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Paramagnetic Polymerized Liposomes: Synthesis, Characterization, and Applications for Magnetic Resonance Imaging

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Abstract: Liposomes are biocompatible materials that show promise as vehicles for drug delivery, inhibitors of cell adhesion, and carriers for the introduction of genetic material into cells. In this paper, we describe the synthesis and characterization of a new class of polymerized liposome particles (paramagnetic polymerized liposome (PPL), Figure 1) that have lanthanide ion chelates as head groups and that can be easily visualized using magnetic resonance imaging (MRI). The R_1 molar relaxivity was found to depend primarily on the linker length (m) and on the surface metal density and only weakly on particle size. PPLs containing 10 mol % of compound **1b** ($m = 2$) and 90 mol % of compound **3** had a $R_1 = 12.2$ mM^{-1} s^{-1}, while PPLs with 10 mol % compound **1a** ($m = 1$) and 90 mol % of compound **3** had a $R_1 = 5.7$ mM^{-1} s^{-1}. PPLs with 10 mol % of compound **1a** and 90 mol % of compound **4** had a $R_1 = 8.9$ mM^{-1} s^{-1}, while PPLs with 50 mol % of compound **1a** and 50 mol % of compound **4** had a $R_1 = 4.3$ mM^{-1} s^{-1}. A biotinylated lipid (compound **2**) was also incorporated into the particle without affecting R_1 relaxivities for use as a marker for histochemical studies. We have also for the first time used atomic force microscopy (AFM) to investigate the size and nature of these particles in an aqueous environment. We feel that these new materials may prove useful for the *in vivo* investigation of liposome formulations as vehicles for therapeutic applications and for evaluating tissue pathology with MRI.

Introduction

Liposomes are biocompatible materials that show promise as vehicles for drug delivery,[1-4] inhibitors of cell adhesion,[5-8] and carriers for the introduction of genetic material into cells.[9-11] We and others have been studying polymerized liposomes as an alternative to classical lipid systems because of their increased physical stability, unique spectroscopic properties, and durability during chemical modification.[6,12-15] In particular, the physical stability of polymerized liposomes, a property arising from the

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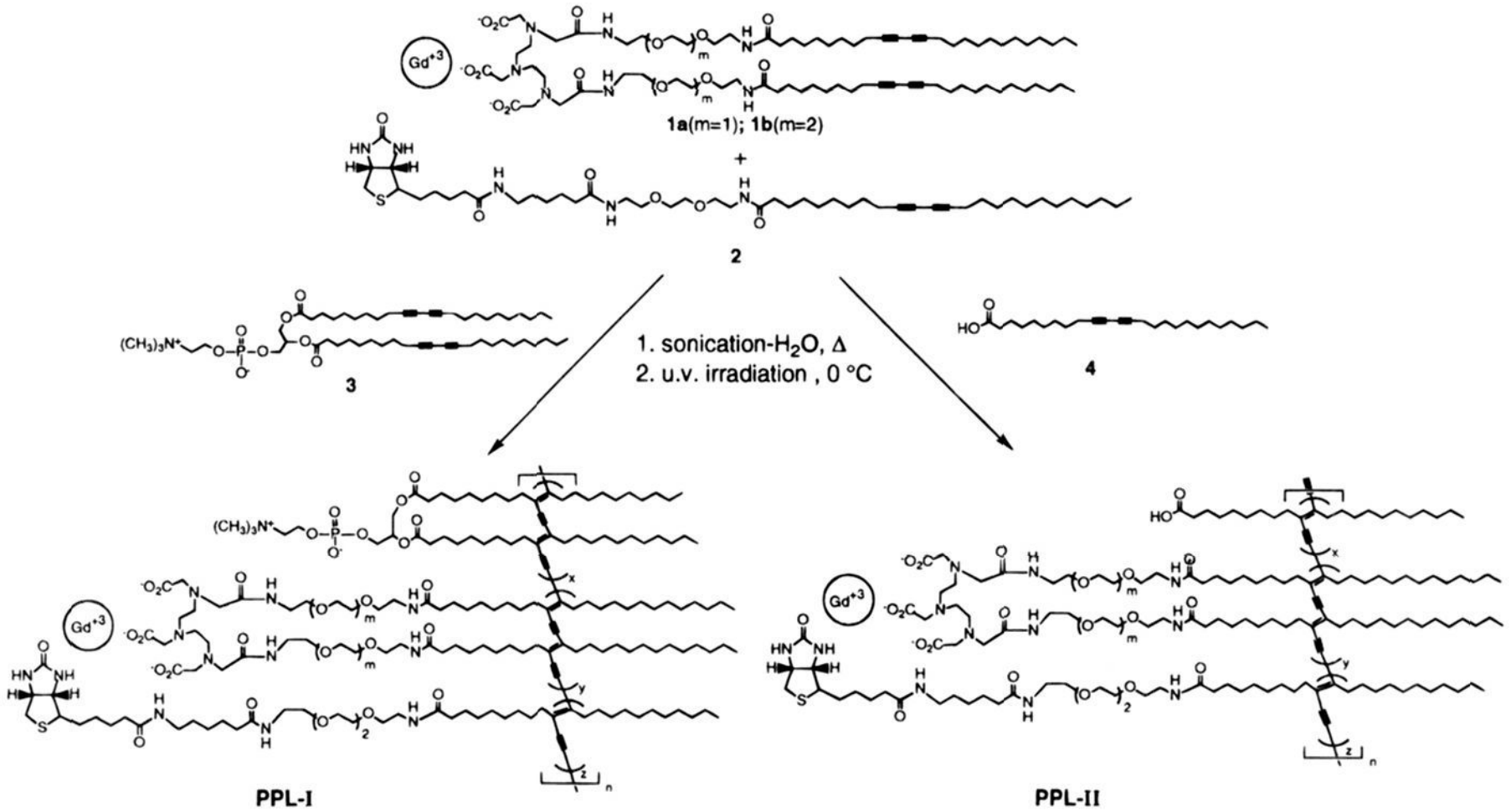

