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The structure of detergent-resistant membrane vesicles from rat brain cells

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ABSTRACT

The size and the bilayer thickness of detergent-resistant membranes isolated from rat brain neuronal membranes using Triton X-100 or Brij 96 in buffers with or without the cations, K^+/Mg^{2+} at a temperature of either 4 °C or 37 °C were determined by dynamic light scattering and small-angle neutron scattering. Regardless of the precise conditions used, isolated membrane preparations consisted of vesicles of ~100 to 200 nm diameter as determined by dynamic light scattering methods, equating to an area of the lipid based membrane microdomain size of 200 to 400 nm diameter. By means of small angle neutron scattering it was established that the average thickness of the bilayers of the complete population of detergent-resistant membranes was similar to that of the parental membrane at between 4.6 and 5.0 nm. Detergent-resistant membranes prepared using buffers containing K^+/Mg^{2+} uniquely formed unilamellar vesicles while membranes prepared in the absence of K^+/Mg^{2+} formed a mixture of uni- and oligolamellar structures indicating that the arrangement of the membrane differs from that observed in the presence of cations. Furthermore, the detergent-resistant membranes prepared at 37 °C were slightly thicker than those prepared at 4 °C, consistent with the presence of a greater proportion of lipids with longer, more saturated fatty acid chains associated with the *Lo* (liquid-ordered) phase. It was concluded that the preparation of detergent-resistant membranes at 37 °C using buffer containing cations abundant in the cytoplasm might more accurately reflect the composition of lipid rafts present in the plasma membrane under physiological conditions.

1. Introduction

Biological membranes are known to be structurally heterogeneous. Each morphologically-distinct membrane is characterised by its own particular compliment of lipids and proteins. Likewise, the arrangement of the constituents of the membrane is not symmetric and this is most conspicuous with the proteins that are vectorally oriented across the membrane. The polar lipids representing the membrane bilayer matrix are also known to be asymmetrically arranged with zwitterionic and glycolipids located on the extracellular surface and acidic lipids confined largely to the cytoplasmic leaflet. Recently attention has been focussed on the lateral heterogeneity of components within membranes. Clear evidence of lateral phase separation of components is seen in polarised cells where the composition and functions of the apical plasma membrane differ greatly from those of the basolateral membrane. More localised features such as coated pits, membrane junctions and the like, which are associated with particular membrane functions, are formed primarily by proteins dedicated to this purpose. The apparent association of functional lipids with particular membrane proteins such as Ca^{2+}-ATPase [1] and cytochrome oxidase [2] is another example of lateral separation driven by specific protein–lipid interactions.

Another process recognised over the past few years is the creation of lateral domains that act as membrane protein filters to select for inclusion proteins mainly involved in transmembrane signalling processes [3], pathogen entry [4,5] and apoptosis [6]. There is considerable controversy over the existence and nature of these, so called, membrane rafts [7], not least the identity of membrane fractions isolated by detergent treatment purporting to represent these domains.

Studies based primarily on model systems consisting of lipid mixtures dispersed in aqueous media have suggested that these domains exist in a liquid-ordered (*Lo*) state distinct from the surrounding fluid lipid (*Ld*). This has been demonstrated in dispersions of saturated molecular species of phosphatidylcholine and sphingomyelin in the presence of cholesterol where domains of *Lo* phase form in an essentially fluid bilayer structure [8,9]. The formation of *Lo* phase is mediated by cholesterol in the outer leaflet of the cell membrane where the zwitterionic phospholipids are located [10]. Ordered lipid phases consisting of unsaturated molecular species of phosphatidylethanolamine and glucocerebroside have been described [11] and it has been inferred that domains located on the cytoplasmic leaflet align with those on the surface to create a signalling platform containing appropriate protein receptors and effectors. It has been demonstrated that domains of *Lo* phase created in giant unilamellar

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vesicles survive brief exposure to non-ionic detergents which solubilize the remaining membrane components allowing the detergent-resistant membrane (DRM) fraction to be isolated [12]. To address critical questions regarding the size, composition and arrangement of components in DRMs, a reliable isolation method is a key step. The detergent treatment approach is known to problematic as, for example, in model systems where it has been shown that detergents can selectively solubilize certain types of lipids, generating pseudo-DRMs [13]. Nevertheless, detergent-free extraction procedures that have been developed to date also present difficulties. For example, the detergent-free methods require operations performed at 4 °C which is known to induce *Lo* phase and create lateral domains in membranes that are not observed at physiological temperatures. This study tests the effect of temperature and detergent type on the structure of the surviving membrane preparations. Our optimised protocol of detergent treatment with Brij-96 at 37 °C demonstrated that the vesicles retain their original asymmetry and can be separated into populations by immuno-adsorption methods which possess either Thy-1 or PrP antigens confirming that components confined to distinct domains on the cell surface remain segregated after treatment with Brij-96 [14].

