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Synthesis and characterization of PEGylated bolaamphiphiles with enhanced retention in liposomes

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ABSTRACT

Long-circulating liposomes are typically prepared with poly(ethylene glycol)- (PEG)- modified lipids, where the lipid portion is inserted in the lipid bilayers as an anchor and the hydrophilic PEG coats the surface to prevent lipidome aggregation and rapid clearance in vivo. However, these steric protection effects are compromised upon systemic administration due to low retention of PEGylated lipids within lipidome membranes upon dilution. Hence, a series of PEGylated bolaamphiphiles (PEG-bolas) were for the first time developed to increase retention in the lipid bilayer, presumably leading to enhanced integrity of the PEG protective layer upon dilution. We hypothesized that PEG-bolas with a sufficiently long hydrophobic domain and rigid central group could predominantly adopt a membrane-spanning configuration, taking full advantage of steric protection offered by PEG and enhanced retention in liposomes enabled by the bola geometry. In this paper, liposomes stabilized by PEG-bolas comprised of a biphenyl core and twelve-carbon alkyl chain not only exhibited similar storage and biological stability compared to conventional PEGylated lipid stabilized liposomes, but also significantly improved retention upon dilution. Our findings facilitate new designs of lipidome-stabilizing agents and can be applied to improve the delivery efficiency of liposomal delivery vehicles in vivo.

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1. Introduction

Liposomes are spherical, enclosed bilayers primarily composed of phospholipids. They have received extensive attention as a promising class of localized drug delivery vehicles that can effectively reduce drug toxicity and increase drug accumulation at pathological sites [1–4]. In addition, liposomes can be utilized to encapsulate both hydrophobic and hydrophilic drugs, in the lipid bilayer and internal aqueous compartment respectively, as biocompatible drug carriers [5]. However, poor colloidal stability (i.e., liposome aggregation) and biological stability (i.e., fast elimination from blood circulation) hamper their wider application as drug delivery systems [6–8].

The formulation of sterically stabilized liposomes, which were coated with poly(ethylene glycol) (PEG), drastically extended their circulation half-life in blood [9,10]. The flexible PEG segments form a hydrophilic spatial barrier that prevents both particle aggregation in vitro and serum protein adsorption, which cause fast blood clearance by the reticuloendothelial system (RES) [6,11,12]. While surface modification of liposomes with PEG can be achieved in different ways, the most widely used approach is to incorporate the PEGylated phosphatidylethanolamine (PE) (e.g., N-(Carbonyl-methoxy(polyethylene glycol))-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, PEG-DPPE, Fig. 1A) during liposome preparation. The lipid portion of PEG-PEs is embedded within the hydrophobic domain of the lipid bilayers as an anchor, with the PEG portion effectively extending to the aqueous bulk solution [6]. As the PEG head group has a much larger size than the hydrophobic acyl moiety, PEG-PEs have superior water-solubility compared to unmodified phospholipids, resulting in diffusion from lipid bilayers upon dilution [13–15]. This dissociation behavior seriously undermines the integrity and stabilizing effects of the PEG coating, leading to premature liposomal drug release and reduced delivery efficiency [15].

Bolaamphiphiles (bolas) have two polar head groups connected by one or two long alkyl chain spacers. Archaeabacteria have membranes rich in bolas and are found to show superior membrane integrity towards harsh conditions compared to conventional phospholipids [16,17]. The unusual stability of the archaeabacteria membrane was ascribed to the bolas that extend completely across the membrane and act as “rivets” to keep the membrane bilayer intact. This property can be attributed to the high activation barrier against pulling the inner hydrophilic end groups translocating through the membrane’s hydrophobic interior [18,19]. Some attempts to incorporate bolas for preparing stable liposomes have shown great promise owing to their good miscibility with lipids and preference for stretched conformation [20–23]. Despite these desirable properties, investigation of bolas has been largely limited as it is difficult to isolate bolas from natural membranes in large quantities; synthetic bola mimics are therefore desired [17,24].

