Macromolecules

ARTICLE

pubs.acs.org/Macromolecules

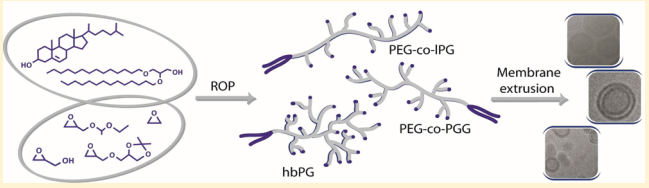
Rapid Access to Polyfunctional Lipids with Complex Architecture via Oxyanionic Ring-Opening Polymerization

Anna Maria Hofmann,[†] Frederik Wurm,[‡] and Holger Frey*[,†]

[†]Institut für Organische Chemie, Johannes Gutenberg-Universität, Duesbergweg 10-14, 55099 Mainz, Germany

[‡]Institut des Matériaux, Laboratoire des Polymères Batiment MXD, Ecole Polytechnique Fédérale de Lausanne (EPFL), Station 12, 1015 Lausanne, Switzerland

ABSTRACT: Polymer-coated liposomes, particularly poly(ethylene glycol) (PEG)-substituted liposomes, have emerged as long-circulating carrier systems for drug delivery and diagnostic purposes. A rapid synthesis of three different types of multifunctional lipids with structurally diverse hydrophilic, polyether-based architectures via one- or two-pot approaches is described. Architectural variation is achieved by the combination of different oxyanionic polymerization strategies and various glycidyl ether building units. Branched polyglycerol lipids have been prepared via cholesterol- or 1,2-bis-*n*-alkyl glyceryl ether-initiated, oxyanionic ring-opening polymerization (ROP) of protected glycidyl ethers and glycidol, respectively. In addition to these polyglycerol-based lipids, we describe the synthesis of multifunctional PEGs as the hydrophilic part of the lipid, which can be compared to conventional stealth lipids, but bear an adjustable number of hydroxyl functions within the PEG backbone. These lipids can be readily obtained by random copolymerization of ethylene oxide and protected glycidyl ethers, such as ethoxyethyl glycidyl ether (EEGE) and isopropylidene glyceryl glycidyl ether (IGG). Polydispersities M_w/M_n of the amphiphilic polyether structures were in the range of 1.04−1.2 for the linear structures and 1.1−1.6 for the hyperbranched lipids. Critical micelle concentrations (CMC) have been determined via the pyrene fluorescence method and were in the range of 1.4−40.7 mg/L, correlated to molecular weight and functionality of the polar polyether segment. Liposomes containing these hydroxy-functional lipids have been prepared via the membrane extrusion method and have been visualized by transmission electron microscopy (TEM) and cryo-TEM.



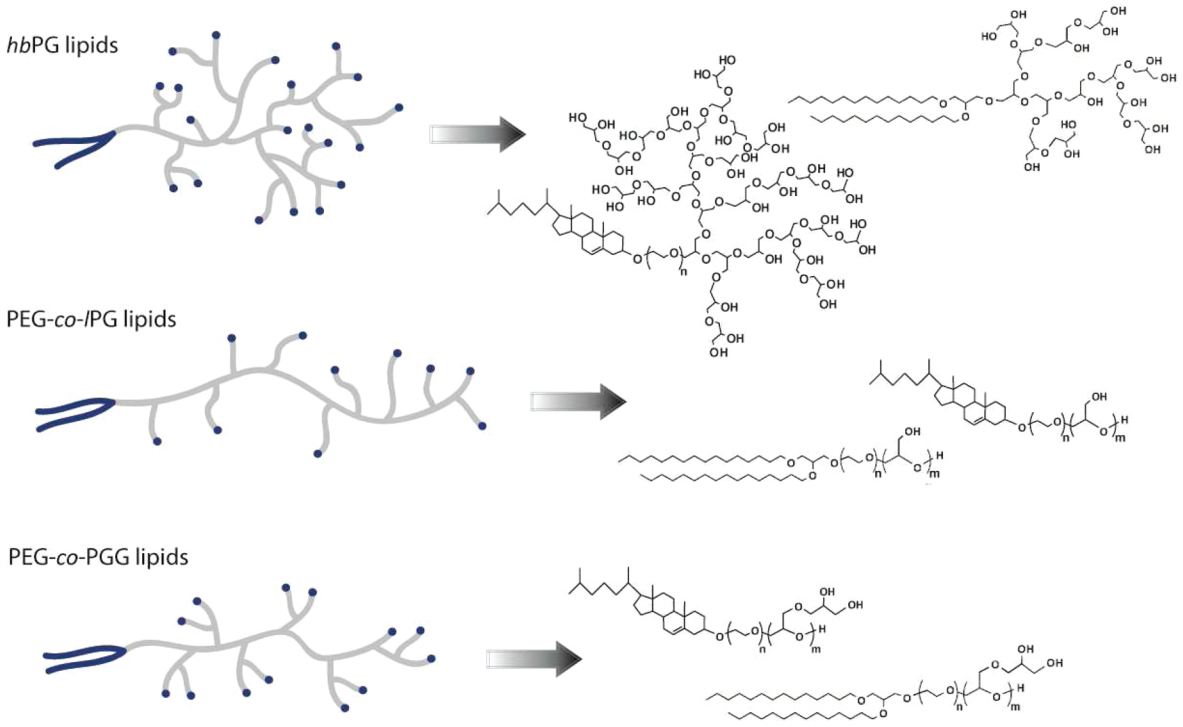
INTRODUCTION

Liposomes have emerged as versatile carrier systems for a variety of drugs, but intravenously administered they undergo fast opsonization and thus are rapidly cleared by macrophages of the mononuclear phagocyte system (MPS). Modification of liposomes with poly(ethylene glycol) (PEG) results in strongly reduced uptake by the liver and in prolonged blood circulation times of the drug carrier system due to the steric hindrance to the MPS.[1−8] Sterically stabilized liposomes have been shown to lower harmful side effects, to protect the active ingredient, and to transport high concentrations of a drug in specific tissues. Furthermore, PEG-coated liposomes show reduced vesicle aggregation and improved stability of the respective liposomal formulations. In addition to PEG, several other hydrophilic polymers have been reported as coatings for long-circulating liposomes, such as poly(oxazoline), poly(*N*-vinylpyrrolidone), poly(vinyl alcohol), poly(amino acid)s, and linear polyglycerol.[9−14] Besides the ability of these sterically stabilized liposomes to target active compounds passively to the site of action due to the enhanced permeability and retention (EPR) effect of solid tumors, the specific delivery of drugs to target sites has been studied in a number of works in the past decades and represents an important topic in ongoing research.[15−17] The attachment of functional ligands, like monoclonal antibodies, peptides, or folate, to the surface of liposomes can lead to efficient recognition and selective binding to specific cells. However, the design of liposomes with prolonged circulation times offering the possibility of facile coupling of targeting or labeling species to the lipids still represents a challenge. The use of commercially available linear PEG for the preparation of stealth-type structures requires postpolymerization derivatization to attach the polymer chain to lipids. Since coupling reactions often rely on tailored functionalities, research efforts have been focused on the synthesis of PEGs with different functional groups including carboxylic, amine, thiol, aldehyde, tosyl, epoxide, or succinimidyl succinate groups, either via modification of commercial PEG diol or PEG monomethyl ether (MPEG) or functional initiation or termination of the anionic ring-opening polymerization of ethylene oxide.[18−23] Further derivatization for labeling of the polymer or specific targeting of the liposomes can be necessary. In order to overcome the lack of multiple functionalities in the PEG chain, the number of end groups can be increased by dendronization of the chain ends. However, this approach often requires demanding multistep syntheses.[24−27]

Linear−hyperbranched block copolymers based on PEG and polyglycerol (PG) were synthesized via oxyanionic ROP of ethylene oxide and subsequent polymerization of protected

Received: February 17, 2011
Revised: May 6, 2011
Published: May 18, 2011

Scheme 1. Schematic Architectures and Chemical Structures of the Three Different Classes of Multifunctional Polyether Lipids Prepared via Lipid-Initiated Oxyanionic ROP: (i) *hb*PG Lipids, (ii) PEG-*co*-*l*PG Lipids, and (iii) PEG-*co*-PGG Lipids



glycidyl ethers, such as ethoxyethyl glycidyl ether (EEGE) or isopropylidene glyceryl glycidyl ether (IGG). After cleavage of the protecting groups, the linear precursor polymers can be used as macroinitiators for the ring-opening multibranching polymerization of glycidol.[28−30] While the above-mentioned synthetic strategies led to copolymers that bear functionalities in one block, random copolymerization of ethylene oxide with different protected glycidyl ethers provides hetero-multifunctional PEGs with functional groups at the polymer backbone.[31−34]

Very recently, we gave a first account of the synthesis of complex lipids based on linear-hyperbranched PEG-*b*-PG block copolymers, which were successfully incorporated in liposomal formulations with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as a colipid.[35] In the current work, we describe a rapid access strategy to three different types of polyglycerol-derived lipids via cholesterol- or 1,2-bis-*n*-alkyl glyceryl ether-initiated oxyanionic polymerization (Scheme 1). Such lipid structures are promising for the preparation of liposomal formulations and can be conveniently coupled to labeling or targeting moieties.[14,36−40] The polymer lipids with polyfunctional polyether structure and systematically varied multifunctional architectures have been included in liposomal membranes with DOPC as colipid. The resulting liposomes have been visualized via transmission electron microscopy (TEM) and cryo-TEM.