The transfer of techniques established in model systems to biomembranes is fraught with difficulties. The structures formed by polar lipids in dispersions are essentially symmetric structures so that mixtures consisting of detergent-soluble and insoluble components are thought to resolve into a soluble fraction and DRMs formed through a reassembly of the insoluble lipids into symmetric bilayers. Under more carefully controlled conditions of detergent treatment it has been shown that, in giant unilamellar vesicles, domains of *Lo* phase pinch off as small vesicles that remain intact while the fluid bilayer dissociates into detergent-stabilized micelles [12]. In this case there is a reasonable expectation that the detergent-resistant vesicles have components that retain their original disposition in the membrane.

The successful isolation of membrane lipid rafts relies on formation of vesicles that represent domains of the parent membrane in their original orientation. Our strategy for isolating membrane rafts is to employ detergent treatments under carefully controlled conditions and to judge success by biochemical and structural analyses [14,15]. In deciding on the conditions of detergent treatment we were mindful that the existence/lifetime of the *Lo* phase is temperature dependent and using conventional methods of treatment at 4 °C may produce DRM fractions that do not exist at physiological temperature. In addition, the cytoplasmic leaflet of biological membranes is replete with acidic lipids that are more fluid in nature and more sensitive to environmental change such as the ionic composition. The use of a buffer containing physiological concentrations of K^+ and Mg^{2+} was reasoned to mimic more precisely the ionic environment of the cytoplasm, potentially protecting the cytoplasmic leaflet of the membrane. Finally, the extracellular environment is invariably oxidising while the cytoplasm is maintained in a reducing state and for this reason a reducing agent is included in buffers used for detergent treatment.

In addition, two non-ionic detergents were examined in the present study for their ability to form DRMs, namely Triton X-100 and Brij 96. Triton X-100 was studied because, although it has variously been reported to disrupt membrane structure [16] and mix DRMs from different membranes [17] it is still widely used to prepare DRMs. Brij 96 was of particular interest in the present study because it has been reported that this surfactant is more protective of the membrane structure in neuronal tissues than Triton X-100 [17] and enables separation of DRMs with lipid composition distinct from the parent membranes [18].

Previous electron microscopic studies, have shown DRM preparations isolated under conventional conditions to be mostly membrane vesicles, or vesicles contained within bigger vesicles, although tubular structures with open ends have also been observed [15,17,19]. Replicas of freeze-fractured DRMs when examined under the electron microscope show membrane planes devoid of membrane-associated particles, consistent with the finding that the proteins in DRMs are mainly GPI-anchored proteins like Thy-1 and PrP of neuronal cells rather than intrinsic membrane proteins that require a rearrangement of membrane lipids to package them into the lipid matrix. Although electron microscopic studies are important in the study of DRM structure, scattering methods offer a number of significant advantages in addition to defining structural parameters including vesicle size distribution, shape and thickness in a non-invasive manner and in an environment more closely mimicking the physiological state. In the present study dynamic light scattering (DLS) has been used to determine the size of the DRM vesicles while small-angle neutron scattering (SANS) was used to assess the bilayer thickness, and where appropriate the *d*-spacing of the stacked DRMs.

2. Materials and methods

2.1. Preparation of detergent resistant membranes (DRMs)

Rat brains of mixed strains, sex and ages were homogenized (Dounce, SLS, UK) at 4 °C in different solubilization buffers; namely standard solubilization buffer (SB) pH 7.4 (250 mM sucrose, 10 mM TrisHCl, 1 mM EGTA) or intracellular solubilization buffer (ISB) pH 7.6 (200 mM sucrose, 10 mM Hepes-KOH, 50 mM KOAc, 1 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 1 mM EGTA). Protease inhibitor cocktail tablets (Roche, UK) and 1 mM phenylmethylsulphonyl fluoride were added fresh to each buffer. All chemical reagents used were of the highest grade available from Sigma-Aldrich (UK) or BDH (UK) and used as received.

