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Membrane Intercalated Particles in Human Erythrocyte Ghosts: Sites of Preferred Passage of Water Molecules at Low Temperature

(membrane proteins/transport/membrane structure/pore/electron microscopy)

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ABSTRACT **Although hydrophilic pores have been inferred to account for characteristics of the movement of water molecules across erythrocyte membranes, no direct evidence has associated such pores with actual structural differentiations within the membrane. Freeze–fracture and freeze–etch studies of isolated erythrocyte membranes show that they are comprised of fluid bilayer domains tranversed by protein-containing intercalations ("membrane intercalated particles"). It has previously been hypothesized that the topology of the polar and apolar spaces of the membrane-intercalated particles was not concentric the hydrophobic spaces being equatorially distributed. In consequence, axial organization of the hydrophilic regions could result in hydrophilic continuity across the membrane and might provide a structural basis for passage of hydrophilic molecules. The present experiments support this hypothesis. It is shown that sublimation at −100° of erythrocyte membrane suspensions (that had been incubated at pH 5.5 to cause aggregation of the membrane particles) results in progressive and selective sinking of the membrane regions comprised of aggregates of intercalated particles, i.e., that sublimation of water molecules occurs preferentially across these membrane regions. These results indicate that, under these experimental conditions, the membrane-intercalated particles provide a preferential structural pathway for passage of water molecules across erythrocyte ghost membranes.**

Although the movement of small hydrophobic molecules across biological membranes can be described as a process of dissolution and diffusion across a continuous hydrophobic matrix of a membrane with uninterrupted bilayer organization, studies of the movement of water and other small hydrophilic molecules across erythrocyte membranes have demonstrated the need for the existence of parallel hydrophilic pathways, i.e., porous membranes have been postulated (1–7). However, although the concept of "equivalent pore" has been introduced to describe operationally membrane components that permit viscous flow* (6, 8), no direct evidence associates the "pores" with actual structural differentiations within the membrane (5).

Recent studies of the structure and fluidity of biological membranes (9–13) have also shown that while a bilayer organization is a structural feature common to most biological membranes, not all of the membrane area is comprised of a bilayer: monistic views of membrane structure (bilayer versus globular) are now reconciled by viewing the plasma membrane as formed by a bilayer continuum, which is interrupted by stable, yet potentially mobile, proteic intercalations (10, 14, 15). Freeze–fracture of plasma membranes clearly illustrates these views because it reveals that the bilayer domains of the membrane (split during the fracture process) are interrupted by numerous particulate components (11, 16). In erythrocyte ghost membranes, freeze–fracture and freeze–etch labeling studies (17–20) demonstrate exclusive association between the membrane particles and A and B antigens, anionic sites, influenza virus, and wheat-germ agglutinin receptors at the outer surface and, also, anionic sites at the inner surface; such studies also show that the particles are the morphological representation of protein-containing sites that are intercalated into and completely traverse the hydrophobic matrix of the bilayer membrane. Because intercalation implies exposure to a heterogeneous environment (successively hydrophilic/hydrophobic/hydrophilic), I proposed (10) that the topology of the hydrophilic and hydrophobic spaces of the membrane intercalated particles is not concentric (as

