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Resolving the Morphology of Peptoid Vesicles at the One Nanometer Length-Scale Using Cryogenic Electron Microscopy

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ABSTRACT

Vesicle formation in a series of amphiphilic sequence-defined polypeptoid block copolymers comprising a phosphonated hydrophilic block and an amorphous hydrophobic block, poly-N-(2-ethyl)hexylglycine-block-poly-N-phosphonomethylglycine (pNeh-b-pNpm) is studied. The hydrophobic/hydrophilic block ratio was varied keeping the total chain length of the copolymers constant. A new approach for characterizing the vesicle membrane morphology based on low-dose cryogenic electron microscopy (cryo-EM) is described. The individual low dose micrographs cannot be interpreted directly due to low signal-to-noise. Sorting and averaging techniques, developed in the context of protein structure determination, were thus applied to vesicle micrographs. Molecular dynamic (MD) simulations of the vesicles were used to establish the relationship between membrane morphology and averaged cryo-EM images. This approach enables resolution of the local thickness of the hydrophobic membrane core at the one nanometer length scale. The thickness of the hydrophobic core of the pNeh-b-pNpm membranes increases linearly with the length of the hydrophobic block.

INTRODUCTION

There is continuing interest in vesicles due to their ability to encapsulate and release drugs and other biomolecules\textsuperscript{1-3}. Vesicles are formed by the self-assembly amphiphilic molecules such as phospholipids (liposomes) and block copolymers (polymerosomes) in water. We use the term vesicles to refer to both systems. The morphology of vesicles has been studied by both scattering and microscopy. Characterization methods in reciprocal space such as small angle x-ray scattering (SAXS) and small angle neutron scattering (SANS) provide information on ensemble-averaged properties such as membrane curvature and thickness.\textsuperscript{4-19} Determining
parameters such as the local thickness of hydrophobic and hydrophilic layers has been outside the scope of these methods.\textsuperscript{4, 8-10, 20-21}

Freeze-fracture and chemical staining are often used sample preparation methods for electron microscopy studies.\textsuperscript{22-24} The resolution of structures determined by these approaches \textsuperscript{25-26, 27-28} is significantly lower than of pioneering work of Lepault \textit{et al.} \textsuperscript{29} who used cryogenic electron microscopy (cryo-EM). Following this study, low-dose cryo-EM is widely used to accurately characterize the morphology of vesicles. In this approach, the natural status of vesicles in solution is preserved as they are frozen and hydrated, and radiation damage minimized.\textsuperscript{29-41} Accurate measurement of membrane thickness is crucial because it controls transport in and out of the vesicle. It also reflects the conformation of molecules in the bilayer. In early work, Tahara \textit{et al.} \textsuperscript{34} proposed that the bilayer thickness of frozen hydrated liposomes can be quantified by comparing cryo-EM micrographs with computed EM images of membrane models. They showed the relationship between bilayer thickness and molecular structure. However, they used simplified membrane models accounting only for phosphorus atoms placed at pre-specified locations to mimic the bilayer membrane. In contrast, Wang \textit{et al.} determined the thickness of the hydrophobic membrane of phospholipid liposomes using radially averaged electron density profiles in cryo-EM micrographs.\textsuperscript{42} They also used molecular dynamics simulations to interpret their data. Bermudez \textit{et al.} also used rotational averaging to determine the bilayer morphology of the poly(ethylene oxide)-\textit{b}-polybutadiene polymersomes\textsuperscript{43}. In their study, the hydrophobic core thickness was calculated by fitting the rotationally averaged profiles to a simple homogeneous density model for the vesicles. Cryo-electron tomography (cryo-ET) has also been used to investigate both liposomes and polymersomes.\textsuperscript{44-46} This approach provides the three-dimensional structure of vesicles but quantification of membrane morphology is limited by low signal-to-noise ratio (SNR).\textsuperscript{27-28}
When dispersed in water, amphiphilic polypeptoid block copolymers self-assemble into a variety of structures such as sheets and cylinders. In this paper we study vesicles formed by polypeptoid diblock copolymers containing a phosphonated hydrophilic block and an amorphous hydrophobic block. We describe a new technique for determining the morphology of vesicle membranes based low-dose cryogenic electron microscopy (cryo-EM). To date, radial averaging of cryo-EM images is the most sophisticated approach for studying vesicle morphology. Unfortunately, information of local variations in membrane thickness within a vesicle is lost when this approach is used. Instead of radial averaging, we leveraged sorting and averaging techniques, developed by the structural biology community for determining protein structure. In this approach, segments of the membranes are sorted into classes and then averaged. Some of the differences in local membrane morphology (especially in polymerosomes) may arise from molecular weight dispersity. We have used a sequence-defined polypeptoid block copolymers with ultra-low molecular weight dispersity to minimize these effects. To gain fundamental insight into the relationship between molecular structure and membrane morphology, we synthesized and characterized a series of polypeptoid block copolymers with different block ratios but identical total chain lengths. Molecular dynamic simulations are used to establish the relationship between membrane morphology and the averaged cryo-EM micrographs. Morphological results obtained by cryo-EM are compared with simulation results.