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Alternatively, PEGylated bolas (PEG-bolas, Scheme 1), which leverage the steric stabilization effects of PEG and robust retention in the lipid bilayer elicited by bola geometry, can be used as liposome stabilizing agents to prepare liposomes with long-lasting PEG coatings. To design and synthesize PEG-bolas, amphiphilic macro-molecules (AMs) (Fig. 1B) with structural similarity to PEGylated lipids were used as the key building blocks. Previously, Tao et al. successfully demonstrated AM’s ability to stabilize liposomes compared to conventional PEG-DPPE, with improved synthetic efficiency [25]. As such, PEG-bolas were synthesized by coupling two AM molecules through their free carboxylic acid groups (Scheme 1). To promote the membrane-spanning orientation in liposomal membranes over U-shaped conformation (Fig. 1C), rigid aromatic groups including benzene (BZ) and biphenyl (BP) were introduced between the hydrophobic domains of AMs for PEG-bolas (Scheme 1) [19,26]. In addition, the lengths of alkyl chains connecting AMs were varied to match the thickness of model 1,2-dipalmitoylphosphatidylcholine (DPPC) liposomes lipid bilayers. It was hypothesized that PEG-bolas with sufficiently long hydrophobic domains could preferentially span the DPPC lipid bilayer with firm association. To test the feasibility of using liposomes stabilized by PEG-bolas as drug carriers, the colloidal and biological stability were systematically evaluated.

2. Materials and methods

2.1. Materials

All reagents and solvents were purchased from Sigma-Aldrich (Milwaukee, WI) and used as received unless otherwise indicated. DPPC and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (sodium salt, PEG-DPPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Lissamine™ rhodamine B 1,2 dihexadecanoyl-sn-glycero-3-phosphoethanolamine (triethylammonium salt, Rh-PE) was purchased from Molecular Probes, Inc. (Eugene, OR). Uranyl acetate was purchased from Electron Microscopy Sciences (Hatfield, PA). Polytetrafluoroethylene (PTFE) syringe filters were purchased from Fisher Scientific (Fair Lawn, NJ). RPMI 1640 media was purchased from ATCC (Manassas, VA).

2.2. Characterization

Proton (1H) nuclear magnetic resonance (NMR) spectra were recorded on a Varian 400 MHz spectrophotometer. Samples (~5 mg/mL) were dissolved in deuterated chloroform (CDCl3) with trimethylsilane (TMS) as an internal reference. The average weight (Mw) and polydispersity indices (PDI) of PEG-bolas and their precursors were determined by gel permeation chromatography (GPC) using a Waters LC system (Milford, MA) equipped with a PLgel
MIXED column (Agilent, Santa Clara, CA). Samples were dissolved at 10 mg/mL in high performance liquid chromatography (HPLC) grade dichloromethane (DCM) and filtered through 0.45 μm PTFE syringe filters prior to injection at a flow rate of 1.0 mL/min. WaterBreeze v3.20 software was used for data collection and processed against a calibration curve derived from broad molecular weight PEG standards (Waters, Milford, MA).

2.3. Synthesis

Polymer 1 was prepared as previously published using 2 kDa PEG [27].

Synthesis of 2. Using 2a as an example, 1 (100 mg, 0.040 mmol) and hydroxyl succinimide (NHS, 5.0 mg, 0.044 mmol) were dissolved in 2 mL anhydrous DCM and 0.2 mL anhydrous dimethylformamide. N,N′-dicyclohexylcarbodiimide (DCC, 1 M in DCM, 0.044 mL) was added dropwise under argon and the reaction stirred for 1 h. In a separate flask, 1,10-diaminodecane (42 mg, 0.24 mmol) was suspended in 0.2 mL DMF and NHS-activated 1 prepared in situ was added via a syringe pump at 1.0 mL/h. The reaction was then allowed to stir for an additional 24 h. The reaction mixture was cooled to −20 °C to remove the insoluble side product dicyclohexylurea (DCU) via vacuum filtration. The filtrate was washed with 0.1 N HCl (2 × 15 mL), saturated sodium bicarbonate (2 × 15 mL), and brine (2 × 20 mL). The combined organic layers were dried over magnesium sulfate (MgSO₄), filtered, and concentrated in vacuo. The crude product was purified by precipitation into chilled diethyl ether (3 × 15 mL) and isolated via centrifugation at 3500 rpm for 5 min (Hettich EBA 12, Beverly, MA).