Figure 1. Formation of paramagnetic polymerized liposomes (PPLs).

increased membrane rigidity conferred by cross-links in the constituent lipids of the liposomes,[13] may reduce the likelihood of undesired phospholipid exchange or fusion with other liposomes or with cell membranes.[16,17] Increased membrane rigidity, furthermore, appears to decrease the extent to which the reticuloendothelial system removes liposomes from the blood, thereby increasing the chances for liposomes to circulate in the blood and to reach their desired destinations.[16,18] Polymerized liposomes also have increased durability during chemical modification relative to classical liposomes.[6] Consequently, biochemical moieties, such as proteins and carbohydrates, and chemical groups, such as chelators of metal ions, may be attached to a liposome carrier. One such chelator, diethylenetriaminepentaacetic acid (DTPA), complexed with the lanthanide ion gadolinium(III), is presently in clinical use as a contrast agent for magnetic resonance imaging (MRI).[19] By incorporating DTPA-conjugated lipids into liposomes, it may be possible to use MRI as a powerful tool for directly assessing the *in vivo* distribution of the liposomes in real time.[20−24] In this paper, we describe the synthesis and characterization of a new class of polymerized liposome particles (paramagnetic polymerized liposome (PPL), Figure 1) that have lanthanide ion chelates as head groups and that can be visualized using MRI. We have also incorporated biotin into the particle for use as a marker for histochemical studies. These new materials should prove useful for the *in vivo* investigation of liposome formulations as vehicles for therapeutic and diagnostic applications and for evaluating tissue pathology with MRI.

Results and Discussion

The lanthanide−lipid conjugates (compounds **1a,b**) for the construction of the PPLs were synthesized as outlined below. Pentacosadiynoic acid (PDA, compound **4**) was treated with *N*-hydroxysuccinimide (NHS) and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) to form the NHS ester, which was treated with the poly(ethylene glycol) (PEG)-diamine linkers ($NH_2(CH_2CH_2O)_2CH_2CH_2NH_2$ (**5a**) and NH_2-$(CH_2CH_2O)_3CH_2CH_2NH_2$ (**5b**)) to give PEG−PDA derivatives (compounds **6a,b**).[6] Compounds **6a,b** were then treated with diethylenetriaminepentaacetic acid dianhydride (DTPAA; compound **7**) in pyridine to form DTPA−bis(PEG−PDA) diamides (compounds **8a,b**). Treatment of **8a** and **8b** with gadolinium

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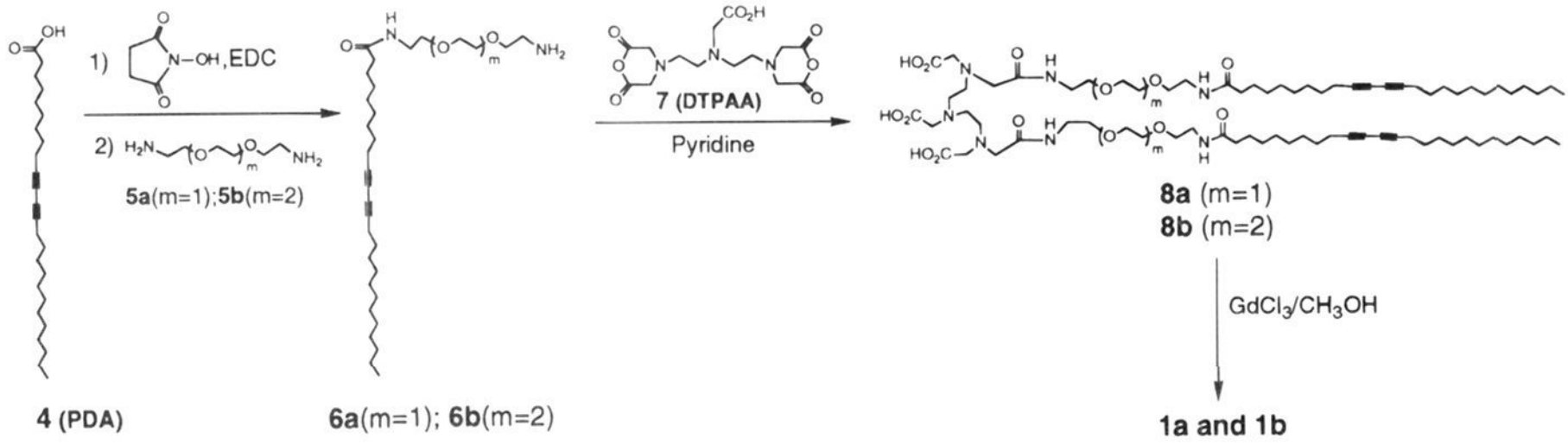
Scheme 1. Synthesis of Lipids **1a,b**



