Synthesis and Purification of Homogeneous Lipid-Based Peptide Nanocarriers by Overcoming Phospholipid Ester Hydrolysis

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ABSTRACT: Despite the therapeutic promise of phospholipid-based nanocarriers, a major obstacle to their widespread clinical translation is a susceptibility to fatty acid ester hydrolysis, leading to lack of quality control and inconsistencies in self-assembly formulations. Using electrospray ionization mass spectrometry fragmentation in combination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, we have demonstrated a method to detect hydrolysis of one or both of the fatty acid esters in a PEGylated phospholipid, DSPE-PEG, in conditions commonly applied during nanocarrier production. Because such carriers are increasingly being used to deliver peptide-based therapeutics, we further investigated the hydrolysis of phospholipid esters in conditions used for solid-phase peptide synthesis and high-performance liquid chromatography of peptides. We ultimately detail a synthetic strategy to reliably produce pure phospholipid–peptide bioconjugates (peptide amphiphiles), while avoiding unintended or unnoticed hydrolyzed byproducts that could lead to polymorphic nanotherapeutics with dampened therapeutic efficacy. We believe that such an approach could help standardize phospholipid–peptide-based therapeutic development, testing, and clinical translation.

1. INTRODUCTION

Peptides are increasingly being used to fill a therapeutic gap between small molecules and biologics, particularly for targeting intracellular protein–protein interactions (PPIs).1 While only one small molecule drug and zero biologics have been approved by the U.S. Food and Drug Administration (FDA) for targeting intracellular PPIs,2 peptides are entering clinical trials in increasing numbers every year, with more than 100 currently under study.1,3 Peptides can harness natural PPI specificity through mimicking a protein’s amino acid sequence and secondary structure. Additionally, peptides can possess the biodegradability and low toxicity associated with biologics, while also having the potency and synthetic accessibility typically associated with small molecules.4 Despite their promise, peptides face a number of obstacles to clinical translation, including rapid clearance, low oral bioavailability, cellular impermeability, and metabolic instability.3,4 Phospholipid-based nanocarriers are one approach being used to overcome these obstacles.5,6

Phospholipid–peptide conjugation to form peptide amphiphiles (PAs) can improve the pharmacologic potential of peptide drugs that would otherwise be clinically unsuccessful, as phospholipids are biocompatible, drive nanoparticle self-assembly, can be modified with functional elements (i.e., therapeutic, diagnostic, or targeting), promote cellular internalization, and extend the circulation half-lives of drugs.5,6–14 Chemical functionalization and purification of phospholipids with peptides, however, exposes them to conditions that can lead to lipid hydrolysis. Hydrolysis byproducts can significantly affect the structure and properties of these nanostructures.1,3,10–12,14–17 Consequently, one of the main obstacles to clinical translation of lipid-based nanocarriers, as recently highlighted by the U.S. FDA, is quality assurance and consistency in self-assembly formulations, particularly as it relates to hydrolysis.18–24

Despite the risks for hydrolysis, there are many examples of lipopeptide synthesis using potentially hydrolytic conditions without documentation of a lack of ester hydrolysis byproducts at the end of synthesis and purification. This can be particularly problematic when a polyethylene glycol (PEG) domain is included in a peptide–lipid conjugate. Here, the molecular weight (MW) becomes polydisperse, and side reactions, including hydrolysis, are more difficult to detect with standard peptide validation techniques such as liquid-chromatography mass spectrometry (LC–MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF–MS), and amino acid analysis (AAA), thereby ultimately limiting quality control.

During the synthesis and purification of a PA nanoparticle containing a p53-reactivating therapeutic peptide conjugated...
to a PEGylated phospholipid, DSPE-PEG, we noticed an uncharacteristic MW signature and investigated its cause and detection. In doing so, we (1) uncovered partial hydrolysis of one or both of DSPE-PEG’s fatty acid esters in conditions commonly applied to DSPE-PEG in the literature, (2) validated a detection method using electrospray ionization mass spectrometry (ESI–MS) fragmentation, and (3) demonstrated a synthetic route to reliably produce pure phospholipid–peptide bioconjugates (PAs) without unintended or unnoticed hydrolysis byproducts that can possibly lead to polymorphic nanotherapeutics with dampened efficacy.

