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1 **ABSTRACT**

2 We report the ability of hydrophobically modified polypeptoids (HMPs), which are
3 amphiphilic pseudo-peptidic macromolecules, to connect across lipid bilayers and thus form
4 layered structures on liposomes. The HMPs are obtained by attaching hydrophobic decyl groups
5 at random points along the polypeptoid backbone. While native polypeptoids (with no
6 hydrophobes) have no effect on liposomal structure, the HMPs remodel the unilamellar
7 liposomes into structures with comparable diameters but with multiple concentric bilayers. The
8 transition from single bilayer to multiple bilayer structures is revealed by small angle neutron
9 scattering (SANS) and cryo-transmission electron microscopy (cryo-TEM). The spacing between
10 bilayers is found to be relatively uniform at ~ 6.7 nm. We suggest that the amphiphilic nature of
11 the HMPs explains the formation of multi-bilayered liposomes; i.e., the HMPs insert their
12 hydrophobic tails into adjacent bilayers and thereby serve as the connective glue between
13 bilayers. At higher HMP concentrations, the liposomes are entirely disrupted into much smaller
14 micelle-like structures through extensive hydrophobe insertion. Interestingly, these small
15 structures can reattach to fresh unilamellar liposomes and self-assemble to form new two-
16 bilayered liposomes. The two-bilayer liposomes in our study are reminiscent of two-bilayered
17 organelles such as the nucleus in eukaryotic cells. The observations have significance to
18 designing new nanoscale drug delivery carriers with multiple drugs on separate lipid bilayers and
19 extending liposome circulation times with entirely biocompatible materials.

1 INTRODUCTION

2 Liposomes are lipid based vesicles that are of significant interest in drug delivery
3 technologies due to their ability to encapsulate drug species and to be taken up by cells through
4 fusion with cell membranes.¹⁻⁵ Ever since their discovery in 1965 by Bangham who noticed the
5 structural similarity to cell membranes, liposomes have been used in biophysical studies of
6 phospholipid bilayers and as cell membrane mimics.⁶⁻⁷ Newer applications of liposomes include
7 their use as coatings on biomedical devices,⁸ in single molecule spectroscopy and biosensing,⁹⁻¹⁰
8 and as submicron encapsulation systems in microfluidics.¹¹

9 In many applications of liposomes, understanding the interaction of these vesicular systems
10 with polymers becomes extremely important, whether to enhance circulation lifetimes, to control
11 drug delivery rates, or to encapsulate liposomes in polymer matrices. This paper describes the
12 interaction of liposomes with a class of synthetic molecules known as polypeptoids that are
13 pseudo-peptidic materials with substituents attached to the nitrogen atoms of the polyglycine
14 backbone.¹²⁻¹³ These polymers have been of recent interest in chemical biology due to their
15 highly designable structure and their structural similarity to polypeptides.¹⁴⁻¹⁵ Unlike
16 polypeptides however, polypeptoids lack extensive hydrogen bonding and backbone chirality
17 due to the N-substitution, leading to a more flexible conformation and an enhanced resistance to
18 proteolysis.¹⁶⁻¹⁸ Due to backbone degradability, biocompatibility, and processability,
19 polypeptoids are of promise in applications related to drug delivery carriers, tissue engineering
20 materials, and smart coatings.¹⁹⁻²¹ However, there is little information in the literature on
21 polypeptoid interaction with lipids and cell membranes.

22 The objective of this study is to define and control the interactions of a model liposome
23 system with two classes of polypeptoids in order to develop new concepts in liposome-polymer

1 systems. The polymers chosen are (1) a polypeptoid containing the methoxyethyl (MeOEt)
 2 groups exclusively as side chains, hereafter denoted as an unmodified polypeptoid (UMP) and (2)
 3 a polypeptoid where about 26% of the MeOEt groups are randomly substituted by the n-decyl
 4 (C10) groups hereafter denoted as a hydrophobically modified polypeptoid (HMP) as shown in
 5 Figure 1a. Both polypeptoids can be synthesized through the synthetic route shown in Figure1b.

Figure 1. (a) Chemical structures of unmodified polypeptoid (UMP), hydrophobically modified polypeptoid (HMP) and L- α -phosphatidylcholine (PC). (b) Synthetic route to the polypeptoids.

