)$ is the fluorescence intensity, τ_i is the fluorescence lifetime and α_i is the amplitude of the fluorescence lifetime. Non-linear least squares and a Marquardt algorithm were used to extract α_i and τ_i using an iterative reconvolution method [19].

Perrin's equation describes the general relation between fluorescence depolarization of a fluorescent molecule and the hydrodynamic properties of the surrounding environment. A general form of the Perrin equation assumes that:

$$r_o/r = 1 + (\tau/\rho) \quad (3)$$

where r_o is the limiting anisotropy when the probe is completely immobilized (0.362 for DPH), r is the steady-state anisotropy, τ is the fluorescence lifetime of the probe in the medium and ρ is the rotational correlation time. Variations in r would imply variations in ρ, the probe mobility, if coincident changes in τ are accounted for [2].

2.6. Thermal transitions in gelatin used to induce changes in proteins and membranes

The hydrophobic probe DPH was incorporated by pre-incubation for 30 min at 30°C into the globular protein BSA and LUVs of DMPC. These were then embedded in gelatin of various concentrations containing 10 mM sodium phosphate buffer, pH 7.4. The experimental strategy used here involved two polymers: (i) the gelling polymer, in which macromolecules were embedded; and (ii) the test macromolecule — BSA or DMPC vesicles. DPH did not give a significant fluorescence signal with gelatin per se as compared with BSA or DMPC vesicles (cf. Fig. 1a). Also, after 30 min of incubation followed by removal of free DPH by Sephadex G50, BSA gave results identical to the unchromatographed BSA. This indicated that DPH would be intercalated within a pocket of BSA. Scatchard analysis according to the Klotz method [20] showed that $\sim$1.8 mol of DPH would bind with 1.0 mol of BSA. The fluorescence was first order with regard to the protein used. Binding studies indicated that the K_a (dissociation constant) was of the order of 3 μM for DPH, and hence approximately four-fold the K_a concentration was used in all measurements with BSA. After embedding the macromolecules in gelatin of various concentrations, ranging from non-gelling to those with distinctly different and increasing T_{gel} (gel-to-sol transition temperature), they were heated at 0.5°C/min and measurements were carried out as described above.

2.7. Turbidity changes

A major advantage of the use of gelatin as an external solute lies in the fact that it is too large to penetrate other proteins or membranes, unlike osmolytes such as glycerol, urea, etc. However, the very large size of gelatin, $\sim$100 000 Da [21], restricts its osmotic pressure contribution to a miniscule level, rendering direct measurements difficult. Melting of gelatin gel should lead to the availability of a relatively large number of monomers [vide supra [8,9]], leading to a modest and yet finite, sharp increase in osmotic pressure, i.e. diminished chemical potential of water. This in turn would reflect on the solubility product of a solution of a relatively insoluble salt such as calcium phosphate, which could be monitored by simple turbidimetry of critically selected concentration ranges. Similarly, decreased chemical potential of water/increased osmolality should also contract entrapped red blood cells, which can also be monitored as enhanced turbidity in association with gelatin transition. Turbidity changes were monitored in a HP 8450A diode-array spectrophotometer for precipitation of calcium phosphate in mixtures of $CaCl_2$ and Na_2HPO_4 at 450