2a. Yield: 91 mg, 88% (white powder). 1H NMR (400 MHz, CDCl₃): δ 6.67 (br, 1H), 6.08 (br, 1H), 5.64 (dd, 2H) 3.58 (br, ~180 H), 3.31 (s, 3H), 3.16 (m, 2H), 2.92 (m, 2H), 2.34 (m, 4H), 1.61 (m, 4H), 1.42 (m, 4H), 1.18 (br, 44H), 0.81 (t, 6H). Mₚ, 2.6 kDa; PDI, 1.1.

2b. Yield: 200 mg, 94% (white powder). 1H NMR (400 MHz, CDCl₃): δ 6.64 (br, 1H), 6.10 (br, 1H), 5.60 (dd, 2H) 3.64 (br, ~180 H), 3.38 (s, 3H), 3.20 (m, 2H), 2.93 (m, 2H), 2.39 (m, 4H), 1.61 (m, 4H), 1.41 (m, 4H), 1.25 (br, 44H), 0.88 (t, 6H). Mₚ, 2.6 kDa; PDI, 1.1.

Synthesis of 3. Using 2-3a as an example, 2a (81 mg, 0.030 mmol) and terephthaloyl chloride (3.0 mg, 0.015 mmol) were suspended in 2 mL anhydrous DCM. Triethylamine (TEA, 8.4 μL, 0.06 mmol) was added, and the reaction stirred overnight. The reaction mixture was washed with 0.1 N HCl (2 × 10 mL), saturated sodium bicarbonate (2 × 15 mL), and brine (2 × 20 mL). The crude product was purified by precipitation into chilled diethyl ether (3 × 15 mL) and isolated via centrifugation at 3500 rpm for 5 min (Hettich EBA 12, Beverly, MA). The product was further purified by placing in a Spectra/Por dialysis tubing (molecular weight cutoff, MWCO 3.5 kDa, Spectrum Laboratories, Inc., Compton, CA) and dialyzing against deionized water for 48 h. The dialyzed solution was then lyophilized to obtain pure L-3.

BZ-3a. Yield: 65 mg, 81% (white powder). 1H NMR (400 MHz, CDCl₃): δ 8.11 (br, 2H), 7.84 (s, 4H), 6.86 (br, 2H), 6.22 (br, 2H), 5.60 (dd, 4H) 3.55 (br, ~360 H), 3.38 (s, 6H), 3.23 (m, 8H), 2.39 (m, 4H), 1.63 (m, 8H), 1.48 (m, 8H), 1.25 (br, 76H), 0.88 (t, 12H). Mₚ, 6.6 kDa; PDI, 1.2.

BZ-3b. Yield: 46 mg, 81% (white powder). 1H NMR (400 MHz, CDCl₃): δ 8.11 (br, 2H), 7.83 (s, 4H), 6.72 (br, 2H), 6.13 (br, 2H), 5.61 (dd, 4H) 3.55 (br, ~360 H), 3.38 (s, 6H), 3.23 (m, 8H), 2.40 (m, 4H), 1.63 (m, 8H), 1.48 (m, 8H), 1.26 (br, 80H), 0.88 (t, 12H). Mₚ, 6.5 kDa; PDI, 1.3.