1 The presence of the hydrophobic alkyl groups leads to the attachment of polymers to the
2 lipid bilayers through the insertion of alkyl groups into the bilayer as a consequence of
3 hydrophobic interaction. Such insertions of hydrophobic groups into the bilayer are inherent
4 properties of polymeric surfactants (polysoaps)²²⁻²³, peptide amphiphiles²⁴ and lipopolypeptoids²⁵.
5 In the case of a lipid conjugated polyethylene glycol (PEG) (such as 1,2-distearoyl-sn-
6 glycerol-3-phosphoethanolamine-N-amino (polyethylene glycol), DSPE-PEG), the polymer chain
7 is attached to the liposome surface through insertion of the hydrophobic distearoyl group into the
8 lipid bilayer.²⁶ This is important for extending the circulation times of liposomes for drug
9 delivery. Bis alkyl hydrophobes attached to PEG have been shown by Auguste and coworkers to
10 bind more strongly to the bilayer in comparison to monoalkyl hydrophobes.²⁷ Work by Shen and
11 coworkers have shown the potential for bis alkyl lipopolymers to tether lipid bilayers to
12 surfaces.²⁸ When multiple alkyl groups are present on long-chain polymers, the liposomes are
13 connected into a network by the polymer chains.²⁹ Hydrophobic interactions are intrinsic in
14 understanding the effects of surfactants on lipid bilayers, where liposomes and cell membranes
15 have been found to break down through fluidization of the bilayer and eventual dissolution of the
16 lipids into mixed lipid-surfactant micelles.³⁰⁻³³

17
18 We have found several unique and distinct features in the interactions of HMPs with
19 liposomes which we elaborate on, in this paper. Specifically, HMPs seem to stitch together
20 vesicle bilayers, leading to multilayered liposomes with small and relatively uniform spacings
21 between their concentric bilayers. Such structures have not been observed before. The formation
22 of these multilayered structures is confirmed by a combination of SANS and cryo-TEM.

EXPERIMENTAL SECTION

Materials.

L- α -phosphatidylcholine (PC) was purchased from Avanti Polar Lipids. Deuterium oxide was purchased from Cambridge Isotope Laboratories. Deionized (DI) water generated by ELGA reverse osmosis water purification system (MEDICA 15BP) with a resistance of 18.2 M Ω ·cm was used in all experiments. All chemicals were used as received unless otherwise noted. *N*-decyl *N*-carboxyanhydride (*N*-decyl NCA) in 53-57% yield and *N*-methoxyethyl *N*-carboxyanhydride (*N*-MeOEt NCA) in 43-49% yield were synthesized by adapting previously reported procedures, as shown in Supporting Information Scheme S1 (route I and II).³⁴⁻³⁵

Synthesis of Polypeptoids.

The polypeptoids were synthesized in a glove box through benzyl amine-initiated ring-opening polymerization of the corresponding *N*-substituted *N*-carboxylanhydride (R-NCA) monomers. The unmodified polypeptoid (UMP), poly(*N*-methoxyethyl glycine) (PNMeOEtG), was synthesized by polymerization of *N*-methoxyethyl NCA, whereas the hydrophobically modified polypeptoid (HMP), poly[(*N*-methoxyethyl glycine)-*r*-(*N*-decyl glycine)] (P(NMeOEtG-*r*-NDG)), was synthesized by copolymerization of *N*-methoxyethyl NCA and *N*-decyl NCA (Figure 1b). Both UMP and HMP are designed to reach polymerization degree of 100. In a typical synthesis of HMP, stock solutions of *N*-methoxyethyl NCA (1.3 ml, 0.4 M, 0.52mmol) and *N*-decyl NCA (0.3 ml, 0.4 M, 0.13 mmol) in THF were mixed in a small vial. Benzyl-NH₂/THF stock solution (70 μ l, 92.7 mM, 6.5 μ mol) was added to the above mixture and heated at 50 °C for 48 h. Aliquots were taken and analyzed by ¹H NMR spectroscopy to check conversion. The polymer was precipitated out by adding excess hexanes, collected by

filtration, and dried under vacuum to obtain the final product as a white solid (61.6 mg, 88.2% yield).

NMR and Size Exclusion Chromatography (SEC) Analysis of the Polypeptoids.

