Published in final edited form as:

Macromolecules. 2018 April 24; 51(8): 3184–3192. doi:10.1021/acs.macromol.8b00361.

Fabrication and Characterization of Hybrid Stealth Liposomes

Kenneth P. Mineart^{*,†}, Shrinivas Venkataraman[‡], Yi Yan Yang[‡], James L. Hedrick[§], Vivek M. Prabhu^{*,†}

[†] Materials Science and Engineering Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, United States

[‡] Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, Singapore 138669, Singapore

[§] IBM Almaden Research Center, 650 Harry Road, San Jose, California 95120, United States

Abstract

Next-generation liposome systems for anticancer and therapeutic delivery require the precise insertion of stabilizing polymers and targeting ligands. Many of these functional macromolecules may be lost to micellization as a competing self-assembly landscape. Here, hybrid stealth liposomes, which utilize novel cholesteryl-functionalized block copolymers as the molecular stabilizer, are explored as a scalable platform to address this limitation. The employed block copolymers offer resistance to micellization through multiple liposome insertion moieties per molecule. A combination of thermodynamic and structural investigations for a series of hybrid stealth liposome systems suggests that a critical number of cholesteryl moieties per molecule defines whether the copolymer will or will not insert into the liposome bilayer. Colloidal stability of formed hybrid stealth liposomes further corroborates the critical copolymer architecture value.

Graphical Abstract

*Corresponding Authors: kpm007@bucknell.edu (K.P.M.), vprabhu@nist.gov (V.M.P.).

Present Address K.P.M.: Department of Chemical Engineering, Bucknell University, Lewisburg, PA 17837.

Notes

The authors declare no competing financial interest.

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.macromol.8b00361.

¹H NMR spectra and size exclusion chromatographs for PEG-P(8C-Chol) copolymers, TEM of PEG-P(8CChol) micelle solutions, further details of SANS modeling, further detail of hybrid stealth liposome colloidal stability (PDF)

INTRODUCTION

Stealth liposomes are aqueous self-assemblies consisting of lipid molecules and a minority quantity of polymer to coat the surface. These vesicles are hollow spherical containers with diameters ranging from tens to hundreds of nanometers, which provides interior space available for loading of hydrophilic payload. The surface-bound polymer chains independently provide the liposomes with steric stability by preventing close approach of liposome bilayers and therefore eliminating the possibility of aggregation or fusion and subsequent precipitation.^{1–3} The polymer molecules additionally afford stealth liposomes their namesake, “stealth”, due to the fact that they induce a thick hydration shell around liposomes, making them nearly undetectable by the body’s clearance mechanisms. This results in drug delivery vehicles that have long circulation times and are effective via the enhanced permeability and retention (EPR) effect.^{4,5} The polymer chains do not greatly change the fluidity of the liposome bilayer,⁶ as other stabilization approaches do,^{7,8} and hence transport of molecules across the bilayer is unaffected. The combination of their cargo space, stability, stealthiness, and fluid bilayers makes stealth liposomes a modern platform for academic studies and next-generation drug delivery vehicles. The potential impact of stealth liposomes is apparent from their first generation use as vehicles for therapeutics including doxorubicin (chemotherapy)^{9,10} and various anti-inflammatories.¹¹

The polymer component in stealth liposomes plays a crucial role in their stability and function. Typically, the polymer of choice for stealth liposome formulation is poly[ethylene glycol] (PEG) due to its well-understood hydrophilicity and biocompatibility, though “second-generation” materials are appearing rapidly as a means to impart stimulus response or site targeting.^{12–15} The attachment of hydrophilic polymer chains to a liposome bilayer can be achieved via either physical adsorption or covalent attachment.^{16–19} The latter approach, which utilizes lipid molecules chemically functionalized at the headgroup (e.g., dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[mPEG-5000] (DPPE-PEG)), has become commonplace because it more strongly tethers the polymer to the liposome surface. Early work on PEG-functionalized lipid inclusion into liposomes identified the approximate molecular weight range (1–10 kDa) and mole fraction (0.01–0.10) at which the hydrophilic polymer serves its intended purpose of stabilizing liposomes without disrupting the bilayer structure.^{20–22} However, it has been noted that increasing functionalized lipid content leads to solubilization of lipid molecules, with a mole fraction >0.05–0.10 resulting in a

measurable quantity of mixed micelles (i.e., micelles containing polymer-functionalized lipid and lipid).²² The molecular transfer from bilayers to micelles stems from the high asymmetry of functionalized lipid molecules, which prefer to form highly curved interfaces over the nearly planar bilayers of liposomes.²³

Therefore, a critical problem in all liposome formulations is reducing or suppressing the loss of molecules to micellization. A number of previous studies have attempted to address this challenge. Frey et al.^{24,25} replaced linear PEG with hyper-branched polyglycerol (hbPG), leading to a larger occupied volume per hydrophilic chain and therefore a decrease in the molar fraction required to stabilize liposomes. However, this approach also greatly increases molecular asymmetry and as a result increases the driving force toward the preference of curved, micellar interfaces. Vesicles based on symmetric block copolymers (i.e., polymersomes^{7,26}) offer a solution that completely eliminates dependence on asymmetric molecules. Unfortunately, the larger hydrophobic segments on the copolymer relative to those on lipids translates to thicker and less fluid bilayers, which greatly hinders the transmembrane diffusion of cargo, rendering them less effective in delivery applications. More recent interest has evolved to vesicles composed of both lipid and symmetric block copolymer (i.e., hybrid polymer/lipid vesicles), which typically contain phasesegregated copolymer and lipid domains in the bilayer due to molecular size mismatch. The lipid domains enable sufficient transmembrane diffusion, and the copolymer domains provide vesicle stability. The size incompatibility of lipids and block copolymers, however, also leads to the presence of undesirable structures, such as worm- and disk-like micelles.^{27–31}

