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Supramolecular assemblies of novel aminonucleoside phospholipids and their bonding to nucleic acids†

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A novel class of aminonucleoside phospholipids has been developed. These molecules could spontaneously assemble into supramolecular structures including multilamellar organization, hydrogels, superhelical strands, and vesicles. Their ability to bind to DNA by hydrogen bonding and π–π stacking interactions was investigated by many means.

Molecular self-assembly plays an important role in biological systems, and is a widely used principle in the preparation of supramolecular structures.[1] Phospholipids are major components of prokaryotic and eukaryotic cell membranes, and they spontaneously organize to form liposomes in aqueous solvents.[2] Liposomes are of great interest as gene transfection agents. A large number of cationic lipids have been designed and assayed in transfection protocols, where nucleic acids bind to cationic liposomes because of the electrostatic interaction between the negatively charged phosphate backbone of DNA and cationic molecules.[3] However, there are still significant shortcomings for cationic lipids because of their cytotoxicity and their possible binding to serum proteins that are mostly negatively charged at physiological pH.[4]

In the last few decades, there has been substantial interest in the design of "nucleoside lipids", which have a double functionality based on the combination of nucleic acids and lipid characteristics.[5] These molecules constructed a large variety of supramolecular systems, and could interact with DNA through H-bonding and π–π stacking interactions in aqueous solvents. The study of mimicking molecular organization that is usually observed in biological systems is of widespread interest for both constructing supramolecular assemblies and biological applications. However, to the best of our knowledge, there has been no report of lipids used for gene delivery, which rely merely on H-bonding and π–π stacking interactions, despite the importance of developing more nucleoside-based lipids.

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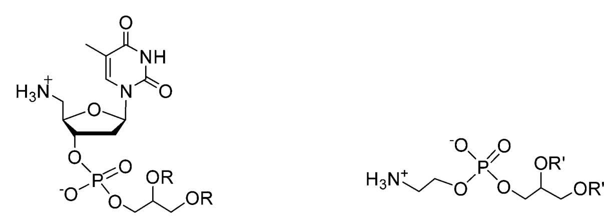
† Electronic supplementary information (ESI) available: Synthetic procedures and characterization data; ^{1}H, ^{13}C and ^{31}P NMR; SEM and TEM images; molecular dynamics simulation and cell experimental details. See DOI: 10.1039/c4cc07538b

Our group has been studying novel gene delivery systems for many years.[6] In this study, we present the synthesis and physicochemical characterization of a novel class of aminonucleoside phospholipids. These phospholipids are analogues of phosphatidyl ethanolamine, where 5′-amino-5′-deoxythymidine was used as the hydrophilic head group instead of ethanolamine. Two kinds of aminonucleoside phospholipids were prepared initially and named DPPAdT and DOPAdT, respectively (Fig. 1). Thymidine was chosen due to its relatively low price and simplicity of its derivatization. Moreover, supramolecular structures formed by aminonucleoside phospholipids were characterized by many means, and their biocompatibility, transmembrane capability, and binding behaviors to DNA were also investigated in this study.

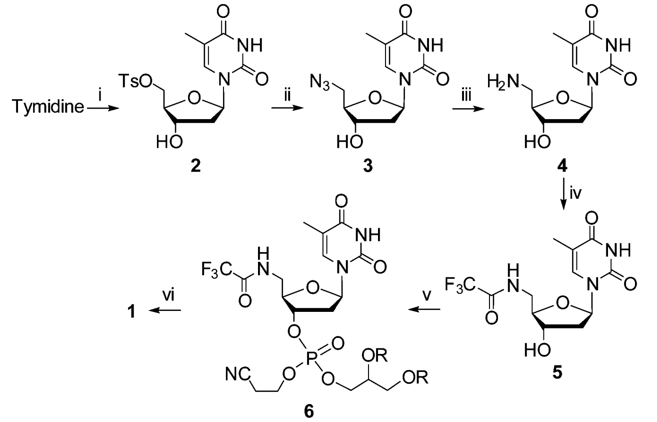
As shown in Scheme 1, thymidine was used as the starting material in the synthesis. After tosylation, azidation[7] and catalytic hydrogenation, 5′-amino-5′-deoxythymidine (**4**) was obtained in a high yield, followed by protection of the resulting amino group with trifluoroacetyl to give intermediate **5**. Then, compound **5** conjugated with trivalent phosphorus reagent and 2,3-bis(alkyloxy)-propanol in one pot to give intermediate **6**. Aminonucleoside phospholipid was obtained after the deprotection of **6**.[8] 2,3-Bis(alkyloxy)-propanol was prepared from glycerol and alkyl bromides (or alkyl alcohol, see Schemes S2 and S3 in the ESI†).[9] Through this synthetic strategy, two kinds of aminonucleoside phospholipids, named DPPAdT and



