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Vesicle formation from a synthetic adenosine based lipid

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Abstract—We report the synthesis of a novel purine based amphiphile; di-oleyladenosinephosphocholine (DOAPC). Light microscopy, TEM and QELS studies on DOAPC in aqueous media support the formation of lamellar systems. These investigations indicate that the presence of adenine does not prohibit the formation of lamellar organizations. Stable small unilamellar vesicles can be prepared by using extrusion techniques.

Multiple noncovalent interactions including hydrogen bonds, π-stacking, and salt bridges play a critical role in the structure and function of many small molecules and macromolecules. By mimicking and manipulating these interactions, chemists have synthesized molecules that self assemble into supramolecular architectures of varied size, shape, and function.[1] For example, the fundamental information units of molecular recognition found in nucleic acids have been inserted in amphiphile structures to create hybrid molecules such as nucleoside based lipids that form supramolecular assemblies in aqueous solution.[2,3] These molecules feature both the molecular characteristics of nucleic acids and the compartmentalization capabilities of lipids. Within this family, natural phosphocholine lipids possessing a uridine head group have been recently reported.[3] Interestingly, these amphiphiles spontaneously formed self-assemblies including vesicles, fibers, hydrogels, and organogels. Because these pyrimidine based amphiphile exhibited unique behaviors we have incorporated a purine moiety in the zwitterionic phosphocholine amphiphile structure to further elucidate the properties of these amphiphiles. To compare the physico-chemical behavior of the adenine and uridine derivatives, the oleyl chains were selected as the hydrophobic segment since a liquid lamellar phase was previously observed at room temperature for the dioleyl uridine amphiphile.[3] In this study, we describe the synthesis and preliminary physico-chemical investigations of a purine nucleoamphiphile possessing two oleyl fatty acid chains.

A nucleoside-phosphocholine amphiphile derived from adenosine was synthesized following a four-step route as shown in Scheme 1. The adenosine acetonide derivative **1** was reacted with an excess of chloro-oxo-dioxaphospholane in THF at 0 °C to afford the phosphate nucleoside derivative **2**. Note that this reaction was selective to the 5′ primary hydroxyl.[4] The phosphorylated intermediate **2** was transferred to a pressure tube and heated for one day with trimethylamine in acetonitrile to give the 2′,3′-(isopropylidene)-5′-(phosphocholine)-adenosine **3**. The 5′-phosphocholine intermediate **4** was obtained after cleavage of the isopropylidene protecting group. The oleyl fatty acids were coupled to **4**. Note that the synthesis of **5** was started from the ammonium salt **4** without protecting the amino group belonging to the nucleobase. The chain grafting reaction was achieved in acidic conditions using an imidazole activation of the fatty acids.[5] Under these esterification conditions the adenosine amino group was protonated, and thus did not participate in the nucleophilic reactions. This synthetic strategy used simple and abundant materials to afford the expected adenosine based amphiphiles **5**.[6]

The adenine-based zwitterionic amphiphile possessing oleyl acid chains self-assembles into liposome-like aggregates when dissolved in aqueous solutions. Similar results were observed for the uridine analog (DOUPC).[3] Supramolecular assemblies were obtained following

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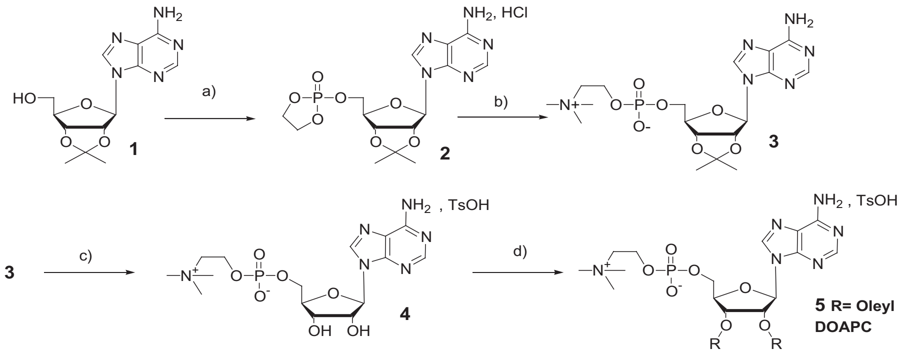

Scheme 1. Synthesis of the dioleyl adenosine phosphocholine (DOAPC). (a) Chloro-oxo-dioxaphospholane, THF, 0 °C, 24 h; (b) trimethylamine, acetonitrile, 75 °C, 24 h (yield for a) and (b): 87%; (c) APTS, MeOH, reflux 4 h (yield = 83%); (d) activated oleyl APTS, DMF (yield = 24%).

four different experimental procedures; (1) formation of a thin film by evaporation under vacuum of an organic solution containing the amphiphile (procedure A), (2) hydration of the amphiphile directly on a glass slide (procedure B), (3) sonication of an aqueous amphiphile solution (procedure C), and (4) high-pressure extrusion of an aqueous amphiphile solution (procedure D).[7] Depending on the polycarbonate filter size, this latter technique (D) afforded unilamellar vesicle sizes from 30 nm to 5 μm.