EXPERIMENTAL SECTION

Instrumentation. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AC 300 spectrometer operated at 300 MHz, employing $CDCl_3$ and DMSO-d_6 (dimethyl sulfoxide) as solvents. ^{13}C NMR spectra (referenced internally to solvent signals) were recorded at 100.15 MHz. FT-IR spectra were recorded on a Nicolet SDXC FT-IR spectrometer equipped with an ATR unit. SEC measurements were carried out in dimethylformamide (DMF) containing 0.25 g/L of lithium bromide. An Agilent 1100 Series GPC setup (gel permeation chromatography) was used as an integrated instrument, including a PSS HEMA column ($10^6/10^5/10^4$ g/mol), a UV-detector (254 nm), and an RI-detector. Calibration was achieved using poly(ethylene glycol) standards provided by Polymer Standards Service. The eluent was used at 50 °C and at a flow rate of 1 mL/min. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-ToF) measurements were performed on a Shimadzu Axima CFR MALDI-ToF mass spectrometer equipped with a nitrogen laser delivering 3 ns laser pulses at 337 nm. α-Cyano-3-hydroxycinnamic acid (CHCA) was used as a matrix. Samples were prepared by dissolving the polymer in methanol at a concentration of 10 g/L. A 10 μL aliquot of this solution was added to 10 μL of a 10 g/L solution of the matrix and 1 μL of a solution of potassium trifluoroacetic acid (KTFA) (0.1 M in methanol as cationization agent). A 1 μL aliquot of the mixture was applied to a multistage target, methanol evaporated, and a thin matrix/analyte film created. Electron microscopy measurements were performed using a transmission electron microscope (FEI, XM12) with an acceleration voltage of 120 kV.

Reagents. All reagents and solvents were purchased from Acros and used as received, unless otherwise mentioned. Cholesterol was purchased from Fluka and stored at 4 °C. Dry solvents stored over molecular sieves were purchased from Fluka. Deuterated $CDCl_3$ and DMSO-d_6 were purchased from Deutero GmbH, dried, and stored over molecular sieves. Ethoxyethyl glycidyl ether (EEGE) was prepared as described in the literature,[41−43] using glycidol and dried over CaH_2 directly before use. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 4.76 ($OCH(CH_3)O$), 3.35−3.90 (OCH_2CH_3 and $OCH_2C_2H_3O$), 3.15 (CH epoxide), 2.61−2.91 (CH_2 epoxide), 1.33 ($OCH(CH_3)O$), 1.19 (OCH_3). Isopropylidene glyceryl glycidyl ether (IGG) was prepared as described in the literature,[29] dried over CaH_2, and freshly distilled before

use. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 4.3 (m, 1H, CH acetal), 4.07 (m, 1H), 3.88−3.39 (m, 6H), 3.17 (m, 1H), 2.81 (t, 1H, CH_2 epoxide), 1.44 (s, 3H, CH_3), 1.38 (s, 3H, CH_3). Glycidol (99% Acros) was purified by distillation from CaH_2 directly prior to use.

Synthesis: Polymerizations. *a. Cholesterol−PGG Precursor Polymers.* Cholesterol was placed in a Schlenk flask, the appropriate amount of $CsOH \cdot H_2O$ (degree of deprotonation 90%) and benzene were added, and the mixture was stirred for 30 min at 60 °C in an argon atmosphere to generate the cesium alkoxide. The initiator was dried in vacuo at 90 °C and dissolved in dry diethylene glycol dimethyl ether under an argon atmosphere (20 wt %), and IGG was added via syringe. The polymerization was carried out for 12 h at 90 °C, a sample was removed for NMR and SEC characterization, and then the isopropylidene protecting groups were cleaved by addition of methanol, water, and acidic ion-exchange resin, stirring for 12 h at room temperature. Filtration and subsequent precipitation in cold diethyl ether resulted in the pure polymer that was dried in vacuo.

The 1,2-bis-*n*-alkyl glyceryl ether-initiated polymerizations could be accomplished in analogy to the previous procedures.

Cholesterol−Poly(isopropylidene glyceryl glycidyl ether). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 5.30 (C═CH cholesterol), 4.14 (m, CHO acetal), 3.96 (m, CH_2O acetal), 3.76−3.22 (polyether backbone; CHO cholesterol), 2.28−0.82 (br, CH_2, CH cholesterol), 1.31−1.26 (br, CH_3 acetal), 0.63 (br, CH_3 cholesterol). The DP_n of PIGG was determined by comparison of the methyl signal (0.63 ppm) of cholesterol with the signals of PIGG at a chemical shift of 4.14 ppm.

Deprotection to Cholesterol−Poly(glyceryl glycidyl ether). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 5.30 (C═CH cholesterol), 4.27 (br, OH), 3.76−3.22 (polyether backbone; CHO cholesterol), 2.28−0.82 (br, CH_2, CH cholesterol), 0.63 (br, CH_3 cholesterol).

1,2-Bis-n-alkyl Glyceryl Ether−Poly(isopropylidene glyceryl glycidyl ether). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 4.15 (m, CHO acetal), 3.97 (m, CH_2O acetal), 3.78−3.22 (polyether backbone; CH_2O, CHO initiator), 1.44 (br, CH_2CH_2O initiator), 1.31−1.26 (br, CH_3 acetal), 1.23 (br, CH_2 initiator), 0.85 (br, CH_3 initiator). The DP_n of PIGG was determined by comparison of methyl (0.85 ppm) and methylene (1.23 ppm) signals of the initiator with the signals for the PIGG block at a chemical shift of 4.15 ppm.

Deprotection to 1,2-Bis-n-alkyl Glyceryl Ether−Poly(glyceryl glycidyl ether). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 4.28 (br, OH), 3.78−3.22 (polyether backbone; CH_2O, CHO initiator), 1.44 (br, CH_2CH_2O initiator), 1.23 (br, CH_2 initiator), 0.85 (br, CH_3 initiator).

b. Hypergrafting of Glycidol. The PGG precursor polymer was placed in a Schlenk flask, $CsOH \cdot H_2O$ (degree of deprotonation 20−30%) and benzene were added, and the mixture was stirred at 60 °C for 30 min under argon atmosphere to generate the cesium alkoxide. After removal of the solvent in vacuo at 90 °C the macroinitiator was dissolved in dry diglyme (20 wt %), heated to 90 °C, and a 20 wt % solution of glycidol in diglyme was added slowly with a syringe pump. The reaction was terminated by addition of an excess of methanol and acidic cation exchange resin. The crude product was filtrated and precipitated into cold diethyl ether. The resulting material was assimilated in methanol and dried in vacuo.

Cholesterol−Hyperbranched Polyglycerol. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 5.30 (C═CH cholesterol), 4.77−4.43 (br, OH, different signals due to hyperbranched PG), 3.76−3.22 (polyether backbone; CHO cholesterol), 2.28−0.82 (br, CH_2, CH cholesterol), 0.63 (br, CH_3 cholesterol).

1,2-Bis-n-alkyl Glyceryl Ether−Hyperbranched Polyglycerol. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 4.77−4.43 (br, OH, different signals due to hyperbranched PG), 3.77−3.22 (polyether backbone; CH_2O, CHO initiator), 1.44 (br, CH_2CH_2O initiator), 1.23 (br, CH_2 initiator), 0.85 (br, CH_3 initiator).

c. Cholesterol−PEG-co-lPG. Cholesterol was placed in a Schlenk flask, the appropriate amounts of $CsOH \cdot H_2O$ (degree of deprotonation 90%) and benzene were added, and the mixture was stirred at 60 °C for 30 min in an argon atmosphere to generate the cesium alkoxide. The initiator was dried in vacuo at 90 °C, and dry tetrahydrofuran (THF) was cryo-transferred to suspend the deprotonated cholesterol. Ethylene oxide was cryo-transferred first to a graduated ampule and then to the Schlenk flask containing the initiator suspension. Subsequently, ethoxyethyl glycidyl ether (EEGE) was added to the reaction mixture via syringe. The polymerization was performed for 12 h at 90 °C in vacuo. After removal of a sample for NMR and SEC characterization the acetal protecting groups were cleaved by addition of methanol, water, and acidic cation exchange resin. The crude product was filtrated and precipitated into cold diethyl ether. The cholesterol-PEG-*co*-*l*PG was assimilated in methanol and dried in vacuo. The 1,2-bis-*n*-alkyl glyceryl ether-initiated polymerizations could be accomplished analogously to the previous procedures.

