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Glass-Like Membrane Protein Diffusion in a Crowded Membrane

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Many functions of the plasma membrane depend critically on its structure and dynamics. Observation of anomalous diffusion in vivo and in vitro using fluorescence microscopy and single particle tracking has advanced our concept of the membrane from homogeneous fluid bilayer with freely diffusing proteins into a highly organized crowded and clustered mosaic of lipids and proteins. Unfortunately, anomalous diffusion could not be related to local molecular details given the lack of direct and unlabeled molecular observation capabilities. Here, we use high-speed atomic force microscopy and a novel analysis methodology to analyze the pore forming protein lysenin in a highly crowded environment and document coexistence of several diffusion regimes within one membrane. We show the formation of local glassy phases, where proteins are trapped in neighbor-formed cages for time scales up to 10 seconds, which had not been previously experimentally reported for biological membranes. Furthermore, around solid-like patches and immobile molecules a slower glass phase is detected leading to protein trapping and creating a perimeter of decreased membrane diffusion.

Keywords: Anomalous Diffusion / Membrane Dynamics / Membrane Domains / High-Speed Atomic Force Microscopy / Single Molecule / Glass-Glass Transition

Graphical Table of Contents
Biological membranes are formed by lipids and proteins (and associated sugars). The composition, relative concentration and density of lipids and proteins define their aggregation and diffusion, which in turn regulate membrane protein distribution and function. In the recent years, evidence has been accumulated that biological membranes are complex and organized, featuring local aggregations of lipids and proteins (rafts) and protein species- and localization-dependent diffusion properties, which regulate the interaction of the membrane components and modulate the biomolecular processes taking place in the membrane.

Diffusion can be Brownian, i.e. the mean-square displacement (MSD) scales linearly with the time of observation. However, the observed diffusion trajectories of lipids and proteins in biological membranes often deviate from Brownian behavior. Indeed, it is observed that MSD of a membrane component has a nonlinear relationship with observation time: these types of anomalous diffusions are termed superdiffusion and subdiffusion when the object goes farther or stays closer to its initial position, respectively, compared to a Brownian movement. Such intricate and diverse diffusion properties at the cell membrane regulate biomolecular traffic and thus molecular encounters and function. We are just at the beginning of understanding membrane architecture and dynamics.

Lysenin is a 33kDa protein extracted from the coleomic fluid of the earthworm Eisenia fetida. It belongs to the family of Pore-Forming Toxins (PFT), and its mechanism of action is common to PFT and is described in six steps: Secretion, binding to the target membrane, two-dimensional diffusion, oligomerisation, assembly in hexagonal close pack, and pre-pore to pore transformation. Besides Lysenin’s affinity for Sphingomyelin (SM), membrane-embedded cholesterol (Chol) has been shown to facilitate the formation of Lysenin oligomers. Lysenin offers hence an excellent system for studying fundamentals of membrane diffusion and aggregation allowing it to interact with mixed SM/Chol bilayers at variable local density.

The general approach to study membrane protein diffusion is fluorescence microscopy combined with single molecule tracking. Fluorescence microscopy allows studying the diffusion of fluorescence-tagged molecules with high temporal resolution and under physiological conditions. Unfortunately, fluorescence microscopy comes with some shortcomings: (i) Only tagged molecules can be studied and are visible as long as they are not bleached, and (ii) the lateral and the spatial resolution are limited to optical diffraction ~200nm (distance between two objects that can be resolved in real space at the same time) and to fitting of the point-spread function ~20nm (typical precision of localization). Furthermore, only one single molecule can be tracked – keeping the environment non-fluorescent in the color of the observed tag is prerequisite for the localization of the molecule of interest.

