Synthesis of Lipid-α-End-Functionalized Chains by RAFT Polymerization. Stabilization of Lipid/Polymer Particle Assemblies

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ABSTRACT: A functional dithioester including a phospholipid moiety (Lipid-DT) has been synthesized in a high yield (71% after purification) from a precursor chain transfer agent (CTA) and fully characterized. The RAFT polymerization of an acrylamide derivative, *N*-acryloylmorpholine (NAM), mediated by this Lipid-DT exhibits a prolonged induction period in comparison with a nonfunctional dithioester. This phenomenon is discussed in terms of steric shielding induced by the bulky lipid moiety of the CTA. Moreover, the lipid moiety located at the α-end of the chains has a strong influence on the size exclusion chromatography analyses in THF using a standard-based calibration, with the suspicion of a retention phenomenon. The well-defined structure of the lipid-end-functionalized polymer chains has been evidenced by MALDI−TOF mass spectrometry. Finally, these chains have been successfully incorporated into lipid/polymer particle assemblies (LipoParticles) that resulted in an improved stabilization in aqueous medium at relatively high ionic strength (300 mM).

Introduction

LipoParticles are organized macromolecular assemblies composed of a polymer core and a lipid shell.[1] These composite structures combine the advantages of particles (mechanical stability, control of the final morphology, easy purification) with the best attributes of lipids (biocompatibility, biomimetic behavior, ability to interact with a wide variety of molecules due to their amphiphilic nature) in biotechnological and biomedical applications *in vitro*[2−6] or *in vivo*.[7−12] However, the elaboration of such lipid/polymer particle assemblies is a challenge. As shown and discussed in previous articles,[13,14] the assemblies composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/1,2-dipalmitoyl-3-trimethylammoniumpropane (DPTAP) lipid mixtures and poly(lactic acid) or poly(styrene) core particles are not stable enough toward ionic strength, leading to strong LipoParticle aggregation (screening of their electrostatic charges). For instance, electrostatic repulsions alone are not efficient enough to stabilize LipoParticles based on poly(lactic acid) particles in aqueous solutions containing more than 10 mM NaCl. By analogy with stealth liposomes where lipid-poly(ethylene glycol), (Lipid-PEG), conjugates have been incorporated in the lipid formulation,[15−18] we demonstrated that Lipid-PEG conjugates did stabilize LipoParticles.[14] In fact, the polymer chains located at the aqueous phase/lipid interface induce repulsive steric forces between assemblies that bring colloidal stabilization.

In such systems, the polymer chain is immobilized by only one anchor point (the lipid end-group) on the lipid membrane and is expected to be extended in the aqueous phase. This should produce a better colloidal stabilization than that provided by polymers physically adsorbed onto the liposome surface by several anchor points. Indeed, Takeuchi et al.[19] have compared the liposome stabilization efficiency of a poly(vinyl alcohol) (PVA) with that of a modified PVA (PVA-R) bearing a long alkyl chain end. The first polymer is immobilized onto the liposome surface by many polymer segments distributed all along the chain, whereas the second one is immobilized by only one anchor point (insertion of the hydrophobic end-group into the liposome membrane). The authors evidenced that—considering a same PVA molar mass—the PVA-coated liposomes presented a thinner interface layer and less protective effects than PVA-R-coated liposomes, confirming the positive effect of the hydrophobically modified polymer end-group.

In the literature, polymer chains modified at one end by a lipid or phospholipid moiety (that will be called lipid-polymer conjugates), have been synthesized using various strategies. One simple strategy involves the postmodification of a preformed polymer chain. Such polymer chain must have a reactive group (−COOH, $-NH_2$, −OH, −SH) at least at one chain-end, either coming from the initiator[20−22] or from a transfer agent.[23,24] This strategy often requires the addition of an excess of the lipid derivative (with a necessary subsequent purification) and the coupling yield is limited by the length of the polymer chain.

Another strategy involves the introduction of the lipid derivative directly in an initiator or a transfer agent further used to carry out the polymerization. This method generally leads to very high chain functionalization yields. However, the chemistry required to introduce the lipid should be compatible with the initiator or the transfer agent, and the lipid should not interfere with the polymerization mechanism nor be degraded. Lipid-modified initiators were synthesized as azo-initiator for conventional free radical polymerization,[25−27] initiator for ring opening polycondensation,[28] or initiators specially designed for controlled radical polymerization (CRP) such as initiators for NMP,[29,30] and initiators for ATRP.[31−33] Conversely, lipid-

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modified transfer agents were synthesized as mercaptan used in conventional free radical polymerization,[34] or as iniferter used to obtain controlled oligomers.[35] However, to our knowledge, phospholipids have not been introduced to date in a reversible chain transfer agent (CTA) to mediate RAFT polymerization.

RAFT polymerization is carried out with thiocarbonylthio compounds (of the general formula Z−C(=S)−SR, known as RAFT agents) which reversibly react with growing radicals via chain transfer reactions.[36,37] Besides exhibiting a narrow molar mass distribution and a controlled chain length, the obtained polymer chains are characterized by the presence of the R and Z groups—from the RAFT agent—at their α- and ω-ends, respectively. Then, modification of the RAFT agent structure appears as a highly powerful means for introducing a molecule of interest at polymer chain-ends.

However, when modifying RAFT agents, the major problem consists in the extreme sensitivity of dithioesters, dithiocarbonates and trithiocarbonates toward amino groups.[38] Consequently, most derivatives were bound to CTA via an ester link.[39−41] We have recently designed new precursor RAFT agents that allow the introduction of amino-derivatives without destruction of the thiocarbonylthio moiety.[42] Using this strategy various biorelated dithiobenzoates were synthesized, bearing for instance a biotin or a sugar linked via a stable amide function, and it was extended for the first time here, to a phospholipid.

