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STRUCTURAL COMPARISONS OF NATIVE AND REAGGREGATED MEMBRANES FROM *MYCOPLASMA LAIDLAWII* AND ERYTHROCYTES BY X-RAY DIFFRACTION AND NUCLEAR MAGNETIC RESONANCE TECHNIQUES

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SUMMARY

1. Reaggregates of Mycoplasma membranes after dispersion in sodium dodecyl sulphate were indistinguishable structurally from native membranes by the criteria of composition, density, and electron microscopy.

2. The X-ray diffraction patterns show that a lipid bilayer structure is reformed in the reaggregates, although the extent of the bilayer regions is probably less than in the native membranes.

3. Probe experiments using nuclear magnetic relaxation measurements confirm that there are extensive interactions between the membrane components which exclude some of the binding sites for the probe molecules that are exposed in the separated lipid and protein components. Nevertheless, the reaggregated structures are readily distinguishable from the native membranes by this probe technique, which indicates that at least some of the membrane proteins must be incorrectly reassembled.

4. The Mycoplasma membranes and their separated components are compared with similar preparations from erythrocyte membranes. The pattern of responses of both membrane systems to perturbation by benzyl alcohol as reported by the relaxation measurements is very similar. The data imply that the membranes have a common element of structural organisation.

INTRODUCTION

There are several reports in the literature that membranes which have been substantially separated into their lipid and protein components can reaggregate under appropriate conditions to structures which are similar to the native membranes[1–7]. The most extensively studied reaggregates are of Mycoplasma membranes dissolved in 10 mM sodium dodecyl sulphate, and subsequently reaggregated by dialysis against a buffer containing Mg^{2+}. However, only limited structural criteria were available to

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compare the native and reaggregated membrane structures. The original evidence that the reaggregates resembled the intact membranes was obtained from electron micrographs which showed that characteristic triple layered structures were reformed in the aggregates[4,8]. More recently RAZIN *et al.*[9] have examined the factors which determine the lipid:protein ratio in the reaggregated structures and the conditions required to obtain the same composition as the native membrane. These reaggregates also have the same buoyant density as their parent membranes, and if hybrid reaggregates are prepared from Mycoplasma strains with different densities, the hybrids have intermediate densities appropriate to their composition[10]. Here we have extended these criteria to more stringent tests of structural organisation[11] based on X-ray diffraction and nuclear magnetic resonance (NMR) techniques.

X-ray diffraction techniques have recently been developed for the examination of random dispersions of membranes[12]. Here we have examined the thermal phase transition which occurs in Mycoplasma lipids in both intact and reaggregated membrane structures[13,14]. Analysis of the low-angle X-ray diffraction bands arising from the lipid bilayer in the membranes above and below the thermal transition enables the extent to which the lipid bilayer structure is restored in the reaggregates to be estimated.

For the NMR experiments we have selected benzyl alcohol as the probe molecule, which has previously been studied in detail in erythrocyte membranes[15,16]. With increasing concentrations of alcohol in the presence of erythrocyte membranes there is a biphasic change in the relaxation rate of the bound alcohol molecules, which can be directly related to the structural perturbations induced in the membrane. This biphasic response is characteristic of a range of intact membranes[17] and is reported here for the Mycoplasma membrane. The intact Mycoplasma membrane is compared with the reaggregated structure and also with similar experiments for erythrocyte membranes.

MATERIALS AND METHODS

(*a*) *Mycoplasma membranes*

Mycoplasma laidlawii, strain B (PG 9) was grown in static 37° cultures on the medium of RAZIN *et al.*[8]. The organisms were harvested by centrifugation at 10000 × *g* for 30 min in the late logarithmic growth phase as estimated from turbidity measurements ($A_{650\,nm}$ = 0.2–0.4; terminal $A_{650\,nm}$ = 0.6–0.7). The membranes were isolated by osmotic lysis and washed 3 times by cyclic centrifugation and resuspension in 1/20 β-buffer (β-buffer is: 0.156 M NaCl, 0.05 M Tris, 0.01 M 2-mercaptoethanol, pH 7.4).