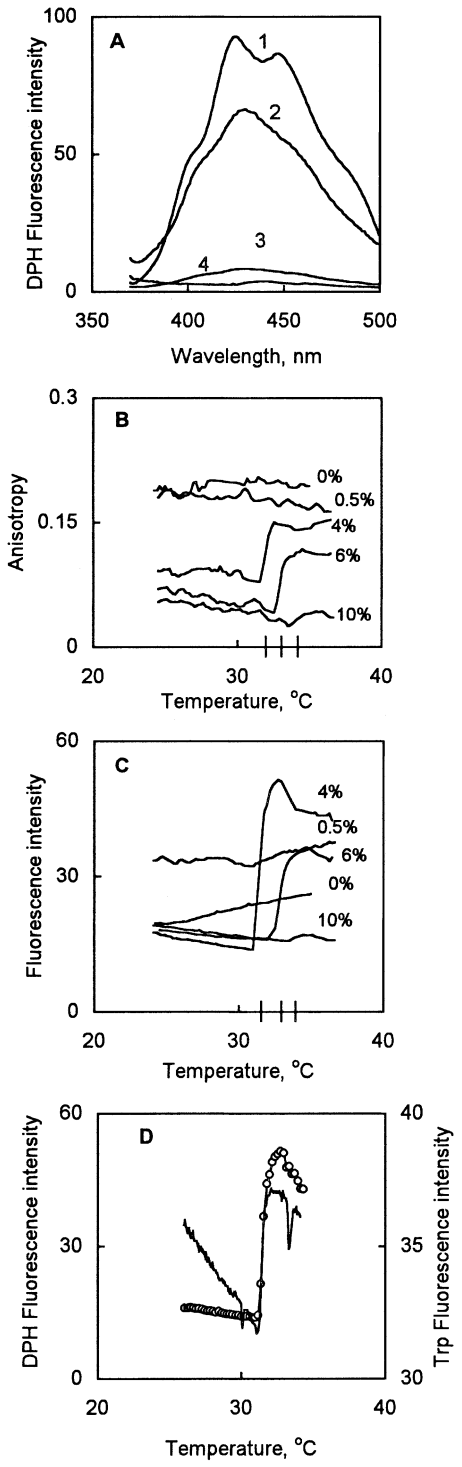

Fig. 1.

nm and erythrocyte volume changes at 520 nm. Temperature ramping was carried out using a HP 89100A temperature controller interfaced to the HP 8450A.

3. Results and discussion

3.1. Gelatin thermal transition reported by DPH incorporated in BSA

DPH yielded a good emission signal upon binding to BSA and was an ideal exogenously added probe to investigate the internal changes. DPH did not yield a measurable fluorescence signal in aqueous solutions, nor with gelatin, indicating that gelatin per se had no measurable interaction with DPH (Fig. 1a). Fig. 1b,c shows the DPH fluorescence intensity and anisotropy in BSA in various concentrations of gelatin as a function of temperature. Both fluorescence intensity and anisotropy reveal the gel–sol transition in gelatin. As a further control, the fluorescence of tryptophan residues of BSA embedded in gelatin was also measured as a function of temperature (Fig. 1d), which shows the identical transition temperature as DPH for 4% gelatin. However, DPH added directly into gelatin did not report the gelatin transition.

The melting point of gelatin was also determined independently as the physical pour-

Fig. 1. Influence of phase transitions in gelatin on fluorescence and anisotropy of probes. (a) Fluorescence emission spectra (arbitrary units) of DPH, excited at 355 nm in DMPC LUV (1), BSA (2), 4% gelatin (3) and in distilled water (4, bottom most line). Transitions as shown by: (b) anisotropy; and (c) fluorescence intensity of DPH incorporated in BSA, embedded in gelatin of various concentrations. The transition points were determined as first derivatives, identified as notches on the *x*-axis. Transition temperatures obtained by fluorescence intensity were, 31.6, 32.9 and 33.9°C; by anisotropy, 32, 33.1 and 34.2°C, respectively, for 4%, 6%, and 10% gelatin gels. (d) Fluorescence intensities of intrinsic tryptophan (—) and exogenously incorporated DPH (o—o) in BSA embedded in 4% gelatin gel show an identical transition point. In all the experiments, BSA was used at 250 μg/ml and the solutions contained 10 mM sodium phosphate buffer pH 7.4.

point. At the concentrations deployed, neither BSA nor DMPC vesicles affected the T_{gel} of gelatin at any gelling concentration of gelatin.

It was observed that as gelatin concentration increased, the steady state anisotropy of BSA significantly decreased more readily in the gel state than in the sol state of gelatin. The phase transition tracked by anisotropy of DPH, therefore, actually reported this differential susceptibility of DPH within BSA to the state of the external environment, gelatin. However, turbidimetry experiments, either of calcium phosphate precipitation or erythrocyte turbidity (embedded in gelatin) hardly showed 0.8% change on transition. Therefore, only small changes in fluorescence were anticipated for DPH, virtually at the limits of measurement. Surprisingly, fluorescence intensity on melting of gelatin abruptly increased by three–four-fold at 4% gelatin concentration, and the magnitude varied biphasically with gelatin concentration.

