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Effective and reversible DNA condensation induced by bifunctional molecules containing macrocyclic polyamines and naphthyl moieties

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ABSTRACT

A series of bifunctional molecules containing macrocyclic polyamines [12]aneN$_3$ and naphthyl moieties **1**–**3**(**a**, **b**) have been designed and synthesized through efficient N-alkylation and copper-mediated alkyne–azide click reactions. Experiments on gel electrophoresis, dynamic light scattering and atomic force microscopy confirmed that **2b** and **3b** with two [12]aneN$_3$ units efficiently induced the DNA condensation at the concentration of 120 μM in less than 5 min. The condensation mechanism was studied by EB displacement fluorescence spectra, viscosity titration, and ionic strength effects. The condensation process was found to be reversible, and the presence of both naphthyl and [12]aneN$_3$ units in the molecules was proved to be necessary for the effective DNA condensation inductions. Cytotoxicity assay showed that the presence of triazole moieties can result in lower toxicity.

1. Introduction

During the past two decades, gene therapy has attracted intensive attention as a promising method for treating intractable genetic disorder, cancer, and heart diseases. The development of effective and safe carrier vectors capable of compacting and protecting DNA for gene therapy has become one of the greatest challenges for chemists and biologists. To this end, rapid advances have recently been made in the design and syntheses of nonviral gene vectors due to their advantages including adjustable structure, nonimmunogenicity, and potential for large-scale production over viral vectors.[1–4] DNA condensation is an essential step for gene therapy, the condensing agents used as nonviral gene vectors reported so far include multivalent cations derived from small organic molecules such as spermine and spermidine,[5] as well as organic polymers such as polyamines,[6,7] polysaccharides,[8] cationic lipids,[9–11] peptides,[12–14] dendrimers[2,15] and metal complexes.[16–20] In spite of the various nonviral vectors that have been investigated, small organic molecules capable of effectively condensing DNA remain largely undeveloped.

Macrocyclic polyamines, such as 1,4,7-triazacyclononane ([9]aneN$_3$), 1,5,9-triazacyclodoecane ([12]aneN$_3$), and 1,4,7,10-tetraazacyclododecane ([12]aneN$_4$), have been widely used in the development of artificial nucleases and drugs.[21–25] With their multiple nitrogen atoms and unfolded conformations, these molecules can serve as suitable cationic units that interact with negative charged phosphate in the DNA backbone and induce desired condensation. More recently, Yu et al. has applied [12]aneN$_4$ units in cationic lipids and organic polymers and proved their applicability as nonviral gene vectors.[26,27] On the other hand, it is well known that large planar aromatic rings such as naphthyl exhibit strong binding ability toward DNA.[28–31] Studies on mechanism of DNA condensation indicate that major factors responsible for effective condensation include electrostatic force, groove binding, and/or intercalation.[2,32–34] Thus effective organic DNA condensing agents should be resulted based on units that engages in electrostatic and other strong binding interactions.

In consideration of all factors mentioned above, herein we report on the preparation of a series of bifunctional organic molecules **1**–**3**(**a**, **b**) (Scheme 1) and their capabilities of inducing DNA condensation. It is anticipated that the cationic [12]aneN$_3$ will be responsible for electrostatic interaction, while the naphthyl unit will bind strongly to DNA molecules via intercalation or groove binding. The two structural units are expected to work synergistically, leading to the effective DNA condensation. Structure–activity relationship of these compounds has been examined by using gel electrophoresis, dynamic light scattering, and atomic force microscopy. The obtained data clearly demonstrates that bifunctional molecules **2b** and **3b** are highly efficient in condensing plasmid DNA into particulate structures. These molecules can thus be used as a new class of effective DNA condensing agents.

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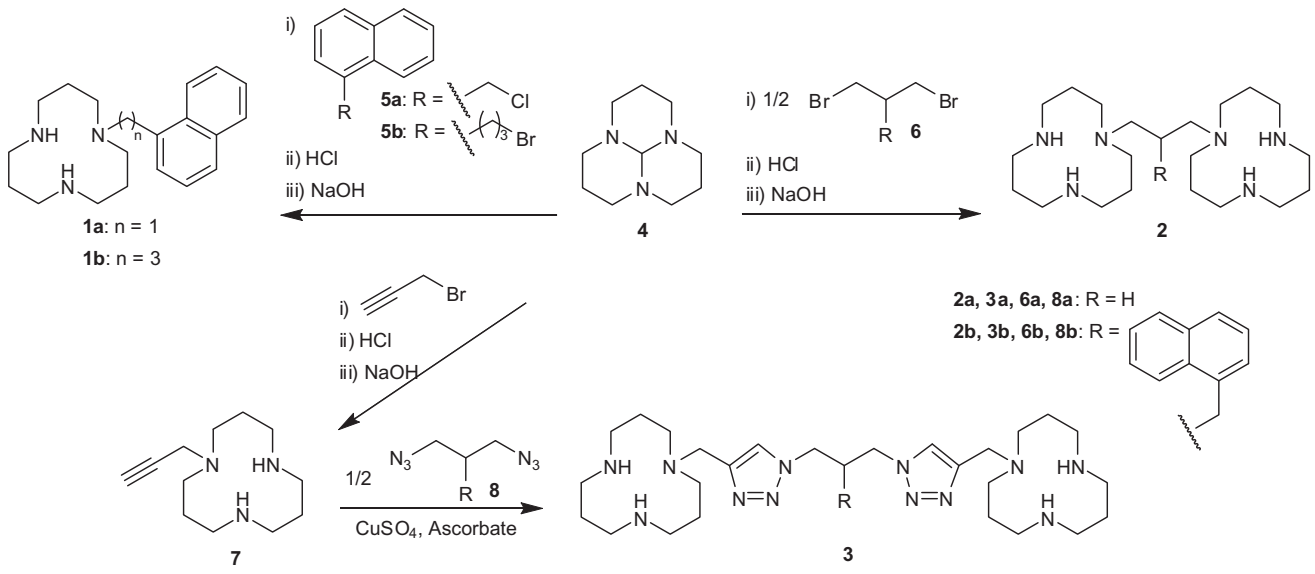

Scheme 1. Syntheses of functional macrocyclic polyamines **1**–**3**(**a**, **b**).