The current study seeks to build upon previous efforts by exploring PEG-containing materials that are less molecularly asymmetric and have multiple bilayer anchor points per molecule. The material selected to achieve these criteria is the block copolymer poly[ethylene glycol]-*b*-poly[cholesteryl 2-oxo-1,3,6-dioxazocane-6-carboxylate] (PEG-P(8C-Chol), Figure 1). The PEG block serves its traditional purpose whereas the cholesteryl-functionalized block offers a handle on simultaneously altering molecule symmetry and the number of anchoring locations per molecule. These features decrease both the entropic and enthalpic driving forces for molecules to leave the bilayer without greatly changing the bilayer thickness of the resultant *hybrid stealth liposomes*. In this manner, we take advantage of the known effects of cholesteryl packing within lipid bilayers^{32–35} to offer a biomimetic solution to the stealth liposome problem (Figure 1).

MATERIALS AND METHODS³⁶

Materials and Synthesis

Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and alkyl tail-deuterated DPPC (i.e., DPPC-d62, denoted dDPPC) were purchased in powder form from Avanti Lipids, Inc. Chloroform (CHCl₃, anhydrous 99.8%) and dimethylformamide (DMF, 99.8%) were purchased from Fisher Scientific, and heavy water (D₂O, 99.8%) was from Cambridge Isotopes. Deionized water (18 MΩ-cm) was obtained from a Milli-Q purification system. For synthesis of the PEG-P(8C-Chol) copolymer, 5000 Da methoxy-PEG macroinitiator (mPEG-OH, RAAP Polymere GmbH), organo-catalyst 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 99%, Sigma-Aldrich), dichloromethane (DCM, >99.8%, Sigma-Aldrich), benzoic

acid (99%, Merck), diethyl ether (ACS grade, Tedia), and hexanes (ACS grade, J.T. Baker) were purchased.

The copolymer poly[ethylene glycol]-*b*-poly[cholesteryl 2-oxo1,3,6-dioxazocane-6-carboxylate] (PEG-P(8C-Chol), Figure 1) was synthesized in a nitrogen-filled glovebox. In general, DBU was used to catalyze the reaction between a mPEG-OH and cholesteryl-functionalized cyclic carbonate monomer. Detailed syntheses are reported elsewhere.³⁷ The reaction was carried out with varying ratios of monomer:macroinitiator in order to control the P(8C-Chol) block degree of polymerization ($X_{n,P(8C-Chol)}$). As a representative example, the synthesis of PEG-P(8C-Chol)_{4,8} was carried out as follows. In a 7 mL vial containing a magnetic stir bar, cholesteryl-functionalized cyclic carbonate monomer (545 mg, 1002.2 μ mol) and mPEG-OH (1006 mg, 201.2 μ mol) were dissolved in dichloromethane (DCM, 2.0 mL). To this solution, DBU (7.6 mg, 50.2 μ mol) was added to initiate polymerization. The reaction mixture was allowed to stir at room temperature for 2 h, at which point 30–50 mg of benzoic acid was added to quench the reaction. The reaction mixture was precipitated three times into diethyl ether:hexanes (60:40 vol:vol), and the sample was dried under vacuum until constant mass was achieved (1.46 g, 94% yield). The purified copolymers were characterized with ¹H NMR and size exclusion chromatography (Figures S1 and S2).

Liposome Preparation

Liposomes were prepared using the film rehydration and extrusion procedure, and copolymer incorporation was accomplished via the postinsertion mechanism. In general, a desired amount of DPPC was dissolved in CHCl₃, which was subsequently removed using a steady stream of ultrahigh-purity N₂, forming a thin, solid film. The films were further dried under vacuum at 55 °C for 18 h. For rehydration, H₂O or D₂O was added to the films, and they were held at 55 °C with periodic agitation over the period of an hour followed by aging at 55 °C overnight. The resulting dispersions were extruded through 400 nm (20 passes), 200 nm (20 passes), and 100 nm (41 passes) polycarbonate filters (Whatman) using an Avanti Mini-Extruder held at 55 °C. The postinsertion of PEG-P(8C-Chol) was carried out by mixing extruded DPPC liposomes with the appropriate amount of separately prepared aqueous polymer solution. For the most part, the precursor copolymer solutions were prepared by direct dissolution in water using a combination of mixing and sonication. The only exception is PEGP(8C-Chol)_{4,8}, which required dialysis from dimethylformamide (DMF). Following initial mixing, the formulations were maintained at 55 °C for 1 h with regular agitation.

Liposome Characterization

Control and modified liposomes were analyzed using a host of techniques to probe their interaction, structure, and colloidal stability. These experimental techniques include isothermal titration calorimetry (ITC), dynamic light scattering (DLS), small-angle neutron scattering (SANS), and cryogenic transmission electron microscopy (cryoTEM).

A TA Instruments Nano ITC equipped with a 1 mL cell and a 250 μ L titration syringe was used for ITC measurements. Extruded DPPC liposome solutions were prepared at 20 mmol/L (mM) and amphiphilic polymer solutions at 0.05 mM (both in H₂O). The liposome

solution was titrated in 5 μL injections into the cell containing copolymer solution. The temperatures of the cell and syringe were maintained at 55 °C throughout experiments, and the instrument was provided >12 h to equilibrate prior to starting trials. A stir rate of 300 rpm was used for all experiments to ensure sufficient mixing. Experiments were repeated in triplicate, and reported data is the average of the trials with error bars reflecting standard deviation.

Aside from ITC, solutions were prepared using the postinsertion protocol described above at sufficient concentration for SANS (25 mM total solids: DPPC + dDPPC + PEG-P(8C-Chol)). These master solutions were formulated with a DPPC:dDPPC molar ratio of 1:6.46, for isotopic matching conditions (for SANS) identified elsewhere,^{38,39} and were prepared in pure D₂O. The master solutions were diluted 10 and 20-fold with D₂O for DLS and cryoTEM, respectively.