Cholesterol−Poly(ethylene glycol)-co-Poly(ethoxyethyl glycidyl ether). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 5.30 (C═CH cholesterol), 4.63 (br, CHO acetal), 3.72−3.21 (polyether backbone; CHO cholesterol), 2.28−0.82 (br, CH_2, CH cholesterol), 1.18−1.06 (br, CH_3 acetal), 0.64 (br, CH_3 cholesterol). DP_n of PEO-*co*-PEEGE was determined by comparison of methyl signal (0.64 ppm) of cholesterol with the signals of the polyether backbone (3.72−3.21 ppm) and the acetal proton at a chemical shift of 4.63 ppm.

Cholesterol−Poly(ethylene glycol)-co-Linear Polyglycerol. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 5.30 (C═CH cholesterol), 4.24 (br, OH), 3.72−3.21 (polyether backbone, CHO cholesterol), 2.28−0.82 (br, CH_2, CH cholesterol), 0.64 (br, CH_3 cholesterol).

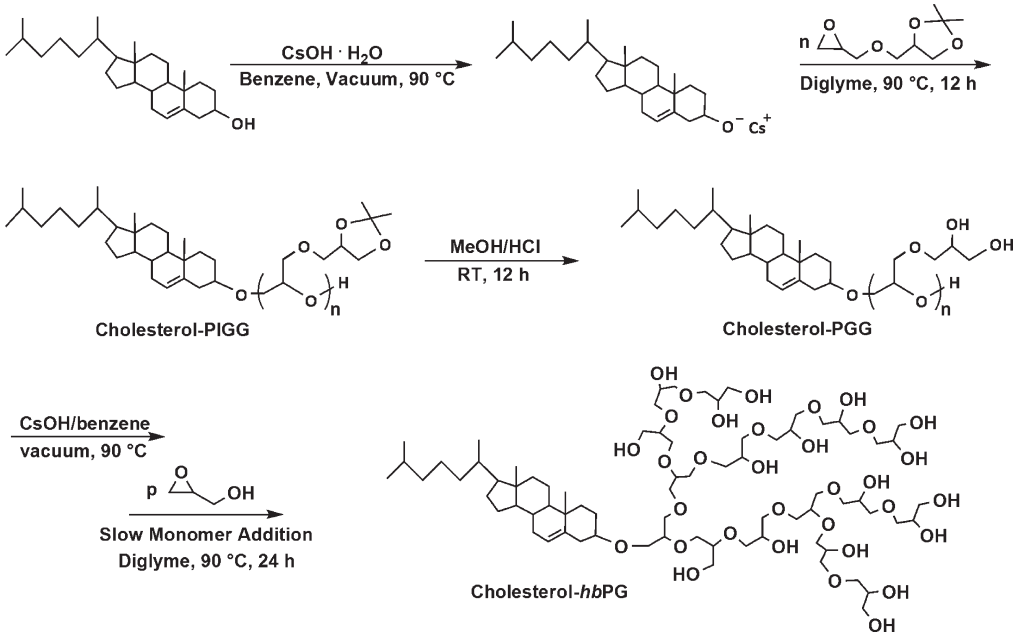
1,2-Bis-n-alkyl Glyceryl Ether−Poly(ethylene glycol)-co-Poly(ethoxyethyl glyceryl ether). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 4.63 (br, CHO acetal), 3.74−3.22 (polyether backbone; CH_2O, CHO initiator), 1.42 (br, CH_2CH_2O initiator), 1.18−1.06 (br, CH_3 acetal; CH_2 initiator), 0.80 (br, CH_3 initiator). DP_n of PEO-*co*-PEEGE was determined by comparison of methyl signals (0.80 ppm) of the initiator with the signals for the polyether backbone (3.72−3.21 ppm) and the acetal proton at a chemical shift of 4.63 ppm.

1,2-Bis-n-alkyl Glyceryl Ether−Poly(ethylene glycol)-co-Linear Polyglycerol. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 4.26 (br, OH), 3.74−3.22 (polyether backbone; CH_2O, CHO initiator), 1.42 (br, CH_2CH_2O initiator), 1.18−1.06 (br, CH_3 acetal; CH_2 initiator), 0.80 (br, CH_3 initiator).

d. Cholesterol−PEG-co-PGG. Cholesterol was placed in a Schlenk flask, and the appropriate amount of $CsOH \cdot H_2O$ (degree of deprotonation 90%) and benzene were added. Then the mixture was stirred for 30 min at 60 °C in an argon atmosphere to generate the cesium alkoxide. The initiator was dried in vacuo at 90 °C, and dry tetrahydrofuran (THF) was cryo-transferred to suspend the deprotonated cholesterol. Ethylene oxide was cryo-transferred first to a graduated ampule and then to the Schlenk flask containing the initiator suspension. Subsequently, IGG was added to the reaction mixture via syringe. The polymerization was performed for 12 h at 90 °C in vacuo. After removal of a sample for NMR and SEC characterization the isopropylidene protecting groups were cleaved by addition of methanol, water, and acidic cation exchange resin. The crude product was filtrated and precipitated into cold diethyl ether. The cholesterol−PEG-*co*-PGG was assimilated in methanol and dried in vacuo.

Cholesterol−Poly(ethylene glycol)-co-Poly(isopropylidene glyceryl glycidyl ether). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 5.31 (C═CH cholesterol), 4.15 (m, CHO acetal), 3.96 (m, CH_2O acetal), 3.70−3.21 (polyether backbone; CHO cholesterol), 2.28−0.82 (br, CH_2, CH cholesterol), 1.30−1.25 (br, CH_3 acetal), 0.64 (br, CH_3 cholesterol). DP_n of PEO−PIGG was determined by comparison of

Scheme 2. Reaction Sequence Developed for the Synthesis of the Series of Cholesterol-Initiated Hyperbranched Polyglycerol (*hb*PG) Lipids



methyl signal (0.64 ppm) of cholesterol with the signals of the polyether backbone (3.70−3.21) and the PIGG at a chemical shift of 4.15 ppm.

Deprotection to Cholesterol−Poly(ethylene glycol)-co-Poly(glyceryl glycidyl ether). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 5.31 (C═CH cholesterol), 4.50 (br, OH), 3.70−3.20 (polyether backbone; CH_2O, CHO cholesterol), 2.28−0.82 (br, CH_2, CH cholesterol), 0.63 (br, CH_3 cholesterol).

Derivatization Reactions. *a. Etherification with Propargyl Bromide.* The respective polymer was dissolved in dry toluene, and sodium hydride was slowly added at 0 °C. The reaction mixture was stirred for 30 min and after slow addition of propargyl bromide allowed to warm up to room temperature. The reaction was quenched with water after stirring at room temperature for 24 h. Removal of the solvent *in vacuo* and precipitation in cold diethyl ether resulted in the pure product.

Cholesterol-hbPG−$OCHH_2C{\equiv}CH$. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 5.30 (C═CH cholesterol), 4.77−4.43 (br, OH, different signals due to hyperbranched PG), 4.31−4.17 ($OCH_2C{\equiv}CH$), 3.76−3.22 (polyether backbone; CHO cholesterol), 2.45 (C≡CH), 2.28−0.82 (br, CH_2, CH cholesterol), 0.63 (br, CH_3 cholesterol).

b. Click Reaction. To a deoxygenized solution of cholesterol-*hb*PG−$OCHH_2C{\equiv}CH$ in DMF $CuSO_4$ and sodium ascorbate were added in catalytic amounts. After addition of azido-rhodamine B dissolved in DMF the reaction mixture was stirred at 80 °C for 48 h. The crude product was purified via dialysis in methanol/water (1:1).