DPH fluorescence intensity in BSA decreased with higher concentrations of gelatin solutions, although the fluorescence intensity profile as a function of gelatin concentration was biphasic in the sol domain of gelatin gels (≥ 35°C). However, DPH anisotropy decreased monotonically with gelatin in the same domain. The decrease in anisotropy of DPH should be considered, in the first instance, in terms of first-order partitioning into gelatin, and possibly associated with specific temperatures as well, such as phase transition, at all concentrations of gelatin. This would account for a decrease in fluorescence intensity as well as anisotropy for DPH, say at ~ 25°C. The range of the dye used would be first order with regard to these measurements, though in a complex system, at high protein densities, a kinetic evaluation would be far too complex to be reliable. The fact that gelatin decreased the fluorescence intensity of DPH at higher concentrations would be in line with a decreased fluorescence intensity and anisotropy being due to loss of the dye into gelatin. Such an explanation would still be consistent with the basic observation that distant phase transitions could be reported by the same dye. However, the empirical evidence weighed against this

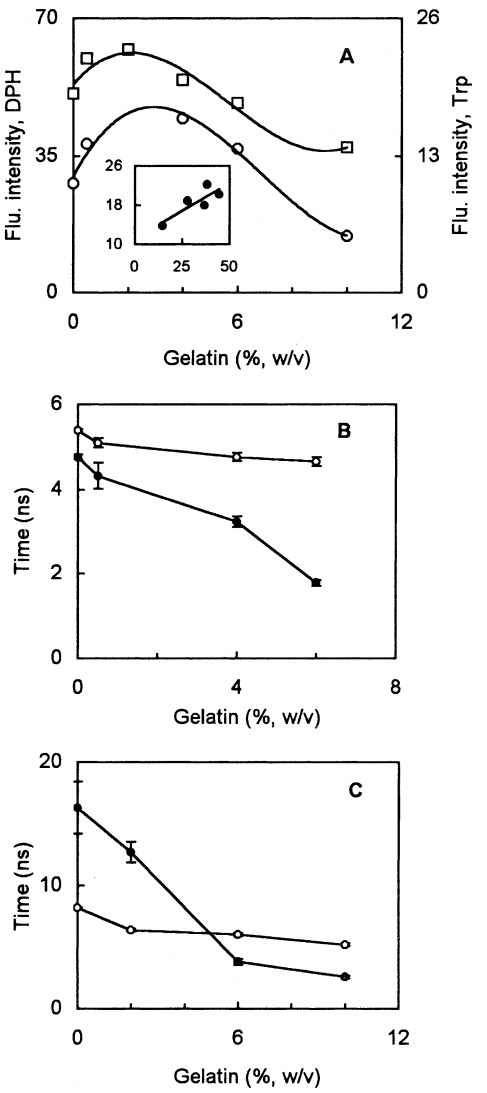

Fig. 2. (a) Fluorescence intensity of intrinsic tryptophan of BSA (squares) and intercalating hydrophobic probe DPH (circles) as a function of gelatin concentration measured at 36 ± 1.0°C. The inset shows that the fluorescence intensity of DPH (x-axis) and of tryptophan (y-axis) in BSA responded similarly as a function of gelatin ($r = 0.84$; $P < 0.01$). Fluorescence lifetime (open circles) and rotational correlation time (filled circles) of: (b) incorporated DPH in BSA; and (c) DMPC vesicles embedded in gelatin gels. All solutions contained 10 mM sodium phosphate buffer pH 7.4. DPH lifetimes in BSA were determined at 25 ± 0.5°C and in DMPC bilayer vesicles at 20 ± 1.0°C. The error bars indicate the standard error of mean of three independent measurements carried out at each concentration of the gel. Error bars are not visible when they are too small.

direct explanation. Fig. 2a shows a plot of fluorescence intensity of intrinsic tryptophan and DPH in BSA, with gelatin concentration, measured at ~36°C. The results were similar for tryptophan and DPH. The fact that tryptophan is covalently bound to BSA means that loss of the probe into the gelatin matrix by way of partitioning does not arise, indicating a plausible local internal perturbation in BSA rather than a loss of the probe to gelatin.

