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Detecting the presence of membrane domains using DSC

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This manuscript is dedicated to the memory of Professor Julian Sturtevant, as a person and as a scientist.

Abstract

Both biological and model liposomal membranes have unequal distribution of molecular components in the plane of the membrane. There is increasing interest to determine the composition and properties of membrane domains enriched with specific molecular components. Several methodologies have been applied to study this. Each has its own advantages and provides a particular kind of information. In the present article, we will focus on the application of differential scanning calorimetry to the determination of the distribution of molecules into membrane domains with particular emphasis on protein and peptide-induced domain formation.

Keywords: DSC; Membrane domains; Rafts; Cholesterol; Phase transition; Cationic peptides

1. Introduction

There is much evidence to indicate that biological membranes are not homogeneous, uniform entities. Cellular and organelle membranes are based on the arrangement of polar lipids in the form of a bilayer containing a significant amount of incorporated protein. These molecular components are not uniformly distributed in the membrane. There are differences in the chemical composition of each of the monolayers that comprise the bilayer of a biological membrane. In addition, in polarized cells, there are different protein and even different lipid components in the apical and basolateral surfaces of polarized cells [1]. Furthermore, on even a shorter length scale, there are clusters of certain membrane components that comprise a membrane domain. Some of these domains form morphologically distinct structures in the membrane, such as caveolae or clatherin-coated pits. However, there are also domains that are not associated with a distinct morphological feature. These domains are thought to be "floating" in the membrane and one type of such a domain has been termed a "raft". There has been much discussion and research into characterizing such domains. They are rich in cholesterol and sphingomyelin and form a bilayer in a liquid-ordered state [2]. Their existence in simple model membranes is well established, but whether such domains or modified forms of these domains actually exist in biological membranes is currently controversial [3–6].

2. Experimental evidence for domain formation

A great variety of methods have been applied to determine the presence of domains in both model and biological membranes. Many of these methods have been discussed in a recent review [7]. In the present article, we will briefly comment on some of these methods, particularly in relation to information that can be obtained using DSC.

Imaging methods using fluorescent microscopy are useful in obtaining direct information about the size and molecular composition of membrane domains. However, it must be recognized that imaging using light microscopy has a comparatively low resolution and can only determine domains larger than about 100 nm in diameter. In addition, fluorescent probes must be introduced that may perturb the system. It has recently been shown, by combining confocal fluorescence microscopy with atomic force microscopy (AFM), that fluorescently labeled lipid molecules sequester to different domains than non-labeled ones [8].

A method that does not require the use of large probes and has been applied to the study of membrane domains is NMR.

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NMR is a very versatile method and many variations have been used to study domain formation. One of the early papers in this area determined the complete phase diagram of DPPC and cholesterol using ^{2}H NMR, analyzing the order parameters of the acyl chain [9]. Recent studies have combined deuterium NMR measurements with ^{1}H MAS/NMR to obtain some information about domains in three component systems exhibiting liquid–liquid immiscibility [10]. A different mechanism for domain formation is through cationic substances binding to the surface of membranes containing anionic lipids and sequestering these lipids into domains. The resulting change in headgroup orientation of the phospholipid, as a consequence of the local charge on the membrane, can be used to reveal the presence of domains [11].

Differential scanning calorimetry (DSC) has been extensively used to study domain formation. Many of the applications of DSC in this area have been through the construction of phase diagrams and their interpretation in terms of phase miscibility. We will focus on examples in which the miscibility of components is assessed by changes in the phase behavior upon addition of a peptide or protein. DSC provides certain advantages over other methods used for observing domain formation. The method does not require the introduction of extraneous probes that might perturb the system. It is also insensitive to the size of the domain. Thus evidence for the presence of small domains, not resolvable with light microscopy, can be obtained. However, because of the insensitivity to domain size, except for very small domains, it is generally not possible to calculate the size of the domain. In addition, unlike imaging methods, evidence from calorimetry is indirect.