DLS was conducted on a Malvern Nano ZS maintained at 25 °C and equipped with a 532 nm laser. The instrument-standard backscattering detector (173°) was used for all data collection. Solutions were analyzed in polystyrene cuvettes, and a viscosity (η) of 1.10 cP⁴⁰ and a refractive index (n_0) of 1.33 were used for D₂O. CryoTEM was conducted on an FEI Titan microscope operated at an accelerating voltage of 300 kV and mounted with a Gatan OneView camera. EMS C-flat TEM grids were plasma-treated for 10 s to increase surface hydrophilicity, and then a thin vitreous sample was prepared by deposition onto the grid (at 25 °C and 85% relative humidity), blotting with filter paper, and plunging into liquid ethane at -174 °C using a Leica EM GP. All samples were imaged within 3 h of cryo-preparation to reduce contamination.

SANS experiments were performed on the NGB 10m beamline at the National Institute of Standards and Technology Center for Neutron Research (NCNR). Solutions were analyzed in Hellma 1 mm quartz cells. Three neutron wavelength/sample-to-detector distance configurations ($\lambda/\lambda_{\text{sd}}$) provided a sufficiently wide q -range (~ 0.005 – 0.5 1/Å): 10 Å/5.2 m (low- q), 5 Å/5.2 m (mid- q), and 5 Å/1.2 m (high- q). All three configurations had wavelength spreads ($\Delta\lambda/\lambda$) of 0.15, and in the case of the mid- q configuration an offset detector position enabled a slightly expanded q -range to be probed. Scattered intensity, $I(q)$, was collected as a function of scattering vector, q , where $q = 4\pi \sin(\theta/2)/\lambda$ and θ is the scattering angle. The collected intensity for each sample was corrected for detector dark current and reduced to solely sample scattering by subtracting the contribution from an empty cell. All intensities were placed on an absolute scale using transmission measurements. Finally, scattering from D₂O was subtracted from dispersions to isolate the scattering from lipid/copolymer assemblies.^{41,42} The error bars in all SANS scattering profiles reflect one standard deviation from the mean scattering intensity.

RESULTS AND DISCUSSION

The exploration of PEG-P(8C-Chol) as a molecular stabilizer for liposomes is conducted using model liposomes composed of the phospholipid dipalmitoylphosphocholine (DPPC, see Figure 1). The synthesis of a series of PEG-P(8C-Chol) copolymers with fixed PEG length (5000 Da) and systematic variation of the P(8C-Chol) length (degree of

polymerization $X_{n,P(8C-Chol)} \approx 1-5$, see Table 1) enables the impact of molecular symmetry and multiple insertion sites per given chain to be probed. Prior to exploring structural and stability implications, the insertion of PEG-P(8C-Chol) into liposome bilayers must be confirmed. ITC enables a direct measurement of thermodynamic changes upon interaction of free molecules with liposomes.^{43,44} Keeping with the protocols developed previously to measure PEG-functionalized lipid incorporation to liposomes,⁴⁵ the current experimental design consists of titrating concentrated liposomes (ca. 120 nm in diameter prepared via rehydration and extrusion) into a dilute copolymer solution at a temperature above the lipid transition temperature ($T = 55\text{ }^{\circ}\text{C}$; $T_m = 41\text{ }^{\circ}\text{C}$, where T_m is the lipid transition temperature).

Integration of the heat flow corresponding to each liposome injection and correction for the effect of dilution (i.e., subtraction of the heat produced when liposomes are titrated into pure water) result in the observed heat (q_{obs}) as a function of liposome concentration in the cell (Figure 2a). Examination of these results immediately suggests that the utilized copolymer grade changes the thermodynamics: An increase in $X_{n,P(8C-Chol)}$ decreases the observed heat produced by liposome–copolymer interactions over the course of the experiment.

More detailed information can be extracted from the raw ITC data through fitting with an appropriate interaction model. Here, we utilize a two-state model consisting of copolymers assembled in micelles as state one and copolymers residing in the bilayers of liposomes as state two. The lipid molecules correspondingly transition from a state of pure lipid bilayers to mixed lipid–copolymer bilayers, but the associated energetics are assumed to be negligible.

This two-state model can be described by the equation⁴⁶

$$q_{obs} = \Delta H \left\{ -\frac{1}{2} + \frac{K([DPPC] + [PEG - P(8C - Chol)]) + [H_2O]}{2\sqrt{K^2([DPPC] + [PEG - P(8C - Chol)])^2 + 2K[H_2O]([DPPC] - [PEG - P(8C - Chol)]) + [H_2O]^2}} \right\} \quad (1)$$

where all concentrations correspond to those in the cell, $[H_2O]$ equals 55,500 mM, H is the average specific enthalpy change, and K is the average partition coefficient, which is further defined as the ratio of the mole fractions (x_i) of PEG-P(8CChol) in the liposome and aqueous/micellar phases:

$$K = \frac{x_{HSL}}{x_{mic}} = \frac{[PEG - P(8C - Chol)]_{HSL}}{([PEG - P(8C - Chol)]_{HSL} + [DPPC])} \times \frac{([PEG - P(8C - Chol)]_{mic} + [H_2O])}{[PEG - P(8C - Chol)]_{mic}} \quad (2)$$

The subscripts HSL and mic refer to the hybrid stealth liposome or micelle assemblies, respectively. Further, the sum of the PEG-P(8C-Chol) concentrations is fixed in order to impart mass balance into the model. For the model to be valid, no mixed micelles can be formed (i.e., all of the lipid molecules remain in the liposome bilayer). While there is no

simple way to prove that this assumption is fulfilled, the model describes the data reasonably well (Figure 2a, solid lines), and therefore the extracted parameters are assessed in terms of the impact of $X_{n,P(8C-Chol)}$ (Figure 2b).

