# High resolution membrane structures within hybrid lipid-polymer vesicles

# revealed by combining x-ray scattering and electron microscopy

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Hybrid vesicles consisting of phospholipids and block-copolymers are increasingly finding applications in science and technology. Herein, small angle X-ray scattering (SAXS) and cryoelectron tomography (cryo-ET) were used to obtain detailed structural information about hybrid vesicles with different ratios of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and poly(1,2-butadiene-block-ethylene oxide) (PBd<sub>22</sub>-PEO<sub>14</sub>,  $M_s = 1800 \text{ gmol}^{-1}$ ). Using single particle analysis (SPA) we were able to further interpret the information gained from SAXS and cryo-ET experiments, showing that increasing PBd<sub>22</sub>-PEO<sub>14</sub> mole fraction increases the membrane thickness from 52 Å for a pure lipid system to 97 Å for pure PBd<sub>22</sub>-PEO<sub>14</sub> vesicles. We find two vesicle populations with different membrane thicknesses in hybrid vesicle samples. As these lipids and polymers are known to homogeneously mix, bistability is inferred between weak and strong interdigitation regimes of PBd<sub>22</sub>-PEO<sub>14</sub> within the hybrid membranes. It is hypothesized that membranes of intermediate structure are not energetically favourable. Therefore, upon formation, each vesicle selects one of these two membrane structures, which are assumed to have comparable free energies. We conclude that, by combining biophysical methods, an accurate determination of the influence of composition on the structural properties of hybrid membranes is achieved, revealing that two distinct membranes structures can coexist in homogeneously mixed lipid-polymer hybrid vesicles.

# 1. Introduction

Vesicles have many applications from microreactors[1] and sensors[2], to drug delivery vectors [3-5] and models for cell membrane research.[6, 7] Lipids and amphiphilic block copolymers are common materials for fabrication of synthetic or model membrane vesicles in the form of liposomes and polymersomes, respectively.[8] More recently, there has been significant interest in hybrid vesicles for technological applications that combine advantageous material properties from blended lipid and block copolymer components.[9-17]

Inclusion of membrane proteins within the vesicle membrane opens a range of potential applications, from carriers and bioreactors for drug delivery to sensors and artificial cells. [6, 18] Classically, membrane proteins have been reconstituted into liposomes because their membranes most closely resemble their native biomembrane environment. [19, 20] However, phospholipid membranes are known to be chemically and structurally labile. For instance, the oxidation of the double bonds of the fatty acid chains in an aerobic environment decreases membrane stability over time, resulting in leakage of contents by the formation of transitory pores and lipid aggregation. Unlike liposomes, polymersomes are more colloidally stable, robust, have a low permeability [21-23] and can be functionalised to tune their properties. [3] Polymersomes have been used for the encapsulation of molecules such as drugs, [5, 24, 25] enzymes, [26] and DNA or RNA fragments [27] and for protein reconstitution, despite their difference in material properties from native membranes such as membrane fluidity and membrane thickness. [28, 29]

However, polymer membranes are not an ideal solution for every application: the inherent biocompatibility and biofunctionality of liposomes [21-23] can be lacking in polymer vesicles. This is especially important for applications requiring protein reconstitution as specific lipid interactions are essential for many membrane proteins to retain functionality. [10] Combining lipids and polymers into a hybrid vesicle is one potential solution to the challenges that arise from using pure systems and membrane proteins have been successfully reconstituted within them. [10, 30-36]

Due to their technological interest, numerous studies report on the physical properties of hybrid vesicles. [5, 11, 12, 31, 37, 38]. Previous studies have shown that 1-palmitoyl-2-oleoyl-snglycero-3-phosphocholine (POPC) and poly(1,2-butadiene-block-ethylene oxide) (PBd-PEO) form well-mixed hybrid vesicles with no evidence of compositional heterogeneities on optical length scales. [37, 39] The favourable mixing of these components has been attributed to the similar Hildebrand solubility parameter of the lipid and amphiphilic block copolymer components, [40, 41] i.e., here PBd mimics the long acyl chains of a phospholipid, while PEO is hydrophilic like the phosphocholine head group. [42] PEO is also known to improve the pharmacokinetic properties of vesicles due to its strong interaction with the hydrating water layer that imparts a stealth effect by suppressing binding of opsonin proteins to its surface. This hinders the recognition of the vesicle as a foreign body by the immune system. [43] Hybrid vesicles containing the lower molecular weight PBd-PEO polymer are more likely to form hybrid vesicles. [39, 44, 45] Other studies have used PBd-PEO of different molecular weights to form membranes suitable for membrane protein reconstitution. [42, 46] Understanding the relationship between structure and material properties is required for the informed design of vesicular properties, including properties required for successful reconstitution of membrane proteins. However, there are currently limited high resolution structures of these hybrid membranes. Here, we have performed an in-depth structural characterisation of hybrid vesicles using cryo-transmission electron microscopy (cryo-TEM), including single particle analysis (SPA), cryo-electron tomograph (cryo-ET) and small-angle X-ray scattering (SAXS). This

structural information of hybrid vesicles of POPC mixed with  $PBd_{22}$ -PEO<sub>14</sub> (M<sub>s</sub> = 1800 gmol<sup>-1</sup>, > 85% 1,2 addition) is used to determine how increasing polymer mole fraction affects the structure and membrane properties of the hybrid vesicles.

