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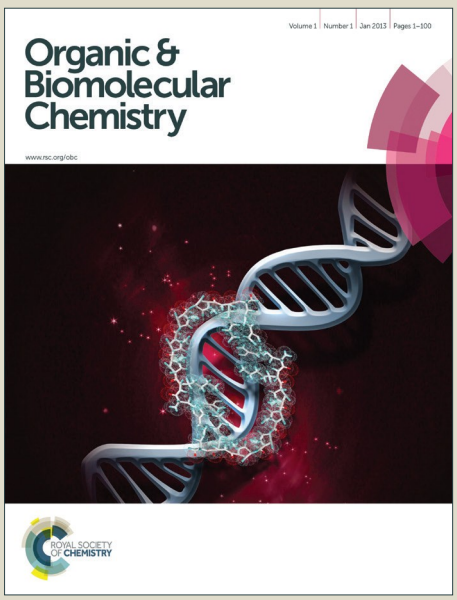

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ARTICLE TYPE

Synthesis of bifunctional molecules containing [12]aneN$_3$ and coumarin moieties as effective DNA condensation agents and new non-viral gene vectors†

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A series of bifunctional molecules with different combinations of macrocyclic polyamine [12]aneN$_3$ and coumarin moietie, **4a**/**b** and **5a**/**b**, were synthesized by the two-step copper(I)-mediated alkyne–azide click reactions between 1,3,5-tris(azidomethy)benzene and boc-protected *N*-propynyl-[12]aneN$_3$/ 7-propynyloxycoumarins. Agarose gel electrophoresis experiments indicated that bifunctional molecules **4b** and **5b** effectively induced complete plasmid DNA condensation at concentrations up to 40 μM. It was found that the structural variation had major impacts on the condensation behavior of these compounds. The electrostatic interaction involving the [12]aneN$_3$ moiety can be compensated by the binding contribution of the coumarin units during the DNA condensation process. These two types of interaction showed different effects on the reversibility of DNA condensation. Results from studies using dynamic laser scattering, atomic force microscopy, and EB replacement assay further supported the above conclusion. Cytotoxicity assay on bifunctional compounds **4a/b** and **5a/b** indicated their lower cytotoxicity. Results from cellular uptake and cell transfection experiments proved that bifunctional compounds **4b** and **5b** successfully served as non-viral gene vectors. Furthermore, methyl substituents attached to the coumarin unit (**4b** and **5b**) greatly enhanced their DNA condensation capability and gene transfection. These bifunctional molecules, with the advantages of lower cytotoxicity, good water solubility, and potential structural modification, will have great potential for the development of new non-viral gene delivery agents.

Introduction

The development of efficient vectors for delivering nucleic acids into cell is critical for gene therapy, which offers new opportunities for treating diseases such as cancers, diabetes, AIDS, and cardiovascular ailments at the level of their origin.[1-3] Major progress has been made in the design and synthesis of non-viral vectors over the last two decades. Various compounds including cationic lipids, polymers, dendrimers, peptides, chitosan, and metal complexes have been synthesized as DNA condensing agents and used as non-viral gene vectors in *in vitro* experiments and for clinical trials.[4-9] Compared to viral gene vectors, these synthetic agents have shown advantages such as biocompatibility, non-immunogenicity, convenience of modification, and potential for large-scale production. However, several barriers, including the lack of reproducible and scalable formulation, low stability in biological fluids, DNA size-dependent delivery, and properly reproducible and efficient transfection, need to be overcome before these synthetic agents can be put into practical application.[10-12] For these reasons, the exploration of new and novel non-viral gene vectors is still much needed.

For the development of new non-viral gene vectors, some common design principles and trends in structure-property relationship have emerged.[11, 13-15] For most gene vectors, the presence of positively charged moieties such as amine or guanidine groups is a prerequisite. In comparison to non-viral gene vectors based on cationic lipids[16, 17] and polymers such as polyethyleneimine (PEI),[18, 19] poly(L-lysine) (PLL),[20, 21] Chitosan,[22] poly-macrocyclic polyamines,[23-26] poly cationic cyclodexytrin,[27, 28] and small interfering RNA (siRNA),[29, 30] small molecules with multi-functional units as non-viral gene vectors have not been extensively explored. It is well known that factors such as hydrophobic, hydrogen-bonding, and π–π stacking interactions can facilitate the interactions between DNA and non-viral gene vectors. Recently we have been working on the design and synthesis of bifunctional molecules containing macrocyclic polyamine [12]aneN$_3$ and aromatic moieties as effective DNA condensing agents,[31, 32] and the further development of such molecules as non-viral gene vectors. To continue our effort in this field, coumarin with its bio-compatibility, planar-structure, and modular versatility[33, 34] has attracted our attention for the development of new bifunction molecules. It is expected that the combination of macrocyclic polyamine [12]aneN$_3$ and coumarin would results in effective DNA condensation agents and potential non-viral gene vectors with lower cytotoxicity and fluorescent imaging property for mechanism study.

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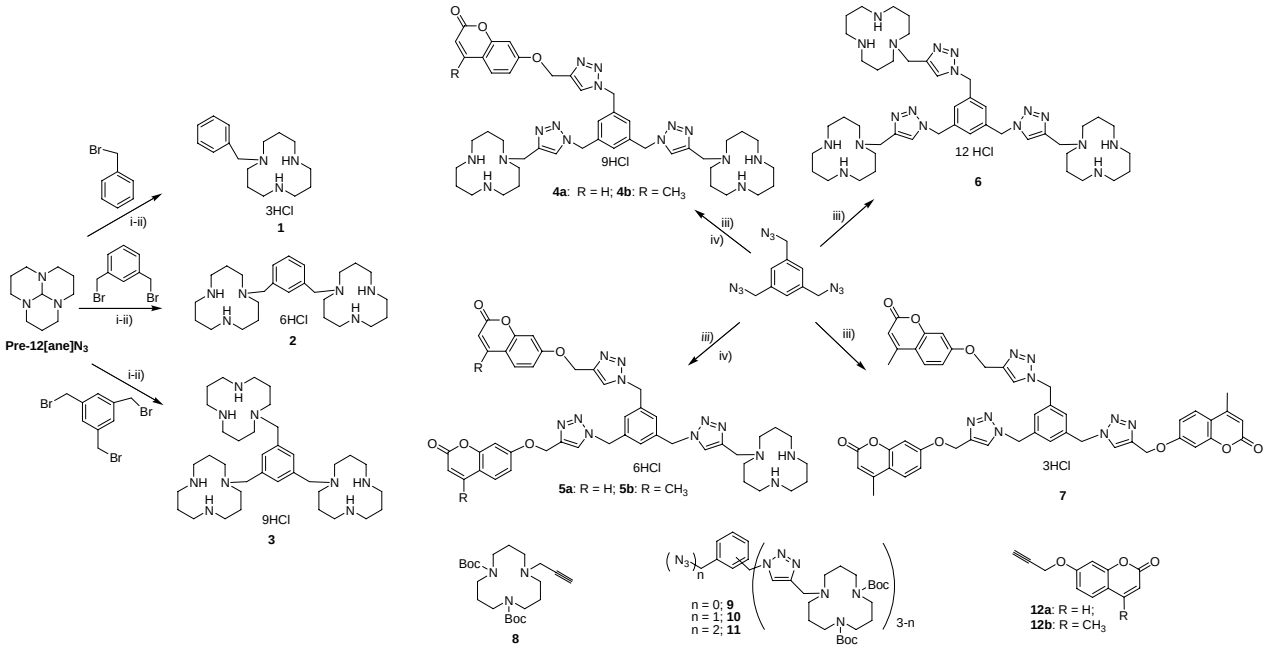

Scheme 1 Structures of compounds **1-6** and their syntheses: i) $CHCl_3$, overnight; ii) 3M HCl, reflux overnight; iii) THF/H_2O, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, overnight; iv) CH_3COCl, CH_3OH. For the step iii) of compound **4a**/**4b**: a) 2 eqv. **8**, b) 1 eqv. **12a**/**12b**; of compound **5a**/**5b**: a) 1 eqv. **8**, b) 2 eqv. **12a**/**12b**; of compound **6**: 3 eqv. **8**; of compound **7**: 3 eqv. **12b**.

With the above consideration, we report here the synthesis of bifunctional molecules bearing macrocyclic polyamine [12]aneN$_3$ and coumarin moieties (Scheme 1) and their application in the condensation of plasmid DNA and gene-transfection. Several control compounds were also synthesized. By varying the combinations of [12]aneN$_3$ and coumarin moieties in the target molecules, the structure-activity relationship of this system was systematically explored. The potential of these small bifunctional molecules to serve as gene vectors was investigated.

