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A Minimal Hydrophobicity Is Needed To Employ Amphiphilic p(HPMA)-co-p(LMA) Random Copolymers in Membrane Research

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Supporting Information

ABSTRACT: Because a polymer environment might be milder than a detergent micelle, amphiphilic polymers have attracted attention as alternatives to detergents in membrane biochemistry. The polymer poly[*N*-(2-hydroxypropyl)-methacrylamid] [p(HPMA)] has recently been modified with hydrophobic lauryl methacrylate (LMA) moieties, resulting in the synthesis of amphiphilic p(HPMA)-co-p(LMA) polymers. p(HPMA)-co-p(LMA) polymers with a LMA content of 5 or 15% have unstable hydrophobic cores. This, on one hand, promotes interactions of the hydrophobic LMA moieties with membranes, resulting in membrane rupture, but at the same time prevents formation of a hydrophobic, membrane mimetic environment that is sufficiently stable for the incorporation of transmembrane proteins. On the other hand, the p(HPMA)-co-p(LMA) polymer with a LMA content of 25% forms a stable hydrophobic core structure, which prevents hydrophobic interactions with membrane lipids but allows stable incorporation of membrane proteins. On the basis of our data, it becomes obvious that amphiphilic polymers have to have threshold hydrophobicities should an application in membrane protein research be anticipated.

To analyze the structure and function of highly hydrophobic membrane proteins *in vitro*, detergents or lipids are traditionally used in membrane biochemistry. However, amphiphilic polymers have attracted attention as an alternative to detergents and lipids, and amphiphilic polymers, the best characterized class of Amphipols, have already been used successfully as stabilizing and nondenaturing solvents in membrane protein research.[1−11] The polymer poly[*N*-(2-hydroxypropyl)-methacrylamid] [p(HPMA)] is a water-soluble, nontoxic, and nonimmunogenic polymer,[12] dating back to the invention of pharmacological active polymers by H. Ringsdorf in the 1970s.[12−16] Recently, p(HPMA) polymers with a narrow size distribution became available[16] and have been modified with hydrophobic lauryl methacrylate (LMA) moieties, resulting in the synthesis of amphiphilic copolymers.[17] p(HPMA)-co-p(LMA) copolymers (Figure 1) self-assemble spontaneously in aqueous solutions into micelle-like aggregates, which allow encapsulation of hydrophobic compounds.[18−21] The formation of p(HPMA)-co-p(LMA) aggregates, as well as its properties, e.g., the size of the aggregates or the composition of the hydrophobic core, might be controlled by the content of the hydrophobic LMA block within the polymer, which is statistically spread across the hydrophilic backbone. Initial studies have indicated that p(HPMA)-co-p(LMA) polymers form aggregates with a micellar substructure, when the polymer contains at least 5 mol % of the hydrophobic LMA side chains.[20] Thus, p(HPMA)-co-p(LMA) polymers might make up a new class of surfactants used for membrane protein solubilization and characterization or the transport of pharmaceutically active, hydrophobic peptides to specific cellular targets. While in the case of a polymer with 5% LMA, the amount of aggregates is very small and the aggregates are rather unstable, incorporation of hydrophobic compounds eventually results in formation of a more stable hydrophobic core.[20] In contrast, polymers containing 10% LMA form stable hydrophobic core structures by themselves.[20] Although the exact structures of these aggregates are still enigmatic, the polymers comply with the conditions that are essential for membrane protein research, as they form a hydrophobic environment in aqueous solutions and thereby mimic a membrane environment, similar to classical detergent micelles. However, detergents typically have high aggregation numbers and critical micelle concentrations (CMCs), and thus, high detergent-to-protein ratios are typically needed to solubilize membrane proteins. This results in an environment of strong entropic forces, which may destabilize or even denature membrane proteins.[22] In contrast, a copolymer environment might be milder than a detergent micelle and thus may destabilize membrane proteins to a lesser extent.

In this study, we have analyzed the properties of p(HPMA)-co-p(LMA) polymers with LMA contents of approximately 5, 15, and 25% (**C5**, **C15**, and **C25**, respectively) for applications

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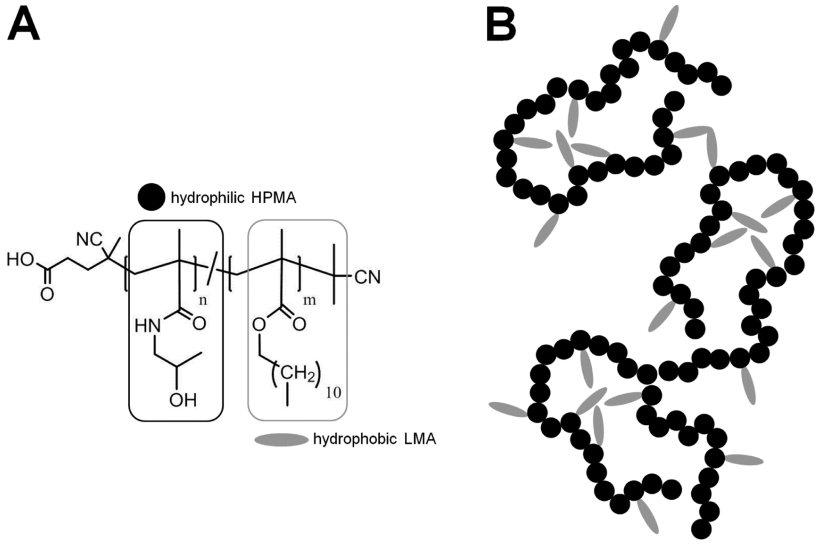

Figure 1. Chemical structure of the p(HPMA)-co-p(LMA) polymer and its aggregate in aqueous solution. (A) Chemical structure of the p(HPMA)-co-p(LMA) random copolymer with its hydrophobic LMA and its hydrophilic HPMA monomers. n represents the mole percent of HPMA and m the mole percent of LMA (5, 15, and 25% in this study). (B) Scheme of a p(HPMA)-co-p(LMA) aggregate composed of three individual copolymer chains in aqueous solution.