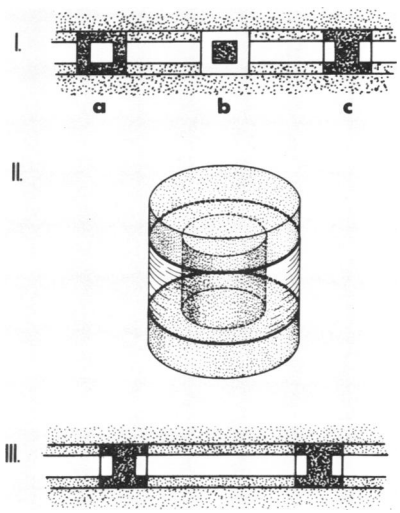
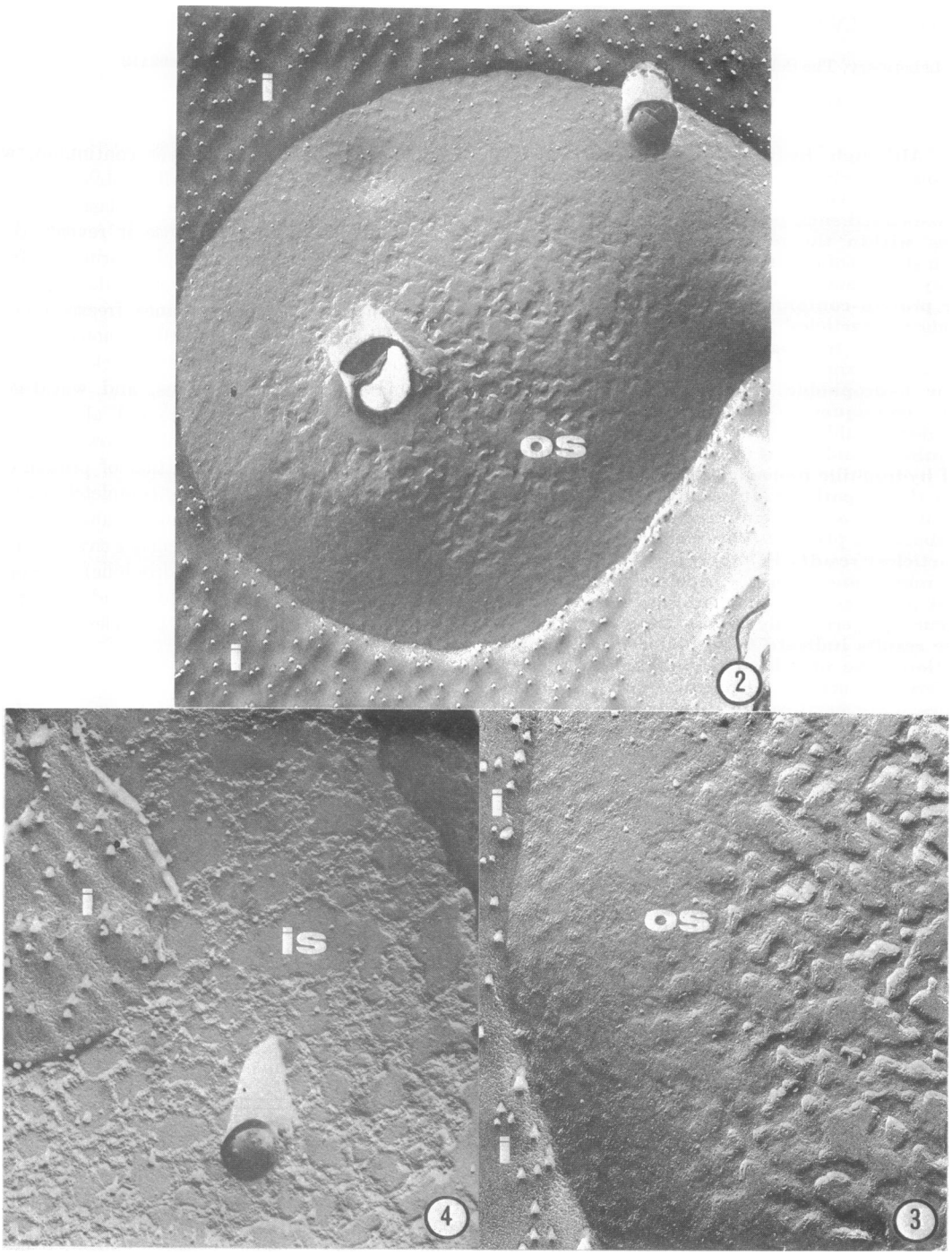


FIG. 1. Topology proposed for hydrophilic (*shaded*) and hydrophobic (*clear*) spaces of a bilayer membrane with membrane intercalated particles (10). Concentric organization of hydrophilic and hydrophobic spaces is unfavorable because it would result in exposure of hydrophilic regions of the particle to a hydrophobic environment (*Ia*) or vice-versa (*Ib*) and, consequently, in entropy reduction. This problem is avoided if the hydrophobic residues are equatorially distributed in a torus in register with the hydrophobic matrix of the bilayer membrane (*Ic, II*). In consequence, axial concentration of polar regions can provide hydrophilic continuity across the membrane (*III*).