Results and Discussion

Syntheses of bifunctional [12]aneN$_3$ compounds

For the preparation of compounds **1**-**3**, pre-[12]aneN$_3$ was prepared according to a literature method[35] and used as the starting material to react with (bromomethyl)benzene, 1,3-bis(bromo-methyl)benzene, 1,3,5-tris(bromomethyl)benzene and followed by acid hydrolysis in 3 M HCl solution (see Scheme 1).

For the synthesis of the bifunctional compounds **4a**/**4b** and **5a**/**5b**, two steps of copper-mediated alkyne–azide click reactions were carried out, using 1, 3, 5-tris(azidomethyl)benzene as the starting material. The synthesis began with Boc-protected *N*-propynyl-[12]aneN$_3$ **8**, which was an important building block for the preparation of multi-[12]aneN$_3$ ligands. The Boc-protected intermediates **9**-**11** were obtained through careful control of the amount of **8** used in the first click reaction. Further click reactions of **10** and **11** with different equivalents of **12a**/**12b** and removal of Boc protecting groups with acetyl chloride in methanol resulted in target compounds **4a**/**4b** and **5a**/**5b**. Compound **6** was obtained directly from compound **9** in high yields after deprotection. Compound **7** was obtained through the one step click reaction of 1, 3, 5-tris(azidomethyl)benzene with 3 equivalents of **12b** (see Scheme 1).

All new compounds were fully characterized by ^{1}H NMR, ^{13}C NMR, IR, ESI, or high-resolution mass spectrometry (details on their characterization are included in the Supplementary data). Compound **7** was found to have a very poor solubility in water and organic solvents, and thus was not further studied.

Gel retardation assay

To assess the DNA condensation ability of the synthesized molecules, gel retardation assays were performed. The effect of [12]aneN$_3$ unit were first evaluated by using compounds **1**-**3**. The results indicated that compound **1**, which contains one [12]aneN$_3$ unit, was not able to condense DNA even at the concentration of 600 μM (Fig. 1a). Compound **2**, which contains two [12]aneN$_3$ units, showed a condensation activity when its concentration was above 300 μM (Fig. 1b). Compound **3**, having three [12]aneN$_3$ units, showed effective condensation activity at 80 μM or above (Fig. 1c), with no form I of plasmid DNA being detected. Compared to **3**, compound **6**, which contains triazol moieties, exhibited an even higher condensing activity, with complete

retardation being observed at less than 60 μM (Fig. 1d). The effect of coumarin moieties was subsequently explored by examining the condensation abilities of compounds **4a**/**4b** and **5a**/**5b**. For **4a** and **5a**, which contain one and two coumarin units, respectively, complete retardation of electrophoresis was not observed even at concentrations up to 100 μM (Fig. 1e and f). However, compounds **4b** and **5b**, which are structurally similar to **4a** and **5a** but with a methyl substituent attached to their coumarin ring, showed effective condensation activity. Complete retardation at 80 μM and 40 μM was observed for **4b** and **5b**, respectively (Fig. 1g and h). Compared to the bifunctional molecules we reported before,[31] the condensation ability of **5b** was further improved.

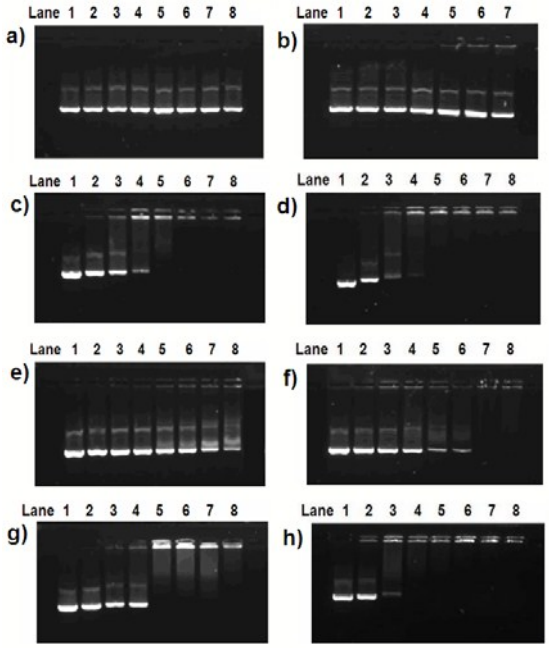

Figure 1 Agarose gel electrophoresis assay to investigate the pUC18 DNA condensation induced by different concentrations of **1** (a), **2** (b), **3** (c), **6** (d), **4a** (e), **5a** (f), **4b** (g) and **5b** (h) in 50 mM Tris-HCl buffer (pH = 7.4). [DNA] = 9 μg/mL, 37 ºC, incubate 1 h. Lane 1: DNA control. (a) lanes 2-8: [**1**] = 50, 100, 200, 300, 400, 500, 600 μM. (b) lanes 2-7: [**2**] = 50, 100, 200, 300, 400, 500 μM. (c-h) lanes 2-8: [**3**] = [**6**] = [**4a**] = [**5a**] = [**4b**] = [**5b**] = 20, 40, 60, 80, 100, 120, 140 μM.

The above results clearly demonstrate that the structures of the designed molecules play an important role for their condensation activities. Firstly, the DNA condensation activity of **3** was much higher than those of **1-2**. The incorporation of triazole moieties (compound **6**) further promoted the condensation effect. The disparities of performance between compounds **3** and **6** can be attributed to their different degrees of protonation; compound **6**, containing additional triazole moieties, should be more protonated. Thus, increasing the number of cationic moieties in the molecules greatly enhanced the condensing activity, which is consistent with those reported in literatures.[7] Secondly **,** the effective DNA condensation activity of **4b** and **5b** can be attributed not only to the electrostatic effects from macrocyclic polyamines but also to the binding interaction between the coumarin moieties and DNA. Compared to the compounds with the same number of [12]aneN$_3$ units, the presence of aromatic units in the molecules greatly promote DNA condensation process, which are consistent with our previous observations.[31, 32] Thirdly, the presence of methyl substituent in the coumarin moieties of **4b** and **5b** further facilitated the condensation abilities of these two compounds when compared to those of **4a** and **5a**. The difference in DNA condensation ability between compounds **4b**/**5b** and **4a**/**5a** may be contributed to enhanced hydrophobic interaction due to the presence of methyl groups in **4b** and **5b**.

The results from gel electrophoresis indicate that replacing one or two [12]aneN$_3$ units in compound **6** with methyl substituted coumarin units led to derivatives with the same or even enhanced DNA condensation capability, suggesting that the coumarin moiety can compensate the binding contribution of the [12]aneN$_3$ unit to a large extent. The incorporation of the coumarin units in the bifunctional molecules also reduced the N/P molar ratio in DNA condensation, which should benefit the reduction of the cytotoxicity of the corresponding compounds. To provide additional insights, the following experiments were performed on compounds **3**-**6**. The results obtained further confirmed the above conclusion.

Dynamic light scattering

DNA condensations in the presence of **3-6** were investigated with dynamic light scattering (DLS) technique. The measurements were performed at 1 μg/mL of DNA in Tris-HCl buffer (6 mM, pH 7.4) at 25.00 ℃ (Figure 2). The obtained results revealed that the effective hydrodynamic diameters of DNA particles condensed by **3**, **6**, and **5b** at different molar ratios of condensing agent vs mononucleoetide ranged from 159 to 346 nm, 94 to 210 nm and 234 to 463 nm, respectively. The sizes of the DNA particles increased with increasing concentrations of the condensing agents. The observation is consistent with examples reported before.[36, 37] Apparently, the particle size plateaued as the molar ratio reached 3.2 and above. The DNA particles condensed by **4a**, **5a** and **4b** at [compound]/[DNA base] molar ratio 3.2 have diameters of 399 nm, 451 nm and 249 nm, respectively (see Supplementary data). The above results also indicate that the more [12]aneN$_3$ units in the condensing agents, more condensed particle were produced. The Zeta potentails of the DNA particles were measured and proved to be close zero, which is somewhat unusual according to our knowledge.

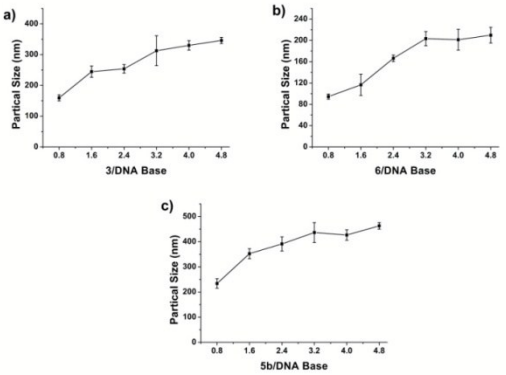

Figure 2 Hydrodynamic diameter distributions of pUC18 DNA particles condensed by **3** (a), **6** (b) and **5b** (c) at different mole ratios (compound/DNA base). The DNA concentration is 1 μg/mL.