2. Results and discussion

2.1. Syntheses of bifunctional molecules

To obtain the bifunctional molecules aimed at the development of new effective DNA condensing agents, two procedures were applied in the syntheses (Scheme 1).

In the first procedure, orthoamide **4** was used as starting material to react with monohalides **5**(**a**, **b**) or dihalides **6**(**a**, **b**) through alkylation reactions. The target compounds **1** and **2** were achieved by hydrolysis of the above resulted stable salts in 3 M HCl solution and further basification with 5 M NaOH. Halides **5b** and **6b** were prepared from compound **5a** through alkylation which was seen in the Supplementary data).

In the second procedure, copper-mediated alkyne–azide click reactions were carried out. As a modular synthetic approach, click chemistry has been explosively applied in all areas of modern chemistry from drug discovery to material science in recent years. The synthesis began with *N*-propynyl-[12]aneN$_3$ **7**, which was an important building block for the preparation of multi-[12]aneN$_3$ ligands and was prepared by propargylation of orthamide **4**. The diazido compounds **8**(**a**, **b**) were easily afforded in high yields by the substitution reactions of corresponding bromide and tosylate with sodium azide. After the click reactions, compounds **3**(**a**, **b**) were converted to their hydrochlorides and further purified by recrystalization, which are stable for long time and easy to be handled.

All new compounds were fully characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy (details on their characterization are included in the Supplementary data).

2.2. Gel retardation assay

To assess the ability of bifunctional molecules **1**–**3** in condensing plasmid DNA, gel retardation assay was performed. Retardation of DNA bands in the gel can indicate the decrease of the negative charge on the plasmid DNA and the formation of large-sized DNA particles.[16,17,35,36] First, we examined the condensation ability of **1a** and **1b**, each of which has one [12]aneN$_3$ unit connected to a naphthyl unit through a methylene (in **1a**) or trimethylene (in **1b**) linker. The obtained results indicate that **1a** could not condense DNA even at a concentration up to 300 μM (Fig. 1a). In contrast, **1b** showed condensation activity when its concentration was above 120 μM, however, with free DNA (Form I) being clearly present even at 300 μM (Fig. 1b). Both compounds **2b** and **3b**, with di-[12]aneN$_3$ and naphthyl moieties in their structures, showed effective condensation activity (Fig. 1c and d). At elevated concentrations of **2b** and **3b** (varied from 0 to 160 μM), the amount of supercoiled closed circular pUC18 DNA (Form I) diminished steadily, and the retardation of DNA became increasingly noticeable. When the concentrations of **2b** or **3b** reached 120 μM, no form I of plasmid DNA could be detected. Instead, the strongly compacted DNA remained in the gel loading wells. Compared to **2b**, compound **3b** which contains triazol moieties exhibited a slightly higher condensing activity. In controlled experiments, **2a** and **3a**, which have two [12]aneN$_3$ units but contain no naphthyl unit, failed to show any condensation effects under identical conditions or even at concentrations up to 300 μM (Fig. S1 in Supplementary data).

The above results clearly demonstrate that the bifunctional structure of **2b** and **3b** plays an important role for the observed high condensation activity. The DNA condensation activity of **2b** and **3b** can be attributed not only to the electrostatic effects from macrocyclic polyamines but also to the binding interaction between the naphthyl moieties and DNA. Besides, the length of the aliphatic linker between the two functional moieties also directly affects the condensing activity (e.g., compound **1** is inactive because its linker is too short). Increasing the number of cationic moieties in the bifunctional molecules greatly enhanced the condensing activity. Finally, the presence of triazole moiety further promoted condensation. Compared to other small organic condensing agents such as spermine, speramidine,[5] and recently developed [12]aneN$_4$ derivative,[26] **2b** and **3b** can induce the condensation of plasmid DNA in less than 5 min, on the micro-molar scale, and in the absence of Mg^{2+} ions. In previous literature, spermine, spermidine or lipo-spermine promoted DNA condensation under concentrations of at least 1 mM and in the presence of Mg^{2+} ions.[5] With these encouraging results, compounds **2b** and **3b** were investigated in more detail.

2.3. Dynamic light scattering

DNA condensations in solution in the presence of **2b** or **3b** were investigated by dynamic light scattering (DLS) technique.[17,33] The measurement was performed at DNA concentration of 4 μg/mL in