* The "equivalent pore radius" defined as "equivalent to radius of a pore in an ideal membrane containing uniform, circular pores in which diffusion and bulk flow may be described by the equations of Fick and Poiseiulle" and designed "to express the passive permeability properties of biological membranes to hydrophilic molecules by a single parameter which best describes the steric and frictional characteristics of the pore" (6, 8).

would be expected if their environment was homogeneous) (Fig. 1, *Ia* and *b*) but that, instead, their polar regions were equatorially concentrated in a torus in register and interacting with the hydrophobic matrix of the bilayer membrane (Fig. 1, *Ic* and *II*). In consequence, axial organization of the polar spaces in the intercalated particle could result in hydrophilic continuity across the membrane and, possibly, a structural basis for the transfer of hydrophilic molecules

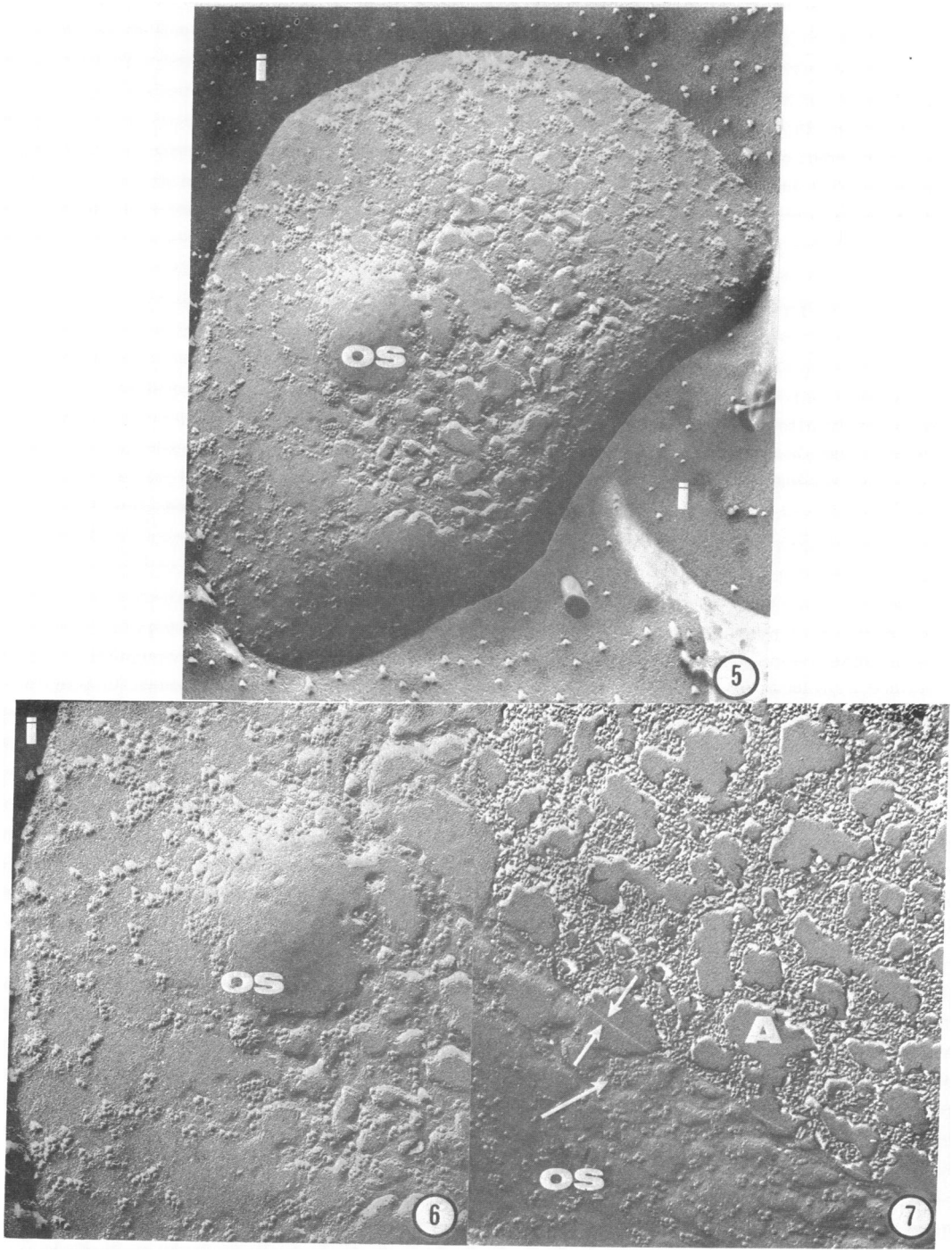


FIGS. 2–4. Human erythrocyte ghosts incubated at pH 5.5 (to cause aggregation of the membrane particles) frozen, fractured, and etched (sublimed) for 10 min at −100°. Figs. 2 and 3: outer (etched) surface (*os*) shows a system of canyons more pronounced at the center (due to convexity of the membrane, this region is subjected to a longer period of sublimation) that is progressively attenuated towards the periphery and, next to the receding ice (*i*) is substituted by a system of slight positive rugosities. Fig. 4: Inner etched surface (*is*); because of concavity of the membrane, the regions at the periphery are now those that are subject to a longer period of sublimation; these are now the depressed regions; the depressions are attenuated towards the center and become clear positive rugosities next to the receding internal ice. (Fig. 2, ×30,000; Figs. 3 and 4, ×55,000).

(Fig. 1, *III*). Experiments that associate sites for preferential passage of water molecules at low temperature (−100°) to the membrane-intercalated particles of human erythrocyte ghosts are reported here.

MATERIALS AND METHODS