Atomic force microscopy

Additional evidence supporting DNA condensation was provided by atomic force microscopy (AFM). Figure 3 shows the typical AFM images of pUC18 in the presence of **3**, **6** and **5b**. The diameters of the nanoparticles condensed by 100 μM of **3**, 80 μM of **6** and 60 μM of **5b** were 141-191 nm, 133-167 nm and 222-444 nm, respectively (Fig. 3a-c). The AFM images clearly demonstrate the DNA condensation ability of **3, 6** and **5b**, which indicate that the more positive charged units in the condensing agents, the more condensed particles there are. The sizes of articles revealed by DLS measurement are larger than those obtained by AFM. This can be attributed to the different sampling conditions adopted in these two different types of techniques.[37]

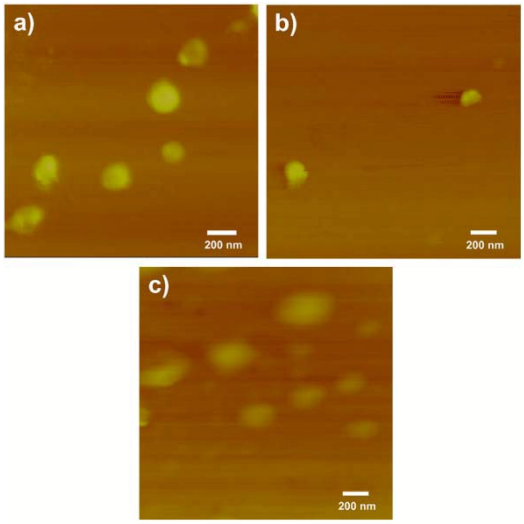

Figure 3 AFM Images of pUC18 DNA (9 μg/mL) and its condensation induced by bifunctional molecules in Tris-HCl buffer (50 mM, pH 7.4): (a) DNA + 100 μM **3**; (b) DNA + 80 μM **6**; (c) DNA + 60 μM **5b**.

EB displacement assay

The binding abilities of compounds **1-6** with DNA were assessed by using the ethidium bromide (EB) displacement assay. It is well known that the emission intensity of EB is enhanced upon intercalating with DNA. Accordingly, quenching of emission due to the displacement of EB by another molecule can be used to evaluate the binding of that molecule to DNA.[38-40] As shown in Figure 4, the emission of the EB-bound CT-DNA was reduced upon the addition of **1-6**. The binding constants were found to be $(2.0 \pm 0.1) \times 10^4$ M^{-1} (**1**), $(6.4 \pm 0.3) \times 10^4$ M^{-1} (**2**), $(2.0 \pm 0.1) \times 10^5$ M^{-1} (**3**), $(1.2 \pm 0.1) \times 10^5$ M^{-1} (**4a**), $(1.4 \pm 0.1) \times 10^5$ M^{-1} (**4b**), $(1.36 \pm 0.09) \times 10^5$ M^{-1} (**5a**), $(2.4 \pm 0.1) \times 10^5$ M^{-1} (**5b**), $(1.8 \pm 0.1) \times 10^5$ M^{-1} (**6**), respectively. The binding constants of **4a**/**b** and **5a**/**b** are similar to those of the naphthelene-derived bifunctional compounds,[31] but smaller than those of the carbozol-derived bifunctional compounds previously reported.[32]

Comparing the binding constants of compounds **1-3** indicates that increasing the number of [12]aneN$_3$ units greatly enhanced the binding strength to DNA, which is consistent with the results from gel electrophoresis assays. The binding constants of compounds **3-6** are very similar, which implies that the electrostatic effect from the [12]aneN$_3$ units and the binding effect from coumarin units can compensate each other in the DNA condensation process.

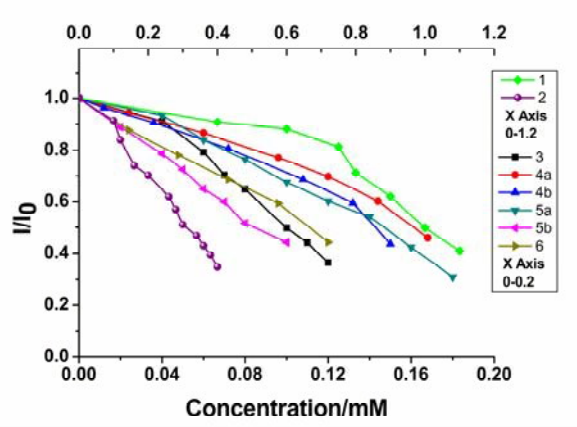

Figure 4 Fluorescence decrease (λ_{ex} = 537 nm, [EB] = 20 μM, [DNA] = 100 μM, 25.0 °C) induced by **1-6** through the displacement of CT-DNA-bound ethidium bromide in the buffer (5 mM Tris-HCl/50 mM NaCl, pH 7.4).

Ionic strength effect

To elucidate the different roles played by [12]aneN$_3$ and coumarin units in DNA condensation, additional experiments were carried out. The effect of ionic strength was first investigated. As shown in Figure 5, the DNA condensation abilities of **3** and **6** were weakened as the concentrations of NaCl increased from 0 to 400 mM, which suggested that the condensing agents bound with the phosphate group of DNA via electrostatic interactions. Because excess salt partially neutralizes the phosphate backbone, which in turn decreases the binding of condensing agents with DNA.[40] The inhibiting effects of NaCl clearly indicate that electrostatic interaction resulted from [12]aneN$_3$ functional units plays important roles in driving the DNA condensation process involving **3** and **6**. For compound **5b**, less ionic strength effect was observed (Fig. 5d), indicating that the driving force in the DNA condensation was mainly from the binding interaction of the coumorin moieties, the electrostatic interaction of the [12]aneN$_3$ unit played a minor role.

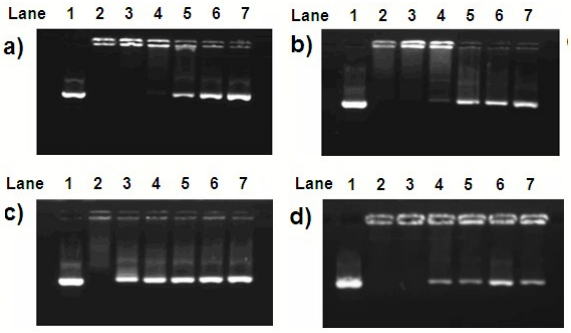

Figure 5 Agarose gel electrophoresis assay to investigate the effect of ionic strength on pUC18 DNA condensation induced by **3**, **6**, **4b** and **5b,** incubation time: 1 h in 50 mM Tris-HCl buffer at 37 ℃. [DNA] = 9 μg/mL, Lane 1: DNA control, lanes 2-7: [NaCl] = 0, 50, 100, 200, 300, 400 mM; [**3**] = 80 μM (a), [**6**] = 60 μM (b), [**4b**] = 80 μM (c), [**5b**] = 60 μM (d).

Reversibility of DNA condensation

The release of DNA from its compact state is very important for efficient nonviral gene vectors. A variety of procedures have been applied to trigger the DNA dissociation from condensing agents, such as pH jump,[41] breaking of linkage,[42, 43] and addition of

additives.[36, 44] In this work, we used high concentration of NaCl solution to release packed DNA. As shown in Figure 6, the condensed DNA could be released after being treated with 100-500 mM of NaCl, indicating that the DNA condensation induced by **3**, **6**, **4b** and **5b** was reversible. Nevertheless, a significant fraction of the condensates still remained in the loading well. Figure 6 also shows that the stability of the condensates is related to the number of [12]aneN$_3$ units in the condensing agents, with more condensed DNA being released for the condensates formed with the bifunctional compounds having more than one [12]aneN$_3$ units, which can be attributed to dissociation triggered by electrostatic interaction. For compound **5b**, the driving force for the DNA condensation should be mainly from the binding interaction associated with the coumarin units. Thus the DNA dissociation was not obvious at high concentration of NaCl. The above result is also consistent with that from the experiment on ionic effect.

