Imaging of Exposed Headgroups and Tailgroups of Phospholipid Membranes by Mass Spectrometry

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Cellular membrane function is driven by a complex set of physical and chemical properties including molecular density, spatial distribution, and molecular orientation in the leaflets of the phospholipid bilayer. Here we show, for the first time, by using secondary ion mass spectrometry (SIMS), that it is possible not only to determine the chemical identity of the membrane molecules but also to determine whether the headgroup or the tailgroup of the phospholipid molecule is exposed to the vacuum. This remarkable signature is demonstrated on both freeze-fractured, frozen-hydrated red blood cell membranes and on synthetic membranes made by Langmuir–Blodgett (LB) techniques.

Our experiments were performed by means of imaging time-of-flight secondary ion mass spectrometry (TOF-SIMS). With this method the specimen surface is probed with a finely focused (200 nm) pulsed Ga^+ ion beam which desorbs ions from a well-defined area.[1] An image is created by rastering the ion beam over the surface and collecting a series of mass spectra at each point. The intensity of the ions as a function of position provides spatial information about the distribution of target molecules. Lipid samples were analyzed at temperatures of less than 170 K to prevent the sublimation of lipid in a vacuum.

The behavior of the SIMS fragment ions from oriented organic films is best revealed using LB technology where the molecular configuration is precisely determined. We constructed oriented LB films of phosphatidylcholine dipalmitoyl (DPPC) such that either a headgroup or a tailgroup is forced toward the air–film interface. To prepare a film with the headgroup pointing away from the substrate surface, a C_{16} alkane thiol monolayer on gold was inserted through a layer of DPPC on a water subphase.[2] The thiol substrate is important because it presents a nonpolar surface that induces physisorption of the lipid tailgroups. To prepare a film with the tailgroup pointing away from the surface, a SiO_2/Si substrate was pulled through a layer of DPPC on the same water subphase. This substrate presents a polar surface that preferentially binds the lipid headgroup. The LB films were made at a highly compressed region of the surface pressure/area isotherm at a constant pressure of 35 mN/m. Each type of sample was confirmed to be one monolayer in thickness using ellipsometry.[3] Random or unoriented DPPC films were prepared by depositing a chloroform solution of DPPC onto a Si wafer under ambient conditions and allowing it to dry.

The relevant portion of the positive ion TOF-SIMS spectra associated with the above preparations is shown in Figure 1. The fragment at m/z 184 corresponds to the well-known phosphocholine headgroup moiety,[4] while the fragments near m/z 311 are associated with the alkyl tailgroup. From the spectra, it is clear that the intensity of specific ions correlates strongly with a specific molecular orientation. In the heads up configuration, m/z 184 is
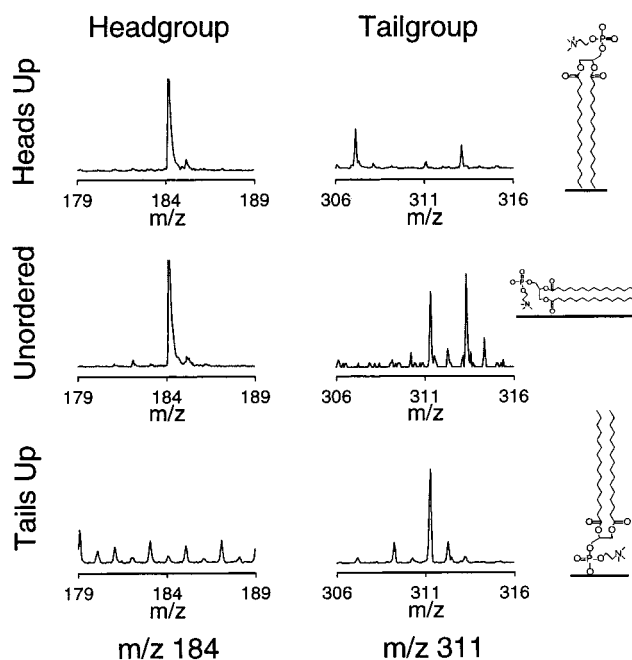
Figure 1. Positive ion SIMS spectra of a DPPC LB film on alkane thiol on Au scaled to 600 counts (top), DPPC deposited from a chloroform solution on SiO_2 scaled to 400 counts (middle, left) 30 counts (middle, right), and a DPPC LB film on SiO_2 scaled to 400 counts (bottom).