3. Experimental considerations

There is no ideal way to mix components to measure domain formation in the mixture. The common method to prepare a sample containing more than one component that is insoluble in water is first to mix them in organic solvent, generally a 2:1 (v/v) mixture of chloroform and methanol and then to evaporate the solvent. Since the components are cosolubilized in the same solution, it is likely that they will form a uniform, homogeneous film upon solvent evaporation. However, this is not a certainty, since there can be preferential precipitation of one of the components during solvent evaporation. This is generally a small effect and is characterized by sensitivity of the properties of the mixture to details of sample preparation, such as the rate of solvent evaporation and the temperature at which the solvent is evaporated. More problematic are substances that are soluble in water and cannot be readily incorporated into the lipid film from organic solvent. This is also the case for proteins that might denature in the presence of organic solvents. Some water-soluble substances can be dissolved in methanol and the methanolic solution added to a solution of the lipid in chloroform/methanol without precipitation. In other cases, however, an aqueous solution of the substance is added to the lipid film during hydration of the lipid to maximize the chance of incorporation into the membrane. Further equilibration of these mixtures can be done by several freeze/thaw cycles. The difficulty with these mixtures of membrane components can be that they are very slow to rearrange to an equilibrium state once they are hydrated.

Another consideration is the range of temperatures over which the sample will be scanned. This is also related to the choice of phospholipid component to be used, since it must have a phase transition temperature within the experimental temperature range. High sensitivity DSC instruments must be maintained between approximately 0 and 100 °C with aqueous samples. Often, one wishes to scan only up to an upper temperature significantly lower than 100 °C to avoid irreversible denaturation of proteins, to prevent dissociation of the peptide or protein from the membrane and to avoid sample degradation. It is a limitation of DSC that the behavior of the system cannot be measured isothermally.

DSC can be done in both heating and cooling directions. This is a good check for reversibility. At more rapid scan rates, samples will often exhibit some hysteresis, which may in part be caused by instrumental factors. For transitions close to 0 °C, it is generally easier of obtain scans in the cooling direction to avoid loss of some data caused by the time required to reach a steady state after a scan has been initiated. In DSC, there is also a trade off between the use of slow scans giving better equilibration at each temperature and fast scans giving higher sensitivity and requiring less time.

4. Role of peptides and proteins in the modulation of cholesterol-rich domains

There is much current interest in biology in the possible role of membrane “rafts”. Much of the research in this area with model systems has been with lipid mixtures of phosphatidylcholine containing unsaturated acyl chain, cholesterol and one of the following high melting lipid components: either natural bovine brain or egg sphingomyelin or palmitoyl sphingomyelin or dipalmitoyl phosphatidylcholine. At certain temperatures and compositions, mixtures of these three lipid components exhibit fluid–fluid immiscibility with the formation of domains enriched in cholesterol and the high melting lipid component. However, proteins comprise a major fraction of the mass of biological membranes and they will also affect the distribution of lipids in the membrane. Furthermore, it seems likely that there are a variety of domains in membranes with different chemical compositions and properties. Even domains classified as “rafts” appear to be heterogeneous [12]. The functional roles of “raft” domains have been associated mainly with proteins that bind to the cytoplasmic leaflet of the plasma membrane that has very little sphingomyelin and therefore the driving force for the formation of domains on this monolayer is likely to be different from that observed with the three component lipid mixtures containing a high melting lipid. In order to assess the roles of proteins in the redistribution of cholesterol in membranes, we have measured the effects of peptides on membranes of 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) and cholesterol. As a pure phospholipid, SOPC has a gel to liquid-crystalline phase transition at about 8 °C. In the presence of cholesterol, the temperature of the transition is not greatly