BP-3a. Yield: 63 mg, 72% (white powder). 1H NMR (400 MHz, CDCl₃): δ 8.09 (d, 2H), 7.80 (d, 2H), 7.62 (m, 4H), 6.66 (br, 2H), 6.12 (br, 2H), 5.54 (dd, 4H) 3.58 (br, ~360 H), 3.31 (s, 6H), 3.16 (m, 8H), 2.33 (m, 4H), 1.54 (m, 8H), 1.42 (m, 8H), 1.19 (br, 76H), 0.81 (t, 12H). Mₚ, 6.6 kDa; PDI, 1.1.

BP-3b. Yield: 146 mg, 80% (white powder). 1H NMR (400 MHz, CDCl₃): δ 8.09 (d, 2H), 7.81 (d, 2H), 7.65 (m, 4H), 6.68 (br, 2H), 6.06 (br, 2H), 5.54 (dd, 4H) 3.59 (br, ~360 H), 3.31 (s, 6H), 3.16 (m, 8H), 2.33 (m, 4H), 1.56 (m, 8H), 1.40 (m, 8H), 1.19 (br, 80H), 0.83 (t, 12H). Mₚ, 6.7 kDa; PDI, 1.1.

2.4. Langmuir monolayer

The surface pressure (π)-area (A) isotherms of PEG-bolases were obtained using a Langmuir surface balance from KSV-Nima (Espoo, Finland) on a subphase of ultra-pure water (resistivity ≥ 18.2 MΩ cm) at ambient temperature (~25 °C). PEG-bolase were dissolved in HPLC grade chloroform at 1 mg/mL. For all experiments, the Teflon trough (Biologic Scientific, MD) and barriers were cleaned with chloroform and rinsed thoroughly with ultra-pure water. The subphase surface was cleaned by aspirating during repeated sweeps of the computer-controlled barriers until negligible π changes were observed. PEG-bolase were spread onto the subphase surface using a digital high precision Hamilton syringe (Ren, NV). After a 10 min delay to allow for complete solvent evaporation, the films were compressed at a rate of 10 mm/min. The surface pressure π was monitored following a Wilhelmy-plate method using a filter paper connected to an electrobalance. Data were collected by KSV-Nima’s LB Control software (v. 3.60).

2.5. Liposome preparation

Liposomes were prepared via a film-extrusion method adapted from established procedures [25]. Briefly, PEG-bolase and DPPC lipids were co-dissolved in chloroform at desired molar ratios (0%, 2%, 4%, 6%), and the solvent removed in vacuo. When necessary, cholesterol (CHO) and Rh-PE was included in this step at 30% and 0.2% respectively. The mixtures were further dried under high vacuum overnight. To the dried lipid film, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM, pH adjusted to 7.4 with 0.1 M NaOH) was added to hydrate and resuspend the lipids at 60 °C. The hydrated lipid films were subjected to five freeze-thaw cycles, freezing in dry ice (~78 °C) and heating in water bath (60 °C) alternatively. Large unilamellar vesicles (LUV) were prepared by extruding 15 times through a 100 nm polycarbonate membrane (Sigma-Aldrich, Milwaukee, WI) using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL) at ~56 °C.

2.6. Liposome morphology and size

Liposomes were visualized by transmission electron microscopy (TEM) with negative staining. A drop of the liposome suspension (~0.1 mg/mL) was deposited onto on a carbon film-coated copper grid. After 60 s, excess solution was removed by tapping the edge of grid with filter paper. A drop of 1% uranyl acetate solution was then applied to the same grid for 60 s. The grid was again tapped dry and further dried in the desiccator overnight. Images were taken on JEOL 1200EX electron microscope (JOEL USA, Inc, Pleasanton, MA).

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posome particle sizes and PDI were assessed by dynamic light scattering (DLS) using a NanoZS90 instrument (Malvern Instruments, Southboro, MA) at room temperature. Each sample was run at a 90° scattering angle in triplicate with 30 measurements per run.

The colloidal stability of various liposome formulations was studied at 4 °C, room temperature, and 37 °C for up to 8 weeks. All experiments were conducted in triplicate. The particle size and PDI of liposomes were monitored by DLS at predetermined time points and reported as mean ± standard deviation of the mean. Results were analyzed by multiple comparison with two-way ANOVA using built-in statistical analysis function of Prism 6 (GraphPad Software Inc., La Jolla, CA). Significance criteria assumed a 95% confidence level (p < 0.05). Standard deviation is reported in the form of error bars.