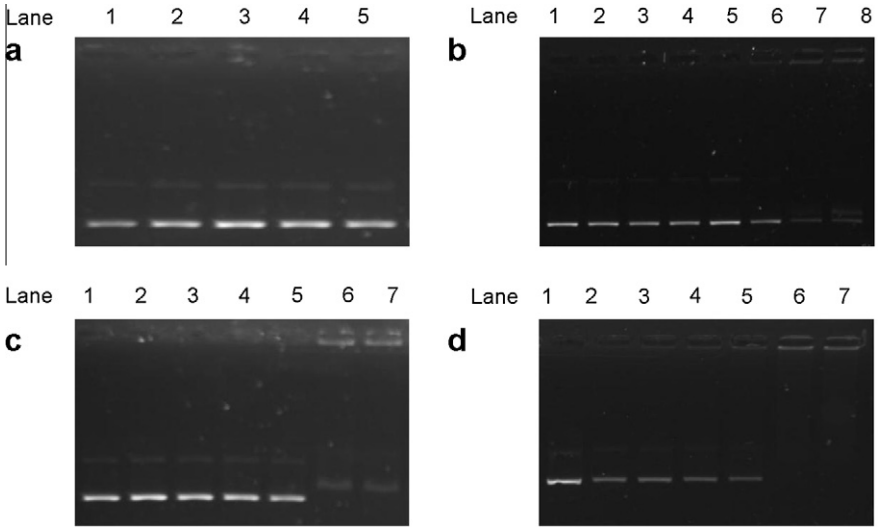

Figure 1. Agarose gel electrophoresis assay to investigate the pUC18 DNA condensation induced by different concentrations of **1a** (a), **1b** (b), **2b** (c) and **3b** (d) in Tris–HCl buffer (50 mM, pH 7.4). (a) lanes 1–5: [**1a**] = 0, 60, 120, 200, 300 μM; (b) lanes 1–8: [**1b**] = 0, 20, 40, 80, 120, 160, 200, 300 μM; (c) lanes 1–7: [**2b**] = 0, 20, 40, 60, 80, 120, 160 μM; (d) lanes 1–7: [**3b**] = 0, 20, 40, 60, 80, 120, 160 μM. The DNA concentration is 9 μg/mL.

Tris–HCl buffer (50 mM, pH 7.4) at 25.00 °C (Fig. 2). The obtained results revealed that the effective hydrodynamic diameters of DNA particles condensed by **2b** at 60 and 120 μM were about 104.8 and 146.0 nm, respectively. The DNA particles condensed by **3b** at the same concentrations have diameters of 180.8 and 221.6 nm. The sizes of the DNA nano-particles increased with the increase of the concentrations of condensing agents, which is consistent with examples reported in literatures.[16]

2.4. Atomic force microscopy

Additional evidence supporting DNA condensation was provided by atomic force microscopy (AFM).[16,18] Figure 3 shows the typical AFM images of pUC18 in the absence and presence of **2b** and **3b**. AFM images in the absence of **2b** and **3b** showed that naked DNA formed closed loops displaying twists of its strands (Fig. 3a). The size of the DNA strands is consistent with that of uncondensed DNA.[17] Upon the addition of 60 μM of **2b**, the DNA molecules were induced to form nanoparticles with a diameter of 47–56 nm (Fig. 3b), along with the presence of some free DNA molecules as relaxed circles with their strands twisted. As the concentration of **2b** was increased to 120 μM, all the DNA molecules were condensed into larger nanoparticles, with diameters between 55–81 nm (Fig. 3c). Similar condensation phenomenon was observed when DNA was treated with **3b**. The diameters of the nanoparticles condensed by **3b** at concentrations of 60 and

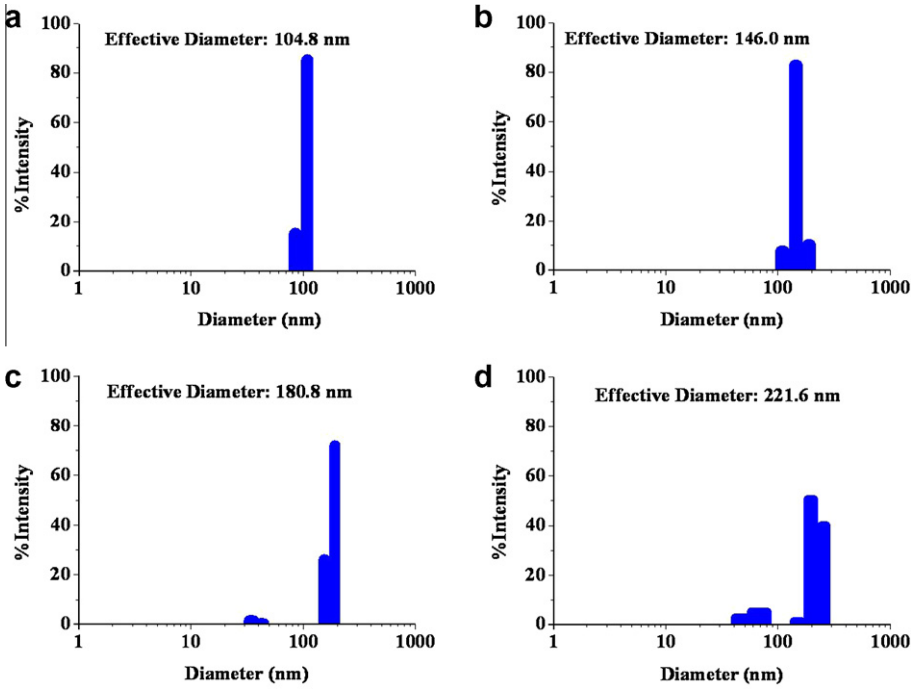

Figure 2. Hydrodynamic diameter distributions of pUC18 DNA particles condensed by (a) 60 μM **2b**, (b) 120 μM **2b**, (c) 60 μM **3b** and (d) 120 μM **3b** in Tris–HCl buffer (50 mM, pH 7.4) at a scattering angle of 90° at 25.00 °C. The DNA concentration is 4 μg/mL.