^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were obtained using a Bruker AV-400 Nanobay spectrometer (400 MHz for ^1H NMR and 100 MHz for $^{13}\text{C}\{^1\text{H}\}$ NMR) at 298 K. Chemical shifts (δ) given in parts per million (ppm) were referenced to proton impurities or the ^{13}C isotopes of deuterated solvents (CDCl_3 , CD_2Cl_2 , and D_2O). ^1H NMR (Supporting Information Figure S1) and $^{13}\text{C}\{^1\text{H}\}$ NMR (Supporting Information Figure S2) of the monomers are included in the Supporting Information, respectively. SEC analysis of the polypeptoids were performed using an Agilent 1200 system (Agilent 1200 series degasser, isocratic pump, autosampler and column heater) equipped with three Phenomenex 5 μm , 300×7.8 mm columns [100 $\text{\AA}$, 1000 $\text{\AA}$ and Linear (2)], a Wyatt OptilabREX differential refractive index (DRI) detector with a 690 nm light source, and a Wyatt DAWN EOS multiangle light scattering (MALS) detector (GaAs 30mW laser at $\lambda = 690$ nm). DMF with 0.1M LiBr was used as the eluent at a flow rate of $0.5 \text{ mL} \cdot \text{min}^{-1}$. The column and detector temperature was set at 25 $^\circ\text{C}$. All data analysis was performed using Wyatt Astra V 5.3 software. The polydispersity index (PDI) were obtained by conventional SEC analysis with a calibration curve. The calibration curve was constructed from twenty three pauci-disperse polystyrene standards ($M_n = 590 \text{ g} \cdot \text{mol}^{-1}$ -1472 $\text{kg} \cdot \text{mol}^{-1}$, Polymer Laboratories, Inc.) using Astra's column calibration template. Relative PDI was then calculated using Astra's conventional calibration template. The sample for SEC analysis was prepared as below: 130 μl reaction mixture of HMP (or UMP) was added to 1 ml of DMF with 0.1 M LiBr and mixed thoroughly. This mixture was directly injected into SEC instrument for analysis.

Liposome Preparation.

L- α -phosphatidylcholine (PC) liposomes were prepared using thin film hydration method.³⁶ 0.1 g PC was first dissolved in a round bottom flask by 15 ml mixed solution of chloroform and methanol at a volume ratio of 2:1. The solvent was then evaporated using a rotary evaporator (Buchi R-205) at room temperature under 100 mbar for 3 hours to form a thin lipid film. The pressure was further reduced to 6 mbar for 30 min to remove solvent residual. The obtained thin lipid film was hydrated with DI water at 50 °C. The aqueous suspension was transferred to a syringe and extruded 21 times through a 100 nm polycarbonate membrane to acquire liposomes with an average diameter of 100 nm.

Preparation of Liposome and Polypeptoid Mixtures.

DI water was used to dilute the liposome stock suspension to 0.5%. HMP solutions at concentrations of 0.25%, 0.5%, 0.75% and 1% were mixed with a 0.5% liposome suspension at 1:1 volume ratio, respectively, to acquire solutions with the liposome concentration fixed at 0.25% and varying concentrations of HMP. The resulting mixtures were labeled as LIP0125HMP, LIP025HMP, LIP0375HMP and LIP05HMP denoting the final overall concentrations of HMP. Liposomes were also incubated with UMP at overall concentrations of 0.25% and 0.5% as negative controls, denoted as LIP025UMP and LIP05UMP, respectively. The liposome solution with the overall concentration of 0.25% was used as the blank control, denoted as LIP. Essentially all samples had the same concentration of lipid (0.25%) and the studies were done with varying concentrations of polymers. All samples were incubated for at least 12 hours prior to instrumental characterization. All concentrations are reported as wt %.

SANS Data Collection and Reduction.

The SANS experiments were carried out on the extended-Q range small angle neutron scattering (EQ-SANS) diffractometer at the Spallation Neutron Source (SNS) at the Oak Ridge National Laboratory (ORNL). All samples were prepared with pure deuterium oxide to generate sufficient scattering contrast. The samples were loaded into 2 mm path-length quartz banjo cells (Hellma, Germany) and placed in a 42-position sample chamber. Measurements were taken at room temperature (20 °C). The instrument was operated in a 60 Hz mode with a neutron wavelength (λ) range of 2.5 Å – 6.1 Å. The sample-to-detector distance was set to 4 m. This configuration provides an effective q-range of 0.009 Å⁻¹ – 0.44 Å⁻¹. The scattering vector, q , is defined by $q = 4\pi[\sin(\theta/2)]/\lambda$ where θ is the scattering angle.

