# Effect of hydration on the structure of caveolae membranes

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*In situ* energy dispersive x-ray diffraction was used to investigate the effect of hydration on the structure of caveolae membranes. The structure of caveolae membrane was found to be strongly dependent on hydration. At low hydration two lamellar phases with distinct repeat spacings were found to coexist with segregated cholesterol crystallites. Upon hydration, the lamellar phases did swell, while diffraction peak of cholesterol crystals disappeared suggesting that cholesterol molecules redistributed homogeneously within the caveolae membrane. At full hydration, unbinding of caveolae membrane occurred. Upon dehydration the system returned to the bound state, demonstrating that the unbinding transition is fully reversible. © *2009 American Institute of Physics*.* [DOI: 10.1063/1.3116615]

Caveolae are cholesterol- and sphingolipid-rich small invaginations (50–100 nm) of the plasma membrane. Their main difference from other membrane domains with similar tasks, the lipid rafts, is the presence of caveolin, an oligomeric protein, which is thought to be the driving-force behind the segregation of cholesterol and other lipid species between the outer and inner leaflet of the bilayer.[1] These flask-shaped structures are considered to be multifunctional organelles with a physiological role depending on cell type and cellular needs.[2] For instance, they are important in the regulation of various signaling[2] and internalization[3] processes. Recent studies[4] showed that, in skeletal muscles, caveolae are involved in water transport as well as in maintaining osmotic hemostasis under abnormal water balance. Mice exposed to dehydration (bred without water for 24 h before being dying) and mice infused with water in the peritoneal cavity for 1 h exhibited very different distribution and density of caveolae in the muscle plasma membrane.[4] These hydration-induced alterations are thought to be important for the massive water movement across the plasma membrane, which requires a route for its molecular transport. However, further study, such as analyses of the structural changes of the caveolar lipid bilayer are required to elucidate the exact functional role of such structures in skeletal muscles.

In this letter we report on the structural changes of caveolae membranes induced by hydration. An experimental setup that allows for *in-situ* energy dispersive x-ray diffraction (EDXD) experiments with a precise control of relative humidity (RH) and temperature was applied.[5]

Caveolae pellets were isolated from lung tissue of 4 months old female mice (strain C57Bl/6J) as elsewhere described.[6] A drop of 100 $\mu$l of the sample solution was carefully spread onto the freshly cleaved surface of ⟨100⟩ oriented silicon wafers. After solvent evaporation, the sample was transferred to the hydration chamber where the dry film was carefully hydrated by vapor. The sample was followed as a function of time over a wide range of RH ($0.4 < \mathrm{RH} < 1$). Each x-ray pattern was collected at room temperature ($T = 300$ K) for $t = 1000$ s. Biological samples are not damaged by EDXD experiments as elsewhere discussed.[7]

Figure 1 (panel a) shows the EDXD pattern of dehydrated caveolae isolated from lung tissue of wild type mice (RH=0.42). As evident, three Bragg peaks were detected that could not be indexed on the basis of any known lipid phase.[8] According to this, the first two peaks of Fig. 1 (panel a) can be interpreted as the first-order reflections of two multilamellar phases with distinct repeat spacing (57.2 and 39.9 Å, respectively). This means that solid-supported caveolae membranes at low hydration most likely contain separated structures with distinct bilayer thicknesses. Such structures as found here are indicated as phase *L* (large *d*-spacing phase) and *S* (small *d*-spacing phase) in the subsequent text. In principle, membranes with distinct repeat spacings as those we found may be due to different domains enriched in specific lipid species.[9] Indeed, in model membranes consisting of ternary mixtures of cholesterol, sphingomyelin or a saturated phospholipid, and an unsaturated phospholipid, liquid-ordered ($L_o$) domains containing cholesterol are rich in saturated lipid, whereas liquid-disordered ($L_d$) domains are rich in unsaturated lipid.[9] Accordingly, our findings may indicate that the thicker *L* phase should be rich in saturated lipids, while the *S* phase should be rich in unsaturated lipids. The higher intensity of the Bragg peak associated to the *L* phase (Fig. 1, panel a) most likely indicates that sphingomyelin and other saturated phospholipids are the main lipid components of caveolar systems. This suggestion is in good agreement with the results of a nuclear magnetic resonance (NMR) study we performed to identify and quantify the lipid species present in the whole caveolae. NMR experiments (data not reported) confirmed that the main lipid components were sphingomyelin (48.4%), phosphatidylcholine (10.6%), phosphatidylethanolamine (24.7%), and cholesterol (16.3%).

