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Controlled Release of Hydrophilic Guest Molecules from Photoresponsive Nucleolipid Vesicles

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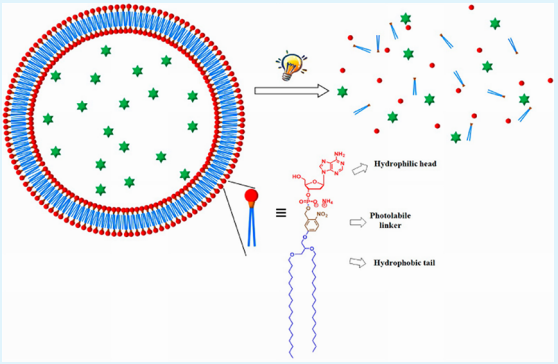
Supporting Information

ABSTRACT: Amphiphilic hybrid nucleolipids bear the structural and functional hallmarks of both lipids and nucleic acids and hold great potential for biotechnological applications. However, further tailoring of their structures and properties for specific applications represents a major challenge. We here report a novel design and synthesis of a light-responsive nucleolipid by introducing an *o*-nitrobenzyl group that acts as a linker between a nucleotide and a lipid. The nucleolipid was applied readily to preparing smart vesicles and encapsulating hydrophilic guest molecules 5(6)-carboxyfluorescein (CF) in their inner aqueous phase. Upon light irradiation, their vesicular structure was disrupted as a result of the photolytic degradation of the nucleotide, resulting in CF release. Furthermore, temporally controlled CF release from these vesicles could be readily realized by turning on and off light. By demonstrating the molecular assembly and photodisassembly cycle, this report aims to stimulate further research exploring practical applications of nucleolipids.



KEYWORDS: *nucleolipid, photolabile, vesicle, controlled release*

1. INTRODUCTION

Nucelolipids are hybrid amphiphiles composed of a lipid covalently anchored to a nucleoside, or a nucleotide, or an oligonucleotide.[1] Due to their inherent characteristics such as biocompatibility and biological functions (e.g., lipid compartmentalization and selective recognition between complementary bases), nucleolipids hold considerable potential in biotechnological applications. They are particularly attractive for fabricating liposomes, nanofibers, and other supramolecular structures.[1−6] Khiati et al. recently demonstrated that anionic nucleolipid-based liposomes were effective for in vitro DNA encapsulation and transfection with enhanced efficacy and little cytotoxicity.[6]

For delivery systems, good biocompatibility and controllable degradability are essential in their clinical uses.[7,8] Despite major advances in the biocompatibility of liposomes, which is typically achieved via the incorporation of biomotifs such as amino acids, peptides, saccharides, nucleosides/nucleotides, and oligonucleotides, the degradation manipulation of novel biomimetic liposomes and the subsequent release control of parent drugs still remain challenging. Disruption of the lipid bilayer walls is regarded as a crucial step for the controlled release. This can be achieved via the alteration of the hydrophilic−lipophilic balance (HLB) of amphiphiles comprising the lipid bilayers, thus disrupting the vesicular architectures formed.[9−11] Accordingly, various responsive units have been incorporated into the molecular structures of the stabilizing lipids and their mimics, and stimuli including pH,[12] redox,[13] temperature,[14] enzyme,[15] and irradiation[16−18] have been used to change the HLB, thereby resulting in the dissociation of liposomes and the concurrent release of guest molecules. Among these methods, light is typically recognized as one of the most effective protocols because it enables effective controls spatially and temporally.[19,20]

By introducing a light-responsive unit, an *o*-nitrobenzyl (*o*-NB) group, at the hydrophobic and hydrophilic interface of a nucleolipid, this work describes the first attempt to design and synthesize an amphiphilic photocleavable nucleolipid (Scheme 1). Similar to common lipids, this new molecule could readily form vesicles, and upon application of an external light, the vesicular structure was completely disrupted. As a result, we could encapsulate hydrophilic guest molecules in the inner aqueous phase of the vesicles and follow their release behavior in response to photoirradiation. By demonstrating the molecular assembly and photodisassembly cycle, this report aims to stimulate further research exploring practical applications of this type of nucleolipids.