High-Speed Atomic Force Microscopy (HS-AFM) offers a novel view of membrane protein architecture and dynamics. It contours proteins with about ~1nm lateral and ~0.1nm vertical resolution in buffer at ambient temperature and pressure, and is subsecondrate. Hence, HS-AFM does not only visualize the movement of unlabeled proteins, but it images all molecules within the membrane. Given this novel and unique feat, HS-AFM allows correlating diffusion to the molecular environment.
RESULTS/DISCUSSION

Here, we use HS-AFM to determine the effect of local molecular crowding on the diffusion behavior of lysenin on supported lipid bilayers (SLB) constituted of SM/Chol 1:1 (Figure 1a, Supplementary Movie 1). As previously reported, the molecules in pre-pore and pore states can be distinguished based on their membrane protrusion height. Both classes of molecules associate and diffuse next to each other as pre-pores inserts into the first leaflet and pores into both leaflets of the membrane. The resolution and signal-to-noise ratio in the HS-AFM movie allows almost each lysenin ring to be clearly depicted over the entire movie (Figure 1a, left 4 panels). In a time average over the entire movie (Figure 1a, right panel) areas of different morphology and dynamics are readily distinguished: In certain areas proteins form stable lattices and are well resolved after averaging. In contrast, in other areas, no structural features are recognizable reporting high dynamics in these locations. In agreement, the standard deviation (SD) map of the pixel height values over the entire movie duration (Figure 1b, left panel) displayed low values (purple) in the hexagonally packed areas, while the SD was high (yellow) in the diffusive areas. Interestingly, at the interface between these domains, well-defined circular patterns were detected, corresponding to lysenin localizations, where the SD-map displayed low to high SD values (Figure 1b, arrowheads). This indicated that molecules at interfaces revealed association times of varying duration from long (well-preserved topography and low SD) to short (undefined topography and high SD). Some of the pores, about 0.5% of all molecules got stuck by interactions with the mica supportand served us as proof that the vast majority of the molecules had motional freedom (Figure 1b, dashed squares, Figure 1c, Supplementary Movie 2). Furthermore, these molecules mimic anchored protein-pickets, and confirm experimentally that immobile molecules can strongly influence the diffusion of its annular environment (Figure 1c): The immobile molecule creates new borders that resemble structurally and dynamically the borders of the crystalline domains.

We analyzed the local dynamics of the proteins, using an approach inspired by Fluorescence Correlation Spectroscopy (FCS). Since in an AFM measurement, the pixel intensity is the molecular height, the pixel value is directly related to the presence of a molecule under the tip. Hence, the time that a pixel keeps a certain value reports directly about the residence time of a protein and a value change reports the diffusion ‘under’ that pixel. A section kymograph (Figure 1d, along the dashed line in Figure 1a) illustrates the power of the approach: Pixels constituting the crystalline areas display constant height values as a function of time (Figure 1d, top), while pixels that are part of the diffusive areas presents rapid changes (Figure 1d, bottom). At the border pixels are occupied with stable molecules for intermediate lag-times (seconds to minutes) in alternation with rapid molecular redistribution (Figure 1d, middle).

In order to analyze the diffusion of molecules ‘under’ each pixel, we calculated the difference of each pixel value \( z_i \) at time \( t \) with respect to a later moment \( t + \tau \) \( \Delta z_i(t, \tau) = z_i(t + \tau) - z_i(t) \). As a result, histograms of the height changes \( \Delta z_i(\tau) \) of
each pixel \((i)\) and varying lag-time \((\tau)\) were obtained. Such histograms, also termed
‘van Hove distributions’, are commonly used in colloidal sciences to extract diffusion
parameters.\(^{21}\) From each van Hove distribution on each pixel, the variance \(V(\tau)\) is
extracted, analogous to the MSD in single molecule tracking,\(^{22}\) following
\[
V(\tau) = \frac{1}{n} \sum_{i=1}^{n} (\Delta z_i(\tau) - m_i(\tau))^2
\]

where \(n\) is the number of frames of each pixel \(i\), and \(m_i\) the pixel mean value. Using this
approach, variance maps for varying \(\tau\) are generated (Figure 2a). As expected, the
variance increases with the \(\tau\) in all the domains, though much less in the crystalline
areas. Whatever the intensity of dynamics, as long as the behavior is Brownian then
the van Hove distribution is Gaussian. In contrast, non-Brownian dynamics, as found for
example in glasses, give rise to non-Gaussian van Hove distributions. This is because
particles in glasses are constraint and transiently caged by the dense packing and
interactions with neighboring particles, but undergo rare large displacements due to
cage rearrangements. To characterize such complex dynamics the Kurtosis \(K\) of the
distribution on each pixel is calculated, following
\[
K(\tau) = \frac{1}{n} \sum_{i=1}^{n} \frac{(z_i(t + \tau) - z_i(t) - m_i)^4}{\left( \frac{1}{n} \sum_{i=1}^{n} (z_i(t + \tau) - z_i(t) - m_i)^2 \right)^2}
\]