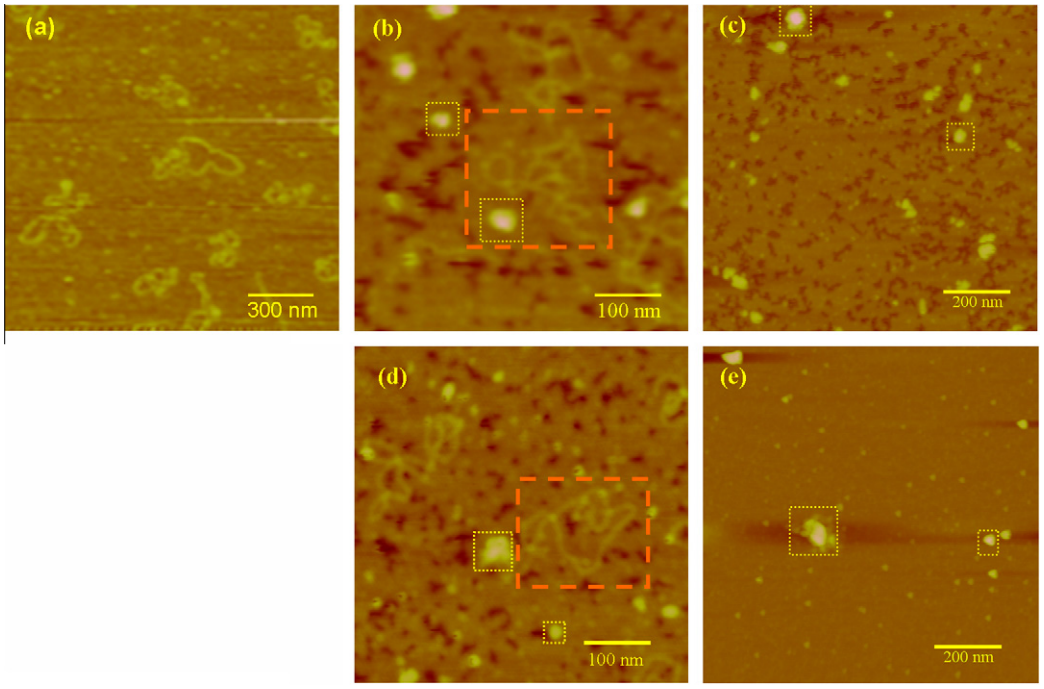

Figure 3. AFM Images of pUC18 DNA (9 μg/mL) and its condensation induced by bifunctional molecules in Tris–HCl buffer (5 mM, pH 7.4): (a) DNA; (b) DNA + 60 μM **2b**; (c) DNA + 120 μM **2b**; (d) DNA + 60 μM **3b**; (e) DNA + 120 μM **3b**.

120 μM were 49–71 and 56–118 nm, respectively (Fig. 3d and e). The AFM images clearly demonstrate the effective DNA condensation ability of both **2b** and **3b**. The sizes of **2b**/**3b**-DNA complex particles revealed by DLS measurement are larger than those obtained by AFM. This can be attributed to the different sampling techniques used in these two different types of experiments.[37] For AFM, the samples were dried, which caused dehydration of the complex and resulted in the decrease in the apparent size of the condensed particle. DLS provides *z* average values in solution in which larger particles contribute more than smaller ones.

2.5. EB displacement assay

The binding ability of compound **2b** and **3b** with DNA were assessed using the ethidium bromide (EB) displacement assay. It is well known that the emission intensity of EB is greatly 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 ability.[38–40] As presented in Figure 4, the emission of the EB-bound CT-DNA was reduced upon the addition of **2b** or **3b**. It was found that the binding constants were $(1.2 \pm 0.1) \times 10^5$ and $(0.98 \pm 0.06) \times 10^5$ M^{-1} for **2b** and **3b**, respectively. The data clearly indicates that the DNA-binding mode of these bifunctional molecules is mainly the groove binding mode.[40] These binding constants are higher than those of previously reported propargylic sulfones containing naphthyl units,[41] which is a clear indication that the [12]andN_3 units can strengthen the binding ability of naphthyl with DNA in **2b** and **3b**.

2.6. Ionic strength effect

To elucidate the different roles played by the two functional units, that is, [12]andN_3 and naphthyl of **2b** or **3b** in DNA condensation, several additional experiments were carried out. The effect of ionic strength in buffer solutions was first investigated. As shown in Figure 5, the DNA condensation ability of **2b** and **3b** was weakened with the increase of NaCl concentrations from 0

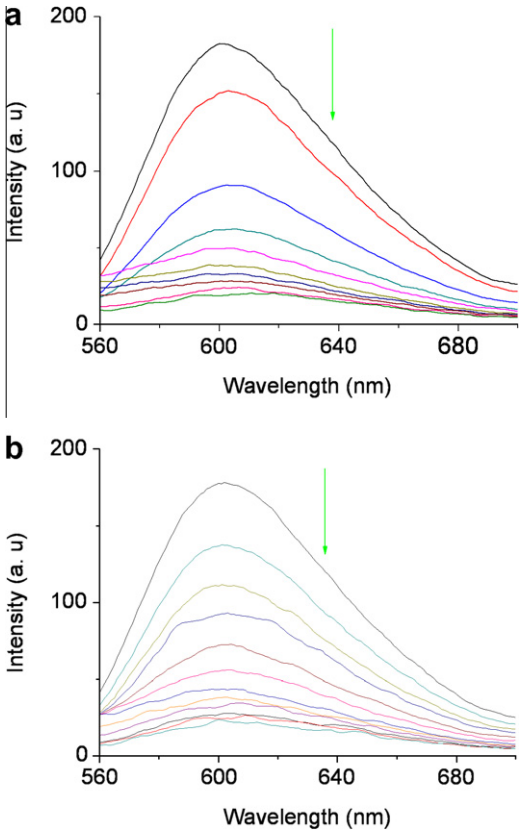

Figure 4. Fluorescence quenching curves of EB bound CT-DNA by **2b** (a) and **3b** (b) in Tris–HCl buffer (50 mM, pH 7.4) (λ_{ex} = 524 nm, [EB] = 4 μM, [DNA] = 80 μM, 25.0 °C). The arrows show the intensity changes on increasing the concentration of the condensing agents form 0 to 0.9 mM.

to 400 mM, which suggested that the condensing agents bound with the phosphate group of DNA via electrostatic interactions.