in membrane protein research. The **C5** polymer interacted with a phosphatidylcholine (PC) model membrane, and the interaction resulted in membrane rupture. While the **C15** polymer had a similar effect on membranes, in the case of the **C25** polymer no influence on the structure of the analyzed model membrane system has been determined. Furthermore, while in **C5** and **C15** polymers formation of a transmembrane (TM) helix dimer structure was not well-supported, the secondary as well as the quaternary structure of this protein was well-preserved in **C25** polymer solutions. Together, our data show that the two p(HPMA)-co-p(LMA) polymers with LMA contents of 5 and 15% have unstable hydrophobic cores, which on one hand promotes interactions of the hydrophobic LMA moieties with membranes but at the same time prevents formation of a membrane mimetic environment that is sufficiently stable for the incorporation of TM proteins. On the other hand, the **C25** p(HPMA)-co-p(LMA) polymer forms a stable hydrophobic core, which prevents hydrophobic interactions with membrane lipids but allows the stable incorporation of membrane proteins. Thus, the **C25** polymer is introduced as a new potential tool in membrane protein research. Furthermore, on the basis of our data for copolymers with increasing LMA contents, it becomes obvious that newly designed polymers must exceed threshold hydrophobicities should an application in membrane protein research be anticipated.

MATERIALS AND METHODS

Materials for Fluorescence and Circular Dichroism Measurements. Peptides corresponding to residues 69−101 of the human glycophorin A (GpA) TM domain (SEPEITLIIFGVMAGVIGTILLISYGIRRLIKK) were custom-synthesized and labeled at the N-terminus with either the donor and acceptor dyes fluorescein (FL) and 5-6-carboxyrhodamine (TAMRA), respectively (Peptide Specialty Laboratories, Heidelberg, Germany).[23] Peptides were dissolved in 2,2,2-trifluoroethanol (TFE) purchased from Sigma-Aldrich (Munich, Germany). 1,2-Dieicosenoyl-*sn*-glycero-3-phosphocholine (20:1 PC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss Rhod PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Laurdan (6-dodecanoyl-*N*,*N*-dimethyl-2-naphthylamine), *n*-dodecyl β-D-maltopyranoside (DDM), and 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) were purchased from Sigma-Aldrich. Amphipol A8-35 was purchased from Affymetrix (Santa Clara, CA). PD10 columns were purchased from GE Healthcare (Buckinghamshire, Great Britain). 8-Aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS), and *p*-xylene-bis-pyridinium bromide (DPX) were purchased from Invitrogen Life Technologies (Carlsbad, CA).

Polymer Synthesis. Polymers **C5**, **C15**, and **C25** were synthesized following previously published procedures[17,24] with some alterations.[20,21]

4-Cyano-4-[(thiobenzoyl)sulfanyl]pentanoic acid, synthesized according to published procedures,[25] was used as a CTA (chain transfer agent).

Pentafluorophenyl methacrylate (PFPMA) was prepared following described procedures.[26,27]

In a typical reaction, 3 g (12 mmol) of PFPMA, 41 mg (0.16 mmol) of CTP with AIBN as an initiator (1:8 AIBN:CTP molar ratio), and the respective amounts of lauryl methacrylate (LMA) (5 mol % for **C5**, 15 mol % for **C15**, and 25 mol % for **C25**) were dissolved in 5 mL of dry dioxane in a Schlenk tube. After three freeze−vacuum−thaw cycles, the reaction mixture was stirred at 65 °C overnight. The p(PFPMA)-co-p(LMA) polymer was precipitated three times in hexane, isolated by centrifugation, and dried for 12 h at 40 °C under vacuum. A pink powder was obtained with 60% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (br t), 1.20−1.75 (br), 2.00−2.75 (br s); ^{19}F NMR (400 MHz, $CDCl_3$) δ −162 (br), −157 (br), −152 to −150 (br).

Postpolymerization Modification to p(HPMA)-co-p(LMA) Random Copolymers. C5 is used as an example to describe the reaction procedure: 280 mg of the precursor polymer (M_n = 23000 g/mol) was dissolved in absolute dioxane, and 48 mg (0.9 mmol) of 2-hydroxypropanamine and 183 mg (1.8 mmol) of triethylamine were added to the reaction

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mixture. The reaction continued overnight at 60 °C. To ensure complete removal of reactive ester groups, an excess of 48 mg of 2-hydroxypropanamine and 183 mg of triethylamine were added again to the reaction mixture. Completion of the reaction was assessed using ^{19}F NMR. The final polymer was precipitated three times in diethyl ether, dissolved in 1 mL of DMSO, and dialyzed against deionized water. A colorless fluffy powder was obtained in 76% yield after lyophilization: ^{1}H NMR (400 MHz, DMSO-*d*) δ 0.85 (br t), 0.90−1.40 (br), 1.6−2.20 (br), 2.75−3.10 (br), 3.50−3.80 (br), 4.60−4.80 (br).

The polymers contain ∼5, ∼15, or ∼25 mol % hydrophobic LMA as verified by NMR. Polymer characteristics are summarized in Table 1. Their molecular weights were determined by gel permeation chromatography in THF.

Table 1. Overview of the C5, C15, and C25 Random Copolymers[a]

polymer	HPMA:LMA ratio	M_n (g/mol)	PDI	ref
C5	95:5	13500	1.20	20, 21
C15	85:15	14000	1.17	21
C25	75:25	17000	1.23	48

[a]Molecular weights (M_n) and the polydispersity index (PDI) were determined by gel permeation chromatography. For the sake of comparison, please note that **C5** and **C15** are identical to **C5** and **C15**, respectively, in refs 20 and 21 and **C25** is comparable to **P2*** in ref 48, which has, however, a higher molecular weight.