Procedures A, B, C, and D can be used to obtain vesicular systems, but heterogeneous multilamellar aggregates are usually observed using the first three conditions (Fig. 2a). For procedure A, a typical experiment involves forming a thin film of phosphocholine derivatives in 50 mL round-bottom flask by dissolving 3 mg of amphiphile **5** in 1 mL of chloroform and subsequently removing of solvent by rotoevaporation. A phosphate buffer (Phosphate buffer 8.3 mM, pH 7.2) is then added and the solution is stirred for 30 min at room temperature. When DOAPC is hydrated directly on a glass slide (procedure B), the hydration phenomenon can be followed by light microscopy. As shown in Figure 1, vesicular organizations and/or lamellar systems are observed for DOAPC. However, small differences in behaviors are noted during the hydration phenomena between DOAPC and DOUPC. The DOUPC samples exhibit rather large vesicles and/or 'worms' like structures, which are slowly hydrated compared to the DOAPC. It appears that the hydration phenomena is faster for DOAPC than for DOUPC as a result of the more hydrophilic character of the DOAPC head group. The protonated amino group affects the hydrophilicity of the polar head in this amphiphile (p*K*a adenosine = 9.2). Typically, DOAPC first forms myelin like organisations (Fig. 1a), which evolve quickly to giant vesicles (Fig. 1b). Procedure C affords heterogeneous unilamellar vesicles. In that case, aqueous solutions of DOAPC are sonicated in a Branson 3200 cleaning bath for 15 min. To prepare homogeneous populations of small unilamellar vesicles the extrusion technique (procedure D) is employed. As shown in Figure 2, extruding DOAPC through a 50 nm polycarcarbonate filter at room temperature affords a vesicle population of 25–65 nm in size with an average particle size of 45 nm as determined by Transmission Electronic Microscopy experiments (TEM). This vesicle population is confirmed by QELS (Quasi Elastic Light Scattering) experiments, which indicate the presence of vesicles in solution having an average diameter ∅ of 33 nm. After 1 h the vesicles composed of adenosine-based lipids are slightly smaller than those prepared from uridine nucleoamphiphiles (∅ = 40 nm) under similar conditions.[3] Likewise the stability of the DOAPC vesicles is similar to DOUPC, with an average diameter of ∅ = 65 nm after 7 days.

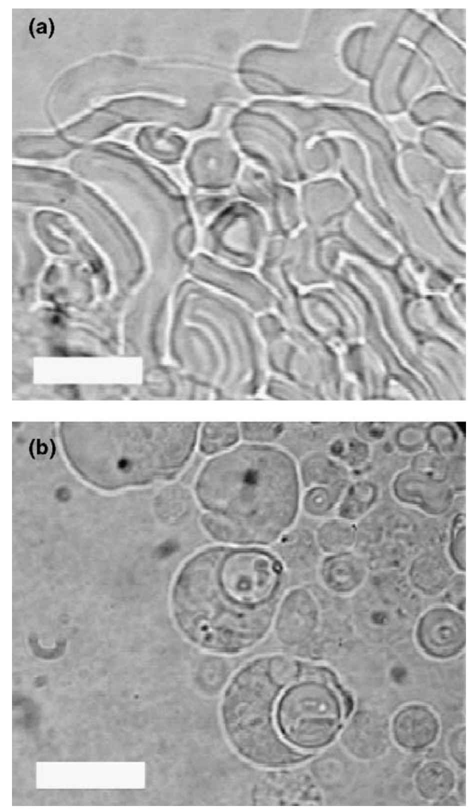

Figure 1. Light micrographs of hydrated DOAPC prepared using procedure (B), (bar 14 μm. Phosphate buffer 8.3 mM, pH 7.2).