2. RESULTS AND DISCUSSION

We attempted to conjugate a therapeutic peptide (p53<sub>14-29</sub>) to DSPE-PEG in an effort to form p53<sub>14-29</sub> PA nanoparticles. The p53<sub>14-29</sub> peptide, in its hydrocarbon stapled form, is known to penetrate cells and reactivate cell death through disruption of the interaction between WT p53 and its endogenous inhibitors, MDM2 and MDM4. A PA consisting of a peptide conjugated to polydisperse DSPE-PEG (~3000 Da) should have MWs spanning approximately 4700–5700 Da with an average of ~5200 Da. MALDI-TOF–MS provided little sensitivity to detect small changes in MW because of side reactions for such polydisperse samples. However, during ESI–MS, the dialkylglycerol portion of DSPE-PEG was artifically cleaved from the rest of the molecule to produce a prominent, monodisperse MW signature at 607 Da (Figure 1). When using MALDI-TOF–MS and ESI–MS at relevant time points. To avoid misleading artifactual fragmentation, MS techniques were performed in parallel with different ionization modes (Figure 1). Our first goal in standardizing the detection of hydrolyzed phospholipids was to establish an inert solvent that could be used to prepare samples for both ESI–MS and MALDI-TOF–MS without affecting DSPE-PEG. Methanol, a polar, protic solvent but poor nucleophile, should not harm DSPE-PEG. To test this, DSPE-PEG was dissolved in methanol and incubated at room temperature (RT) for up to 72 h. MALDI-TOF–MS showed no observable effect of methanol on DSPE-PEG at RT for at least 72 h, and the polydisperse MW distribution remained centered around the expected average MW, 2867 Da (Figure 2a). ESI–MS of these samples measured an m/z pattern reflective of intact DSPE-PEG, with polydisperse MW distributions centered around m/3, m/4, and m/5, and a strong ionization artifact peak at 607 Da corresponding to the existence of double-tailed DSPE-PEG (Figure 2b). Therefore, methanol had no effect on DSPE-PEG and was used during MS characterization of all subsequent samples.

Because peptides are commonly conjugated to lipids in an increasing variety of biotherapeutic applications, we next tested the compatibility of DSPE-PEG with solid-phase peptide synthesis (SPPS) conditions. Following synthesis, peptides on resin are subjected to a trifluoroacetic acid (TFA) cleavage cocktail to remove them from solid-phase support and deprotect their amino acid side chains. The removed side-chain protecting groups generate highly reactive carbocations, necessitating the presence of nucleophilic scavengers [e.g., water and trisopropylsilane (TIS)]. Because this strong aqueous acid solution could theoretically hydrolyze fatty acid esters within DSPE-PEG, we monitored DSPE-PEG stability in a TFA cleavage cocktail for up to 2 h, the minimum length of time generally used to remove peptides from resin at preparative scales. MALDI-TOF spectra detected a MW distribution shifted to the left by approximately 266 Da after 1 h, corresponding to the hydrolysis of one stearic acid from DSPE-PEG (Figure 2c). The MW distribution continued to shift leftward after 2 h of incubation, centering around an average MW corresponding to the loss of two stearic acid molecules from DSPE-PEG. ESI–MS confirmed fatty acid ester hydrolysis with a new peak appearing at 341 Da, corresponding to the hydrolyzed ionization fragment (Figure 2d). These results demonstrate that DSPE-PEG is incompatible with solid phase conjugation to peptides using commonly available acid-labile resins and side-chain protecting groups. Fatty acid esters would also not be assumed to be compatible with SPPS conditions.

One strategy to avoid this obstacle to lipid-based peptide nanocarrier synthesis is to exclude water and other nucleophiles from the TFA cleavage cocktail. However, such a strategy would generate a new risk of undesired side reactions between the amino acid side chains and highly reactive carbocations generated during amino acid deprotection. Another potential strategy is to identify nucleophilic scavengers that react readily with carbocations but not esters. However, this brings an increased risk of side reactions that would be buried within the polydispersity of a PEGylated lipid’s MW during standard LC–MS, MALDI-TOF–MS, and AAA validation.