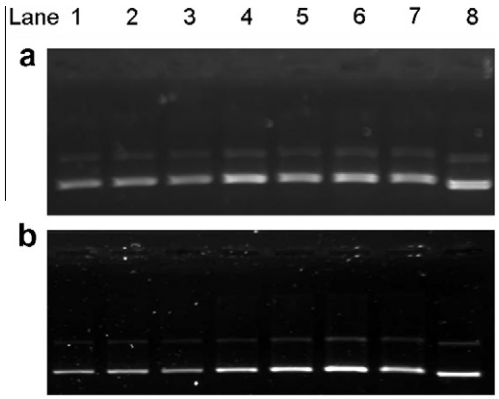

Figure 5. Agarose gel electrophoresis assay to investigate the effect of ionic strength on pUC18 DNA condensation induced by **2b** (a) and **3b** (b) in Tris–HCl buffer (50 mM, pH 7.4): lane 1–7, [NaCl] = 0, 10, 50, 100, 200, 300, 400 mM; lane 8, DNA control. The concentration of DNA and the condensing agents are 9 μg/mL and 80 μM, respectively.

Because excess salt partially neutralizes the phosphate backbone, which in turn decreases the binding of condensing agents with DNA.[42,43] Nevertheless, DNA condensation was still obvious as the concentration of NaCl raised above that of physiological value. The inhibiting effects of NaCl clearly indicate that electrostatic interaction resulted from [12]aneN$_3$ functional units plays important roles in driving the condensation process.

2.7. Viscosity titration assay

Viscosity titration assay is always used to determine the binding mode of small molecules with DNA. The relative specific viscosity of DNA gives a measurement on the increase of DNA length resulted from the separation of base pairs caused by intercalation, a classical DNA intercalating compound like EB showed significant increase in the viscosity of the DNA solutions.[44,45] In contrast, groove binding and electrostatic binding do not show pronounced effect on the viscosity. As presented in Figure 6, neither **2b** nor **3b** showed significant effect on the viscosity of DNA. Thus the intercalating binding mode should be ruled out. Groove binding mode between macrocyclic polyamines appended with arylmethyl and DNA have been reported a few times in the literatures.[46,47] Together with the results of ionic strength effect assay, EB displacement experiments, and structure activity relationship in the gel electrophoresis study, we speculate that **2b** and **3b** bind with DNA by both electrostatic interaction and groove binding mode.

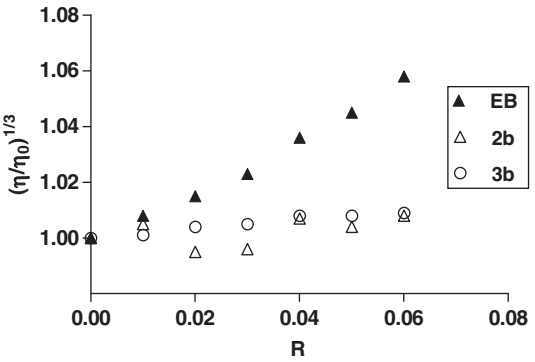

Figure 6. Relative specific viscosities of CT-DNA at 37 °C in 10 mM Tris–HCl buffer (pH 7.4) as the function of the concentration ratio (R) of condensing agents and CT-DNA (500 μM).

2.8. Reversibility of DNA condensation

The release of DNA from its compact state is very important for efficient nonviral gene vectors.[37,48] Thus the ability to reversibly condense DNA is a prerequisite for being an effective vector, that is, good condensing agents should not only have the ability to condense DNA, but also should release DNA from the condensates under proper conditions. A variety of procedures have been applied to trigger the DNA dissociation from condensing agents, such as pH jump,[49] linkage break,[50] and addition of additives.[16,26,51] Here we used high concentration of NaCl solution to release the DNA. As shown in Figure 7 of gel electrophoresis, the condensed DNA could be released after being treated with 300 mM of NaCl, indicating that the DNA condensation induced by **2b** and **3b** was reversible. Obviously, since the release of the condensed DNA in the cell is a complicated process,[1] our experiment intends to show that the condensation process is potentially reversible. Such release may be attributed to the electrostatic competition between condensing agents and NaCl with phosphate backbone of DNA.

2.9. Cytotoxicity assay

The cytotoxicity of **2b** and **3b** was evaluated by MTT assay against HepG2 and T98G cell lines according to the procedure in the literature.[33] As presented in Figure 8, **2b** exhibited high cytotoxicity towards both HepG2 and T98G, so it is hard to use **2b** as a gene vector. In contrast, **3b** showed only a weak toxicity towards HepG2 and did not show obvious cytotoxicity towards T98G. Even when the concentration of **3b** increased to 120 μM, the viabilities of HepG2 and T98G were 64% and 88%, respectively. Since the minimum concentration of **3b** needed for DNA condensation is below 120 μM, it might be suitable for the development of nonviral gene vector.