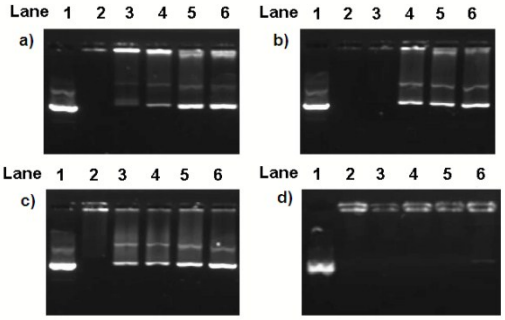

Figure 6 Agarose gel electrophoresis assay to investigate the reversibility of DNA condensation induced by **3**, **6**, **4b** and **5b** in Tris-HCl buffer (50 mM, pH 7.4) at 37 ℃. [DNA] = 9 μg/mL, Lane 1: DNA control, lanes 2-6: [NaCl] = 0, 100, 200, 300, 500 mM; [**3**] = 80 μM (a), [**6**] = 60 μM (b), [**4b**] = 80 μM (c), [**5b**] = 60 μM (d).

As a short summary from the above experimental results, the effective DNA condensation abilities of the bifunctional compounds **4b**/**5b** resulted from two aspects: elelctrostatic interactions with DNA through positive charged [12]aneN$_3$ and triazole units, which is a prerequisite for the condensation; π-π and hydrophobic interactions from coumarin units, which further promoted the condensation process. These two types of interactions showed different effects on the reversibility of DNA condensation, and supported the general mechanism of DNA condensation of most non-viral gene vectors.[31, 32, 45]

Cytotoxicity assay

The cytotoxicity of **1-6** was evaluated by MTT assay against Hela and A549 cell lines based on reported procedure.[31, 46] As shown in Figure 7, compounds **1-6** show weak toxicities towards Hela and A549 cells. Even at the concentration of 100 μM, the viabilities of Hela and A549 were all as high as 75%. For compound **3-6**, the viabilities of Hela and A549 were as high as 85%. Since the minimum concentrations of **3-6** needed for DNA condensation are well below 100 μM, the obtained results on cytoxicity showed that these compounds are suitable for the development of non-viral gene vectors.

Cellular uptake study

To examine the possibility of using the bifunctional compounds as non-viral vectors for gene delivery, cellular uptake studies with Hela cells was carried out by using fluorescein isothiocyanate (FITC)-labeled dsDNA condensates. Although FITC is mostly used for amine-labeling, it is also used for DNA or RNA labeling in cellular uptake experiments,[47, 48] which appear as green dots under fluorescence microscope and is also commercially available. To investigate the cellular uptake and the location of the condensates, the nuclei of Hela cells were stained with DAPI, a nucleus-specific blue fluorescence dye. Preliminary experiments revealed that the presence of DOPE, a common helper lipid that enhances gene transfection efficiency, was necessary for cellular uptake. In the absence of DOPE, no cellular uptake of FITC-DNA was found (see Figure S4a). A molar ratio of 1:1 (condensing agent to DOPE) had stronger cell uptake than other ratios (see Figure S4). In addition, the cellular uptake of NLS-bound condensates was notably improved when compared with the NLS-free ones.

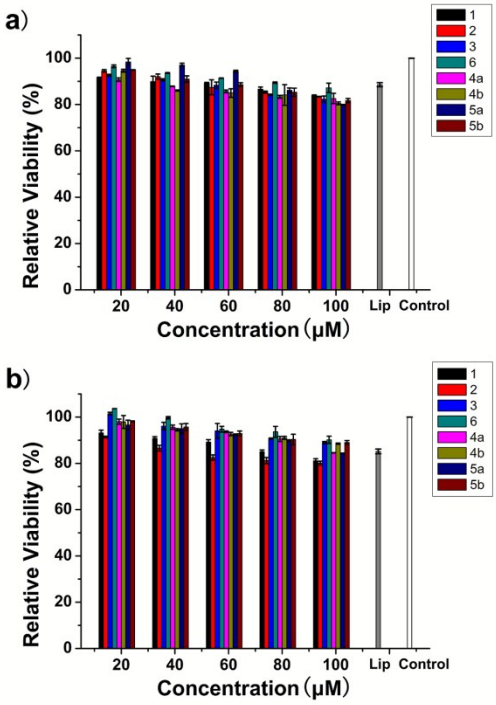

Figure 7 The cytotoxicity of **1-6** toward Hela (a) and A549 (b), respectively, after incubation at 37 ℃ for 24 h.

Figure 8 shows green fluorescent spots observed in the images, which indicates that the cellular uptake of the FITC-DNA condensates containing the bifunctional compounds was successful. In contrast, the image of the control without DNA condensates did not show any observable green fluorescent spots (Fig. 8i). Thus, chemical structure and concentration of the condensing agents have an obvious influence on cellular uptake. Green fluorescent spots increased as the concentration of **4b** was increased from 40 to 80 μM (Fig. 8a, b). More green fluorescent spots for **5b** were found at 20 or 40 μM than those at 60 μM (Fig. 8c-e). Compounds **5a** (Fig. 8f), **6** (Fig. 8g) and **3** (Fig. 8h) showed very poor efficiency for cellular uptake.

The images of cellular uptake demonstrate that the FITC-labeled dsDNA condensates induced by the synthesized bifunctional compounds were able to enter into the cytoplasm and to be distributed around the nuclei, the condensates formed with

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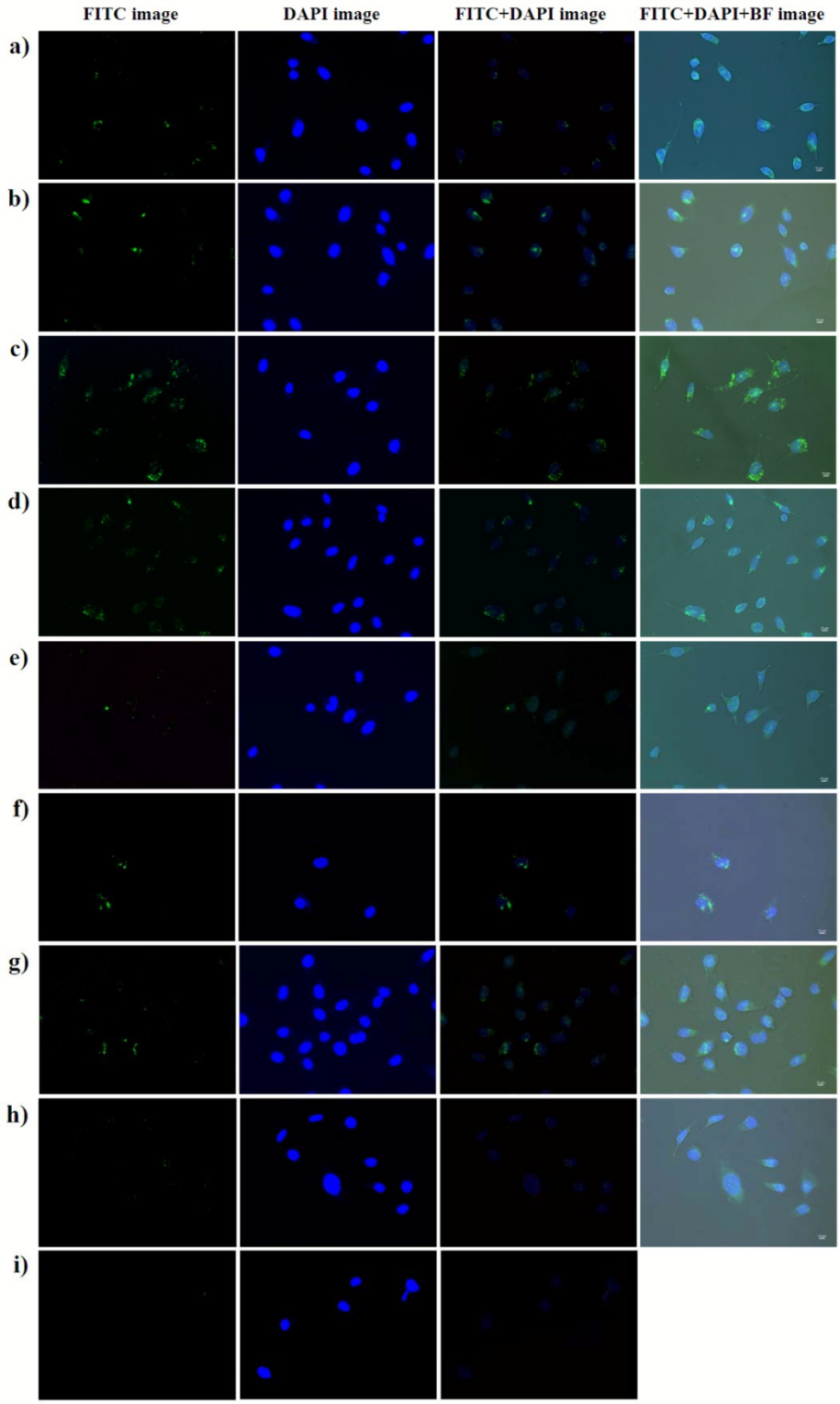

Figure 8 Fluorescence microscope image (40×) of Hela cells transfected with FITC-DNA. The condensing agents/DOPE ratio is 1:1, NLS-bound, the concentration of FITC-DNA was 5 μg/dish. (a-b) **[4b]** = 60, 80 μM, respectively; (c-e) **[5b]** = 20, 40, 60 μM, respectively; (f) **[5a]** = 80 μM; (g) **[6]** = 60 μM; (h) **[3]** = 80 μM; (i) NLS-plasmid assemblies control.

