FEBS Letters 556 (2004) 260–264 FEBS 27947

# Phospholipid phase transitions in homogeneous nanometer scale bilayer discs

Andrew W. Shaw[a], Mark A. McLean[b,c], Stephen G. Sligar[a,b,c,*]

[a] Department of Chemistry, University of Illinois, Urbana, IL 61801, USA
[b] Department of Biochemistry, University of Illinois, Urbana, IL 61801, USA
[c] Beckman Institute, University of Illinois, Urbana, IL 61801, USA

Received 29 August 2003; revised 9 October 2003; accepted 20 October 2003

First published online 8 December 2003

Edited by Felix Wieland

**Abstract** **Nanoscale protein supported phospholipid bilayer discs, or Nanodiscs, were produced for the purpose of studying the phase transition behavior of the incorporated lipids. Nanodiscs and vesicles were prepared with two phospholipids, dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine, and the phase transition of each was analyzed using laurdan fluorescence and differential scanning calorimetry. Laurdan is a fluorescent probe sensitive to the increase of hydration in the lipid bilayer that accompanies the gel to liquid crystalline phase transition. The emission intensity profile can be used to derive the generalized polarization, a measure of the relative amount of each phase present. Differential scanning calorimetry was used to further quantitate the phase transition of the phospholipids. Both methods revealed broader transitions for the lipids in Nanodiscs compared to those in vesicles. Also, the transition midpoint was shifted 3–4°C higher for both lipids when incorporated into Nanodiscs. These findings are explained by a loss of cooperativity in the lipids of Nanodiscs which is attributable to the small size of the Nanodiscs as well as the interaction of boundary lipids with the protein encircling the discs. The broad transition of the Nanodisc lipid bilayer better mimics the phase behavior of cellular membranes than vesicles, making Nanodiscs a 'native-like' lipid environment in which to study membrane associated proteins.**


*Key words:* Phospholipid; Phase transition; Laurdan; Generalized polarization; Differential scanning calorimetry

## 1. Introduction

In a recent publication, a method to produce homogeneous nanoscale phospholipid bilayer structures referred to as Nanodiscs was described [1]. The process is initiated by mixing detergent solubilized phospholipids with a class of proteins termed membrane scaffold proteins (MSPs) engineered for disc formation and purification. Removal of the detergent through dialysis or interaction with hydrophobic beads initiates a self-assembly process by which the discoidal nanoscale phospholipid bilayers are produced. Size exclusion chromatography is employed to separate the Nanodiscs from any protein–lipid aggregates formed during the process. The resulting discs contain approximately 160 phospholipids in a bilayer encircled by two MSPs and measure 9.5–10 nm in diameter and approximately 5.5 nm thick [1]. This assembly process is depicted in Fig. 1.

Nanodiscs provide supported membrane-like structures that are soluble and stable and are therefore of great interest in the study of membrane associated proteins. The absence of detergents and equivalent access to both sides of the membrane invoke Nanodiscs as an attractive alternative protein solubilization system over micelles or liposomes. This system has already been used to solubilize membrane proteins with different levels of membrane association, such as the seven transmembrane bacteriorhodopsin [2] and heterologously expressed cytochrome P450s [3]. In order to precisely understand the properties of membrane associated proteins incorporated into Nanodiscs, the physical characteristics of these nanoscale structures, and in particular the phospholipid bilayer enclosed therein, must be well understood. One of the most important physical properties of a lipid system is its gel to liquid crystalline phase transition behavior. This communication explores the characteristics of the phase transitions of two phospholipids, dipalmitoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidylcholine (DMPC), incorporated into Nanodiscs. The transitions are characterized using two techniques: generalized polarization (GP) of laurdan fluorescence and differential scanning calorimetry (DSC).

Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) is a

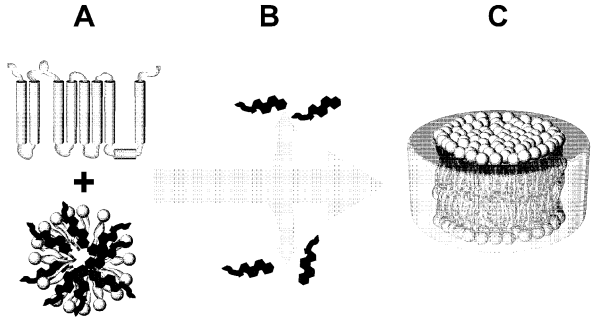


Fig. 1. Formation of Nanodiscs occurs by mixing MSP with detergent solubilized phospholipids (A) followed by removal of the detergent (B). The result is a discoidal phospholipid bilayer encircled by protein measuring 9.5–10 nm in diameter (C).

*Corresponding author. Fax: (1)-217-265 4073.
*E-mail address:* s-sligar@uiuc.edu (S.G. Sligar).

*Abbreviations:* MSP, membrane scaffold protein; DPPC, dipalmitoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; DPH, 1,6-diphenyl-1,3,5-hexatriene; GP, generalized polarization; DSC, differential scanning calorimetry

0014-5793/03/$30.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01400-5

fluorescent probe which incorporates readily into lipid bilayers with its fluorescent head group at the hydrophobic/hydrophilic interface of the lipids and its hydrophobic tail embedded with the acyl chains of the phospholipids [4,5]. The fluorescent properties of laurdan are extremely sensitive to solvent relaxation [6], giving large shifts in its peak emission wavelength as the level of hydration in the lipid bilayer changes [5,7,8]. It is known that the lipid bilayer has a greater level of hydration when the lipids are in the disordered $L_a$ confirmation favored above the transition temperature compared to the partially ordered $L_b$ confirmation favored below the transition temperature [9]. Thus, as the lipid environment becomes more hydrated during its gel to liquid crystalline phase transition, the maximum emission wavelength of laurdan shifts from about 440 nm to 490 nm when excited at 340 nm [8]. The emission spectra for laurdan in DPPC vesicles and Nanodiscs at various temperatures are shown in Fig. 2. Parasassi et al. have shown that the relative amount of each phase present in the system can be determined by the GP value of laurdan. GP is defined as: $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$, where $I_{440}$ and $I_{490}$ are the emission intensities at 440 nm and 490 nm respectively with excitation at 340 nm [8].

The use of laurdan in studying phase state is advantageous over other fluorescent probes such as 1,6-diphenyl-1,3,5-hexatriene (DPH) and parinaric acid in that GP can be used to quantify the amount of gel and liquid crystalline phases in a heterologous lipid system [8]. The anisotropy of DPH has been used to investigate membrane phase state, but it has been pointed out that this measurement is partially dependent on temperature [10]. Resolution of coexisting phases would require multiple time sensitive fluorescence measurements with a large number of parameters [11]. This makes quantification of phase states with this probe extremely difficult in most systems. Parinaric acid presents similar problems as its emission is characterized by three exponentials with temperature dependent amplitudes [12]. The steady state emission of laurdan, however, is dependent on polarity of its environment, not temperature, making it sensitive to the phase state of lipid and allowing for simple quantification of gel and liquid crystalline phases present in a system [13]. Therefore, laurdan GP is now widely used to examine phase states and transitions in a variety of lipid systems, from liposomes composed of synthetic lipids [14,15] to cellular membranes [16–18].

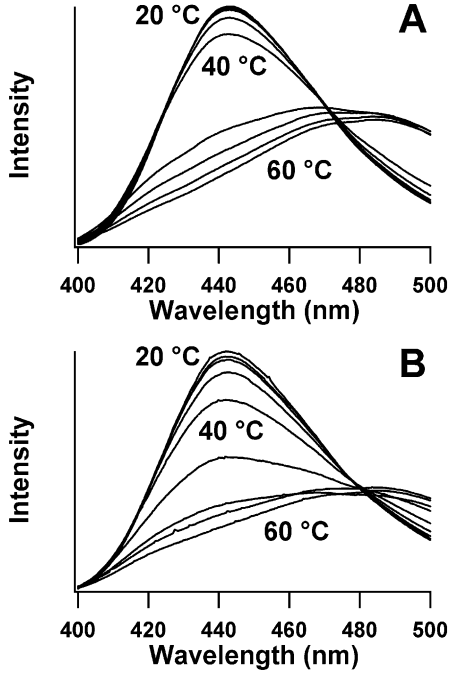


Fig. 2. Laurdan emission spectra (excitation wavelength = 340 nm) taken at 5° increments from 20°C to 60°C. A: DPPC vesicles. B: DPPC Nanodiscs.