1. Aminonucleoside phospholipid
R= $-CH_2(CH_2)_{14}CH_3$ **DPPAdT**
$-CH_2(CH_2)_7CH{=}CH(CH_2)_7CH_3$ **DOPAdT**

Phosphatidyl ehanolamine

Fig. 1 Chemical structures of aminonucleoside phospholipids and phosphatidyl ethanolamine.



Scheme 1 Synthesis of aminonucleoside phospholipids. Reaction conditions: (i) TsCl, Py, 0 °C–r.t., 12 h, 81%; (ii). NaN_3, DMF, 70 °C, 12 h, 85%; (iii) H_2, Pd/C, MeOH, 5 h, 95%; (iv) CF_3COOEt, MeOH, Ar_2, −78 °C, 1 h, 80%; (v) $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, $HOCH_2CH(OR)CH_2OR$, 1*H*-tetrazolium, DMF, r.t.; (vi) NH_3/MeOH, r.t.

DOPAdT, were easily prepared in high yields using readily available starting materials (Scheme 1).

This synthetic strategy used readily available starting materials, and it is easy to prepare a large amount of derivatives. According to the preparation method, many supramolecular structures could be formed by aminonucleoside phospholipids in aqueous solvent, which have been studied by many means.

Multilamellar organization was obtained by direct hydration of DPPAdT in water. In this method, the suspending liquid of DPPAdT in water was sonicated at 70 °C for 10 min, and aged at 25 °C for 1 week. The scanning electron microscopy (SEM) image showed a stacking multilamellar membrane structure (Fig. 2a, also see Fig. S1, ESI†). The UV band at 260 nm of this structure was monitored as a function of temperature, and an obvious hyperchromic shift was observed at about 42 °C. Meanwhile, the absorbance of 5′-amino-5′-deoxythymidine at the same concentration did not vary with temperature (Fig. 2b). This result indicated the disassembly of this multilamellar organization above the phase transition temperature (42 °C).

DPPAdT also forms a hydrogel at room temperature. When the suspending liquid of DPPAdT with a concentration higher than 6 wt% in water was sonicated at 70 °C for 10 min, and then

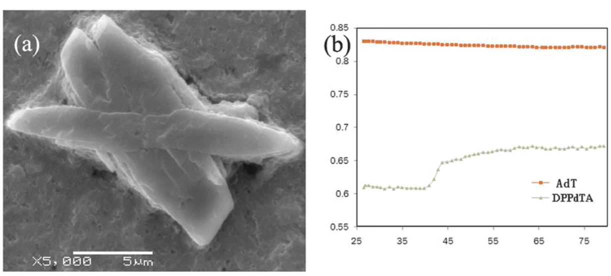


Fig. 2 (a) SEM image of the multilamellar suprastructure of DPPAdT (bar = 5 μm). (b) Absorbance of 5′-amino-5′-deoxythymidine (AdT, 100 μM) and the multilamellar suprastructure of DPPAdT (100 μM) at 260 nm *versus* temperature.