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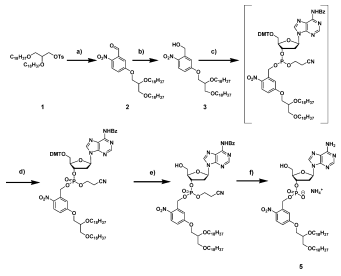
Scheme 1. Synthesis route of PLA[a]



[a](a) 5-Hydroxy-2-nitrobenzylaldehyde, K_2CO_3, DMF, 90 °C, 16 h; (b) $NaBH_4$, THF, room temperature, 1 h; (c) 5′-DMT protected nucleobase phosphoramidite, 5-ethylthiotetrazole, THF, room temperature, 6 h; (d) H_2O_2 in THF, room temperature, 1 h; (e) Cl_3CCOOH in CH_2Cl_2 (10%), room temperature, 0.5 h; (f) $NH_3{\cdot}H_2O$ in sealed tube, 80 °C, 12 h.

2. EXPERIMENTAL SECTION

2.1. Materials. All chemicals were purchased from Sigma-Aldrich and Alfa Aesar and used as received unless otherwise stated. Tetrahydrofuran (THF) was refluxed over and distilled from potassium benzophenone prior to use. Water used in all experiments was Millipore Milli-Q deionized (18.2 MΩ cm).

2.2. Nucleolipid Synthesis. The synthesis routes of photoresponsive lipid adnosine (PLA) and lipid adnosine (LA) are shown in Scheme 1 and Scheme S1 in the Supporting Information.

Compound 2. 5-Hydroxy-2-nitrobenzaldehyde (1.0 g, 6 mmol), 1,2-*o*-dioctadecyl-rac-glycerol tosylate (compound **1**, 4.4 g, 6 mmol), and 5.0 g K_2CO_3 were dissolved in 60 mL of *N*,*N*-dimethylformamide (DMF), followed by stirring in an oil bath (90 °C) for 16 h. The reaction mixture was poured in water and then extracted with ethyl acetate twice. The resultant crude product was purified by column chromatography with a mixture of hexane/ethyl acetate (7:1) as eluent to produce a yellow solid with a yield of 81%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.48 (1H, s, ArC*H*O), 8.13−8.16 (1H, d, J = 9.2 Hz, Ar*H*), 7.36−7.37 (1H, d, J = 3.2 Hz, Ar*H*), 7.16−7.18 (1H, dd, J = 3.2 Hz, J = 9.2 Hz, Ar*H*), 4.14−4.27 (2H, m, CH_2O), 3.46−3.80 (7H, m, CH_2O + C*H*O), 1.55−1.57 (4H, m, CH_2), 1.21−1.27 (64H, m, CH_2), 0.86−0.89 (6H, m, CH_3). MALDI-TOF MS calcd for $C_{46}H_{83}NO_6$, 746.1; found 768.6 (M + Na^+); 619.6 (lipid + Na^+).

Compound 3. Compound **2** (1.8 g, 2.4 mmol) was dissolved in 30 mL of anhydrous THF under ice bath, followed by the addition of 92 mg of $NaBH_4$. The mixture solution was stirred at room temperature for 1 h, and the excess $NaBH_4$ was then quenched by 1 M NH_4Cl. After the organic phase was separated, the crude product was recrystallized with ethyl acetate to produce a yellow solid with a yield of 88%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.15−8.17 (1*H*, d, J = 9.2 Hz, Ar*H*), 7.25−7.26 (1H, d, J = 2.8 Hz, Ar*H*), 6.90−6.93 (1H, dd, J = 2.8 Hz, J = 9.2 Hz, Ar*H*), 4.98 (2H, s, ArCH_2O), 4.14−4.22 (2H, m, CH_2O), 3.43−3.63 (7H, m, CH_2O + C*H*O), 1.55−1.57 (4H, m, CH_2), 1.21−1.27 (64H, m, CH_2), 0.86−0.89 (6H, m, CH_3). MALDI-TOF MS calcd for $C_{46}H_{85}NO_6$, 748.1; found 770.6 (M + Na^+); 619.6 (lipid + Na^+).