Human erythrocyte ghosts were prepared from fresh human blood (type O, Rh-positive) (21). For each experiment, 50 μl of packed ghosts was incubated in 15 ml of 8 mM



FIGS. 5–7. Human erythrocyte ghosts incubated at pH 5.5, anionic sites labeled with ferritin of high isoelectric point (pI 9.5), frozen, fractured, and etched for 10 min at −100°. The basic ferritin molecules bind exclusively anionic sites on the membrane particles and are used to show that the depressed areas correspond to membrane regions containing particles. Figs. 5 and 6: At the center, ferritin is confined to canyons; towards the periphery it follows attenuated depressions; next to the receding ice, it overlays positive rugosities (Fig. 6, *left*). Fig. 7: The random network patterns of aggregated particles on fracture face *A* also show marked depression; they are continued on the etched surface (*os*) by a system of canyons that is labeled with ferritin (*arrow*). No increased discontinuity is observed on smooth regions (bilayer membrane domains (*opposing arrows*) from fracture face *A* (inner half of the bilayer membrane) to etched surface *os* (surface of intact bilayer membrane), as might be expected if sublimation also occurred through the bilayer regions. (Fig. 5, ×40,000; Figs. 6 and 7, ×70,000).

potassium phosphate buffer–1 mM $CaCl_2$ at pH 5.5 for 30 min at 30° to aggregate the membrane particles (10). The ghosts were washed twice in a 1:20 dilution of the buffer and frozen in the liquid phase of partially solidified Freon 22 cooled by liquid nitrogen. In other experiments, the membrane particles of ghosts were labeled by addition to cooled ghost (2°) suspensions of 20 μl of a solution of high isoelectric point (pI 9.5) ferritin (19). Freeze–fracture and etching were carried out in a Balzers device (stage temperature −100°). After freeze–fracture, the specimens were allowed to sublime ("etch") for 10 min at −100°. The specimens were shadowed and the replicas were recovered, cleaned, and observed. The micrographs are mounted with shadow from bottom to top and show the outer (Figs. 2, 3, and 5–7), and inner surfaces (Fig. 4), and fracture face *A* (Fig. 7) (see refs. 11 and 19 for interpretation).