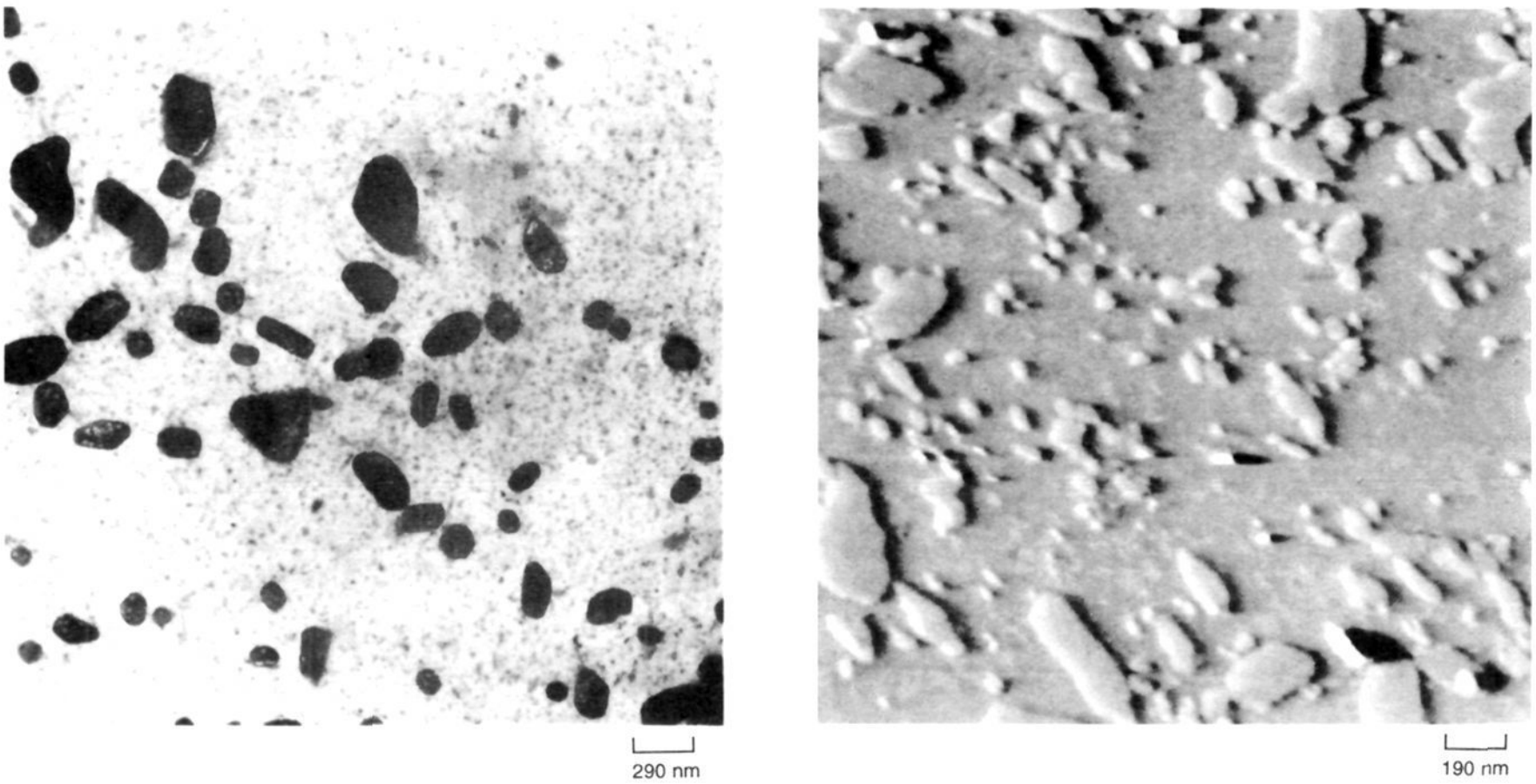
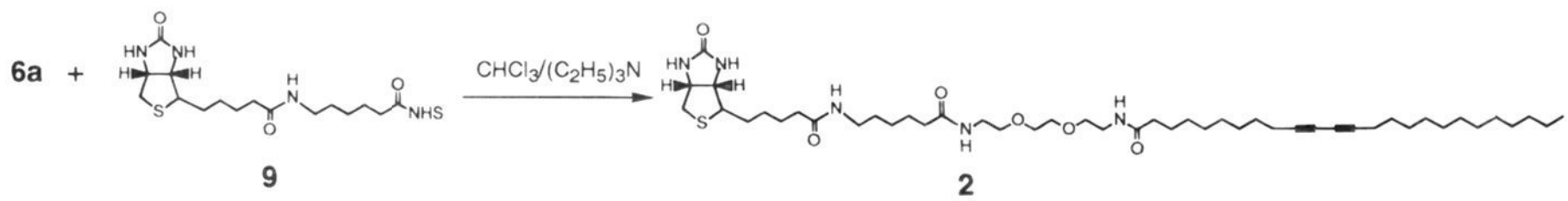


Figure 2. (left) Transmission electron micrograph (TEM) of PPLs. A dilute solution of nonextruded PPLs (lipid concentration, 15 μM) was deposited onto the specimen grid of a transmission electron microscope, freeze-dried, and stained with OsO_4. The micrograph was then taken at a magnification of 21000× (reproduced at 80% of original size). (right) Atomic force micrograph (AFM) of PPLs. Freshly cleaved Mica was covered with a dilute solution of nonextruded PPLs (lipid concentration, 15 μM) for 1−2 min, then rinsed with distilled water and covered with several drops of distilled water. The images were then obtained and processed on an Explorer Life Sciences model 200 atomic force microscope (Topometrix).