Fluorescence Measurements/CMC Determination. Stock solutions were prepared by dissolving the amphiphilic polymers, which were placed in a volumetric flask, in water (Milli-Q) to give a final concentration of 500 mg/L. These stock solutions were diluted in order to yield solutions varying in polymer concentration from 500 to 0.01 mg/L. Pyrene dissolved in THF was added to the sample solutions in sufficient amount to give a pyrene concentration of 6×10^{-7} g/L. The samples were allowed to equilibrate for 24 h at room temperature prior to fluorescence measurements. Fluorescence measurements were carried out with an emission wavelength of 372 nm. The fluorescence intensities at excitation wavelengths of 339 and 335 nm have been determined.

Liposome Preparation. Liposomes consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and amphiphilic *hb*PG, PEG-*co*-*l*PG, and PEG-*co*-PGG copolymers were prepared via the membrane extrusion method. Solutions of DOPC (100 g/L) and the respective polymer (10 g/L) in chloroform were blended at a molar ratio of 98:2, and the organic solvents were removed under reduced pressure. The lipid film was hydrated with tris(hydroxymethyl)aminomethane/citrate (tris3citrate) buffer (50 mM, pH 7.2) to a final lipid concentration of 40 g/L. The mixture was vortexed to yield multilamellar vesicles (MLV), and subsequent extrusion of the solution afforded the liposome formulations.

■ RESULTS AND DISCUSSION

Synthesis and Characterization of Polyglycerol-Based Lipids. In a recent paper, we presented the first account of the synthesis of multifunctional lipids based on linear-hyperbranched PEG-*b*-PG block copolymers for liposomal application.[35] In the current work, several accelerated synthetic strategies have been developed that allow rapid access to complex, amphiphilic polyether architectures. The synthesis of a series of hyperbranched polyglycerol (*hb*PG)-derived lipids was carried out in a rapid two-pot approach using combined oxyanionic polymerization of different epoxide monomers (Scheme 2). In a first step the polymerization of isopropylidene glyceryl glycidyl ether (IGG), initiated directly with cholesterol or previously synthesized 1,2-bis-*n*-alkyl glyceryl ether, leads to a multifunctional amphiphilic macroinitiator after acidic cleavage of the acetal protecting groups. This linear structure can be used for the ring-opening multibranching polymerization of glycidol following a slow monomer addition protocol. The preparation of the linear precursor polymer is necessary to obtain optimal conditions for the hypergrafting of glycidol and to ensure low polydispersity. Partial deprotonation of the large number of hydroxyl groups in combination with the slow monomer

dx.doi.org/10.1021/ma200367c |*Macromolecules* 2011, 44, 4648–4657

Table 1. Characterization Data for Hyperbranched Polyglycerol Lipids (*hb*PG Lipids)

no.	polymer	initiator	M_n (th), g/mol	M_n[a] (NMR), g/mol	M_n[b] (SEC), g/mol	PDI[b]	CMC, mg/L
1	*hb*PG_{25}	cholesterol[c]	1900	2200	1300	1.20	1.4
2	*hb*PG_{30}	cholesterol[c]	2400	2600	1400	1.63	4.5
3	*hb*PG_{35}	cholesterol[c]	2800	3000	1500	1.18	8.3
4	*hb*PG_{42}	bis(tge)[c]	3400	3600	1600	1.10	15.5
5	*hb*PG_{31}	bis(hge)[c]	2700	3100	1500	1.51	40.7

[a] M_n determined via ^{1}H NMR spectroscopy. [b] M_n and M_w determined via SEC-RI in DMF with poly(ethylene glycol) standards. [c] CsOH used as initiator. tge = tetradecyl glyceryl ether, hge = hexadecyl glyceryl ether.

addition permits good control over the alkoxide concentration leading to growth of all chain ends, which narrows the molecular weight distribution and prevents undesired homopolymerization of the cyclic inimer glycidol. Since the concentration of alkoxides decreases in the course of the grafting reaction, a sufficient amount of deprotonated hydroxyl groups at the beginning of the polymerization, i.e., a multifunctional macroinitiator, is mandatory.[44] This synthetic approach resulted in a number of well-defined amphiphilic *hb*PG structures (Table 1), which show monomodal molecular weight distributions at all stages of the synthesis (Figure 2) and low polydispersities in a range of 1.1−1.6. The degree of polymerization was determined via ^{1}H NMR spectroscopy, integrating the methyl groups of the initiator (cholesterol: 0.63 ppm; 1,2-bis-*n*-alkyl glyceryl ether: 0.85 ppm) and the acetal protecting groups for the precursor polymers or the polyether backbone for *hb*PGs. With respect to potential applications of the amphiphilic hyperbranched polymers in liposomal formulations the molecular weights of the polymers were adjusted in the range of 2000−3000 g/mol, representing a DP_n of glycidol of 28−40. It is known that liposomes containing linear PEG in this molecular weight range show increased blood circulation times due to an effective protection of the liposome by a steric barrier as well as sufficient anchoring of the PEG-lipid in the liposomal membrane.[7,45−47]

Characterization data for all polymers are summarized in Table 1. Comparison of the molecular weights determined via SEC in DMF using PEG standards with the molecular weights obtained from ^{1}H NMR spectroscopy (Table 1) demonstrates a general underestimation by SEC. This is attributed to the strong influence of the hyperbranched, globular polyglycerol on the hydrodynamic radius of the polymers. Incorporation of the initiator moiety in every polymer chain formed is crucial for the amphiphilic character of the resulting lipids. Full incorporation was evidenced via MALDI-ToF mass spectrometry for all structures prepared (Figure 1). The use of lipophilic initiators, such as the commercially available cholesterol or 1,2-bis-*n*-alkyl glyceryl ethers, not only permits incorporation of the polymers in liposomal membranes but also simplifies the synthetic approach, since no additional postpolymerization derivatization with such units is required.

Synthesis and Characterization of PEG-*co*-*l*PG Lipids. Although the above-mentioned strategy allows good control over molecular weights and afforded low polydispersities for the amphiphilic *hb*PGs, we aimed at shortening the synthetic approach to multifunctional linear polymer−lipids to one single polymerization step. The random copolymerization of ethylene

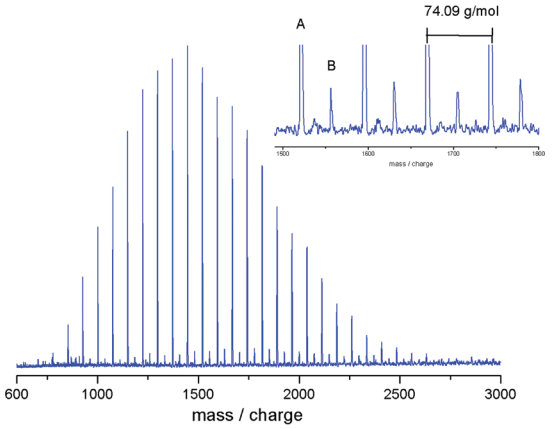


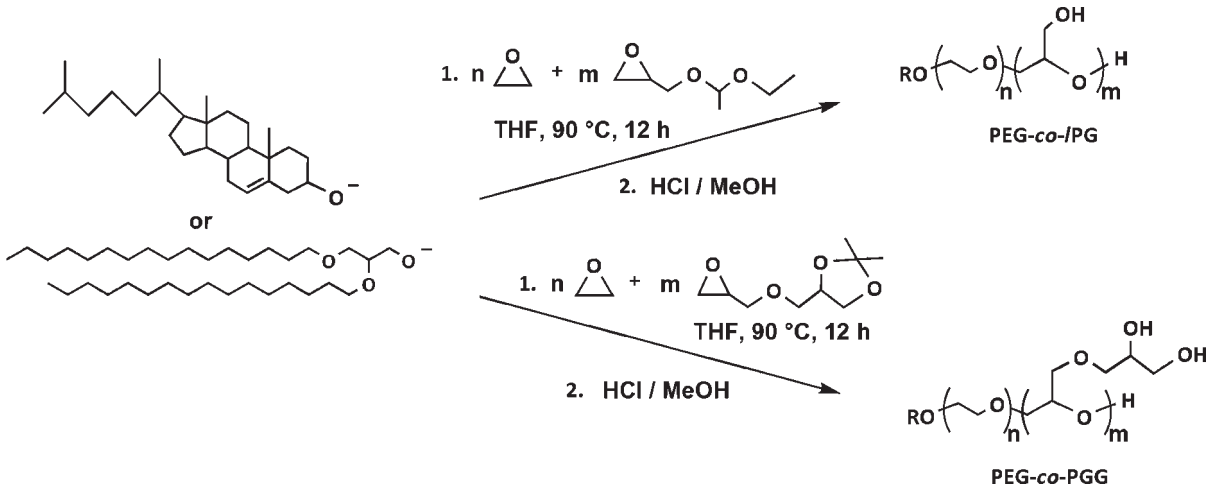
Figure 1. MALDI-ToF spectrum of amphiphilic hyperbranched polyglycerol (cf. compound **1**, Table 1) initiated with cholesterol. A: main distribution with sodium as a counterion. B: subdistribution with cesium as a counterion.