The various lipid-end-functionalized chains reported in the literature correspond to several kinds of polymers, either pH-sensitive (poly(acrylic acid)),[22,25,34] thermosensitive (poly(*N*-isopropylacrylamide)),[24,34] able to recognize sugars (poly(acrylamidophenylboronic acid)),[27] biocompatible (poly(*N*-vinylpyrrolidone)[23] and glycopolymers[26,29−31,35]) or biodegradable (poly(caprolactone)[21] and polypeptides[20,28]). Concerning the selection criteria for the polymer to sterically stabilize LipoParticles, the polymer chains should be water soluble, highly flexible, biocompatible and pharmacology acceptable. It has been established that poly(*N*-acryloylmorpholine), poly(NAM), presented all these properties, similarly to PEG.[43,44] In addition, poly(NAM) can be synthesized in a control fashion by the RAFT process.[45−47]

In this article, we describe the first synthesis of a phospholipid-functionalized CTA suitable for RAFT polymerization and its application for the synthesis of phospholipid-end-functionalized poly(NAM) chains. Analytical techniques such as NMR, SEC and MALDI−TOF MS were used to investigate the possible influence of the lipid moiety on the kinetics and on the structure of the resulting chains. Finally, the potential of several lipid-end-functionalized poly(NAM) samples to stabilize LipoParticles in aqueous medium at relatively high ionic strength was assessed.

Experimental Section

Materials. *N*-Acryloylmorpholine (NAM) (Aldrich, 97%) was distilled under reduced pressure (120 °C, 10 mmHg). 2,2′-Azobis(isobutyronitrile) (AIBN) (Fluka, 98%) was purified by recrystallization from ethanol. 1,4-Dioxane (Acros, 99%) was distilled over $LiAlH_4$ (110 °C). Tetrahydrofuran (Aldrich, >99.5%), triethylamine (Aldrich, ≥99.5%), trioxane (Acros, 99%), sodium chloride (Merck) and other materials were all used without further purification. Precursor RAFT agent **1** and Morpholine−DT were synthesized according to a previously published procedure.[42]

The zwitterionic lipid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, $C_{40}H_{80}NO_8P$), was purchased from Sigma Chemical Co. (St. Louis, MO). The cationic lipid, 1,2-dipalmitoyl-3-trimethylammoniumpropane (DPTAP, $C_{38}H_{76}NO_4Cl$), the aminated lipid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(hexanoylamine) (Lipid-NH_3^+, $C_{44}H_{85}NO_9P$), and the PEGylated lipid, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-5000] (Lipid-PEG_{113}), were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). All lipids were used without further purification.

Anionic poly(styrene) particles were purchased from Interfacial Dynamics Co. (Portland, OR) and used as supplied. Particles are functionalized with sulfate groups and described as surfactant-free. Their charge number per particle, surface charge density, and specific surface area are 4.2×10^4, 2.6 $\mu C \cdot cm^{-2}$, and 2×10^5 $cm^2 \cdot g^{-1}$, respectively (as given by the manufacturer). The mean particle diameter determined by the supplier from transmission electron microscopy is 290 nm (standard deviation of 8 nm). The mean hydrodynamic diameter determined by quasi-elastic light scattering (QELS) was 308 ± 3 nm with a Poly value of 0.03 (see definition in the QELS section).

Vesicles and lipid/polymer particle assemblies (LipoParticles) were prepared in ultrapure water (Milli-Q quality, 18.2MΩ/cm, Millipore Co., Bedford, MA).

Lipid-DT Synthesis. In a 100 mL round-bottomed flask equipped with a magnetic stirrer, Lipid-NH_3^+ (200 mg, 0.25 mmol) and precursor RAFT agent **1** (89 mg of raw product corresponding to 0.25 mmol of pure dithioester) were dissolved in chloroform (14 mL). A solution of triethylamine (54 mg, 0.67 mmol) in 2 mL of chloroform was separately prepared. The latter was added in three fractions (about 20 min between each addition) to the reaction mixture. The reaction mixture was stirred at 30 °C for 2 h. Then, it was washed three times with 60 mL of distilled water and twice with 60 mL of NaCl solution (100 $g \cdot L^{-1}$). The organic phase was diluted with acetone (to induce precipitation of the residual NaCl) and filtrated. The usual drying step was avoided since Lipid-DT was found to stick to magnesium sulfate powder. After solvent removal, column chromatography (Silica gel 60, Merk) was performed (pure acetone, then pure ethyl acetate to remove residual water and impurities, then mixture of chloroform/ethanol: 80/20 v/v) to afford an orange-red oil (final yield 71%). Thin layer chromatography performed in several solvent mixtures showed only one spot.

^{1}H NMR 200 MHz ($CDCl_3$, RT, ppm) (see Figure 1 for proton assignment): 0.87 (t, H19); 1.24 (H18); 1.56 (H8 and H17); 1.65 (d, H5); 2.26 (H9 and H16); 3.21 (H7); 3.48 (H11); 3.95, 4.14 and 4.35 (H12, H13 and H15); 4.68 (q, H4); 5.23 (H14); 6.98 and 7.26 (H6 and H10); 7.36 (dd, H2); 7.53 (dd, H1); 7.97 (d, H3).

^{13}C NMR 50 MHz ($CDCl_3$, RT, ppm) (see Supporting Information for carbon assignment): 14.11 (C30 and C31); 16.66 (C7); 22.70 (C24 and C25); 24.91 (C13); 29.40 (C28 and C29); 29.74 (C26 and C27); 31.94 (C22 and C23); 34.11; 34.30 and 35.55 (C10, C11 and C12); 39.66 and 40.54 (C9 and C15); ∼48.5 (C6); 62.66; 63.67; 64.48 and 70.35 (C16, C17, C18 and C19); 127.19 (C3); 128.43 (C2); 132.87 (C1); 144.34 (C4); 170.68 (C8); 173.25; 173.59 and 174.94 (C14, C20 and C21); 227.14 (C5).