2.7. Retention of PEG-bolas upon dilution

The retention of PEG-bolas in DPPC liposomes upon dilution was investigated by 1H NMR spectroscopy with modified procedures [13,28]. The study was performed with DPPC liposomes with and without 2 mol% stabilizing agents (i.e., PEG-bolas and PEG-DPPE) at 37 °C. Liposome samples (30 mM) were diluted 10 times with HEPES buffer under stirring and transferred into a Slide-A-Lyser cassette (MWCO 20 kDa, ThermoFisher Scientific, Waltham, MA), where the unincorporated PEG-bolas were removed by dialysis against HEPES buffer. An aliquot of diluted liposomes (1.5 mL) was taken at predetermined time points for up to 48 h. The samples were lyophilized and then dissolved in CDCl3 for NMR characterization. The ratio of the choline methyl proton of DPPC (∼3.4 ppm) to the methylene proton of PEG (∼3.6 ppm) was obtained by 1H NMR spectroscopy before and after dilution, which correlated to the incorporated ratio of stabilizing agents to lipids in the bilayers. The result was normalized to the initial incorporated ratio and reported as % PEG-bolas retention.

2.8. Uptake of PEG-bolas stabilized DPPC liposomes by macrophages

The phagocytic uptake of PEG-bolas stabilized DPPC liposomes was determined with human monocytes derived macrophages (HMDMs). HMDMs were isolated and cultured from human buffy coats (New York Blood Center, Long Island City, NY) as previously published [29]. For fluorescence microscopy, 3.75 × 105 cells were seeded in 8-well Lab-Tek™ and incubated at 37 °C for 24 h prior to use. DPPC/CHO liposomes (with or without 6 mol% liposome stabilizing agents) containing 0.2 mol% Rh-PE were suspended in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 300 μM and an aliquot (250 μL) was added to designated wells. Following 5 h incubation, the cells were washed with cold phosphate buffered saline (PBS, pH 7.4) three times and fixed with 1% paraformaldehyde before imaging on a Leica TCS SP2 confocal microscope (Leica Microsystems, Buffalo Grove, IL).

3. Results and discussion

3.1. PEG-bolas synthesis and characterization

The primary goal of this work was to design and synthesize novel PEG-bolas that could effectively span lipid bilayers, which was accomplished coupling two AMs together with a hydrophobic domain as shown in Scheme 1. As only bolas with sufficiently long hydrophobic domains (i.e., molecular length of bolas’ hydrophobic portion close to membrane thickness) can extend across membrane [26], diaminoalkanes with varying alkyl chain lengths (C10 and C12) were used as building blocks. In addition, central rigid groups (i.e., phenyl and biphenyl) were introduced into PEG-bolas’ hydrophobic domains to promote a membrane-spanning conformation over a U-shaped conformation [19,26].

To ensure monocoupling of 1, diaminoalkane was used in large excess amount (6 eq.) and NHS-activated 1 (1 eq.) was added slowly with diluted concentration. The chemical structure of amine-terminated 2 was confirmed by 1H NMR, as indicated by the appearance of methylene next to the amine at ∼3.2 ppm, while GPC was used to assess molecular weight and verify the monoconjugation of diaminoalkane. To synthesize PEG-bolas (L-3), 2 eq. of 2 was reacted with 1 eq. of acyl chlorides of aromatic central linkers in the presence of TEA. The appearance of aromatic peaks (7.5–8.5 ppm) together with a roughly twofold molecular weight increase corroborated the successful synthesis of L-3, and narrow polydispersities (PDI < 1.3) indicated the complete conversion of the reaction.