Cryo-transmission electron microscopy (cryo-TEM) is ideally suited to determine the size and shape of vesicles [47-49] and, after post-acquisition computation, resolve high resolution 3D density maps.[50, 51] Membrane thickness from cryo-TEM is usually measured between the outer edges of the contrast interfaces in images without consideration of the hydrophobic core. [47, 52, 53] More detailed information can be obtained with single particle analysis (SPA), which is more commonly used to determine structural features in proteins.[54-57] In SPA, multiple instances of the sample are imaged aligned, classified and combined to give a higher-resolution image [58] whereas cryo-electron tomography (cryo-ET) collects multiple images of the same specimen are taken at different angles, which can then be reconstituted into a 3D volume representing the same. Besides EM techniques, SAXS is an ensemble technique that has been used to confirm the size and shape of vesicle samples [59, 60] and the average electron density profile (EDP) of lipid membranes, [59-61] unveiling, under optimised conditions, the membrane thickness with a precision of a few Ångströms.[62, 63] However, the resultant scattering pattern is not always straightforward to model without some *a priori* structural information that constrain model fits to physically relevant solutions. [31, 50, 51]

While cryo-TEM and SAXS are powerful techniques on their own, their combined use offers several advantages as the information gained from one technique informs and helps optimise the other. For example, fully reconstructed 3D structures obtained using cryo-TEM have been used to obtain theoretical SAXS curves, which can be compared to experimental SAXS profiles. [51] Therefore, the analysis of SAXS data can be refined with cryo-EM data to give the most probable ensemble structure. [51] Here, we combine these techniques to provide high resolution membrane structures in hybrid vesicles and show the coexistence of two membrane thickness populations in

hybrid systems. This bimodal bilayer thickness distribution, in what are known to be well-mixed membranes, implies that structures of intermediate membrane thickness are energetically unfavourable.

### 2. Results and Discussion

# 2.1 Cryo-ET images show that membrane thickness increases with block copolymer composition

Large unilamellar vesicles (LUVs) with increasing PBd<sub>22</sub>-PEO<sub>14</sub> mole fraction were formed by extrusion (through 100 nm track-etched pores) and mixed with colloidal gold, which act as fiducial markers for tomogram alignment. From the tomogram, transmembrane line profiles of individual vesicles were collected in the same direction (inside to outside) to allow differentiation between the inner and outer membrane leaflets (data available in Supplementary Information Section 1). These line profiles were then averaged to find the electron intensity profile for a vesicle composition as shown in **Figure 1**. The electron intensity profiles were used to infer structural features in the bilayer, such as the membrane thickness from the full width half maximum (FWHM), and the hydrophobic core thickness using peak-to-peak distance.

As the PBd<sub>22</sub>-PEO<sub>14</sub> mole fraction increases, the membrane thickness increases (Figure 1). A large increase in membrane thickness (from 57 to 78 Å and 83 to 97 Å) and hydrophobic core (from 36 to 50 Å and 53 to 66 Å) is observed between 25 and 50 mol% and 75 and 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub>, respectively. From 0 to 25 mol% PBd<sub>22</sub>-PEO<sub>14</sub> samples, we observe only a small increase in membrane thickness, suggesting that at low ratios, the polymer largely adapts to the intrinsic lipid bilayer thickness. In hybrid vesicles with >25 mol% PBd<sub>22</sub>-PEO<sub>14</sub>, the lipids instead appear to adapt to the thicker polymer membrane structure. 50 and 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> compositions have a broad

distribution in membrane thickness measurements, with some thicknesses comparable with that of a lipid membrane, and others close to that of a polymer membrane, as shown in Figure 1g.



Figure 1. **Electron density profiles of hybrid vesicle membranes obtained by cryo-ET.** Cryo-ET images of a) POPC, b) 25%, c) 50%, d) 75% and e) 100% PBd<sub>22</sub>-PEO<sub>14</sub> vesicle compositions (scale bar is 750 Å). The graphs show f) the average electron intensity profile across the vesicle membrane, and g) membrane thickness measurements (outer edges of contrast images in a-e) of individual membranes within 0-100% PBd<sub>22</sub>-PEO<sub>14</sub> vesicle samples.

The hydrophobic core thickness (peak-to-peak measurements) follows the same general trend as the membrane thickness: there is an increase in hydrophobic core thickness as the polymer fraction increases (see SI Section 2). The hydrophobic core measurement delivers particularly useful information, when setting up an appropriate membrane model for fitting the SAXS pattern (see below).

The peak intensity values of individual vesicles were further used to determine, whether there was a transmembrane asymmetry in the different leaflets of the membrane (method and data available in SI Section 3). Consistently significant differences in electron intensity between the inner

and outer peaks would be indicative of membrane asymmetry. However, this fractional difference (relative difference between electron density) was less than 5% for all vesicle populations, including those composed of pure lipid or pure polymer, suggesting that none of the hybrid membranes had significant transverse compositional asymmetry. We attribute the slight electron density asymmetry that is observed in all vesicles to the curvature of the membrane, where the inner leaflet is slightly compressed when compared to that of the outer leaflet.

#### 2.2 Two populations of different membrane thickness are observed in the hybrid vesicles

The membrane thicknesses shown in Figure 1 have a broad distribution for 50 and 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> vesicles. When explored more closely, two groups of vesicles within these compositions begin to emerge: population 1 with a membrane thickness < 60 Å and population 2 with a membrane thickness > 70 Å, as shown in Figure 1g. The presence of two populations within each sample is significant to within a 95% confidence using a Tukey and Bonferroni ANOVA analysis. When compared with the pure systems, population 1 in both 50 and 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> compositions has similar membrane thicknesses as 0% PBd<sub>22</sub>-PEO<sub>14</sub>, suggesting a thinner lipid-bilayer-like membrane structure, while measurement of population 2 vesicles infer a thicker polymersome-like membrane.

#### 2.3 Coexisting membrane domains of different thickness are not observed in individual vesicles

Computational analysis was used to determine whether there were lateral domains of different thickness forming in the membrane of different vesicle compositions (see SI Section 4). [64] The analysis compares the apparent membrane thickness along a section of a hybrid membrane with the bilayer thickness measurements of a pure lipid and pure polymer vesicle and computes the likelihood that a measurement in the hybrid membrane falls within the thickness distribution attributed to the pure lipid or pure polymer vesicle. The hybrid thickness measurements are sorted into thick, polymersome-like (> 70 Å) and thin (< 60 Å) membrane thickness categories.



Figure 2. Hybrid vesicles have homogeneous membrane structures with no evidence of phase separated domains. The original and Canny-filtered cryo-ET images with the measurement lines and determined phase as a colour map of % likelihood the bilayer is in a polymersome-like phase of a) 25 mol% PBd<sub>22</sub>-PEO<sub>14</sub>, b) 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> (population 1), c) 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> (population 2), d) 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> (population 2). Blue indicates 0% likelihood that membrane is in polymersome-like phase, while red indicates 100% likelihood that membrane is in polymersome-like phase.