observed, while in the tails up configuration, the m/z 311 species predominates. The peak at m/z 311, previously unreported, has the molecular formula $C_{19}H_{35}O_3$ as determined from its exact mass. These results are consistent with predictions of molecular dynamics computer simulations.[5] Although there have been other reports which indicate the SIMS ion yield intensities depend on which functional groups are exposed to the ion beam,[6] we believe these results are the first to indicate that specific fragment ions are associated with specific configurations.

It would be of special interest to utilize this mass spectral signature to probe the structure of intact biological membranes. To test this idea, we have examined the surface of rapidly frozen human red blood cells that have been freeze fractured directly in the vacuum environment of the mass spectrometer. These types of cells make an excellent model system since they have no internalized membranes and have been extensively characterized by many techniques.[7] We have employed freeze fracture to prepare these samples since previous studies have shown that this protocol is the most effective procedure for exposing an unperturbed membrane surface.[8]

The red blood cell samples were prepared in a sandwich-style holder consisting of two Si wafers spaced apart by polystyrene beads 8 μm in diameter. After being rapidly frozen in liquid propane and introduced into the spectrometer, the wafers were split apart, and the sample was fractured to expose a fresh surface for analysis. Details have been presented elsewhere.[8] The fractured cell surface could potentially consist of cells with (i) lipid molecules with headgroups exposed if the fracture occurred at

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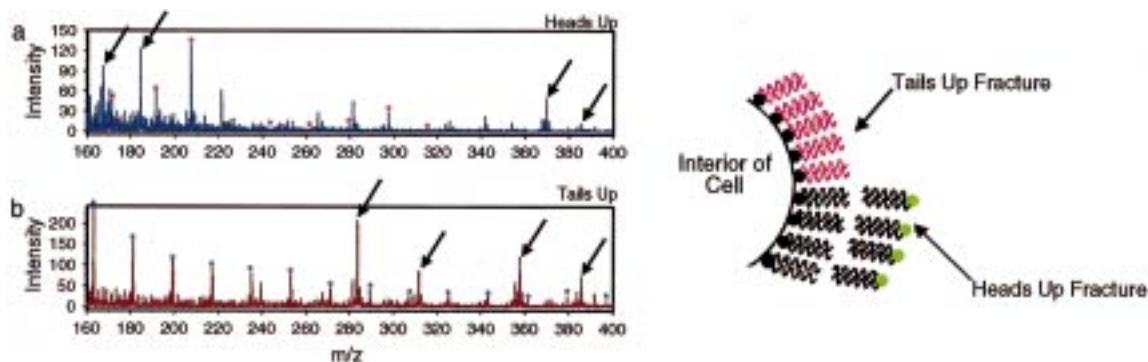(7) Op den Kamp, J. A. F. *Annu. Rev. Biochem.* **1979**, *48*, 47.
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Figure 2. (a) Positive ion SIMS spectrum of freeze-fractured, frozen-hydrated red blood cell outer membranes (heads-up fracture). The mass peaks which start at m/z 189 and are separated by 18 amu are a result of water clustering with SiOH of the formula $SiOH(H_2O)_n^+$ and are denoted by *. Headgroup-related peaks are denoted by arrows at m/z 166 and m/z 184. Cholesterol-related ions $(M)^+$ and $(M - OH)^+$ are also denoted at m/z 386 and m/z 369, respectively. (b) Positive ion SIMS spectrum of freeze-fractured, frozen-hydrated red blood cell inner membranes (tails-up fracture). The mass peaks, which start at m/z 181 are assigned to water clusters of the formula $H(H_2O)_n^+$ and are labeled with a *. Tailgroup-related peaks are seen at m/z 283 $(C_{18}H_{35}O_2)^+$ and m/z 311. Cholesterol-related ions $(M)^+$ and $(M - C_2H_5)^+$ are also shown at m/z 386 and m/z 357, respectively. Both spectra were recorded from a few dozen red blood cells over a field of 143 by 120 μm. The results reported in (b) were found at least an order of magnitude less frequently than those reported in (a), and only occurred when employing densely packed cells. For these samples, chemical mapping yields a homogeneous distribution of m/z 283 and 311.

the water–membrane interface, (ii) lipid molecules with tailgroups exposed if the fracture split the lipid bilayer itself, or (iii) a cross section exposing a region of the cell interior with only a ring of lipid bilayer around the cell.[9] There is also a possibility of biasing the position of the fracture plane by adjusting the size of the beads,[10] but we have not yet extensively tested this idea.