Because water is a critical solvent commonly used for phospholipid self-assembly, conjugation, and purification, we next tested the stability of DSPE-PEG in de-ionized water and

![Figure 1. Acid-catalyzed ester hydrolysis of DSPE-PEG generates shifts in MW signatures. Hydrolysis of one or both esters in DSPE-PEG generates shifts in average MW, observable by MALDI-TOF–MS, and in the MW of an alkylglycerol ionization fragment, observable by ESI–MS. Thereby, MALDI-TOF–MS was used to qualitatively measure shifts in the average MW of polydisperse MW distributions, while ESI–MS was used to detect the presence of the hydrolyzed alkylglycerol portion of the molecule at 341 Da.](image-url)
common buffers used for peptide purification. The rate of phospholipid ester hydrolysis is minimized at pH 6.5 and greatly accelerated at higher or lower pH.\(^{15,28,29}\) MALDI-TOF analysis of DSPE-PEG dissolved in unbuffered, ultrapure Milli-Q water revealed a distribution of MWs 532 Da smaller than DSPE-PEG after 72 h at RT, corresponding to hydrolysis of

Figure 2. MALDI-TOF and ESI–MS reveal phospholipid ester hydrolysis when DSPE-PEG is exposed to a TFA cleavage cocktail used to remove peptides from solid-phase support. (a,b) Esters of DSPE-PEG are stable in methanol at RT. (a) MALDI-TOF shows a time-independent polymeric MW distribution centered at the average MW of 2867 Da, corresponding to an intact dialkylglycerol portion of DSPE-PEG and polymeric distributions centered around the expected values of \(m/3, m/4,\) and \(m/5\). (c,d) After incubation with a TFA cocktail commonly used to remove peptides from solid-phase support, MALDI-TOF and ESI–MS both demonstrate leftward shifts in the MW of DSPE-PEG, corresponding to ester hydrolysis. (c) MALDI-TOF shows a leftward shift in the MW distribution of 266 Da per hydrolyzed ester, as indicated by each red arrow. (d) In ESI–MS, the signature ionization fragment at 607 Da shifts to 341 Da, corresponding to phosphodiester fragmentation during ionization of the alkyl-glycerol portion of DSPE-PEG with only one fatty acid tail. In agreement with the MALDI-TOF data, this hydrolyzed fragment appeared as early as 30 min of treatment, with the signal increasing over 2 h.

Figure 3. Phospholipid esters are hydrolyzed by unbuffered and acidic water, and hydrolysis is accelerated by heat. (a–c) Esters of DSPE-PEG are hydrolyzed in unbuffered water, and hydrolysis is accelerated by heat. (a) DSPE-PEG dissolved in ultrapure Milli-Q water for 72 h at RT results in a leftward shift in MW on MALDI-TOF corresponding to the loss of two stearic acid molecules (−266 Da per hydrolyzed ester, as indicated by each red arrow). (b) Heating to 60 °C accelerates this loss. (c) Analysis of the 2 h, 60 °C sample using ESI–MS confirms hydrolysis with a shift of the signature ionization fragment from 607 to 341 Da. (d–f) Acidic HPLC buffer and heat each increase the rate of hydrolysis of the esters of DSPE-PEG. (d) After DSPE-PEG was incubated in acidic HPLC buffer, MALDI-TOF shows a leftward shift in MW corresponding to the loss of two stearic acid molecules (−266 Da per hydrolyzed ester, as indicated by each red arrow). (e) Identical sample was heated to 60 °C, and hydrolysis was detectable as early as 30 min. (f) As confirmed with ESI–MS, the signature ionization fragment of 607 Da shifts to 341 Da.
both esters (Figure 3a). This was accelerated when samples were heated to 60 °C with a leftward shift appearing in the MALDI-TOF spectra after only 2 h (Figure 3b). ESI–MS confirmed the presence of hydrolysis byproducts after 2 h with a peak appearing at 341 Da (Figure 3c). Unbuffered water, therefore, is not sufficient for preventing hydrolysis of phospholipid esters such as in DSPE-PEG.