The colour maps overlaying the filtered images in Figure 2 give the probability of the bilayer measurement corresponding to a thicker polymersome-like phase. Blue indicates 0% likelihood that the membrane is in the polymersome-like phase, while red indicates 100% likelihood that the membrane is in the polymersome-like phase. Our analysis occasionally assigns 'thickness' to one leaflet only, rather than across two leaflets (bilayer), yielding anomalous results with the wrong colour assignation. Values are discarded by visual inspection of the measurement lines of Canny-filtered images, removing erroneous values. For all vesicles, the colour map shows that the membrane thickness is consistent throughout the vesicle, so the absence of different membrane thickness suggests POPC and PBd<sub>22</sub>PEO<sub>14</sub> are homogenously distributed within the membrane of vesicles in both thin and thick membrane populations.

**2.4 Vesicle diameters correlate with membrane thickness for 75 mol% polymer hybrid vesicles** All PBd<sub>22</sub>-PEO<sub>14</sub> samples were extruded using a membrane with a 100 nm pore size, yet Z-averages from dynamic light scattering (provided in SI Section 5) and visual observation of cryo-TEM images indicate that the vesicles have a monomodal size distribution with average diameters > 100 nm. Initially DLS shows there is a small decrease in Z-average between 0 mol% and 25 mol% PBd<sub>22</sub>-PEO<sub>14</sub> samples (131 nm, PDI 0.106 and 103 nm, PDI 0.120, respectively), and then the Z-average increases with increasing PBd<sub>22</sub>-PEO<sub>14</sub> fraction to 141 nm (PDI 0.097) in the 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub> sample. 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> sample had a high PDI of 0.236, suggesting that this sample was significantly more polydisperse than the other compositions.

As tomography has shown that there are two bilayer thickness populations in 50 and 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> samples, the thickness might be related to the vesicle diameter (i.e., curvature), since  $\kappa \propto d^2$ , where  $\kappa$  is the membrane bending rigidity and *d* is the membrane thickness.[21, 65] To test this, vesicle diameter measurements were taken for every vesicle across 5 tomograms (188 vesicles in 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> and 117 vesicles in 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub>) and categorised into thin or thick bilayer populations based on visual inspection.

As the difference between the membrane thickness of a thick and thin population is quite large, visual sorting is possible. The histograms in Figure *3* c,d show the fraction of vesicles of thick or thin membrane thickness sorted by size. Upon first inspection, thin and thick membranes appear to coincide with smaller or larger vesicles in 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> samples. However further analysis shows that this difference is not statistically relevant (p > 0.1; Tukey & Bonferroni ANOVA). As shown in Figure 3c**Error! Reference source not found.**, the reason for the perceived difference is the presence of a large number of small vesicles with thick bilayer measurements. In contrast, thin membranes do significantly correlate with smaller vesicles and thicker membranes with larger vesicles in the 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> sample (p < 0.05; Tukey & Bonferroni ANOVA) as no small vesicles with thick membranes were observed. These observations are consistent with vesicle size being dictated by the higher bending rigidity of the vesicle population with thicker membranes.



Figure 3. **Two distinct membrane populations are observed at some hybrid vesicle compositions.** Electron intensity profiles of 0%, 100% and population 1 and 2 of a) 50 mol% and b) 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> vesicle compositions. Population 1 (Pop. 1) of both 50 and 75 mol% compositions has a profile similar to 0% indicating a thin membrane, while population 2 (Pop. 2) of both compositions is a thick membrane. The histograms of the vesicle diameters of the thick and thin populations in c) 50 mol% and d) 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub>.

# 2.5 Cryo-ET results refine the ensemble analysis of SAXS data

Figure 4 shows that as the polymer mole fraction increases, that the scattering intensity increases in the SAXS patterns at low q (< 0.08 Å<sup>-1</sup>). We note that as long as no diffraction peak is present (no underlying ordered structure exists), scattering patterns from SAXS must be fitted with a

model in order to account for unilamellar membrane information. [50] Therefore, no contribution from a structure factor was expected such that the SAXS profiles can be fitted with a form factor alone. The form factors used were adapted from a Gaussian-based model described previously. [66, 67] By using results from cryo-ET, physically relevant models were developed. We note that the vesicles used for SAXS analysis were dilute (6.57 mM) and displayed a PDI < 0.25 from Dynamic Light Scattering (SI Section 5).

Tomography has shown 50 and 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> samples contain a thick and thin membrane populations. Therefore, SAXS patterns of these samples were fitted with models based on coexistence of two vesicle populations with thin membranes ( $F_{thin}$ ) and thick membranes ( $F_{thick}$ ).

For thin membranes, a 3-Gaussian form factor  $(F_{thin})$  model is applied:

$$F_{thin}(q) = 2\pi \left( 2\sigma_H \exp\left(-\frac{\sigma_H^2 q^2}{2}\right) \cos(qz_H) - \sigma_C \rho_R \exp\left(-\frac{\sigma_C^2 q^2}{2}\right) \right)$$
 Eq 1

where two Gaussians are used to represent polar headgroups at  $\pm z_H$  and one Gaussian to model the methyl trough region at the bilayer midplane. The widths of these Gaussians are given by  $\sigma_H$  and  $\sigma_C$ , respectively. The ratio of the headgroup amplitude against the negative methyl group amplitude is the minimum to maximum electron density contrast, given as  $\rho_R = \rho_C / \rho_H$ .

To account for the broad hydrophobic core observed using tomography of samples for pure polymer membranes (PBd<sub>22</sub>-PEO<sub>14</sub>), a 4-Gaussian form factor  $F_{thick}$  was applied:

$$F_{thick}(q) = 2\pi \left( 2\sigma_H \exp\left(-\frac{\sigma_H^2 q^2}{2}\right) \cos(qz_H) - 2\sigma_C \rho_R \exp\left(-\frac{\sigma_C^2 q^2}{2}\right) \cos(qz_C) \right) \qquad \text{Eq } 2$$

where the broad hydrophobic core region is represented by two Gaussians at  $\pm z_c$  with similar width of  $\sigma_c$  and the position of the polar groups are given also by two Gaussians at  $\pm z_H$ . The minimum to maximum electron density contrast here is given by  $\rho_R = \rho_c / \rho_H$ . We note, that adding more fitting parameters (additional Gaussian distributions) to the SAXS model did not statistically improve the fits.