3.2. Monolayer study

The behavior of four different PEG-bolas at the air/water interface was studied to shed light on their preferential conformations (see Fig. 2) [25,30]. Initially, the surface pressure of PEG-bola monolayers gradually increased over a relatively large surface area with compression (i.e., reduction of area per molecule), which was considered to be a process to compress the entangled PEG chains [13]. The following steep increase in surface pressure was observed at 360 Å²/molecule from BZ-linked PEG-bolas with 650 Å² and 1250 Å² noted for BP-3a and BP-3b, respectively. This change indicated the phase transition from expanded liquid phase to compressed solid phase. For PEG-bolas based on a phenyl linker with two PEG segments (2 kDa), their molecular areas were less than 2-fold smaller than that of PEGylated lipids with the same molecular weight (260 Å²) [13]. Thus, it is possible these components are not sufficiently rigid and can adopt bent-shaped (i.e., U-shaped) conformations where PEG chains are compressed to form a condensed structure. In contrast, BP-based PEG-bolas had molecular areas larger than 520 Å² as mentioned above. These differences are likely due to their long hydrophobic domains in extended conformation conferred by the rigid biphenyl linker, such that molecular area is not limited by the maximum cross-sectional area of the PEG chain.

![Fig. 2. π – A isotherms of PEG-bola monolayers at the air/water interface (curves are composites measured using low-to-high surface concentrations of PEG-bolas).](image-url)
3.3. Liposome colloidal stability

PEG-bolas were then incorporated into DPPC liposomes as stabilizing agents. Retaining uniform liposome sizes upon storage is crucial in developing robust drug delivery vehicles as an increase in particle size (>200 nm) generally results in rapid capture by RES in vivo with reduced circulation time and altered biodistribution [31]. In addition, liposome aggregation can cause premature drug release and compromise delivery efficiency [32]. Therefore, the colloidal stability of DPPC liposomes stabilized by PEG-bolas was evaluated for up to 8 weeks by DLS. Furthermore, the influence of PEG-bolas’ structural parameters on their liposome-stabilizing effects was established.

DPPC liposomes with or without varied mol% of PEG-bolas (2%, 4%, 6%) were prepared systematically via a well-established film-extrusion method [25]. The highest incorporation ratio was kept under 8% to avoid potential phase separation of the liposome membrane or micelle formation [6,33]. The incorporation of PEG-bolas at different mol% had marginal influence on their particle sizes as shown in Fig. 3A. While all fresh formulations displayed particle sizes between 150 and 165 nm with narrow size distribution (PDI < 0.2), liposomes with more than 2% of PEG-bolas resulted in slightly larger sizes. The visual turbidity of samples coupled with TEM images ascertained the successful preparation of uniform LUVs.

To compare the physical stability of liposomes made from different PEG-bolas, the liposomes were stored at refrigerated temperature (4 °C), room temperature, and 37 °C respectively in triplicate, and their particle sizes as a function of storage time was monitored. The liposome-stabilizing effects of PEG-bolas were compared to the commercially available PEGylated lipid PEG-DPPE [34,35]. At 4 °C, all PEG-bolas stabilized formulations, except for ones stabilized by BZ-3a, exhibited comparable stability to control PEG-DPPE (data not shown). Size increased less than 6% for up to 8 weeks. At this storage temperature, even 2% stabilizing agent provided sufficient steric stabilization to prevent appreciable particle aggregation and fusion.

In contrast, under physiological temperature, liposome size changed to varying degrees for different formulations. For example, liposomes stabilized by 6% BZ-3a and BP-3a, which were comprised of same decacyl chains, experienced notable size differences by 8 weeks (Fig. 4). In particular, the size rapidly increased from 164 nm to 179 nm after 1 week and eventually grew to 230 nm by week 8 for BZ-3a, whereas no considerable size increase happened until week 4 for BP-3a, suggesting better protection effects of PEG-bolas with BP linker. Notably, when comparing different alkyl chain lengths (BZ-3a vs. BZ-3b, BP-3a vs. BP-3b), PEG-bolas with dodecyl chain were more effective at preventing liposome aggregation upon long time storage. Similar to control PEG-DPPE, sizes of liposomes stabilized by BZ-3b and BP-3b remained constant in the experiment, which is highly desirable for delivery systems. Collectively, the results suggested both alkyl lengths and central linker types had substantial influences on PEG-bolas’ stabilizing efficiency; PEG-bolas comprised of dodecyl chains and/or biphenyl linker exhibited better stabilizing effects. Similar trends were also observed under room temperature.