Pyrene Fluorescence Spectroscopy. Critical aggregate concentrations of the polymers, i.e., the equivalent of CMC values characterizing detergent micelle formation, were determined as described in detail recently.[28] Briefly, a stock solution of the **C15** and **C25** copolymers was prepared at a concentration of 0.1 g/L by dissolving the polymers in DMSO. The polymer stock solution was subsequently diluted to 10 different concentrations down to 1×10^{-6} g/L using an aqueous NaCl solution. We then prepared each sample by dropping carefully 40 μL of a pyrene solution (2.5×10^{-5} mol/L in acetone) into an empty vial, evaporating the acetone by gently heating at 50−60 °C, adding 2 mL of one of the polymer solutions, and stirring the closed and light-protected vials for 48−72 h at 50−60 °C. The final concentration of pyrene in water thus reached 5.0×10^{-7} mol/L, which is slightly below the pyrene saturation concentration in water at 22 °C. Steady-state fluorescence spectra of the air-equilibrated samples were recorded using an LS 50 B Perkin-Elmer luminescence spectrometer (right angle geometry, 1 cm × 1 cm quartz cell) using the following conditions: excitation at 333 nm and slit widths of 10 nm for excitation and 2.5 nm for emission. The intensities of the fluorescence emission at 372 and 383 nm were then evaluated. The decrease for 372 and 383 nm versus the logarithmically plotted polymer concentration was used to determine the critical aggregation concentration.

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded on a Jasco J-815 spectropolarimeter at 25 °C in a step-scan mode using 0.1 cm path length quartz cells from Hellma (Müllheim, Germany). The concentration of the unlabeled GpA peptide was 5 μM. Data points were collected at a resolution of 1 nm, an integration time of 1 s, and a bandpass of 1 nm. Each spectrum shown resulted from at least three averaged scans from which buffer scans were subtracted. To measure how well the respective polymers dissolve the GpA TM helix, the peptides were mixed together with the polymers in organic solvent and dried in a gentle stream of nitrogen with subsequent desiccation overnight. The GpA TM domain was reconstituted in 10 mM phosphate buffer (pH 7.4). As a positive control, the peptides were reconstituted in a 5 mM DDM detergent solution, which is known to stabilize α-helical structures and to solubilize the GpA TM domain very well. Prior to CD measurements, samples were incubated for 2 h at 37 °C followed by sonication for 10 min and centrifugation at 16100*g* for 10 min. Secondary structure contents were predicted with DICHROWEB using both soluble and TM proteins as a reference data set (CDSSTR method, reference set 7).[29,30]

Fluorescence and Förster Resonance Energy Transfer Measurements. Steady-state fluorescence measurements were performed using the FL-labeled GpA TM helix (0.25 μM) mixed with the respective polymer (5−50 μM). For Förster resonance energy transfer (FRET) measurements, equal concentrations of FL- and TAMRA-labeled GpA TM domains were used. Concentrations of the peptide stock solutions were determined from absorbance measurements in a Perkin-Elmer Lambda 35 UV−vis spectrophotometer. In all experiments, we used a concentration of 0.25 μM for each of the two labeled GpA peptides. Peptides dissolved in TFE and the p(HPMA)-co-p(LMA) polymer dissolved in ethanol were mixed, and organic solvents were removed in a gentle stream of nitrogen gas. Residual solvent traces were removed by vacuum desiccation overnight. The dried peptide−polymer film was then hydrated in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. After incubation at 37 °C for at least 2 h, sonication for 10 min, and centrifugation at 16100*g* for 10 min, steady-state fluorescence measurements were performed at 25 °C on a Horiba Fluoromax 4 system with both excitation and emission slits set to 2 nm. The excitation wavelength was 439 nm, and emission spectra were recorded from 480 to 650 nm.

The sensitized donor emission was calculated using the FRET pair fluorescence intensities at 520 and 575 nm according to

$$E_{sens} = F_{575\,nm}/F_{520\,nm} \quad (1)$$

Increasing E_{sens} ratios indicate an increase in the extent of GpA dimerization. The FL spectrum yields an E_{sens} ratio of only ∼0.3, defining the lowest possible ratio at which no dimer is present.

Generalized Polarization. Large unilamellar vesicles (LUVs, 1 mM) of 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine (20:1 PC) containing the fluorescent lipid probe Laurdan (2 μM) were prepared by hydration of the dried lipid film in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, followed by eight freeze−thaw cycles. The polymers were prepared as described above, yielding a polymer concentration of 100 μM, and titrated into the LUV-containing solutions. Steady-state fluorescence measurements were performed after polymer addition, mixing, and incubation at 25 °C. The excitation wavelength was set to 350 nm, and Laurdan emission spectra were recorded from 400 to 550 nm at a slit width of 1 nm for both excitation and emission slits. GP values were calculated using the following equation:

$$GP = (F_{435\,nm} - F_{490\,nm})/(F_{435\,nm} + F_{490\,nm}) \quad (2)$$

Low GP values indicate a low level of membrane lipid order, whereas increasing GP values imply the membrane is becoming more rigid because of the attachment of copolymers.

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Liposome Content Release. DOPC and Liss Rhod PE (1:1000) were mixed in organic solvent, dried under a nitrogen stream, and desiccated overnight. The lipid film was then hydrated with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, the fluorescent dye ANTS (12.5 mM), and the quencher DPX (45 mM). Unilamellar vesicles, having a concentration of 0.5 mM, were prepared by performing eight freeze−thaw cycles. Nonencapsulated dye and quencher molecules were removed from the LUVs on a PD10 gel filtration column (filled with Sephadex G-25 M, GE Healthcare). Elution fractions were collected on 96-well plates, and ANTS and Liss Rhod fluorescence were detected in a Fluostar Omega microplate reader (BMG Labtech) to identify the fractions containing the loaded lipid vesicles. Immediately before the measurement, the vesicles loaded with ANTS and DPX were diluted with buffer [10 mM HEPES buffer (pH 7.4) and 150 mM NaCl] and mixed with a polymer solution (100 μM stock, prepared as described above) to a final lipid concentration of 0.1 mM and polymer concentrations of 5 and 20 μM. Because of the mixing of the compounds prior to the measurement, the dead time of the measurement was approximately 10 s, which is neglected in the final data analysis. Fluorescence measurements were performed at 25 °C on a Horiba Fluoromax 4 spectrofluorimeter. The excitation wavelength was set to 360 nm, and fluorescence emission was recorded at 530 nm, with both slits set to 5 nm. For each measurement, positive (addition of 1% Triton X-100) and negative (liposomes without polymer) controls were performed. Assuming that the amount of leakage L is 0 in negative control N and 1 (100%) in positive control P, the time-dependent leakage, induced by the addition of polymer, can be calculated using fluorescence intensity I at 530 nm of each sample S and the following eq 3 according to ref 31