A single model was used to fit the pure lipid and polymer systems (the 3 Gaussian and 4 Gaussian functions of Eq 1 and 2, respectively). However, for the hybrid POPC-PBd<sub>22</sub>-PEO<sub>14</sub> membranes, a combination of the two models was required to account for contributions from both membrane structures. Led by the measurements obtained using cryo-ET, two models were fitted to the hybrid membranes resulting in both thick (Eq. 2) and thin (Eq. 1) membrane electron density profiles for each hybrid vesicle sample. For hybrid samples, a weighting factor, *w*, was applied for the fraction of the thick membrane vesicles in the hybrid sample (with (1 - w) indicating the fraction of thin membrane vesicles):

$$F_{hybrid} = (1 - w)F_{thin} + w F_{thick}$$
 Eq 3

The output gives two electron density profiles, which correspond to the thin membrane and thick membrane vesicles within the hybrid samples, respectively. Finally, the scattered intensity was fitted by the equation:

$$I(q) = F(q)^2/q^2 \qquad \qquad \text{Eq 4}$$

with F(q) being the form factor as described in Eq 1-3 and the  $1/q^2$  the Lorentz correction applied for planar structures.[66, 68]



Figure 4. Membrane electron-density profiles of hybrid vesicles from SAXS using fitting models refined by cryo-ET data. Fitted SAXS profiles of a) 0%, b) 25 mol%, c) 50 mol%, d) 75 mol% and e) 100% PBd<sub>22</sub>-PEO<sub>14</sub> and resultant electron density profiles. Thin and thick membrane populations were found in PBd<sub>22</sub>-PEO<sub>14</sub> hybrid vesicle samples, which agrees with the results obtained from cryo-ET.

While the thin membrane electron density profile appears unchanged with increasing PBd<sub>22</sub>-PEO<sub>14</sub> mole fraction, the peak positions and trough depth increase with increasing PBd<sub>22</sub>-PEO<sub>14</sub> mole fraction in the thick membrane model. Figure 4 shows that initially the minima in the thick membrane electron density profiles have a similar depth to the thin membrane models (Figure 4b). These minima increase in depth as the polymer fraction in the sample increases (Figure 4c and d). This confirms that lipids are present in the thick membranes of the hybrid vesicles, and as the lipid fraction in the entire sample decreases, their presence in the thick membranes also decrease.

PBd22-PEO14 (mol%)	Cryo-ET		SAXS	
	Thin membrane	Thick membrane	Thin membrane	Thick membrane
	(%)	(%)	(%)	(%)
0	100	0	100	0
25	100	0	56	44
50	58	42	25	75
75	63	36	15	85
100	0	100	0	100

Table 1. The fractions of thin and thick membranes in P	OPC/PBd22-PEO14 hybrid vesicle				
compositions found by cryo-ET and SAXS analysis.					

The percentage of thin and thick membranes in each sample was also calculated (see parameter *w* in Eq. 3) and compared to the fractions of thin and thick membranes found from cryo-ET images as shown in Table 1. Although cryo-ET showed no thick membrane population in 25% PBd<sub>22</sub>-PEO<sub>14</sub> hybrid vesicles, SAXS indicates that the two membrane populations also coexist within this hybrid composition. Overall, the fraction of thin membranes in each composition decreased as the polymer mole fraction increased, from 56% in the 25 mol% PBd<sub>22</sub>-PEO<sub>14</sub> sample to 15% in the 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> sample. As SAXS is an ensemble technique with high statistics, (10<sup>10</sup> vesicles), compared to the low statistics of analysing individual in cryo-ET images, (10<sup>2</sup> vesicles), the population fraction analysis is more accurately obtained from the SAXS data. However, the cryo-ET data was essential for determination of the SAXS fitting models used. Also of note is the observation that thick membrane fractions determined from Cryo-ET samples are consistently lower than those obtained by SAXS. This is consistent with thicker, more rigid membranes leading to, on average, larger vesicles (Section 2.4 **Vesicle diameters correlate with membrane thickness for 75 mol% polymer hybrid vesicles**), where larger vesicles are known to be more commonly excluded from the thin vitrified ice films, (10<sup>2</sup> nm), of cryo-EM samples.

# 2.6 Comparing results from cryo-ET, SAXS and SPA provides deeper insight into hybrid membrane structures

To further support the SAXS fits and interpretation, SPA was used to obtain and group together multiple cryo-TEM projection images of 0%, 50% and 100% PBd<sub>22</sub>-PEO<sub>14</sub> hybrid vesicles to give a high-resolution image of sections of membrane for each sample as shown in Figure 5.



Figure 5. **Single particle analysis of electron micrographs reveals detailed membrane structure.** Cryo-EM SPA images of a) POPC, 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> b) Population 1 and c) Population 2 (thin and thick respectively) and d) 100% PBd<sub>22</sub>-PEO<sub>14</sub> vesicle compositions.

A fast Fourier transform (FFT) of 10 cryo-ET images resulted in a scattering pattern similar to the SAXS scattering curves (see SI Section 6). These FFT patterns were also fitted with either the 3-Gaussian model (Eq 1) for thin membranes or the 4-Gaussian model (Eq 2) for thick membranes to give an electron intensity profile of the membrane that can be compared to the electron density and intensity profiles from SAXS and cryo-TEM techniques.

We can now compare electron density and electron intensity profiles obtained from SAXS, cryo-ET and SPA (Figure 6). A quantitative analysis using feature positions was carried out to compare these profiles directly: peak-to-peak measurements were taken from all electron profiles to determine whether the data acquired in the SAXS, cryo-ET, FFT of cryo-ET images and SPA correspond to the same structure.