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5b seemed to be more effective. Thus the incorporation of coumarin units in the condensation agents promotes cellular uptake, and methyl groups attached to the coumarin (compounds **4b** and **5b**) units strengthen the uptake process, which can be correlated to the effective condensation of DNA resulting from the presence of the methyl groups.

Cell transfection

In order to visualize the transfection activity of the bifunctional compounds, transfection experiments with the A549 cell line by using a plasmid DNA construct encoding enhanced green fluorescent protein (pEGFP) were carried out. As shown in Fig. 9, similar to results from the cellular uptake experiments, the transfection efficiency also varied with the structures and concentrations of the condensing agents. The condensates with **5b** showed the better transfection efficiency among the condensing agents tested (Fig. 9a-c). For transfection with **4b** and **5a**, fewer green spots were observed (Fig. 9d, e). The condensates with **6** and **3** showed almost no green spots (Fig. 9f-h). Nevertheless, the density of transfected cells with **5b** was less than that observed in the experiment with the commercially available Lipofectamine 2000^{TM} (Fig. 9i). To be noticed, the presence of DOPE is necessay for the cell transfection, the experiments without DOPE showed alomost no cell transfection (results not shown here).

The above results indicate that the combination of coumarin and [12]aneN$_3$ moieties is necessary to achieve effective transfection. The combination of macrocyclic polyamines and aromatic moieties not only resulted in efficient DNA condensation but also led to nonvial gene vectors. As small organic molecules, these compounds have great potential to be further optimized for high efficient and targeted delivery of genes.

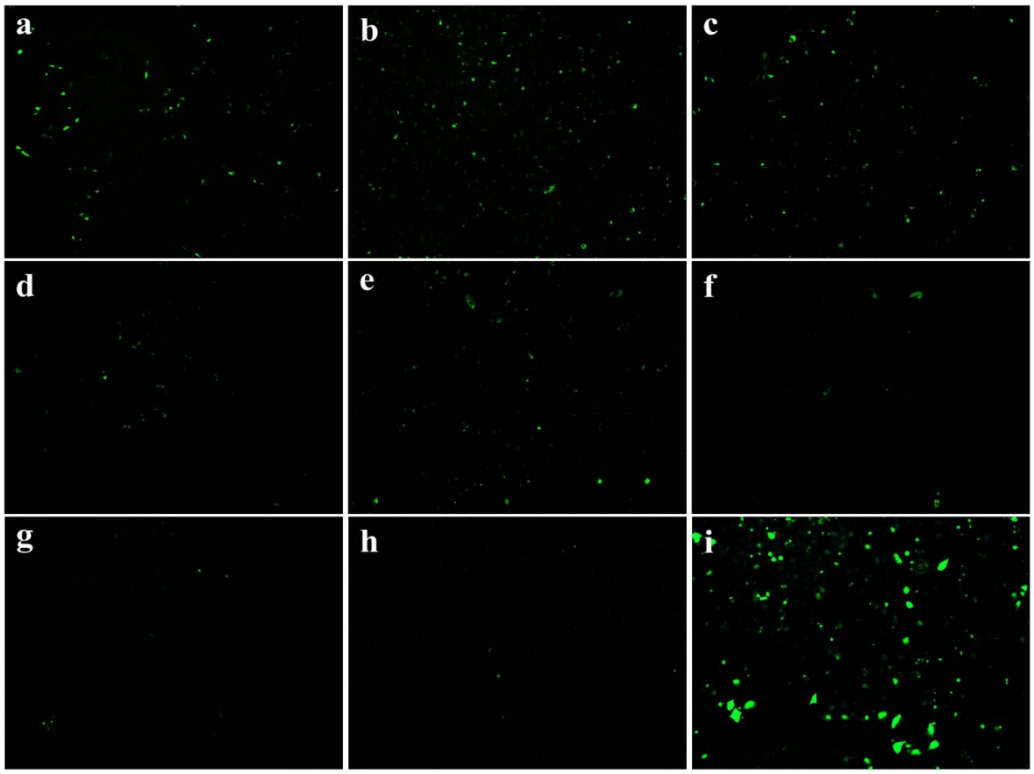

Figure 9 Fluorescence microscope image (10×) of pEGFP-transfected A549 cells. The condensing agents/DOPE ratios were 1:1, NLS-bound, and the concentration of pEGFP-N1 DNA was 5 μg/dish. (a-c) **[5b]** = 20, 40, 60 μM, respectively; (d) **[4b]** = 80 μM; (e) **[5a]** = 80 μM; (f) **[6]** = 60 μM; (g) **[3]** = 80 μM; (h) NLS control; (i) Lipofectamine 2000^{TM}.

Conclusions

Macrocyclic polyamine [12]aneN$_3$ derivatives **1-6** were synthesized as potential DNA condensation agents and non-viral gene vectors. Studies based on agarose gel electrophoresis experiments, AFM and DLS assays clearly indicate that the combination of [12]aneN$_3$ and coumarin moieties could effectively induce plasmid DNA condensation at low concentrations, complete retardation can be achieved at 80 μM and 40 μM for **4b** and **5b**, respectively. Effects of ionic strength and EB replacement assay demonstrate that bifunctional compounds **4a/b** and **5a/b** could interact strongly with DNA due to electrostatic and other binding interactions. The electrostatic interaction imparted by [12]aneN$_3$ moiety can be compensated by

the binding effect from coumarin units for DNA condensation. These different interactions lead to different reversibility for DNA condensation in the presence of high concentration of NaCl. MTT assay on bifunctional compounds **4a/b** and **5a/b** revealed low cytotoxicity. Cellular uptake and cell transfection experiments proved that bifunctional compound **5b** can act as a nonviral gene vector, although its efficiency is lower than Lipofectamine 2000™.

The present work proved that proper combination of structural moieties engaging in electrostatic, π-π stacking and perhaps hydrophobic interactionscan lead to effective DNA condensation agents and nonviral gene vectors with low cytotoxicity and good water solubility. In comparison to cationic lipids and polymeric nonviral gene vectors, these small organic bifunctional molecules have great potential to be further optimized for highly efficient and targeted delivery of nucleic acids.

Experimental

1. Physical Measurements

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance III 400 MHz spectrometer at 25 ºC. Chemical shifts were referenced on residual solvents peaks. The infrared spectra were taken on a Nicolet 380 spectrometer in the range of 4000-400 cm^{-1}. Mass spectra were acquired on a Waters Quattro Mocro spectrometer and high resolution mass spectra were acquired on a Waters LCT Premier XE spectrometer. Electrophoresis apparatus was a BG-subMIDI sub marine system (BayGene Biotech Company Limited , Beijing, China). Bands were visualized by UV light and recorded on a UVP EC3 visible imaging system.

Hydrodynamic diameters were determined using a Brookhaven ZetaPlus Partical Size and Zeta Potential Analyzer. Atomic force microscopy (AFM) images were obtained with a Veeco NanoScope IIIa atomic force microscope. Fluorescence spectra were measured on a Varian Cary Eclipse spectrometer. UV-Vis spectra were measured on a Varian Cary 300 UV-Vis spectrophotometer using solutions in 1.0 cm quartz cuvettes.

2. Chemicals

Electrophoresis grade agarose, 6 × loading buffer (30 mM EDTA, 40% glycerol, 0.03% xylene cyanol FF, and 0.05% bromophenol blue), Goldview II, Ethidium bromide (EtBr), 4,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium boromide (MTT), calf thymus DNA (CT-DNA) and plasmid DNA (pUC 18) were purchased from Solarbio Company (Beijing, China). Dioleoylphosphatidylethanolamine (DOPE) was from Santa Cruz Biotechnology, NLS (nulear localiazation sequence) peptide (PKKKRKV) was from GLS (Shanghai, China). Plasmid pEGFP-N1 vector was from Clontech (Palo Alto, CA, USA). Lipofectamine 2000™ and fluorescein isothiocyanate (FITC)-labeled double-stranded DNA oligomer 5′-GGTCGGAGTCAACGGA-TTTGGTCG-3′ (FITC-DNA) were from Invitrogen (Life technologies, Mauricio Minotta, USA). The concentrations of DNA were determined by NanoVue™ ultraviolet spectrophotometer. Ultrapure milli-Q water (18.25 MΩ) was used in all DNA condensation assays. All solvents and other reagents were of analytical grade and were used as received.