oxide with ethoxyethyl glycidyl ether (EEGE), recently studied in detail by our group,[32] offers several advantages in comparison to other strategies. In the field of drug-delivery systems and especially of sterically stabilized liposomes, poly(ethylene glycol) is one of the best examined and most often used polymers. The introduction of additional hydroxyl functions via random copolymerization of ethylene oxide and the protected glycidyl ether and acidic hydrolysis allows further derivatization with targeting or labeling moieties in analogy to the above-described amphiphilic hyperbranched polyglycerols. The synthesis of linear PEG-*co*-*l*PG lipids (Scheme 3) has been carried out using cholesterol or 1,2-bis-*n*-alkyl glyceryl ethers as initiator and potassium methanolate or cesium hydroxide monohydrate as deprotonating agent. The use of both initiating systems led to good control of molecular weights and the degree of functionalization. As expected for a linear copolymer, complex lipids based on PEG and linear PG with narrow molecular weight distributions were obtained. Polydispersities were generally lower (M_w/M_n < 1.2) than for the amphiphilic hyperbranched structures (Figure 2).

Characterization data of all PEG-*co*-*l*PG copolymer lipids are listed in Table 2. Again, the molecular weights determined via SEC in DMF using PEG standards are underestimated in comparison to the values determined by ^{1}H NMR spectroscopy. This can be explained by the different chemical structure of the copolymer lipids in comparison with the calibration standard PEG (Table 2). The influence of the unusual, complex architecture of the polymers on the hydrodynamic radii can lead to elution times varying with molecular weights as well as with molecular structure due to the amphiphilic character and high number of hydroxyl groups. This represents a problematic issue for molecular weight determination from SEC. This structural influence differs for PEG-*co*-*l*PG and PEG-*co*-PGG with varying molecular weights. However, the overall molecular weights of the copolymer lipids were adjusted to about 3000 g/mol in view of liposome formation.[7,45−47]

In contrast to the series of amphiphilic hyperbranched polyglycerol lipids, the degree of functionalization for the lipids presented in Table 2 can be varied independently of the molecular weight of the polymer. The composition of the PEG-*co*-*l*PG copolymers has been varied in the range of 18−55% glycerol incorporation.

Scheme 3. Reaction Sequence for the Synthesis of PEG-*co*-*l*PG Copolymer Lipids and PEG-*co*-PGG Copolymer Lipids Initiated with Cholesterol or 1,2-Bis-*n*-hexadecyl Glyceryl Ether



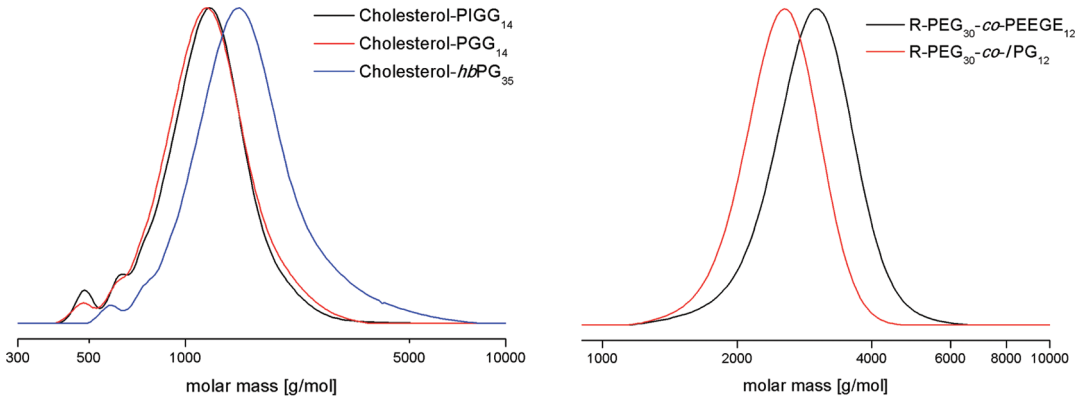


Figure 2. Left: SEC molecular weight distribution (RI detection, PEG standards) of PIGG initiated with cholesterol before (black) and after removal of the isopropylidene protecting groups (red) and after hypergrafting of glycidol (blue, cf. compound **3**, Table 1). Right: SEC molecular weight distribution (RI detection, PEG standards) of random PEG-*co*-*l*PG copolymers initiated with 1,2-bis-*n*-hexadecyl glyceryl ether (R) before (black) and after removal of the acetal protecting groups (red, cf. compound **6**, Table 2).

The degree of polymerization and functionalization was determined via ^{1}H NMR spectroscopy, integrating the methyl groups of the initiator (cholesterol: 0.63 ppm; 1,2-bis-*n*-alkyl glyceryl ether: 0.85 ppm), the acetal protecting groups of the comonomer, and the polyether backbone (Figure 3). The random distribution of the comonomer units within the PEG backbone was confirmed via ^{13}C NMR analysis, relying on the study of the triad sequence distribution, which evidence random incorporation of the monomers in the polymer chain.[32,48] As an example, Figure 4 shows the ^{13}C NMR spectra of 1,2-bis-*n*-tetradecyl glyceryl ether-PEG$_{30}$-*co*-*l*PG$_{20}$ (cf. compound **4**, Table 2) measured in DMSO-d_6, including the enhanced regions related to the signals of the PEG (E) and PG (G) backbone. The signal of the E-E-E triad (I), which is due to both methylene groups of an EO unit adjacent to two other EO units, exhibits an intensity similar to the signals of the E-E-G, G-E-E, and G-E-G triads, in which at least one glycerol unit is located adjacent to the central EO unit. This confirms the random incorporation of the functional comonomer within the polyether chain.

Synthesis of PEG-*co*-PGG Lipids. While the copolymerization of ethylene oxide and the above-mentioned ethoxyethyl glycidyl ether (EEGE) results in linear PEG structures with randomly distributed hydroxyl groups at the polymer backbone, the use of isopropylidene glyceryl glycidyl ether (IGG) leads to the incorporation of two adjacent hydroxyl groups in the resulting glycerol side chains for each comonomer in the polymer chain.[33] Thus, following an analogue synthetic approach, copolymers with varied architectures can be obtained by use of the comonomer IGG, which in addition to etherification or esterification of single hydroxyl functions permits the further derivatization of the two vicinal hydroxyl groups via reaction of ketones or aldehydes to cyclic acetal or ketal structures. Size exclusion chromatography was carried out for all samples, again demonstrating narrow molecular weight distributions with polydispersity indices within the range of 1.1−1.2 for this third class of polyether lipids (Table 2, compounds **8**−**13**). As expected, molecular weights obtained from SEC measurements in DMF (PEG standards) deviate from the values determined via ^{1}H NMR

Table 2. Characterization Data of PEG-*co*-*l*PG Lipids and PEG-*co*-PGG Lipids

no.	polymer	initiator	M_n (th), g/mol	M_n[a] (NMR), g/mol	M_n[b] (SEC), g/mol	PDI[b]	EEGEor IGG cont, %	CMC, mg/L
1	PEG_{20}-*co*-lPG_{25}	cholesterol[c]	2800	3100	1600	1.18	55	8.8
2	PEG_{32}-*co*-lPG_{18}	cholesterol[c]	3000	3100	1800	1.15	36	8.7
3	PEG_{36}-*co*-lPG_{35}	cholesterol[c]	3100	4600	2000	1.13	49	13.9
4	PEG_{30}-*co*-lPG_{20}	bis(tge)[d]	3000	3300	2000	1.07	40	15.6
5	PEG_{23}-*co*-lPG_{13}	bis(hge)[d]	2300	2500	2300	1.12	36	5.8
6	PEG_{30}-*co*-lPG_{12}	bis(hge)[d]	2600	2800	3100	1.04	29	12.1
7	PEG_{46}-*co*-lPG_{10}	bis(hge)[d]	3000	3000	3000	1.04	18	10.5
8	PEG_{19}-*co*-PGG_{9}	cholesterol[c]	2700	2600	1000	1.21	32	9.9
9	PEG_{30}-*co*-PGG_{8}	cholesterol[c]	2900	2900	1000	1.20	21	13.4
10	PEG_{18}-*co*-PGG_{12}	cholesterol[c]	3000	3000	2000	1.17	40	7.1
11	PEG_{29}-*co*-PGG_{11}	cholesterol[c]	2900	3300	1800	1.06	28	10.7
12	PEG_{12}-*co*-PGG_{17}	cholesterol[c]	3000	3400	1900	1.15	59	19.1
13	PEG_{12}-*co*-PGG_{18}	cholesterol[c]	3000	3600	1000	1.13	60	15.3

[a] M_n determined via ^{1}H NMR spectroscopy. [b] M_n and M_w determined via SEC-RI in DMF with poly(ethylene glycol) standards. [c] MeOK used as deprotonation agent. [d] CsOH used as a deprotonation agent. tge = tetradecyl glyceryl ether, hge = hexadecyl glyceryl ether.