**EXPERIMENTAL SECTION**

**Polypeptoid synthesis and self-assembly**

A series of sequence-defined amphiphilic polypeptoid block copolymers, poly-$N$-(2-ethyl)hexylglycine-$block$-poly-$N$-phosphonomethylglycine (pNeh$_m$-$b$-pNpm$_n$), were synthesized as described in our previous work. The characteristics of the copolymers ($m$, $n$, molecule weight, dispersity index, and molecular purity) are given in Table 1. The chemical structure of the
copolymer is shown in Figure 1A. The series in Table 1 enable quantification of the effect of block copolymer composition (the ratio \( m/n \)) at a fixed chain length of 36 \((m + n = 36)\), as depicted in Figure 1B. Vesicles were prepared by dissolving these amphiphilic polypeptoids in a tetrahydrofuran (THF)/water mixture (50:50 by volume), followed by slow evaporation of the THF at room temperature for 72 hours. The initial concentration of solutions was 5 mg/mL. The final concentration of the peptoid/water solutions after THF removal was 10 mg/mL.

**Cryo-EM characterization and classification**

Gold lacey carbon grids (Ted Pella Inc.) were glow discharged in air for 15 s in a plasma chamber to make the surfaces hydrophilic. Three microliters of a peptoid vesicle suspension was added onto the front side and blotted from the both sides for 3 s using Vitrobot (FEI Inc.) at 20 °C in air with 100% relative humidity (RH). The grids were rapidly frozen by plunging into liquid ethane and stored in liquid nitrogen. Micrographs of frozen hydrated vesicles were collected using JEOL-3100FSC transmission electron microscopy (JEOL Inc. Japan) at 300 KeV with the energy filter slit width at 35 eV. Movies were recorded at 20K magnification on the JEOL-3100FSC by a K2 direct electron detector in counting mode. The accumulated dose for each movie was \( \sim 40 \, \text{e}/\text{Å}^2 \). The dose fractionation movies were aligned and averaged using Motioncorr2. The Contrast Transfer Function (CTF) and defocus values were obtained by using the program gCTF. The level of defocus varied from 0.8 to 2.0 \( \mu\text{m} \) as suggested by the CTF estimations (Table S1). The small square boxes (80 nm, 50nm and 28 nm respectively) were extracted using the program Relion along the vesicle membrane in the CTF corrected micrographs.

The boxes were classified using the software package Relion 2.0. Relion uses the maximum-likelihood approach for sorting cryo-EM micrographs. This approach has proven to be particularly useful in the classification of
structurally heterogeneous data from biomolecules. The intensity in the boxes is first normalized. Reference-free class averages are obtained in a completely unsupervised manner by starting multiple references from average images of random sets of the normalized images in the extracted boxes. All images are compared to all references in all possible orientations and probability weights are calculated for each possibility instead of assigning images to one particular class or orientation. Class averages are then calculated as weighted averages over all possible assignments. The number of classes requested is set by the user: fewer boxes participate in each class with increasing the number of classes. Averaging over a small number of boxes leads to noisy averages, which result in suboptimal alignment and classification. For the boxes obtained from the vesicles, we found that 6 classes gave the most informative results.