Figure 6. **Correlating membrane structural features across the different SAXS and electron microscopy analyses.** In 0 mol% PBd<sub>22</sub>-PEO<sub>14</sub> membranes, there is excellent correspondence between the a) SAXS electron density profile and the b) cryo-ET electron intensity profile, as well as the electron profiles resulting from c) FFT analysis of the cryo-ET images and d) SPA. In the thin population of 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> vesicles there is also excellent correspondence in peak-to-peak

distances between the e) SAXS electron density profile and the f) cryo-ET electron intensity profile, as well as electron profiles resulting from g) FFT analysis of the cryo-ET images and h) SPA. Although for the thick 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> membranes, the i) SAXS electron density profiles do not correspond with j) cryo-ET electron intensity profiles. However, the k) FFT analysis of the cryo-ET images results in a profile that has excellent correspondence to i). Further analysis with l) SPA gives a result that combines some of the features seen in both SAXS and cryo-ET (i&j) profiles, including a higher density peak in the hydrophobic core region indicated by the green circle. Like thick 50 PBd<sub>22</sub>-PEO<sub>14</sub> membranes, 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub> membrane m) SAXS electron density profiles do not correspond with n) cryo-ET electron intensity profiles. However, o) FFT analysis of the cryo-ET images results in a profile that has excellent correspondence to m). Further analysis with p) SPA gives a result that combines the features seen in both SAXS and cryo-ET (m&n) profiles including a higher density peak in the hydrophobic core region indicated by the green circle. Comparing the pure POPC lipid profiles, the peak-to peak positions from both SAXS, cryo-ET, FFT and SPA have excellent correspondence, where peak positions are closely aligned as shown by Figure 6a-d. In the SAXS profile, the peak-to-peak distances in POPC lipid membranes is 36 Å, while for the cryo-ET profile, the peak-to-peak distance is 34 Å (see SI Section 7). The FFT and SPA profiles give peak-to-peak measurements of 35 Å and 41 Å, respectively. All these measurements are comparable to the peak-to-peak and membrane thickness measurements of POPC lipid membranes found previously using SAXS and cryo-TEM: in SAXS, the peak-to-peak distance was estimated to be approximately 38 Å, [66, 68, 69] while measurements of cryo-TEM images gives a membrane thickness of 37 Å. [47]

Both SAXS and cryo-ET reveal two bilayer populations within the 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> hybrid vesicles, one population presenting a thinner membrane < 65 Å (Figure 6e-h), and the other with a thicker membrane > 65 Å (Figure 6i-l). For thin membranes (< 65 Å) of 50 mol% PBd<sub>22</sub>PEO<sub>14</sub> vesicles, the peak-to-peak positions from both SAXS, cryo-ET, FFT and SPA also have excellent correspondence. Peak positions are closely aligned as shown by Figure 6e-h. In the SAXS profile, the peak-to-peak distances for the thin membranes is 36 Å, while for the cryo-ET profile, the peak-to-peak distance is 36 Å. The FFT and SPA profiles give peak-to-peak measurements of 40 Å and 39 Å, respectively. The FFT profile in Figure 6g also shows a small electron dense peak within the centre of the trough region, potentially indicative of homogenous hybrid membranes as well as interdigitation within these membranes. As previous studies have shown well mixed hybrid vesicles form from POPC/PBd<sub>22</sub>PEO<sub>14</sub> mixtures, polymers present in both the inner and outer bilayer leaflets of these thin membranes would tangle to reduce exposure of their hydrophobic region to water. This region would then have a higher electron density due to interdigitation, than if the polymer leaflets were completely segregated.

There is a significant disparity between the appearance and peak-to-peak measurements between the SAXS electron density profile and the electron intensity profile obtained by cryo-ET for the thick membranes in the 50 mol% hybrid sample (Figure 6i and 6j, respectively). The SAXS profile suggests that the thick membrane population has a peak-to-peak thickness of 110 Å, while cryo-ET suggests a measurement of 60 Å. SAXS electron density profiles also show a small electron dense peak within the centre of the trough region, which is not present in the cryo-ET electron intensity profile. Initially this feature was ignored, since it results from the choice of using two Gaussian distributions for resembling the hydrophobic core (note, in a comparable strip model this feature does not appear, see SI Section 8). However, Figure 6k of the FFT profile from the cryo-ET images also shows the same feature but has a larger peak-to-peak measurement of 140 Å, which once more justifies the choice of our 4-Gaussian model. This suggests that the information collected by SAXS is present in the cryo-ET images, but it is too noisy (or heterogeneous) to be clearly resolved during real-space analysis. Interestingly, this central peak seen in Figure 6i and Figure 6k is also potentially indicative of interdigitation within thick 50 mol% PBd<sub>22</sub>PEO<sub>14</sub> membranes.

The electron intensity profile resulting from SPA in Figure 6l combines features of the results seen in SAXS, cryo-ET and FFT analysis. The SPA profile in Figure 6l has an inner peak-to-peak distance of 60 Å, which is very well comparable to cryo-ET results of 60 Å, and aligns with the hydrophobic core region (56 Å) in the SAXS profile (its borders are determined by the zero-point positions of the profiles). The SPA profile also potentially has two slight shoulders at approximately  $\pm$  60 Å, which aligns with the peaks at  $\pm$  55 Å,  $\pm$  70 Å and  $\pm$  70 Å seen in SAXS, FFT and cryo-ET profiles, respectively. The 4-Gaussian model used for thick 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> membranes does not account for these two additional positive electron density contributions in this structural region, which is why this particular feature cannot be reproduced in the SAXS or the predicted scattering pattern from a FFT of cryo-ET images.