On the other hand, the identification of the third diffraction peak onto the EDXD pattern of Fig. 1 (panel a) is straightforward. It is due to formation of cholesterol crystals embedded within (or in contact with) caveolae membrane.[10–12] Such crystals contain a pseudobilayer structure with repeating distance approximately 34 Å.

Closing the hydration chamber windows, the adsorption of water onto the sample immediately proceeded[13] and was followed as a function of time and RH. Upon progressive hydration, the system underwent a remarkable structural rearrangement as evident from representative EDXD patterns

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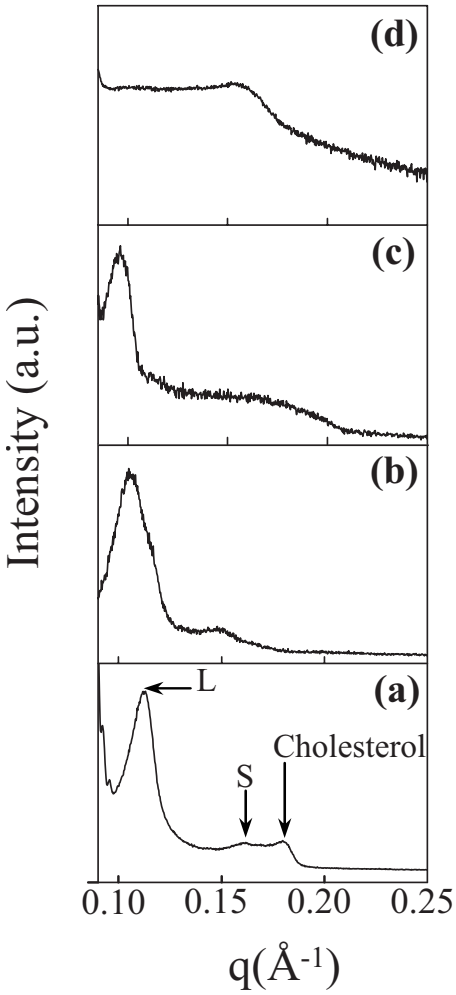


FIG. 1. Representative EDXD patterns of solid-supported caveolae membranes as a function of increasing hydration: RH=0.42 (panel a), RH =0.95 (panel b), RH=0.99 (panel c), and RH>0.99 (panel d). The two Bragg peaks indicated as *L* and *S* were interpreted as the first-order reflections of two phase-separated lamellar structures, while the peak labeled as "cholesterol" is due to cholesterol crystallites in contact with caveolae membranes.

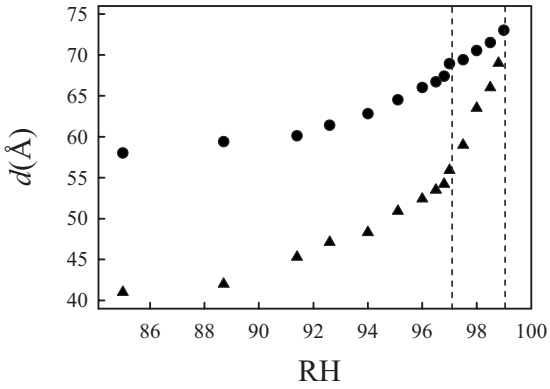


FIG. 2. Evolution of the lamellar repeat spacing, *d*, of both the *L* (circles) and *S* (triangles) phases as a function of RH. At RH=0.988 *d*-spacing assimilation was observed. Dashed lines separate three distinct regimes of hydration.