Compound 4 (LA). *N*6-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine-3′-*O*-[*O*-(2-cyanoethyl)-*N*,*N*′-diisopropylphosphoramidite] (2.0 g, 2.33 mmol), 5-ethylthio-1*H*-tetrazole (600 mg, 4.6 mmol), and 1, 2-*o*-dioctadecyl-rac-glycerol (1.2 g, 1.6 mmol) were dissolved in anhydrous THF under nitrogen. The reaction mixture was stirred at room temperature for 6 h, followed by adding 5 mL of H_2O_2 (30% in water). After 5 min of oxidation, 10 mL of 10% trichloroacetic acid (TCA) solution in CH_2Cl_2 was added, and the solution was stirred for an additional 5 min. Excess TCA was then quenched by saturated $NaHCO_3$ aqueous solution. The isolated organic phase was washed with H_2O and evaporated under vacuum to obtain the crude product. The crude product was dissolved in 25 mL of $NH_3{\cdot}H_2O$ (17% in water) and heated at 80 °C under stirring in a sealed tube for 12 h. The final product (white solid) was obtained by column chromatography with a mixture of 20:1 CH_2Cl_2/methanol as eluent. Yield: 44%. ^{1}H NMR (400 MHz,$CDCl_3$) δ: 8.18 (1H, m, Ar*H*), 8.01 (1H, m, Ar*H*), 6.20−6.21 (2H, m, CH_2O), 3.47−3.67 (2H, m, CH_2O), 4.73−4.75 (2H, m, CH_2O), 4.04−4.21 (3H, m, CH_2O + C*H*O), 3.46−3.80 (6H, m, CH_2O + C*H*O), 1.57−1.59 (4H, m, CH_2), 1.21−1.28 (60H, m, CH_2), 0.86−0.89 (6H, m, CH_3). MALDI-TOF MS calcd for $C_{49}H_{95}N_6O_8P$, 927.3; found 932.7 (M − NH_4^+ + Na^+); 954.7 (lipid − NH_4^+ + 2Na^+), 970.7, (M + K^+ + Na^+).

Compound 5 (PLA). *N*6-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine-3′-*O*-[*O*-(2-cyanoethyl)-*N*,*N*′-diisopropylphosphoramidite] (2.0 g, 2.33 mmol), 5-ethylthio-1*H*-tetrazole (600 mg, 4.6 mmol), and compound **3** (1.2 g, 1.6 mmol) were dissolved in anhydrous THF under nitrogen. The reaction mixture was stirred at room temperature for 6 h, followed by adding 5 mL of H_2O_2 (30% in water). After 5 min of oxidation, 10 mL of 10% TCA in CH_2Cl_2 was added, and the solution was stirred for an additional 5 min. Excess TCA was then quenched by saturated $NaHCO_3$ aqueous solution. The isolated organic phase was washed with H_2O and evaporated under vacuum to obtain the crude product. The crude product was dissolved in 25 mL of $NH_3{\cdot}H_2O$ (17% in water) and heated at 80 °C under stirring in a sealed tube for 12 h. The final product (yellow solid) was obtained by column chromatography with a mixture of 20:1 CH_2Cl_2/methanol as eluent. Yield: 30%. ^{1}H NMR (400 MHz, $CDCl_3$ + MeOD) δ: 8.37 (1H, s, Ar*H*), 8.15−8.17 (1H, d, J = 9.2 Hz, Ar*H*), 8.18 (1H, s, Ar*H*), 7.58−7.59 (1H, d, J = 2.8 Hz, Ar*H*), 6.92−6.95 (1H, dd, J = 2.8 Hz, J = 9.2 Hz, Ar*H*), 5.44−5.47 (2H, m, ArCH_2O), 4.19−4.37 (3H, m, CH_2O + C*H*O), 3.44−3.89 (10H, m, CH_2O + C*H*O), 3.01−3.07 (2H, m, CH_2), 2.65−2.72 (2H, m, CH_2), 1.55−1.57 (4H, m, CH_2), 1.21−1.27 (64H, m, CH_2), 0.86−0.89 (6H, m, CH_3). MALDI-TOF MS calcd for $C_{56}H_{101}N_7O_{11}P$, 1078.4; found 1105.7 (M − NH_4^+ − H^+ + 2Na^+).

