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ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · JUNE 2008

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## Asymmetric Droplet Interface Bilayers

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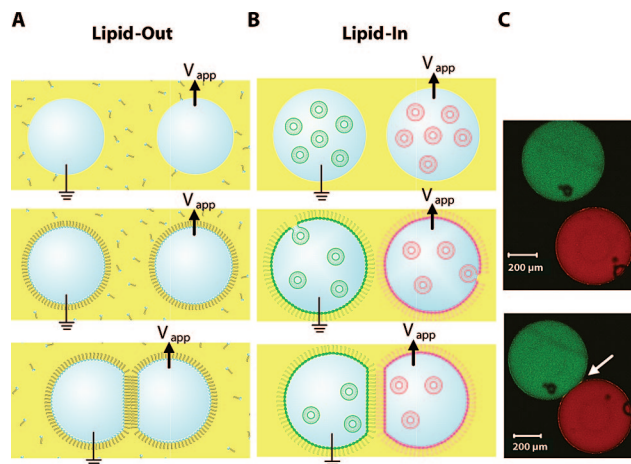
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The lipid compositions of the inner and outer leaflets of cell membranes differ.<sup>1</sup> For example, in the outer membrane of Gram-negative bacteria, the outer leaflet is largely composed of lipopolysaccharides, whereas the inner leaflet only includes phospholipids.<sup>2</sup> In the plasma membranes of eukaryotic cells, phosphatidylcholine and sphingomyelin predominate in the outer leaflet while aminophospholipids are primarily in the cytosolic leaflet.<sup>1</sup> The biological prevalence of asymmetric lipid bilayers has galvanized the exploitation of model systems, such as planar bilayers,<sup>2</sup> supported bilayers,<sup>3</sup> and vesicles,<sup>4</sup> to investigate the effects of lipid asymmetry on the function of membrane proteins. However, the study of pores and ion channels by single-channel electrical recording using these systems is difficult. Electrical recording in supported bilayers requires access to the tiny ionic reservoir beneath the bilayer, which is quickly depleted when a constant potential is applied. Planar bilayers are useful for single-channel recording, but they are delicate and short-lived. Furthermore, once a planar bilayer breaks, the lipids mix and asymmetry is lost. Such experiments must be restarted, rendering the use of asymmetric planar bilayers tedious.

Recently, we and others developed the droplet interface bilayer (DIB),<sup>5–7</sup> which is formed by joining lipid monolayer-encased aqueous droplets submerged in an oil–lipid mixture (lipid-out DIBs, Figure 1A). The resulting bilayer is robust and can readily incorporate ion channels and pores. Electrodes can be inserted into the droplets to enable single-channel recording. Since the lipid-out DIB technique uses lipids dissolved in the external oil bath, all droplets are surrounded by an identical monolayer. In this paper, we extend the DIB method by moving the lipids from the oil phase into the aqueous phase as vesicles (lipid-in DIBs, Figure 1B). The vesicles fuse at the oil–water interface to form a monolayer. Advantages of lipid-in DIBs over lipid-out DIBs include reduced experimental cost, since lipids are not included in the bulk oil phase, and a broader range of bilayer compositions, since a greater number of lipids can form vesicles than are soluble in oil. Moreover, by creating droplets with different vesicle compositions, droplets in the same bath can be encased by different monolayers, enabling the formation of asymmetric bilayers (Figure 1B,C).

Asymmetric droplet interface bilayers (a-DIBs) are stable for days to weeks (Supporting Information) and can be separated and re-formed repeatedly. The area of DIBs can be modulated easily,<sup>6</sup> which is advantageous for single-channel studies; initially, the bilayer area can be made large to maximize the chance of protein insertion and then reduced to minimize the probability of further incorporation.<sup>8</sup> The spontaneous transmembrane flip-flop of phospholipids is slow, with a half-life on the order of hours to days.<sup>9</sup> Experiments using fluorescently tagged lipids confirm that the exchange of phospholipids across