Scheme 2. Synthesis of Lipid **2**



trichloride ($GdCl_3$) in methanol resulted in the neutral amphiphilic lanthanide chelates, compounds **1a** ($m = 1$) and **1b** ($m = 2$).

The biotinylated lipid conjugate (compound **2**) was synthesized by treating the commercially available biotinamidocaproic acid *N*-hydroxysuccinimide ester (compound **9**) with compound **6a** in chloroform and triethylamine.

To control the surface density of the metal ion on the PPL, either compound **1a** or **1b** was mixed in organic solvent (chloroform or methylene chloride) with a matrix lipid of either diacetylenic phosphatidylcholine (DAPC (**3**), Avanti Polar Lipids, Birmingham, AL) or compound **4**.[25,26] To ensure the miscibility of the two diacyl lipids, we conducted Langmuir trough experiments on mixtures of compounds **1b** and **3**. The resulting pressure−area isotherms showed that compound **3** was miscible with **1b** up to 15 mol % of **1b**, above which the pressure−area isotherm showed evidence of phase separation of the two lipids. In contrast, previous Langmuir studies with

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compound **4** showed no evidence of phase separation with a variety of derivatized lipids.[6,12]

To form the PPLs, organic solvent was removed from the mixture of compounds **1a** or **1b** and **3** or **4** by evaporation, and the dried lipid film was hydrated to a known lipid density (30 mM acyl chain) using aqueous buffer or deionized water. The resulting suspension was then sonicated at temperatures above the gel−liquid crystal phase transition (compound **3**, $T_m \cong 37$ °C;[27] compound **4**, $T_m \cong 64$ °C[28]) using a probe-tip sonicator. Sonication yielded a milky suspension after 1 min, at which point the size of unpolymerized DAPC-containing liposomes could be controlled by extrusion.[29] For nonextruded liposomes, sonication was continued for 1 h, resulting in a clear colorless solution of emulsified lipid vesicles, or liposomes. The liposomes were then polymerized by cooling the liposome solution (either extruded or nonextruded) to 0 °C on a bed of wet ice and irradiating the solution at 254 nm with a hand-held UV lamp. The resulting DAPC−PPLs (**PPL-I**) were yellow-orange in color and had two visible absorption bands centered at 490 and 535 nm arising from the conjugated eneyne diacetylene polymer.[26] PDA−PPLs (**PPL-II**) were dark blue, with absorption bands at 544, 588, and 638 nm (λ_{max}), but gentle heating turned them red (absorption maxima at 498 and 538 nm).[6] The colors and absorption spectra of the liposomes were not affected by the presence or absence of extrusion.

Using transmission electron microscopy (TEM) and atomic force microscopy (AFM), we determined that the unextruded liposomes were 30−200 nm in diameter (Figure 2) and that liposomes extruded through filters with nominal pore diameters of 100 nm were 100−150 nm in diameter. With TEM using OsO_4 staining, the PPLs appeared as discrete dark ellipsoids and were easily distinguished from dust and buffer crystal artifacts.[30] Using the same TEM protocol, however, we could not visualize unpolymerized liposomes.[31] With AFM, the PPLs also appeared as flattened ellipsoids with a height of 10 nm and in-plane dimensions approximately the same as those estimated from TEM but with a greater abundance of particles on the lower end of the size spectrum.[32] The AFM images, furthermore, were obtained in aqueous solution on the surface of cleaved Mica. We therefore believe that the AFM images provide a more accurate description of the distribution of liposome sizes. To our knowledge this also represents the first AFM images of synthetic liposomes in an aqueous environment.[33]

In order to assess the utility of PPLs in MRI studies, we first constructed DAPC−PPLs (**PPL-I**) in sizes ranging from 30 to 200 nm in diameter using 2.5−15 mol % of compound **1a** or **1b** and 85−97.5 mol % of compound **3**. These PPLs were extruded at temperatures greater than T_m through polycarbonate filters with defined porosity. Interestingly, the R_1 molar relaxivity was found to depend primarily on the linker length (m) and only weakly on particle size. The PPLs containing 10% of compound **1b** ($m = 2$) and 90% of compound **3** had a $R_1 = 12.2$ mM^{-1} s^{-1}. This was in contrast to PPLs containing 10% of compound **1a** ($m = 1$) and 90% of compound **3**, where the $R_1 = 5.7$ mM^{-1} s^{-1}. In the former case we believe that the metal ion is suspended farther off the surface of the PPL, allowing greater aqueous accessibility to the lanthanide ion and hence greater relaxivity. Therefore, by providing a spacer (e.g., PEG linker) to separate the metal from the surface of the liposome, one can control the relaxivity of the PPL. Of note, the commercial Gd-DTPA conjugate in clinical use (Magnevist, Berlex Laboratories, Wayne, NJ) has a $R_1 = 4.24$ mM^{-1} s^{-1} under the conditions used in our studies. The greater lipid miscibility provided by the PDA matrix allowed investigation of liposomes with higher surface densities of lanthanide. PDA−PPLs (**PPL-II**) were constructed with 10, 30, or 50 mol % of compound **1a** and 90, 70, or 50 mol % of compound **4**, respectively. PDA−PPLs with 10% and 30% of compound **1a** showed similar R_1 relaxivities of 8.9 and 8.7 mM^{-1} s^{-1}, respectively. However, liposomes with 50% compound **1a** had a lower relaxivity of 4.3 mM^{-1} s^{-1}. We feel that this dependence of R_1 on surface metal density is primarily due to the overlap of the hemispheres of water relaxed by individual ions because of their close proximity at high lanthanide surface densities.