3. Synthesis

Details for the preparation and characterization of compounds **1**, **2**, **8-12** can be found in the Supplementary data.

(1) *Preparation of compound **3***

To a solution of Pre-12[ane]N_3 (0.96 g, 5.2 mmol) in $CHCl_3$ (40 mL) was added 1,3,5-tris(bromomethyl)benzene (0.47 g, 1.3 mmol) and stirred at room temperature for 24 h. Then the resulting dark yellow precipitates were filtered, washed with $CHCl_3$ (6 × 30 mL)，and then dried to afford **3Br-3**. The resulting solid **3Br-3** was dissolved in 3M HCl (36 mL) and refluxed overnight. After that the solvents were removed under reduced pressure. The resulting solid was washed with ethanol and ether, respectively, then dried in vacuum to give **3** as dark yellow solid.

3Br-3): M.p.:132 -133 ℃. ^{1}H NMR (400 MHz, D_2O) δ 8.11 (s, 3H), 7.14 (s, 3H), 3.96 (s, 6H), 3.85 – 3.70 (m, 6H), 3.63 – 3.49 (m, 6H), 3.45 – 3.29 (m, 12H), 3.15 – 2.99 (m, 6H), 2.64 – 2.45 (m, 6H), 2.35 – 2.12 (m, 12H), 1.51 – 1.27 (m, 6H). ^{13}C NMR (101 MHz, D_2O) δ 156.66, 134.77, 132.45, 55.40, 53.82, 52.34, 42.51, 22.34, 19.46. ESI-MS Calcd. for $C_{39}H_{66}N_9^{3+}$: 660.5(M), found: 220.4.

3): 0.72 g, Yield: 58%. M.p.: 210 -211 ℃. ^{1}H NMR (400 MHz, D_2O) δ 7.78 (s, 3H), 4.45 (s, 6H), 3.46 – 3.40 (m, 24H), 3.40 – 3.22 (m, 12H), 2.39 – 2.32 (m, 6H), 2.31 – 2.23 (m, 12H). ^{13}C NMR (101 MHz, D_2O) δ 135.74, 131.27, 57.62, 47.22, 42.40, 41.23, 20.53, 17.76. IR (KBr, cm^{-1}): 3416, 2952, 2752, 2633, 1612, 1583, 1454, 1427, 1384, 1067. HR-MS Calcd. for $C_{36}H_{70}N_9$ $(M+H)^+$: 628.5754, found: 628. 5770.

(2) *Preparation of compounds **4a, 4b***

Boc-protected compound **10** (0.30 g, 0.28 mmol) and compound **12a** (0.09 g, 0.31 mmol) or **12b** (0.10 g, 0.34 mmol) were added into THF/H_2O (v/v = 2:1, 15 mL), $CuSO_4{\cdot}5H_2O$ (8.4 mg, 0.03 mmol) and sodium ascorbate (11.4 mg, 0.06 mmol) were also added into the solution as catalysts. The mixture was stirred over night at room temperature, saturated with NaCl, and extracted with ethyl acetate. The organic layers were washed once with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude products were purified by flash chromatography on silica gel with CH_2Cl_2 /Acetone (1.5:1) to yield the Boc-protected compound. Then the above product was dissolved in CH_3OH (30 mL), and acetyl chloride (15 mL) was dropped into the solution at ice bath. After 2 h the solvent was removed under reduced pressure, and the remaining solid was washed with ether and dried in vacuum.

4Boc-4a): 0.27 g, Yield: 76%. M.p.: 93 ℃-94 ℃. ^{1}H NMR (400 MHz, Acetone) δ 8.17 (s, 1H), 7.92 (s, 2H), 7.90 (s, 1H), 7.61 (d, *J* = 8.6 Hz, 1H), 7.30 (s, 2H), 7.24 (s, 1H), 7.06 (d, *J* = 2.3 Hz, 1H), 7.00 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.23 (d, *J* = 9.5 Hz, 1H), 5.65 (s, 2H), 5.62 (s, 4H), 5.30 (s, 2H), 3.78 (s, 4H), 3.39 – 3.19 (m, 16H), 2.47 – 2.34 (m, 8H), 1.90 – 1.79 (m, 12H), 1.43 (s, 36H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.46, 161.12, 156.54, 155.90, 143.72, 143.42, 137.20, 136.94, 131.03, 129.08, 128.99, 127.54, 123.57, 113.67, 113.18, 113.01, 102.28, 79.70, 62.45, 53.57, 53.41, 49.56, 47.47, 45.59, 44.35, 28.64, 27.87. IR (KBr, cm^{-1}): 2975, 2929, 1735, 1688, 1613, 1477, 1413, 1366, 1165,

1129, 1049. ESI-MS Calcd. for $C_{65}H_{97}N_{15}O_{11}(M+2H)^+$: 1263.7, found: 1263.6.

4a): 0.20 g, Yield: 80 %. M.p.: 165 ℃-166 ℃. ^{1}H NMR (400 MHz, D_2O) δ 8.07 (d, *J* = 4.6 Hz, 3H), 7.69 (d, *J* = 9.5 Hz, 1H), 7.33 (d, *J* = 8.7 Hz, 1H), 7.24 (s, 1H), 7.12 (s, 2H), 6.78 (dd, *J* = 8.6, 2.3 Hz, 1H), 6.56 (d, *J* = 2.0 Hz, 1H), 6.11 (d, *J* = 9.5 Hz, 1H), 5.54 (s, 2H), 5.50 (s, 4H), 5.16 (s, 2H), 4.12 (s, 4H), 3.36 – 3.25 (m, 16H), 3.08 – 2.97 (m, 8H), 2.30 – 2.21 (m, 4H), 2.16 – 2.02 (m, 8H). ^{13}C NMR (101 MHz, D_2O) δ 164.27, 160.53, 154.43, 145.52, 143.05, 136.82, 136.35, 129.54, 127.82, 127.54, 126.47, 125.52, 113.47, 113.20, 111.94, 101.90, 61.26, 53.23, 49.21, 46.72, 43.47, 41.98, 19.87, 19.27. IR (KBr, cm^{-1}): 3429, 2972, 1723, 1614, 1458, 1130, 1052. HRMS Calcd. for $C_{45}H_{64}N_{15}O_3(M+H)^+$: 862.5317, found: 862.5322.

4Boc-4b): 0.23 g, Yield: 65%. M.p.: 96 ℃-97 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H), 7.52 (d, *J* = 8.8 Hz, 1H), 7.37 (s, 2H), 7.10 (s, 3H), 6.94 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.90 (d, *J* = 2.4 Hz, 1H), 6.14 (d, *J* = 1.1 Hz, 1H), 5.49 (s, 2H), 5.47 (s, 4H), 5.24 (s, 2H), 3.76 (s, 4H), 3.35 – 3.25 (m, 16H), 2.46 – 2.41 (m, 8H), 2.40 (d, *J* = 1.0 Hz, 3H), 1.90 – 1.76 (m, 12H), 1.44 (s, 36H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.07, 161.01, 156.26, 155.04, 152.53, 144.32, 143.50, 137.15, 136.67, 127.32, 127.24, 125.77, 123.45, 122.77, 114.04, 112.50, 112.17, 101.98, 79.27, 62.21, 53.93, 53.40, 53.15, 49.70, 46.88, 45.38, 43.87, 28.46, 27.27, 26.06, 18.61. IR (KBr, cm^{-1}): 3434, 2970, 2924, 1734, 1685, 1617, 1385, 1163. ESI-MS Calcd. for $C_{66}H_{99}N_{15}O_{11}(M+2H)^+$: 1277.8, found: 1277.6.