MALDI−TOF mass spectrometry: characteristic ion $[M + Na]^+$, $C_{53}H_{93}N_2O_{10}PS_2Na$; calculated monoisotopic mass 1035.6 mass units; found 1035.6 mass units (the corresponding spectrum is available in the Supporting Information).

Fast atom bombardment (FAB) mass spectrometry: characteristic ion $[M + H]^+$, $C_{53}H_{94}N_2O_{10}PS_2$; calculated 1013.6088 mass units; found 1013.6077 mass units.

Polymerization Procedure. NAM (1 g, 7.1 mmol), Lipid-DT (50.2 mg, 0.05 mmol), AIBN (0.77 mg, 0.005 mmol), dioxane (3.54 mL), and trioxane (0.05 g, internal reference for ^{1}H NMR determination of monomer consumption) were introduced in a Schlenk tube equipped with a magnetic stirrer. The mixture was degassed by five freeze−evacuate−thaw cycles and then heated under nitrogen using a thermostated oil bath (90 °C). Periodically, samples were withdrawn from the polymerization medium for analyses.

Monomer conversion was determined by ^{1}H NMR spectroscopy using a Bruker AC 200 spectrometer (200 MHz), by comparison of one vinylic proton (5.7 ppm) with trioxane (5.1 ppm).[48] Typically, 400 μL of d-chloroform was added to 200 μL of each sample.

Polymers were precipitated in a large volume of diethyl ether, recovered by centrifugation, and finally dried under vacuum.

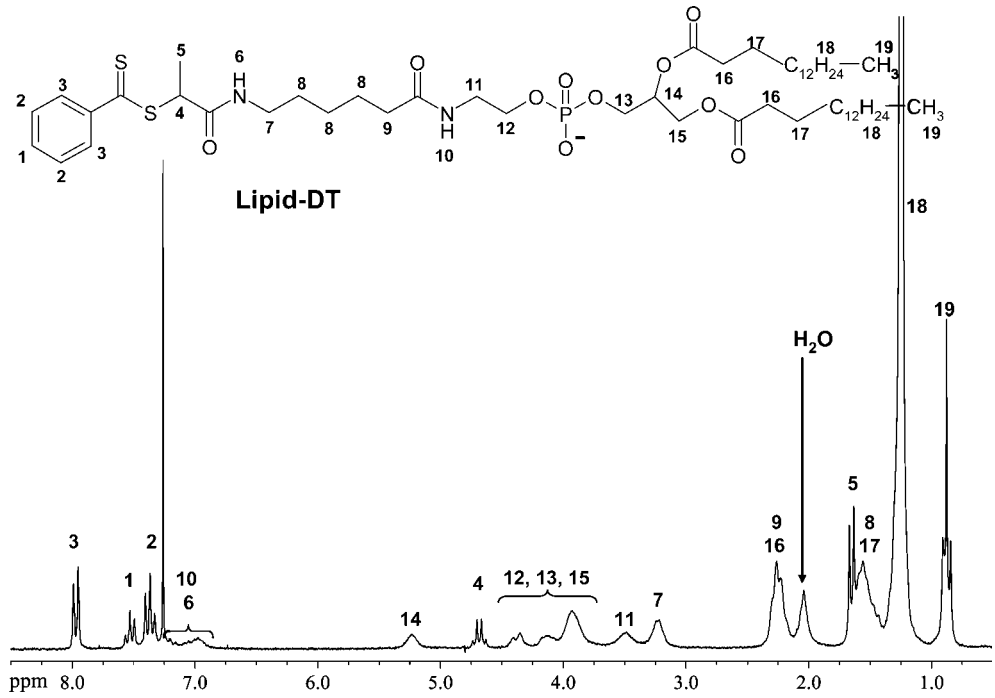


Figure 1. ^{1}H NMR spectrum (200 MHz, anhydrous $CDCl_3$, RT) of purified Lipid-DT. ^{13}C NMR spectrum is available in Supporting Information.

Vesicle Preparation. Lipid mixtures (DPPC/DPTAP/lipid−polymer conjugate, (50 − *x*)/50/*x*, mol/mol/mol) were dissolved in chloroform, and the solvent was then removed by rotary evaporation to get a homogeneous and thin lipid film. Large multilamellar vesicles (LMV) were obtained by adding ultrapure water to the film, and by stirring this mixture in a water bath at 70 °C. Smaller vesicles were made by disruption of LMV suspension using a bath sonicator thermostatted at 70 °C (Branson 3510, Branson Ultrasonics Co., Danbury, CT).

LipoParticle Preparation. LipoParticles were synthesized by adding a suspension of sonicated vesicles (10-fold excess) to a dispersion of poly(styrene) particles in ultrapure water. The mixture was vortexed for 1 h at 70 °C. Under such conditions, the adsorption and spreading of highly curved vesicles on the particles leads to a lipid shell formation around the particle surface.[1,13,49] Then, dispersions were centrifuged twice at 9000*g* for 30 min to remove free lipids. Finally, the ionic strength was adjusted at 300 mmol·L^{-1} with a NaCl solution.