Reaggregated membranes. Membranes in 1/20 β-buffer at a concentration of approximately 4 mg protein per ml were dissolved by bringing the suspension to 10 mM sodium dodecyl sulphate at room temperature. After 0.5 h the solubilised material, which consists of dissociated lipid and protein, is centrifuged at 100000 × *g* and the small pellet of black material is discarded (this material is present in the growth medium). The clear supernatant is dialysed against 3 changes of 1/20 β-buffer over a period of 3 days at 4°. The dialysate becomes somewhat turbid as reaggregation proceeds, but the full turbidity of the original suspesion is not recovered. The reaggregated structures are known to be smaller than the native membranes by a factor

of 2–10 times, and contain residual sodium dodecyl sulphate, which is not more than 0.5 % of the membrane dry weight.

Separated lipid and protein fractions. Lipids were extracted from the Mycoplasma membranes using 90 % acetone (v/v) at 4°. After stirring for 1 h at room temperature the protein was centrifuged at 5000 × *g* for 30 min and the supernatant acetone solution containing the lipid was decanted. The acetone was removed under vacuum in a rotary evaporator and the lipid was resuspended in the required buffer solution by agitation. The protein pellet was washed with 90 % acetone, resuspended in 1/20 β-buffer, and the residual acetone removed by dialysis against the same buffer solution.

During NMR experiments this protein suspension rapidly became clumped in the spinning sample tube, which caused large variations in the observed line widths. A more dispersed preparation of the membrane protein was prepared by a butanol separation procedure similar to the method described by MADDY[18] for erythrocyte membrane proteins, as modified by REGA *et al.*[19]. Over 95 % of the Mycoplasma membrane protein is obtained in aqueous solution and is stable under the NMR experimental conditions.

(*b*) *Erythrocyte membranes*

The preparation of erythrocyte membranes by a modification of the method of DODGE *et al.*[20] and the separation of the lipid and protein components by butanol are described in detail elsewhere[21].

Reaggregated membranes were prepared by a procedure due to ZAHLER[22] in which the original membranes are dissolved in methyl cellosolve ($HO \cdot CH_2CH_2OCH_3$) at pH 2 and allowed to reaggregate by dialysis against an aqueous 10 mM Tris buffer, pH 7.4, at 4°.

(*c*) *X-ray diffraction*

Membrane samples were concentrated by centrifugation and sealed in thin-walled glass capillaries 1 mm in diameter. The capilliaries were mounted in a temperature controlled specimen chamber on an Elliot toroidal point-focussing X-ray camera. Exposures of 2–12 h were taken at various temperatures. In several instances the initial exposure conditions were repeated after a series of exposures and no changes in the initial and final patterns were detected. Films were measured with a travelling microscope and a Joyce–Loebl microdensitometer.

(*d*) *Nuclear magnetic resonance*

Benzyl alcohol in a ^2H_2O buffer containing 45 mM NaCl, 30 mM sodium acetate, 5 mM sodium phosphates and 1 mM NaN_3; pH 7.4 was used in all experiments. Line width measurements of the phenyl protons were made on a Varian 100-MHz NMR spectrometer at a sweep speed of 0.4 Hz/sec and at a radiofrequency power level which caused no detectable saturation. Experiments were performed at 25° unless otherwise stated. The concentration of the membrane preparations was generally adjusted to 1.0 % (w/w) except for samples of membrane protein, reaggregated membranes, and membranes pretreated with a lytic benzyl alcohol concentration, where it was convenient to use lower sample concentrations which caused less broadening of the phenyl resonances. From the measured line widths of these samples, values were calculated for 1.0 % (w/w) sample concentrations by assuming a linear relationship between

resonance broadening and sample concentration. This has been rigorously established elsewhere for all the erythrocyte membrane preparations over the sample concentration ranges used in these experiments[23]. The line width of the phenyl resonance of benzyl alcohol free in solution was taken as 0.70 Hz, and measurements were corrected for instrumental broadening from the measured line widths on the internal acetate reference in each sample. These did not differ by more than 0.1 Hz from the acetate line width in the standard ^2H_2O buffer in the absence of membrane samples. The line widths in different membrane preparations may then be compared directly at a standard sample concentration of 1.0 % (w/w).

For some experiments, membranes (native and reaggregate) were pretreated by dialysis against 300 mM benzyl alcohol in standard buffer until equilibrated. The alcohol was then removed by dialysis against the same buffer until the residual alcohol concentration was less than 1 mM.