Total membrane fractions were obtained by centrifugation of the homogenates at 30,000 $\times g_{av}$ for 40 min at 4 °C. The supernatant was discarded and the top layer of the pellet, i.e. the total membrane fraction, (the bottom layer was comprised of nuclei and cell debris) was carefully re-suspended, washed with homogenizing buffer and the protein concentration determined using a BioRad protein assay kit (BioRad, UK). The protein concentration of the total membrane fraction was adjusted to 5 mg/mL.

Samples of the total membrane fractions were depleted in myelin. This depletion was accomplished by loading the total membrane fractions, isolated as above, on top of a 15–50% sucrose step gradient made in the appropriate homogenizing buffer and centrifuging (Beckman Ultracentrifuge, UK) at 300,000 $\times g_{av}$ for 1 h at 4 °C. Myelin, which floats above 25% sucrose, was discarded. Western blotting of myelin associated glycoprotein (MAG) antibody showed that the heavy membrane fractions, collected at the interface between 30% and 50% sucrose contained little myelin; this heavy membrane fraction was used to isolate DRMs.

The total membrane fractions (with and without myelin depletion) were subjected to treatment using either of the two detergents, namely Triton X-100 (CalBiochem, UK) or Brij 96 (Fluka, UK) dissolved in either SB or ISB at 4 °C for 15 min or at 37 °C for 5 min. The concentration of Triton X-100 used for membrane solubilization was 1% w/v while 0.5% w/w Brij 96 was used. The solubilized membrane fractions were diluted 1:1 with 80% sucrose in the relevant buffer, loaded at the bottom of the centrifuge tubes, overlaid with a linear sucrose gradient of 5% to 30% sucrose and centrifuged (Beckman Ultracentrifuge) at 250,000 $\times g_{av}$ for 18 h at 4 °C.

Sequential aliquots of the gradients were collected from the top of the centrifuge tubes and analyzed by Western blotting using antibodies to two GPI-anchored proteins, namely Thy-1 (OX7, [20]) and PrP (SAF32, SPI-BIO, France). Fractions recovered from the gradients of between 10% and 25% sucrose were found to be enriched in both Thy-1 and PrP, and were combined to produce DRMs.

2.2. Dynamic light scattering

The diffusion coefficient of the DRMs was routinely determined using a BIC Zetaplus sizer (Brookhaven Instruments Corp, USA) fitted

with a 5 mW He–Ne laser operating at a wavelength of 677 nm at a measurement angle of 90° and a temperature of 25 °C. Prior to measurement, the vesicular samples were diluted by a factor of 10–20 with buffer and filtered (200 nm polycarbonate Millipore filters). At these concentrations the sizes of the vesicles recorded were independent of concentration. The measured vesicle diffusion coefficient was converted to a hydrodynamic size using the Stokes Einstein equation assuming spherical vesicles and using the measured viscosity (calibrated (British Standard) Cannon-Manning Viscometer) of the final suspending medium, which contained about 2% sucrose. The distribution of sizes in the preparation was determined using multimodal analysis. The diffusion coefficient of filtered (100 nm polycarbonate Millipore filters) micelles of Brij 96 and Triton-X 100 was determined using a ALV DLS5000 light scattering instrument (GmbH Company, Germany) using the goniometer stage from 30° to 150° in every 10°, and a wavelength of 633 nm.

2.3. Analysis of DRM size distribution by filtration and Western blotting

In order to determine whether the structures within μm size range detected in DRM fractions by DLS contained any DRM markers, the DRMs were also subjected to filtration through 450 nm polycarbonate Millipore filters under the application of gentle pressure with a syringe. It was assumed that this process did not result in any change in vesicle size distribution. The presence or absence of the raft marker proteins Thy-1 and PrP was determined by Western blotting of the DRMs before and after filtration.

2.4. Small-angle neutron scattering

SANS measurements were performed at 25 °C on the LoQ beam line at the ISIS pulsed neutron source (Rutherford-Appleton Laboratory, Didcot, UK). DRMs for SANS measurements were obtained from several DRM preparations, which were pooled and centrifuged at 45,000 $\times g_{av}$ for 2 h, after which time the pellet was washed twice with D_2O (99%, Sigma) containing 200 mM sucrose. DRMs floated to the top of the solution because of the higher density of D_2O (1.12 g/mL) and as a consequence the DRMs were carefully aspirated using a syringe. D_2O recovered from a second wash was used as the blank. The aspirated membranes were resuspended in D_2O-sucrose to give suspensions of protein concentrations of about 2 mg/mL and 0.2 mg/mL. DRMs were placed in scrupulously cleaned disc-shaped fused silica cells of 2 mm path length and neutron scattering intensity measured at the two concentrations to ensure the absence of inter-particulate interactions.