Biotinylated PPLs (89 mol % of **3** or **4**, 10 mol % of **1a** or **1b**, and 1 mol % of **2**) were formed as described above. We incorporated biotin into these liposomes both to demonstrate the ease of modifying their surface with biologically important ligands and to allow for the *in vitro* evaluation of the biodistribution of the particles using standard histochemical techniques based on avidin and streptavidin.[34] Treatment of the biotinylated PPL preparations with avidin caused a reaction that precipitated the liposomes from solution. Examination of the supernatant by gel electrophoresis showed no free avidin in the reaction mixture. Addition of 1% biotin to either the DAPC or PDA liposome system had no effect on R_1 relaxivities.

In summary, we have synthesized and characterized a new paramagnetic particle in which we can control particle size, metal density, metal topography, and surface functionality. These materials have excellent R_1 relaxivities for magnetic resonance imaging. We have also used AFM to investigate the size and nature of these particles in an aqueous environment. Investigation on the use of PPLs for *in vivo* MRI contrast enhancement and evaluation of tissue pathology is currently in progress.[35]

Experimental Section

General Methods. All solvents and reagents used were of reagent grade. Solvent evaporations were performed under reduced pressure provided from house vacuum or a Welch direct drive vacuum pump at ≤40 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL-200 at 200 and 50 MHz or a Varian XL-400 at 400 and 100 MHz, respectively, in $CDCl_3$, CD_3OD, or blends thereof as described for each case. (Note: although soluble in $CDCl_3$, the addition of CD_3OD to the lipids inhibits formation of inverted micelles and thus provides sharper spectra.) Spectra were referenced to residual $CHCl_3$ (7.25 ppm) for ^{1}H experiments and the center line of $CDCl_3$ (77.00 ppm) for ^{13}C

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(29) We were unable to extrude PDA-containing liposomes through the polycarbonate filters used.

(30) As the discrete dark ellipsoids were heated by the electron beam, a phase transition occurred, distinguishing them from dust and buffer crystal artifacts.

(31) It is speculated that the unpolymerized liposomes formed a film of lipid on the surface of the Formvar coating of the TEM specimen grid; this is based on the apparently smoother texture of the Formvar coat when TEM of unpolymerized liposomes was attempted. This observation confers additional confidence in the identity of the dark ellipsoids as PPLs.

(32) Polymerized liposomes lacking the DTPA chelate lipid **1a** or **1b** did not produce AFM images using this protocol. This observation, as well as the flattened appearance of the PPLs, suggests that DTPA chelates to the magnesium on the cleaved Mica, since flattening of the PPLs would maximize the number of DTPA−Mica interactions. These observations suggest that DTPA may be able to serve as a unique functionality for the attachment of biomolecules to the surface of cleaved Mica for AFM imaging.

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experiments. Fast atom bombardment ionization mass spectrometry was performed on ZAB2-EQ or VG-70SE instruments (University of California, Berkeley). TLC was performed on glass-backed Merck 60 F254 (0.2 mm; EM Separations, Wakefield RI) and the developed plates were routinely dipped in or sprayed with ceric sulfate (1%) and ammonium molybdate (2.5%) in 10% aqueous sulfuric acid and heated to ≈150 °C. Other developers included 0.2% isatin in acidified ethanol (general use), iodine (general use), 2% ninhydrin in 4% aqueous pyridine (for amines), 0.2% (dimethylamino)cinnamaldehyde in acidified ethanol (for biotin), and ultraviolet light (for chromophores).

***N*-Succinimidyl 10,12-Pentacosadiynoic Acid Ester (5).** The title compound was prepared in a fashion similar to that described previously.[6] Compound **4** (Lancaster; 10.0 g, 26.7 mmol), *N*-hydroxysuccinimide (NHS, Aldrich; 5.00 g, 43.4 mmol) and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC, Aldrich; 6.01 g, 31.3 mmol) were stirred in 650 mL of CH_2Cl_2 at room temperature and shielded from light. The reaction was followed by TLC ($CHCl_3$/MeOH, 8/1) and deemed complete after approximately 5 h. The solution was then washed with water, 1% HCl, saturated sodium bicarbonate, and brine. The organic phase was then dried with $MgSO_4$, filtered, and concentrated under reduced pressure to yield the desired succinimidyl ester as a slightly yellow solid (10.84 g, 23.0 mmol, 86%). The product had physical properties in agreement with those reported previously.[6]