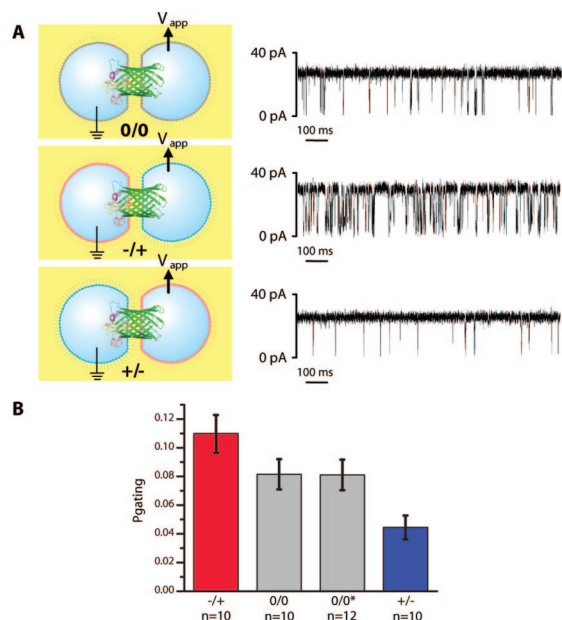


**Figure 1.** Lipid-out and lipid-in droplet interface bilayer formation. (A) Symmetric lipid-out DIB formation (previously described;<sup>5,6</sup> not used in this paper). Top: Two aqueous droplets are deposited in a lipid–hexadecane solution. (B) Asymmetric lipid-in DIB formation. Top: Two aqueous droplets containing lipid vesicles of different compositions are deposited in a hexadecane reservoir. (A and B) Middle: A monolayer spontaneously forms at the oil–water interface. Bottom: Monolayer-encased droplets are brought together to form a stable bilayer. (C) Scanning confocal fluorescence *z*-slices of two droplets containing fluorescently tagged lipids (Supporting Information). The top-left droplet contains 2 mM DPhPC vesicles doped with 2 mol % of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(carboxyfluorescein), and the bottom-right droplet contains 2 mM DPhPC vesicles doped with 1 mol % of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl). An asymmetric DIB (arrow) forms when the droplets are brought into contact.

a-DIBs is slow (Supporting Information), suggesting that bilayer asymmetry is maintained for at least several hours.

Asymmetric DIBs provide an optimal platform for studying the behavior of transmembrane pores and channels with respect to bilayer leaflet composition. Outer membrane protein G (OmpG) is a monomeric porin from *Escherichia coli*; the structure of OmpG has recently been elucidated by X-ray diffraction and NMR spectroscopy.<sup>10–12</sup> The 14 strands in OmpG's  $\beta$ -barrel are connected by negatively charged loops on the extracellular side and short turns on the periplasmic side. It was recently shown that movements of the extracellular loops cause the porin to spontaneously fluctuate among open, partially closed, and fully closed states: a behavior known as gating.<sup>13,14</sup> Spontaneous gating activity can be quantified by using the mean gating probability ( $P_{gating}$ ), which is defined as the time a pore spends in partially or fully closed states divided by the pore open time.

When OmpG was inserted into a lipid-in DIB composed of the neutral lipid 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), we found that the observed  $P_{gating}$  was the same as that in a planar bilayer<sup>15</sup> with the same lipid composition



**Figure 2.** OmpG behavior in symmetric and asymmetric bilayers. (A) Current traces showing OmpG gating. Top: neutral DIB (0/0). Middle: insertion from the negative side of the asymmetric DIB (-/+). Bottom: insertion from the positive side of the asymmetric DIB (+/-). A potential of +50 mV was applied. The buffer used was 10 mM HEPES, 200 mM KCl, pH 7.0. Protein was in the grounded droplets. (B) Comparison of mean gating probabilities ( $P_{\text{gating}}$ ) of OmpG in a symmetric neutral planar bilayer (0/0\*), symmetric neutral lipid-in DIB (0/0), and asymmetric DIBs (-/+ and +/-).  $P_{\text{gating}}$  is defined as the ratio of the time a pore resides in partially or fully closed states to the pore open time. Error bars represent one standard error and are based on recordings from at least 10 pores.