## 2. Materials and methods

### 2.1. Materials

Synthetic DPPC and DMPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Lipids were stored in chloroform stock solutions at −20°C. Laurdan was obtained from Molecular Probes (Eugene, OR, USA) and stored in a methanol stock solution at −20°C. MSP was expressed recombinantly in *Escherichia coli* and purified by previously described methods [1].

### 2.2. Vesicle preparation

For vesicle preparation, laurdan was added to a lipid solution of either DPPC or DMPC to give a lipid to probe molar ratio of 200:1. This solution was dried using nitrogen and placed under vacuum overnight to ensure complete removal of solvents. The lipids were then resuspended in standard buffer (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 0.01% $NaN_3$, pH 7.4). This lipid solution was taken through approximately 10 freeze–thaw cycles by immersing the solution in a dry ice/ethanol bath with subsequent thawing under hot running water. The lipids were extruded at least 10 times through two 0.1 μm track etched polycarbonate membranes using a two syringe lipid extruder (Avanti Polar Lipids). Extrusion was carried out near the phase transition temperature of the lipid (37°C and 25°C for DPPC and DMPC respectively). This extrusion technique has been shown to produce unilamellar vesicles with a mean diameter of 80 nm [19]. Vesicles were stored at the phase transition temperature of the lipid and were used within 48 h.

### 2.3. Nanodisc preparation

For Nanodisc preparation, lipids and laurdan were mixed and dried in the same manner as vesicle preparation. Resuspension was performed by adding 50 mM sodium cholate in buffer to give a lipid concentration of 25 mM. The lipids were heated and sonicated until completely solubilized. MSP was then added to give a protein:lipid:probe molar ratio of 2:200:1. This mixture was incubated for 4 h near the phase transition temperature of the respective lipid. A 20 h dialysis procedure, performed at the same temperature, was used to remove the cholate. Using radiolabeled cholate, this dialysis procedure was shown to remove greater than 99.5% of the detergent (data not shown). The samples were sized on a Superdex 200 HR 10/30 column (Amersham Biosciences, Piscataway, NJ, USA), and the 10 nm fractions were taken and combined. Nanodisc concentrations were determined using $A_{280}$ (MSP $\varepsilon_{280} = 24\,700$ $cm^{-1}$ $M^{-1}$) correcting for the $A_{280}$ of laurdan ($\varepsilon_{280} = 24\,000$); laurdan concentrations were determined by $A_{364}$ ($\varepsilon_{364} = 20\,000$) where the absorption of MSP is negligible.

### 2.4. Fluorescence measurements

Fluorescence measurements were obtained using an F-3010 Fluorimeter (Hitachi, Tokyo, Japan). Samples were heated and cooled using a thermoelectric Peltier junction (All Electronics, Van Nuys, CA, USA) controlled by feedback from an YSI 400 series thermistor probe (Cole-Parmer, Vernon Hills, IL, USA) embedded in the sample holder. All vesicle and Nanodisc samples contained approximately 150 μM lipid. Samples were constantly stirred during measurements, and temperature readings were obtained directly from the sample using an immersible thermocouple (Cole-Parmer).