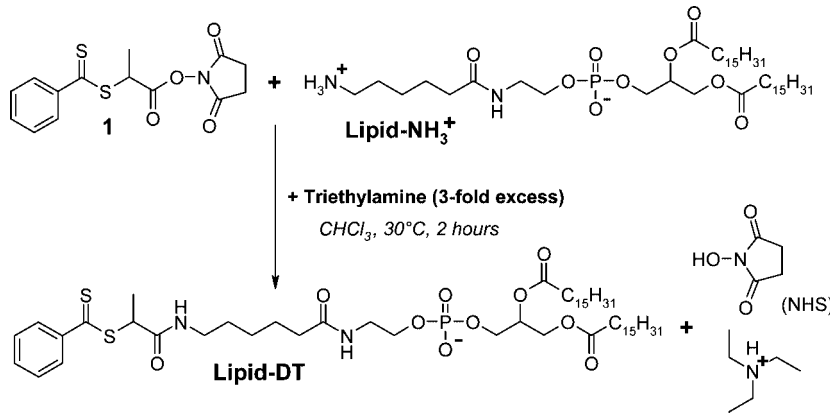
Characterization Methods. *Aqueous Size Exclusion Chromatography Coupled with Light Scattering Detection (ASEC/LSD).* This was performed using a Waters 510 pump and two Waters Ultrahydrogel columns (2000 and 500 Å). Online double detection was provided by a differential refractometer (DRI Waters 410) and a three-angle (47°, 90°, 130°) MiniDAWN light scattering photometer (Wyatt Technologies), operating at 690 nm. Analyses were performed by injection of 200 μL of polymer solution (5 mg·mL^{-1}) in a borate buffer (pH = 9.3, 0.05 mol·L^{-1}) (Morpholine−poly(NAM) solution is clear but Lipid−poly(NAM) solution is cloudy), previously filtered (0.22 μm Millipore GS filter) and used as eluent at a flow rate of 0.5 mL·min^{-1} (35 °C). The specific refractive index increment (dn/dc) for poly(NAM) in the same eluent (0.163 mL·g^{-1}) was previously determined with a NFT ScanRef monocolor interferometer operating at 633 nm. The molar mass and polydispersity data were determined using the Wyatt ASTRA SEC/LS software package.

Size Exclusion Chromatography in THF. (SEC/THF) was performed using a Waters 1515 isocratic HPLC pump (flow rate = 1 mL·min^{-1}) and a Waters column (Styragel HR4E, reticulated polystyrene). Detection was performed using a Waters 2410 refractive index detector. Analyses were performed by injection of 20 μL of polymer solution (5 mg·mL^{-1}) in THF. The molar mass and polydispersity data were determined using the Waters Breeze software package. The system was calibrated by using narrow poly(NAM) samples (previously synthesized by the RAFT process with *tert*-butyl dithiobenzoate as chain transfer agent).[46]

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. (MALDI−TOF MS) measurements were performed with a Voyager-DE STR (Applied Biosystems, Foster City, CA). This instrument was equipped with a nitrogen laser (wavelength 337 nm). The accelerating voltage was 20 kV. The positive ions were detected in all cases. The spectra were the sum of 200 shots, and an external mass calibration was used (mixture of peptide standards, Sequazyme kit). Samples were prepared by dissolving the polymer in tetrahydrofuran (THF) at a concentration of 10 g·L^{-1}. The matrix was α-cyano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich), used without further purification and dissolved in THF (10 g·L^{-1}). Matrix and polymer solutions were mixed at a volume ratio of 9:1, some NaI was added to the sample solution and 1 μL of the resulting mixture was deposited onto a stainless steel target and dried before insertion into the ion source chamber. In the case of Lipid-DT, samples were prepared by dissolving Lipid-DT in methanol at a concentration of 10 g·L^{-1}. The matrix was 2,5-dihydroxybenzoic acid (DHB, Sigma-Aldrich), used without further purification and dissolved in methanol (10 g·L^{-1}). Matrix and Lipid-DT solutions were mixed at a volume ratio of 9:1; cation-exchange resin DOWEX 50W-X8 was added to the sample solution.

Quasi-Elastic Light Scattering (QELS). Mean hydrodynamic diameters (D_h) and mean size distributions (Poly) were determined at 25 °C by QELS (Zetasizer 3000 HS, Malvern Instrument, U.K.). The measurement angle was 90°, the laser was a helium−neon type operating at 633 nm, solvent refractive index and viscosity at 25 °C were 1.33 and 0.8904 cP, respectively. The Poly value is a dimensionless measurement of the distribution broadness (defined by μ_2/Γ^2, where μ_2 is the second cumulant of correlation function fitted by the cumulant analysis and Γ is the average decay rate). For a monodisperse sample, the Poly value should theoretically be zero. In practice, for a "monodisperse" latex, the Poly value lies between 0 and 0.05.[50] Poly values above 0.15 cannot be considered as absolute, however, relative comparisons between samples can still be performed below 0.50. Samples were diluted in a 1 mmol·L^{-1} NaCl solution before measuring the intensity of autocorrelation functions. Typically, five independent measurements

Scheme 1. Lipid-DT Synthesis from Precursor Dithioester 1 and Lipid-NH_3^+



were recorded to obtain a mean hydrodynamic diameter and a mean Poly value.

Results and Discussion

Lipid-DT Synthesis and Characterization. The strategy to synthesize a functional dithioester from a precursor dithioester and an aminated compound has been published previously.[42] That strategy was extended here to an ammonium carrying compound such as a Lipid-NH_3^+ (Scheme 1). As the latter is not reactive in the presence of the precursor dithioester **1**, triethylamine was added in the reaction mixture to induce its *in situ* deprotonation and the quantitative formation of a lipid-carrying dithioester (Lipid-DT). Most of the side-products (*N*-hydroxysuccinimide (NHS) and triethylammonium ion) were removed by aqueous washes. In comparison with the general purification procedure,[42] these washing steps were much longer due to the amphiphilic nature of the Lipid-DT. Silica gel column chromatography purification afforded a pure Lipid-DT with a final yield of 71%, similar to those reported for the synthesis, from the same precursor dithioester, of others biorelated CTAs (66−72%).[42]

The synthesis yields of others lipid-functionalized thiocarbonylthio compounds reported in the literature are in the same range or lower: 66% (without purification via silica gel chromatography) for the phospholipid-functionalized *N*,*N*-diethyldithiocarbamate iniferter (insensitive to aminolysis) synthesized by Kitano et al.,[35] and 15% (after purification) for a trithiocarbonate bearing two C18 alkyl chains synthesized by Kujawa et al.[51] In the latter case, this low yield is probably explained by the competitive aminolysis of the trithiocarbonate moiety (an excess of aminated lipid was used). Our strategy, based on the use of an activated ester and a stoechiometric amount of aminated-lipid, enables one to prevent this unwanted aminolysis.