RESULTS

Examination of extensive areas of the outer surface exposed by sublimation revealed a system of depressions—"canyons"—pronounced in the central regions of the exposed membrane and progressively attenuated as the pheripheric regions are approached (Figs. 2 and 3). At the immediate periphery, i.e., next to the receding ice, the system of canyons was substituted by a system of rugosities showing slight positive relief (Fig. 3). The surface patterns of meandering canyons and rugosities were strinkingly similar to those displayed by the aggregates of membrane intercalated particles on fracture face *A* (Fig. 7) caused by incubation at pH 5.5 (10). Proof that the system of canyons and rugosities corresponded to the membrane regions comprised of particle aggregates was obtained by selectively labeling anionic sites at the outer surface regions of the membrane-intercalated particles with a ferritin derivative with high isoelectric point (19); the positively charged label could be exclusively traced onto the system of canyons and reliefs (Figs. 5–7).

The decreased depth and—eventually—inversion of the canyons at the peripheric surface regions paralleled the length of exposure to sublimation, as the central regions corresponded to the regions first uncovered during the lowering of the ice surface and, consequently, were exposed to a longer period of etching. On the contrary, regions at the periphery were exposed to progressively shorter periods of sublimation, with the areas immediately contiguous with the receding ice representing surface areas just uncovered and, thus, exposed to a very short period of sublimation.

Observation of the effect of etching on fracture face *A* also showed depression of the membrane particles (Fig. 7), as first reported by Engström (22). In regions where fracture face *A* could be observed in continuity with the outer (etched) surface (Fig. 7), continuity of the patterns of aggregation of the membrane particles on the fracture face with the system of canyons at the surface provided further clear evidence that sinking of the membrane was confined to membrane regions comprised of particle aggregates. Depression, even if to a lesser extent, of smooth (bilayer) regions of the membrane was not possible to establish due to the absence of a nonsublimable standard to which they would have to be referred. However, circumstantial evidence suggested that lowering of the smooth regions did not occur: observation of contiguous smooth regions on fracture face *A* and the outer surface did not show a discontinuity greater than that which is due to the splitting of the membrane (Fig. 7, opposed arrow). Should lowering occur on the smooth regions it would be more pronounced on the fracture face (which represents half of the split membrane) than on the etched surface.

Observation of the inner surface of erythrocyte ghost membranes showed also a similar system of depressions and rugosities (Fig. 4). However, because of the concavity of the membrane, the regions of the periphery were exposed to the longest period of sublimation, whereas near the center the sublimation period was shortest, and a "puddle" of ice that had not sublimed could occasionally be observed (Fig. 4, *i*). As illustrated elsewhere (19), the rugosities that correspond to the membrane particles at the inner surface are much clearer than at the outer surface, probably due to the association of other membrane components at the inner surface. Observation of fracture face *B* (which represents the outer half of the membrane) was not possible due to the complete disruption of this face upon exposure to an extended period of sublimation (19).

DISCUSSION

The present experiments show that exposure of erythrocyte ghost membranes at −100° results in selective sinking of the membrane regions comprised of aggregates of membrane intercalated particles. Although sinking of the particulate regions occurs either in intact or fractured membranes (face *A*), no evidence for a similar effect is detected on the smooth, nonparticulate regions, i.e., on membrane areas with an uninterrupted bilayer organization. Sinking of the particulate regions directly follows the period of exposure to sublimation.