Both H and K display meaningful trends as the hydrophobic P(8C-Chol) block of the copolymer is lengthened. First, the H values remain negative and decrease in magnitude with increasing $X_{n,P(8C-Chol)}$, suggesting that the cholesterol-rich environment within micelle cores becomes similar to the liposome bilayer core region in terms of intermolecular interactions. This is sensible because the precursor PEG-P(8C-Chol) micelles grow in size with $X_{n,P(8C-Chol)}$, which will be the focus of a separate study. Second, K stays relatively constant around 7.0×10^5 for PEGP(8C-Chol)_{1,4} through PEG-P(8C-Chol)_{3,7} and then drops precipitously to 1.1×10^5 for PEG-P(8C-Chol)_{4,8} reflecting a substantial decrease in the transfer of PEG-P(8C-Chol) molecules into liposome bilayers. Using eq 2, the partition coefficients can be converted to the fraction of PEG-P(8C-Chol) chains that exist in liposomes: ≈ 0.552 for $1.4 \leq X_{n,P(8C-Chol)} \leq 3.7$ and 0.172 for $X_{n,P(8C-Chol)} = 4.8$ (for a 95:5 molar ratio of DPPC to PEG-P(8C-Chol)) further emphasizing the difference.

We now direct focus to the hybrid stealth liposome structure. Based on the ITC results, the liposome solutions exhibit two regimes of PEG-P(8C-Chol) incorporation: Regime 1: PEGP(8C-Chol)_{1,4}-PEG-P(8C-Chol)_{3,7} where high insertion occurs. Regime 2: PEG-P(8C-Chol)_{4,8} where less insertion takes place. Subsequent discussion is divided into these two groupings.

Regime 1: PEG-P(8C-Chol)_{1,4}-PEG-P(8C-Chol)_{3,7}

Liposomes containing PEG-P(8C-Chol) are fabricated via the postinsertion mechanism in which pure DPPC liposomes are first rehydrated and extruded, and then PEG-P(8C-Chol) is added. In keeping with previous formulation guidelines, the liposomes prepared and discussed henceforth are composed of 5 mol % PEG-P(8C-Chol). All liposome assembly steps described were carried out at 55 °C.

Cryogenic transmission electron microscopy (cryoTEM) captures size and shape of assembled structures in an aqueous environment. The sole structures observed for liposomes prepared in regime 1 are single-walled (i.e., unilamellar) vesicles (Figure 3).

Vesicles with both rounded and faceted shape can be seen in micrographs for all of the HSLs imaged, which is expected based on previous studies for both pure liposomes and stealth liposomes prepared below the lipid T_m (the liposome solutions were quenched from room temperature into liquid ethane).^{47,48} It is a bit surprising that nothing resembling PEG-P(8C-Chol) micelles (Figure S3) are detected in the micrographs since ITC results suggest that approximately half of the PEG-P(8C-Chol) molecules remain excluded from bilayers. This may either reflect an underestimation of PEG-P(8C-Chol) incorporation by the ITC model or inability of cryoTEM images to detect the comparatively tiny micelles alongside liposomes ($\sim 10\times$ smaller radius).

The hybrid stealth liposomes in micrographs can be analyzed in terms of their diameter (exterior of bilayer to exterior of bilayer) and do not appear to change with variation in the PEG-P(8C-Chol) grade used for preparation (Figure 4).

Dynamic light scattering (DLS) conducted on the same solutions immediately after preparation confirms the polymer-independent liposome diameter (Figure 4). To be clear, the DLS data reflect slightly larger dimensions because they are a measure of the hydrodynamic diameter, which includes the extended PEG chains and any hydration layers on the liposomes. While precise values of liposome bilayer thickness are limited due to image resolution, it is clear that the bilayers are relatively uniform in thickness and have little dependence on the PEG-P(8C-Chol) copolymer incorporated. Taken in sum, these results indicate that the liposomes are not substantially altered during the incorporation of copolymer molecules; e.g., no partial bilayer dissolution and reformation occur.

Small-angle neutron scattering (SANS) serves as an outstanding complementary technique to cryoTEM since it offers Fourier-space information on a globally averaged set of liposomes. While the q -range (inversely proportional to real space) utilized in the current SANS experiments is limited in ability to provide information regarding liposome size, it is very sensitive to structural changes on the size scale of the liposome bilayer and polymer radius of gyration (R_g) as previously indicated.^{38,39} Therefore, it is expected that the current scattering experiments (see Materials and Methods for details) will be sensitive to the incorporation of PEG-P(8C-Chol) into liposome bilayers and any corresponding dimension changes. The SANS profiles for each of the liposomes discussed above are presented in Figure 5 along with the unmodified parent liposomes (pure DPPC). Qualitative observation of these data shows a strongly contrasting feature difference in the range of $q = 0.04\text{--}0.1 \text{ \AA}^{-1}$ between the “standard” liposomes and those with PEG-P(8C-Chol). The peaks at this location, which are attributed to the liposome bilayer in the current neutron scattering contrast matching scheme,^{38,39} become broader and less intense in the presence of the copolymers. This is due to the polymer chains extending from the liposome surface of the liposomes.