### 2.5. DSC

DSC experiments were carried out to further characterize the phase transition. Vesicles and Nanodiscs used in DSC experiments were prepared in an identical manner except 20 mM KPi, 150 mM NaCl, pH 7.4 was used as the buffer system and laurdan was not added to

the lipid mixture. Concentrations of discs and vesicles were 6.5 mM and 2.0 mM respectively, based on lipid. Heating curves were recorded using a Microcal MCS Differential Scanning Calorimeter (Microcal, Northampton, MA, USA) set at a scan rate of 70°C/h. Nearly identical thermograms were obtained at a scan rate of 35°C/h (data not shown). Curve fitting was performed using the software package Origin from Microcal utilizing a non-two-state transition model. In a non-two-state transition model, the calorimetric enthalpy is determined only by the area under the curve for a given transition while the van't Hoff enthalpy is determined only by the shape of the curve. When the calorimetric enthalpy and the van't Hoff enthalpy are different the transition is considered 'non-two-state' or 'cooperative'.

## 3. Results

Fig. 3 shows the GP data from laurdan incorporated in DMPC (panel A) and DPPC (panel B) liposomes and Nanodiscs. The data shown were obtained by increasing the temperature, although identical results were obtained with decreasing temperatures. There are two notable differences between the transitions of the phospholipids in vesicles and Nanodiscs. First, the transition temperatures for both lipids are shifted roughly 3–4°C higher in Nanodiscs versus vesicles. Table 1 displays the transition temperature of DMPC and DPPC in each system as found by laurdan GP and DSC. The second difference is the width of the transitions, as DPPC vesicles show a dramatic change in the GP value at the melting temperature (Fig. 3B). This is contrasted by the gradual change seen in Nanodiscs over a span of about 20°C. DMPC vesicles also display a sharp inflection in the GP curve at the transition temperature while the decrease in GP is more gradual in the corresponding Nanodisc structures (Fig. 3A). This change, however, is not as great as that observed in DPPC vesicles. In all samples, GP values continue to decrease

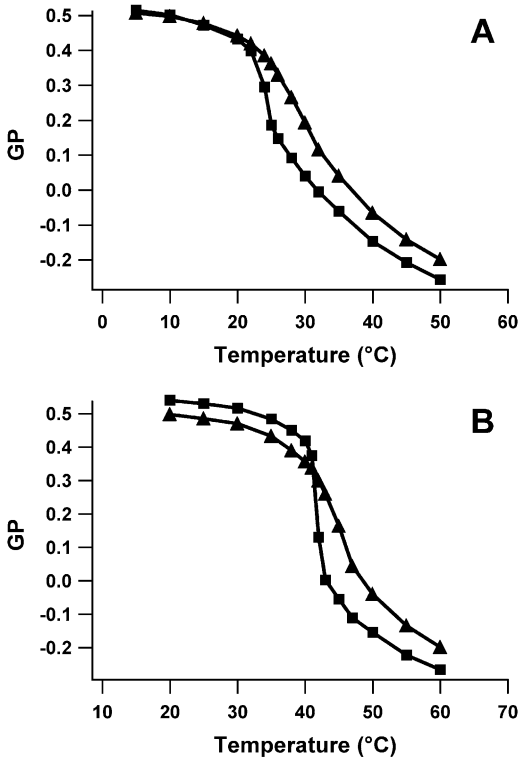


Fig. 3. GP values of laurdan in: (A) DMPC vesicles (■) and Nanodiscs (▲); (B) DPPC vesicles (■) and Nanodiscs (▲).

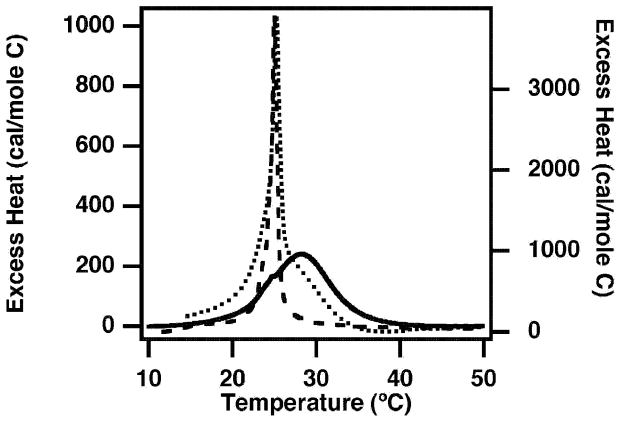


Fig. 4. DSC curves for DMPC discs (solid line), DMPC vesicles (dashed line) and DMPC discs after scanning to 90°C (dotted line). The scale on the left is for discs while the scale on the right is for vesicles.