**Molecular dynamics simulation and EM image simulation**

Initial coordinates for molecular dynamic simulations were generated with all backbones in the *cis*-amide conformation. For the monolayer and bilayer, two chains containing side chains of all Neh or all Npm were randomly distributed within the monolayer and bilayer. The dihedral potential for the amide bonds in the Npm residues were reduced to facilitate faster isomerization. Topology information and psf generation were performed using autopsf in VMD. Water molecules were added using the autosal plugin in VMD. Simulations were run in NAMD with CHARMM force-field parameters similar to MFTOID. Simulations were run at 300 K for at least 19 ns. We calculated projected potential images from the relaxed atomic models. These simulated EM images were computed using the multislice method and scattering potentials given by Kirkland. All multislice simulations used the same microscope parameters as in the experiment.

**RESULTS AND DISCUSSION**
Typical low-dose cryo-EM images obtained from our peptoid vesicles are shown in Figure 1C through 1F. Classical vesicles are obtained when the hydrophilic chain length $n$, is between 18 and 6. Reducing $n$ from 6 to 4 results in morphological change; a network morphology is obtained in \( \text{pNeh}_{32}\text{-b-pNpm}_4 \). In spite of the variety of morphologies seeing in Figures 1C through F, the membranes are clearly seen in all cases. The interaction of high-energy electrons with soft materials is well-described by the weak-phase object approximation.\(^{66}\) In these systems, contrast is produced by defocusing the objective lens.\(^{66}\) Consequently, all of the micrographs used in this study were obtained under defocused conditions; images obtained in the absence of defocus were uninterpretable. The contrast transfer function (CTF) and defocus values used in this study are given in the supplementary information (Figure S1 to S4 and Table S1.)

We use a typical vesicle obtained from \( \text{pNeh}_{26}\text{-b-pNpm}_{10} \) to describe the details of our approach. This vesicle is shown in Figure 2A. Also shown in Figure 2A is an overlaid black circle concentric with the vesicle, which helps visualize the vesicle’s deviations from perfect circularity. Some of these deviations may be caused by neighboring vesicles: note the additional vesicle located in the lower right corner of the micrograph in Figure 2A. In addition to shape variation, thickness variations were also evident in some of the vesicles. This is demonstrated using images of two portions of the \( \text{pNeh}_{26}\text{-b-pNpm}_{10} \) vesicle, labeled by the arrows in Figure 2A. Enlarged views of the membrane in the vicinity of the arrows are shown in Figures 2B and 2C. The portion of the membrane in Figure 2C is visibly thicker than that in Figure 2B.

In spite of working under defocused conditions, the contrast between the vesicles and their surroundings is low due to our use of low electron doses. Previous studies have used rotationally averaged intensity as a function of the radial coordinate of micrographs to increase signal-to-noise \(^7,^{34,67}\). The result rotational averaging on the vesicle in Figure 2A is presented in Figure 2D (top curve). The intensity profiles obtained in the two boxes
identified in Figures 2B and 2C are also shown in Figure 2D. These profiles are more noisy due to the fact that they were obtained after averaging the limited data contained in each box. All of the curves in Figure 2D show two minima, representing the locations of the edges of the membrane. The locations of these minima, shown by the arrows in Figure 2D, are similar for the rotationally averaged and box B curves. The nominal membrane thickness (8.2 nm) obtained by rotational averaging is similar to that obtained from box B. The nominal membrane thickness from box C is 11.2 nm, which differs substantially from the rotationally averaged thickness and that of box B. It is evident that important local thickness variations in membrane thickness may be lost if the images are rotationally averaged. An approach to overcome this limitation is described below.

The classification method, established by the structural biology community for determining the atomic arrangement within proteins, was adapted in this study. We used the program Relion for classifying portions of the vesicle micrographs. In this program, the micrographs are divided into boxes that are classified and averaged. In the case of proteins, there are clear guidelines for determining the appropriate box size, which is governed by the size of the molecule. The guidelines for determining the box size that should be used for classification of vesicle membranes have, however, not yet been established. We thus performed the classification and averaging process using different box sizes. In Figure 3A, we show averaged micrograph obtained with a relatively large box size (80 nm). A circular mask with a diameter of 70 nm was used to focus on the membrane (rather than extraneous signals near the corners of the box). Figure 3A shows the class average with the highest probability. The averaged image of the vesicle membrane in Figure 3A appears blurry at the edges. This artifact is due to differences in the local curvature of the membranes that are not properly averaged in this large box. Reducing the box size to 50 nm with a 40 nm diameter mask reduces the blurriness at the edges but does not eliminate it
The blurriness is absent when 28 nm boxes with 28 nm masks are used (Figure 3C). All of the analysis that follows is based on the mask size of 28 nm.