The SANS intensities, $I(Q)$ – recorded as a function of the scattering vector, Q ($Q=4\pi\sin(\theta/2)/\lambda$) – were normalised according to sample transmission, and corrected by application of an instrument resolution function. Backgrounds from D_2O recovered in the second wash of DRMs were subtracted as appropriate. All fitting procedures included flat background corrections to allow for any mismatch in the incoherent and inelastic scattering between the sample and solvent. Fitted background levels were always checked to ensure that they were of a physically reasonable magnitude (for the various systems studied here, lying in the range 0.01–0.03 cm^{-1}).

Given the Q range employed in these SANS experiments – and given that the vesicle diameters were generally larger than 100 nm – the vesicles were approximated as randomly oriented infinite planar sheets of thickness τ, for which the scattering is [21,22]:

$$P(Q) = 2\pi(\Delta\rho)^2 S\tau^2 \cdot 1/Q \cdot \sin^2(Q\tau/2)/(Q\tau/2)^2$$

where $\Delta\rho$ is the neutron scattering length density difference between sheet and solvent and S is the area of sheet per unit sample volume [21,22]. Since the scattering is only significant in the direction normal to the sheet, this sheet model can then be extended via simple one-dimensional Fourier transforms.

In those cases where the vesicle suspensions consisted of a mixed population of unilamellar and multilamellar vesicles, the SANS data were modelled assuming the presence of (isolated) infinite planar sheets as well as one-dimensional paracrystals (or stacks) of these [21–23]. The scattering from individual layers was modelled as described above with successive layer spacings chosen at random from a Gaussian distribution. The structure factor for the paracrystal has parameters: M, the number of layers in the stack; D, their mean separation; and $\sigma(D)/D$, the width of the Gaussian distribution in the plane positions [23]. In the case of unilamellar vesicles, the lamellae were treated as sheets of uniform scattering length density, with a mean lamellar thickness of L, and a Schultz polydispersity characterised by $\sigma(L)/L$. The model fits were performed using the FISH software [24] — with least squares refinement of the seven parameters, L, $\sigma(L)/L$, M, D and $\sigma(D)/D$, together with the absolute scale factors for the unilamellar and multilamellar vesicles. L and $\sigma(L)/L$ were constrained to be the same for the isolated and stacked lamellae.

Scattering scaled with protein concentration suggesting the absence of any significant inter-vesicular interactions under the conditions of the study. Therefore both protein concentrations were fitted using the same model parameters but with adjustment of the scale factors for the unilamellar and mutilamellar vesicle components by the protein concentration.

3. Results and discussion

3.1. Size of DRMs

The rationale for characterising the size of DRM vesicles is that the surface area of the vesicles, based on direct observations of the detergent action on giant unilamellar vesicles [12], is related to the raft domain area of the parent membrane. The size of the DRMs prepared from total membrane fractions obtained from rat brain were routinely characterised using DLS together with multimodal analysis. Preliminary dynamic light scattering studies showed that, at a given temperature, there was no significant difference in the hydrodynamic size of micelles formed by Triton X-100 and Brij 96 in either of the two buffers used for DRM preparation. From this it may be inferred that the differing effects of the two buffers on the membrane is responsible for differences in the DRM preparations rather than any action of the buffers on the detergents. The results of a size analysis study are given in Table 1 presented as the relative number of particles present in the most frequent size range. The values shown in Table 1 are typical of those obtained from many repeat preparations of DRM. Despite the polydispersity of size of the DRM preparations, it can be seen that, regardless of the method of preparation, the majority of DRMs (generally between 97 and 99% of the particles present) were in the size range 130–380 nm. The other few percent of the particles were larger and in the μm size range. Unfortunately DLS can not be used to determine the structure of the DRMs. However as electron micrograph studies of the morphology of DRMs prepared from erythrocyte membranes [19] and rat basophilic leukaemia cells [16] have concluded that most structures present were vesicles, it is likely that

Table 1
Range of diameters of DRMs determined by DLS

Sample	Temperature	Size range of major population/nm	Relative population size/%
Tx100, SB	4 °C	140–200	99
	37 °C	130–190	98
Tx100, ISB	4 °C	220–380	97
	37 °C	130–240	99
Brij 96, SB	4 °C	170–250	92
	37 °C	130–190	97
Brij 96, ISB	4 °C	200–340	99
	37 °C	160–240	98

the DRMs in the present study were also predominately vesicular in nature.