$$L(t) = [I_S(t) - I_N(t)]/[I_P(t) - I_N(t)] \quad (3)$$

ANS Fluorescence Measurement. ANS is a widely used fluorescent dye, the fluorescence emission of which dramatically increases after binding to hydrophobic regions of molecules and assemblies. It is used as an extrinsic tool in studies regarding protein folding and formation of hydrophobic assemblies, such as micelles.[32−34] Changes in the spectral shape and fluorescence intensity of ANS can be attributed to the formation or collapse of hydrophobic regions in the analyzed molecules, which are the copolymer assemblies in this case. Polymers **C5**, **C15**, and **C25** were dissolved as described above in 10 mM HEPES (pH 7.4), 150 mM NaCl buffer, which additionally contained 5 μM ANS probe. During the measurements, the temperature was increased from 25 to 80 °C in 5 °C steps. For each temperature, ANS emission spectra were recorded from 450 to 600 nm after excitation at 374 nm (both slits set to 4 nm).

RESULTS AND DISCUSSION

Interaction of the p(HPMA)-co-p(LMA) Polymer with Model Membranes. Almost 20 years ago, amphiphilic polymers were introduced for the first time in membrane protein research, and the class of so-called Amphipols is currently the best studied and most widely used. However, as there still is a need to develop new detergents and/or surfactants for membrane protein research, new amphiphilic polymers might enter the field. Recently, it has been concluded that **C5** and **C10** p(HPMA)-co-p(LMA) polymers interact with membranes,[20] and moreover, **C10** interacts with blood serum components, such as VLDL particles.[19] However, a potential application of p(HPMA)-co-p(LMA) polymers in membrane biochemistry has never been elucidated. The three p(HPMA)-co-p(LMA) polymers used in this study differ in the contents of their hydrophobic LMA moieties, which affects the stability of the polymer aggregates in aqueous solution. While the polymer with 5% LMA appears to form aggregates, the amount of aggregates is very small and the aggregates are rather unstable,[20] whereas polymers containing >10% LMA form a stable hydrophobic core.[20] As described in Materials and Methods, by monitoring changes in pyrene fluorescence emission, we determined the critical aggregation concentrations of the **C15** and **C25** polymers to be 59 and 10 nM, respectively. These concentrations are far below the polymer concentrations used in our measurements, and hence, these polymers form stable aggregates under our experimental conditions. p(HPMA)-co-p(LMA) polymers with 10 and 15% LMA are believed to interact with membranes of living cells.[21] However, the exact mode of interaction remains elusive, and it is currently unclear whether the polymers interact directly with membrane lipids or whether membrane association and/or integration is mediated by membrane-associated proteins. To elucidate a direct interaction of the polymers with membrane lipids, we measured interactions of selected p(HPMA)-co-p(LMA) polymers with LUVs by monitoring the fluorescence emission of the dye Laurdan, a fluorescent membrane probe, which detects changes in lipid packing and membrane fluidity. As shown in Figure 2, upon addition of both polymers **C5** and **C15** to LUVs, the Laurdan GP value increased strongly, and thus, both polymers interact significantly with the membrane lipids. It is noteworthy that after addition of the polymers to the LUV-containing solution, the GP values changed immediately, and no additional changes were observed when the fluorescence was followed for

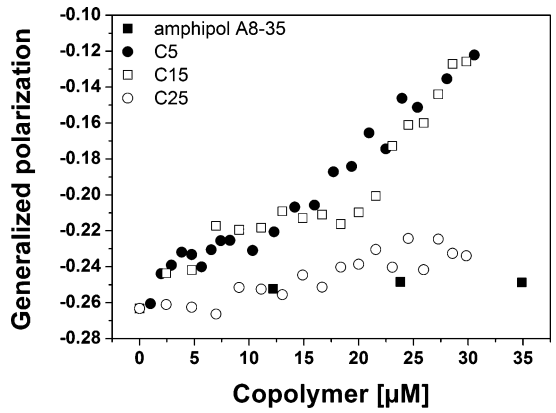

Figure 2. Interactions of the polymer with model membranes monitored by Laurdan generalized polarization. Generalized polarization (GP) was determined using the Laurdan fluorescent probe (2 μM) embedded in 1 mM 20:1 PC LUVs upon addition of the p(HPMA)-co-p(LMA) polymers at 25 °C. Polymers were titrated into the LUV solution, and the Laurdan probe was excited at 350 nm. Fluorescence emission was recorded from 400 to 550 nm. An increase in the GP value reflects a decrease in membrane fluidity and thus an increased level of bilayer lipid order. The large increase in GP in the case of polymers **C5** and **C15** indicates a significant interaction of the polymers with the membranes. For the sake of comparison, GP values monitored in the presence of Amphipol A8-35, which is known to stabilize membrane proteins very well and is able to form a stable hydrophilic core, are shown. Both, Amphipol A8-35 and the **C25** polymer hardly influence the GP value and thus only marginally influence the membrane structure.

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an additional 60 min (data not shown). In contrast to the **C5** and **C15** polymers, **C25** and Amphipol A8-35, which was used as a control, appear to hardly interact with the model membrane, as the determined GP values increased only slightly upon addition of increasing amounts of the **C25** polymer to the liposomes (Figure 2). It is notable that also in LUVs composed of a PC lipid with a shorter acyl chain length (DOPC), as well as LUVs composed of the negatively charged lipid DOPG, significant interaction of **C5** and **C15**, but not of **C25** or Amphipol A8-35, with the membrane was observed (Figure S1 of the Supporting Information), indicating that membrane interaction does not strictly depend on the lipid species. To elucidate whether membrane interaction of the polymers interferes with membrane stability, e.g., resulting in leakage of encapsulated vesicle content, we next monitored the release of soluble liposome content upon polymer addition (Figure 3).