RESULTS

(*a*) *Electron microscopy*

Sectioned and positively stained samples of the Mycoplasma membranes and reaggregates used in these experiments both showed triple-layered structures confirming previous observations on similar preparations by RAZIN *et al.*[8] and ENGELMAN *et al.*[3]. On this evidence alone it would not be possible to distinguish the ultrastructure of intact and reaggregated membranes with any confidence. Similarly it was not possible to distinguish the membranes and reaggregates structurally by negative staining with ammonium molybdate, although it was clear that the reaggregates were considerably smaller than the intact membranes. This is consistent with their relative light scattering properties. On the other hand the reaggregates are readily distinguished from similarly stained preparations of the separated lipid and protein fractions so that the microscopic evidence is consistent with extensive interactions between the components in the reaggregates.

(*b*) *Density gradient centrifugation*

Samples of the original and reaggregated structures were centrifuged to approximate equilibrium in sucrose density gradients. In both preparations a single band was obtained; the approximate buoyant densities were 1.170 and 1.165 g/cm^3 for the original and reaggregated structures, respectively. This result was taken with the electron microscopic data show that the reaggregated structure is very similar to that observed in previous work. The density gradient experiments also confirm the coherent nature of the reaggregates, which contain virtually all of the lipid and protein of the original membranes.

(*c*) *X-ray diffraction*

The thermal phase transition of the fatty acid chains in the membrane lipids recently described by STEIM *et al.*[13] and ENGELMAN[14] provides a new criterion for comparing the original and reaggregated structures. In X-ray diffraction patterns from dispersions of membranes, a marked change occurs in the wide-angle pattern through the phase transition. Below the transition there is sharp, strong line at 4.15 Å which arises from the regular hexagonal packing of the fatty acid chains; above the transi-

tion there is a very broad, strong band centered at 4.6 Å which arises from the more liquid-like packing of the chains. The thermal phase transition is quite broad (approximately 10°), but the high temperature end can be detected accurately from the disappearance of the 4.15-Å line.

In addition to these changes in the fatty acid chain diffraction, there are changes in the low angle pattern which accompany the transition. Both above and below the transition there are series of bands which arise from the electron density profile of the lipid bilayer in the membrane[12]. In particular, the positions of the second band seen above the transition, and the second and third bands below the transition are at spacings which are submultiples of the distance between the centres of the lipid headgroups of the bilayer.

The thermal phase transition was observed in both the original and the reaggregated structures used in the present experiments. In both samples it was marked by a sharp 4.15-Å line below the transition and a broad band at 4.6 Å above the transition as described above, and the high temperature end of the transition occurred at $23 \pm 2°$ in both samples. There are no significant differences between the samples in the dimensions calculated for the width of the bilayer from the spacings of the low angle diffraction pattern.

The corrected half-width of the 4.15-Å line is related to the size of the ordered region giving rise to the diffraction[24]. In the original membranes below the phase transition the half-width is approximately 0.2 Å; in the reaggregate it is approximately 0.4 Å. The increased half-width implies that the ordered regions in the reaggregate giving rise to the 4.15 Å line are smaller than in the original membrane.

In addition to the lipid bilayer diffraction there is a broad band at 10 Å which does not change during the thermal transition in the native membranes. It is not present in patterns from isolated lipids, and is attributed to the non-lipid portion of the

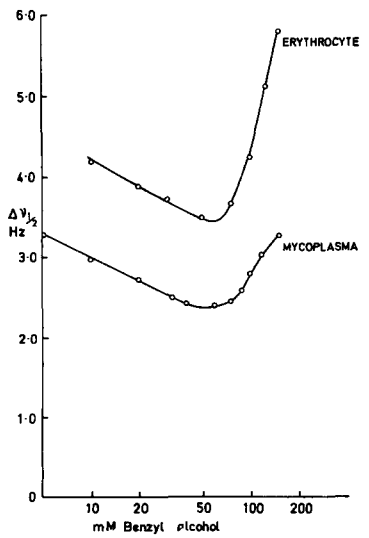

Fig. 1. Line widths ($\Delta\nu_{1/2}$) of the phenyl protons of benzyl alcohol in the presence of 5.0% (w/w) membrane suspensions as a function of alcohol concentration at 25°.

membrane, most likely to the protein component. The 10-Å diffraction is present in the patterns from reaggregated structures, but its intensity is reduced relative to the lipid diffraction when compared with original membranes.