The red blood cells yield two different characteristic mass spectra after freeze fracture which we propose arise from the two possible lipid orientations in the membrane as seen in Figure 2 (situations i and ii). These spectra contain mass peaks corresponding to those observed for DPPC, cholesterol, and water clusters. Choline-containing lipids, along with cholesterol, are intrinsic components of the outer red blood cell membrane and should dominate situation (i).[7] The spectrum shown in Figure 2a, most commonly observed in our studies, exhibits a strong lipid fragment ion at m/z 184. The lipid components of this spectrum also match most closely the expected spectrum for choline-containing lipids in the heads-up configuration. The spectrum shown in Figure 2b, however, exhibits a prominent lipid fragment at m/z 311, characteristic of palmitic acid tailgroups. Palmitic acid groups are the most common fatty acids of lipids in human red blood cells.[11] In this case, the red blood cell membrane bilayer is apparently split during fracture, exposing the fatty acid chains.

The behavior of the SIMS spectrum of cholesterol in these fractured red blood cells potentially provides analogous information about how this molecule is bound in the phospholipid matrix. Cholesterol is thought to align in the cell membrane with its hydroxyl group in the proximity of the ester oxygen atoms of the lipid and the terminal alkane chain near the nonpolar tailgroups of the lipid.[12] Cholesterol is present in human red blood cell membranes in a 0.95 molar ratio to phospholipids.[13] Note that in Figure 2a, the $(M)^+$ ion of cholesterol is present at m/z 386 and that a major fragment ion is seen at m/z 369 corresponding to $(M - OH)^{+}$.[14] In Figure 2b, however, when the hydroxyl end of the cholesterol molecule is proposed to be near the substrate surface and the alkyl chain is directly exposed to the ion beam, the prominent fragment ion is $(M - C_2H_5)^+$ at m/z 357 with the $(M - OH)^+$ ion almost absent. At this point, oriented films of
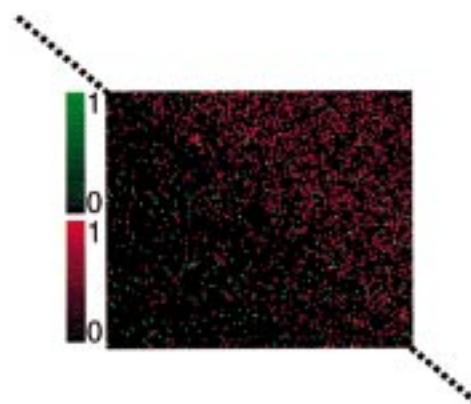
Figure 3. Positive ion SIMS image of the boundary between tails-up DPPC LB film and disordered DPPC film on SiO_2. The tailgroup at m/z 311 is shown in green, and the headgroup at m/z 184 is shown in red. The field of view is 143 by 120 μm. The dotted line approximates the boundary.

cholesterol are unavailable for proving this hypothesis, although our data certainly point to an approach which is amenable to directly determining the configuration of molecules within the membrane bilayer.

These spectral features provide sufficient intensity to acquire molecule-specific images of intact freeze-fractured, frozen-hydrated membranes.[8,15] An imaging example is displayed in Figure 3, which depicts the boundary between the ordered and disordered part of a DPPC film on SiO_2/Si. Below the boundary the deposit is an LB film which is well-ordered in the tails-up configuration.[3] Above the boundary the layer is disordered since these DPPC molecules are deposited onto the substrate before film compression in the LB trough. The disorder is evidenced by the presence of headgroup and tailgroup signal above the boundary and mainly tailgroup signal below the boundary. This image shows the sensitivity of SIMS to orientation and the potential to image different orientations on the same cell.

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