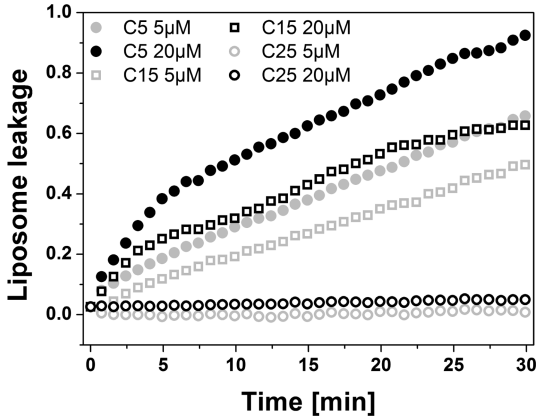

Figure 3. Release of soluble content encapsulated in LUVs after the addition of **C5**, **C15**, and **C25** copolymers. DOPC (0.1 mM lipid) liposomes loaded with the fluorescent dye ANTS (12.5 mM) and the quencher DPX (45 mM) were treated with 5 or 20 μM copolymers (**C5**, **C15**, or **C25**). The fluorescence of the ANTS dye increased upon leakage of the DOPC liposomes. As a positive control representing 100% leakage, 1% Triton X-100 was added to the liposomes. As a negative control, the fluorescence of the pure liposomes was monitored over the same period of time. Liposome leakage was calculated using the fluorescence intensities at 530 nm and eq 3. Polymer **C25** induces no leakage at either tested polymer concentration, whereas **C15** and even more significantly **C5** induce leakage in a concentration-dependent manner, indicating interactions of the **C5** and **C15** polymers with the membranes.

Addition of **C25** to LUVs loaded with a fluorophore and quencher did not show any increase in fluorescence emission, further supporting the idea that **C25** does not significantly interact with membranes and demonstrating that **C25** does not affect liposome stability. In contrast, when the **C5** polymer was added, a large increase in fluorescence emission was observed because of the destabilization of the LUV structure and the release of liposome content. Also, addition of **C15** resulted in the release of liposome content, and thus, this polymer also affects liposome stability and membrane integrity, although to a lesser extent than the **C5** polymer. We can therefore conclude that **C5** and **C15**, but not **C25**, interact with membranes and destabilize the vesicular membrane structure.

Potentially, because of the increased hydrophobicity of the **C25** polymer, this polymer forms aggregates with a more stable hydrophobic core. To test this assumption, we incorporated the hydrophobic fluorescent probe ANS into the polymer aggregates and measured ANS fluorescence emission at increasing temperatures. Melting of the hydrophobic aggregate core structure, into which ANS is incorporated, will result in a decrease in fluorescence emission, due to ANS release. As shown in Figure 4, increasing the temperature of a **C25** polymer solution resulted in a decreased ANS fluorescence. While melting of **C15** polymer aggregates was similar to that of **C25**, in the case of **C5** the ANS fluorescence essentially does not change. However, while the initial ANS fluorescence was high in the case of **C25**, indicating stable incorporation of the probe into a hydrophobic core structure, the ANS fluorescence was significantly lower in the case of the **C15** polymer, and no increased ANS fluorescence was observed in the **C5** polymer solution. Thus, this polymer does not form a hydrophobic core structure that is sufficiently stable to incorporate the hydrophobic ANS probe, which is in line with recent observations.[20]

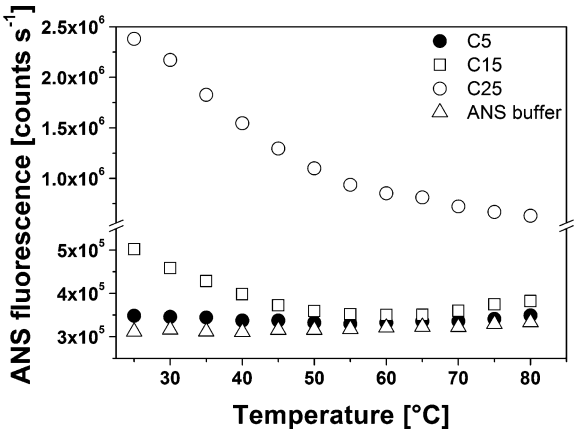

Figure 4. Temperature-dependent fluorescence changes of the hydrophobicity-sensitive dye ANS in copolymer solutions. Copolymers **C5**, **C15**, and **C25** (20 μM) were dissolved in 10 mM HEPES (pH 7.4), 150 mM NaCl buffer containing 5 μM ANS. The fluorescence emission of the ANS dye is sensitive to changes in the hydrophobicity of its environment. The stability of the polymers' hydrophobic core was tested by thermally unfolding the polymer assemblies. ANS was excited at 374 nm, and the fluorescence intensities measured at 490 nm are plotted vs temperature. Polymer **C25** shows the highest fluorescence at room temperature, indicating the formation of a hydrophobic core, which is more stable than in the case of the **C15** and **C5** polymer solutions, where only little fluorescence emission was observed. Higher temperatures lead to a decreased fluorescence emission due to destabilization of the hydrophobic polymer assembly cores. **C25** preserves a hydrophobic environment much better than the other polymers, even at 80 °C.

Together, these data show that the **C25** polymer forms the most stable hydrophobic core structure, which is significantly more stable than that observed in the case of the two remaining polymers. While the **C15** polymer also appears to form a (marginally stable) hydrophobic core, in the case of the **C5** polymer, the results indicate that the polymer aggregates are not stable. The **C25** polymer aggregates do not expose hydrophobic structures and remain stable even in the presence of lipids. Consequently, while these data indicate that **C25** cannot be used to efficiently extract lipids or proteins from biological membranes, the stability of the **C25** hydrophobic core might be beneficial for incorporating and stabilizing membrane proteins.

Solubilization of the GpA TM Peptide and Secondary Structure Formation. As at least the **C15** and **C25** polymers

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appear to form hydrophobic cores, we next studied how the individual polymers solubilize a TM protein. Therefore, we first monitored solubilization of the 33-amino acid hydrophobic human erythrocyte GpA TM peptide by following the fluorescence signal of a fluorescein (FL)-labeled GpA TM helix in solution after addition of increasing polymer concentrations. The labeled peptide (0.25 μM) was dissolved in different concentrations of **C5**, **C15**, and **C25** p(HPMA)-co-p(LMA), reaching a final polymer concentration of 50 μM. In all three polymer solutions, the intensity of the fluorescence signal increased nonlinearly with an increasing polymer concentration, and a maximal fluorescence yield was reached at polymer concentrations exceeding 20−25 μM (Figure 5).