3. Conclusion

With the aim to develop new effective DNA condensing agents and nonviral gene vectors, a series of small organic bifunctional molecules have been designed and synthesized by the combination of macrocyclic polyamine [12]aneN$_3$ unit for charge neutralization of negative phosphate and naphthyl moieties for DNA binding. Studies on gel electrophoresis, dynamic light scattering, atomic force microscopy, fluorescence spectra, and viscosity titrations showed that bifunctional compounds **2b** and **3b** can effectively

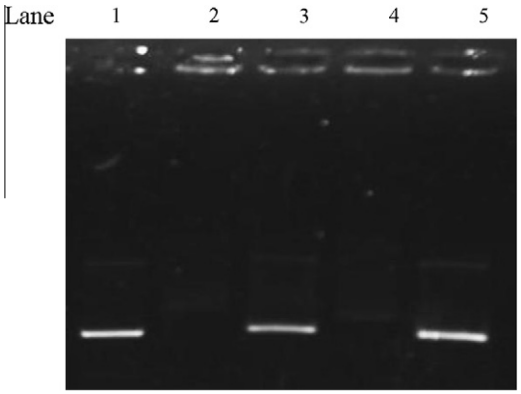

Figure 7. Agarose gel electrophoresis assay to investigate the reversibility of DNA condensation induced by **2b** and **3b** in Tris–HCl buffer (50 mM, pH 7.4): lane 1, DNA control; lane 2, DNA + **2b**; lane 3: DNA + **2b**, then treated with NaCl; lane 4, DNA + **3b**; lane 5: DNA + **3b**, then treated with NaCl. The concentrations of DNA, the condensing agents and NaCl are 9 μg/mL, 120 μM and 300 mM, respectively.

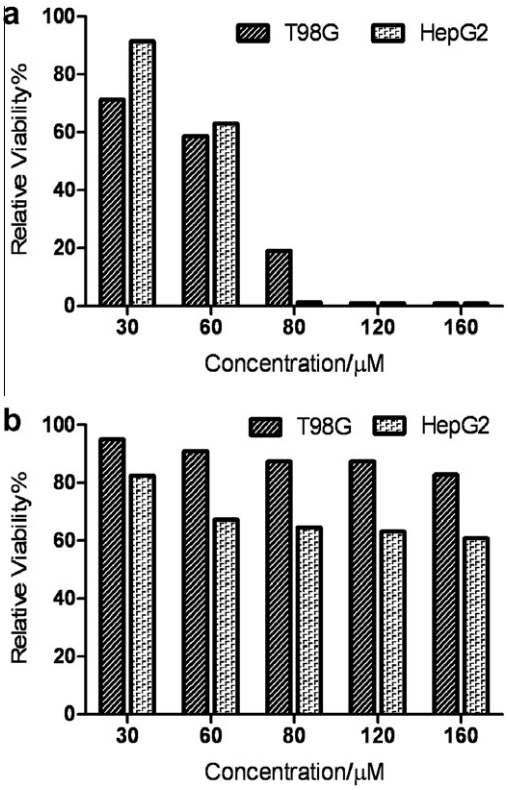

Figure 8. The cytotoxicity of **2b** (a) and **3b** (b) toward T98G and HepG2, respectively, after incubation at 37 °C for 24 h.

induce the DNA condensation at concentrations below 120 μM in less than 5 min and in a reversible way. Structure variations and mechanism investigations have confirmed that the presence of both the naphthyl and di-[12]aneN_3 functional units are necessary for the condensation activities. This work clearly demonstrated that these small molecules containing both electrostatic neutralization and groove binding functional units are a new type of effective agents capable of condensing DNA. Research aimed to better understand the condensation process and to develop the compounds into nonviral gene vectors is underway.

4. Experimental section

4.1. Materials and methods

Calf thymus DNA (CT-DNA) and pUC18 DNA were purchased from Solarbio company. The concentrations of CT-DNA were determined by UV spectroscopy at 260 nm, taking 6600 M^{-1} cm^{-1} as the molar absorption coefficient. All solvents and reagents were of analytical grade and were used as received. Ultrapure milli-Q water (18.25 MΩ) was used in all DNA condensation assays. ^1H and ^{13}C NMR spectra were obtained on a Bruker Avance III 400 MHz spectrometer at 25 °C. Chemical shifts were referenced on residual solvents peaks. The infrared spectra were taken on a Nicolet Magna 750 spectrometer. Mass spectra were acquired on a Waters Quattro Mocro spectrometer and high resolution mass spectra were acquired on a Waters LCT Premier XE spectrometer. Hydrodynamic diameters were determined using a DynaPro Nanostar dynamic laser light scattering apparatus. AFM images were obtained with a Veeco NanoScope IIIa atomic force microscope. UV–vis spectra were measured on a Varian Cary 300 UV–vis spectrophotometer using solutions in 1.0 cm quartz cuvettes. Fluorescence spectra were recorded on a Varian Cary Eclipse Spectrometer. The viscosity was measured using a Ubblehode viscometer.

4.2. Syntheses

Compounds **2a**,[52] **4**[53] and **8a**[54] were synthesized according to literature methods. Details for the preparation and characterization of compounds **5b**, **6b**, **7** and **8b** can be found in the Supplementary data. A typical procedure for the preparation of compounds **1a**, **1b** and **2b** was described for the synthesis of **1a**, **3a** and **3b** were prepared in a similar way.

4.2.1. 1-(1-Naphthylmethyl)-1,5,9-triazacylododecane (1a)

To a solution of 1-chloromethylnaphthalene (0.89 g, 5.0 mmol) in acetonitrile (25 mL) was added **4** (0.91 g, 5.0 mmol). The reaction was heated to reflux for 24 h and then cooled down to room temperature. After evaporation of the solvents, the resulting solid was dissolved in aqueous solution of HCl (3.0 N, 10 mL) and the mixture was refluxed overnight. After which time the solution was cooled down and the solvents were removed under reduced pressure. The resulting solid was recrystallized with ethanol to give **1a** as its hydrochloric salt, white powder (1.87 g, 89%). ^1H NMR (400 MHz, D_2O, 25 °C): δ 8.10 (m, 3H), 7.69 (m, 2H), 7.58 (m, 2H), 4.84 (s, 2H), 3.47 (br, 4H), 3.31 (br, 8H), 2.19 ppm (br, 6H); ^{13}C NMR (101 MHz, D_2O, 25 °C): δ 133.89, 131.37, 129.45, 127.85, 126.95, 125.72, 122.72, 55.94, 47.39, 42.47, 41.18, 20.66, 17.79 ppm; IR (KBr): $\bar{\nu}$ 780 (w), 806 (w), 1071 (w), 1459 (m), 1596 (m), 2752 (s), 2951 (s), 3411 cm^{-1} (m); HRMS (ES): *m*/*z*: calcd for: $C_{20}H_{30}N_3$: 312.2440; found 312.2444 $[M+H]^+$.