The reduction of SANS data was performed using MantidPlot software following standard procedures.³⁷ The data were corrected for instrument dark current, detector sensitivity, incident beam normalization, sample transmission and solvent background. A calibrated standard provided by ORNL, Porasil B, was used to obtain the scale factor which was used to convert the data into absolute intensity units (cm⁻¹). Reduced data were azimuthally averaged using MantidPlot to generate scattering intensity per unit volume, $I(q)$, as a function of q .

Cryo-TEM Imaging.

Cryo-TEM imaging was done on an FEI G2 F30 Tecnai TEM operated at 150 kV. To prepare the sample, a 300-mesh lacey carbon grid (Electron Microscopy Sciences) was picked up by a tweezer and mounted on the plunging station of an FEI Vitrobot. 10 µl of the solution was applied to the grid. The excess liquid was blotted by filter paper attached to arms of the Vitrobot

for 2 seconds to form a thin film. The sample was then vitrified by plunging into liquid ethane. The vitrified sample was finally transferred onto a single tilt cryo specimen holder for imaging.

RESULTS AND DISCUSSION

Polypeptoid Characterization through ^1H NMR and Size Exclusion Chromatography (SEC).

The compositions of UMP and HMP were determined by ^1H NMR spectroscopy (Supporting Information Figure S3). The number average degree of polymerization (DP) of UMP was determined by the integration of methylene group in the backbone at 4.52, 4.41, 4.18, 4.10 ppm relative to the integration of benzyl end-group at 7.24 and 7.31 ppm. The DP of HMP was determined by the integration of methyl group in the side chain and the integration of methylene groups in the backbone relative to the integration of benzyl end-group at 7.24 and 7.31 ppm. The integrations give UMP a DP of 106 and HMP a DP of 100 containing 26 mol % *N*-decyl glycine units, corresponding to molecular weight of 12509 g/mol for UMP and 13939 g/mol for HMP. The size exclusion chromatographic (SEC) analysis indicated the monomodal and narrow distribution of polymer molecular weight for both UMP and HMP (Supporting Information Figure S4), with low PDI of 1.09 and 1.08, respectively.

Small Angle Neutron Scattering (SANS).

As the first step to the characterization of the interactions between the polymers and liposomes, we carried out SANS experiments on the individual systems with the results shown in Figure 2. As shown here, the data are separated by a scale factor of 0.1 for clarity and ease in visualization especially at the larger q values. The scattering intensity, $I(q)$, is determined by the

1 structure factor, $S(q)$, and the form factor, $P(q)$. In dilute, noninteracting system, $S(q)$ is close to
2 1. Therefore, $I(q)$ is determined by $P(q)$.³⁸

5 **Figure 2.** Scattering profiles of 0.25% liposome (LIP, a), 0.5% HMP (050HMP, b), 0.25% HMP
6 (025HMP, c), 0.5% UMP (050UMP, d) and 0.25% UMP (025UMP, e) in D₂O. The curves are
7

scaled and separated by a factor of 0.1. The solid lines are best-fit results to the corresponding models. The inset shows the Guinier plots ($\ln(I)$ vs. q^2) of the HMP and UMP samples.

Curve a in Figure 2 shows the scattering profile of 0.25% liposome suspension (LIP). For a detailed analysis of the form factor, the scattering data was fitted to the PolyCoreMultiShell model developed by the National Institute of Standards and Technology (NIST) using IgorPro software. When N (model parameter, number of shells) is fixed at 1, $P(q)$ can be described as³⁹⁻⁴⁰

$$P(q) = \frac{scale}{V_{shell}} \left[\frac{3V_1(\rho_1 - \rho_2)J_1(qR_1)}{qR_1} + \frac{3V_2(\rho_2 - \rho_{solv})J_1(qR_2)}{qR_2} \right]^2 + bkg \quad (1)$$

where $scale$ is a scale factor,³⁹ V_{shell} is the volume of the shell, V_1 is the volume of the core, V_2 is the total volume, R_1 is the radius of the core, R_2 is the outer radius of the shell, ρ_1 is the scattering length density of the core, ρ_2 is the scattering length density of the shell, ρ_{solv} is the scattering length density of the solvent, bkg is the background level, and $J_1(x) = (\sin x - x \cos x)/x^2$. With polydispersity in the core radius being considered, the form factor is averaged over the Schultz distribution³⁸

$$P(q) = \int f(R)P(q, R) dR \quad (2)$$

$$f(R) = \left(\frac{z+1}{R_0} \right)^{z+1} \frac{R^z}{\Gamma(z+1)} \exp \left[- (z+1) \frac{R}{R_0} \right] \quad (3)$$