***N*-(8′-Amino-3′,6′-dioxaoctyl)-10,12-pentacosadiynamide (6a).** Compound **5** was dissolved in CH_2Cl_2 (250 mL) and then slowly added, in a dropwise fashion, to a stirring solution of 1,8-diamino-3,6-dioxaoctane (9.13 g, 61.6 mmol; Texaco) in CH_2Cl_2 (110 mL) over a 16 h period at room temperature, shielded from light. The resulting solution was concentrated to a thick slurry and chromatographed on silica gel using a gradient of $CHCl_3$/MeOH (25/1 to 8/1). The homogeneous fractions were pooled and evaporated under reduced pressure to give the desired lipid as a white solid (4.40 g, 38.1%) which was used immediately in the next step. Care should be exercised with this product as it spontaneously polymerizes in the solid state when pure. It is more stable in solution at 4 °C. ^{1}H-NMR (200 MHz, $CDCl_3$/CD_3OD (1/1)): δ 3.36 (mult, 4H), 3.28 (t, J = 7 Hz, 4H), 3.31 (2t, J = 7 Hz, 2 × 2H), 1.95 (t, J = 7 Hz, 4H), 1.91 (t, J = 7 Hz, 2H), 0.95 to 1.4 (mult, 32H), 0.59 (t, J = 7 Hz, 3H). ^{13}C{^{1}H}-NMR (50 MHz, $CDCl_3$/CD_3OD (1/1)): δ 174.3, 76.8, 76.6, 71.5, 71.2, 69.6, 69.2, 69.1, 69.0, 64.8, 64.7, 40.3, 38.7, 38.6, 35.7, 31.4, 30.7, 29.1, 28.9, 28.8, 28.7, 28.5, 28.4, 28.3, 28.2, 27.8, 25.3, 22.1, 18.5, 13.3. FAB(+)-MS (nitrobenzyl alcohol) for $C_{31}H_{56}N_2O_3$: m/z (ion) 505 (M + 1), 400 (M − $O(CH_2)_2O(CH_2)_2NH_2$).

***N*-(11′-Amino-3′,6′,9′-trioxaundecyl)-10,12-pentacosadiynamide (6b).** This compound was prepared in a fashion similar to that described above for the tris(ethylene glycol)-diamine analog and has been previously described.[6] A solution of **5** (4.34 g, 9.2 mmol; prepared as described above) in 80 mL of $CHCl_3$ was added dropwise to a stirring solution of 1,11-diamino-3,6,9-trioxaundecane (7.78 g, 40.5 mmol) in 80 mL of $CHCl_3$. Silica gel chromatography as above gave 1.93 g (38%) of the desired lipid. The product had physical properties in agreement to those reported previously.[6]

***N*,*N*-Bis[[[[(13′,15′-pentacosadiynamido-3,6-dioxaoctyl)carbamoyl]methyl](carboxymethyl)amino]ethyl]glycine (8a).** Compounds **6a** (4.40 g, 8.78 mmol) and **7** (1.56 g, 4.37 mmol) were stirred in pyridine (25 mL) overnight at room temperature, shielded from light. The solvent was evaporated and the residue coevaporated to dryness with methanol twice to give an oil free of pyridine. The residue was dissolved in acetone and the product allowed to precipitate from solution after overnight storage at 4 °C. Filtration gave the desired chelator lipid as a white amorphous powder containing minor impurities (3.30 g, 55%). Pure lipid (1.55 g, 26%) was crystallized from methanol (40 mg/mL, mp 128.5−129.5 °C (dec)). ^{1}H-NMR (200 MHz, $CDCl_3$/CD_3OD (1/1)): δ 3.1−3.5 (mult, 38 H), 2.81 (t, J = 7 Hz, 4H), 1.94 (t, J = 7 Hz, 8H), 1.91 (t, J = 7 Hz, 4H), 1.32−0.91 (mult, 64H), 0.59 (t, J = 7 Hz, 6H). ^{13}C{^{1}H}-NMR (50 MHz, $CDCl_3$): δ 174.1, 173.6, 170.9, 169.8, 77.6, 77.4, 70.3, 70.1, 70.0, 69.8, 69.4, 65.4, 57.3, 56.6, 52.9, 50.4, 39.2, 36.5, 31.9, 29.6 (2C), 29.5, 29.3 (2C), 29.1, 29.0, 28.9 (2C), 28.8, 28.7, 28.6, 28.5, 28.4 (2C), 25.8, 22.6, 19.2 (2C), 14.1. FAB(+)-MS (nitrobenzyl alcohol) for $C_{76}H_{131}N_7O_{14}$: m/z (ion) 1367 (M + 1), 747 (M + 1 − $CH_3(CH_2)_{11}C_4(CH_2)_8CONH((CH_2)_2O)_2CH_2)_2$-$NHCOCH_2NHCH_2CO_2H$), 646 (747 − $C_4H_7NO_2$), 400 ($CH_3(CH_2)_{11}C_4$-$(CH_2)_8CONH((CH_2)_2)$. High-resolution FAB(+)-MS: m/z 1366.9830 (calcd for M + 1, m/z 1366.9832).