When \(K=3\), then the distribution in the van Hove plot is Gaussian and the underlying
motion is Brownian. Deviations from \(K=3\) are associated with anomalous diffusion, and
typically \(K>3\) are signature of the cooperative behavior in glasses.\(^{23}\)
Plotting \(K\) on each pixel as a function of varying \(\tau\), areas of non-Brownian dynamics are
highlighted (Figure 2b). Within the fluid areas, we find subregions, upper left corner,
which display a Kurtosis \(\sim 3\) at all \(\tau\), corresponding to free diffusion. However, most of
the fluid domains revealed Kurtosis significantly \(>3\) at short lag-times, and \(\sim 3\) when
analyzed over longer \(\tau>8\)s, corresponding to the average trapping time of particles in
this glass phase. Beyond this \(\tau\) the glass behaves like a Brownian fluid. The
interfaces between fluid and crystalline domains displayed high Kurtosis values \(>4\) even
over extended lag-times indicating that molecules are trapped for varying durations
and eventually up to minutes. The crystalline domains despite their low dynamics
displayed \(K\sim 3\), and correspondingly statistically to an area of Brownian diffusion.
To better understand the relationship between the structure and dynamics of these
domains,\(^{24}\) we undertook two types of analysis: First, for a more detailed
comprehension of the crystalline domains we performed negative stain electron
microscopy (EM) (Supplementary Figure S1a) combined with single particle analysis
(Supplementary Figure S1b), and cryo-EM (Supplementary Figure S1c) combined with
electron crystallography (Supplementary Figure S1d) of lysenin as individual molecules
and in the crystalline arrangement, respectively. Both approaches depicted lyseninas a
nonamericopore with two concentric density rings. The 9-fold symmetric molecule
assembles with \(p3\)-symmetry in the ‘hexagonal packing’ with \(a=b=12\)nm and \(\gamma=120^\circ\), in
agreement with HS-AFM (Figure 1a). In such an arrangement onelysenin ring occupies a
membrane area \(A = 2 \cdot (\sqrt{3}/4 \cdot a \cdot b)\) of 125nm\(^2\) corresponding to an area fraction(\(\phi\)
of 0.91. Second, for a more detailed comprehension of the fluid domains, we performed Delaunay triangulation and Voronoi tessellation (Supplementary Figure S2a). In the case of a 2D-lattice, each molecule has 6 nearest neighbors and the Voronoi cells are hexagons with 125nm² area. In the fluid domain, the average distance between molecules is 16.5±3.5nm, with about 5.5 nearest neighbors, and pores occupy areas up to 250nm², an approximate area fraction of 0.45 without short- or long-range order (Supplementary Figure S2b). Furthermore, the shape factor $\xi$ of the Voronoi cells have been analyzed: Each cell is characterized by a shape factor $\xi = (C^2/4\pi A)$, where $C$ is the perimeter and $A$ the area of the Voronoi cell. In the range $\phi=0.60-0.71$ a bimodal distribution of the shape factor is observed, meaning that molecules in the same density range have different number of neighbors, characteristic of phase transitions (Supplementary Figure S3). Having in hand methodologies to characterize diffusion properties (Figure 2) and local protein density (Supplementary Figure S2a) ‘under’ each pixel, we had competence to determine how diffusion properties scale and change as function of local membrane structure. Plotting the variance of all pixels in the movie as a function of the Voronoi cell area that comprises each pixel and as a function of varying $\tau$, a segregation of the data occurred (Figure 3a). A large number of data points gather at a Voronoi cell size of 125nm² and small variance that remains unchanged with increasing lag-time; these pixels are part of the crystalline area. In contrast, pixels in areas of low protein density and Voronoi cell size >200nm² show high variance that further increases with $\tau$. The Kurtosis of the crystalline area, Voronoi cell sizes of 125nm², is stable at a value of ~3, Brownian rattling (Figure 3b). However, pixels that locate in the fluid areas with low protein density and Voronoi cell area >200nm² depict wide spread Kurtosis values with many significantly >3at short $\tau$.