(d) Nuclear magnetic resonance

The line width of the phenyl proton resonance of benzyl alcohol in 1.0 % (w/w) concentrations of erythrocyte and Mycoplasma membranes is shown in Fig. 1. For both membranes the line width decreases with increasing alcohol concentration, passes through a minimum at about 60–80 mM benzyl alcohol, and increases again at higher concentrations. Concentrations of benzyl alcohol above 80 mM causes lysis of intact erythrocytes and irreversibly inhibit Mycoplasma growth.

It has been shown previously that the decrease in line width in the prelytic concentration range for erythrocyte membranes corresponds to a decreasing relaxation rate of the bound alcohol molecules[15]. This implies that the mobility of the alcohol molecules within the membrane increases with increasing alcohol concentration, and it was inferred that the membrane components themselves become more fluid as a result of perturbation by the alcohol. The upswing in the lytic concentration range was attributed to the exposure of new binding sites which are inaccessible in the prelytic concentration range and are located predominantly on the membrane protein. These conclusions were based on line width measurements of the phenyl protons in the presence of separated membrane lipid and protein fractions, and when similar experiments were performed on Mycoplasma lipid and protein fractions it was found that they showed the same pattern of response as the erythrocyte fractions (Fig. 2). For both membrane systems the lipid and protein curves show a monotonic decrease in

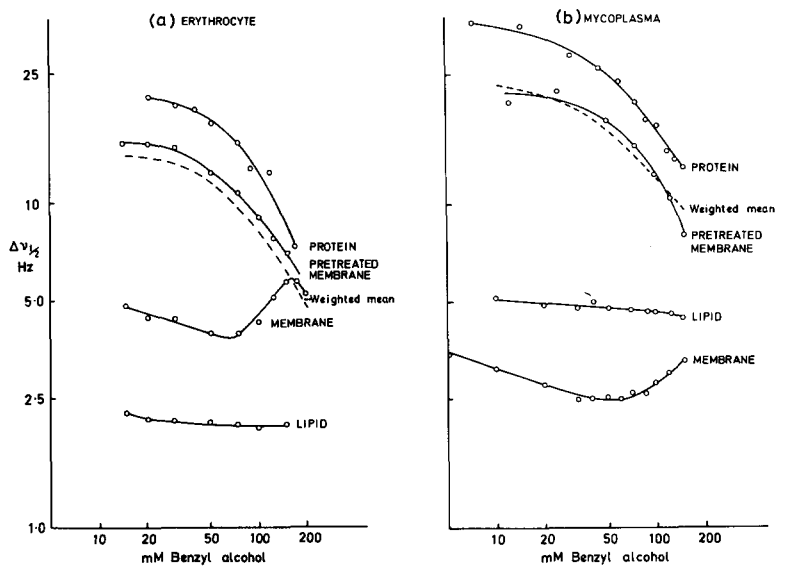

Fig. 2. Line widths of the phenyl protons of benzyl alcohol in the presence of various membrane preparations at 25°. The linewidths are all corrected to 1.0 % (w/w) as described in the text. The dash curves are the weighted mean linewidths of the separated components corresponding to the composition of the intact membranes. (a) Erythrocytes. (b) Mycoplasma.

line width with increasing concentration quite distinct from the intact membrane curves. For both membranes the protein also causes a much larger broadening effect than the lipid. The main qualitative difference between the erythrocyte and Mycoplasma membranes is the reversal in relative line broadening from the membrane and the separated lipid component. The factors determining binding and resonance line widths in lipids are discussed elsewhere[25].

If we calculate the line width corresponding to the lipid–protein composition of the intact membranes, this is substantially greater than the line width for the intact membranes in the prelytic concentration range (Fig. 2). Above approximately 150 mM benzyl alcohol the two curves are coincident for the erythrocyte membrane. However, much higher concentrations of alcohol ($\approx$ 300 mM) are required to induce the same degree of dissociation in the Mycoplasma membrane.