A more quantitative description of liposome structure can be captured through the use of a form factor model to fit the SANS profiles. While the simplest fitting approach consists of a single structure, we were unable to effectively capture the SANS profile features in any of the liposome solutions using solely the form factor for polymer-functionalized liposomes (Figure S4). This may be anticipated since the ITC experiments discussed above suggest that not all of the PEG-P(8C-Chol) collocates in liposome bilayers. By combining the modified liposome model with scattering data from copolymer micelle solutions (eq 3), we are able to capture the data features more appropriately. The overall scattering intensity, $I(q)$, is calculated in the model via

$$I(q) = \psi_{\text{HSL}} P_{\text{HSL}}(q) + \psi_{\text{mic}} I_{\text{mic}}(q) + \text{bkg} \quad (3)$$

where ψ_{HSL} and ψ_{mic} are weighting functions that describe the number density of hybrid stealth liposomes and micelles in solution, respectively, $P_{\text{HSL}}(q)$ is the polymer functionalized liposome form factor,³⁸ $I_{\text{mic}}(q)$ is experimental SANS data for pure PEG-

P(8C-Chol) assemblies in solution (Figure S5), and bkg is background. The model is further confined by conservation of PEG-P(8C-Chol) using the aforementioned parameters. The structural information embedded within

$P_{\text{HSL}}(q)$ (i) reiterates that the liposome bilayers remain constant at 5.1 nm regardless of the PEG-P(8C-Chol) copolymer added, (ii) indicates that the PEG block R_g remains relatively constant around 2.0 nm as expected (R_g for 5 kDa PEG $\approx$ 3.1 nm),⁴⁹ and (iii) informs that the molar portion of PEG-P(8C-Chol) in liposomes is 3.0–3.6% (out of a maximum 5% by formulation) and decreases slightly with increasing $X_{\text{n,P(8C-Chol)}}$. It is possible that the estimated quantity of PEGP(8C-Chol) in liposomes is an underestimate since the diskshaped micelles that potentially reside in solution alongside the liposomes produce similar scattering signal as would the segregation of the copolymer molecules within the liposome bilayer.⁵⁰

Regime 2: PEG-P(8C-Chol)_{4.8}

Examination of structural data for the PEG-P(8C-Chol)_{4.8} copolymer mixed with liposomes reemphasizes its difference compared with the other molecular stabilizers. Cryo-micrographs of liposome/ PEG-P(8C-Chol)_{4.8} solutions display a number of liposomes but also contain a moderate quantity of oblong assemblies having dense cores (Figure 6). Close examination of the oblong assemblies alongside images from deposited solutions containing only PEG-P(8C-Chol)_{4.8} suggests that these objects are PEG-P(8C-Chol) micelles that have resisted incorporation into liposome bilayers. Further details regarding the unique structure of copolymer micelles will be the topic of a future study. The liposome diameters as measured from cryoTEM images reflect no change from the other liposome solutions (Figure 4). DLS measurements of the average assembly size also appear to have little difference from the remainder of the solutions studies; however, the greater presence of micelles likely influences this data as suggested by the larger polydispersity.

The impact of less PEG-P(8C-Chol) collocated in liposomes is also apparent from SANS data. The feature changes that correspond to polymer chains grafted on the liposome surface are still noticeable albeit at a far lesser extent than those from those liposomes in regime 1 (Figure 7a). Fitting the DPPC/ PEG-P(8C-Chol)_{4.8} SANS profile using the same model as above results in similar structural features: a bilayer thickness of 5.1 nm and PEG R_g of 2.0 nm.

However, the molar fraction of PEG-P(8C-Chol) in liposomes is reduced to 0.8 mol % (out of a maximum 5%). The formulated mole fraction of PEG-P(8C-Chol) and that quantified in liposomes can be used to approximate the molar fraction of PEG-P(8C-Chol) molecules that reside in bilayers with the remaining molecules residing in copolymer micelles. As in ITC assessment, a striking binary behavior is noted between the copolymers in regimes 1 and 2 (Figure 7b): the majority of copolymers with $X_{\text{n,P(8C-Chol)}} = 3.7$ are located in liposomes (>60%) whereas only 15% of those with $X_{\text{n,P(8C-Chol)}} = 4.8$ participate.

Liposome Colloidal Stability

As made clear above, there is a larger contrast in the quantity of PEG-P(8C-Chol) collocated in liposomes between regimes 1 and 2. It is expected that this difference also influences the

steric stability of hybrid stealth liposomes, which can be monitored using DLS on the same solutions over a period of time. For example, in pure DPPC liposomes it is anticipated that the unprotected exterior surfaces will lead to liposome aggregation with time, ultimately resulting in non-negligible precipitation. The DLS data for the unmodified liposomes clearly show this behavior (Figure 8 and Figure S7). Following only 2 weeks, the distribution has shifted to larger size and breadth, and after 3 weeks a nontrivial quantity of precipitation is visible in the bottom of the DLS cuvette. Size distributions for modified liposomes using copolymers in regime 1, on the other hand, undergo no detectable change over a period of 35 weeks (Figure 8 and Figure S7) as is expected for stealth liposomes (Figure S6). The addition of PEG-P(8C-Chol)_{4,8} results in increases of average size and distribution breadth even faster than the pure DPPC control system. It is not currently understood why PEG-P(8CChol)_{4,8} accelerates the instability of liposomes in solution, but it may be postulated that the abundance of remaining copolymer micelles serve as nucleation sites for liposome agglomeration.

CONCLUSIONS

The present study provides a novel mechanism to stabilize stealth liposomes, specifically against loss to micellization, by incorporation of a cholesteryl-functionalized diblock copolymer. The copolymer offers two molecular features that make it advantageous versus traditionally used polymer-functionalized lipids: (i) it is more molecularly symmetric, and (ii) it has multiple bilayer insertion groups per molecule. In the current study, a critical point of ca. 4–5 cholesteryl groups per copolymer chain was determined as the transition point between substantial and minimal copolymer insertion into bilayers. Measurements of liposome–copolymer interaction and resultant structure independently arrive at this conclusion and the consequence on colloidal stability is clear: copolymers with $X_{n,P(8C-Chol)}$ below the critical point stabilize liposomes in solution and those with $X_{n,P(8C-Chol)}$ above it accelerate their instability. Further development of the hybrid stealth liposome platform as an effective route to stabilize liposomes will lead to its implementation as a potential alternative to conventional stealth liposomes. This unique approach may create a number of additional exploratory opportunities through the connectivity of cholesteryl groups such as controlled phase separation and heterogeneously tailored membrane fluidity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Ron Jones for SANS support, Alline Myers for aid in cryoTEM experiments, and Rob Brinson for ITC training and input. K.P.M. acknowledges postdoctoral research support from the National Research Council Research Associateship Program. S.V. and Y.Y.Y. acknowledge funding support from the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology, and Research), Singapore.