In summary, a convenient synthetic route involving unprotected intermediates for the preparation of adenosine-based amphiphiles is reported. This strategy should extend to other fatty acids chain length including linear,

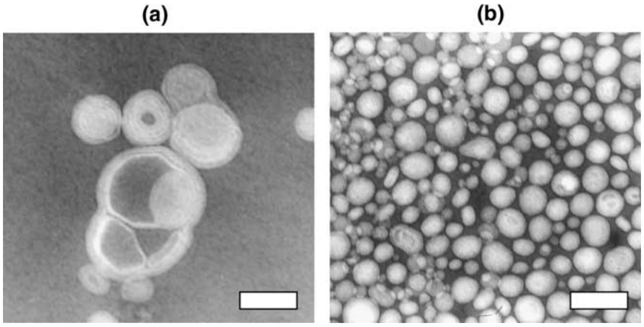

Figure 2. TEM images obtained from hydrated DOAPC, (a) multi lamellar vesicles (MLV, procedure A) and (b) SUV population (procedure D, extrusion filter 50 nm). Negative staining with ammonium molybdate 1% in water, phosphate buffer 8.3 mM, pH 7.2, bar = 150 nm.

branched, saturated, and polyunsaturated hydrocarbon chains. The preliminary physico-chemical investigations indicate similar aggregation properties as DOUPC. The adenine moiety does not prohibit the formation of lamellar phases. Light microscopy experiments reveal the formation of worms like structures and giant vesicles, whereas the extrusion of DOAPC in aqueous solution leads to homogeneous and stable populations of small unilamellar vesicles. Amphiphilic structures possessing an increase number of molecular features for molecular interactions provide opportunities to further characterize the relationship between molecular structure and supramolecular assemblies, mimic natural systems for basic studies, and create nanometers to micrometer objects for use in biotechnological and medical applications.

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6. Preparation of 2′,3′-(isopropylidene)-5′-(oxo-dioxaphospholane)-adenosine **2**; freshly distilled THF (25 mL) was added under argon to adenosine acetonide (800 mg, 2.60 mmol). The mixture was cooled down to 0 °C and 2-chloro-2-oxo-1,3,2-dioxaphospholane (360 μL, 1.5 equiv, 3.91 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 15 h. The adenosine acetonide oxo-dioxaphospholane hydrochloride salt was precipitated. The supernatant was removed and the precipitate was eluted with 10 mL of dry acetonitrile. This solution was directly used without further purification in the following step. 2′,3′-(Isopropylidene)-5′-(phosphocholine)-adenosine **3**; Anhydrous trimethylamine (10 mL) was condensed at −50 °C under argon in a pressure tube. The solution containing **2** was added to the cold trimethyl amine. The reaction mixture was then maintained at 75 °C under stirring for 24 h. After evaporation at room temperature of the residual trimethylamine and filtration, a white solid was isolated. The crude material was washed in acetonitrile and purified by reverse phase chromatography (eluant: H_2O/MeOH, 8:2). 1.072 g of a hygroscopic solid **3** were obtained after drying under high vacuum (Yield: 87%). R_f: 0.29 (reverse phase, MeOH/H_2O: 8:2). ^{1}H NMR (300 MHz, DMSO): δ 1.32 (s, 3H, CH_3), 1.54 (s, 3H, CH_3), 3.10 (s, 9H, $N(CH_3)_3$), 3.49 (m, 2H, NCH_2), 3.77 (t, 2H, $J = 4.8$ Hz, CH_2O), 4.01 (m, 2H, $H5'$), 4.34 (m, 1H, $H4'$), 5.04 (dd, 1H, $J = 2.1/6.0$ Hz, $H2'$ or $H3'$), 5.39 (dd, 1H, $J = 2.8/6.0$ Hz, $H2'$ or $H3'$), 6.16 (d, 1H, $J = 2.8$ Hz, $H1'$), 7.38 (s, 2H, NH_2), 8.17 (s, 1H, $H8$), 8.49 (s, 1H, $H2$). ^{13}C NMR (75 MHz, DMSO): δ 25.09 (CH_3), 26.96 (CH_3), 52.99 ($N(CH_3)_3$), 58.21 ($C5'$), 64.42 (CH_2O), 65.32 (NCH_2), 81.57 ($C3'$), 83.37 ($C2'$), 85.13 ($C4'$), 89.11 ($C1'$), 112.91 (OCO), 118.69 ($C5$), 139.62 ($C8$), 149.02 ($C4$), 152.68 ($C2$), 155.95 ($C6$). ^{31}P NMR (121 MHz, DMSO): −0.954 ppm. High resolution FAB MS (MH+) (theoretical = 473.1914, observed = 473.1947).
5′-(Phosphocholine)-adenosine **4**; 2′,3′-(isopropylidene)-5′-(phosphocholine)-adenosine (150 mg, 1 equiv, 0.315 mM) and *para*-toluene sulfonic acid monohydrate (181 mg, 3 equiv, 0.950 mM) were dissolved in 25 mL of methanol. The mixture was heated for 4 h, solvent was then evaporated under vacuum and the crude product was dissolved in 4 mL of anhydrous ethanol. After crystallization in ethanol/ethyl acetate (95:5). The supernatant is removed. 113 mg of a white and hygroscopic solid **4** were isolated. (Yield: 59%). R_f: 0.75 (reverse phase, MeOH/H_2O: 8:2).^{1}H NMR (300 MHz, DMSO): δ 2.28 (s, 3H, CH_3), 3.11 (s, 9H, $N(CH_3)_3$), 3.63 (t, 2H, $J = 4.9$ Hz, NCH_2), 4.16 (m, 2H, $H_{4'}$ and $H_{3'}$), 4.22 (m, 1H, $H_{2'}$), 4.30 (m, 2H, $H_{5'}$), 4.56 (t, 2H, $J = 4.9$ Hz, CH_2O), 6.00 (d, 1H, $J = 5.1$ Hz, $H_{1'}$), 7.12 (d, 2H, $J = 7.7$ Hz, H arom), 7.51 (d, 2H, $J = 8.1$ Hz, H arom), 8.53 (s, 1H, H8), 8.68 (s, 1H, H2). ^{13}C NMR (75 MHz, DMSO): δ 20.74 (CH_3), 52.98 ($N(CH_3)_3$), 59.86 (C5′), 64.67 (CH_2O), 66.05 (NCH_2), 69.95 (C3′), 73.67 (C2′), 83.06 (C4′), 87.78 (C1′), 118.66 (C5), 125.43 (2C, CH arom.), 128.15 (2C, CH arom.), 137.98 (C8), 142.01 (C arom.), 145.06 (C arom.), 145.39 (C4), 148.33 (C2), 150.27 (C6). ^{31}P NMR (121 MHz, DMSO): −1.557 ppm. High resolution FAB MS (MH+) (theoretical = 433.1601, observed = 433.1616).
Bis-(2′,3′-oleoyl)-5′-(phosphocholine)-adenosine **5**; carbonyldiimidazole (172 mg, 1 equiv, 1.06 mM) was added to leic acid (337 μL, 1 equiv, 1.06 mM) in 5 mL of anhydrous DMF under an atmospheric pressure of argon (release of