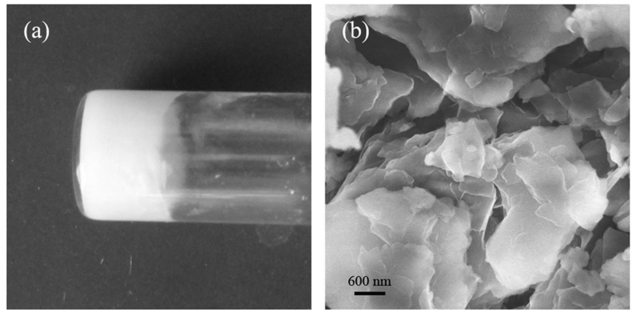


Fig. 3 (a) Photograph of DPPAdT hydrogel (6 wt%). (b) SEM image of a freeze-dried DPPAdT hydrogel (bar = 600 nm).

cooled to room temperature, an opaque hydrogel was obtained (Fig. 3a).

This hydrogel turned into a fluid liquid crystal at the temperature above 42 °C. The SEM image of a freeze-dried DPPAdT hydrogel showed a lamellar structure (Fig. 3b). Spontaneous hydrogel formation from low molecular weight hydrogelators offer several advantages to the currently more prevalent polymer gels, so it has emerged as an important class of biomaterials for medical applications.[10]

A superhelical structure was detected in the aqueous solution of DPPAdT. Biological polymers such as nucleic acids and proteins possess molecular helicity as their most basic property. Investigation of various helical strands that assemble spontaneously in aqueous solution has been of interest for decades.[11] To prepare the superhelical structure, the suspending liquid of DPPAdT in aqueous solution (below 6 wt%) was sonicated at 70 °C for 10 min, and aged at 60 °C for up to 2 days, then cooled

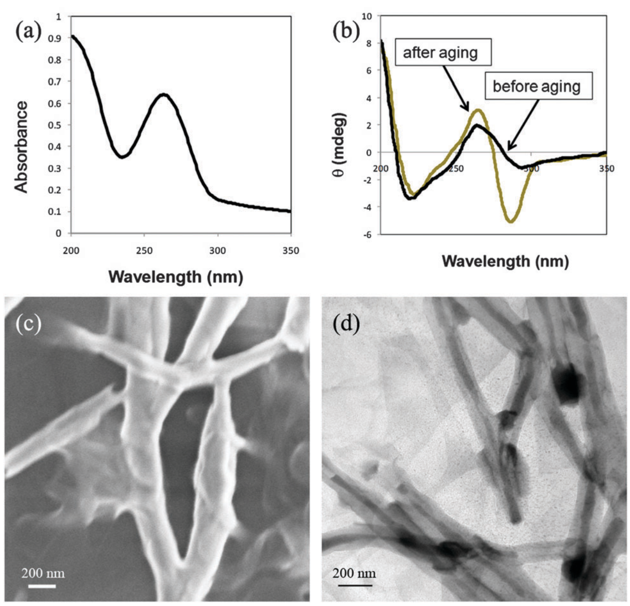


Fig. 4 (a) UV-vis absorption spectrum of DPPAdT in aqueous solution (100 μM). (b) CD spectra of DPPAdT in aqueous solution (1 mM) before and after aging at 60 °C for 2 days. (c) SEM image of superhelical strands formed from DPPAdT (bar = 200 nm). (d) TEM image of superhelical strands formed from DPPAdT (bar = 200 nm).

to room temperature. The UV-vis absorption spectrum showed a maximum absorption peak at 260 nm and no chromophoric absorption at around 280 nm (Fig. 4a). This coincides with the CD spectrum of DPPAdT aqueous solution before aging, which showed negative and positive inflections at 210 and 260 nm, respectively. After aging at 60 °C for 2 days, the CD spectrum of DPPAdT solution showed an extra negative inflection at 280 nm (Fig. 4b), indicating that a chiral supramolecular structure was formed. The SEM image of this structure showed a cross-linking superhelical network (Fig. 4c, also see Fig. S2, ESI†). The helical strand is left-handed with a diameter of *ca.* 200 nm. These results were further supported by the transmission electron microscopy (TEM) image, which also showed the cross-linking strand network with an average width of 200 nm. (Fig. 4d, also see Fig. S3, ESI†).