3.4. Retention of PEG-bolas in liposome upon dilution

The dissociation behavior of PEGylated lipids from liposomal membranes upon drastic dilution (i.e., systemic administration) severely compromises the integrity of PEG coatings on liposome surfaces, negatively impacting their performance in vivo [13–15]. As such, the retention of PEG-bolas was studied using DPPC liposomes with 2% PEG-bolas, and the incorporation ratio was examined before and after 10-fold dilution with buffer following literature procedures [13], which mimics extensive dilution. This low incorporation ratio could minimize the PEG chain-chain entanglement on the liposome surface, and thus better reflect the strength of interactions between PEG-bolas and lipid bilayers [6,36]. Due to the poor colloidal stability of liposomes stabilized by BZ-3a, this compound was not further investigated.

As shown in Fig. 5A, the methylene proton of PEG was well resolved from choline methyl proton of DPPC and thus their relative ratio was used to calculate the incorporation ratio, which was normalized to 100% at time zero and presented as % polymer retention (Fig. 5B). Upon 10-fold dilution, PEG-DPPE experienced a 10% instant loss at 4 h while negligible dissociation was observed for all PEG-bolas. By 48 h, only 83% of PEG-DPPE remained embedded within DPPC liposomes. This effect is consistent with data reported by Tao et al. as PEGylated lipids with small hydrophobic domains suffer from poor retention on dilution [25]. In contrast, PEG-bolas exhibited enhanced retention in lipid bilayer and underwent much slower dissociation. Specifically, BP-3b with a dodecyl chain and biphenyl linker maintained the highest incorporation ratio (94%) over 48 h, highlighting its promise as a liposome stabilizing agent for use in vivo to minimize premature drug release and improve delivery efficiency. It is plausible that the PEG-bolas with biphenyl linkers are more likely to adopt membrane-spanning conformation and dodecyl chain provides a longer hydrophobic domain that fits better within the DPPC lipid bilayer.

![Fig. 3. Particle sizes of freshly prepared DPPC liposomes stabilized by different ratios of 3-4. (A) and representative TEM images of DPPC liposomes stabilized with BP-3b at 6% (B).](image-url)
3.5. Biological stability of liposomes

A surface modification with a PEG layer is commonly used to provide steric protection of liposomes, preventing serum protein ab.

Fig. 4. Particle sizes of DPPC liposomes stabilized by 6% PEG-bolas or PEG-DPPE upon storage at 37 °C for 8 weeks.

Fig. 5. 1H NMR spectrum of BP-3b in DPPC liposomes with peaks used for incorporation ratio estimation (A). Retention of polymers incorporated at 2% in DPPC liposomes after 10-fold dilution with HEPES buffer (B).

Fig. 6. Fluorescent images of HMDMs showing uptake of liposomes labeled with 0.2% Rh-PE after 5 h incubation. Control DPPC/CHO (A) and DPPC/CHO stabilized with 6% PEG-DPPE (B) and BP-3b (C).
4. Conclusion

In this work, PEG-bolas were for first time proposed and successfully synthesized as PEGylated lipid alternatives in preparing highly stable liposomes. The colloidal stability of DPPC liposomes stabilized with various PEG-bolas, coupled with their retention ability, implies that both central rigid core and the alkyl chain length had substantial influences on their potential as a candidate for producing long-circulating properties, and therefore, this work facilitates a new design and structure of a candidate for producing long-circulating properties. Currently, PEG-bolas stabilize liposomes under evaluation as antitumor drug carriers and will be reported in future work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcis.2016.07.013.

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