almost asymptotically well into the liquid crystalline region. This behavior is not observed in the gel phase where GP values are fairly constant in each sample. The decreasing values observed at higher temperatures are the result of the formation of new water containing sites in the bilayer which increase the relaxation of the laurdan excited state [17]. The phospholipids do not allow a significant amount of water to penetrate the bilayer in the gel phase, accounting for the level GP readings at the lowest temperatures of each scan. This asymmetry of the GP curves may explain why the transition temperatures found by this method are slightly higher than those found by DSC (see Table 1).

It is also apparent that the GP values in the liquid crystalline phase are higher for both lipids in Nanodiscs than their respective vesicles. Because GP is additive in its values for each phase present, it would appear that a portion of the lipid in Nanodiscs is not behaving as if it were in the liquid crystalline phase but remaining in a more rigid state, hindering the permeation of water into the bilayer. Indeed, it has been shown that there is a lipid population which associates with integral membrane proteins, forming a region of 'boundary lipid' with completely distinct thermodynamic properties. For instance, Tall et al. observed that in reconstituted high density lipid particles the one to two rings of phospholipid adjacent to the apolipoprotein adopt this state [20]. Furthermore, in natural membranes fluorescence resonance energy transfer (FRET) between the tryptophan residues of acetylcholine receptors and laurdan probes within the adjacent lipid belt has shown that the GP of such laurdan was indeed higher than bulk laurdan, indicating that the boundary lipid was in a more rigid state [21]. The higher liquid crystalline GP values in Nanodiscs are apparently the result of this 'boundary lipid' remaining in a more ordered state. The lipid ordering phe-

Table 1
Transition temperatures (°C) of DMPC and DPPC vesicles and Nanodiscs as determined by laurdan GP and DSC

| | | Laurdan GP | DSC |
|---|---|---|---|
| DMPC | Vesicles | 27.0 | 25.0 |
| | Nanodiscs | 31.5 | 28.0 |
| DPPC | Vesicles | 42.0 | 41.5 |
| | Nanodiscs | 45.5 | 44.5 |

Table 2
Calorimetric and van't Hoff enthalpies for DMPC vesicles and Nanodiscs obtained from a fit using a non-two-state transition

| | | Calorimetric enthalpy (kcal/mol) | van't Hoff enthalpy (kcal/mol) | Ratio |
|---|---|---|---|---|
| DMPC | Vesicles | 3.8 | 657 | 172 |
| | Nanodiscs | 2.3 | 73 | 32 |
| DPPC | Vesicles | 5.3 | 470 | 88 |
| | Nanodiscs | 2.4 | 116 | 48 |

The ratio of the van't Hoff enthalpy and the calorimetric enthalpy is a measure of the size of the cooperative unit.

nomenon is noticeable in this Nanodisc experiment even without the use of FRET measurements due to the extremely high portion of boundary lipid in the discoidal bilayers. Taking an area of 0.65 $nm^2$ per DPPC molecule [22] and 80 DPPC molecules in one leaflet of a Nanodisc yields a lipid disc with an area of 52 $nm^2$ and, thus, a radius of 4.1 nm. Subtracting the size of one lipid, to account for an outer lipid ring, leaves a disc with radius of 3.3 nm which would contain approximately 53 lipids. Therefore, the outermost ring should contain about 27 lipids, or 34% of the total lipid present in the Nanodisc. Since previous studies have shown the amount of boundary lipid to be greater than the lipid in the outer ring [20,23], the expected percentage of lipid acting as boundary lipid in Nanodiscs would be somewhat higher.