where f is the distribution function, R is the core radius, R_0 is the average core radius, $\Gamma(x)$ is the gamma function, and z is the polydispersity index. The polydispersity, p , is related to z through

$$p = \frac{1}{\sqrt{z+1}} \quad (4)$$

The scattering profile of the liposome system (Figure 2, curve a) indicates a q^{-2} dependence in the decay at low q range, which is characteristic of non-interacting liposomes.³² The solid line

shows the best-fit of the scattering data of liposome. The fitting results show a liposome diameter of 99.4 nm with a polydispersity of 0.17. The shell thickness, or the lipid bilayer thickness, is 3.68 nm, which is close to the reported value in the literature.⁴¹⁻⁴²

We found that the scattering curves for HMP samples were best fitted to the Flexible Cylinder model.⁴³⁻⁴⁴ For UMP, the curve was also fitted by the Gaussian Coil model, but we retained the excellent fit of the Flexible Cylinder model to provide a direct comparison of parameters with HMP. In both cases we assumed that the contour length $L \gg$ the Kuhn length b . The results are presented in Figure 2 as solid black lines. L was calculated based on polymerization degree and was fixed in the Flexible Cylinder model calculations to estimate b and the axial radius of the polymer aggregates, R_{cyl} . The radius of gyration, R_g , of each sample was also calculated from the model fit results.⁴³ The results are included in Table 1.

Table 1. Model Fitting Parameters for SANS Data

Sample	Components		R_{cyl}^a (nm)	L^b (nm)	b^c (nm)	R_g (nm)
025HMP	-	0.25% HMP	1.7	38.0	0.76	3.0
050HMP	-	0.5% HMP	1.7	38.0	1.08	3.4
025UMP	-	0.25%UMP	0.6	39.4	1.00	3.0
050UMP	-	0.5% UMP	0.6	39.4	0.87	2.9
LIP050HMP	0.25% liposome	0.5% HMP	2.0	38.0	3.43	4.7

^a R_{cyl} : cylinder radius; ^b L : contour length; ^c b : Kuhn length; ^d R_g values calculated based on fitting results of Flexible Cylinder model.

The inset to Figure 2 shows the empirical Guinier plots where data in the range of $0.00014 \text{ \AA}^{-2} < q^2 < 0.0016 \text{ \AA}^{-2}$ were used in the calculation for R_g . The results of the Guinier analysis are listed in Table 1 and show very reasonable agreement with the R_g values calculated from the

1 model fitting results. To summarize Table 1, the values of R_g between UMP and HMP show
2 insignificant differences at both concentrations and we infer that the state of the polymers over
3 this range of concentration remains essentially the same. The fact that the form factor fits the low
4 q range in these dilute systems may additionally imply that there is minimal intermolecular
5 aggregation. As shown in subsequent discussion, significant changes in R_g values are obtained
6 through the interaction of HMP with lipid bilayers.

7 To explore the interactions between liposomes and polypeptoids, we prepared solutions
8 containing 0.25 % liposomes with 0.25 % and 0.5% UMP as control samples. The SANS data for
9 these samples (LIP025UMP and LIP050UMP, respectively) are shown in Figure 3. At low q , the
10 slope of the scattering curve remains -2 when liposomes are mixed with UMPs, which is
11 indicative that the bilayer structure is maintained when liposome is incubated with UMPs. We
12 note that the scattering intensity of the mixtures is essentially just the concentration weighted
13 summation of the individual components with the scattering from the liposomes dominating the
14 pattern at low q . These observations indicate that there is no structural change in the system
15 induced by UMP and that UMP and liposomes exist as independent entities in solution.

16

Figure 3. Scattering profiles of 0.25% liposomes incubated with 0% UMP (LIP, a), 0.25% UMP (LIP025UMP, b) and 0.5% UMP (LIP050UMP, c). The scattering curves are separated by a scale factor of 0.1 for clarity. Addition of UMP has little effect on the scattering pattern which is essentially a linear summation of the scattering data of the individual components.