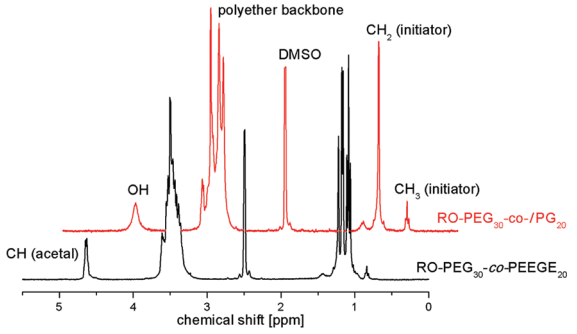


Figure 3. ^{1}H NMR spectra (300 MHz, DMSO-d_6) of PEG-*co*-*l*PG copolymer (cf. compound **4**, Table 2) with 1,2-bis-*n*-tetradecyl glyceryl ether as initiator before (bottom) and after (top) removal of acetal protective groups.

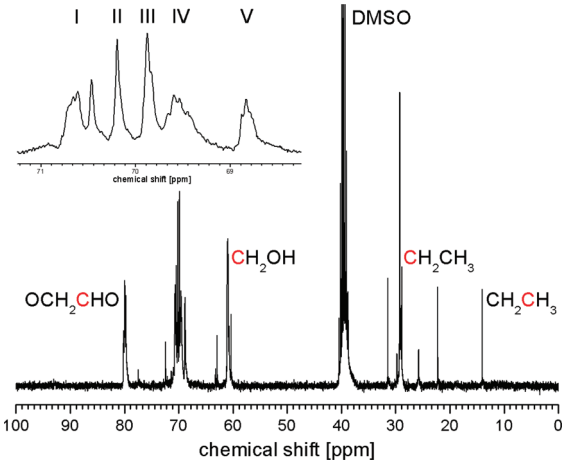


Figure 4. ^{13}C NMR spectra (75.5 MHz, DMSO-d_6) of PEG-*co*-*l*PG copolymer (cf. compound **4**, Table 2) with 1,2-bis-*n*-tetradecyl glyceryl ether as initiator. Inset for enlarged region: relevant EO centered triads, demonstrating random copolymerization.

measurements in DMSO-d_6. The underestimation of the values by SEC becomes more relevant with increasing amount of IGG. This can be attributed to a lower hydrodynamic radius despite incorporation of comonomers with a higher molecular weight. The degree of polymerization and the degree of functionalization were determined via ^{1}H NMR spectroscopy by integrating the methyl groups of the initiator (cholesterol: 0.63 ppm), the acetal protecting groups of the comonomer, and the polyether backbone. The molecular weights of the PEG-*co*-PGG copolymers were adjusted to 3000 g/mol, and the amount of comonomer was varied in the range of 21−60% IGG incorporation. Successful removal of the protecting groups was monitored by ^{1}H or ^{13}C NMR spectroscopy, since signals that are due to protons of the isopropylidene groups disappear after acidic hydrolysis. In analogy to the PEG-*co*-*l*PG lipids, the random distribution of the IGG units within the polymer was evidenced by investigation of the triad sequence distribution via ^{13}C NMR analysis.

Derivatization. Besides facile further modification the main advantage of the novel polymeric lipids is their large number of hydroxyl functionalities that clearly distinguishes them from "classical" stealth type PEG lipids. Although hydroxyl groups can be conveniently addressed by a variety of reactions, facile synthetic approaches under mild and neutral reaction conditions are often required. To this end, alkinyl-functionalized amphiphilic *hb*PGs, PEG-*co*-*l*PGs, and PEG-*co*-PGG have been prepared by etherification with propargyl bromide. Facile attachment of targeting or labeling moieties, such as rhodamine B, can be accomplished using the respective azide in an azido−alkyne Huisgen cycloaddition, as is shown in Figure 5. The amount of rhodamine B incorporated in the polyether lipids is determined by the number of alkyne functionalities. This is adjusted by the equivalent of propargyl bromide used in the etherification reaction and has been varied from an average of one to four functionalities. Obviously, these values represent average numbers of alkyne moieties that were controlled by the stoichiometry employed. Following this example, functional derivatives can be incorporated in liposomal formulations based on these novel polyether-modified lipids. Facile attachment of fluorescent dyes onto liposomal surfaces via the reaction of alkinyl-functionalized cholesterol−PEG-PGs and azido-derived fluorescent dyes has been carried out in order to evidence encapsulation of siRNA into polyether-modified liposomes. The preparation of siRNA

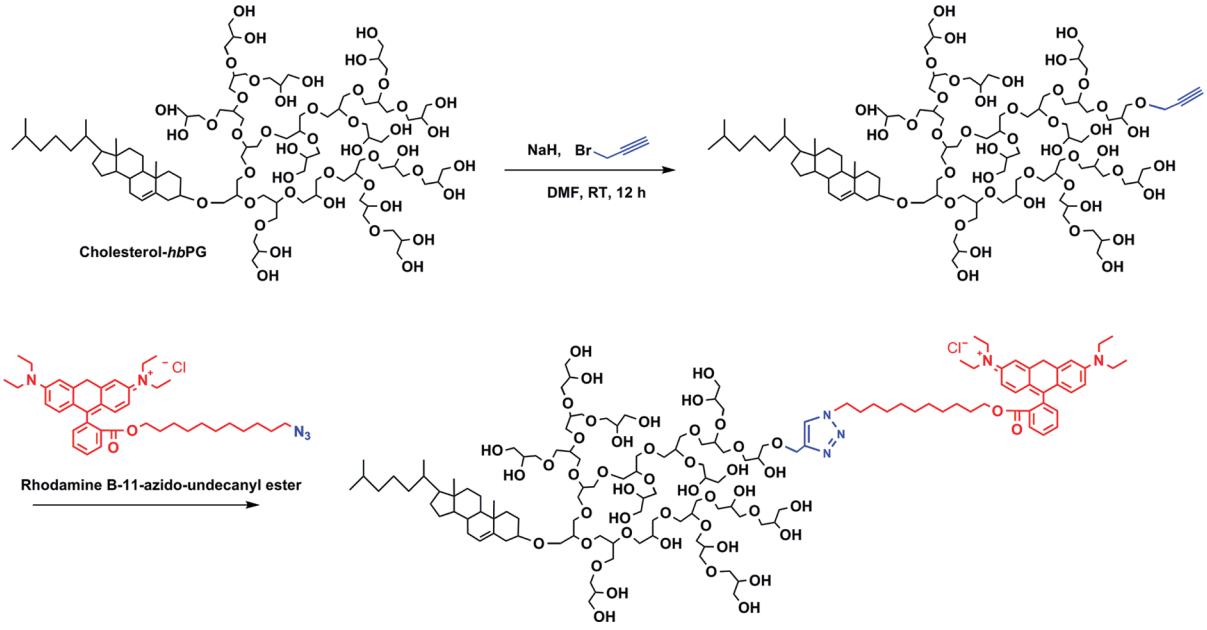


Figure 5. Reaction sequence for the synthesis of rhodamine B-labeled cholesterol-*hb*PG lipids via click chemistry.