reported in Fig. 1. Three distinct hydration regimes were identified. In the first regime ($0<t<25.000$ *s*; $0.42<\mathrm{RH}<0.97$) both lamellar phases did swell with increasing RH as shown by the shift of Bragg peaks to lower *q* values (Fig. 1, panel b). In this regime, the diffraction peak of cholesterol crystallites disappeared suggesting that, upon progressive hydration, cholesterol molecules are getting redistributed homogeneously within the caveolae membrane plane. In the second regime ($25.000<t<30.000$ *s*; $0.97<\mathrm{RH}<0.99$) Bragg peak of the *S* phase approaches the *d* spacing of the *L* phase and finally, for RH=0.988, merges with the strong peak of the *L* phase (Fig. 1, panel c). The remaining diffraction peak slightly moved to lower *q* values indicating that caveolae membranes persist in absorbing water. In the third regime ($t>30.000$ *s*; RH>0.99), Diffraction peaks disappeared and the system exhibited pure diffuse scattering due to the caveolae bilayer form factor (Fig. 1, panel d) indicating that the interbilayer positional correlations were completely lost.[14,15] This finding means that, upon hydration, unbinding transition of caveolae membrane occurs. However, upon dehydration the system returns to the bound state, the EDXD patterns (not reported) being practically indistinguishable from those collected upon hydration at the same RH, demonstrating that the unbinding transition is fully reversible.

In Fig. 2 evolution of *d* spacing of both *L* (circles) and *S* (triangles) phases with RH is reported. The lamellar *d* spacing of both the lamellar phases increased monotonously with RH. For RH~0.988, *d*-spacing assimilation was observed. This finding could indicate a hydration-induced remixing of lipid species in the plane of the membrane. Otherwise, it could also mean that *L* and *S* phase domains remain separated with repeat spacings becoming similar with increasing RH. Indeed, in the miscibility gap of phospholipid/cholesterol mixtures $L_o$ and $L_d$ domains coexist with similar *d* spacings, and hence only an average Bragg peak is observed by x-ray diffraction.[12]

Furthermore, we also observed that the lamellar *d* spacings did not seem to reach any finite swelling limit. Such a swelling behavior is due to the electrostatic repulsion between opposing charged membranes and has been reported several times in the literature.[16]

Hydration can affect the long range order of multilamellar systems.[14] In our EDXD experiments, we observe that the diffraction intensity rapidly decreased with RH. However, the intensity decrease is strongly affected by water adsorption and cannot be used as an argument for a decaying long range order. On the other hand, the full width at half maximum (FWHM) of the Bragg peaks was found to be roughly constant. Since the FWHM of a diffraction peak is related to the average domain size of the multilayers, $L_m$, the latter observation is likely to mean that $L_m$ did not vary remarkably over the investigated range of RH. To have a direct estimation of the long range order in the multilamellar *L* phase, we used the Debye–Scherrer relation, $L_m=2\pi/\Delta q$, where $\Delta q=\sqrt{(\mathrm{FWHM})^2_{\mathrm{exp}}-(\mathrm{FWHM})^2_{\mathrm{beam}}}$, $(\mathrm{FWHM})_{\mathrm{exp}}$ is the experimental width of the (001) Bragg peak and $(\mathrm{FWHM})_{\mathrm{beam}}$ is the width of the intrinsic instrumental resolution function $[(\mathrm{FWHM})_{\mathrm{beam}}\sim 8\times 10^{-4}\ \text{Å}^{-1}]$. Given the calculated average domain size ($L_m\sim 700$ Å) and the lamellar *d* spacing ($d\sim 60$–$70$ Å), we estimate a number of about ten lamellae per scattering domain.

In conclusion, we have characterized the structure of caveolae membranes over a wide range of RH. The structure of caveolae membrane was found to be strongly dependent on hydration. At low hydration (RH~0.4) caveolae membrane

assemblies are composed of segregated lipid domains with different lamellar periodicities in contact with cholesterol crystallites. According to literature reviews, such phase-separated domains most likely reflect the coexistence of liquid-ordered domains containing cholesterol being rich in saturated lipid, and liquid-disordered domains being rich in unsaturated lipid.

Upon hydration, cholesterol crystals disintegrated and cholesterol molecules most likely redistributed homogeneously within the caveolae membrane plane. Simultaneously, both the lamellar phases did swell up to a single broad scattering signal was observed suggesting a complete remixing of lipid species in the plane of the membrane. At full hydration, unbinding transition of caveolae occurred as shown by the observation of pure diffuse scattering due to the caveolae bilayer form factor. Furthermore, EDXD has revealed itself as a powerful tool to investigate the effect of hydration on the structure of caveolae membranes.

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