From Table 1 it can be seen that the size of DRMs prepared at 4 °C tended to be slightly larger than those prepared at 37 °C. This small difference in size could be due to the fact that the liquid-ordered (*Lo*) domains present in the parental membrane are larger when detergent-resistant membranes are prepared at 4 °C rather than at 37 °C. Another factor to be considered is that, because the aqueous solubility of the detergents decreases with increasing temperature, i.e. the detergent becomes more hydrophobic at higher temperatures, the detergent micelles increase in size with temperature, forming larger micelles which may not solubilize the various components of the biomembrane as readily [25]. It may also be inferred that the difference in size of the DRMs isolated by treatment at the two preparation temperatures is due, in part, to differences in micelle size/structure and to membrane fluidity. Buffer composition also did not significantly affect vesicle size in as far as DRMs prepared using the ISB buffer were only slightly larger than those prepared using the SB buffer. The effect of buffer on size, was most pronounced for DRMs made at 4 °C, although these differences were small and probably not significant given the polydispersity of vesicle size.

From the DLS results there appears to be no obvious effect of detergent type on the size of the DRMs prepared under identical conditions. It has been previously reported [15] that DRMs prepared at 4 °C using Brij 96 were similar to those formed by Triton X-100. We could not detect any significant difference in size between the DRMs prepared using these two surfactants in the present study.

Myelin has been observed in electron micrographs of DRMs and is known to be of μm dimensions. To establish whether the larger particles observed in the light scattering study were myelin, DRMs were prepared under conditions of myelin depletion. Myelin is a problem when preparing DRMs from neuronal cells because it co-floats with the DRM fraction in sucrose density gradients due to its relatively low density. In these experiments, Brij 96 was used to obtain the myelin-free DRM. Table 2 gives the size of the myelin-depleted DRMs. Even in these myelin-depleted DRMs there were, however, still some particles present in the μm range. As can be seen the average size of the DRMs tended to decrease regardless of the precise conditions used for their isolation. This may be due to a more efficient solubilization of the parental membrane in the absence of the lipid-rich myelin. Indeed it is known that myelin is more difficult to solubilize than neuronal membranes due to their higher lipid content [26]. These studies suggest that myelin is probably present in the DRM preparations but it represents only a relatively small fraction (typically less than a few percent) of the total number of particles present.

To further test whether the small number of larger particles present in the DRM fraction were myelin, DRMs (non-myelin depleted) were passed through a 450 nm polycarbonate membrane filter (larger than the largest DRM reported using laser light scattering) using gentle hand-applied pressure so as not to break up the myelin and ensure that any myelin particles are retained on the filter. From experience of liposome preparation, it was considered unlikely that the DRM are reduced in size during passage through the filter. The amounts of the raft markers, Thy-1 and PrP, present in the DRM preparations, before and after filtration, were assessed by Western blotting (Fig. 1). From inspection of Fig. 1 it can be seen that, upon comparing the intensities of both raft markers, their presence in both DRM preparations did not change significantly, suggesting that Thy-1 and PrP resided in the DRMs of size smaller than the 450 nm diameter fraction.

Table 2
Range of diameters of myelin-depleted DRMs determined by DLS

Sample	Temperature	Size range of major population/nm	Relative population size/%
Brij 96, SB	4 °C	110–140	98
	37 °C	130–190	99
Brij 96, ISB	4 °C	120–200	98
	37 °C	120–180	99

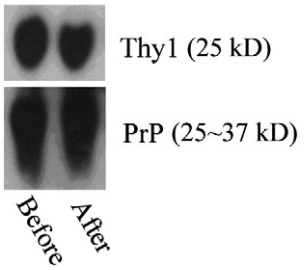

Fig. 1. Comparison of the relative amounts of Thy-1 and PrP before and after filtration through a 450 nm polycarbonate membrane by hand pressure with the aid of a syringe. Light density fractions were taken from gradient made from ISB at 37 °C with 1% Triton X-100. Total membrane preparation without myelin depletion was used.