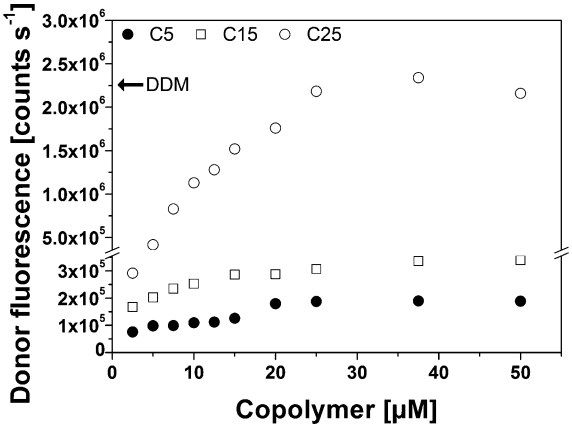

Figure 5. Fluorescence emission of the donor-labeled GpA TM peptide solubilized in a **C5**, **C15**, or **C25** polymer solution. Fluorescein-labeled GpA peptides (0.25 μM) were solubilized in the **C5**, **C15**, or **C25** p(HPMA)-co-p(LMA) polymer, as described in Materials and Methods. The peptide fluorescence intensities at 520 nm increase with increasing polymer concentrations to a value monitored after dissolving the fluorescein-labeled GpA peptides in 5 mM DDM (marked by the arrow). The maximal fluorescence emission is ~7-fold higher in the **C25** polymer solution than in the **C5** and **C15** solutions, indicating that the **C5** and **C15** polymers solubilize the GpA TM peptide less well than **C25**.

While the intensity of the fluorescence signal measured in the **C15** solution was ~2 times higher than determined in **C5**, the measured fluorescence intensities of the FL-GpA peptide in the presence of both **C5** and **C15** were at least 7-fold lower than for the **C25** solution. Here, at a **C25** polymer concentration of ~25 μM, the signal intensity reached a value comparable to the value obtained in DDM solutions. This analysis clearly indicates that the **C25** polymer is most efficient in solubilizing a simple TM protein. However, the results do not allow us to draw any conclusions about the structure and stability of TM proteins solubilized in the individual polymers. Thus, the question of how stable a given TM protein structure will be in the individual polymer solutions arose. Therefore, we next monitored by CD spectroscopy whether the p(HPMA)-co-p(LMA) polymers promote formation of the GpA TM helix secondary structure, compared to the mild and widely used detergent DDM. As shown in Figure 6A, in all three tested p(HPMA)-co-p(LMA) polymers, 5 μM GpA TM domain might be solubilized and the expected α-helical structure forms in all tested polymer solutions. The α-helix contents, calculated from the CD spectra, were comparable to the value obtained after solubilizing the GpA TM helix in 5 mM DDM. However, different polymer concentrations were needed to achieve

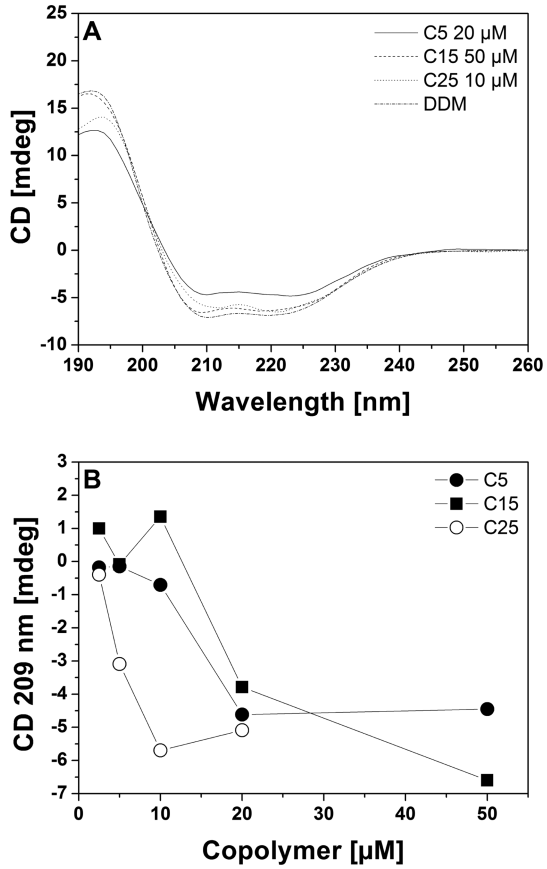

Figure 6. Far-UV CD spectra of the GpA TM domain solubilized in **C5**, **C15**, and **C25** p(HPMA)-co-p(LMA) polymer solutions. (A) The GpA TM domain (5 μM) was solubilized in **C5**, **C15**, and **C25** p(HPMA)-co-p(LMA) polymers at different concentrations. Spectra were recorded at 25 °C in phosphate buffer (pH 7.4). As a positive control, the GpA TM domain has been solubilized in 5 mM DDM detergent, which is known to support the formation of α-helices. (B) CD signal intensity at 209 nm, a characteristic minimum for α-helical protein structures, plotted vs polymer concentration. The corresponding value of the GpA TM helix solubilized in DDM is ~7 mdeg. The CD spectrum of GpA in 50 μM **C25** could not be measured, because of the high background absorption at low UV wavelengths. The CD spectra demonstrate the good solubility of the GpA TM helix in **C25**, even at low polymer concentrations. In the case of **C5** and **C15**, higher concentrations are needed to solubilize the GpA TM domain at the same level as the DDM detergent. The helical content of the GpA TM domain (5 μM) was 87% in 20 μM **C5** and 50 μM **C15** and 92% in 10 μM **C25** and 5 mM DDM, as determined using the DICHROWEB algorithm (CDSSTR).

proper CD spectra and thus to properly stabilize the α-helical structure of the peptides. In polymer **C5**, the best CD signal has been monitored at a polymer concentration of 20 μM, whereupon the CD signal did not improve further. However, at no concentration did the signal intensity at 209 nm reach the value obtained after solubilization of the GpA TM peptides in DDM. In the case of 50 μM **C15**, the CD spectrum was comparable to that monitored in a DDM solution or in 10 μM **C25**, indicating the proper formation of the peptides' secondary structure. However, at **C5** and **C15** polymer concentrations of <20 μM, essentially no CD signal was detected (Figure 6B), once again demonstrating the inability of these polymers to dissolve the peptides at such low concentrations (compare above). In contrast, the **C25** polymer efficiently solubilized the

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GpA TM domain at a concentration as low as ~10 μM, and the GpA TM domain was fully solubilized and correctly folded, as indicated by the predicted α-helix content of 92%. Higher concentrations of the **C25** polymer did not improve the CD signal further.