4.2.2. 1-[3-(1,5,9-Triazacylododec-1-yl)propyl]naphthalene (1b)

Yield 72%; ^1H NMR (400 MHz, D_2O, 25 °C): δ 8.00 (d, J = 8.3, 1H), 7.86 (d, J = 8.0, 1H), 7.74 (d, J = 8.2, 1H), 7.48 (m, 2H), 7.42–7.23 (m, 2H), 3.25–3.06 (m, 16H), 2.11–1.91 ppm (m, 8H); ^{13}C NMR (101 MHz, D_2O, 25 °C): δ 136.12, 133.63, 131.07, 128.91, 127.24, 126.68, 126.57, 126.17, 125.92, 123.47, 54.42, 47.13, 41.98, 41.15, 28.73, 24.45, 20.54, 17.51 ppm; IR (KBr): $\bar{\nu}$ 781 (w), 792 (w), 1068 (w), 1365 (w), 1459 (m), 1589 (m), 2755 (s), 2950 (s), 3431 cm^{-1} (m); HRMS (ES): *m*/*z*: calcd for: $C_{22}H_{34}N_3$: 340.2753; found 340.2754 $[M+H]^+$.

4.2.3. 1,3-Bis-(1,5,9-triazacylododec-1-yl)-2-(1-naphthylmethyl) -propane (2b)

Yield 72%; ^1H NMR (400 MHz, D_2O, 25 °C): δ 7.98 (d, J = 8.2 Hz, 1H), 7.83 (d, J = 8.0 Hz, 1H), 7.73 (d, J = 7.8 Hz, 1H), 7.50 (dt, J = 15.0, 7.0 Hz, 2H), 7.35 (m, 2H), 3.64 (m, 2H), 3.48–2.80 (br, 28H), 2.62 (s, 1H), 1.50–2.25 ppm (m, 12H); ^{13}C NMR (101 MHz, D_2O, 25 °C): δ 133.76, 133.68, 131.34, 129.09, 128.38, 127.90, 126.93, 126.38, 125.94, 123.58, 55.70, 49.89, 47.26, 46.17, 42.08, 41.30, 40.86, 36.69, 33.03, 20.73, 18.96, 16.85 ppm; IR (KBr): $\bar{\nu}$ 742 (w), 802 (w), 1005 (w), 1066 (w), 1366 (w), 1456 (m), 1588 (m), 2790 (s), 2949 (s), 3419 cm^{-1} (m); HRMS (ES): *m*/*z*: calcd for: $C_{32}H_{55}N_6$: 523.4488; found 523.4508 $[M+H]^+$.

4.2.4. 1,3-Bis-[4-(1,5,9-triazacylododec-1-ylmethyl)-1,2,3-triazol-1-yl]propane (3a)

To a solution of **7** (0.42 g, 2 mmol) in H_2O/*tert*-butanol (3/3, 6 mL) were added **8a** (0.13 g, 1 mmol), $CuSO_4$ (16 mg, 0.1 mmol), and sodium ascorbate (40 mg, 0.2 mmol) under argon. The solution was stirred at room temperature for 24 h. *Tert*-butanol was evaporated in vacuo, and aqueous ammonia (25%, 10 mL) was added. The solution was extracted with CH_2Cl_2 (3 × 15 mL); the combined organic phase was dried over Na_2SO_4, filtrated and concentrated in vacuo. The residue was dissolved in 20 mL acetone; then adjusted the pH to about 2 with hydrochloric acid. Immediately upon addition of hydrochloric acid, a white precipitate formed. The mixture was filtrated and recrystallized with ethanol to give **3a** as its hydrochloric salt, white powder (0.61 g, 73%). ^1H NMR (400 MHz, D_2O, 25 °C): δ 8.09 (s, 2H), 4.42 (t, J = 6.6, 4H), 4.19 (s, 4H), 3.23

(m, 24H), 2.51 (m, 2H), 2.14 (m, 12H); ^{13}C NMR (101 MHz, D_2O, 25 °C): δ 139.10, 126.64, 49.02, 47.58, 47.26, 43.31, 41.94, 29.41, 19.94, 19.12 ppm; IR (KBr): $\bar{\nu}$ 745 (w), 849 (w), 915 (w), 996 (w), 1056 (m), 1224 (w), 1359 (w), 1459 (m), 1584 (m), 2758 (s), 2956 (s), 3422 cm^{-1} (s); HRMS (ES): *m*/*z*: calcd for: $C_{27}H_{53}N_{12}$: 545.4516, found 545.4528 $[M+H]^+$.