4b): 0.20 g, Yield: 91%. M.p.: 176 ℃-177 ℃. ^{1}H NMR (400 MHz, D_2O) δ 8.08 (d, *J* = 10.9 Hz, 3H), 7.29 (d, *J* = 8.9 Hz, 1H), 7.22 (s, 1H), 7.17 (s, 2H), 6.68 (dd, *J* = 8.9, 2.4 Hz, 1H), 6.40 (d, *J* = 2.3 Hz, 1H), 5.89 (s, 1H), 5.54 (s, 2H), 5.47 (s, 4H), 5.11 (s, 2H), 4.12 (s, 4H), 3.37 – 3.25 (m, 16H), 3.12 – 2.96 (m, 8H), 2.44 – 2.19 (m, 4H), 2.12 (s, 3H), 2.12 – 2.03 (m, 8H). ^{13}C NMR (101 MHz, D_2O) δ 163.85, 160.30, 155.92, 153.66, 139.41, 136.77, 136.38, 127.94, 127.78, 126.48, 126.30, 125.54, 113.78, 113.09, 110.52, 110.42, 101.76, 61.17, 53.24, 49.23, 46.84, 43.42, 42.02, 19.92, 19.33, 17.77. IR (KBr, cm^{-1}): 3430, 2969, 2750, 1717, 1613, 1458, 1391, 1144, 1072, 1053. HR-MS Cacld. for $C_{46}H_{66}N_{15}O_3(M+H)^+$: 876.5473, found: 876.5470.

(3) *Preparation of compounds **5a, 5b***

Boc-protected compound **11** (0.20 g, 0.31 mmol) and compound **12a** (0.24 g, 0.84 mmol) or **12b** (0.21 g, 0.74 mmol) were added into THF/H_2O (v/v = 2:1, 30 mL), $CuSO_4{\cdot}5H_2O$ (7.6 mg, 0.03 mmol) and sodium ascorbate (12.1 mg, 0.06 mmol) were also added into the solution as catalysis. The mixture was stirred over night at room temperature, saturated with NaCl, and extracted with ethyl acetate. The organic layers were washed once with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude products were purified by flash chromatography on silica gel with CH_2Cl_2 /Acetone (1:1) to yield the Boc-protected compound. Then the above product was dissolved in CH_3OH (30 mL), and acetyl chloride (15 mL) was dropped into the solution at ice bath. After 2 h the solvent was removed under reduced pressure, and the remaining solid was washed with ether and dried in vacuum.

2Boc-5a): 0.22 g, Yield: 68%. M.p.: 107 ℃-108 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 – 7.59 (m, 4H), 7.39 (s, 1H), 7.37 (s, 2H), 7.14 (s, 3H), 6.91 (d, *J* = 8.6 Hz, 2H), 6.88 (s, 2H), 6.24 (d, *J* = 9.5 Hz, 2H), 5.50 (s, 4H), 5.48 (s, 2H), 5.24 (s, 4H), 3.76 (s, 2H), 3.37 – 3.25 (m, 8H), 2.50 – 2.34 (m, 4H), 1.91 – 1.76 (m, 6H), 1.43 (s, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.12, 160.85, 156.17, 155.46, 143.37, 137.11, 136.64, 128.93, 127.60, 127.49, 123.60, 122.81, 113.16, 112.83, 112.71, 101.75, 79.18, 62.10, 53.28, 53.04, 49.53, 46.67, 45.28, 43.74, 28.37, 27.17, 25.95. IR (KBr, cm^{-1}): 3428, 2970, 1725, 1682, 1617, 1384, 1125, 1048. ESI-MS Calcd. for $C_{55}H_{66}N_{12}O_{10}(M+2H)^+$: 1054.5, found: 1054.3.

5a): 0.20 g, Yield: 80%. M.p.: 183 ℃-184 ℃. ^{1}H NMR (400 MHz, D_2O) δ 8.12 (s, 1H), 7.94 (s, 2H), 7.40 (d, *J* = 9.5 Hz, 2H), 7.21 (s, 2H), 7.03 (d, *J* = 8.5 Hz, 2H), 6.88 (s, 1H), 6.49 (d, *J* = 8.7 Hz, 2H), 6.18 (s, 2H), 5.87 (d, *J* = 9.4 Hz, 2H), 5.42 (s, 2H), 5.34 (s, 4H), 4.79 (s, 4H), 4.09 (s, 2H), 3.40 – 3.28 (m, 8H), 3.13 – 2.88 (m, 4H), 2.36 – 2.23 (m, 2H), 2.17 – 1.98 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 161.17, 160.41, 155.35, 144.41, 142.36, 137.24, 129.65, 127.65, 127.40, 125.20, 113.00, 112.79, 112.71, 101.65, 61.75, 56.14, 52.49, 48.69, 46.15. IR (KBr, cm^{-1}): 3430, 2921, 1725, 1618, 1384, 1125, 1059. HR-MS Cacld. for $C_{45}H_{49}N_{12}O_6(M+H)^+$: 853.3898, found: 853.3903.

2Boc-5b): 0.24 g, Yield: 75%. M.p.: 108 ℃-109 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 2H), 7.51 (d, *J* = 8.8 Hz, 2H), 7.38 (s, 1H), 7.14 (s, 3H), 6.93 (d, *J* = 8.8 Hz, 2H), 6.87 (s, 2H), 6.13 (s, 2H), 5.50 (s, 6H), 5.24 (s, 4H), 3.77 (s, 2H), 3.40 – 3.21 (m, 8H), 2.50 – 2.41 (m, 4H), 2.39 (s, 6H), 1.94 – 1.75 (m, 6H), 1.43 (s, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.15, 161.08, 161.05, 156.38, 155.13, 152.61, 152.57, 137.29, 136.86, 127.67, 127.60, 125.84, 123.59, 114.14, 112.57, 112.29, 102.08, 102.06, 79.39, 62.34, 53.50, 53.28, 49.89, 45.50, 44.03, 28.56, 27.42, 26.23, 18.66. IR (KBr, cm^{-1}): 3433, 2973, 1724, 1684, 1614, 1386, 1146, 1071. ESI-MS Calcd. for $C_{57}H_{70}N_{12}O_{10}(M+2H)^+$: 1082.5, found: 1082.6.

5b): 0.12 g, Yield: 50%. M.p.: 198 ℃-199 ℃. ^{1}H NMR (400 MHz, DMSO) δ 9.56 – 9.31 (m, 3H), 8.33 (s, 2H), 7.68 (d, *J* = 8.4 Hz, 2H), 7.27 (s, 3H), 7.12 (s, 2H), 7.02 (d, *J* = 7.6 Hz, 2H), 6.22 (s, 2H), 5.62 (s, 6H), 5.26 (s, 4H), 3.47 – 3.05 (m, 12H), 2.39 (s, 6H), 2.23 – 1.86 (m, 6H). ^{13}C NMR (101 MHz, DMSO) δ 161.00, 160.20, 154.64, 153.48, 142.33, 137.20, 128.82, 127.49, 127.25, 126.55, 125.10, 113.42, 112.64, 111.34, 101.60, 61.68, 56.08, 52.43, 18.56, 18.17. IR (KBr, cm^{-1}): 3434, 1721, 1619, 1384, 1110, 1075. ESI-MS Cacld. for $C_{47}H_{54}N_{12}O_6(M+H)^+$: 882.4, found: 882.3. HR-MS Cacld. for $C_{47}H_{53}N_{12}O_6(M+H)^+$: 881.4211, found: 881.4209.

(4) *Preparation of compound **6***

Boc-protected compound **9** (0.45 g, 0.30 mmol) was dissolved in CH_3OH (30 mL), acetyl chloride (15 mL) was added dropwisely into the solution at ice bath. After 2 h the solvent was removed under reduced pressure, and the remaining solid was washed with ether and dried in vacuum to give **6** as white solid.

6): 0.38 g, Yield: 97%. M.p.: 186 ℃-188 ℃. ^{1}H NMR (400 MHz, D_2O) δ 8.20 (s, 3H), 7.26 (s, 3H), 5.63 (s, 6H), 4.31 (s, 6H), 3.40 – 3.28 (m, 24H), 3.26 – 3.10 (m, 12H), 2.32 – 2.22 (m, 6H), 2.22 – 2.10 (m, 12H). ^{13}C NMR (101 MHz, D_2O) δ 138.84, 136.47, 127.88, 127.01, 53.41, 48.83, 47.44, 43.15, 41.84, 19.98, 18.99. IR (KBr, cm^{-1}): 3424, 2956, 2793, 1613, 1585, 1458, 1365,

1232, 1126, 1058, 746. HR-MS (m/z) Cacld. for $C_{45}H_{79}N_{18}$ $(M+H)^+$: 871.6735, found: 871.6730.