3.2. Bilayer structure

The internal structure of DRM, prepared using the various preparation conditions was investigated using SANS, along with that of their parental membranes. Note that because the average size of the DRM (as determined by DLS) were greater than 100 nm it was not possible to determine their size using SANS. One consequence for neutron scattering of their large size was that it was possible to model the DRMs as single sheets (or bilayers) and/or stacks of sheets. Using this approach in analyzing the SANS data meant that, while it was possible to show that the DRM consisted of lipid bilayers, it was not possible to prove (using SANS) that the DRM were in the form of vesicles. As the DLS experiments showed that the amount of myelin in the DRM preparations was low, any contaminating myelin was not expected to contribute significantly to neutron scattering intensity from the sample. Reassuringly for each preparation tested, the I_0 of the original DRM sample and its diluted form scaled by a factor of 10 (data not shown). The same parameters could be used to fit the SANS data for the original and diluted DRMs, with the exception of the absolute scale factors for the unilamellar (sheet) and multilamellar structures (stack), which scaled with concentration. The SANS data and the corresponding model fits obtained for the original DRMs and the total (parental) membrane are shown in Fig. 2, and the best fitted values of the model parameters are presented in Table 3.

Several observations can be made from these results. Firstly, the architecture of the crude extracts of the total rat brain membrane obtained in the two buffers was almost identical with bilayer thickness about 4.5 nm, and d-spacing about 7.2 nm. Secondly the amount of stacks present in the sample relative to sheets was relatively low. This observation was not surprising, as unlike the DRMs, the parental membrane preparations had not been exposed to the high-speed centrifugation step, which was considered to increase the likelihood of the formation of oligolamellar structures [19].

Interestingly the bilayer thickness of all the DRM preparations ranged from 4.6 to 5.0 nm, varying only slightly with the different isolation conditions. The fact that the thickness of the DRM bilayers were slightly greater than those of the parental membranes is probably a consequence of the fact that the DRMs consist primarily of lipids in an *Lo* phase whereas the parental membrane contains lipids both in an *Lo* and a fluid phase, an observation supported by studies investigating model membrane systems [27] which have reported that the *Lo* phase, as a consequence of the longer, more saturated hydrocarbon chains present, has a greater thickness than fluid phase bilayers. Although, because of the complexity of actual biomembranes and the fact that both the *Lo* phase and the *Ld* phase