Liposomes are of great interest as gene transfection agents, drug delivery vehicles, and as models for biological membranes. Using the film hydration method, DOPAdT formed an ordered spherical-like nanostructure (liposomes or micelles, not just nanoparticles) in PBS buffer. The average particle size was 229.7 nm, and the zeta potential value was −31.5 mV, which was close to a neutral state with slightly negative charges distributed around these formations. Besides, nanostructure formation was also confirmed by SEM and TEM experiments. Hence, the formation of liposomal vesicles was confirmed by the particle size (∼200 nm, too big for micelles) and TEM image (showed a depression in the centre of almost every particle). In addition, the particle size in SEM and TEM images did not match well; this result may be attributed mainly to different sample preparation processes and the explanation in detail is given in the ESI† Section 3.4 (Fig. 5, also see Fig. S4, ESI†).

Aminonucleoside phospholipids are of potential use in gene delivery. Actually, the interaction between aminonucleoside phospholipids and single- or double-stranded DNA was studied. These interactions are based on π–π stacking and many base-pairing modes, including the canonical Watson–Crick or Hoogsteen non-traditional base-pairing motifs.[12]

CD spectroscopy was used to study the interactions between DPPAdT and polyadenylic acid (polyA, 20 bps). The CD spectrum of polyA is shown in Fig. 6a, and the addition of DPPAdT (base ratio = 1 : 1) led to minor changes immediately. However, annealing of this mixture resulted in distinctive changes in the CD spectrum (the decrease of the CD intensity at around 220 nm and the increase at around 270 nm), suggesting the formation of a polyA–DPPAdT complex. Molecular dynamics simulation was also used to study the thermodynamic stability of the polyA–DOPAdT complex. The final stable structure of the DOPAdT–polyA complex is shown in Fig. 6b. We can see that the Watson–Crick base-pairing interaction of the complex was well maintained. Take the 10th deoxyadenosine (from the 5′ end) and the corresponding DOPAdT molecule binding to it for an example, the lengths of N–H and O–H hydrogen bonds were 1.910 Å and 1.926 Å, respectively (Fig. S8, ESI†), which were close to that in the canonical Watson–Crick base-pairing modes (1.832 Å and 1.930 Å, respectively).[13]

The interaction between aminonucleoside phospholipids and double-stranded DNA was investigated by comparison of the AFM images of calf thymus DNA in the absence and presence of DPPAdT.[14] As shown in Fig. 7a, the AFM image of the intact DNA revealed the configuration of dispersive chains. After the addition of DPPAdT, the originally loose DNA was condensed to thick curve-like arrays with an average width of 100 nm (Fig. 7b), the size of which could be endocytosed efficiently by various cells.[3d] Considering that aminonucleoside phospholipid is electronegative under our experimental conditions, its attachment to double-stranded DNA should be mainly based on π–π stacking and H-bonding interactions. This may be a novel potential gene cargo, avoiding the cytotoxicity and immunogenicity of diverse cationic non-viral vectors in gene delivery currently reported.

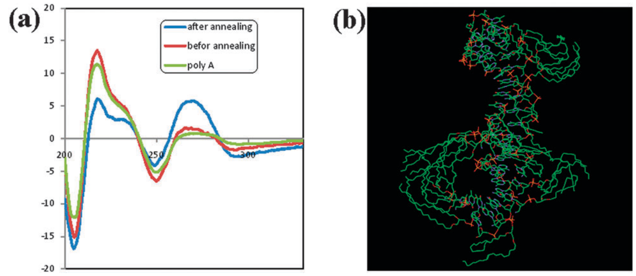


Fig. 6 (a) CD spectra of the solution of polyA (20 bps, 50 μM), the mixture solution of DPPAdT with polyA (base ratio = 1 : 1) before and after annealing. (b) Molecular dynamics simulation result of polyA–DOPAdT complex.