Figure 4 illustrates the scattering patterns of mixtures of liposomes and HMP, and it is immediately clear that there are significant changes in the patterns in comparison to the scattering from LIP. With the addition of HMP, the slope of the scattering curve at the low q range flattens significantly, indicating changes in the liposome bilayer structure. Along with this, a significantly decreased intensity at low q range was observed for each sample with HMP added compared to the control sample (Supporting Information Figure S5 shows the unscaled data for the plots of Figure 4). We additionally see a Bragg diffraction peak around $q=0.092 \text{ \AA}^{-1}$ at an HMP concentration of 0.125% which shifts to slightly higher q and decreases in intensity with increased addition of HMP. The drop in scattering intensity at low q with the addition of more scattering entities (HMPs) and the observation of the Bragg peak are counter intuitive and are not

1 easily fit using models for neutron scattering profiles. The drop in scattering intensity points to a
2 decrease in the number density of the larger scattering entities (liposomes) and the Bragg peak
3 suggests the occurrence of layered structures with relatively uniform spacing. But to better
4 understand these SANS observations, we have used cryo-TEM, and the imaging results for all
5 samples used in SANS analysis are described below.

6

Figure 4. SANS data of 0.25% liposomes incubated with 0% HMP (LIP, a), 0.125% HMP (LIP0125HMP, b), 0.25% HMP (LIP025HMP, c), 0.375% HMP (LIP0375HMP, d) and 0.5% HMP (LIP050HMP, e). The scattering curves are separated by a scale factor of 0.1 for clarity. The black solid lines show best-fit results to the corresponding models. The inset shows the Guinier plot of the scattering data of LIP0125HMP giving an R_g of 4.7 nm.

Cryo Transmission Electron Microscopy of Microstructures

Figure 5a and b show cryo-TEM images of the control samples containing liposomes alone (Figure 5a) and liposomes incubated with UMP (Figure 5b) where we have done higher resolution imaging to verify the unilamellarity of the liposomes. The clear observations are that the unilamellar PC liposomes are unchanged by the addition of UMP, in agreement with the SANS results. It is, therefore, evident that the liposomes are stable in the presence of UMPs and that liposome-UMP interactions do not affect liposome structure.

Figure 5. Cryo-TEM images of 0.25% liposome incubated in DI water (LIP, a), 0.25% UMP (LIP025UMP, b), 0.125% HMP (LIP0125HMP, c), 0.25% HMP (LIP025HMP, d) and 0.5% HMP (LIP050HMP, e), and corresponding high magnification images below them (red square). While addition of UMP has no effect on the liposome structure, addition of HMP to liposome suspension results in a transition from unilamellar structure to two-bilayer (c) and multiple-bilayer (d) structures, and finally a solubilization of liposome (e). The inset in c indicates liposomes with multiple layer structures also exist in LIP0125HMP sample but with a small population. Scale bar of the inset is 50 nm.

Figure 5c to 5e illustrates the cryo-TEM images of liposomes incubated with varying concentrations of HMP. With 0.125% HMP addition, while there is no significant change in the size of liposomes, there is the interesting visualization of a second lipid layer, with the higher resolution image of Figure 6c showing that the layer is incomplete. We also observe liposomes with multiple bilayers (inset to Figure 5c). With cryo-TEM it is not possible to determine the

number density of imaged objects as number density varies at different locations on the grid. Nevertheless, we observed a reduced density of liposomes over the TEM field of view. The observation of a reduced number density becomes more pronounced with the addition of HMP to a concentration of 0.25%, and again we see the presence of liposomes with multiple bilayers (Figures 5d). In all these cases, the number of bilayers varies, and the formed additional layers are not necessarily complete and uniform. The average distance between each two adjacent layers (center to center distance) in the high magnification image of Figure 5d was measured using Nano Measure software. Multiple measurements were taken at different locations, and the results were averaged. The measured average spacing distance is 6.8 ± 0.9 nm, which agrees very well with the observed Bragg peak at a q value of $0.092 \text{ \AA}^{-1}$ corresponding to a d spacing of 6.8 nm. To our knowledge, this is the first observation of liposomes with two or more bilayers with a relatively uniform spacing of a few nm.

When the HMP concentration is further increased to 0.5%, the SANS data shows the loss of the Bragg peak (curve e in Figure 4). On the cryo-TEM, we are unable to see any liposomes in the TEM field of view indicating a complete disassembly of liposomes. However, we see clear evidence of small nanoscale structures (Figure 5e). A close examination of the images reveals that some of the shapes in the 2-D image appear somewhat circular with dimensions around 5 nm, and some appear elongated as small wormlike structures with a 5 nm thickness and a length of 10-15 nm. The circular dots typically have a higher contrast perhaps indicative of a coiled globular structure or of rods vitrified with axes parallel to the beam. The scattering curve at this HMP concentration can be again modeled through the Flexible Cylinder model (Table 1). The R_g value calculated from the model fitting result is 5.1 nm, while the Guinier plot (inset to Figure 4) shows the R_g value of 4.7 nm and we note that these values are much smaller than liposomal