Regarding the synthesis of lipid-carrying initiators for other CRP processes such as NMP and ATRP, three studies can be mentioned: (1) a *N*,*N*-di(octadecyl)amino-substituted alkoxyamine initiator, synthesized by Götz et al. (98% yield with a 4-fold excess of the initial lipid compound),[30] (2) various ATRP lipoinitiators based on monoalkyl and dialkyl *N*-substituted 2-bromopropionamide, prepared by Theato et al. (yield ranging from 42 to 55%),[33] (3) an ATRP initiator based on a "Span" compound (lipid-functionalized carbohydrate derivative), synthesized by Bon et al. (yield not mentioned).[31,32]

The structure of the Lipid-DT was confirmed by MALDI−TOF and FAB mass spectrometry (see experimental part) although these analyses do not give information about the nature of the initial counterion of the phosphate moiety because of the possibility of ion exchange (see Figure 2 in Supporting Information).^{1}H NMR analysis was also in agreement with the expected structure (Figure 1) and confirmed the good purity of the product. A significant signal that shifted upfield when temperature was raised to 40 °C, was observed at 2.1 ppm. It was related to the presence of water. Since the Lipid-DT had been thoroughly dried under vacuum and since that signal remained when anhydrous $CDCl_3$ was used for the NMR analysis, we suggested that water molecules were strongly associated with Lipid-DT molecules. According to the integral value,[52] there would be ∼1.35 H_2O molecules (or ∼0.9 H_3O^+ molecule) per Lipid-DT molecule.

Polymerization Mediated by Lipid-DT. Polymerization of NAM mediated by Lipid-DT (Table 1) was performed in dioxane at 90 °C in the presence of AIBN as initiator ([NAM] = 1.6 mol·L^{-1}, $[CTA]_0/[AIBN]_0$ = 10), using optimized conditions for *tert*-butyl dithiobenzoate.[46] Targeted M_n values at full conversion were 51 000, 22 200 and 9500 g·mol^{-1}, respectively, for runs P1, P2, and P3, to subsequently study a potential influence of the molar mass on the steric stabilization of the lipid/polymer particle assemblies. A reference experiment, P0, was performed under the same conditions as P1 except the use of Morpholine-DT.[42] Both CTAs are dithiobenzoates with

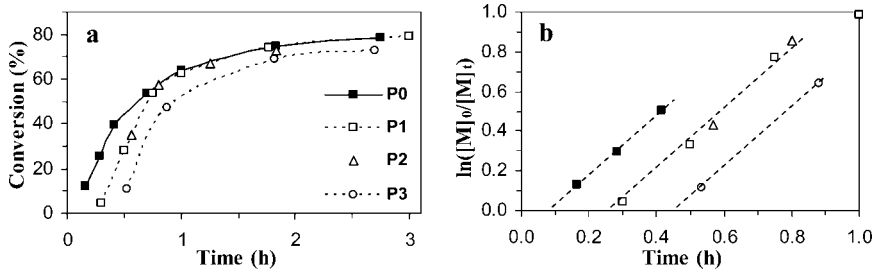
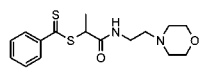
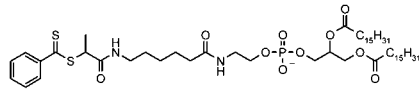


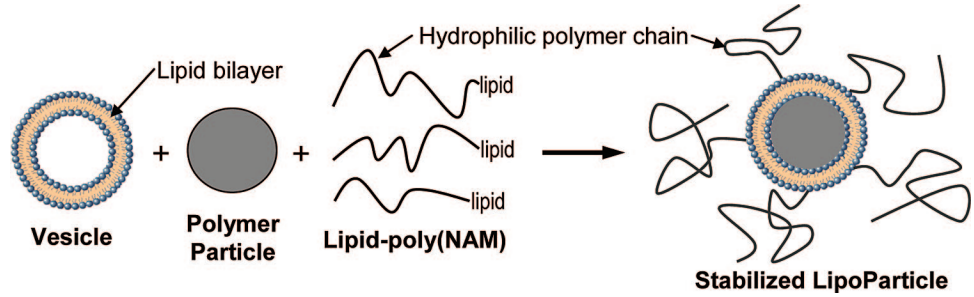
Figure 2. Kinetics plots (a) and ln($[M]_0/[M]$) vs time plots (b) for *N*-acryloylmorpholine (NAM) polymerization mediated by Morpholine-DT (P0; ■) or Lipid-DT (P1−3; □; △; ○) in dioxane at 90 °C targeting various molar masses at 100% conversion: 51 000 g·mol^{-1}(■ and □), 22 200 g·mol^{-1}(△); 9500 g·mol^{-1}(○); $[NAM]_0$=1.6 mol·L^{-1}, $[CTA]_0/[AIBN]_0$ = 10.