The sorting and averaging procedure described above resulted in the identification of three prominent classes shown in Figure 4A. We call the 3 classes 1a, 1b, and 2. Classes 1a and 1b have similar membrane thicknesses (about 10 nm), while class 2 has a membrane thickness of 12 nm. Classes 1a and 1b differ slightly in the radius of curvature and the projected density at the inner edge of membrane. The values reported in the Figure 4A correspond to the probability of finding these classes in our data set. These three classes accounted for 98% of the data. The three other classes that represent 2% of the data are shown in SI (Figure S5). The locations of the three prominent classes in the vesicle membrane shown in Figure 2A are indicated by different colors in Figure 4B. Most of the class 2 boxes are separated from class 1 boxes. This indicates localized variations in membrane thickness.

We use molecular dynamics (MD) simulations to interpret the observed micrographs in terms of membrane morphology. In Figure 5A, we show one of the initial configurations of the membrane formed by $\text{pNeh}_{26-b-pNpm}_{10}$. We defined a Cartesian reference frame (see Figure 5), and the projections in Figure 5 correspond to the $x$-$y$ plane. Not surprisingly, this projection is very similar to the projection in the $y$-$z$ plane (see Figure S6 in SI). The water molecules and the side chains on both hydrophilic and hydrophobic blocks are not shown in Figure 5 for clarity. In the initial configuration, the hydrophobic chains emanating from opposing sides of the membrane are interdigitated to form a monolayer. The MD simulations were run for 19 ns and a typical relaxed molecular configuration is shown in Figure 5B. The final configurations of the hydrophobic pNeh chains are more disordered than the initial configurations but the interdigitated character of the
The hydrophobic membrane core is retained. The hydrophilic brushes are heterogeneous with pockets of pure water separated by pockets of high concentrations of pNpm chains; the pockets of pure water appear blank in Figure 5B. In Figure 5C, we show a second initial configuration of the membrane. In this configuration, the hydrophobic chains emanating from opposing side of the membrane form a bilayer. In other words, the hydrophobic chains are not interdigitated. The MD simulations were run for 95 ns and typical relaxed molecular configurations thus obtained are shown in Figure 5D. The molecules adopt a tilted configuration relative to the plane of the membrane (the membrane is nearly flat on length scales comparable to the membrane thickness). The thicknesses of the relaxed monolayer and bilayer hydrophobic cores are 8.6 and 13.1 nm, respectively.

We arrived at the procedure for analyzing the micrographs by simulating images of the relaxed membranes obtained by MD simulations described above. In Figure 6 we show the locations of all the atoms in pNeh_{26}-b-pNpm_{10} chains in the simulated relaxed membrane, as all of them contribute to the electron micrograph. The hydrophilic brush is shown by itself in Figure 6A. The hydrophobic core is shown by itself in Figure 6B. The entire membrane is shown in Figure 6C. Computed electron micrographs at zero defocus (in-focus micrographs) of the structures shown in Figures 6A through C are shown in Figures 6D through F. Computed electron micrographs of the entire membrane at defocus values of 1.5, 1.6 and 1.7 µm are shown in Figures 6G through I. These are the defocus values used in the experiments. The computed images in Figures 6G through I were obtained after removing the water molecules from the box. The vertical dashed lines in Figures 6A through C indicate average properties of the MD simulated membrane. This was done to clarify the relationship between membrane morphology and captured images. The green vertical lines in Figure 6J represent the averaged thickness of hydrophobic pNeh core. The position of the interface between hydrophobic core and hydrophilic brushes,
which can be identified for each molecule in the simulations precisely, deviates significantly from the dashed green lines due to the roughness of the interface. The simulated averaged hydrophobic core thickness, \( t \) is 8.6 nm. It is relatively easy to locate the membrane core and hydrophilic brushes on the computed zero defocus images of the individual components shown in Figures 6D and E. However, Figure 6F shows that there is no contrast between the hydrophilic and hydrophobic chains at zero defocus. The contrast between the membrane and the background is significantly improved with the introduction of defocus (Figures 6G through I).