REFERENCES

- (1). Lasic DD; Needham D The “Stealth” Liposome: A Prototypical Biomaterial. *Chem. Rev* 1995, 95 (8), 2601–2628.

- (2). Immordino ML; Dosio F; Cattel L Stealth Liposomes: Review of the Basic Science, Rationale, and Clinical Applications, Existing and Potential. *Int. J. Nanomed* 2006, 1 (3), 297–315.
- (3). Nag OK; Awasthi V Surface Engineering of Liposomes for Stealth Behavior. *Pharmaceutics* 2013, 5 (4), 542–569. [PubMed: 24300562]
- (4). Woodle MC Surface-Modified Liposomes: Assessment and Characterization for Increased Stability and Prolonged Blood Circulation. *Chem. Phys. Lipids* 1993, 64 (1–3), 249–262. [PubMed: 8242837]
- (5). Nel A; Ruoslahti E; Meng H New Insights into “Permeability” as in the Enhanced Permeability and Retention Effect of Cancer Nanotherapeutics. *ACS Nano* 2017, 11 (10), 9567–9569. [PubMed: 29065443]
- (6). Pantusa M; Bartucci R; Marsh D; Sportelli L Shifts in ChainMelting Transition Temperature of Liposomal Membranes by Polymer-Grafted Lipids. *Biochim. Biophys. Acta, Biomembr* 2003, 1614 (2), 165–170.
- (7). Discher DE; Ahmed F Polymersomes. *Annu. Rev. Biomed. Eng* 2006, 8 (1), 323–341. [PubMed: 16834559]
- (8). Wang W; Qi H; Zhou T; Mei S; Han L; Higuchi T; Jinnai H; Li CY Highly Robust Crystalsome via Directed Polymer Crystallization at Curved Liquid/Liquid Interface. *Nat. Commun.* 2016, 7, 10599. [PubMed: 26837260]
- (9). Martin F; Woodle M; Redemann C; Yau-Young A Liposomes with Enhanced Circulation Time and Method of Treatment. *US20010051183 A1*, 12 13, 2001.
- (10). Schilt Y; Berman T; Wei X; Barenholz Y; Raviv U Using Solution X-Ray Scattering to Determine the High-Resolution Structure and Morphology of PEGylated Liposomal Doxorubicin Nanodrugs. *Biochim. Biophys. Acta, Gen. Subj* 2016, 1860 (1), 108–119.
- (11). Woodle MC; Martin FJ; Huang SK Method of Treatment of Inflamed Tissues. *US5356633 A*, 10 18, 1994.
- (12). Leroux J-C; Roux E; Le Garrec D; Hong K; Drummond DC N-Isopropylacrylamide Copolymers for the Preparation of pH Sensitive Liposomes and Polymeric Micelles. *J. Controlled Release* 2001, 72 (1), 71–84.
- (13). Harris JM; Chess RB Effect of Pegylation on Pharmaceuticals. *Nat. Rev. Drug Discovery* 2003, 2 (3), 214–221. [PubMed: 12612647]
- (14). Manjappa AS; Chaudhari KR; Venkataraju MP; Dantuluri P; Nanda B; Sidda C; Sawant KK; Ramachandra Murthy RS Antibody Derivatization and Conjugation Strategies: Application in Preparation of Stealth Immunoliposome to Target Chemotherapeutics to Tumor. *J. Controlled Release* 2011, 150 (1), 2–22.
- (15). Adamiak L; Touve MA; LeGuyader CLM; Gianneschi NC Peptide Brush Polymers and Nanoparticles with Enzyme-Regulated Structure and Charge for Inducing or Evading Macrophage Cell Uptake. *ACS Nano* 2017, 11 (10), 9877–9888. [PubMed: 28972735]
- (16). Klibanov AL; Maruyama K; Torchilin VP; Huang L Amphipathic Polyethyleneglycols Effectively Prolong the Circulation Time of Liposomes. *FEBS Lett.* 1990, 268 (1), 235–237. [PubMed: 2384160]
- (17). Kostarelos K; Tadros TF; Luckham PF Physical Conjugation of (Tri-) Block Copolymers to Liposomes toward the Construction of Sterically Stabilized Vesicle Systems. *Langmuir* 1999, 15 (2), 369–376.
- (18). Wang J-Y; Chin J; Marks JD; Lee KYC Effects of PEO– PPO–PEO Triblock Copolymers on Phospholipid Membrane Integrity under Osmotic Stress. *Langmuir* 2010, 26 (15), 12953–12961. [PubMed: 20666423]
- (19). Allen TM; Cullis PR Liposomal Drug Delivery Systems: From Concept to Clinical Applications. *Adv. Drug Delivery Rev* 2013, 65 (1), 36–48.
- (20). Edwards K; Johnsson M; Karlsson G; Silvander M Effect of Polyethyleneglycol-Phospholipids on Aggregate Structure in Preparations of Small Unilamellar Liposomes. *Biophys. J* 1997, 73 (1), 258–266. [PubMed: 9199790]
- (21). Rovira-Bru M; Thompson DH; Szleifer I Size and Structure of Spontaneously Forming Liposomes in Lipid/PEG-Lipid Mixtures. *Biophys. J* 2002, 83 (5), 2419–2439. [PubMed: 12414678]