Table 1. RAFT Polymerization Conditions Used for Synthesis of the Lipid-Poly(NAM) Conjugates[a]

	CTA		Polymers
Exp	Name and structure	M_{CTA} (g.mol^{-1})	Targeted M_n** (g.mol^{-1})
P0	Morpholine-DT 	338	50, 300
P1	Lipid-DT	1012	51, 000
P2			22, 200
P3			9, 500

[a] Experimental conditions: solvent = dioxane; [NAM] = 1.6 mol·L^{-1}; $[CTA]_0/[AIBN]_0 = 10$; temperature = 90 °C. The double asterisk in the body of the table indicates targeted M_n at 100% conversion = $[NAM]_0/[CTA]_0 \times M_{NAM} + M_{CTA}$, where $[NAM]_0$ and $[CTA]_0$ are the initial concentration of monomer and chain transfer agent and M_{NAM} and M_{CTA} are their respective molecular weights.

Scheme 2. Schematic Representation of the Sterically Stabilized LipoParticles



a similar structure of the linking moiety of the R group. Then, comparison between P0 and P1 should reflect the possible effect of the phospholipid moiety on the RAFT polymerization.

NAM polymerization mediated by dithiobenzoates is retarded[45,46,53] as reported for several RAFT polymerizations mediated by various dithiobenzoates.[54−56] Here, the rate retardation effect seems similar whatever the dithiobenzoate (Figure 2b, same initial slope of the $\ln([M]_0/[M])$ versus time plots). However, the polymerizations mediated by Lipid-DT exhibit longer induction periods (~15 min for P1−P2, ~25 min for P3) than the reference experiment with Morpholine-DT (~6 min) or similar experiments with *tert*-butyl dithiobenzoate (~8−10 min).[46]

Since a strong reduction or even a disappearance of the induction period (where conversion remains below a few percents (2−3%)) was observed when a macro-CTA was used,[46,56,57] it has been related to the consumption period of the initial dithiobenzoate. However, the consumption period may be longer than the induction period. For instance, in experiment P3, traces of the initial CTA were detected by ^{1}H NMR up to 30% conversion (40−45 min) while the induction period is ~25 min. Hypothetical explanations are still subject to debate.[58]

In the case of a system exhibiting an induction period, a major influence of the reinitiation rate coefficient (k_i', see Scheme 2 in Supporting Information) on the duration of the CTA consumption period has been evidenced by McLeary et al.[59] Generally, a variation of electronic, polar and steric parameters of the reinitiating radical R° may modify k_i'. Considering the two dithiobenzoates synthesized from precursor **1**, their structural similarity in the close environment of the dithioester function (up to the δ position) implies an electronic similarity. Then, electronic effects are probably not modifying k_i' value to a significant extent. However, in the case of the Lipid-DT, not only the molecular weight of the corresponding R° is high (859 g·mol^{-1}) but the polymerization solvent (dioxane) is not the best for phospholipids (Lipid-DT is partially soluble in dioxane at room temperature whereas Morpholine-DT is fully soluble). Similarly to a polymer chain in a bad solvent, the alkyl chains of the lipid may collapse increasing the steric hindrance around R°. Consequently, k_i' value would be reduced.

If such a steric shielding is operative and reduces k_i' value, the propagation rate coefficient (k_p) would also be affected during the first monomer additions (k_{p1}, k_{p2},..., k_{pi}). Since poly(NAM) is in a good solvent in dioxane, the chain is not likely to collapse on the lipid chain-end. Hence, as chain grows, the oligoradical end moves away from the lipid moiety and the steric shielding effect is supposed to decrease. Actually, k_p is chain length dependent: after the first experimental evidence and *ab initio* molecular orbital calculations supporting the decrease of k_p for the addition of the first ~5 monomer units,[60,61] it has been found to be effective on a longer polymerization degree range (>100).[62,63] As proposed by Olag et al.,[62] the physical reasons would be a 'progressive shielding of the active center by the tailing rest of the growing chain'. Our above-mentioned hypothesis about a possible effect of the bulky lipid moiety on the rate coefficients may modulate the usual chain length dependence of k_p—with opposite consequences on k_{pi} as chain grows—for very low polymerization degrees.

Characterization of Lipid-α-End-Functionalized Chains. In aqueous solution, phospholipid end-functionalized poly(NAM) chains display an amphiphilic character and self-assemble.[64] As a result, the aqueous size exclusion chromatography/light scattering detection (ASEC/LSD) system previously used to characterize morpholine-, biotin- or sugar-functionalized poly(NAM) chains was not suitable here.[42,53] Contrary to some poly(acrylamide) derivatives such as poly(*N*-isopropyl acrylamide) and poly(*N*,*N*-diethyl acrylamide),[65−67] analysis of

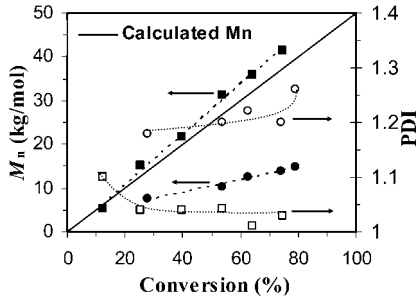


Figure 3. Evolution of molar masses (closed symbols) and polydispersity indexes (PDI, open symbols) versus conversion for *N*-acryloylmorpholine (NAM) polymerization mediated by Morpholine-DT (squares, exp. P0, data from ASEC/LSD analyses) or Lipid-DT (circles, exp. P1, data from SEC/THF analyses) in dioxane at 90 °C; $[NAM]_0 = 1.6$ mol·L^{-1}; $[CTA]_0/[AIBN]_0 = 10$; $[NAM]_0/[CTA]_0 = 355$; calculated $M_n = [NAM]_0/[CTA]_0 \times M_{NAM} \times$ conversion; ($M_{NAM} = 141$ g·mol^{-1}).