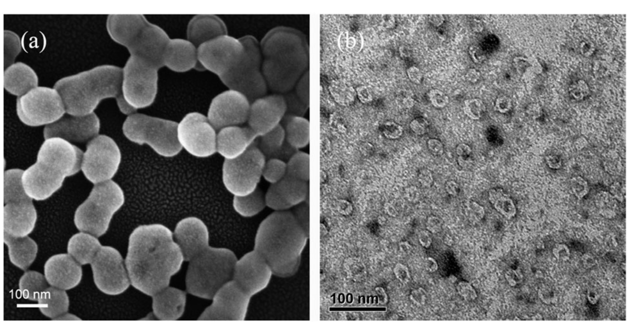


Fig. 5 (a) SEM image of DOPAdT liposomes (bar = 100 nm). (b) TEM image of DOPAdT liposomes (bar = 100 nm).

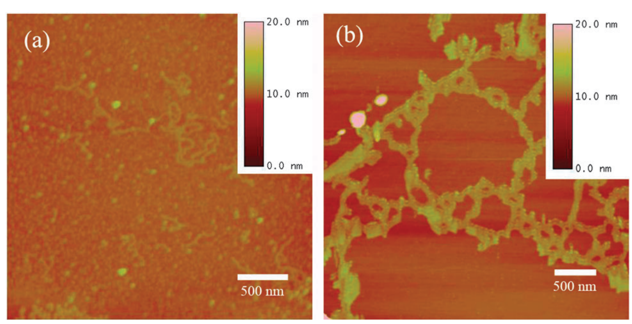


Fig. 7 AFM images of (a) calf thymus DNA (2 ng μL^{-1}) and (b) the mixture of calf thymus DNA (2 ng μL^{-1}) and DPPAdT (84 ng μL^{-1}) in aqueous solution (bar = 500 nm).

To verify the biocompatibility of aminonucleoside phospholipids, we added DPPAdT or DOPAdT to the culture of Human Embryonic Kidney 293 cells, and cell proliferation was measured using a cell counting kit-8 (CCK-8). According to the CCK-8 assay[15] shown in Table S2 (ESI†) and Fig. S5 (ESI†), after being incubated with aminonucleoside phospholipids for 24 h, the cell viability remained nearly 100%, and the addition of DOPAdT at a concentration of 100 μM even stimulated the cell proliferation. These results proved that aminonucleoside phospholipids are fully biocompatible.

Evaluation of the cellular uptake of aminonucleoside phospholipids was performed by encapsulating coumarin as the fluorescent probe.[16] As shown in Fig. S6 and S7 (ESI†), after 4 h incubation, the fluorescence intensities of MCF-7 breast cancer cells after applying free coumarin, coumarin-phosphatidylcholine (EPC) liposomes, coumarin-DOPAdT liposomes and blank medium were 224, 245, 317 and 5, respectively. These results demonstrated that DOPAdT liposomes significantly enhanced the cellular uptake of coumarin, exhibiting high transmembrane capability.

In the study of transfecting polyA, however, the result was not parallel to coumarin. It could be due to the electrostatic repulsion of phosphate anions between polyA and DOPAdT relatively hindering the interaction of gene and materials, for which the potential value of liposomes was −31 mV. New thymine-glyceride analogues based on DOPAdT excluding the phosphate moiety are yet to be developed to improve the efficient delivery of gene drugs in further research.

In conclusion, we have developed a novel class of aminonucleoside phospholipids, which spontaneously assemble into various supramolecular structures in aquatic media, including multilamellar organization, hydrogels, superhelical strands, and vesicles. These molecules have good biocompatibility, high transmembrane capability, and can form complexes with single- and double-stranded DNA by π–π stacking and hydrogen bond networks. This work has opened up interesting avenues for the development of novel supramolecular systems, and for the design and use of novel non-viral vectors in gene delivery. Further studies on the application of aminonucleoside phospholipids in gene delivery are ongoing in this laboratory.

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