**Supported Lipid Bilayer Asymmetry is Impacted by Lipid-substrate Interactions.**

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**Abstract:** The asymmetric arrangement of phospholipids in the two leaflets of a eukaryotic plasma membrane is an integral part of cellular function. But the inter-leaflet distribution of lipids in supported lipid bilayer (SLB) was rarely understood, due to the ultra-thin thickness of a bilayer. However, the bilayer unzipping assay developed in our group is a promising technique to study lipid asymmetry. This assay is capable of decoupling the two leaflets of a SLB into two monolayers. After separating two leaflets apart, we applied fluorescence microscopy and High-Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS) to quantify the lipids on two unzipped monolayers individually. Surprisingly, we found that distribution of phosphatidylserine (PS) and phosphatidylethanolamine (PE) lipids were asymmetric in SLBs, with more distributed on the lower leaflet. And we revealed that hydrogen bonding and electrostatic interactions between the lipid head group and glass surface are the major factor to induce PS, PE asymmetry. Moreover, by altering salt concentration, pH and buffer conditions, we have the ability to modulate the lipid asymmetry in SLBs.