Together, these results indicate that the **C25** polymer aggregates have a stable hydrophobic core, which allows solubilization of TM proteins already at low polymer concentrations, whereby the protein's secondary structure remains fully preserved.

Stability of the GpA Quaternary Structure in Polymer Solutions. These results allow the classification of the propensity of the three analyzed p(HPMA)-co-p(LMA) polymers to solubilize a TM protein, as well as their abilities to support and/or preserve the secondary structure of TM helices. However, defined interactions of various individual TM helices are typically involved in the formation of a final TM protein structure. As formation of larger TM helix bundles is mostly mediated by defined TM helix–helix interactions, the GpA TM helix dimer can serve as an excellent probe for studying the impact of a lipid, detergent, or polymer environment on the formation of sequence-specific TM helix oligomers. To study GpA TM helix dimerization, the donor-labeled FL-GpA and the acceptor-labeled TAMRA-GpA were used in FRET measurements. The distance of the N-termini in the GpA TM helix dimer is approximately 10 Å, which is far below the Förster radius of the FRET pair dyes (49–54 Å).[35] Therefore, upon formation of the GpA TM helix dimer, the acceptor fluorescence emission at 575 nm is sensitized because of the resonance energy transfer from the donor to the acceptor. This FRET-based assay has already been used in several detergents and artificial membranes to study the impact of different environmental factors on the stability of an oligomeric TM protein.[22,23,36−40] In Figure 7, we compare the energy transfer (sensitized emission) at increasing polymer concentrations, which reflects the dimerization propensity of the GpA TM helix in the different polymer environments. The ratio of the FRET pair fluorescence intensities at 575 and 520 nm has a theoretical minimum, which is defined by the ratio of these wavelengths in the pure FL-GpA spectrum, because the FL fluorescence emission at 575 nm is not zero in absence of the acceptor dye. This minimal ratio is on the order of ~0.3, whereas higher ratios indicate formation of GpA dimers or higher-order oligomers. Figure 7A shows normalized FRET pair emission spectra in the three polymers, at polymer concentrations of 2.5–50 μM. With increasing polymer concentrations, the sensitized acceptor emission increases in the **C15** and **C25** polymers to a particular concentration (~15 μM **C15** and ~10 μM **C25**). A further increase of the polymer concentration did not further change the sensitized acceptor emission significantly. In the **C5** polymer, formation of GpA oligomers was essentially not observed, and with increasing polymer concentrations, the FRET pair ratio increased only modestly (from 0.3 to 0.45). In the **C15** polymer, oligomerization of the GpA TM domain was slightly enhanced compared to that with the **C5** polymer, resulting in an ultimately reached FRET pair ratio of 0.5 (Figure 7B). However, in both **C5** and **C15** polymer solutions, the maximal FRET pair ratios were rather low, compared to values obtained in DDM micelles (~1.3) or in **C25** polymer solutions. Thus, the GpA TM helix dimer is dramatically more stable in **C25** than in the polymers with lower LMA contents. Up to a **C25** polymer concentration of ~10 μM, the FRET pair ratio

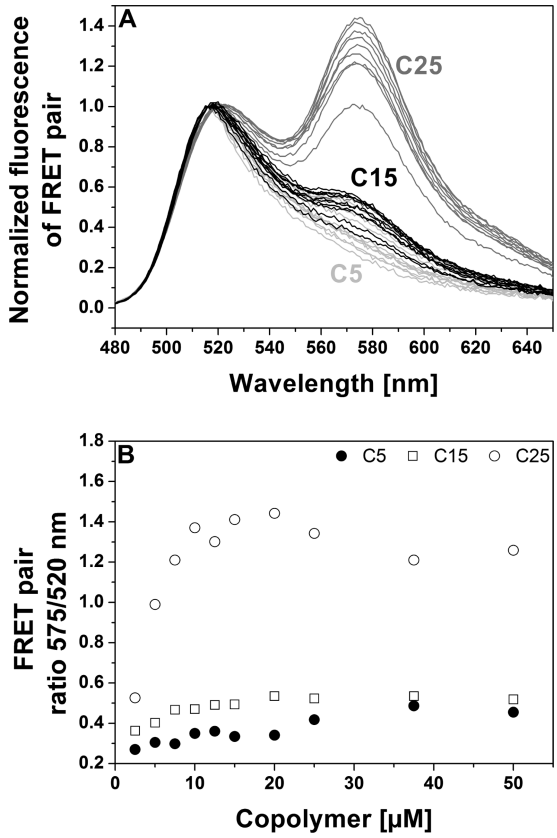

Figure 7. Dimerization of the GpA TM helix in **C5**, **C15**, and **C25** p(HPMA)-co-p(LMA) polymer solutions. (A) Donor- and acceptor-labeled peptides (0.25 μM each) were mixed at a 1:1 ratio and solubilized in the **C5**, **C15**, and **C25** polymers. At increasing polymer concentrations (2.5–50 μM), FRET pair emission spectra were recorded at 25 °C after excitation at 439 nm, and spectra were normalized at 520 nm. The increasing acceptor emission at 575 nm indicates an increased level of dimerization of the GpA TM domain. (B) Ratio of the fluorescence emission measured at 575 and 520 nm as a function of polymer concentration. While the **C5** and **C15** polymers essentially do not promote dimerization, the **C25** polymer facilitates dimerization well, even at very low concentrations. The dimerization propensity of the GpA TM helix in polymer **C25** is equal to the dimerization propensity in 5 mM DDM.

increased significantly from 0.5 to 1.4 and did not increase further afterward. However, at **C25** polymer concentrations exceeding 25 μM, the FRET pair ratio marginally decreased, which might indicate destabilization of the GpA TM helix dimer at higher polymer concentrations. This has been observed before at increasing concentrations of various detergents.[22,23,37,40] To exclude the possibility that the energy transfer arose because of unspecific aggregation of the labeled peptides, we measured energy transfer in polymer aggregates at changing acceptor mole ratios. As shown in Figure S2 of the Supporting Information, the FRET pair ratio, i.e., the energy transfer efficiency, linearly depends on the acceptor mole fraction, and this is only the case when the formed oligomer is a dimer, as derived in detail in, e.g., refs. 37 and 41.