Similar differences are seen when evaluating the profiles of 100 mol% PBd<sub>22</sub>PEO<sub>14</sub> membranes. Again, the SAXS data indicates much larger peak-to-peak distances compared to the cryo-ET profiles (108 Å and 66 Å, respectively), as seen in Figure 6m and Figure 6n. As shown by

Figure 6m and Figure 6o, the peak-to-peak distance in the profile from the FFT correspond to the peaks found from the SAXS scattering curves (108 Å from both techniques). A closer inspection of the SAXS and FFT also reveals a small electron dense peak in the centre of the trough region, within the hydrophobic core. Again, this suggests that the information collected by SAXS is present in the cryo-ET images, but it is too noisy to be clearly resolved by our real space analysis of these images. The SPA profile of 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub> in Figure 6p also shows a small electron dense peak within the centre of the trough region. Comparing the SPA profile of thick membranes, this central peak has a greater intensity in 100 mol% PBd<sub>22</sub>PEO<sub>14</sub> membranes than in thick 50 mol% PBd<sub>22</sub>PEO<sub>14</sub> membranes. As this peak appears indicative of interdigitation within the membranes, perhaps the low peak intensity seen in the thick 50 mol% PBd<sub>22</sub>PEO<sub>14</sub> membrane profile is due to an increased free volume in the hydrophobic core of these membranes due to the presence of shorter lipid molecules, reducing the extent of polymer interdigitation in the membrane mid-plane.

In Figure 6m the hydrophobic core region in the SAXS electron density profile (59 Å) corresponds with the inner peak-to-peak distance in cryo-ET and SPA profiles in Figure 6n and p (66 Å and 63 Å, respectively). Previously the hydrophobic core region for a similar polymer, PBd<sub>23</sub>-PEO<sub>16</sub>, was simulated to be 63 Å.[53] Although the PBd<sub>23</sub>-PEO<sub>16</sub> polymer used in the previous study is slightly larger than PBd<sub>22</sub>-PEO<sub>14</sub> used here, there is good agreement between these previous simulations and our experimental peak to peak measurements from cryo-ET and SPA.

The cryo-ET profile in Figure 6n also shows a shoulder forming on the outer edges of these peaks at approximately  $\pm$  80 Å. Although the position of this shoulder does not align with the outer peak at  $\pm$  59 Å in the SPA profile, Figure 6p, this outer peak in the SPA profile is directly between the peak and shoulder positions in the cryo-ET profile. The same observation can be made when comparing the SAXS profile with the cryo-ET profile, where the SAXS peak position at  $\pm$  54 Å is directly between the peak and shoulder in the cryo-ET profile. As mentioned, the 4-Gaussian model, used here for 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub> membranes, does not account for these two additional positive

electron density contributions in this structural region, which is why this particular feature cannot be reproduced, and may require application of an asymmetric membrane model. Comparing the SPA profile of thick membranes, these outer peaks have a greater intensity in 100 mol% PBd<sub>22</sub>PEO<sub>14</sub> membranes than in thick 50 mol% PBd<sub>22</sub>PEO<sub>14</sub> membranes. The lower intensity of the outer peaks in the thick 50 mol% PBd<sub>22</sub>PEO<sub>14</sub> membrane profile is consistent with there being less polymer in these membranes, than the 100% polymer membranes. The inner peaks of the SPA profile in Figure 6p could represent the hydrophobic/hydrophilic interface between the hydrophobic polybutadiene (PBd) and hydrophilic polyethylene oxide (PEO) blocks, while the outer peak may represent the centre of the PEO corona.

#### **2.7 Discussion**

A fully extended PBd<sub>22</sub>-PEO<sub>14</sub> polymer is estimated to have a maximum bilayer thickness of approximately 209 Å, and a hydrophobic core of 111 Å. Although polymers can form an extended, segregated brush conformation, this conformation is entropically highly unfavourable. The polymer block chains can also collapse to protect the hydrophobic region, [52] and the hydrophobic polymer chains can become entangled and interdigitate, thus decreasing the bilayer thickness. [70] Hence, the fully extended bilayer and hydrophobic core thickness provide physical limits for the underlying system. The collapsed polymer conformation can be modelled using a polymer random walk model (freely jointed chain), where the root mean squared end-to-end distance of the polymer is given by  $\langle R^2 \rangle^{\frac{1}{2}} = N^{\nu-1}L$ . Here *N* is the number of Kuhn segments, *v* is the Flory exponent and *L* is the polymer contour length. For polybutadiene, (1,2 addition), the Kuhn length is 13.7 Å,[71] while for poly ethylene oxide, the Kuhn length is 8 Å.[72] Under the assumption that the polymers are acting as ideal chains (similar to their configuration in a polymer melt) that do collapse and fully interdigitate, the Flory exponent is given by v = 0.5, leading to a bilayer thickness of 95 Å and hydrophobic core thickness of 55 Å for  $PBd_{22}$ -PEO<sub>14</sub> membranes. However, this random walk model has to be used with great care as it is most accurate for the statistics of longer polymers, where the number of Kuhn segments, *N*, is much larger.

The peak-to-peak measurements from SAXS, cryo-ET, FFT and SPA profiles of 100 mol% PBd<sub>22</sub>PEO<sub>14</sub> and thick 50 mol% PBd<sub>22</sub>PEO<sub>14</sub> membranes can be compared with the theoretical values obtained from a polymer random walk model. The peak-to-peak values from all techniques are all below the predicted hydrophobic thickness for fully segregated polymer leaflets, but above the thickness calculated from fully interdigitated membranes (55 Å), suggesting PBd<sub>22</sub>-PEO<sub>14</sub> forms only partially interdigitating membranes.

SAXS appears more sensitive to the electron density at the centre of the PEO hydrophilic corona and can collect data from the entire sample, approximately ranging from 10<sup>10</sup>-10<sup>13</sup> vesicles at once. However, the scattering pattern can be problematic to model without *a priori* structural information. [50] Cryo-ET and SPA images provide high contrast images with a large gradient in electron intensity at the hydrophilic-hydrophobic interface of the membranes, however the results are always specific with respect to the analysed vesicles rather than representative of the whole sample. Therefore, SAXS remains an important characterisation technique, when considering the ensemble structure in the sample.