- (22). Johnsson M; Edwards K Liposomes, Disks, and Spherical Micelles: Aggregate Structure in Mixtures of Gel Phase Phosphatidylcholines and Poly(Ethylene Glycol)-Phospholipids. *Biophys. J* 2003, 85 (6), 3839–3847. [PubMed: 14645073]
- (23). Israelachvili JN *Intermolecular and Surface Forces*, 3rd ed.; Academic Press, Burlington, MA, 2011.
- (24). Hofmann AM; Wurm F; Hühn E; Nawroth T; Langguth P; Frey H Hyperbranched Polyglycerol-Based Lipids via Oxyanionic Polymerization: Toward Multifunctional Stealth Liposomes. *Biomacromolecules* 2010, 11 (3), 568–574. [PubMed: 20121134]
- (25). Müller SS; Dingels C; Hofmann AM; Frey H PolyetherBased Lipids Synthesized with an Epoxide Construction Kit: Multivalent Architectures for Functional Liposomes. In *Tailored Polymer Architectures for Pharmaceutical and Biomedical Applications*; ACS Symposium Series; American Chemical Society: 2013; Vol. 1135, pp 11–25.
- (26). Discher BM; Won Y-Y; Ege DS; Lee JC-M; Bates FS; Discher DE; Hammer DA Polymersomes: Tough Vesicles Made from Diblock Copolymers. *Science* 1999, 284 (5417), 1143–1146. [PubMed: 10325219]
- (27). Chemin M; Brun P-M; Lecommandoux S; Sandre O; Le Meins J-F Hybrid Polymer/Lipid Vesicles: Fine Control of the Lipid and Polymer Distribution in the Binary Membrane. *Soft Matter* 2012, 8 (10), 2867–2874.
- (28). Le Meins J-F; Schatz C; Lecommandoux S; Sandre O Hybrid Polymer/Lipid Vesicles: State of the Art and Future Perspectives. *Mater. Today* 2013, 16 (10), 397–402.
- (29). Pippa N; Kaditi E; Pispas S; Demetozos C PEO-b-PCLDPPC Chimeric Nanocarriers: Self-Assembly Aspects in Aqueous and Biological Media and Drug Incorporation. *Soft Matter* 2013, 9 (15), 4073–4082.
- (30). Tuyen Dao TP; Brûlet A; Fernandes F; Er-Rafik M; Ferji K; Schweins R; Chapel J-P; Fedorov A; Schmutz M; Prieto M; Sandre O; Le Meins J-F Mixing Block Copolymers with Phospholipids at the Nanoscale: From Hybrid Polymer/Lipid Wormlike Micelles to Vesicles Presenting Lipid Nanodomains. *Langmuir* 2017, 33 (7), 1705–1715. [PubMed: 28128560]
- (31). Naziris N; Pippa N; Chrysostomou V; Pispas S; Demetozos C; Libera M; Trzebicka B Morphological Diversity of Block Copolymer/Lipid Chimeric Nanostructures. *J. Nanopart. Res.* 2017, 19 (10), 347.
- (32). Kessel A; Ben-Tal N; May S Interactions of Cholesterol with Lipid Bilayers: The Preferred Configuration and Fluctuations. *Biophys. J* 2001, 81 (2), 643–658. [PubMed: 11463613]
- (33). Carrion C; Domingo JC; de Madariaga MA Preparation of Long-Circulating Immunoliposomes Using PEG–cholesterol Conjugates: Effect of the Spacer Arm between PEG and Cholesterol on Liposomal Characteristics. *Chem. Phys. Lipids* 2001, 113 (1–2), 97–110. [PubMed: 11687230]
- (34). Rao Z; Taguchi T Spectroscopic Studies on Interactions between Cholesterol-End Capped Polyethylene Glycol and Liposome. *Colloids Surf., B* 2012, 97, 248–253.
- (35). Ma C-C; He Z-Y; Xia S; Ren K; Hui L-W; Qin H-X; Tang M-H; Zeng J; Song X-R α , ω -Cholesterol-Functionalized Low Molecular Weight Polyethylene Glycol as a Novel Modifier of Cationic Liposomes for Gene Delivery. *Int. J. Mol. Sci* 2014, 15 (11), 20339–20354. [PubMed: 25383677]
- (36). Certain commercial equipment and materials are identified in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation by the National Institute of Standards and Technology (NIST) nor does it imply that the material or equipment identified is necessarily the best available for this purpose.
- (37). Venkataraman S; Mineart KP; Prabhu VM; Hedrick JL; Hedrick JL; Yang YY Cholesterol Functionalized Aliphatic N-Substituted 8-Membered Cyclic Carbonate. *Polym. Chem*, 2018, DOI: 10.1039/C8PY00406D.
- (38). Arleth L; Vermehren C An Analytical Model for the SmallAngle Scattering of Polyethylene Glycol-Modified Liposomes. *J. Appl. Crystallogr* 2010, 43 (5), 1084–1091.
- (39). Mineart KP; Kelley EG; Nagao M; Prabhu VM Processing–structure Relationships of Poly(Ethylene Glycol)-Modified Liposomes. *Soft Matter* 2017, 13 (31), 5228–5232. [PubMed: 28730191]