Decreased anisotropy would indicate enhanced tumbling of the DPH molecule (= decreased rotational correlation time, computed from Perrin's equation) within the pocket of binding, provided associated changes in the fluorescence lifetimes are accounted for. Fluorescence lifetime of DPH in BSA decreased only marginally (consistent with a decrease in steady-state fluorescence intensity) with gelatin concentration. However, the changes in anisotropy were large enough to indicate plausible changes in rotational correlation time (Fig. 2b). The observations were similar, even with DMPC vesicles (Fig. 2c). All the fluorescence decay curves were fitted to a heterogeneous decay with two lifetime components. The shorter (3.1–4.0 ns) and the longer (8.0–9.6 ns) lifetimes contributed to the mean lifetime in the ratio of 0.66–0.76 and 0.24–0.34, respectively, in BSA. In the case of DMPC vesicles, the shorter (3.3–5.1 ns) and the longer (9.3–13.3 ns) lifetimes contributed to the mean lifetime in the ratio of 0.3–0.45 and 0.55–0.7, respectively. The mean lifetime was adopted to infer the changes in rotational correlation time [22,23], since both the components were arising from bound fractions rather than free probe in water (which has < 2.0 ns, cf. [24]). A reason for decreasing rotational correlation time would be the formation of voids in dehydrating media, proposed earlier [25]. The present discussion necessarily restricts itself to the tracking of events outside the polymer BSA by a probe well inside the BSA and other polymeric matrices, such as DMPC vesicles. Specific interactions between BSA/DMPC and gelatin appeared untenable to account for these phenomena, since essentially similar phenomena were also observed in agarose media (data not shown), although these were not of great analytical use, since the transitions were considerably more complex [26].

Although proteins in their tertiary structure exhibit a fascinating similarity to gels [27], unlike gelatin, BSA per se does not possess a gel–sol-like transition in its native state, nor does it show any transition in the temperature range 25–35°C and at pH ~ 7.4 adopted in these experiments (cf. [28]). While HSA (human serum albumin) and BSA are known to attain different forms (such as expanded, fast, normal, basic and aged forms, depending on pH, classified on the rotational times and access to ligands), BSA remains in 'N' (normal) form under the present experimental conditions [28]. If the embedded polymer also has a phase transition, which one would be reported by DPH and why?

3.2. Differential reporting of melting behaviour in DMPC vesicles

DMPC vesicles exhibit phase transitions, characterized by the decreasing fluorescence intensity and anisotropy of DPH [17,18]. We scanned these vesicles in gelatin such that their corresponding phase transition temperatures did not overlap. Fig. 3a shows that anisotropy of DPH in DMPC vesicles corresponded primarily to the melting of DMPC (~23.75°C). The presence of gelatin did not affect the DMPC phase transition, as it could be compared with the transition in the absence of gelatin (inset, Fig. 3a). The anisotropy did not report a clear transition for the gelatin and was markedly noisy. On the other hand, fluorescence intensity per se exhibited a clear transition (Fig. 3b) corresponding to the melting of gelatin (28.0°C), while DMPC phase transition was nearly masked. The absence of gelatin did not give such a sharp change in fluorescence intensity (inset, Fig. 3b). Thus, fluorescence intensity and anisotropy, although apparently interdependent signals, yielded unique and independent information with regard to the milieu of the probe, proximal as well as more distant!

The DPH fluorescence yield and anisotropy decrease in DMPC vesicles due to phase transition is consistent with many previous reports [17,18,29]. The explanation itself is direct: phase