4.2.5. 1,3-Bis-[4-(1,5,9-triazacylododec-1-ylmethyl)-1,2,3-triazol-1-yl]-2-(1-naphthylmeth-yl)propane (3b)

Yield 96%; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 7.78 (m, 3H), 7.63 (m, 2H), 7.49–7.39 (m, 2H), 7.33–7.25 (m, 1H), 7.22 (d, J = 6.8 Hz, 1H), 4.50 (d, J = 6.4 Hz, 4H), 3.98 (s, 4H), 3.39–3.13 (br, 16H), 3.10–2.73 (br, 11H), 2.19 (br, 4H), 2.03 ppm (br, 8H); ^{13}C NMR (101 MHz, D_2O, 25 °C): δ 138.19, 133.54, 133.40, 130.94, 128.74, 127.67, 127.42, 127.22, 126.45, 126.17, 125.77, 123.20, 52.46, 48.96, 46.93, 43.26, 41.91, 39.80, 33.91, 19.97, 19.12 ppm; IR (KBr): $\bar{\nu}$ 742 (w), 804 (w), 1053 (w), 1227 (w), 1458 (m), 1594 (m), 2754 (s), 2951 (s), 3422 cm^{-1} (s); HRMS (ES): *m*/*z*: calcd for: $C_{38}H_{61}N_{12}$: 685.5142; found 685.5123 $[M+H]^+$.

4.3. Agarose gel electrophoresis

Negatively supercoiled pUC18 DNA (9 ng/μL) was treated with the condensing agents in Tris buffer (50 mM, pH 7.4) at room temperature with a total volume of 20 μL. After incubation for 5 min, 2 μL of loading buffer (10 mM Tris–HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol, 60 mM EDTA) was added to the mixtures. The solutions were analyzed by electrophoresis for 5 min at 80 V and then 30 min at 100 V on a 0.7% agarose gel in 1× TAE buffer. The gel was stained with Goldview II and photographed on an UVP EC3 visible imaging system.

4.4. Dynamic light scattering

Dynamic laser light scattering (DLS) equipment was used to determine the average size of DNA nanoparticles condensed by different agents at 25.00 °C. The scattering angle was set to 90°. Typically, 10 runs were measured for each solution, with the average of all the runs reported. DNA solutions (4 μg/mL) were prepared in the presence of condensing agents (Tris buffer 50 mM, pH 7.4) with deionised Milli-Q water (18.25 MΩ). The mixture was allowed to stand for 5 min at room temperature and then 40 μL of the solution was transferred into the standard quartz cuvette for measurement.

4.5. Atomic force microscopy

A stock solution of pUC18 DNA (450 μg/mL) was diluted to 9 μg/mL (Tris buffer 5 mM, pH 7.4, $MgCl_2$ 1 mM) in the absence and presence of macrocyclic polyamine. $MgCl_2$ were used optimally in order to facilitate the adhesion of DNA onto mica for better viewing. Then the corresponding solutions were left to equilibrate at 37 °C for 5 min. Subsequently, samples were studied by AFM in air condition. 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 10 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 8 μL of DNA solution was spotted onto the pretreated mica and incubated for 5 min. After which 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. Scan 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.

4.6. 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 buffer (50 mM, pH 7.4) at room temperature. CT-DNA (80 μM) was first treated with EB (ethidium bromide, 4 μM), then the condensing agents was added and incubated for 2 min prior to measurement (excitement wavelength 524 nm). The apparent binding constant (K_{app}) was calculated using the equation: K_{EB}·[EB] = K_{app}·[condensing agent], where $K_{EB} = 1.0 \times 10^6\ M^{-1}$, [EB] = 4 μM, and [condensing agent] was the concentration at which a 50% reduction of the fluorescence had occurred.

4.7. Ionic strength effect

The influence of ionic strength on DNA condensation was investigated by agarose gel electrophoresis at room temperature. pUC18 DNA (9 μg/mL) was treated with the condensing agents in Tris buffer (50 mM, pH 7.4) over a range of NaCl concentrations. After incubation for 5 min, the solutions were analyzed by electrophoresis as the method mentioned previously.

4.8. 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 buffer (50 mM, pH 7.4). pUC18 DNA (9 μg/mL) was first treated with the condensing agents (120 μM). After incubation for 5 min to facilitate the condensation, the solutions were treated with NaCl (300 mM) and analyzed by electrophoresis as the method mentioned previously.

4.9. Viscosity titration assay

The viscosity measurement was carried out using a Ubblehode viscometer in a constant temperature bath at 37.0 ± 0.1 °C and a stopwatch. CT-DNA (500 μM) was used in Tris–HCl buffer (10 mM, pH 7.4). The data were presented as $(\eta/\eta_0)^{1/3}$ versus [condensing agent]/[DNA], where η is the specific viscosity of DNA in the presence of condensing agents and η_0 is the specific viscosity of DNA alone in Tris–HCl buffer. Specific viscosity values were calculated from the observed flow time of DNA solutions (t) corrected for the buffer alone (t_0), $\eta = (t - t_0)/t_0$.

4.10. Cytotoxicity assay

The cytotoxicity of compound **2b** and **3b** toward HepG2 and T98G cell lines were tested by MTT assays (MTT = 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (15%, v/v) and T98G cells were cultured in minimum essential medium (MEM, Gibco) supplemented with fetal bovine serum (10%, v/v) in a humid atmosphere containing 5% CO_2 at 37 °C. After 48 h of incubation in the medium, the cells were seeded in 96-well plates at 5×10^5 cells per well and cultured for another 24 h. Then the cells were treated with different concentrations of **2b** and **3b** for 24 h, after which time the medium was removed and 10 μL of MTT (5 mg/mL) was added to wells along with 90 μL of culture medium. The cells were incubated for 4 h and the MTT containing medium was replaced with 110 μL of DMSO. Finally the plates were oscillated for 10 min to fully dissolve the formazan crystal formed by living cells

in the wells. The absorbance of the purple formazan was recorded at 490 nm using a Bio-Rad 680 plate reader. The relative viability of the cells was calculated based on the data of four parallel tests by comparing to the controls.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.11.069.

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