In analogy to typical MSD vs lag-time plots from single particle tracking, we plotted variance vs lag-time. Our approach has the advantage that proteins can be grouped together as a function of the local density in which they evolve. In general, the variance increases with lower local protein density (larger Voronoi cell). The slope of the variance as a function of $\tau$ is somewhat steeper at 240nm²/molecule compared to areas at 125nm²/molecule at short $\tau$, flattening at longer $\tau$. However, at intermediate protein density of 185nm²/molecule the slope is increased, especially for longer $\tau$, indicating particular diffusion of molecules at this density (Figure 3c, left). When plotting the variance vs Voronoi cell area for different $\tau$, we observe a biphasic, maybe triphasic, behavior: Low variance for the crystalline areas with 125nm² to 165nm² per molecule, high variance for fluid areas with 200nm² to 250nm² per molecule, and a steep variance increase for intermediate molecular density regions with 165nm² to 200nm² per molecule. Around Voronoi areas of 185nm² a weak variance plateau at short $\tau$, is found (Figure 3c, right).

Plotting the Kurtosis as a function of lag-time for the different Voronoi cells revealed three different populations of Brownian and non-Brownian dynamics (Figure 3d, left): Densely packed molecules have Kurtosis ~3, almost independent of $\tau$. When the local protein density is lower than 165nm² per molecule, then the Kurtosis raises abruptly to a completely non-Brownian regime. A third population is found when the protein density loosens further with Voronoi cells of 200nm² and larger: In this
regime, the Kurtosis is significantly non-Brownian at τ<10s, above this lag-time
diffusion is Brownian. 10s seems to be the average trapping time of lyseninin the
glassy-fluid phase. This striking triphasic behavior and its transitions are best visualized
when plotting the Kurtosis as a function of Voronoi cell area (Figure 3d, right):
Molecules evolving in a density regime of 165nm$^2$ to 200nm$^2$ membrane area (peaking
at 185nm$^2$) display non-Brownian diffusion characteristics basically independent of the
time span. These characteristics around $\phi \sim 0.61$ are in good agreement with theory and
simulation of 2D-glasses.$^{27, 28}$

HS-AFM features the advantage to visualize (i) non-labeled molecules and (ii) not only
single molecules but all molecules in the membrane. An approach inspired by FCS is
used to detect dynamics ‘under’ each pixel with nanometer resolution, of particular
importance in crowded systems. Qualitatively similar results were obtained
from automated single particle tracking (Supplementary Figure S4). However
considerable problems occur when tracking densely packed molecules, where the
motion of the molecules is comparable to the inter-particle distance. Furthermore, a
single local density cannot be attributed to a molecule trajectory as the molecule may
diffuse through heterogeneous domains. In contrast, the pixel-by-pixel
analysis approach used here (Figure 3) provides the possibility to correlate density and
diffusion.

CONCLUSIONS

In summary, the membrane contains four phases with different diffusion dynamics: At
$\phi$ ranging from 0.91 to 0.68 (solid phase), the proteins are essentially crystalline and
rattle around their position with Brownian dynamics. At borders of the solid phase, the
proteins evolve at $\phi$ between 0.68 and 0.56 (sliding glass) and are caged up to minutes.
Morphologically, this glassy area resembles a ‘sliding puzzle’ where moving complexes
occupy defined positions. At $\phi$0.56 to 0.45 (fluid glass) diffusion is fluid yet molecules
are caged at shorter time periods <10s. Calculating SD maps running over 10s of
movie acquisition revealed the presence of spatially correlated dynamics represented
by SDFluctuation waves (Supplementary Movie 4). This glassy phase is characterized
by lack of short-range order. At $\phi$ below 0.45 (liquid phase) proteins diffuse freely. Such
a coexistence of several glasses has been described in colloidal systems.$^{29, 30}$

In a crowded mosaic biological membrane, molecules diffuse in an environment with
$\phi \sim 0.5$ containing stable domains.$^{1, 4}$ Furthermore, molecular heterogeneity favors the
occurrence of glass dynamics.$^{24}$ Specialized membranes, e.g., photosynthetic
membranes$^{32}$ or retinal disk membranes$^{32, 33}$ may be even more crowded and comply to
cooperative rearrangements during their functional tasks.