(5) *Preparation of compound* ***7***

1,3,5-tris(azidomethyl)benzene (0.32 g, 1.33 mmol) and compound **12b** (0.85 g, 3.97 mmol) were added into THF/H_2O (v/v = 2:1, 30 mL), $CuSO_4 \cdot 5H_2O$ (45.1 mg, 0.18 mmol) and sodium ascorbate (88.3 mg, 0.44 mmol) were also added into the solution as catalysis. The mixture was stirred at room temperature for 36 h. The solvent was then removed under reduced pressure, the remaining solid was washed with water, methanol, dichloromethane, THF and dried in vacuum.

7)：0.77 g, Yield: 65%. ^{1}H NMR (400 MHz, DMSO) δ 8.30 (s, 3H), 7.65 (d, *J* = 8.5 Hz, 3H), 7.27 (s, 3H), 7.09 (s, 3H), 7.00 (d, *J* = 7.8 Hz, 3H), 6.19 (s, 3H), 5.61 (s, 6H), 5.24 (s, 6H), 2.37 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 160.95, 160.04, 154.57, 153.25, 142.30, 137.18, 127.38, 126.36, 124.96, 113.31, 112.49, 111.26, 101.50, 61.66, 52.37, 18.03. IR (KBr, cm^{-1}): 1712, 1618, 1385, 1289, 1199, 1147, 1070, 1051, 847. HR-MS (m/z) Cacld. for $C_{48}H_{39}N_9Na_2O_9^{2+}$: 931.2666 (M+2Na), found: 464.9942.

4. Agarose gel electrophoresis

Negatively supercoiled pUC18 DNA (9 μg/mL) was treated with the condensing agents in Tris-HCl buffer (50 mM, pH 7.4) at room temperature with a total volume of 20 μL. After incubation for different times, 4 μL of 6 × loading buffer was added to the mixtures. The solutions were analyzed by electrophoresis for 40 min at 85 V on a 0.7% agarose gel in 1 × TAE buffer. The gel was stained with 2 μL of 5000 × Goldview II and photographed on an UVP EC3 visible imaging system.

5. Dynamic light scattering

Zeta Potential Analyzer was used to determine the average size of DNA nanoparticles condensed by different agents at 25.0 ℃. The scattering angle was set to 90°. DNA solutions (1 μg/μL) were prepared in the presence of condensing agents (Tris-HCl buffer 6 mM, pH 7.4) with deionised Milli-Q water (18.25 MΩ). The mixture was allowed to stand for 15 min at room temperature and then 400 μL of the solution was transferred into the standard quartz cuvette for measurement. Data were based on triplicate independent experiments.

6. Atomic force microscopy

A stock solution of pUC18 DNA (400 μg/mL) was diluted to 9 μg/mL (Tris buffer 50 mM, pH 7.4) in the presence of each compound **3**, **6** and **5b**. Then the corresponding solutions were left to equilibrate at 37 ℃ for 1 h. Freshly cleaved mica was used as substrate for all AFM imaging. Pretreatment of mica was necessary to promote electrostatic immobilization between the condensates and mica. Thus 15 μL of a 10 mM $NiCl_2$ solution was deposited for 2 min onto the surface of mica. The mica was then thoroughly rinsed with pure water to prevent the formation of salt crystals on the surface. A total of 10 μL of DNA solution was spotted onto the pretreated mica and incubated for 5 min. After that the mica was thoroughly rinsed with water and dried under a gentle steam of argon. AFM images were obtained in the air at room temperature with a Veeco atomic force microscope. Scans were run at a rate of 1-3 Hz operating in tapping mode using conical-shaped Si tips integrated to nano-crystalline Si cantilevers with an average resonance frequency of 280 kHz. The images were analyzed with the software accompanying with the imaging module.

7. EB displacement assay

A Cary Eclipse Luminescence Spectrometer was used for the EB displacement assay to confirm the DNA binding ability of the DNA condensing agents in Tris-HCl buffer (5 mM, pH 7.4) with 50 mM NaCl at room temperature. CT-DNA (100 μM) was first treated with EB (ethidium bromide, 20 μM), then the condensing agents was added and the sample was incubated for 2 min prior to measurement (excitement wavelength 537 nm). The apparent binding constant (K_{app}) was calculated using the equation: $K_{EB} \times [EB] = K_{app} \times [\text{condensing agent}]$, where $K_{EB} = 1.0 \times 10^6\ M^{-1}$, [EB] = 20 μM, and [condensing agent] was the concentration at which a 50% reduction of the fluorescence had occurred.

8. Ionic strength effect

The influence of ionic strength on DNA condensation was investigated by agarose gel electrophoresis at room temperature. pUC 18 DNA (9 μg/mL) was treated with the condensing agents (80 μM for **3**, **4b** and 60 μM for **6**, **5b** respectively) in Tris-HCl buffer (50 mM, pH 7.4) over a range of NaCl concentrations. After incubation for 1 h, the solutions were analyzed by electrophoresis as described previously.

9. Release of the compact DNA

The reversibility of DNA condensation induced by the condensing agents was investigated by agarose gel electrophoresis at room temperature in Tris-HCl buffer (50 mM, pH 7.4). pUC 18 DNA (9 μg/mL) was first treated with the condensing agents (80 μM for **3**, **4b** and 60 μM for **6**, **5b,** respectively). After incubation for 1h to facilitate the condensation, the solutions were treated with NaCl at different concentrations for another 1 h and analyzed by electrophoresis as the method mentioned previously.

10. Cytotoxicity assay

The cytotoxicity of compound **1-6** toward Hela and A549 cell lines were tested by MTT assays (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Both cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (FBS, 10%, v/v) in a humid atmosphere containing 5% CO_2 at 37 ºC. After 48 h of incubation in the medium, the cells were seeded in 96-well plates at 5000 cells and 100 μL medium per well and cultured for another 24 h. Then the cells were treated with different concentrations of **1-6**, blank, and Lipofectamine 2000^{TM} in 100 μL DMEM, 10 μL DMEM with 10% FBS was added to each well 4 h later, and cells were further cultured for 20 h. After that the medium was removed and 20 μL of MTT (5 mg/mL) was added to wells, the cells were incubated for another 4 h. Finally MTT was replaced with 200 μL of DMSO, the plates were oscillated for 10 min to fully dissolve the formazan crystals formed by living cells in the wells. The absorbance of the purple formazan was recorded at 490 nm using a Thermo Scientific Multiskan GO. The relative viability of the cells was calculated based on the data of five parallel tests by comparing to the controls.

11. Preparation of DNA condensates

1 μmol condensing agents were mixed with different mole ratio of DOPE in 1 mL Tris-HCl buffer (10 mM, pH 7.4), respectively. The mixtures were sonicated for 20 min in a bath sonicator at 60℃ and stored at 4℃.

To prepare the DNA condensates, 0.03 μM NLS was mixed with 5 μg FITC-DNA or pEGFP-N1 DNA before addition of the condensing agents/DOPE complexes of varied concentrations (20-100 μM), then diluted and mixed in DMEM by pipetting up and down several times to a total volume of 500 μL. After that the resulting mixtures were incubated at room temperature for about 30 min. A commercially available transfection reagent Lipofectamine 2000^{TM} was utilized for comparison. The DOPE or NLS-free DNA condensates and NLS-plasmid assemblies were utilized as controls.

12. Cellular uptake study

The cellular uptake of FITC-labeled dsDNA condensates was observed by fluorescence microscope. Hela cells were cultured in DMEM medium supplemented with 10% FBS in a humid atmosphere containing 5% CO_2 at 37 ºC. The cells were seeded in Glass Bottom Cell Culture Dishes at 1000 cells per dish and cultured for 24 h. After washed three times with DMEM, the cells were treated with freshly prepared FITC-DNA condensates and the controls (500 μL). The blue fluorescence dye DAPI (5 μg/mL) was also added to each dish for nuclear staining. After that the cells were cultured for 4 h. Finally, the cells were washed for 6 times with PBS buffer, observed using a Zeiss Inverted Fluorescence Microscope with a 40× objective and DAPI filter for DAPI (blue), GFP filter for FITC (green), respectively.

13. Cellular transfection

To examine the expression of the internalized DNA, A549 cells were transfected by the condensates containing pEGFP-N1. Cells were seeded in Glass Bottom Cell Culture Dishes at 80000 cells per dish and cultured until reach 80% cell confluence at 37 ºC in a humid atmosphere containing 5% CO_2. Before transfection, the medium was washed three times with DMEM, and treated with freshly prepared pEGFP-DNA condensates and the controls (500 μL). After 4 h under standard culture conditions, the medium was replaced with 500 μL of fresh DMEM medium containing 10% FBS and cultured for another 20 h. Then, the cells were washed for 3 times with PBS, and observed under Zeiss Inverted Fluorescence Microscope with a 10 × objective to examine the expression of the intracellular EGFP.

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Notes and references

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