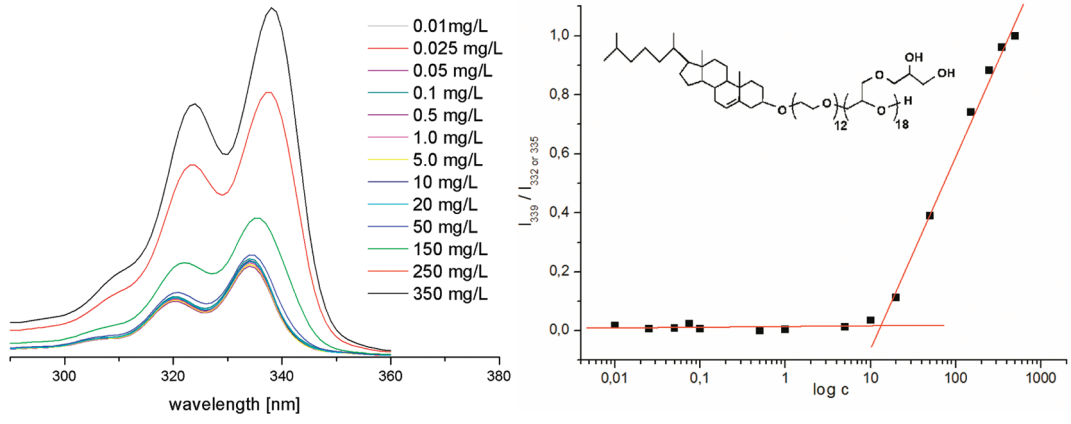


Figure 6. Left: excitation spectra of pyrene in water containing cholesterol-PEG$_{12}$-*co*-*l*PG$_{18}$ (cf. compound **13**, Table 2) at a concentration of 0.01 mg/L. Right: CMC determination using pyrene fluorescence excitation spectra at an emission wavelength of 372 nm.

complexes and a detailed investigation of their cellular uptake will be presented in a separate study devoted to cellular transport and targeting.

CMC Determination via Pyrene Fluorescence Measurements. At first glimpse, the hydrophilic part of the amphiphilic polyether structures developed may appear to be large in comparison to the respective hydrophobic segment. However, the degree of polymerization of the hydrophilic polyether segment was chosen to be analogous to PEG-based "stealth" lipids. In order to assess the amphiphilicity of the polyether structures developed in this work, the critical micelle concentrations (CMC) of all amphiphilic polymers and copolymers have been determined using an established fluorescence technique based on pyrene. This extremely hydrophobic dye is preferentially incorporated in the interior of micelles. The onset of micelle formation can be observed in a shift of the fluorescence excitation spectra of the samples at an emission wavelength of 372 nm.[49,50] In the concentration range of aqueous micellar solutions, a shift of the excitation band in the 335 nm region toward higher wavelengths confirms the incorporation of pyrene in the hydrophobic interior of micelles (Figure 6). The ratio of the fluorescence intensities at 339 and 335 nm was used to quantify the shift of the broad excitation band. The critical micelle concentrations determined from the crossover point in the low concentration range (Figure 6) are summarized in Table 1 (*hb*PG lipids) and Table 2 (PEG-*co*-*l*PG and PEG-*co*-PGG lipids). Generally, the measured CMCs of the random copolymer lipids are in the range of 5−20 mg/L. Since the EO units show similar hydrophilic properties as the glyceryl units and the ratios between the lipophilic and the hydrophilic fractions of all copolymers were adjusted in a similar range, only a slight increase of the CMC with increasing molecular weight can be noticed. This trend is most pronounced in the series of amphiphilic *hb*PG homopolymers. The CMC for the cholesterol-initiated hyperbranched polyglycerol lipids increases from 1.4 mg/L for *hb*PG$_{25}$ with a molar mass of 2200 g/mol to 4.5 mg/L for *hb*PG$_{30}$ (2600 g/mol) and to 8.3 mg/L for

dx.doi.org/10.1021/ma200367c |*Macromolecules* 2011, 44, 4648–4657

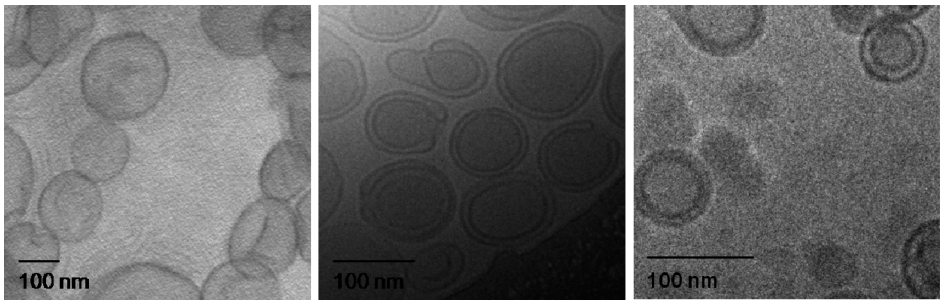


Figure 7. Left: TEM image of liposomes containing DOPC and cholesterol-PEG_{20}-*co*-*l*PG_{25} (compound **1**, Table 2) in a molar ratio of 98:2 (stained with uranyl acetate). Middle and right: cryo-TEM image of liposomes containing DOPC and cholesterol-PEG_{32}-*co*-*l*PG_{18} (compound **2**, Table 2) in a molar ratio of 98:2 (stained with uranyl acetate).

hbPG_{35} (3000 g/mol). The growing steric demand of the hyperbranched polyglycerol segment with increasing degree of polymerization leads to formation of micelles at higher concentrations. We tentatively ascribe this to improved solubility of the amphiphilic molecules due to a lower ratio of lipophilic and hydrophilic moieties.

Liposome Preparation and Characterization. Liposome formulations containing the amphiphilic polyglycerols or respectively the PEG-*co*-*l*PG and PEG-*co*-PGG copolymer lipids have been prepared via the membrane extrusion method. Solutions of DOPC (100 g/L) and the respective polymer (10 g/L) in chloroform were blended at a molar ratio of 98:2, and the organic solvents were removed under reduced pressure. The lipid film was hydrated with tris(hydroxymethyl)aminomethane/citrate (tris3citrate) buffer (50 mM, pH 7.2) to a final lipid concentration of 40 g/L. The mixture was vortexed to yield multilamellar vesicles (MLV), and subsequent extrusion and further dilution of the solution afforded the liposome formulations. The aqueous solutions were drop-cast onto hydrophilized copper grids and dried at room temperature before measurement. Cryo-TEM images (Figure 7) show that unilamellar liposomes could be formed with particle sizes varying in a range of 70−100 nm. In this manner all novel polyether lipids developed in this study, i.e., *hb*PG, PEG-*co*-*l*PG, and PEG-*co*-PGG based lipids, were incorporated into liposomal membranes. Both cholesterol and the 1,2-bis-*n*-alkyl glyceryl ethers are able to stabilize the attachment of the polyether lipids with molecular weights varying from 2200 to 4600 g/mol into lipid bilayers based on DOPC.

CONCLUSION

We have developed rapid 2−3 step syntheses for three different types of amphiphilic polyether-based complex lipids with architectural variation of the hydrophilic segment. The syntheses rely on the anionic ring-opening polymerization of EO, glycidol, and protected glycidyl ethers (EEGE or IGG) and can be performed in two-step protocols to obtain (i) amphiphilic *hb*PG lipids or in a single reaction step, generating (ii) amphiphilic linear multifunctional PEGs with either isolated or (iii) vicinal hydroxyl moieties at the backbone. Two vicinal hydroxyl groups have been incorporated in the PEG structure via random copolymerization of ethylene oxide with isopropylidene glyceryl glycidyl ether (IGG) and subsequent acidic hydrolysis, which opens options for derivatization via formation of cyclic acetals or ketals with the respective ketones or aldehydes. All strategies developed are based on the direct use of the lipophilic segment as initiator for the oxyanionic polymerization and generate multifunctional amphiphilic structures. Potentially toxic moieties like amines or aromatic linkages are avoided. As an example for further functionalization the hydroxyl groups have been derivatized with rhodamine B via a two-step protocol, based on etherification with propargyl bromide and subsequent click reaction with rhodamine B-azide.

We have also investigated the critical micelle concentrations (CMCs) of the complex lipids and were able to show that all lipids can be incorporated into liposomes, which were visualized via TEM and also by cryo-TEM. In a recent paper the properties of the complex polyether lipids described here have been studied with respect to Langmuir and Langmuir−Blodgett films, however, without description of the synthesis.[51]

Because of the analogy of the polymer architectures of these PEG-*co*-*l*PG lipids and conventional stealth lipids based on PEG, liposome formulations are expected to show similar stealth effects with respect to protein binding and blood circulation times, but furthermore can be easily modified, e.g., by etherification or esterification. Currently, we are studying the application of these lipids for targeted drug transport, emphasizing receptor-mediated delivery strategies.

AUTHOR INFORMATION

Corresponding Author

*E-mail: hfrey@uni-mainz.de.

ACKNOWLEDGMENT

The authors thank Daniel Loeper and Eric Hoffmann for their valuable technical assistance, Eva Hühn for liposome preparation, and Robert Branscheid for cryo-TEM measurements. A.M. H. acknowledges the graduate school of excellence MAINZ (Material Sciences in Mainz) for a fellowship and valuable financial support. F.W. is grateful to the Alexander von Humboldt Foundation for a Feodor Lynen fellowship at the EPFL.