Consequently, the **C25** polymer with a hydrophobic LMA content of 25% appears to be well suited to solubilize the GpA TM helix as well as to stabilize tertiary and quaternary contacts of TM proteins.

p(HPMA)-co-p(LMA) C25 Keeps TM Proteins in Solution and Preserves Quaternary Contacts. The **C5**

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polymer has the smallest amount of hydrophobic LMA side chains, and the hydrophobic nature of the three analyzed compounds increases with an increasing LMA content. As determined in this study, only the **C5** and **C15** polymers interact with lipid bilayers and disturb the membrane structure. In contrast, addition of the **C25** polymer to model membranes did not alter the spectroscopic properties of the membrane probe or disrupt the membrane, and thus, the polymer does not appear to interact with the membranes. Most likely, the **C5** and **C15** polymers interact with the bilayer via their hydrophobic moieties, i.e., via their LMA side chains. The stronger the hydrophobic core of the polymer, and thus the more the hydrophobic side chains are shielded from the surrounding medium, the less they interact with the model membranes. As seen in the ANS measurements, the **C5** and **C15** polymer aggregates do not form highly stable hydrophobic core structures, which shield the hydrophobic portions of the molecules from the surrounding medium, but certain hydrophobic regions appear to be free to interact with membrane lipids. In contrast, the hydrophobic moieties of the **C25** polymer do not interact with the membrane, as the increased LMA content results in the formation of a rather stable hydrophobic core, which shields the hydrophobic structures and disables hydrophobic membrane contacts. However, the propensity of the individual polymers to interact with membranes is inversely proportional to their propensity to solubilize and stabilize TM proteins. Polymer **C5** solubilizes the GpA membrane protein less effectively than the remaining polymers, and the **C15** polymer was only slightly effective. Only the **C25** polymer rendered the GpA TM helix soluble and preserved the structure of the TM helix dimer. It is noteworthy that the **C25** polymer was approximately as efficient as Amphipol A8-35, which is known to solubilize and stabilize membrane protein oligomers well.[2,4,5,8−11,42−47]

Together, these results indicate that **C25** forms a stable hydrophobic core, and formation of such stable core structures appears to be a fundamental prerequisite for successfully solubilizing and stabilizing membrane proteins in aqueous solutions.

CONCLUSION AND PERSPECTIVE

In this analysis, we have tested three amphiphilic copolymers, having increasing hydrophobicities. These results indicate that only at a LMA concentration of 25% is the p(HPMA) copolymer able to form a stable hydrophobic core, into which hydrophobic TM proteins might be incorporated. Thus, there appears to be a threshold in the molecular hydrophobicity of polymers for their application in membrane protein research. At lower hydrophobicities, the polymers were not able to properly solubilize the TM protein and the structure of the TM proteins was not well preserved. Thus, an LMA content of >15% is needed to properly incorporate a TM protein. However, while these polymers allow the analysis of TM proteins in solution, such polymers do not significantly interact with membrane lipids (compare Figures 2 and 3) and thus do not alter the structure of biological membranes or extract lipids and proteins from membranes. Thus, using **C25** polymers together with living cells eventually will allow researchers to monitor interactions of soluble domains of TM proteins (solubilized in **C25**) with proteins at a eukaryotic plasma membrane, without interacting with and influencing the structure or stability of the cell membrane. When a TM protein is solubilized in conventional detergents, (i) the detergents will be diluted below their CMC and thus the micellar structures will collapse and/or (ii) the detergent molecules will affect the molecular structure of the biological membrane system. As the amphiphilic **C25** polymer prevents such devastating effects, this detergent substitute might allow future *in vitro* studies with membrane proteins, which were not feasible before.

ASSOCIATED CONTENT

Supporting Information

Interaction of polymers with DOPC and DOPG membranes monitored by Laurdan generalized polarization (Figure S1) and stoichiometry of GpA association (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.S., D.S., and R.Z. designed the research. M.H. and M.A. synthesized polymers and determined CMC and CAG values. M.S. performed all other described experiments. M.S. and D.S. analyzed data. M.S., D.S., and R.Z. wrote the manuscript.

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Notes

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ABBREVATIONS

CAG, critical aggregation concentration; DOPG, 1,2-Dioleoyl-sn-glycero-3-phosphtidylglycerol; p(HPMA), poly[*N*-(2-hydroxypropyl)-methacrylamid]; LMA, lauryl methacrylate; CMC, critical micelle concentration; PC, phosphatidylcholine; **C5**, p(HPMA)-co-p(LMA) copolymer with 5% LMA; **C10**, p(HPMA)-co-p(LMA) copolymer with 10% LMA; **C15**, p(HPMA)-co-p(LMA) copolymer with 15% LMA; **C25**, p(HPMA)-co-p(LMA) copolymer with 25% LMA; TM, transmembrane; GpA, human glycophorin A; FL, fluorescein; TAMRA, 5-6-carboxyrhodamine; TFE, 2,2,2-trifluoroethanol; Laurdan, 6-dodecanoyl-*N*,*N*-dimethyl-2-naphthylamine; 20:1 PC, 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Liss Rhod PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt); ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; DPX, *p*-xylene-bis-pyridinium bromide; DDM, *n*-dodecyl β-D-maltopyranoside; CTA, chain transfer agent; PFPMA, pentafluorophenyl methacrylate; CTP, chain transfer polymer; AIBN, 2,2′-azobis(2-methylpropionitrile); DMSO, dimethyl sulfoxide; PDI, polydispersity index; CD, circular dichroism; FRET, Förster resonance energy transfer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GP, generalized polarization; LUV, large unilamellar vesicle; ANS, 8-anilino-1-naphthalenesulfonic acid ammonium salt; NMR, nuclear magnetic resonance.

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