We can obtain experimental curves similar to the calculated curves for both membranes simply by pretreating the membranes with 200 mM benzyl alcohol, which is the maximum accessible concentration. After reducing the alcohol concentration the experimental line width curves are as shown in Fig. 2. We conclude that for both membranes pretreatment with 300 mM benzyl alcohol causes irreversible changes in the membrane structures so that they both interact with benzyl alcohol essentially as the sum of their separated lipid and protein components. Pretreatment of either membrane with prelytic alcohol concentrations causes negligible changes in the biphasic curves of the intact menbrane structures. By this criterion the interaction of benzyl alcohol with the membranes is fully reversible in the prelytic concentration range. In the lytic concentration range the upswing in the membrane curve reflects the irreversible exposure of new binding sites. The experiments in Fig. 2 indicate that only the membrane protein can provide this increase in broadening so that the new

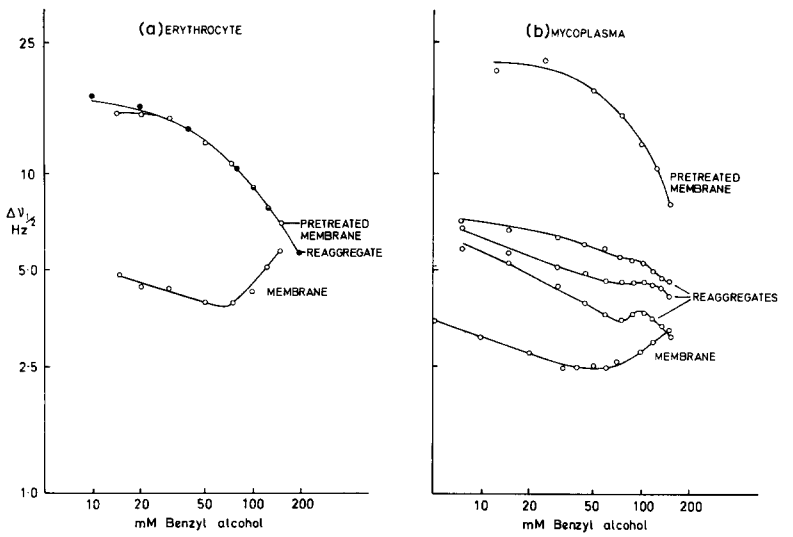

Fig. 3. Line widths of the phenyl protons of benzyl alcohol in the presence of reaggregated membrane structures at 25°. Corresponding curves for intact membranes and membranes pretreated with 300 mM benzyl alcohol, all at 1.0% (w/w), are included for comparison (see text for details of membrane preparations). (a) Erythrocytes. (b) Mycoplasma.

binding sites must be mainly localised on the protein component of both membranes. Further confirmation of this interpretation has been obtained for erythrocyte membranes by measuring the partition of benzyl alcohol into each of the erythrocyte membrane preparations[16] in Fig. 2a.

Thus far the two membranes appear to be very similar qualitatively in their interaction with benzyl alcohol. However, when reaggregates of the two membrane systems are compared we find significant qualitative differences. The line widths of benzyl alcohol in the presence of erythrocyte and Mycoplasma reaggregates are compared with their respective pretreated membranes in Fig. 3. The erythrocyte reaggregate curve is virtually coincident with the pretreated membrane curve and it is clear that the formation of lipid–protein aggregates has not significantly restricted the interaction of the structure with benzyl alcohol. Thus the curve is almost the same as the calculated mean curve for the separated components. The best curves for the Mycoplasma reaggregates show substantially less broadening than the pretreated Mycoplasma membranes and there is also a small but significant inflection in the curve in the region of 60–80 mM benzyl alcohol. It is, however, still easily distinguishable from the biphasic curve for the intact membrane where the upswing is much more pronounced and the line widths are significantly narrower.

In less successful reaggregation experiments the inflection is smaller or non-existent, and the curves lie closer to the pretreated membrane curve with the full complement of binding sites in which the line widths are much broader (Fig. 3). We therefore have evidence for gradations of structural reformation which in the best experiments to date fall well short of complete reconstitution of the native structure as judged by the line width measurements. It should be noted that the greater variability in the curves from reaggregates compared with all the other preparations may reflect the sensitivity of the reaggregation process to the precise physical conditions under which it occurs; on the other hand it may depend critically on the residual concentration of sodium dodecyl sulphate which is not removed by dialysis. In either case, the reaggregation could not be controlled sufficiently to give exactly reproducible results.