2.3. PLA and LA Vesicles Preparation. Ten mg of PLA was well dissolved in 10 mL of the mixture of $CHCl_3/CH_3OH$ (v/v = 9:1) in a 50 mL round-bottom flask. The solvent was removed by rotary evaporation under reduced pressure to form a thin layer on the flask's wall. After drying overnight under vacuum, 5 mL of HEPES buffer containing 10 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 100 mM 5(6)-carboxyfluorescein (CF) was added, followed by shaking in a water bath (80 °C). After all the lipid film was removed from the flask's wall, the suspension was transferred to a clean thick wall tube. The tube was placed in liquid nitrogen and a water bath (80 °C) alternately for 10 cycles to obtain vesicles. To remove nonentrapped CF, the vesicle solution was loaded on the Sephadex G50 column (1 × 30 cm), with HEPES as the eluent. The vesicle-containing fraction was collected and kept in the dark. LA vesicles were prepared in a similar way.

2.4. Photocontrolled CF Release. The release of entrapped CF molecules from nucleolipid vesicles was studied by using the fluorescence dequenching method at 25 °C. The aqueous solution of CF-loaded vesicles, stored in a 1 mm quartz cuvette, was exposed to UV light (100 mW/cm^2) with a band path filter (350−380 nm) for different times, as shown in Figure S1 in the Supporting Information. The fluorescence intensity change at 517 nm (excitation 492 nm) was followed on a Hitachi F2500 fluorescence spectrophotometer, as a function of UV irradiation time. The CF release (%) was calculated by use of the following equation: CF release (%) = $(I_t - I_0)/(I_\infty - I_0) \times 100$, where I_0 is the initial fluorescence intensity, I_t is the fluorescence intensity at time t, and I_∞ is the fluorescence intensity when all the CF molecules were released from vesicles, which was realized by the addition of 0.4% (the final concentration) Triton X-100 and heating for an additional 30 min. Note that, as 0.4% Triton X-100 had a negative effect on the fluorescence intensity of CF (suppression of 20% in our case), the used I_∞ value was corrected. The maximum loading of CF in these vesicles was around 60 mmol of CF per mol of lipid.

2.5. Instrumental Characterization. The size and size distribution of vesicles were assessed by using cryogenic transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS). Cryo-TEM samples were prepared in a controlled-environment vitrification system (CEVS).[21] Five μL of the vesicle solution was

pipetted on a TEM copper grid and then blotted with two pieces of filter paper. After about 5 s, the samples were quickly plunged into a reservoir of liquid ethane (cooled by nitrogen) at −165 °C. The vitrified samples were stored in the liquid nitrogen until they were transferred to a cryogenic sample holder (Gatan 626) and followed with a JEOL JEM-1400 TEM (120 kV) at about −174 °C. The phase contrast was enhanced by underfocus. The images were recorded on a Gatan multiscan CCD and processed with Digital Micrograph. DLS measurements were performed on a Malvern Zetasizer Nano ZS with a 173° scattering angle in a 45 μL quartz cuvette at 25 °C, and all of the results were presented as the average of at least six replicates.

Fluorescence microscopy was performed on a Leica DMI 3000B fluorescence microscope and a Nikon A1 confocal microscope at room temperature. A 20 μL solution containing CF loaded vesicles was dropped onto a slide and covered with a coverslip and then observed with an oil immersion lens under blue exciting light at 488 nm. UV−visible absorbance was recorded on a Shimadzu UV 2450 spectrophotometer at room temperature.