Peptides carrying positive charges are most often purified via HPLC using acidic pH 2–3 buffer, imposing another obstacle for purification of PA conjugates, as this acidic pH should accelerate the rate of ester hydrolysis. To test this, we dissolved DSPE-PEG in a commonly used HPLC buffer (water + 0.1% formic acid, pH 2.7) at both RT and 60 °C. There was no detectable MW shift in MALDI-TOF MS at RT for 2 h, but after 72 h the MW shifted to the left, corresponding to hydrolysis of both fatty acid esters (Figures 1 and 3d). When DSPE-PEG was heated to 60 °C in acidic HPLC buffer, MALDI-TOF revealed a leftward shift starting as early as 30 min (Figure 3e). ESI–MS again confirmed the formation of hydrolysis byproducts with the presence of an ionization fragment at 341 Da (Figure 3f). In contrast, when DSPE-PEG was dissolved in pH 7.4 phosphate-buffered saline (PBS) buffer, hydrolysis was absent at any time point or temperature, as measured by MALDI-TOF and ESI–MS (Figure 4a–c). Therefore, while water and heat are both risks for phospholipid ester hydrolysis, this possibility can be mitigated during HPLC purification by using a neutral pH buffer, lower temperatures, and/or shorter exposure times.

By avoiding the hydrolysis-inducing conditions described above, we generated a pure DSPE-PEG PA nanoparticle with the pS34–29 peptide conjugated to DSPE-PEG maleimide via an N-terminal thiol linker. In summary, we cleaved the peptide from the resin before conjugating the phospholipid to avoid exposing the esters to TFA. We then conjugated DSPE-PEG to the peptide in neutral buffered aqueous solution. Lastly, we avoided hydrolysis during HPLC purification by (1) avoiding high temperatures, (2) buffering the fractions to neutral pH immediately upon elution, and (3) rapidly removing the solvent by rotary evaporation and lyophilization.

Following purification, LC–MS showed only one peak with UV absorbance at 280 nm, indicating a solitary pure product. Here, the two intact lipid tails, rather than the polydisperse PEG, was the predominant driver of the hydrophobic interaction between the DSPE-PEG moieties and the LC column, as reflected in the elution of single peak at ~94% methanol. The corresponding ESI–MS signal had a peak at 607 Da, indicating an intact DSPE-PEG tail with no detectable hydrolysis fragments (Figure 5a). MALDI-TOF revealed a polydisperse MW distribution centered around 5205 Da, the expected average MW of the PA (Figure 5b). There was also a second distribution of MWs that was smaller by 607 Da, corresponding to the same artificial fragmentation of DSPE-PEG’s phosphodiester bond. Lastly, a closer inspection of the exact MWs within the polydisperse distribution revealed an exact match to the calculated MWs of this PA with 44, 45, or 46 PEG units, with the expected spacing of 44 Da (Figure 5c). These PAs formed round, homogenous nanoparticle micelles that were strikingly monodisperse, properties ideal for preclinical testing and clinical translation (Figure 5d,e).

3. CONCLUSIONS

This study highlights the pH- and temperature-dependence of phospholipid ester hydrolysis, raising unique concerns for newly developed phospholipid–peptide conjugates. Unlike SPPS for peptides, there are currently no universal protocols for conjugating, purifying, or otherwise handling phospholipid-based drug delivery systems, and many reports subject phospholipid esters to conditions known to promote their hydrolysis without explicit documentation of final product purity. This is the first report to our knowledge showing the significant limitations that exist when employing widely used peptide synthesis and purification workflows for phospholipids. We believe that techniques such as those presented here should be adapted, especially when a polydisperse polymer, such as PEG, is included in a bioconjugate in advance of preclinical testing.