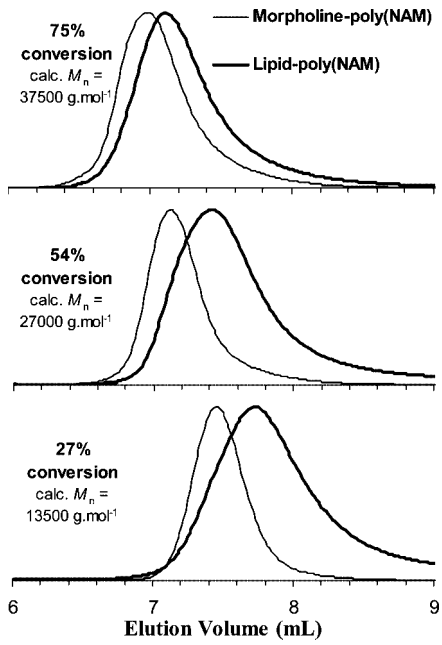


Figure 4. Evolution of the SEC chromatograms (eluent = THF, see Experimental Section) with conversion for NAM polymerization mediated by Morpholine-DT (normal line) or Lipid-DT (bold printed line) in dioxane at 90 °C; $[NAM]_0 = 1.6$ mol·L^{-1}; $[CTA]_0/[AIBN]_0 = 10$; $[NAM]_0/[CTA]_0 = 355$; calculated $M_n = [NAM]_0/[CTA]_0 \times M_{NAM} \times$ conversion ($M_{NAM} = 141$ g·mol^{-1}).

poly(NAM) samples by SEC in a THF phase seemed appropriate. Moreover, Lipid-poly(NAM) chains dissolve well in THF and calibration can be achieved with narrow poly(NAM) samples to analyze the Lipid-poly(NAM) samples (Figure 3). Results for experiment P1 indicate a linear increase of molar masses with conversion. However, the M_n values are significantly lower than the calculated ones and the PDI values (around 1.2) are higher than those obtained in the case of the Morpholine-poly(NAM) samples (P0). The question is whether there would be a loss of control of the polymerization when Lipid-DT is used as CTA?

Comparison of the SEC chromatograms of Morpholine- and Lipid-poly(NAM) samples at the same (increasing) conversions leads to two systematic observations (Figure 4): the chromatograms of Lipid-poly(NAM) are (1) shifted toward higher elution volumes (smaller apparent molar masses), and (2) broader than those of Morpholine-poly(NAM). Both effects are amplified at low conversion (i.e., for shorter chains where the lipid moiety gets more importance). Even for chains of relatively high molar masses, the lipid chain-end has a strong influence on the elution volume: at 75% conversion (calculated $M_n = 37\,500$ g·mol^{-1}), the lipid moiety accounts for less than 2 wt.−% of the whole chain but, according to Figure 3, the corresponding (apparent) M_n value is almost three times smaller than that of the Morpholine-poly(NAM) chains. Further analysis (using a triple detection: differential refractometer/light scattering/differential viscosimeter) indicated that the M_i value (molar mass of a thin chromatogram slice, n_i, $M_n = \sum(n_i \times M_i)/\sum n_i$) remained almost constant along the chromatogram trail.[68] These observations support the hypothesis of a retention phenomenon of the lipid-end-functionalized chains on the solid phase of the chromatographic system used.

Good solvation of the polymer chains is essential in SEC: by avoiding interactions of polymer chains with the solid phase (i.e., strongly limiting enthalpic partition of polymer species between the mobile phase and the solvated stationary phase), only entropic effects are operative in the partition equilibrium that leads to separation of the macromolecules in terms of their molecular size expressed as hydrodynamic volume.[69,70] A possible effect of the polymer chain-ends can be anticipated for chains of low molar masses. In our case, since THF is a poor solvent for lipid, interactions between lipid moieties and solvent molecules are probably too low to prevent interaction of the lipid moieties with the hydrophobic solid phase. Then, chromatographic analyses of the Lipid-poly(NAM) samples in THF result much probably from a combination of size exclusion chromatography (SEC) (entropic partition) and interaction chromatography (IC) (enthalpic partition).

Even if a moderate solvation of the lipid moiety would prevent interaction with the solid phase, conformation of the lipid-end-functionalized chains in THF is likely to be quite different from that of non-end-functionalized chains. According to Berek,[71] changes in macromolecule conformation may involve large entropic effects and then large differences in the hydrodynamic volume. This alone effect could make the poly(NAM) calibration curve unsuitable. The present study is an experimental confirmation that careful choice of the calibration curve must be done when α-functionalized chains are analyzed. According to the observed retention phenomenon, the obtained M_n values are strongly underestimated while the PDI values are overestimated (although still <1.25).

Consequently, another technique, MALDI−TOF mass spectrometry, was used to investigate the molar mass data (sample at 47% conversion, from P3). First, the results infirmed an eventual loss of control during polymerization since the main chain structure (determined in the reflectron mode) corresponds to chains with a lipid-α-end and a dithiobenzoate-ω-end, as expected. For instance, a molar mass of 4443.4 mass units was obtained for a calculated value of 4443.5 mass units (polymerization degree of 24; a detailed chain-end analysis will be published in a forthcoming paper). More interestingly, an experimental M_n value of 4900 g·mol^{-1} was determined on the spectrum in the linear mode (Figure 5), very close to the calculated one (5000 g·mol^{-1}) and with a low PDI value (1.10), confirming the efficient control of NAM RAFT polymerization mediated by Lipid-DT.