3. RESULTS AND DISCUSSION

As photochemical reactions of the *o*-NB containing compounds are usually rapid and efficient, with little competing side reactions, the *o*-NB moiety has been extensively used as a photosensitive protecting group, particularly in the synthesis of caged compounds in biological and physiological studies.[22,23] As shown in Scheme 1, the photolabile nucleolipid synthesis was started from the tosylation of 1,2-*o*-dioctadecyl-rac-glycerol, followed by the etherification with 5-hydroxy-2-nitrobenzaldehyde. After reduction of benzaldehyde, the resulting 2-nitrobenzyl alcohol group was allowed to react with 5′-DMT protected nucleobase phosphoramidite under slightly acid conditions in anhydrous THF, giving rise to phosphite, immediately followed by the oxidation with H_2O_2 at room temperature to obtain more stable cyanoethyl protected phosphate. The DMT on the 5′ of ribose was removed by TCA in CH_2Cl_2, and the cyanoethyl group on phosphate was aminolyzed to give the final PLA. As a control, LA without the photoresponsive group was also synthesized following a similar strategy. The products were characterized by ^{1}H NMR and MALDI-TOF MS, confirming their correct molecular structures and high purities (see the Supporting Information for NMR and MS spectra).

The water-soluble fluorescein (CF) was used as the model guest molecule. To obtain CF-loaded PLA and LA vesicles, we applied the simple freezing and thawing method, and the nonentrapped CF molecules were separated from the CF-loaded vesicles by gel filtration. Cryo-TEM imaging characterization indicated that these vesicles are spherical and unilamillar (Figure 1). Both cryo-TEM and DLS revealed that, apart from a small amount of large vesicles with diameters around 1000 nm (indicated by red arrows in Figures 1 and 2), most of CF-loaded PLA vesicles have diameters in the range of 200−400 nm. In contrast, the diameters of most of the CF-loaded LA ones are in the range of 100−200 nm. Note that there was no significant change in the diameters of nucleolipid vesicles before and after CF-loading (Figure S2 in the Supporting Information), suggesting little influence of CF molecules on these vesicular structures. Importantly, fluorescence microscopic imaging revealed explicit CF encapsulation into the inner aqueous phase of both PLA and LA vesicles (Figure 3a,c). The fluorescence intensity within individual loaded vesicles was weak due to the self-quenching of high concentration of CF in the closed vesicles. However, high quality confocal images could still be taken. The inset shown as Figure 3a and the

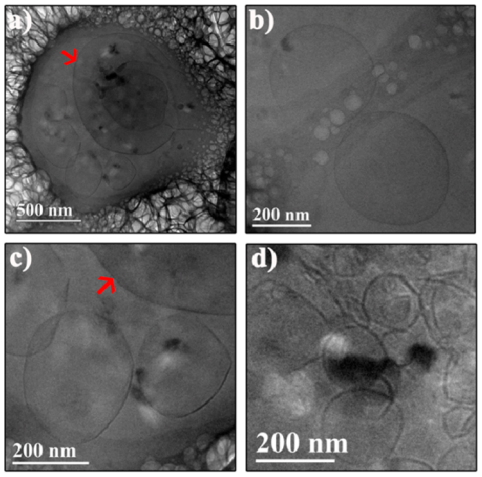


Figure 1. Cryo-TEM images of CF-loaded (a, b, and c) PLA and (d) LA vesicles.