4. METHODS

4.1. Materials. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[azido(polyethylene glycol)-2000] (DSPE-PEG(2000)-azide) was purchased from Avanti Polar Lipids, Inc. Methanol, TFA, and PBS were purchased from Fisher Scientific. Milli-Q water was filtered through a 0.2 μm filter. All standard amino acids, N-methylpyrrolidone (NMP), and dichloromethane (DCM) were purchased from Gyros Protein Technologies with standard TFA-labile protecting groups. Fmoc-beta-alanine-OH was purchased from Novabiochem. Formic acid, piperidine, N,N-diisopropylethylamine (DIPEA), acetic anhydride (Ac2O), ethanedithiol (EDT), TIS, and tris(2-carboxyethyl)phosphine (TCEP) were purchased from...
their corresponding Na\textsuperscript{+} adducts are also visible at +23 Da. (d) PEG units. These peaks have the expected PEG spacing of 44 Da, and reveals peaks matching the expected MWs of PAs with 44, 45, or 46 phosphodiester bond. (c) Zooming-in on the MALDI-TOF spectrum the PA. A secondary distribution is also visible, approximately 607 Da centered at approximately 5205 Da, the expected average MW of ESI shows one peak with 280 nm absorbance, and the corresponding acid (Mpa(Trt)-OH) was purchased from Bachem. 3-Tritylsulfanyl-propionic acid (Mpa(Trt)-OH) was purchased from EMD Millipore. 3-Tritylsulfanyl-propionic acid was synthesized with an N-terminal thiol linker using SPPS conjugated using neutral Bu.NF.

Figure 5. Buffered synthesis and purification conditions generate pure DSPE-PEG-PAs with no detectable hydrolysis byproducts. Peptide p53\textsubscript{14-29} was synthesized with an N-terminal thiol linker using SPPS followed by RP-HPLC purification. DSPE-PEG-maleimide was then conjugated using neutral buffer, and the resulting PA was purified using mild RP-HPLC conditions. (a) LCMS of the pure PA fractions shows one peak with 280 nm absorbance, and the corresponding ESI–MS signal at 607 Da confirms that it has an intact DSPE-PEG tail. (b) MALDI-TOF shows a polydisperse MW distribution, centered at approximately 5205 Da, the expected average MW of the PA. A secondary distribution is also visible, approximately 607 Da smaller, corresponding to artifactual fragmentation of the PA’s phosphodiester bond. (c) Zooming-in on the MALDI-TOF spectrum reveals peaks matching the expected MWs of PAs with 44, 45, or 46 PEG units. These peaks have the expected PEG spacing of 44 Da, and their corresponding Na\textsuperscript{+} adducts are also visible at +23 Da. (d) Transmission electron microscopy (TEM) imaging reveals PA self-assembly into spherical micelles (115 000 × magnification). (e) Histogram of hydrodynamic radius from DLS measurements shows a monodisperse size distribution with D\textsubscript{h} = 12.94 nm and PDI = 0.19.

Sigma-Aldrich. (7-Azabenzotriazol-1-yl oxy)-trispyrrolidinophosphonium hexafluorophosphate (PyAOP) was purchased from EMD Millipore. 3-Tritylsulfanyl-propionic acid (Mpa(Trt)-OH) was purchased from Bachem.

4.2. DSPE-PEG Hydrolysis Tests. For each solvent to be tested, 10 mg of DSPE-PEG was weighed into a 1.5 mL Eppendorf tube and dissolved in 1 mL of solvent. The solution was then split into two Eppendorf tubes, each 500 \( \mu \)L. One tube was left at RT, and the other was incubated at 60 °C. At each measured time point, 100 \( \mu \)L of each sample was transferred into a new Eppendorf tube for solvent removal. For TFA and methanol samples, the solvent was quickly evaporated under a gentle stream of blowing nitrogen. For other aqueous solvents, the sample was rapidly frozen in liquid nitrogen and lyophilized.

4.3. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Dried samples were dissolved in 100 \( \mu \)L of methanol, plated with dihydrourylenzoic acid matrix, and analyzed using the Bruker UltraFlextreme MALDI-TOF-TOF in the University of Chicago’s Mass Spectrometry Core Facility. To avoid fragmentation artifacts during ionization, the laser power was set using pure DSPE-PEG dissolved in methanol to a level that allowed for sufficient ionization without fragmentation artifacts. The same laser power was then applied to all samples.