In Figure 6J, we plot 1-D intensity profiles across the computed images as a function of \( y \), by averaging the image intensities along the \( x \) direction. Each plot in Figure 6J is labeled to indicate the computed image that used for averaging. Also shown in Figure 6J are dashed lines indicating the expected locations of the hydrophobic core (the locations of the vertical lines in Figures 6A through C). The top three curves represent the zero defocus images. The intensities in these images fluctuate around zero in both core and brushes. It is clear that the computed zero defocus images contain no signatures of the membrane, in spite of removing all of the water molecules from the simulation box. The bottom three curves in Figure 6J show the 1-D intensity profiles of images obtained under defocus conditions used in the experiments. The profiles obtained at defocus values between 1.5 and 1.7 \( \mu \)m are qualitatively similar. These profiles contain clear signatures of the membrane morphology. The edges of the hydrophobic core are given by the locations of the crossings of the intensity profile and horizontal baseline. The intensity profiles in Figures 6G through I have oscillations in the vicinity of the crossings. This is due to Fresnel fringes that are obtained due to sharp changes in electron density at the interfaces in the simulations. Note that the inner valleys are located within the hydrophobic core. These valleys represent the dark bands seen in the micrographs (Figure 4A). The thickness of the hydrophobic core is thus the sum of the inner grey band and the outer dark bands. The bright Fresnel fringe size in Figure 6J is coincident with the
expected size of the hydrophilic brush. However, since the Fresnel fringe is arises entirely due to the defocus value used, we conservatively conclude that the size of the hydrophilic brush cannot quantitatively be determined in our system.

The thickness of the hydrophobic core in Class 1 pNeh$_{26}$-b-pNpm$_{10}$ images, $t$, is $10.2$ nm (the distance between the dashed lines in Figure 7A). A similar analysis on Class 2 pNeh$_{26}$-b-pNpm$_{10}$ images gives $t = 12.8$ nm as shown in Figure 7B. The experimentally determined values of $t$ are in reasonable agreement with the thicknesses of the hydrophobic core obtained from the monolayer and bilayer simulations (Figure 5), 8.6 and 13.1 nm, respectively.

In Figure 8A-C, we show the comparison between the dominant averaged EM images corresponding to block copolymers pNeh$_{18}$-b-pNpm$_{18}$, pNeh$_{30}$-b-pNpm$_{6}$ and pNeh$_{32}$-b-pNpm$_{4}$. Images of vesicles used are given in Figure S1 through S4 in SI. The series of peptoids used here enable study of the effect of block copolymer composition on vesicle membrane morphology at fixed chain length ($m+n=36$). Also shown in Figure 8 are the corresponding 1-D intensity profiles corresponding to the averaged EM image (see Figure S7 through S9 for sorted and averaged EM images). The methodology described above was used to determine the hydrophobic core edges which are indicated by the dashed vertical lines in Figure 8. The corresponding MD simulations can be found in Figure 5, and Figures S6 and S10. The effect of molecular structure on membrane thickness obtained by this analysis is summarized in Table 2.

The dependence of membrane thickness on chain length of a series of homologous amphiphiles is often described by power laws. For the peptoids in this study, one expects a relationship of the type

$$t = km^b.$$ 

The prefactor $k$ depends on the parameters like the molecular flexibility and thermodynamic interactions between the blocks themselves and those between the blocks and water. The exponent $b$ reflects the molecular
conformations with in the hydrophobic core. If the chains in the core obey random walk statistics then \( b = 0.5 \). Larger values are obtained if the chains adopt extended configurations. In Figure 9, we plot \( t \) versus \( m \) obtained from cryo-EM experiments and MD simulations. The simulations give an exponent \( b = 0.92 \pm 0.18 \), while experiments give \( b = 1.0 \pm 0.13 \). The exponents indicate that the pNeh chains in the hydrophobic core adopt extended configurations. The experimentally determined pre-factor \( k \) is \( 0.52 \pm 0.32 \) nm, while the MD simulations give \( 0.31 \pm 0.13 \), suggesting that the chain conformations in the vesicles are more extended than those obtained by MD simulations. It is evident that more elaborate simulations are necessary to accurately predict the conformations of peptoid molecules in vesicles. The MD simulations were, however, essential for developing robust algorithms for interpreting the classified and sorted cryo-EM images.