affected, but the melting transition becomes broader and has a lower enthalpy. This behavior is commonly observed with mixtures of cholesterol with several different species of phosphatidylcholine. We can use this system to determine if cholesterol redistributes in the membrane when another component is added. A more non-uniform distribution of cholesterol in the membrane will result in the formation of domains that are depleted of cholesterol, relative to the rest of the membrane. Since cholesterol causes a broadening of the transition and lowering of the enthalpy, the formation of cholesterol-depleted domains will cause this phase transition to sharpen and have a measurably larger enthalpy. This will be the case only if the additive causes the redistribution of cholesterol without greatly affecting the phase transition properties of the cholesterol-depleted domains. This is the case for a peptide segment of the HIV protein that preferentially interacts with cholesterol-rich domains and does not affect the phase transition of pure SOPC or of cholesterol-depleted domains [13]. In cases in which cholesterol redistributes because a peptide binds preferentially to a cholesterol-depleted domain, also favoring the redistribution of cholesterol, the cooperativity and enthalpy of the SOPC transition is not increased [14,15].

Another manifestation of the redistibution of cholesterol in mixtures of SOPC and cholesterol is that cholesterol can pass its limit of solubility in the cholesterol-rich domain. It has been found that cholesterol forms anhydrous crystals when its concentration in bilayers of SOPC exceeds a mol fraction of about 0.6 [16]. It should be noted, however, that the formation of cholesterol crystals is a good criterion for showing the unequal interaction of a substance with cholesterol-rich vs. cholesterol-poor domains; however, both cases will result in the redistribution of cholesterol and the possible formation of cholesterol crystals. Furthermore, if the additive favors cholesterol-rich domains, it will induce the formation of cholesterol crystals only for the cholesterol in that domain that does not interact directly with the additive. An additional consideration is the nature of the cholesterol crystals. Cholesterol can form both anhydrous crystals as well as crystals of cholesterol monohydrate [17]. Anhydrous cholesterol crystals undergo a polymorphic transition at 38 °C on heating and at 22 °C on cooling. This hysteresis is characteristic of anhydrous cholesterol crystals and is a good criterion for its identity. The hysteresis is insensitive to scan rate [18]. In addition to anhydrous cholesterol crystals, cholesterol monohydrate crystals can also form from bilayers of phosphatidylcholine that pass the solubility limit of cholesterol [19]. These crystals are detected calorimetrically by their conversion to crystals of anhydrous cholesterol at about 80 °C. The rehydration of anhydrous cholesterol, even in the presence of excess water, is a very slow process. However, in the case of peptides and proteins that induce changes in cholesterol distribution, heating to over 80 °C can cause denaturation and/or dissociation of the peptide from the membrane. It is therefore often experimentally difficult to accurately assess the amount of cholesterol monohydrate crystals formed using DSC. These crystals, however, can be detected by ^{13}C MAS/NMR or by diffraction.

5. Cationic protein segments induce the lateral segregation of anionic and zwitterionic lipids

There are many examples in the literature where proteins with cationic clusters or divalent cations induce the segregation of anionic and zwitterionic lipids. In particular, the recruitment of phosphatidylinositol (4,5) bisphosphate by proteins with cationic clusters has been demonstrated [20–22]. In addition, there can be more bulk separation of anionic and zwitterionic lipid. DSC can also be used to demonstrate this kind of phase segregation. Such evidence was used to explain the membrane-lytic activity of an anti-microbial peptide [23]. In that work, it was demonstrated that the more potent anti-microbial peptide was more efficient in segregating dipalmitoyl phosphatidylethanolamine from cardiolipin. This resulted in a change of the DSC of the pure lipid from a single broad peak into a curve with two clearly resolved components, one of which corresponded to almost pure dipalmitoyl phosphatidylethanolamine. The cardiolipin component did not exhibit a phase transition above 0 °C, but when mixed with dipalmitoyl phosphatidylethanolamine it lowered and broadened the phase transition of this lipid. This is a general method to look at peptide or protein-induced phase separation, even when one of the phospholipid components does not exhibit an observable phase transition.

6. Summary

Domain formation in biological membranes is an important feature to spatially organize interacting molecules and modify the environment and hence the activity of certain membrane proteins. DSC can be applied to study the rearrangement of lipid components caused by the presence of a protein. Two examples in which this has been done are the redistribution of cholesterol caused by the presence of protein and the rearrangement of lipids as a consequence of electrostatic interactions.

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