4.4. Electrospray Ionization Mass Spectrometry. Dried samples were dissolved in 100 \( \mu \)L of methanol for ESI–MS via an Agilent 6130 LCMS. The ionization conditions were set using pure DSPE-PEG dissolved in methanol to a level that allowed for sufficient ionization without fragmentation artifacts. The same ionization conditions were then applied to all samples. The mobile phase was 50% water + 0.1% formic acid, 50% methanol, with a flow rate of 0.4 mL/min. The MS signal was acquired in positive mode, and the settings in the Agilent software were set as follows: fragmentor = 100, gain = 2.00, threshold = 100, step size = 0.10.

4.5. Solid-Phase Peptide Synthesis. The p53\textsubscript{14-29} peptide with N-terminal thiol and flexible linker was synthesized with sequence MPA(bAla)GG(bAla)-LSQETFSDLWKLLPEN-NH\textsubscript{2}. The peptide was synthesized manually in a peptide synthesis vessel from Chemglass using standard Fmoc SPPS protocols on Agilent AmphiSpheres 40 RAM resin. Before and after each reaction, the resin was washed extensively with NMP and DCM. Fmoc deprotection was accomplished with 2 × 10 min reactions with 25% piperidine in NMP, and deprotection was confirmed via the Kaiser Test. Each amino acid (10X with respect to (w.r.t.) resin substitution) and PyAOP (10X w.r.t. resin substitution) were dissolved in NMP immediately before use and activated by DIPEA (20X w.r.t. resin substitution) immediately before addition to the reaction vessel. Coupling was allowed to proceed until the Kaiser Test was clear. After each coupling, a capping solution (4:1:0.1 NMP/Ac\textsubscript{2}O/DIPEA) was applied to the resin for 10 min to cap any unreacted amines. As the final coupling, the thiol linker (Mpa(Trt)-OH) was added to the N-terminus of the peptide using the same reaction as the amino acids. After the synthesis, the resin was washed extensively with DCM and dried completely. The peptides were then cleaved from the resin using 94/2.5/2.5/1 TFA/H\textsubscript{2}O/EDT/TIS for 2.5 h. The TFA solution was removed by precipitating the peptides in ice cold diethyl ether, centrifuging the precipitate, and the resulting PA was puriﬁed via the Kaiser Test. Each amino acid (10X with respect to (w.r.t.) resin substitution) was dissolved in NMP immediately before use and activated by DIPEA (20X w.r.t. resin substitution) immediately before addition to the reaction vessel. Coupling was allowed to proceed until the Kaiser Test was clear. After each coupling, a capping solution (4:1:0.1 NMP/Ac\textsubscript{2}O/DIPEA) was applied to the resin for 10 min to cap any unreacted amines. As the final coupling, the thiol linker (Mpa(Trt)-OH) was added to the N-terminus of the peptide using the same reaction as the amino acids. After the synthesis, the resin was washed extensively with DCM and dried completely. The peptides were then cleaved from the resin using 94/2.5/2.5/1 TFA/H\textsubscript{2}O/EDT/TIS for 2.5 h. The TFA solution was removed by precipitating the peptides in ice cold diethyl ether, centrifuging the precipitate, removing the supernatant, and allowing the pellet to dry at RT. The peptides were resuspended in 1:1 (H\textsubscript{2}O + 0.1% formic acid)/acetonitrile with TCEP for a few hours to ensure complete thiol reduction before HPLC purification.

4.6. Reverse-Phase HPLC (RP-HPLC) Purification. HPLC purification was performed on a Shimadzu HPLC—
MS system using a Waters column, C8, XBridge BEH OBD Prep Column, 19 mm × 150 mm, 5 μm particle size, and 130 Å pore size. Methanol and acetonitrile were HPLC-grade and purchased from Fisher Scientific. Formic acid was purchased from Sigma-Aldrich. Water was Milli-Q filtered. All peptides were purified using water + 0.1% formic acid and acetonitrile as the mobile phases, with the column temperature at 60 °C. After elution, the acetonitrile was removed by rotary evaporation, and the samples were immediately lyophilized to minimize disulfide formation. All PAs were puriﬁed using water + 0.1% formic acid and methanol as the mobile phases at 25 °C. Immediately after elution, the fractions were buffered with 1 M ammonium bicarbonate buffer, pH 6.8. The methanol was then removed by rotary evaporation with the heat bath set no higher than 30 °C, and the samples were immediately lyophilized.