***N*,*N*-Bis[[[[(13′,15′-pentacosadiynamido-3,6,9-trioxaundecyl)carbamoyl]methyl](carboxymethyl)amino]ethyl]glycine (8b).** This compound was prepared in a fashion similar to that described above for the tris(ethylene glycol)-diamine analog. Compounds **6b** (1.93 g, 3.5 mmol) and **7** (0.504 g, 1.41 mmol) were stirred at room temperature overnight in pyridine (40 mL). The pyridine was removed under vacuum and the residue stored as a methanolic solution. Analytically pure samples of the product were obtained when needed by preparative thin layer chromatography using $CHCl_3$/MeOH/H_2O/HOAc (73/22/4/1) as eluent and $CHCl_3$/MeOH (7/1) to extract the lipid from the silica. ^{1}H-NMR (200 MHz, $CDCl_3$/CD_3OD (1/1)): δ 3.1−3.5 (mult, 42H), 2.96 (t, J = 7 Hz, 4H), 2.55 (mult, 4H), 2.03 (t, J = 7 Hz, 8H), 1.97 (t, J = 7 Hz, 4H), 1.02−1.45 (mult, 64H), 0.66 (t, J = 7 Hz, 6H). ^{13}C{^{1}H}-NMR (50 MHz, $CDCl_3$): δ 174.1, 173.6, 170.9, 169.8, 77.6, 77.4, 70.3, 70.1, 70.0, 69.8, 69.4, 65.4, 57.3, 56.6, 52.9, 50.4, 39.2, 36.5, 31.9, 29.6 (2C), 29.5, 29.3 (2C), 29.1, 29.0, 28.9 (2C), 28.8, 28.7, 28.6, 28.5, 28.4 (2C), 25.8, 22.6, 19.2 (2C), 14.1.

***N*,*N*-Bis[[[[(13′,15′-pentacosadiynamido-3,6-dioxaoctyl)carbamoyl]methyl](carboxymethyl)amino]ethyl]glycine−Gadolinium Complex (1a) and *N*,*N*-Bis[[[[(13′,15′-pentacosadiynamido-3,6,9-trioxaundecyl)carbamoyl]methyl](carboxymethyl)amino]ethyl]glycine−Gadolinium Complex (1b).** As a general procedure, the appropriate chelator lipid (**8a** or **8b**) and $GdCl_3$·$6H_2O$ (0.95 −0.98 equiv) were refluxed for 30 min in methanol with the pH adjusted to 7 using sodium methoxide (0.1 M in methanol). The solvent was evaporated, and resulting lanthanide chelate lipids (**1a** (m = 1), **1b** (m = 2)) were then stored as methanolic solutions at 4 °C, shielded from light. The identities of the synthesized chelates were confirmed by FAB(+)-MS which always gave strong signals (base peak) for the respective molecular ions. The observed molecular ion cluster patterns always matched computer-simulated patterns. FAB(+)-MS for compound **1a** gave m/z 1522.1 (M + 1) (for the major Gd^{3+} isotope) (calcd for **1a** + H, 1522.1), with the cluster pattern matching the Gd isotope distribution pattern. FAB(+)-MS for compound **1b** gave m/z 1610.4 (M + 1) (for the major Gd^{3+} isotope) (calcd for **1b** + H, 1610.4) and 1632.4 (M + Na) (for the major Gd^{3+} isotope), with the cluster patterns matching the Gd isotope distribution pattern.

***N*-[(Biotinamidocaproamido)-3,6-dioxaoctyl]-10,12-pentacosadiynamide (2).** Biotinamidocaproic acid *N*-hydroxysuccinimide ester (compound **9**) (25 mg, 55 mmol; Sigma) and compound **6a** (30.9 mg, 61 mmol) were stirred overnight at room temperature in 6 mL of chloroform containing 100 mL of triethylamine. TLC ($CHCl_3$/MeOH/H_2O/HOAc, 74/22/3/1) indicated the complete consumption of the biotinylating agent (R_f = 0.56) to give the desired product (R_f = 0.63). A small amount of methanol was added to solubilize the products. The solution was then concentrated and applied as a thin band to a preparative TLC plate (Merck 60; 1 mm, 20 × 20 cm). The plate was eluted with $CHCl_3$/MeOH/H_2O/HOAc (80/20/2/0.5) and the product extracted from the silica using CH_2Cl_2/MeOH (9/1). Filtration, evaporation, and drying *in vacuo* gave the desired product as a white homogeneous solid (35.9 mg, 77%). ^{1}H-NMR (400 MHz, $CDCl_3$/CD_3OD (3/1)): δ 4.33 (1H, dd, $J_{1a,2}$ = 4.9, $J_{1b,2}$ = 0, $J_{2,4}$ = 7.9, H_2), 4.14 (1H, dd, $J_{4,5}$ = 4.6, $J_{2,4}$ = 7.9, H_4), 3.45 (4H, s, OCH_2CH_2O), 3.38 (4H, t, J = 5.3, OCH_2CH_2N), 3.23 (4H, t, J = 5.2, OCH_2CH_2N), 3.00 (3H, t, J = 7, CH_2 and H_5), 2.75 (1H, dd, $J_{1a,2}$ = 4.9, $J_{1a,1b}$ = 12.8, H_{1a}), 2.55 (1H, d, $J_{1a,1b}$ = 12.8, $J_{1b,2}$ = 0, H_{1b}), 2.07 (4H, t, J = 7), 2.01 (6H, 3t, $J \sim 7$, $-CH_2C{=}O$), 1.08−1.55 (44H, mult), 0.71 (3H, J = 7.0, CH_3). ^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$/CD_3OD (3/1)): δ 174.4, 174.2, 174.0, 164.0, 77.2, 69.7 (2C), 69.5, 69.4, 65.0, 64.9, 61.6, 59.8, 55.3, 40.0, 38.8 (2C), 38.7 (2C), 36.0, 35.7, 35.4, 31.6, 29.3 (3C), 20.0 (2C), 28.9, 28.8, 28.6, 28.5, 28.4, 28.1, 28.0 (2C), 27.8, 26.0, 25.5, 25.3, 24.9, 22.3, 18.8, 13.7. FAB(+)-MS (NBA) for $C_{47}H_{81}N_5O_6S$: m/z (ion) 844.5 (M + 1), 400.3 ($CH_3(CH_2)_{11}C_4(CH_2)_8$-$CONHCH_2CH_2^+$), 383.2 (biotin-$NH(CH_2)_5CONHCH_2CH_2^+$). High-resolution FAB(+)-MS: m/z 844.59858 (calcd for $C_{47}H_{81}N_5O_6S$ + H, m/z 844.59918).