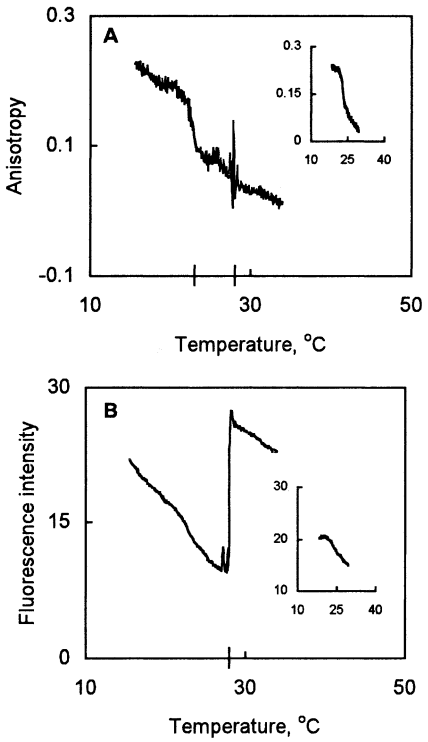

Fig. 3. Phase transitions in gelatin and DMPC lipid bilayer vesicles. Phase transitions as shown by: (a) anisotropy; and (b) fluorescence intensity of DPH incorporated in DMPC LUVs, embedded in 2% (w/v) gelatin. Insets in (a) and (b) illustrate the phase transition of DMPC in the absence of gelatin, respectively. Transition points are shown as notches on the *x*-axis to indicate the gel–sol transition of DMPC (23.75°C) and of gelatin (28°C).

transitions are associated with large structural perturbations leading to massive changes in conductance in the bilayer phase [30,31]. A decrease in fluorescence is consistent with hydration and a decrease in anisotropy directly relates to the induction of defects/flaws/voids consequent to the density fluctuations that underlie the transitional state [30,32,33]. On the other hand, the sudden and sharp change in DPH fluorescence intensity corresponding to the T_{gel} indicates the influence of the physical state of the ambient gelatin.

Gelatin per se offers so little osmotic pressure (a 10% solution gave 67 ± 3 mOs/kg), and yet it would bind a great deal of available water. It is instructive to recall that high molecular-weight polymer solutes, such as polyethylene glycol (PEG), bind large amounts of water molecules per monomer. For example, PEG 6000 binds almost all the available water in a ~ 45–50% (w/v) solution (cf. [34,35]). Thus, the dehydrating effect of gelatin as a bulk effect must be distinguished from its contribution to osmotic changes, both of which could play a role at the phase transition. The sudden and sharp increase in fluorescence intensity and anisotropy of DPH in BSA precisely reported the gelatin transition. It should be noted that the temperature range adopted in these experiments (18–35°C) does not adversely affect these biological materials to warrant such drastic changes.

Melting of gelatin sharply reflected in the solubility product of calcium phosphate by approximately 0.8% (data not shown, vide supra). Melting is also associated with a sharp decrease in viscosity. The increase in fluorescence of DPH observed with BSA and liposomes upon gelatin melting is opposite in direction with regard to viscosity changes in gelatin medium. Fluorescence intensity is directly related to viscosity changes [36]. Thus, gelatin, by virtue of its melting behaviour, yielded evidence that the relevant variable is not viscosity. For reasons of magnitude alone, osmotic pressure (≤ 70 mOs/kg) appears to be inadequate as the proximate force for the changes induced in DPH and tryptophan within the BSA.

The experimental system appears to offer novel experimental strategies for a number of questions of cell biological interest. The use of a non-penetrating, non-interactive and optically clear external protein such as gelatin could be a new addition to the existing tools for the study of macromolecular interactions. Viscosity [37,38], osmotic pressure [5], hydration [39], poly-ionic interactions [40] and a variety of factors have been implicated in protein structure and function. Clear thermodynamic interpretations have often been difficult to achieve and even hazardous, largely owing to the difficulty in identifying the proximate forces responsible (cf. [41]). Gelatin, with its ability to impart specific physical variations in the environment of a polymer, would permit exploration by fluorescence and other methodolo-

gies of a variety of processes in which solvent water is expected to play a major role, the major handle being its concentration-dependent phase transition. Structural perturbations (mediated by high solute concentration or physically by gravitational fields or dehydration) and their physiological implications in lipid bilayer vesicles, mitochondrial and bacterial bioenergetics have been documented [34,35,42–44]. In attempting to delineate the osmotic effects of solutes, great caution has been expressed for the need to keep the concentration of large polymers such as proteins very low, to avoid anomalous effects [3,45]. On the other hand, studies with gelatin indicate the exciting possibility of solvent sharing among proteins as the basis of several of solute effects. Thus, solvent sharing at high protein concentrations in terms of structural perturbations and thermal stability to proteins and membranes could be of high physiological relevance, which would need investigation.

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