1 dimensions but appreciably larger than the values for UMP and HMP alone. These suggest that
2 the liposomes are converted into such new nanoscale structures upon addition of HMPs. We also
3 note that HMP when added to the hydrating solution at 0.5 wt% prevents the formation of the
4 large liposomes typically observed prior to extrusion and leads to the formation of small
5 fragments similar to Figure 5e, thus indicating the generality of the phenomenon of liposome
6 disruption.

7 Both SANS and cryo-TEM reveal the important observation that HMP disrupts liposomes
8 and at sufficiently high concentrations completely disassembles these vesicular structures. But
9 most importantly, it is an additional conclusion that at intermediate concentrations, the HMPs
10 induce the liposomes to fuse which leads to the creation of two or more bilayer structures on
11 surviving liposomes. We propose that it is the formation of such multilayer liposomes that gives
12 rise to the Bragg peak in the scattering.

13 While the above results relate to equilibrated samples, cryo-TEM makes it possible to gain
14 an understanding of the dynamics of the structural transformations by contacting the liposomes
15 with HMP and rapidly vitrifying the systems at varying incubation times. Thus, 0.25% liposome
16 was incubated with 0.25% HMP (LIP0250HMP) for 1 min, 20 min, and 45 min after which they
17 were vitrified for cryo-TEM imaging. The sample incubated for 1 min (Figure 6a) shows
18 significant deformation of liposomes and open bilayer structures. After 20 min (Figure 6b), we
19 see evidence of the formation of two bilayer structures and liposome rearrangement. At this stage,
20 while the liposomes are multilayered, the spacing between bilayers is not uniform and
21 considerably larger than the equilibrium value. At 45 min, liposomes with multi-layered structure
22 are observed (Figure 6c) with the bilayer spacing down to ~ 7 nm. This result suggests that the
23 formation of multilayer liposomes undergoes a stepwise process. After HMP incorporates in the

bilayers, liposomes first partially break up and form open bilayer structures or large sheets (incomplete liposomes). The incomplete liposomes attach to surviving liposomes through hydrophobe insertion into bilayers to form multilayer structures. HMP thus serves as a connective material between the liposome bilayer and additional bilayers. This is distinctly different from small molecule surfactants (with single hydrophobic tail) that simply fluidize and eventually break up liposomes into mixed micelles of surfactant and lipid. [24, 27]

Figure 6. (a-c) Cryo-TEM images of 0.25% liposomes incubated with 0.25% HMP (LIP025HMP) for 1 min (a), 20 min (b) and 45 min (c). In a, arrows show deformed liposomes and open bilayer structures; in b, arrows show fragments of liposomes and the inset shows the two bilayers of the starred liposome (scale bar is 50 nm); and in c, arrows show liposomes with multilayered structures. (d-f) Cryo-TEM images of 0.25% liposomes incubated with 0.5% HMP (LIP050HMP) for 1 min (d), 20 min (e) and 45 min (f). Arrows in d show open bilayer structures; arrows in e show small fragments and the sample has no remnant intact liposomes; and in f, small nanoscale structures are observed (typical structures are pointed to by the arrows).

Figure 6d-f illustrate the identical experiments but with 0.5 % HMP (LIP050HMP). Within 1 minute of mixing, broken liposomes with open structures are observed (Figure 6d). After 20

1 min of incubation, we see the coexistence of small fragments and debris (Figure 6e). With 45
2 min of incubation (Figure 6f), a complete breakdown of the liposomes occurs with the same
3 suspended nanoscale structures observed as in Figure 5e.