HS-AFM allows correlating structure with diffusion behavior, and glassy diffusion is only
detectable when both movement and environment are simultaneously assessed.
Therefore, biologists may have missed glass-like diffusion in crowded membranes due
to the technical limitation of only tracking single molecules. Given the
crowdedness of cellular membranes, we hypothesize that glassy dynamics might be a
frequent feature of membrane protein regulation in vivo.
METHODS/EXPERIMENTAL

Protein purification
A cDNA fragment, coding for Lysenin (GenBank: BAA21518.1, GenScript, USA) and cloned into pET28a vector at BamHI and Hind III, and this vector transformed into BL21(DE3) strain (New England BioLabs France, Evry, France). The transformed cells were inoculated into 1 liter of LB medium containing 100 μg/ml kanamycin sulfate, and incubated at 37 °C while shaking at 200 rpm until the OD$_{600}$ value reached 0.6. For induction of Lysenin expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at final concentration of 0.5 mM, and cells shaken overnight at 20°C and 200 rpm. The bacteria were collected by centrifugation at 2000g for 10 minutes, and disrupted with 3 probe sonicator intervals of 15 seconds sonication and 30 seconds ice cooling each. The resulting suspension was shaken at 4°C for 30 minutes in Triton X-100 at 0.1% and RNase/DNase at 10 μg/ml concentration. The mixture was centrifuged at 10,000 g for 30 minutes. The supernatant (volume: 9 ml) was collected and mixed with 1 ml of metal chelating resin, chelating sepharose Fast flow (GE Healthcare France), composed of chelating cobalt in 100 mM NaCl, Hepes-NaOH, pH 7.5. Lysenin binding was performed by 1 hour incubation at 4°C with gentle shaking, and the resulting resin centrifuged at 100 g for 1 minute to discard the supernatant. The resin was washed with fresh 14 ml of 100 mM NaCl, 100 mM imidazole-HCl, Hepes-NaOH, pH 7.5 by 3 centrifuge/washing cycles. Lysenin was eluted in 1 ml of 100 mM NaCl, 250 mM imidazole-HCl, Hepes-NaOH, pH 7.5. To eliminate the imidazole, the eluate was dialyzed against 1 liter of 100 mM NaCl, Hepes-NaOH, pH 7.5. The resulting protein sample was directly used for the HS-AFM experiment.

Sample preparation for High-speed atomic force microscopy (HS-AFM) observation
Egg Sphingomyelin (SM) and Cholesterol (chol) (Avanti Polar Lipids, Alabama, USA) were used to form giant unilamellar vesicles (GUVs) at a molar ratio SM:Chol 1:1 through electroswelling. Of each lipid 10 µl at 3 mM dissolved in chloroform: methanol 3:1 were deposited in two glass plates coated with indium tin oxide with 70-100 Ω resistivity (Sigma-Aldrich) and placed 60 minutes in the desiccator for complete solvent evaporation. A U-shaped rubber piece of ~1 mm thickness was sandwiched between the two indium tin oxide coated slides. The so-formed chamber was filled with ~400 µl of 200 mM sucrose solution and exposed to 1.5 V sinusoidal 10 Hz AC current for 3 hours followed by squared 5 Hz AC current for 15 minutes, at 55°C. GUVs were harvested from the chamber. To form the supported lipid bilayers (SLBs) for HS-AFM, 1 µl of GUV solution was placed on a 1.5 mm-diameter freshly cleaved mica disk covered with 1 µl of phosphate buffer saline (PBS) and incubated for 30 minutes. To remove lipid that was not firmly attached the SLB was intensely rinsed with PBS. Once the bilayer was formed, 1 µl of purified lysenin was incubated for 15 minutes. Excess of protein was again rinsed with PBS.