Steric Stabilization of Lipid/Polymer Particle Assemblies by Lipid-End-Functionalized Chains. The solid core of lipid/polymer particle assemblies (LipoParticles) confers to the adsorbed lipid layers a mechanical stability, a controlled morphology and a narrow size distribution. However, the major drawback of LipoParticles is their poor colloidal stability especially at relatively high ionic strength. Introduction of a given fraction of a modified lipid (with a hydrophilic polymer chain) in the formulation is expected to provide steric stabilization (Scheme 2), as already demonstrated for lipo-

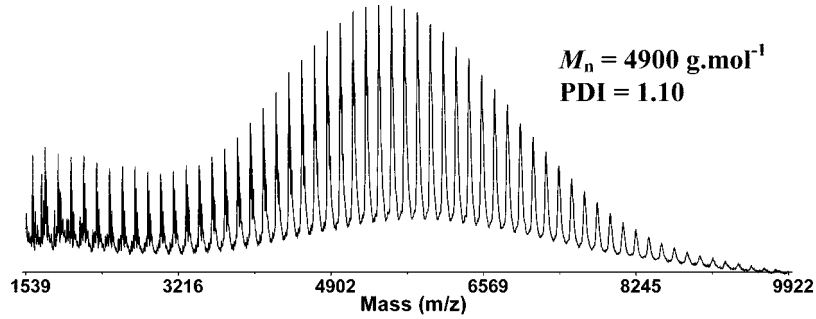


Figure 5. Positive ion MALDI−TOF mass spectrum in the linear mode (with CHCA matrix and NaI) of Lipid-poly(NAM) sample from experiment P3 (47% conversion, calculated M_n = 5000 g·mol^{-1}). Calculated M_n = $[NAM]_0 M_{NAM}$ × Conversion/$[CTA]_0$ + $M_{lipid\text{-}DT}$ (M_{NAM}=141 g·mol^{-1}; $M_{lipid\text{-}DT}$=1013 g·mol^{-1}).

Table 2. Mean Hydrodynamic Diameter (D_h) and Size Distribution (Poly) Values after 20 h in 300 mmol·L^{-1} NaCl Solution for Various Formulations of LipoParticles

modified LipoParticles			QELS data	
	polymer	mol %[a]	D_h (nm)	Poly
LP0	without lipid-polymer chains		2610	1.00[b]
LP1	$PNAM_{28}$	1	2150	1.00
LP2	$PNAM_{28}$	5	710	0.29
LP3	$PNAM_{\mathbf{109}}$	**1**	**490**	**0.16**
LP4	$PNAM_{109}$	5	950	0.92
LP5	$PEG_{\mathbf{113}}$	**5**	**350**	**0.15**

[a] Percentage in the initial formulation. [b] 1.00 = maximum value provided by the QELS technique (see Experimental Section).

somes.[14,23,72−74]

In order to evaluate the interest of Lipid-poly(NAM) conjugates as stabilization agents for LipoParticles, two Lipid-poly(NAM) samples of increasing chain length were tested, $PNAM_{28}$ (polymerization degree of 28 from polymerization P3) and $PNAM_{109}$ (polymerization degree of 109 from polymerization P2). They were compared to a Lipid-PEG (PEG_{113}), classically used.[14] Moreover, effect of the lipid-polymer fraction in the lipid formulation (1 or 5 mol %) was investigated. The size and size distribution data of the resulting assemblies after 20 h in a relatively high ionic strength solution (300 mmol·L^{-1} NaCl) are reported in Table 2.

At this relatively high ionic strength, the LipoParticles were truly instable and large aggregates were present (LP0). Addition of 1 mol % of $PNAM_{28}$ did not have any effect (LP1), however a significant improvement (smaller aggregates and lower polydispersity) was noticed when the amount of $PNAM_{28}$ was increased to 5 mol % (LP2). When using longer chains ($PNAM_{109}$), 1 mol % seemed enough to get an even better stabilization (LP3). In that case, an increase of the amount of Lipid-poly(NAM) from to 1 to 5 mol.−% did not bring additional stabilization (LP4). Finally, the comparison with Lipid-PEG indicated that 5 times less chains of $PNAM_{109}$ than PEG_{113} were necessary to get a similar reduction of polydispersity (LP3 and LP5), even after 4 days (data not shown). Such results using Lipid-poly(NAM) as a stabilization agent for LipoParticles are promising, especially since NAM may also be polymerized as a copolymer (by the RAFT process) which opens the way to introducing various molecules of interest along the polymer hairs at the periphery of the LipoParticles.

Conclusions

A new functional RAFT agent bearing a phospholipid moiety has been synthesized with a 71% yield after purification and characterized by FAB mass spectrometry, ^{1}H and ^{13}C NMR. This lipid−dithiobenzoate leads to an efficient control of the RAFT polymerization of *N*-acryloylmorpholine (NAM). In comparison with other functional dithiobenzoates,[42] an increase in the induction period has been observed, attributed to a reduced reinitiation rate coefficient due to the bulky lipid radical fragment. This is an additional example where a steric effect can induce modifications on the RAFT polymerization kinetics. For instance, it was recently pointed out in the case of NAM polymerization mediated by various functional CTAs, that steric effects induced by the nature of the reinitiating group could prevent an over-retardation phenomenon due to irreversible termination onto intermediate radicals.[53]

The resulting lipid-functionalized poly(NAM) chains exhibit an amphiphilic character and have been successfully used to stabilize LipoParticles assemblies in relatively high ionic strength aqueous solutions. Due to the large range of polymerizable monomers together with the potential of functionalization offered by the RAFT process, lipid-functionalized CTA open the way to easily adjust the surface properties of LipoParticles, as it was already achieved for liposomes (introduction of pH-responsive, temperature-responsive or recognition properties, for instance).[25,28,34,35]

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Supporting Information Available: Figures showing the ^{13}C NMR spectrum of purified Lipid-DT and positive ion MALDI−TOF mass spectrum of Lipid-DT and schemes showing the fragmentation of Lipid-DT in the spectrometer and the general RAFT mechanism. This information is available free of charge via the Internet at http://pubs.acs.org.

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