The presence of thin and thick membrane populations in hybrid lipid-block copolymer vesicle samples might be interpreted as poor mixing between these components and a strong segregation into different vesicles. However, previous studies utilising POPC and PBd<sub>22</sub>-PEO<sub>14</sub> have reported good mixing between these components into hybrid vesicles with a monomodal distribution of lipids and polymers centred around the sample mean. [37] Giant unilamellar vesicles (GUVs), created by the electroformation method, have been studied containing a labelled polymer and lipid-like fluorescent probe, where these components were well mixed across 25 mol%, 50 mol% and 75 mol%

components with a single broad distribution of relative fluorescence intensities across the GUV population. [37] Similarly, Lim *et al.* used flow cytometry of LUVs containing mixtures of POPC and PBd<sub>22</sub>-PEO<sub>14</sub> containing fluorescent lipid and polymer probes to show good mixing between these components in individual large unilamellar vesicles (formed by thin film rehydration and extrusion) in their samples. [39]

Assuming good mixing of lipid and polymer components between individual vesicles is given (with a monomodal relative distribution of components centred on the mean sample composition), a bistability in membrane structure between the thin and thick configurations is implied, dictated by the preferred structure of the lipids or the polymers. It can be inferred that membranes of intermediate structure between these two states are not energetically favourable and hence the vesicle selects a membrane structure in one of these two states upon formation. The homogeneity of the membrane structures in individual vesicles also implies that coexistence of these two membrane configurations in a single vesicle is also not energetically favoured, likely due to the line tension that would arise between these domains. This observation has interesting implications for the application of hybrid vesicles in biotechnology and nanomedicine, where two distinct membrane structures can coexist, which could give rise to differential vesicle properties in a single sample.

#### 3. Conclusion

Generally, the membrane thickness was found to increase with increasing polymer fraction, from 52 Å for a pure lipid system to 97 Å for pure PBd<sub>22</sub>-PEO<sub>14</sub> vesicles. Overall, the bilayer thickness measurements from cryo-ET intensity profiles and SAXS electron density profiles for pure lipid and pure polymer membranes found in this investigation broadly corresponds with measurements determined previously by cryo-TEM,[47] SAXS or SANS[68, 73] and simulation.[53] Here, the hydrophobic thickness of the membrane, previously under-considered in cryo-TEM membrane

measurements, [47, 52, 53] have been resolved using cryo-ET, as well as adding new details, including the presence of 2 bilayer populations in POPC/PBd<sub>22</sub>-PEO<sub>14</sub> hybrid samples, which has been utilised to optimise the SAXS model.

In 50 mol% and 75 mol% PBd<sub>22</sub>-PEO<sub>14</sub> / POPC hybrid vesicles, two thickness populations were found using cryo-ET: vesicles could be categorised by a visibly thick or thin membrane and homogeneity within these populations were confirmed using an automated analysis. SAXS confirms that the hybrid PBd<sub>22</sub>-PEO<sub>14</sub> samples comprise two populations: a thin membrane with an electron density profile similar to a lipid bilayer and a thick membrane with an electron density profile similar to a pure polymer membrane. An FFT of the cryo-ET images also confirmed the peak positions found in the SAXS electron density profile.

SPA of 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> hybrid vesicles confirms a homogeneous distribution within a single vesicle. Previous literature has shown good mixing between these lipid and polymer components in hybrid membranes with a monomodal distribution of lipids and polymers between different vesicles centred on the mean composition. The cryo-EM and cryo-ET data also shows no evidence of phase separation into polymer-rich and lipid-rich domains within individual vesicles of hybrid samples. Together, this strongly implies a bistability between two membrane structures that are close in free energy: thin membranes with a structure dictated by the preferred bilayer ordering of the lipids and thicker membranes with the membrane structure dictated by the partially interdigitated bilayer structure of the block copolymers. Intermediate membrane structures do not appear to be energetically favourable for these hybrid lipid-block copolymer blends. We also find that it is energetically unfavourable for domains of thin and thick membrane structures to coexist in the same vesicle, likely due to the high line tension of domain boundaries. As such, each vesicle selects between the lipid-like thin membrane structure or the polymer-like thick membrane structure during formation. This selection may be partially driven by differences in the relative vesicle compositions

across the width of the monomodal mixing distribution of these components that has been previously reported.[37, 39]

These novel findings on the membrane structure of hybrid POPC/ PBd<sub>22</sub>-PEO<sub>14</sub> vesicles have implications for their technological applications. For example, it is interesting to note that previous work inserting cytochrome *bo<sub>3</sub>* into these vesicle compositions finds that optimal stability of this enzyme's function is within 50 mol% PBd<sub>22</sub>-PEO<sub>14</sub> hybrid vesicles.[10] Two distinct membrane structures exist at this composition: future work could investigate the distribution of these membrane proteins between thick and thin hybrid vesicle membrane populations and how each of these contribute to enzymatic function and stability. Different coexisting membrane populations will also have implications for the use of hybrid vesicles in drug delivery, where membrane thickness would influence the release kinetics of bioactive compounds. A distribution of release rates within a formulation might be beneficial for drug pharmacokinetics in some long-acting delivery applications.

#### 4. Materials & Methods

#### **4.1 Materials**

Diblock copolymers poly(butadiene-block-ethylene glycol) (PBd-b-PEO) with total molecular weights of 1800 g.mol<sup>-1</sup> were purchased from Polymer Source, Inc. (Montreal, Canada). PBd<sub>22</sub>-PEO<sub>14</sub> (PDI 1.01) has a hydrophobic butadiene block of 1200 g.mol<sup>-1</sup> (> 85% 1,2 addition) and a hydrophilic ethylene glycol block of 600 g.mol<sup>-1</sup>. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) in chloroform was purchased from Avanti Polar Lipids (AL, USA) while protein A (10 nm colloidal gold) was purchased from Insight Biotechnology (Wembley, UK). Sodium chloride (NaCl), Sodium hydroxide (NaOH) hydrogen peroxide and 4-(2-hydroxyethyl) -1- piperazine ethane sulfonic acid (HEPES) were bought from Thermo Fisher Scientific (Loughborough, Leicestershire, UK). Chloroform was purchased from VWR International Ltd. (Lutterworth, UK) and Filtered MilliQ water (filtered and deionised water 18.2 M $\Omega$  cm at 25°C) was used for buffer and sample preparation.