High-speed atomic force microscopy (HS-AFM)
HS-AFM movies were acquired with an Ando-type setup equipped with a super luminescent diode (emission wavelength: 750 nm; EXS 7505-B001, Exalos, Schlieren, Switzerland) and a digital high-speed lock-in Amplifier (Hinstra, Transcommers, Budapest, Hungary). 8μm-long cantilevers with spring constant $k = 0.15$ Nm$^{-1}$, resonance frequency $f_r = 500$-700 kHz and quality factor $Q = 1.5$ in liquid (USC-1.2, NanoWorld, Neuchâtel, Switzerland), featuring an electron beam deposition (EBD) tip, were used. For high-resolution imaging the tip was sharpened by helium plasma etching using a plasma cleaner (Diener electronic, Ebhausen, Germany), resulting in a final tip radius tip of about 2 nm, as judged from analysis of the indentation inside the Lysenin rings. Amplitude modulation was used for imaging with free amplitude of ~1.2 nm and operating set point amplitude of ~0.9 nm. Under these conditions we estimate the applied force following $F = (k/\text{set point}) \times (A_0 (1− A_s/A_0) + h_0 \sin(\theta/2))$, where $A_0$ is the free amplitude, $A_s$ is the setpoint amplitude, $h_0$ is the step height of the sample, and $\theta$ is the phase delay of the feedback. Under our imaging conditions $F = 44pN$. All experiments were performed at room temperature and in physiological buffer.

**High-speed atomic force microscopy (HS-AFM) image treatment**

Image treatment was limited to the correction of a first-order XY plane fit and XY drift correction of the HS-AFM movie.

**High-speed atomic force microscopy (HS-AFM) data analysis**

The HS-AFM movie is considered a four dimensional matrix with lateral dimensions $X$ and $Y$, height dimension $Z$, and a time $t$. Time is subdivided in time-intervals $\tau$, the shortest $\tau$ is the time passing between the acquisitions of two subsequent frames. From this matrix, the height changes $\Delta z$ as a function of varying lag-time $\tau$, was calculated by subtraction, following $\Delta z_i(t, \tau) = z_i(t + \tau) − z_i(t)$ on each pixel. So-called van Hove plots, i.e., the histogram distribution of the height variations was calculated for each pixel. Following, the shape of the van Hove plots for each $\tau$ was analysed according two parameters: Variance $V$ and Kurtosis $K$. While the variance informs about the width of the van Hove plots, hence about the intensity of motion, the Kurtosis reports about the non-Gaussianity, hence about non-Brownian behaviour. Each of these steps is performed for every lag-time $\tau$ and for every pixel. This data treatment resulted in variance and Kurtosis maps as shown in figure 2. Variance and Kurtosis were calculated using pre-built functions in Matlab (Mathworks, Natick, USA).

To determine the local density, Voronoi tessellation was calculated from the localization of all particles. In order to determine the localization of all molecules in all frames, cross-correlation searches between a 360-fold symmetrized Lysenin ring (artificial reference) and each movie frame was performed. This resulted in cross-correlation maps of each frame that featured about 700 cross-correlation peaks each. Peak searches allowed the localization of about 740000 molecules in the movie (about 700 in each frame). A lab-developed package integrated in ImageJ was used for the cross-correlation analysis and trajectory extraction. Using the particle localizations, Voronoi tessellation was calculated using a pre-built function in Matlab (Mathworks, Natick, USA).
Combining, the two above-described analysis allowed correlating variance and Kurtosis with local protein density and evaluating how diffusion properties scale as a function of membrane structure.

The area fraction $\phi$ was calculated taking into account that the area occupied by a Lysenin ring is $A_{(mol)} = \pi d^2 = 113nm^2$ (where $d$ is the diameter of the lysenin ring, i.e., center-to-center distance in the crystal packing), and the unit cell area of the hexagonal close packing is $A_{(unit\ cell)} = 2\left(\frac{\sqrt{3}}{4} ab\right) = 124.7nm^2$, resulting in $\phi = 0.906899$. For our analysis $\phi$ is the ratio between $A\ (mol)$ and the unit cell or Voronoi cell in the real HS-AFM movie, in which it is located.