Preparation of Paramagnetic Polymerized Liposomes. Appropriate amounts of purified lipid components (**1a,b**, **2**−**4**) dissolved in organic solvents (CH_2Cl_2 or $CHCl_3$ and CH_3OH in a ratio of 10:1)

were combined. The solvents were evaporated and the residue dried *in vacuo* while shielded from light. Distilled and deionized water was added to yield a heterogeneous solution 30 mM in diacetylene. To obtain a clear homogeneous solution, the lipid/water mixture was then sonicated with a probe-tip sonicator for at least 1 h at the maximum power setting at which no frothing occurred and at which there was minimal disturbance of the solution surface.[36] Throughout sonication, the pH of the solution was maintained at 7−8 with NaOH, and the temperature was maintained above the gel−liquid crystal phase transition point (T_m) with the heat generated from sonication. To polymerize the liposomes, the liposome solution was transferred to a petri dish resting on a bed of wet ice, cooled to 0 °C, and irradiated at 254 nm for at least 1 h with a hand-held UV lamp placed ≅1 cm above the petri dish, yielding PPLs. The PPLs were then filtered through a 0.2 μm filter and collected.

To control liposome size by extrusion, the extent of sonication was reduced to the minimum amount of time necessary (approximately 1 min) to form a homogeneous, milky emulsion. This emulsion was then transferred to a thermobarrel extruder (Lipex Biomembranes, Vancouver, BC, Canada) fitted with two stacked polycarbonate filters with various nominal controlled pore diameters (30, 50, 80, 100, and 200 nm) (Poretics, Inc., Livermore, CA). The extruder was equilibrated to 57 °C and pressurized with N_2 to 50−100 psi. The emulsion was then passed through the membranes a minimum of 10 times, resulting in a slightly opalescent solution. Afterward, this solution was polymerized as described above for nonextruded liposomes.

Transmission Electron Microscopy. Positively stained transmission electron microscopy grids were prepared by placing 5 μL of a dilute PPL solution (lipid concentration, 15 μM) onto Formvar coated, glow discharge ionized, 400 mesh nickel electron microscopy specimen grids. The solution was freeze-dried by placing the specimen grid first in liquid N_2 and then in a vacuum chamber. The resulting unsaturated lipids were stained with OsO_4 by vapor deposition—the specimen grids were placed on a nylon screen suspended 4 cm over a 2% aqueous solution of OsO_4 in a Teflon-sealed glass chamber for 10 min. The samples were then allowed to out-gas in a fume hood for 30 min prior to imaging. All samples were examined with a Phillips 410 transmission electron microscope using a beam voltage of 80 kV.

(36) We found that frothing of the solution during sonication introduced air into the solution and decreased the effectiveness of sonication, resulting in liposomes larger than desired.

Atomic Force Microscopy. Samples for atomic force microscopy (AFM) in solution were prepared by covering freshly cleaved Mica with a dilute PPL solution (lipid concentration, 15 μM) for 1−2 min. The solution was recovered by pipet and the Mica surface rinsed with a stream of distilled water. The Mica was mounted on the AFM head and covered with several drops of distilled water. AFM images were obtained on an Explorer Life Sciences Model 200 atomic force microscope (Topometrix, Santa Clara, CA) located at the Topometrix facility in Santa Clara. The AFM was operated in "contact" mode using the minimum force necessary to prevent hopping of the cantilever tip. The raw images were "flattened" either line-by-line or through a user-defined baseline plane, as appropriate, using the software supplied with the Topometrix instrument.

Relaxivity Measurements. Relaxivity measurments were performed on a 2.0 T OMEGA CSI MR imager (General Electric, Fremont, CA) at ambient temperature. PPL samples were diluted to concentrations of 500, 250, 125, and 62.5 μM Gd^{+3} in 2 mL polyethylene sample vials. For R_1 measurements, the samples were imaged collectively at TR values of 3600, 1800, 900, 450, and 300 ms with an 18 ms TE, 1 NEX, and two dummy scans prior to acquisition. The resulting images were analyzed on a pixel-by-pixel basis to a single exponential using the program "t1img" (Glover, G.; Macfall, J. Personal communication). These T_1 values were averaged over at least 24 pixels in the center of each sample and plotted as $1/T_1$ vs $[Gd^{+3}]$. The slope of this line was the molar relaxivity, R_1.

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