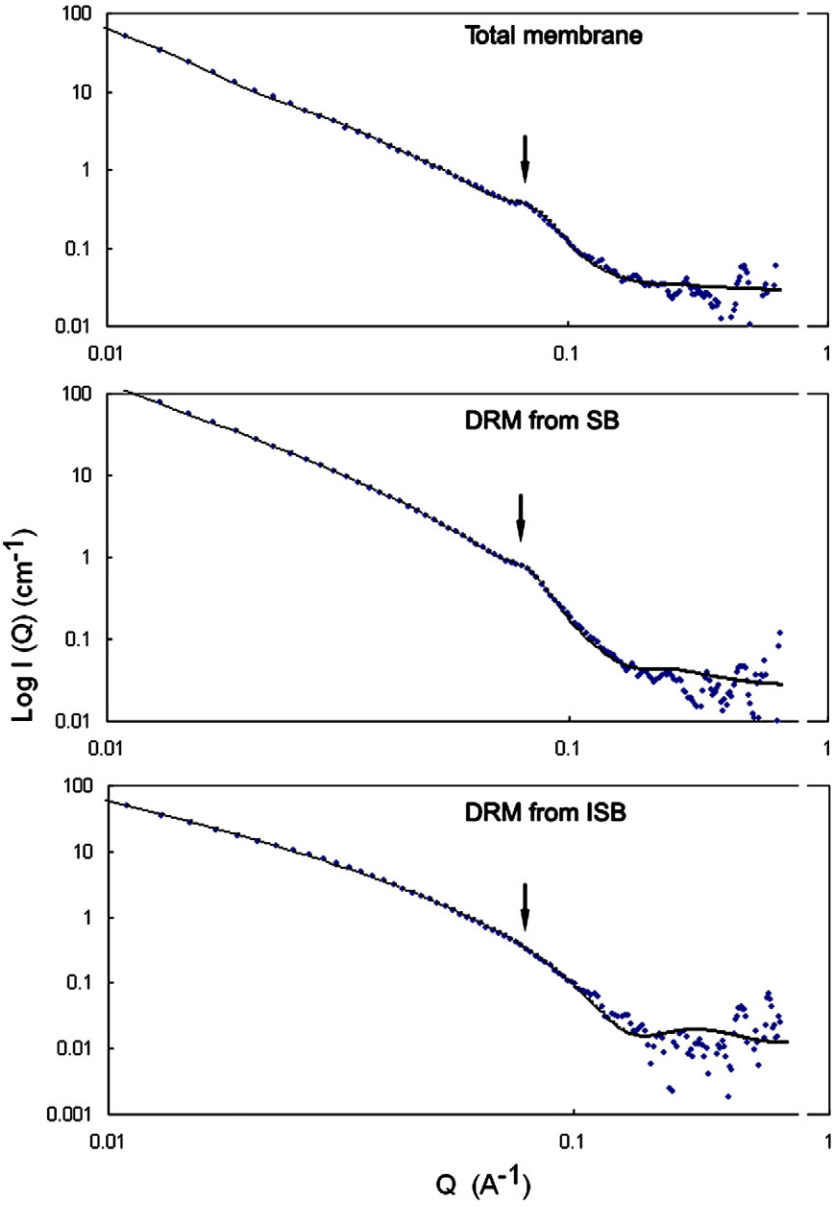

Fig. 2. SANS intensity curves recorded from suspensions of total membrane homogenate and DRMs prepared using Brij 96 at 37 °C with SB or ISB. Decreased amount of multi-lamellar structure in DRMs from ISB was observed. Arrows indicate the Q-spacing corresponding to the d-spacing of stacked membranes.

are composed of many molecular species of lipids, the actual difference between the two phases may not be as obvious as that seen in more simple model systems. It can also be observed from Table 3 that DRMs prepared at 37 °C were slightly thicker than those prepared at 4 °C, suggesting the presence of a greater amount of lipids with longer, more saturated fatty acid chains associated with the *Lo* phase. This observation implies that the detergent micelles are less able to solubilize *Lo* phase lipids at a temperature of 37 °C than 4 °C.

Another interesting observation is the fact that when SB was used to isolate the DRMs a high percentage of the lipid present was in the

Table 3
Structural parameters of parental cell membranes and DRMs prepared under various conditions (derived from FISH model fitting of their SANS data)[a]

	Sample	$L\times10$/nm	$\sigma(L)\times10$/nm	M	$D\times10$/nm	$\sigma(D)\times10$/nm	Stack:bilayer
Total membrane	SB	45.0 (0.5)	0.20 (0.04)	4 (0.01)	71.7 (0.6)	0.05 (0.04)	1:4
	ISB	45.3 (0.4)	0.14 (0.03)	4 (0.02)	72.5 (0.6)	0.05 (0.03)	1:4
DRMs Brij 96, 4 °C	SB	46.4 (0.3)	0.15 (0.02)	4 (0.02)	76.1 (0.9)	0.05 (0.05)	1:12
	ISB	45.7 (0.3)	0.23 (0.02)	0	–	–	–
DRMs Brij 96, 37 °C	SB	49.7 (0.2)	0.18 (0.01)	6 (0.08)	76.4 (0.4)	0.11 (0.01)	1:6
	ISB	46.4 (0.4)	0.10 (0.05)	0	–	–	–
DRMs Triton X-100	4 °C, SB	48.0 (0.6)	0.23 (0.03)	6 (0.01)	75.6 (0.3)	0.04 (0.02)	1:2
	37 °C, ISB	48.5 (0.3)	0.17 (0.02)	0	–	–	–

[a] SANS measurements for all samples were recorded at 25 °C. Parameters: L: bilayer thickness, $\sigma(L)$: polydispersity on bilayer thickness, M: number of lamellae in bilayer stack, D: d-spacing in bilayer stack, $\sigma(D)$: polydispersity on d-spacing, stack:bilayer: ratio of oligolamellar:unilamellar vesicles in sample. Figures given in parentheses indicate the standard errors on the fitted parameter values (derived from the least-squares variance–covariance matrix).