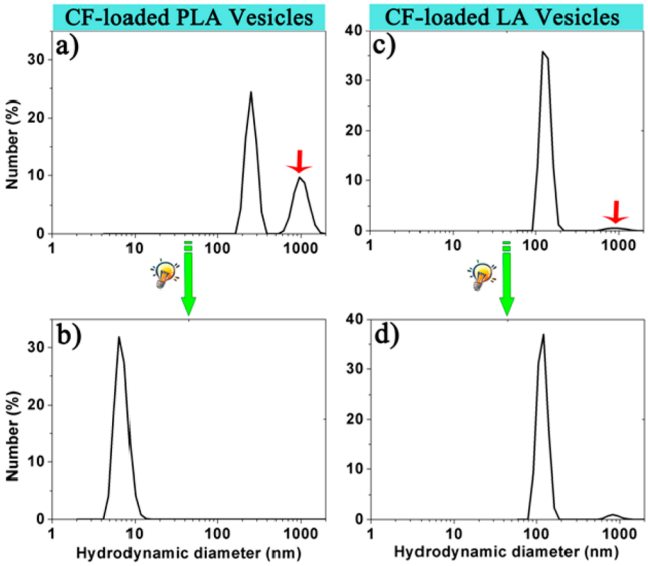


Figure 2. DLS profiles of CF-loaded (a and b) PLA and (c and d) LA vesicles in aqueous solution before (the upper panel) and after (the bottom) 10 min of UV irradiation.

movie (Movie S1 in the Supporting Information) revealed a spherical morphology and a homogeneous fluorescent distribution within a typical loaded PLA vesicular particle.

To assess photolability of the PLA vesicles, their aqueous solution was subjected to UV irradiation (350−380 nm, 100 mW/cm^2; see Figure S1 in the Supporting Information for the UV irradiation geometry). Simultaneously, their UV−vis absorbance was followed with irradiation time. As shown in Figure 4, a continuous increase of the absorbance at 260 nm and an accompanying drop around 325 nm were noticeably observed, indicative of the photolytic degradation of PLA molecules via the cleavage of the ester bonds.[11,24−26] Note that the UV−vis absorbance did not change significantly after 6 min of UV irradiation, suggesting that the majority of the PLA molecules had been photolyzed rapidly. The photoinduced cleavage reaction led to a dramatic change in HLB and alteration of the stability of vesicles. Indeed, DLS analysis revealed the complete disruption of PLA vesicles after 10 min of UV irradiation (Figure 2b). Instead, smaller aggregates with

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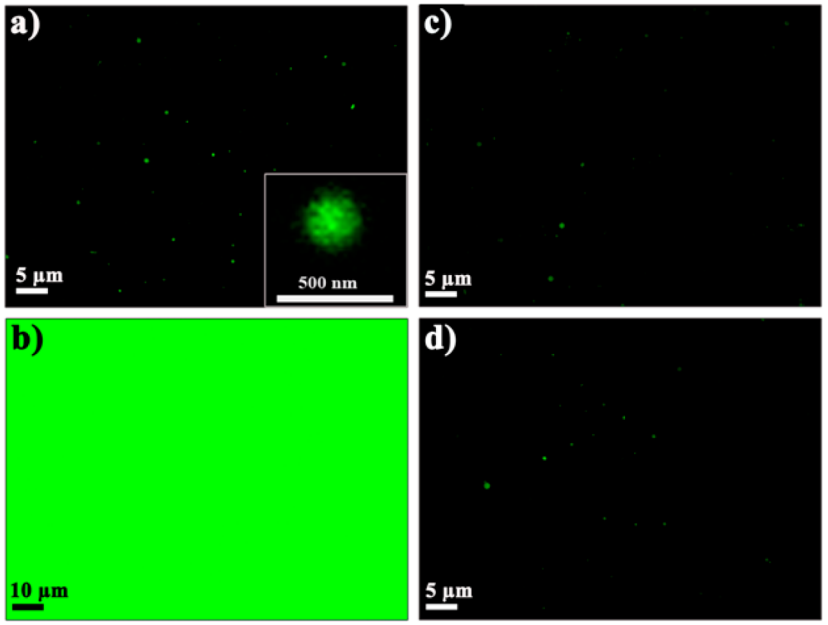

Figure 3. Fluorescence microscopic images of CF-loaded (a and b) PLA and (c and d) LA vesicles before (the upper panel) and after (the bottom) 5 min of UV irradiation. Inset in (a): fluorescent confocal microscopic image of a CF-loaded PLA vesicle before UV irradiation.