CONCLUSIONS

We studied the morphology of self-assembled membranes formed by a series of sequence-defined amphiphilic pNeh\(_m\)-b-pNpm\(_n\) polypeptoid block copolymers in water. The total chain length of the peptoid samples used this study was identical \((m+n=36)\) but various block ratio was varied from 18/18 to 32/4. Peptoid vesicles, which form when \( n \) exceeds 4 in this series, may enable access to properties that are not accessible to either liposomes or polymerosomes due to the presence of the glycine backbone in the hydrophobic block. The tunability of of peptoids enables further control over physical characteristics. The ultra-low molecular weight dispersity makes them attractive candidates for studying the relationship between molecular structure and self-assembly. The morphology of the vesicles was characterized by low-dose cryo-EM imaging with contrast generated by defocus. Image sorting and averaging techniques, developed for the determination of protein structure, were modified to analyze the vesicle images. We used MD simulations to interpret the averaged cryo-EM images.
Analysis of the computed images indicated that the dark bands in the vesicle micrographs are part of hydrophobic core. They arise due to necessity for using a defocused configuration, and concomitant presence of Fresnel fringes in the micrographs. The membranes seen in our samples were dominated by monolayers. The thicknesses of the hydrophobic cores of the membranes increase linearly with the length of the hydrophobic block (m), indicating that the chains are in extended conformations.

ASSOCIATED CONTENT

Supporting Information

Supporting Figures S1 to S11 and Table S1

5 relaxed atomic models after MD simulation in PDB format

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NOTES

The authors declare no competing financial interest.

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### Table 1. Characteristics of the diblock polypeptoids pNeh\textsubscript{m}-b-pNpm\textsubscript{m}

<table>
<thead>
<tr>
<th>Polypeptoids</th>
<th>m</th>
<th>n</th>
<th>molar mass(^1) (g/mol, calc/obs)</th>
<th>Disperstiy(^2)</th>
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<tr>
<td>pNeh\textsubscript{18}-b-pNpm\textsubscript{18}</td>
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<td>18</td>
<td>2941.4/2941.4</td>
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<tr>
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<td>5823.8/5824.4</td>
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<td>6</td>
<td>5969.9/5970.9</td>
<td>1.0001</td>
</tr>
<tr>
<td>pNeh\textsubscript{32}-b-pNpm\textsubscript{4}</td>
<td>32</td>
<td>4</td>
<td>6042.5/6043.4</td>
<td>1.0002</td>
</tr>
</tbody>
</table>