(compare 0/0\* and 0/0 in Figure 2B). This observation indicates that the presence of vesicles does not alter the gating behavior of the protein. The buffering conditions used in all experiments were 10 mM HEPES, 200 mM KCl, pH 7.0. Experimental details are provided in the Supporting Information.

We studied the effect of charged asymmetric bilayers on the spontaneous gating behavior of OmpG by forming a-DIBs with vesicles composed of 10 mol % of dimethyldioctadecylammonium bromide (DDAB, positively charged) in DPhPC and 10 mol % of 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DPPG, negatively charged) in DPhPC. The charges of the bilayer leaflets is denoted by “X/Y”, where “X” is the leaflet charge on the side of protein insertion and “Y” is the charge on the opposing leaflet.

When OmpG inserted from the negative side of the asymmetric bilayer (-/+), the frequency of gating increased relative to the neutral bilayer (0/0) (Figure 2,  $P_{\text{gating}}^{-/+} > P_{\text{gating}}^{0/0}$ , 95% confidence).<sup>16</sup> On the other hand, when the protein inserted from the positive side of the a-DIB (+/-), the frequency of gating decreased relative to the neutral bilayer (0/0) (Figure 2,  $P_{\text{gating}}^{+/-} < P_{\text{gating}}^{0/0}$ , 99% confidence). The insertion of OmpG from opposite sides of the charged a-DIB yielded significantly different gating behaviors (Figure 2,  $P_{\text{gating}}^{-/+} > P_{\text{gating}}^{+/-}$ , >99% confidence). When the orientation of OmpG in the bilayer is such that the extracellular loops are on the side of insertion (grounded droplet), the open pore conductance level is quieter at positive potentials relative to that at negative potentials.<sup>13</sup> We found that OmpG always inserted into DIBs with this orientation. This is unlike experiments using planar bilayers, where both pore orientations are observed. Our results suggest that the +/- bilayer interacts with the negatively charged loops

such that the positively charged leaflet reduces gating. In contrast, the -/+ bilayer interacts with the negatively charged loops such that the negatively charged leaflet increases gating.

Here, we have shown that the formation of asymmetric droplet interface bilayers is straightforward and reliable. In the lipid-out method of forming DIBs, some lipids could not be incorporated into the bilayer because they were not soluble in the oil phase.<sup>5,6</sup> In contrast, vesicles can be formed from most types of lipids and lipid-based macromolecules, such as lipopolysaccharides, expanding the range of lipid-in DIBs that might be created to either model the natural asymmetry in biological membranes or create novel artificial membranes with desired properties. The two leaflets of droplet interface bilayers can be readily separated and examined independently, which may be advantageous for studies such as the characterization of enzyme-catalyzed transmembrane lipid transfer.<sup>17</sup> Unlike other extant methods of creating artificial asymmetric bilayers,<sup>2–4</sup> asymmetric DIBs enable single-channel electrophysiological measurements over a period of hours to days (Supporting Information). Both  $\alpha$ -helical and  $\beta$ -barrel membrane proteins have been successfully reconstituted in a-DIBs. For example, we explored the chlorella virus PBCV-1 potassium channel, Kcv, and found that it functions in charged a-DIBs (Supporting Information), suggesting that it will be possible to study biomedically relevant channels such as voltage-gated potassium channels.<sup>18</sup>

**Acknowledgment.** The Kcv channels were a gift from Amy Mason. The authors thank Dr. Dirk Aarts for kindly allowing us to use his scanning confocal microscope, and Dr. Andrew Heron for helpful discussions. W.L.H. was supported by a Rhodes Scholarship. This work was funded by a Royal Society–Wolfson Research Merit Award (H.B.), the Medical Research Council (H.B.), and the National Institutes of Health (H.B.).

**Supporting Information Available:** Experiments illustrating persistence of bilayer asymmetry, electrical recordings of Kcv in charged a-DIBs, and experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA802089S