- (40). Cho CH; Urquidi J; Singh S; Robinson GW Thermal Offset Viscosities of Liquid H₂O, D₂O, and T₂O. *J. Phys. Chem. B* 1999, 103 (11), 1991.
- (41). Glinka CJ; Barker JG; Hammouda B; Krueger S; Moyer JJ; Orts WJ The 30 m Small-Angle Neutron Scattering Instruments at the National Institute of Standards and Technology. *J. Appl. Crystallogr* 1998, 31 (3), 430–445.
- (42). Kline SR Reduction and Analysis of SANS and USANS Data Using IGOR Pro. *J. Appl. Crystallogr* 2006, 39 (6), 895–900.
- (43). Freire E; Mayorga OL; Straume M Isothermal Titration Calorimetry. *Anal. Chem* 1990, 62 (18), 950A–959A.
- (44). Heerklotz H The Microcalorimetry of Lipid Membranes. *J. Phys.: Condens. Matter* 2004, 16 (15), R441–R467.
- (45). Wu G; Lee KYC Interaction of Poloxamers with Liposomes: An Isothermal Titration Calorimetry Study. *J. Phys. Chem. B* 2009, 113 (47), 15522–15531. [PubMed: 19863124]
- (46). Heerklotz H; Lantzsch G; Binder H; Klose G; Blume A Thermodynamic Characterization of Dilute Aqueous Lipid/Detergent Mixtures of POPC and C12EO8 by Means of Isothermal Titration Calorimetry. *J. Phys. Chem* 1996, 100 (16), 6764–6774.
- (47). Blaurock AE; Gamble RC Small Phosphatidylcholine Vesicles Appear to Be Faceted below the Thermal Phase Transition. *J. Membr. Biol* 1979, 50 (2), 187–204.
- (48). Hirst LS; Ossowski A; Fraser M; Geng J; Selinger JV; Selinger RLB Morphology Transition in Lipid Vesicles Due to InPlane Order and Topological Defects. *Proc. Natl. Acad. Sci. U. S. A* 2013, 110 (9), 3242–3247. [PubMed: 23401499]
- (49). Devanand K; Selser JC Asymptotic Behavior and LongRange Interactions in Aqueous Solutions of Poly(ethylene oxide). *Macromolecules* 1991, 24 (22), 5943–5947.
- (50). Dao TPT; Fernandes F; Er-Rafik M; Salva R; Schmutz M; Brûlet A; Prieto M; Sandre O; Le Meins J-F Phase Separation and Nanodomain Formation in Hybrid Polymer/Lipid Vesicles. *ACS Macro Lett.* 2015, 4 (2), 182–186.

Figure 1. Chemical structure for the lipid DPPC and the novel, multilipid copolymer PEG-P(8C-Chol). Included on the right is a schematic showing a hybrid stealth liposomes at the liposome and bilayer scale. Values for the P(8C-Chol) block degree of polymerization, $m (= X_{n,P(8C-Chol)})$, can be found in Table 1 along with polydispersities.

Figure 2.

(a) Observed heat (q_{obs}) for titration of DPPC liposomes into aqueous PEG-P(8C-Chol) at 55 °C. The various grades are colored coded with matching labels and include solid lines indicating fits using the two-state model described in the main text. The data are shifted vertically by increments of 0.1 for clarity, and dashed lines show the shifted value of 0 for each. (b) Thermodynamic parameters, average specific enthalpy (ΔH), and partition coefficient (K) provided by the two-state model.

Figure 3. CryoTEM micrographs of liposomes formulated with 5 mol % PEG-P(8C-Chol)_{1.4} (top), PEG-P(8C-Chol)_{2.8} (middle), and PEGP(8C-Chol)_{3.7} (bottom).

Figure 4. Liposome hydrodynamic diameter ($2R_H$) from DLS and bilayer-to-bilayer diameter from TEM micrographs (D_{TEM}) for liposomes containing each PEG-P(8C-Chol) copolymer as labeled. Bars indicate the cumulant fit ($2R_H$, solid) and average (D_{TEM} , striped) sizes with error bars reflecting polydispersity for DLS and standard deviation for TEM.

Figure 5.

SANS results obtained for liposomes containing various PEG-P(8C-Chol) copolymers in regime 1 with 5 mol % copolymer loading (color coded and labeled). Data sets are shifted vertically for clarity using the indicated shift factors. The overlaid solid green lines are fits using the model described in the main text.

Figure 6. CryoTEM micrograph of liposomes formulated with 5 mol % PEG-P(8C-Chol)_{4.8}. The inset is a negatively stained TEM micrograph (reverse contrast of main image) for the same copolymer solution in the absence of DPPC (i.e., a pure copolymer dispersion). Arrows highlight the presence of cylindrical micelles in both images.

Figure 7.

(a) SANS results obtained for liposomes containing 5 mol % PEG-P(8C-Chol)_{4,8} alongside control (DPPC) liposome data (color coded and labeled). The liposome data set is shifted vertically for clarity using the indicated shift factor. The overlaid solid green lines are fits using the model described in the main text. (b) Percentage of PEG-P(8C-Chol) copolymers that are incorporated into liposomes as a function of their $X_{n,P(8C-Chol)}$ as extracted from the SANS model and ITC partition coefficient (color coded and labeled).

Figure 8.

Hydrodynamic diameter ($2R_H$) distributions for liposomes containing 5 mol % of the indicated PEG-P(8C-Chol) copolymer as well as for a control system composed of pure DPPC. The overlaid distributions for each liposome were collected at 0, 1, 2, 3, 6, and 35 weeks (transition from black to green color, respectively) with exceptions for the control liposomes and PEG-P(8C-Chol)_{4.8} containing liposomes, which were terminated at 2 weeks due to precipitation.

Table 1.A Summary of PEG-P(8C-Chol) Copolymer Characteristics^a

notation	M_w (kDa)	$X_{n,P(8C-Chol)}$	
PEG-P(8C-Chol) _{1,4}	5.7	1.4	1.11
PEG-P(8C-Chol) _{2,2}	6.2	2.2	1.11
PEG-P(8C-Chol) _{2,8}	6.5	2.8	1.12
PEG-P(8C-Chol) _{3,3}	6.8	3.3	1.13
PEG-P(8C-Chol) _{3,7}	7.0	3.7	1.12
PEG-P(8C-Chol) _{4,8}	7.6	4.8	1.13

^aMolecular weight (M_w) and P(8C-Chol) degree of polymerization ($X_{n,P(8C-Chol)}$) were obtained via ¹H NMR, and copolymer polydispersity () was determined by size exclusion chromatography.