\(^1\) Determined by electrospray ionization (ESI) mass spectrum.
\(^2\) PDI is estimated as described by the ESI and matrix assisted laser desorption/ionization mass spectrometry (MALDI) data.\(^70\)
Figure 1. A. Chemical structure of amphiphilic polypeptoid; B. Schematic cartoon of molecular structure represents the amphiphilic polypeptides with same chain length but different block ratios. C-F. Cryo-EM micrographs of frozen hydrated polypeptoid vesicles. C. pNeh\textsubscript{18}-b-pNpm\textsubscript{18}; D. pNeh\textsubscript{26}-b-pNpm\textsubscript{10}; E, pNeh\textsubscript{30}-b-pNpm\textsubscript{6}; F, pNeh\textsubscript{32}-b-pNpm\textsubscript{4}. The arrows point to the vesicle used in further analysis (Figures 2-4).
Figure 2. A. Cryo-EM micrographs of a frozen hydrated polypeptoid vesicle. Same vesicle is indicated by a thick arrow in Figure 1B. The dark circle is drawn to be concentric with the vesicle. The center of this circle is used to obtain the rotational average of the signal intensity. B. Enlarged cryo-EM micrograph near arrow 1 in Figure 2A. C. Enlarged cryo-EM micrographs near arrow 2 in Figure 2A. D. Line profiles showing the signal intensity as the function of radial distance corresponding to the boxes in Figures 2B and 2C. Also shown is the rotationally averaged signal intensity as the function of radial distance for the entire vesicle. Green arrows indicate the nominal positions of inner and outer dark regions in membrane. These arrows do not represent the edges of hydrophobic membranes.
Figure 3. Sorted and averaged images of boxes extracted from vesicles. **A.** Image shows averaged vesicle membrane using a 80 nm box and 70 nm diameter circular mask. Arrows point to fuzzy images that indicate this box size is too big. **B.** Image shows averaged vesicle membrane using a 50 nm box and 40 nm diameter circular mask. **C.** Image shows averaged vesicle membrane using a 28 nm box and 28 nm diameter circular mask.
Figure 4. A. Averaged high-resolution electron micrographs of boxes extracted from vesicles after classification. Classes 1a and 1b have very similar membrane thicknesses (collectively class 1) while class 2 has a larger membrane thickness. The probability of finding each class in the 4 vesicles is given in the boxes. B. The locations of the boxes along the vesicle perimeter are shown, k blue is class 1 while light green is class 2. Note that the membrane is thick in the top right quadrant.
Figure 5. Molecular dynamic simulations of \( \text{pNeh}_{26-b}\text{-pNpm}_{10} \). **A.** Initial configuration of monolayer membrane; **B.** relaxed simulation of monolayer membrane after 19 ns; **C.** Initial configuration of bilayer membrane; **D.** relaxed simulation of bilayer membrane after 95 ns. Blue chains represent the hydrophilic pNeh backbones and green chains represent the hydrophobic pNpm backbones. Hydrophobic core thickness, \( t \), is 8.6 and 13.1 nm in monolayer and bilayer membrane respectively.
Figure 6. A. Typical configuration of hydrophilic pNpm brush in relaxed monolayer simulations of pNeh<sub>26</sub>-b-pNpm<sub>10</sub> shown in blue. B. Typical configuration of pNeh hydrophobic core. C. The whole relaxed membrane. D. Computed in-focus EM image of the pNpm brush in A. E. Computed in-focus EM image of the pNeh core in B. F. Computed in-focus EM image of the whole membrane in C. G-I. Computed EM images of whole membrane at defocus values indicated in the figures. J. Averaged intensity profiles across the width of images D through I. Green dash lines indicate the interface between pNpm and pNeh; t represents the thickness of hydrophobic core. The number on the left for each curve in J represents the defocus value used for image computation.
**Figure 7.** 

A. Averaged cryoEM image in class 1a of pNeh$_{26}$-b-pNpm$_{10}$ (left panel) and averaged intensity profile across the width of image (right panel). 

B. Averaged cryoEM image in class 2 of pNeh$_{26}$-b-pNpm$_{10}$ (left panel) and averaged intensity profile across the width of image (right panel). Green dashed lines indicate the edges of the hydrophobic core of the membranes.
Figure 8. **A.** Averaged cryoEM image in class 1a of pNeh$_{18}$-b-pNpm$_{18}$ (left panel) and averaged intensity profile across the width of image (right panel). **B.** Averaged cryoEM image in class 1a of pNeh$_{30}$-b-pNpm$_{6}$ (left panel) and averaged intensity profile across the width of image (right panel). **C.** Averaged cryoEM image in class 1a of pNeh$_{32}$-b-pNpm$_{4}$ (left panel) and averaged intensity profile across the width of image (right panel). Green dashed lines indicate the thickness of the hydrophobic core.
Table 2. Morphology of monolayer vesicle membranes by simulations and cryo-EM

<table>
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<tr>
<th>Images</th>
<th>pNeh&lt;sub&gt;18&lt;/sub&gt;-b-pNpm&lt;sub&gt;18&lt;/sub&gt;</th>
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<th>pNeh&lt;sub&gt;30&lt;/sub&gt;-b-pNpm&lt;sub&gt;6&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>Thicknesses of hydrophobic core of monolayer membrane simulations (nm)</td>
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<td>8.6</td>
<td>9.0</td>
<td>10.0</td>
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<tr>
<td>Thicknesses of hydrophobic core in averaged cryo-EM image of the dominant class, (nm)</td>
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<td>10.2</td>
<td>11.8</td>
<td>13.7</td>
</tr>
<tr>
<td>% boxes in dominant cryo-EM class (class 1)</td>
<td>99%</td>
<td>79%</td>
<td>85%</td>
<td>89%</td>
</tr>
</tbody>
</table>
Figure 9. A dependence of the thickness of the hydrophobic core of membranes, $t$, on the number of Neh repeat units in the chain, $m$, obtained by cryo-EM experiments, are compared with MD simulation results. The lines are power law fits through the data. The power law exponents for both data sets are about 1.