DISCUSSION

Previous studies by MOROWITZ and co-workers[3,4,8] have shown that sodium dodecyl sulphate dissolves the membrane of *Mycoplasma laidlawii* to give separate protein- and lipid-detergent complexes of small size. When the detergent is removed in the presence of Mg^{2+}, a reaggregated structure is obtained which is similar to the original membrane in gross composition, buoyant density, and morphology observed by electron microscopy. The reaggregates we have examined in the present study have been shown to resemble the original membranes by the same criteria and were indistinguishable from the reaggregated structures described previously. It is important to establish whether correct self-assembly resulting in the original structural relationships of the membrane components has occurred, since the ability to reconstitute membranes properly would greatly facilitate a detailed description of structural organisation. For example, this would enable each membrane component to be labelled in turn with appropriate spectroscopic probes in known positions before reconstitution, overcoming the main problem in the use of empirical probes as structural deter-

minants that their precise localisation in the structure is unknown. Certainly the criteria for reconstitution which have previously been applied to Mycoplasma and other membrane reaggregates have been based only on minimal requirements of the gross structural features of the membrane. Here we have applied criteria based on the use of probe molecules as described elsewhere[11].

The X-ray diffraction analysis did not reveal any major differences between dispersions of the native and reaggregated Mycoplasma structures. The thermal phase transition is seen in both structures and occurs over the same temperature range. Bands appear in the low angle patterns above and below the thermal phase transition in the same positions for both native and reaggregated membranes. The data show that the lipid bilayer present in the membrane is largely restored in the reaggregate, although the increased half-width of the 4.15-Å diffraction line below the transition indicates that the bilayer regions are smaller in extent in the reaggregates. The diffraction at 10 Å, which most probably arises from the membrane protein, is somewhat reduced in intensity in the reaggregate. This reduction in intensity could indicate that the original organisation of the membrane proteins is not fully restored, but a firm interpretation cannot be made until more is known about the origin of the 10-Å diffraction.

In contrast to the X-ray diffraction data, the evidence from the NMR experiments enables us to distinguish clearly intact and reaggregated membrane structures by their interactions with benzyl alcohol. The magnetic resonance curves indicate that the erythrocyte reaggregates retain most of the binding sites available in the separated components and this accounts for the monotonic form of the reaggregate curve. On the other hand the curves show that there is a substantial restriction on interaction with benzyl alcohol in the Mycoplasma reaggregates, and strongly suggest that this is mainly due to the occlusion of some of the binding sites on the membrane protein which are accessible in the separated component. We consider that in the Mycoplasma reaggregates the lipid and protein are so organised that the number and type of binding sites available to benzyl alcohol are intermediate between the full complement in the separated components, and the restricted sites available in the intact membrane. It is likely that this intermediate nature of the Mycoplasma reaggregate accounts for the gross similarities previously reported between the reaggregated and native membrane structures. They are most clearly distinguished by the absence of the large biphasic response in the NMR curve for benzyl alcohol which we take as an essential feature of the native membrane structure. The appearance of a small inflection in the magnetic resonance curve of the Mycoplasma reaggregate may result from a small fraction of structures resembling those of the native membrane. Based on the magnitude of the inflection there cannot be more than 10–20 % of such structures in the reaggregates.

The striking similaries between the form of the curves for both erythrocyte and Mycoplasma membranes and their derived preparations is surprising at first sight, because of known differences in their lipid composition, and also presumably of their protein components. The magnetic resonance curves depend in the first instance on the binding of the alcohol molecules to the various membrane preparations, and secondly on the rotational motion of the bound alcohol molecules. What the results indicate is that these binding characteristics of benzyl alcohol to both membranes are similar. Elsewhere, we have established that the binding capacity of the erythrocyte membrane is determined by the organisation of the components in the structure[16], so

that in this respect the erythrocyte and Mycoplasma membranes must have substantial elements of structure organised in a similar way and with comparable responses to perturbation.

Finally, we consider that more detailed information could be obtained from probe techniques if specific components of the native membrane structure could be labelled at known positions with probe groups. The failure of the radical attempt at reconstituting Mycoplasma membranes described here means that the procedure does not allow the required specific labelling of membrane components mentioned earlier to be achieved, since the labels will not report on a native membrane structure. We think that the likelihood of a successful reconstitution of this kind is very low when the number of components involved is considered. For example, there are at least 10–20 protein components and correct self-assembly under an arbitrary set of conditions appears improbable. This suggests to us that conservative techniques for removing membrane components and replacing them, after labelling, into the remaining template structure is a more effective strategy and we intend to explore this further.

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