REFERENCES

(1) Immordino, M. L.; Dosio, F.; Cattel, L. *Int. J. Nanomed.* **2006**, *1*, 297–315.

(2) Allen, C.; Dos Santos, N.; Gallagher, R.; Chiu, G. N. C.; Shu, Y.; Li, W. M.; Johnstone, S. A.; Janoff, A. S.; Mayer, L. D.; Webb, M. S.; Bally, M. B. *Biosci. Rep.* **2002**, *22*, 225–250.

(3) Allen, T. M.; Cullis, P. R. *Science* **2004**, *303* (5665), 1818–1822.

(4) Lasic, D. D.; Martin, F. J.; Gabizon, A.; Huang, S. K.; Papahadjopoulos, D. *Biochim. Biophys. Acta, Biomembr.* **1991**, *1070*, 187–192.

(5) Lasic, D. D.; Papahadadjopoulos, D. *Science* **1995**, *267*, 1275–1276.
(6) Klibanov, A. L.; Maruyama, K.; Torchilin, V. P.; Huang, L. *FEBS Lett.* **1990**, *268*, 235–237.
(7) Blume, G.; Cevc, G. *Biochim. Biophys. Acta, Biomembr.* **1990**, *1029*, 91–97.
(8) Lasic, D. D.; Needham, D. *Chem. Rev.* **1995**, *95*, 2601–2628.
(9) Woodle, M. C.; Engbers, C. M.; Zalipsky, S. *Bioconjugate Chem.* **1994**, *5*, 493–496.
(10) Torchilin, V. P.; Shtilman, M. I.; Trubetskoy, V. S.; Whiteman, K.; Milstein, A. M. *Biochim. Biophys. Acta* **1994**, *1195*, 181–184.
(11) Torchilin, V. P.; Levchenk, T. S.; Whiteman, K. R.; Yaroslavov, A. A.; Tsatsakis, A. M.; Rizos, A. K.; Michailova, E. V.; Shtilman, M. I. *Biomaterials* **2001**, *22*, 3035–3044.
(12) Takeuchi, H.; Kojima, H.; Yamamoto, H.; Kawashima, Y. *J. Controlled Release* **2001**, *75*, 83–91.
(13) Romberg, B.; Oussoren, C.; Snel, C. J.; Hennink, W. E.; Storm, G. *Pharm. Res.* **2007**, *24*, 2394–2401.
(14) Maruyama, K.; Okuizumi, S.; Ishida, O.; Yamauchi, H.; Kikuchi, H.; Iwatsuru, M. *Int. J. Pharm.* **1994**, *111*, 103–107.
(15) Roberts, W. G.; Palade, G. E. *Cancer Res.* **1997**, *57*, 765–772.
(16) Allen, T. M.; Sapra, P.; Moase, E.; Moreira, J. o.; Iden, D. *J. Liposome Res.* **2002**, *12*, 5–12.
(17) Dvorak, H.; Nagy, J.; Dvorak, J.; Dvorak, A. *Am. J. Pathol.* **1988**, *133*, 95–109.
(18) Pasut, G.; Veronese, F. M. *Prog. Polym. Sci.* **2007**, *32*, 933–961.
(19) Zalipsky, S. *Bioconjugate Chem.* **1995**, *6*, 150–165.
(20) Thompson, M. S.; Vadala, T. P.; Vadala, M. L.; Lin, Y.; Riffle, J. S. *Polymer* **2008**, *49*, 345–373.
(21) Herman, S.; Hooftman, G.; Schacht, E. J. *Bioact. Compat. Polym.* **1995**, *10*, 145–187.
(22) Vadala, M. L.; Thompson, M. S.; Ashworth, M. A.; Lin, Y.; Vadala, T. P.; Ragheb, R.; Riffle, J. S. *Biomacromolecules* **2008**, *9*, 1035–1043.
(23) Zeng, F.; Allen, C. *Macromolecules* **2006**, *39*, 6391–6398.
(24) Pasut, G.; Scaramuzza, S.; Schiavon, O.; Mendichi, R.; Veronese, F. M. *J. Bioact. Compat. Polym.* **2005**, *20*, 213–230.
(25) Choe, Y. H.; Conover, C. D.; Wu, D.; Royzen, M.; Gervacio, Y.; Borowski, V.; Mehlig, M.; Greenwald, R. B. *J. Controlled Release* **2002**, *79*, 55–70.
(26) Hawker, C. J.; Chu, F.; Pomery, P. J.; Hill, D. J. T. *Macromolecules* **1996**, *29*, 3831–3838.
(27) Taton, D.; Saule, M.; Logan, J.; Duran, R.; Hou, S.; Chaikof, E. L.; Gnanou, Y. *J. Polym. Sci., Part A: Polym. Chem.* **2003**, *41*, 1669–1676.
(28) Wurm, F.; Nieberle, J.; Frey, H. *Macromolecules* **2008**, *41*, 1184–1188.
(29) Wurm, F.; Nieberle, J.; Frey, H. *Macromolecules* **2008**, *41*, 1909–1911.
(30) Wurm, F.; Klos, J.; Räder, H. J.; Frey, H. *J. Am. Chem. Soc.* **2009**, *131*, 7954–7955.
(31) Koyama, Y.; Umehara, M.; Mizuno, A.; Itaba, M.; Yasukouchi, T.; Natsume, K.; Suginaka, A.; Watanabe, K. *Bioconjugate Chem.* **1996**, *7*, 298–301.
(32) Mangold, C.; Wurm, F.; Obermeier, B.; Frey, H. *Macromol. Rapid Commun.* **2010**, *31*, 258–264.
(33) Mangold, C.; Wurm, F.; Obermeier, B.; Frey, H. *Macromolecules* **2010**, *43*, 8511–8518.
(34) Obermeier, B.; Wurm, F.; Frey, H. *Macromolecules* **2010**, *43*, 2244–2251.
(35) Hofmann, A. M.; Wurm, F.; Hühn, E.; Nawroth, T.; Langguth, P.; Frey, H. *Biomacromolecules* **2010**, 568–574.
(36) Kainthan, R. K.; Janzen, J.; Levin, E.; Devine, D. V.; Brooks, D. E. *Biomacromolecules* **2006**, *7*, 703–709.
(37) Kainthan, R. K.; Janzen, J.; Kizhakkedathu, J. N.; Devine, D. V.; Brooks, D. E. *Biomaterials* **2008**, *29*, 1693–1704.
(38) Wilms, D.; Stiriba, S.-E.; Frey, H. *Acc. Chem. Res.* **2010**, *43*, 129–141.
(39) Calderón, M.; Quadir, M. A.; Sharma, S. K.; Haag, R. *Adv. Mater.* **2009**, *22*, 190–218.
(40) Jones, A. T.; Gumbleton, M.; Duncan, R. *Adv. Drug Delivery Rev.* **2003**, *55*, 1353–1357.
(41) Fitton, A. O.; Hill, J.; Jane, D. E.; Millar, R. *Synthesis* **1987**, 1140–1142.
(42) Tokar, R.; Kubisa, P.; Penczek, S.; Dworak, A. *Macromolecules* **1994**, *27*, 320–322.
(43) Dworak, A.; Walach, W.; Trzebicka, B. *Macromol. Chem. Phys.* **1995**, *196*, 1963–1970.
(44) Sunder, A.; Hanselmann, R.; Frey, H.; Mulhaupt, R. *Macromolecules* **1999**, *32*, 4240.
(45) Ahl, P. L.; Bhatia, S. K.; Meers, P.; Roberts, P.; Stevens, R.; Dause, R.; Perkins, W. R.; Janoff, A. S. *Biochim. Biophys. Acta, Biomembr.* **1997**, *1329*, 370–382.
(46) Allen, T. M.; Hansen, C. *Biochim. Biophys. Acta, Biomembr.* **1991**, *1068*, 133–141.
(47) Bedu-Addo, F. K.; Huang, L. *Adv. Drug Delivery Rev.* **1995**, *16*, 235–247.
(48) Hamaide, T.; Goux, A.; Llauro, M. F.; Spitz, R.; Guyot, A. *Angew. Makromol. Chem.* **1996**, *237*, 55–77.
(49) Wilhelm, M.; Zhao, C. L.; Wang, Y.; Xu, R.; Winnik, M. A.; Jean Luc Mura, G. R.; Croucher, M. D. *Macromolecules* **1991**, *24*, 1033–1040.
(50) Wolf, F. K.; Hofmann, A. M.; Frey, H. *Macromolecules* **2010**, *43*, 3314–3324.
(51) Reuter, S.; Hofmann, A. M.; Busse, K.; Frey, H.; Kressler, J. *Langmuir* **2011**, *27*, 1978–1989.