Because, in these frozen membrane suspensions, depression of a surface region implies the removal of the underlying ice, these results demonstrate that, in erythrocyte membranes incubated at pH 5.5 and rapidly frozen in liquid Freon, sublimation of water molecules at −100° preferentially or exclusively occurs through membrane regions comprised of membrane particle aggregates. In consequence, the present results indicate that, at −100°, the membrane intercalated particles provide a preferential (or exclusive) pathway for the transfer of water molecules. As the transfer of water molecules across pathways of molecular dimensions largely depends on the geometry of both water molecules and membrane matrix, and is further modulated by chemical interactions (hydrogen bonding) (1, 23–25), it is likely that, in the absence of drastic temperature-dependent conformational alterations of the molecular components represented by the particles, such an axial pathway may also operate above the freezing point of water. Further extrapolation for a higher temperature is not possible because little can be hypothesized about the process of transfer of water molecules at low temperature; also, at the molecular level, little is known about the characteristics of water transfer at "physiologic" temperatures. The chemistry and geometry of the relevant regions of the membrane matrix are unknown and, within a pore of molecular dimensions, the physical properties of water are not clearly understood (5).

Alternatively, it may be argued that freezing of the membrane could disrupt the association of proteic components of the particles with neighboring lipids creating a system of molecular fissures across which water molecules could escape. This suggestion is unlikely in view of recent studies by Hubbell and collaborators (26) on the properties of phospho-

lipid bilayers containing rhodopsin that demonstrated that the interactions between rhodopsin and neighboring phosphatidylcholine molecules were stronger than the lipid–lipid interactions along the bilayer, even at temperatures below the thermal phase transition of the lipid (Dr. Wayne Hubbell, personal communication). As rhodopsin and the components of the membrane particles are topologically similar (both are stabilized within the membrane by hydrophobic and hydrophilic interactions, both interrupt its bilayer domains, and both originate particles when freeze–fractured), it is probable that the components of the intercalated particles in erythrocyte ghost membranes are also intimately associated with neighboring lipids and, consequently, that molecular fissures suitable for the escape of water molecules are not formed. Furthermore, although lattice discontinuities (27–29) might be observed in bilayer systems containing a single component (possibly through formation of small crystallites), they are not expected in erythrocyte membranes where, due to heterogeneity of the hydrocarbon chains, thermal phase transitions are not observed, the viscosity increasing gradually at progressively lower temperatures (glass formation) (30).

The present results are consistent with a model for topology of hydrophilic and hydrophobic spaces in membrane intercalated particles in which axial organization and, eventually, hydrophilic continuity are a consequence of equatorial distribution of the hydrophobic spaces of the membrane (10, 13). Other observations also indicate involvement of membrane particles in transmembrane phenomenona: (*i*) gap junctions, which consist of specialized aggregates of membrane particles, are the only type of intercellular junction in cell cultures where electrotonic coupling can be demonstrated (31, 32), and provide pathways of low electrical resistance and allow for the passage of small molecules and, possibly, biologically significant macromolecules (33, 34); (*ii*) membrane particles are also a prominent component of septate junctions, where they may also provide a structural basis for ionic coupling (35); (*iii*) circumscribed aggregates of membrane particles were reported in regions of postsynaptic membranes characteristic of the specific synapse site, and may correspond to the region of differential sensitivity of the postsynaptic membrane to specific transmitter molecules (36). Myelin membranes, which show very high electrical resistance, are virtually devoid of particles (37).

Because membrane particles are common to the membranes of all prokaryotic and eukaryotic cells observed (myelin excepted), their early appearance in the evolutionary process is likely. Generality of occurrence, heterogeneity of shape and size (even within a limited membrane region), and association with various functions indicate that them embrane intercalated particles do not correspond to a single structure but, instead, represent a differentiation intercalated across a bilayer domain with a topological organization that may be favorable to the transfer of hydrophilic molecules.

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