**Sample preparation for transmission electron microscopy (TEM) observation**

Adsorption and oligomerization of Lysenin on sphingomyelin-containing lipid monolayer were performed in custom-designed Teflon wells of 4mm in diameter and 1mm in depth. 0.5μl of lipid solution (Sphingomyelin/Phosphatidylcholin 1:4, Avanti Polar Lipids, Alabama, USA) at 0.1mg/ml in chloroform were spread on 15μl of Lysenin at 50μg/ml and incubated for 1 hour at room temperature to reconstitute oligomers or overnight to form 2D-crystal patches. The interfacial surface formed by the lipid monolayer and the adsorbed protein was transferred to carbon-coated grids and analyzed by transmission electron microscopy.

**Negative stain transmission electron microscopy (TEM) and image processing of single Lysenin oligomers**

For imaging of negatively stained samples, the grid was washed with three droplets of pure water and subsequently negatively stained with 2% (w/v) uranyl-acetate. The prepared grids were imaged using a Philips CM10 TEM (FEI Company, Eindhoven, the Netherlands) operated at 80kV. The images were recorded by the 2k x 2k side-mounted Veleta CCD camera (Olympus, Germany) at magnification of 130 000 x. Under these conditions the pixel size at the sample level is 3.7 Å. Image processing was achieved with EMAN2 software package. The images were CTF (contrast transfer function) corrected and the particles were semi-automatically selected. The ‘e2refine2d’ program was used to classify the particles, and produce reference-free class averages. The most populated class represented the top view.

**Cryo Transmission electron microscopy (cryo-TEM) and image processing of 2-crystal patches of Lysenin**

For cryo-TEM, the grid was blotted with Whatman filter paper and vitrified through plunging it into liquid nitrogen cooled liquid ethane using a vitrobot (FEI company, Netherlands). Frozen grids were transferred into a Philips CM200-FEG electron microscope using a Gatan 626 cryo-holder. Electron micrographs were recorded at an accelerating voltage of 200kV and a nominal magnification of 50 000 x, using a low-dose system (10 e⁻/Å²) and keeping the sample at liquid nitrogen temperatures.
Defocus values were around -2.5 µm. Micrographs were recorded on a 4K x 4K CMOS camera (TVIPS, Germany). The pixel size at the sample level is 2.1 Å. The 2D-crystal images were selected based on the presence of diffraction patterns with well-defined spots and further treated using the 2dx software.  

**ASSOCIATED CONTENTS**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI:  

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Figure 1) Lysenindynamics is location dependent. a) Four left panels: HS-AFM movie frames (Supplementary Movie 1) of Lysenin in asphingomyelin/cholesterol (1:1) bilayer. Right panel: Time-averaged frame displaying the positional stability and the high mobility of proteins in the solid and fluid domains (full false color scale: 10nm). b) Left: standard deviation (SD) map of the pixel height values (t=0-505s). Lattice borders with well-defined molecular positions of varying SD (arrows). SD maps (t=0-168s, center) and (t=168-336s, right): At t=168s allocation (dashed squares) drastically changed dynamics (full false color scale: 0.3<SD<1.6nm). c) Individual frames t=90s and t=276s of the molecular organization corresponding to the outlines in b), see also supplementary movie 2. Right: SD map (t=168-336s) of this membrane region displaying the annular alteration of diffusion dynamics around the stuck molecule. d) Kymograph (of the white dashed line in a). Stable (top), highly mobile (bottom), and molecules switching between stability and high mobility (middle) are visible during the entire movie.
Figure 2) Detection of areas of non-Brownian dynamics. a) Variance ($V$) of the distribution of height changes (false color scale: $0<V<2\text{nm}^2$). b) Kurtosis ($K$) (non-Gaussianity) of the distribution of the height changes (false color scale: $2.5<K<5.0$).
Figure 3) Diffusion as function of local density. a) Distribution of Variance and b) Kurtosis for all pixels as a function of Voronoi cell area and lag time (color indicates the concentration of data points in the plot area from blue (low abundance) to red (high abundance)). c) Variance and d) Kurtosis as function of lag time and Voronoi cell area: Each point is the median of all pixels with the same characteristics.