4 Based on the SANS and cryo-TEM observations we propose the following mechanism for
5 the results of liposome-HMP interactions as shown in Figure 7. At the lower HMP
6 concentrations, a fraction of the liposomes is broken into large fragments by the insertion of
7 hydrophobes that have a sufficient local concentration in the bilayer to fluidize and disrupt the
8 bilayer. Such softening of lipid bilayers through hydrophobe insertion is considered the dominant
9 mechanism of liposome disruption by surfactants where the traditional model involves
10 dissolution of liposomes into mixed lipid-surfactant micelles.^[35-38] Polymeric hydrophobes-
11 containing amphiphiles such as HMPs are clearly distinct as they can bridge between lipid
12 bilayers. Thus at the lower HMP concentrations, fragments with attached HMPs can reattach to
13 remnant intact liposomes either through insertion of some of the free hydrophobes into the
14 bilayer of intact liposomes or through interaction with other HMP chains that may be attached to
15 the unbroken liposome (Figure 7b). We propose that the attachment of these fragments may
16 continue to the formation of multiple layers and the spacings are sufficiently periodic to give rise
17 to the constructive interference in neutron diffraction leading to the observed peak at a d spacing
18 of 6.8 nm. At high HMP concentrations, incorporation of additional HMPs into lipid bilayers
19 results in a solubilization of lipid bilayers by forming lipid-HMP small elongated structures that
20 are modeled by flexible cylinders.

Figure 7. (a) Schematic of the formation of multiple-bilayer liposomes and the solubilization of liposomes induced by HMP (scale bars in all the images are 50 nm). (b) Illustration of HMP bridging the fragments and lipid bilayer of a liposome by hydrophobe insertion forming two-bilayer structure.

The hypothesis that fragments with the hydrophobes containing polymer can be hooked on to lipid bilayers was tested through the reverse experiment of contacting the completely broken down liposome system as shown in Figure 5e with a suspension of fresh intact liposomes. The experiment was carried out by mixing the LIP050HMP solution with a fresh batch of liposomes (LIP) at a volume ratio of 1:2 (Figure 8a). Remarkably, incomplete layers form on the surface of the new liposome, and it is difficult to visualize any remaining broken up structures (Figure 8b). The two-bilayer structure is therefore reconstructed. The continuity of the second layer implies

the possibility of surface diffusion of the fragments that first attach to the intact liposomes and that upon contact, connect to each other through hydrophobe insertion (Figure 8c).

Figure 8. (a) Schematic of the experiment showing addition of a lipid-HMP complex solution (LIP050HMP) to a fresh liposome suspension (LIP) at a volume ratio of 1:2. (b) Cryo-TEM images show lipid-HMP complex (i), fresh liposome (ii) and reconstructed bilayers on bare liposomes (iii). The inset in iii shows the reconstructed bilayer on liposome (identified by the asterisk with scale bar 25 nm). (c) Schematic of building up bilayer structure from lipid-HMP complexes. When the lipid-HMP complexes contact bare liposomes, HMP incorporates into the bilayers by hydrophobe insertion into the fresh bilayers. The assembly of such attachment results in formation of additional lipid bilayers.

CONCLUSIONS

In this study, we have demonstrated that the introduction of decyl groups to a hydrophilic polypeptoid backbone can induce interactions between the modified polypeptoid and liposomes through the insertion of hydrophobes into the lipid bilayer. This leads to a breakdown of the liposome structural integrity and a gradual solubilization of the liposome into mixed aggregates of lipids and the modified polypeptoid. In this respect, the phenomenon is similar to the interaction of molecular surfactants with liposomes. A distinct difference however, is the ability of the HMP polymeric amphiphile to attach fragments onto existing and surviving liposomes to create additional layers on the surface of liposomes. The polymer chain serves as the connecting material between lipid containing fragments and liposomal bilayers allowing a buildup of bilayers on the surface of a liposome. In contrast to small molecule surfactants, such polymeric surfactants have membrane interaction properties that may be adjusted both through polymer concentration and the relative degree of hydrophobe substitution on the chain backbone.

The potential to build bilayers onto liposomes using a designed connective polymeric amphiphile has significant technical implications. These concepts may have relevance in the attachment of drug-containing lipid entities to cell membranes and to vesicle systems. The ability of HMPs to remodel liposomes is a novel aspect of multilayer self-assembly with significant applications to using liposomes as multiple drug carriers. For example, it may be possible to load the interior of the liposome with water soluble drugs, and the bilayer with a hydrophobic drug. Subsequently the patchwork addition of a second bilayer containing additional drug or diagnostic components may lead to multilamellar liposomes with multiple therapeutics or sensing agents. We also note that the double bilayer structure has a resemblance to those found in organelles

1 such as in the nuclear membrane of eukaryotic cells, although the bilayer spacing here is smaller.
2 Our continuing research seeks to understand these effects in realizing new applications.

3
4 ***Supporting Information.*** NMR spectrum of HMP and UMP, SEC chromatograms of HMP and
5 UMP, unscaled SANS data of liposomes incubated with varying concentrations of HMPs at low
6 q range.

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