Fig. 4 presents DSC data for DMPC vesicles and Nanodiscs. As has been well documented [24,25], the vesicles display a characteristic sharp transition (gel to liquid crystalline phase) at 25°C and a pretransition near 14°C. For DMPC in Nanodiscs the main transition shifts to 28°C and broadens dramatically. The broadening of the transition is indicative of the loss in cooperativity of the phase transition. A simple measure of this cooperativity is the ratio of the van't Hoff enthalpy, which is the enthalpy per mole cooperative unit, and the calorimetric enthalpy, which is the enthalpy per mole lipid. Fitting the data to a non-two-state transition shows that the cooperativity of DMPC in Nanodiscs is decreased nearly six-fold compared to that of vesicles (Table 2). The size of the Nanodiscs alone constrains the size of the cooperative unit to a maximum of 160 lipids. The enthalpy ratios suggest that the cooperative unit in vesicles is 172 lipids while in Nanodiscs the unit decreases to 32 lipids, demonstrating that the boundary lipid further constrains the cooperativity of the transition. The magnitude of the calorimetric enthalpy of the phase transition in Nanodiscs is much lower than that observed in vesicles. This can also be explained by the presence of the non-melting boundary lipid interacting with the MSP. Since the boundary lipid does not melt in the main phase transition, the measured enthalpy is lower, reflecting only the phase transition of the core lipids. When we compare the calorimetric enthalpies of DMPC vesicles and DMPC discs we see that the value in Nanodiscs is only 60% of that in vesicles. This is consistent with a boundary layer of lipid consisting of 40% of the total lipid incorporated into the Nanodiscs. Similar results have been described for apo-AI and apo-AII discoidal particles [26,27]. The phase transition is completely reversible, and the samples can be rescanned repeatedly as long as the temperature remains below 80°C which is the onset of the disruption of the Nanodisc structure (data not shown). Fig. 4 also shows a thermogram of Nanodiscs after scanning to 90°C. It is clear that the main phase transition is now centered at 25°C and a pretransition similar to pure DMPC vesicles is observed. DPPC discs display very similar trends showing a decrease in cooperativity compared to vesicles and are summarized in Table 2.

## 4. Discussion

Previous studies of high density lipid particles reconstituted with the major lipoprotein apo-AI show the gel to liquid crystalline transition is shifted to higher temperatures and broadened significantly compared to liposomes. These findings were explained by a loss in cooperativity of the transition due to a much smaller cooperative unit compared to vesicles and by the fact that there is a significant amount of boundary lipid that is interacting with the protein scaffold [20]. This explanation is supported by the finding that increasing protein to lipid ratios in vesicles results in increased broadening of the transition [28]. In this study, purified Nanodiscs display broad phase transitions and shifted melting temperatures. This is not surprising due to the fact that one Nanodisc only contains approximately 160 lipids per disc (80 per leaflet) which would dictate that fewer lipids could participate in the 'cooperative unit' giving rise to broadened transitions. With nearly 40% of the lipids participating in the boundary layer, the Nanodisc preparations mimic biological membranes which also have a high percentage of boundary lipids due to the enormous amount of proteins embedded within the bilayer.

The phase behavior of Nanodiscs demonstrates that the incorporated phospholipids retain the basic thermodynamic properties of a lipid bilayer. Furthermore, the lipid bilayer of Nanodiscs surpasses that of vesicles in mimicking biological membranes. The multitude of proteins found in biological membranes interacts with surrounding lipids, contributing to their altered phase behavior. Similar protein–lipid interactions perturb the phase transition of phospholipids in Nanodiscs. The thermodynamic behavior of the phospholipids and the remarkable self-assembly of Nanodiscs provide an excellent platform for studying formerly insoluble membrane proteins in a soluble system with properties much like those of a cellular membrane.

*Acknowledgements:* This work was supported by the Nanoscale Science and Engineering Initiative of the National Science Foundation under NSF Award Number EEC-0118025 and by grants from the NSF DMI9986595 and the National Institute of Health GM33775. The authors thank Aretta Weber for her administrative assistance and Timothy Bayburt and Yelena Grinkova for helpful input and discussions.

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