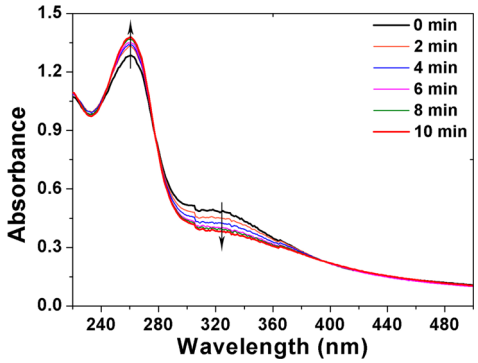

Figure 4. UV−vis spectra of PLA vesicles in aqueous solution upon UV irradiation for different time intervals (0−10 min). Note that no CF-loading was performed with these vesicles to eliminate the interference with the spectra from the dye.

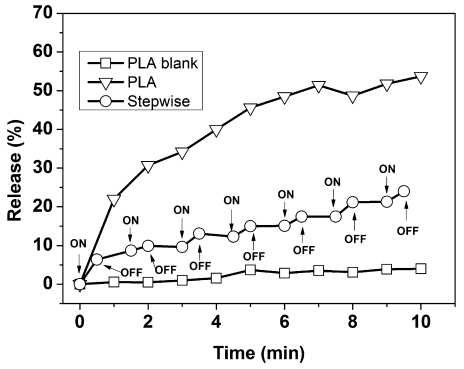

Figure 5. CF release profiles from CF-loaded PLA vesicles under different conditions.

diameters around 7 nm appeared, consistent with the proposed generation of the cleaved products incorporating the dye derivatives (Figure 2b); fluorescent microscopy indicated the release of the entrapped CF into the surrounding hydrophilic milieu, resulting in significantly enhanced fluorescence over the whole field (Figure 3b). In contrast, there is almost no change at all in the DLS profiles and fluorescent images of LA vesicles before and after UV irradiation (Figure 2c,d; Figure 3c,d), indicating their evident stability under UV irradiation due to the absence of the photoresponsive *o*-NB group in LA. This control experiment also showed that under the current conditions the UV irradiation had negligible disruptive interference to CF as far as the stability of the CF-loaded LA vesicles is concerned.

Finally, the photoresponsive release of entrapped guest molecules from PLA vesicles was assessed by measuring the emission fluorescence increase at 517 nm (excitation at 492 nm). As shown in Figure 5 (down triangle), the CF release (%) showed a gradual increase with time and attained a plateau level after irradiation for 6 min, consistent with the observed UV−vis absorbance changes. In contrast, CF release from these photocleavable vesicles was well suppressed in the dark (Figure 5, squar). We then followed the CF release by alternately switching on and off the UV irradiation. Once the UV was off, the CF release also ceased. Upon UV switching on, the CF release was triggered and showed a steady increase (Figure 5, circle). This stepwise release profile clearly demonstrated a temporally controlled release mode, enabling great potential of such nanocarriers in releasing guest molecules with precise time dependent dosage control.[27] As expected, there was no dye release from LA vesicles upon irradiation (Figure S3 in the Supporting Information).

4. CONCLUSION

We have reported the design and synthesis of a novel photocleavable nucleolipid molecule. Similar to common lipids, the nucleolipid could readily form stable vesicles and entrap hydrophilic guest molecules such as CF. Light-induced cleavage of the molecule caused the dissociation of the liposome structure, resulting in the controlled CF release. Temporally controlled CF release from such vesicles could be readily realized by turning on and off the UV light. Given that

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nucleotide-based lipids possess some physically and biologically inherent features such as biocompatibility and biospecificity, the present study provides a paradigm for designing stimuli-responsive smart bionanomaterials for more effective drug loading and release. Further studies that are taking place in our laboratory include its cytotoxicity assessment and in vitro drug release.

■ ASSOCIATED CONTENT

Supporting Information

LA synthesis scheme, ^{1}H NMR and MS spectra of PLA and LA, UV irridation geometry, DLS profiles of unloaded PLA and LA vesicles, CF release profiles from LA vesicles, and the confocal movie of a CF-loaded PLA vesicle. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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