4.7. Conjugation of DSPE-PEG-Maleimide to Thiol-Peptide. DSPE-PEG-maleimide and the thiol-pS31-29 peptide (3:1) were each dissolved in dimethylsulfoxide (DMF) at 37.5 and 50 mM, respectively. The peptide solution was diluted in 0.1 M sodium phosphate buffer, pH 7.4, and DSPE-PEG was then added to the mixture. The ﬁnal reaction mixture was 1:1 DMF/(sodium phosphate buffer) with 5 mM peptide and 15 mM DSPE-PEG maleimide. The reaction was allowed to proceed for 1 h and then injected into the HPLC for puriﬁcation.

The DMF allowed for increased concentration of the reaction mixture and increased reaction rate, and the water with pH 7.4 sodium phosphate buffer served to (1) maintain the speciﬁcity of the thiol–maleimide reaction, (2) prevent maleimide hydrolysis while thiol conjugation proceeded to completion, and (3) prevent DSPE-PEG ester hydrolysis. After 1 h, the conjugation was complete according to LC–MS evaluation. We then puriﬁed the PA from the reaction mixture using RP-HPLC at 25 °C with water + 0.1% formic acid and methanol as the mobile phase solvents. We buffered the collected fractions immediately upon elution using 1 M ammonium bicarbonate buffer, pH 6.8. We then immediately removed the methanol using a rotary evaporator and removed the water by lyophilization.

4.8. Analytical Liquid Chromatography and Mass Spectrometry. Analytical LC–MS of the PA was performed on an Agilent 6130 LCMS system in the University of Chicago’s Mass Spectrometry Facility, using a Waters column, C8, XBridge, 4.6 mm × 150 mm, 5 μm particle size, and 130 Å pore size. The ESI–MS conditions were the same as used in part D, except that the fragmentor was increased to 250 to successfully ionize the PA. The mobile phase solvents used were water + 0.1% TFA and methanol at a total ﬂow rate of 1 mL/min. The method used an isotropic phase at 20% methanol from 0 to 2 min, a gradient from 20 to 80% methanol from 2 to 5 min, 80 to 100% methanol from 5 to 15 min, washing at 100% methanol from 15 to 30 min, followed by equilibration at 20% methanol from 30 to 45 min. The dwell volume from the pumps to the UV detector for this machine was measured to be approximately 3 mL.

4.9. Micelle Formation and Dynamic Light Scattering. PAs were dissolved in DMSO to form a 10 mM stock solution, followed by dilution to 100 μM in PBS. Following micelle formation, the nanoparticles were ﬁltered through a 0.2 μm ﬁlter. A correlation function was measured using a Wyatt Mobius Dynamic Light Scattering (DLS) in the Polymer Size Characterization Suite (sponsored in part by Wyatt Technol-

ogy Corp.) at the University of Chicago’s Institute for Molecular Engineering. The correlation function was used to find the hydrodynamic diameter (D_h) and polydispersity index (PDI) using a cumulant analysis. The average values from 15 measurements were used.

4.10. Transmission Electron Microscopy. Samples were prepared and imaged by the Advanced Electron Microscopy Core Facility at the University of Chicago. Grids (continuous carbon on 200-mesh copper grids—EMS CF200-CU) were glow-discharged for 30 s. The sample (100 μM) was applied soon after for 1 min. The excess sample was blotted off. The grids were stained with two washes of 0.75% uranyl formate and 45 s of 0.75% uranyl formate. Each was blotted oﬀ. Grids were imaged on a Tecnai G2 F30 (FEI) electron microscope operating at 300 kV.

Author Contributions
M.R.S., M.V.T., and J.L.L. conceived the work. M.R.S. and S.P.Y. performed the experiments. Data analysis was performed by M.R.S. and J.L.L. The manuscript was written by M.R.S. and J.L.L. with comments and inputs from all authors.

Notes
The authors declare no competing financial interest.

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