carbon dioxide). After stirring 2 h at room temperature, the reaction mixture was transferred in a round bottom flask enclosing 5′-(phosphocholine)-adenosine (tosylate salt, 320 mg, 0.5 equiv, 0.53 mM) and *para*-toluene sulfonic acid (274 mg, 1.5 equiv, 1.06 mM) in 5 mL of anhydrous DMF. The mixture was stirred for 24 h at room temperature under argon (pH is maintained under 7). The DMF was removed under high vacuum and the crude product was purified by exclusion chromatography Sephadex LH20, DCM/MeOH 5:5. 123 mg of an highly hygroscopic product was isolated. (Yield: 23%). R_f: 0.16 (reverse phase, DCM/MeOH: 5:5). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.85 (m, 6H, CH_3), 1.25 (m, 40H, CH_2), 1.58 (m, 4H, $CH_2CH_2CO_2$), 1.98 (m, 8H, $CH_2CH{=}CH$), 2.17 (m, 4H, CH_2CO_2), 3.20 (s, 9H, $N(CH_3)_3$), 3.66 (m, 2H, CH_2N^+), 4.03 (m, 2H, H5′), 4.24 (m, 3H, H4′, and CH_2OP), 5.32 (m, 4H, $CH{=}CH$), 5.56 (m, 2H, H2′, and H3′), 5.81 (m, 1H, H5), 6.12 (m, 1H, H1′), 7.27 (m, 2H, NH_2), 8.22 (s, 1H, H8), 8.63 (s, 1H, H2). ^{13}C NMR(75.468 MHz, $CDCl_3$): δ 14.07 (CH_3), 22.62 (CH_2CH_3), 24.84 ($CH_2CH_2C{=}O$), 24.90 ($CH_2CH_2C{=}O$), 27.18 (CH_2CH), 29.03–29.72 $(CH_2)_n$, 31.85 ($CH_2CH_2CH_3$), 33.80 ($CH_2C{=}O$), 33.99 ($CH_2C{=}O$), 54.13 $N^+(CH_3)_3$, 59.35 (CH_2O), 63.50 ($C_{5'}$), 65.97 (N^+CH_2), 69.36 ($C_{3'}$), 71.80 ($C_{2'}$), 82.94 ($C_{4'}$), 85.06 ($C_{1'}$), 118.96 (C5), 129.53 ($CHCH_2$), 129.99 ($CHCH_2$), 139.23 (C8), 149.97 (C4), 153.29 (C2), 155.98 (C6), 172.28 (C=O), 172.54 (C=O). ^{31}P NMR (121 MHz, $CDCl_3$): −0.810 ppm. FAB MS = 961.

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