## **5.2 LUV formation**

Large unilamellar vesicles (LUVs) were prepared by the thin film rehydration and extrusion method. To generate different hybrid vesicle compositions, relative volumes of POPC (32 mM) and the polymer (6.57 mM) in chloroform were measured using a Hamilton syringe into a glass vial. The solutions were dried in a vacuum desiccator to give a lipid/polymer film and then rehydrated with 1.0 mL of aqueous solution of 40 mM HEPES and 20 mM sodium chloride buffered to pH 7.4. The films were incubated at 50 °C for 5 minutes and vortexed for 1 minute. The suspensions were then frozen in liquid nitrogen, thawed in a 60 °C water bath and vortexed for 10 seconds. This cycle was repeated 5 times. Suspensions were extruded 11 times at room temperature through 100 nm polycarbonate membrane filter using a LiposoFast Basic Extruder. The samples were then kept at 4 °C until further analysis.

# **5.3 SAXS at the Diamond Light Source**

Samples were placed directly into quartz glass capillary tubes (0.01 mm wall thickness, 1.5 mm outer diameter) for measurement in the Diamond Light Source (Harwell Campus, Oxford, UK) I22 beamline at 17 keV with a sample to detector length of 6.7 m.[74] Scattering profiles were radially averaged and the capillary scattering as well as the background buffer profile was subtracted upon acquisition using the Diamond Light Source software, DAWN.[75] The *q*-calibrant was silver behenate. The vesicle scattering profiles were fitted using an in-house code running on MatLAB, which can be obtained by contacting the corresponding author.

#### **5.4 Cryo-electron tomography**

Protein A conjugated 10 nm gold nanoparticles (Insight Biotechnology) were used to act as fiducial markers when processing the tomograms. The markers were added following LUV formation. First

the Protein A 10nm gold solution was concentrated using a centrifuge (XG, 30 minutes) and resuspended in a solution of 40 mM HEPES and 20 mM sodium chloride buffered to pH 7.4. 10  $\mu$ L of suspended protein A was added to 10  $\mu$ L of LUV sample. Copper Quantifoil grids (400 mesh, 2/2) were glow discharged for 33 seconds at 10 mA using a Cressington208 carbon coater. 3  $\mu$ L of the samples were then placed directly on the grid which was blotted and vitrified using a FEI Vitrobot mk IV using a blot time of 6 seconds and a blot force of 6 at 4 °C and 100% relative humidity. The samples were kept under liquid nitrogen until required.

For cryoEM imaging, a FEI GII Titan Krios microscope with a Bioquantum K2 (Gatan) was used. Tilt series were collected using Tomo software with defocus set to 3  $\mu$ m. The grids were exposed to an electron dose of 18 e<sup>-</sup>.Å<sup>-2</sup>.s<sup>-1</sup> and 61 images were taken at 2° increments and ×25,000 nominal magnification to create the tomogram. The final resolution for these images were 0.1855 pixel.Å<sup>-1</sup>.

The collection of images (movies) were motion corrected and tomograms were reconstructed with eTOMO software from the IMOD suite. Using protein A as the fiducial markers, the tomogram was aligned and applied with back projection and 10 iterations of SIRT to obtain a final reconstruction. Further analysis was carried out using FIJI software for bilayer measurements and a python code for phase determination.

#### **5.5 Single Particle Analysis**

In addition to cryo-ET and SAXS analysis on all PBd<sub>22</sub>-PEO<sub>14</sub> vesicle compositions, single particle analysis (SPA) was performed on 0%, 50% and 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub> sample to confirm the polymer membrane structure. Copper Quantifoil grids (400 mesh, 2 by 2  $\mu$ m) were glow discharged for 33 seconds at 10 mA using a Cressington208 carbon coater. 0%, 50% and 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub> samples were then placed directly on the grid using a FEI Vitrobot mkIV using a blot time of 6 seconds and a blot force of 6 at 4 °C and 100% relative humidity. Samples were vitrified using liquid ethane and were stored in liquid nitrogen until imaged. Samples were visualised using a a FEI GII Titan Krios microscope equipped with a Falcon 4 DED and selectris energy filter. Automated single particle data collection was carried out using EPU software (Version 2.11.0). Micrographs were collected at 105k with a pixel size of 1.2 Angstroms. An exposure time of 4.16 seconds gave a dose of 9.31 electrons per pixel per second, generating a total dose of 27 electrons per Angstroms<sup>2</sup> over 1001 EER frames. Micrographs were motion corrected using RELIONs own implementation and CTF estimated using CTFFind 4.1.

From these corrected micrographs particles were manually selected, extracted and 2D classified. From the 2D projection images of 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub> vesicles, images were grouped into three classes of 100 mol% PBd<sub>22</sub>-PEO<sub>14</sub> membrane section which were then combined to reconstruct a composite image of the membrane structures within the sample.

#### 5.6 Structural bilayer analysis

#### **5.61 SAXS**

Electron density profiles extracted from the SAXS fits were also used to determine bilayer thickness and hydrophobic core measurements. Bilayer thickness was measured using the peak-to-peak value while hydrophobic core borders were defined by the zero-point positions of the electron density profiles.

## 5.62 Tomograms and SPA images

Tomograms and composite images obtained by SPA were further analysed using FIJI and OriginPro. Using FIJI, lines (5 pixels thickness) were always drawn from inside the lumen to the external buffer regions to differentiate the inner and outer leaflets in the final electron intensity profile. Per vesicle, at least 15 lines were drawn across the bilayer at various slices in a tomogram. The line profiles were adjusted on OriginPro to overlap at the central hydrophobic core, baseline corrected, averaged and normalised to give an electron intensity profile for each vesicle composition. Membrane measurements of structural features in profiles from tomograms were taken: bilayer thickness was the full width half maximum (FWHM) between the baseline to the peak; and the hydrophobic core thickness was the peak-to-peak distance.

## 5.63 Automated analysis using Python of tomography images

Full description and mechanics of the code can be found in reference [64]. Briefly, the tomogram slices were first Canny filtered using FIJI. The apparent bilayer thickness is defined as the minimum distance between pixels on the inner and outer leaflet. Bilayer measurements from 20-30 images of each pure system were used to determine which observed distances in the hybrid compositions were categorised into polymer-like or lipid-like phases. The domains were mapped on to the individual tomograms of each hybrid vesicle composition by calculating the probability of each observed distance corresponding to either the polymer-like or lipid-like phase.

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