form of stacked bilayers. This is shown by the existence of a d-spacing, the value of the average number of bilayers in the stack (M) and the ratio of stacked/planar bilayers. The amount of lipid in the form of a stack was greatest when Triton X-100 was used to prepare the DRMs. It had been anticipated that membrane stacking of the DRMs may occur due to the use of centrifugation to pellet the DRMs as it has been observed in X-ray studies that prolonged ultracentrifugation orients DRMs into stacked lamellar arrays [19]. The d-spacing observed in the DRMs prepared using SB was considerably greater than that seen in the total membrane fraction from which the DRMs were prepared, i.e. ~7.6 to 7.2 nm. This difference could be due to a selective enrichment of particular lipids and/or proteins in the DRM fraction compared to the parent membrane. For example, it known that glycosphingolipids and GPI-anchored proteins, nearly all of which are known to be glycoproteins, are enriched in DRMs [28]. The highly hydrophilic nature of the large carbohydrate head groups may result in greater hydration forces of repulsion between bilayers than would be the case for phospholipid head groups and non-glycoproteins.

A striking feature in Table 3 is that there is no evidence of stacked bilayers when ISB was used to isolate the DRMs, irrespective of the temperature of preparation. The reason for this is not clear at present. It is well known that the balance between the attractive and repulsive forces of the membrane bilayers determines whether membrane stacks are formed, and that these forces are affected by the composition of both the buffer and the membrane. It is clear from our studies that the nature of the buffer influences the formation of membrane stacks, although it is noteworthy that the two buffers produced no obvious difference between the isolated parental membranes prepared in respect to their ability to form stacked arrays. The formation of single bilayer DRMs using the ISB is particularly encouraging, especially as the ISB is believed to be more protective of membrane structure that Triton X-100.

4. Conclusions

The structural characteristics of a range of DRM preparations have been examined using a combination of light and small-angle neutron scattering methods in order to develop optimal conditions for the isolation of DRMs. The use of light scattering did not reveal any differences in the various DRMs due to the high polydispersity of DRM size. However, when ISB, a buffer protective of the cytoplasmic leaflet lipids of the membrane, and detergent treatment at 37 °C were used almost all the DRMs were in the size range 130 to 240 nm. Assuming the DRMs are spherical and predominately unilamellar in nature, their surface area matches the predicted domain size of rafts on the biological membrane [29]. DRMs prepared at physiological temperatures were slightly smaller in size compared with those prepared at 4 °C.

SANS was used to determine the internal structure of the DRMs. Although SANS studies have been previously reported investigating the *Lo* phase, they have all been performed using model membrane systems. This study represents the first SANS examination of DRMs prepared from biological membranes and several meaningful observations can be made. Compared with the parental membranes, DRMs possessed slightly thicker bilayers and when isolated in SB, greater d-spacings. Furthermore, DRMs isolated from biomembranes at physiological temperatures rather than conventionally at 4 °C were slightly thicker than those isolated from membranes at lower temperatures. Taken together these observations suggest that DRMs isolated at a higher temperature contain a greater amount of *Lo* phase lipid. The greater d-spacing observed when SB was used implies a possible enrichment in the DRMs of gangliosides and GPI-anchored proteins, resulting in greater repulsive forces between adjacent biomembranes. Indeed the presence of relatively small amounts of lipid containing large hydrophilic polyoxyethylene head groups in artificial bilayers has been shown to increase the d-spacing compared to bilayers containing only phospholipids [29,30]. These observations suggest that 37 °C is the preferred temperature for preparation of DRMs.

Most significant, however, was the observation that the tendency of DRMs to form stacked arrays depended on the buffer used for their isolation with DRMs prepared in ISB (a buffer that mimics the cytoplasmic environment) being only unilamellar in nature and therefore more representative of the state of the membrane *in vivo*. This is an interesting result that implies that the constituents of the DRMs or their arrangement within the DRMs are different. As DLS analysis failed to reveal any significant structural changes of micelles in different buffers, the tendency of DRMs isolated in the different buffers to form stacked arrays was most likely due to the effect of buffers on the biomembranes *per se*. This effect needs to be explored in greater detail and may be a consequence of the effect of cations (Mg^{2+} and K^{+}) and reducing agent DTT in the ISB on the biomembrane. The use of ISB as the preferred buffer to prepare DRMs is supported by our earlier observations that DRM fractions prepared using ISB contained lipids and proteins associated with the cytoplasmic surface of the membrane [14,15] and may more accurately reflect the original structure of lipid-based microdomains in biomembranes.

From the present study, although it is not possible to say whether Brij 96 or Triton X-100 produces DRMs more representative of the rafts present in intact biomembranes, there is evidence to suggest that the preparation of DRMs at 37 °C using ISB may better reflect the structure of the rafts under physiological conditions.

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