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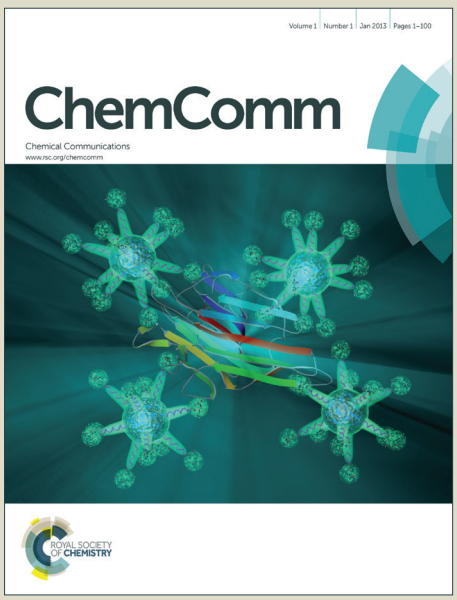

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Chemical Communications ChemComm

Cite this: DOI: 10.1039/c0xx00000x

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Communication

TACN-based oilgomers with aromatic backbones for efficient nucleic acid delivery†

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Cationic oligomers with rigid aromatic backbone were first applied as non-viral gene delivery vectors. These materials showed better DNA condensation ability than their flexible analogues. *In vitro* transfection experiments revealed that the materials with more rigid backbone exhibited considerably higher TE and lower cytotoxicity than 25 KDa PEI.

Design of safe and efficient gene vectors is currently an urgent challenge for gene therapy. In the past decades, various cationic polymers have emerged as one type of promising non-viral gene vector candidates.[1] In contrast to viral vectors, polycations have low immunogenicity and high nucleic acid loading capacity, and they can be prepared reproducibly, designable, inexpensively, and in large-scale.[2] Cationic polymers can electrostatically compact nucleic acids into nanoparticles termed "polyplexes".[3] The condensation may facilitate cellular uptake through various endocytic pathways, and inhibit intra- or extracellular enzymatic degradation.[4] Common cationic polymers such as polyethyleneimine (PEI),[5] Superfect,[6] and chitosan[7] have been used commercially and academically for gene delivery. It should be noted, however, that despite remarkably development of cationic polymeric gene vectors, therapeutic applications of such biomaterials remain limited as a result of their inadequate safety and transfection performance.[8]

One most used strategy to enhance the transfection efficiency (TE) and biocompatibility of polycationic gene vectors is the graft of functional groups, such as PEG,[9] hydrophobic groups,[10] and targeting ligands.[11] These modifications may improve the stability, cellular interaction and uptake, endosomal escape, and intracellular unpacking of the polyplexes. Among these methods, hydrophobic modification of cationic polymers has been established as an effective and straightforward approach to improve membrane binding and gene transfection.[12] So far, most current hydrophobic modification studies intensively aim at conjugation with aliphatic alkyl groups that vary in length and number.[13] Nevertheless, the effect of alkyl modification on the transfection is still controversial.[14] Although some more rigid moieties such as aromatic ring,[15] cholesterol,[16] folate,[17] and glucocorticoid ligand[18] have been attached to polycations, polymeric gene vectors with aromatic backbones were seldom studied.

Our group recently developed non-viral gene vectors with the structures based on 1, 4, 7-triazacyclononane (TACN) due to its unique characteristics, especially pH buffering capacity.[19] Herein, we report a new type of TACN-based oligomers with aromatic phenyl groups in the backbone (Fig. 1). Results revealed that the aromatic moiety plays important role in the gene transfection process, and much higher TE and lower cytotoxicity were obtained by comparison with the "golden standard" 25 KDa branched PEI.

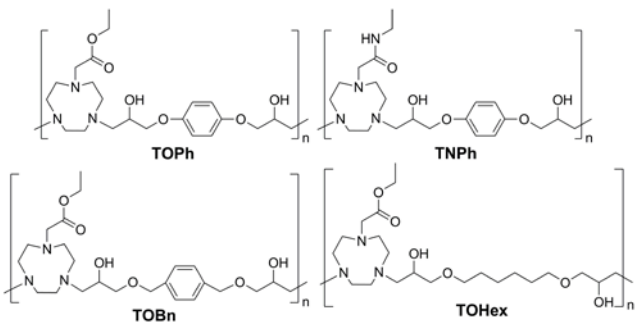

Fig. 1 Chemical structures of TACN-based oligomers.

The title oligomers were prepared through the polymerization between mono-substituted TACN and diglycidyl ether (see ESI† for details). Three diglycidyl ether bridges with varied rigidity (**TOPh** > **TOBn** > **TOHex**) were introduced for the purpose of elucidation of the structure-activity relationship (SAR). Furthermore, amide-contained analog (**TNPh**) was also prepared for comparison. ^{1}H NMR (400 MHz) spectra of the four novel compounds were in full accordance with the expected structures. MALDI-TOF MS results revealed similar molecular weights (Mw ≈ 4800) and low polydispersity indexes (PDI: 1.03 ~ 1.11) for all oligomers.

The polyplexes formed at various oligomer/DNA weight ratios (w/w) were subjected to agarose electrophoresis to detect the amount required to inhibit the migration of plasmids. As shown in Fig. 2A, it was found that all the materials can effectively bind DNA and completely retard DNA migration at the w/w ratio of 8. Increase of the number of carbon atoms in the bridge (**TOBn**) might result in lower positive charge density, leading to relatively weaker DNA binding ability. To quantitatively study the effect of the backbone structure of oligomers on their DNA affinity, ethidium bromide (EB) exclusion assay was carried out (Fig. 2B). Results showed that all newly synthesized linear oligomers could dramatically decrease the fluorescence intensity caused by the

intercalation of EB toward DNA base pairs. With the increase of weight ratio, the relative fluorescence intensity became approximately stable at w/w ratios of ~4, indicating a nearly full DNA condensation. The oligomer with more rigid backbone (**TOPh** and **TNPh**) showed better DNA binding ability, and **TOHex**, with a flexible long chain bridge, showed obviously lower fluorescence quenching ability. These findings are in agreement with theoretical calculations on the simulations of the interactions between rigid/flexible dendrimers and nucleic acids.[20] Delicate changes from ester to amide on the side chain of TACN may slightly increase the DNA binding ability, which is in accordance with the results obtained in gel retardation assays.

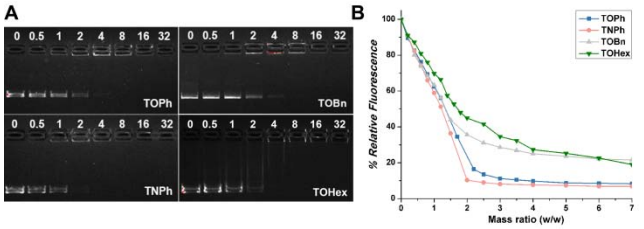

Fig. 2 (A) Agarose gel electrophoresis of cationic oligomers complexed with DNA to form polyplexes at different weight ratios. In each image, the first lane is DNA control. (B) Fluorescence quenching assay of EB/DNA by addition of title oligomers.

The properties of the formed polyplexes were firstly studied by using dynamic light scattering (DLS) to measure their particle sizes and zeta potentials. As shown in Fig. 3A, all four oligomers are able to condense DNA into nanosized particles (~50 to ~400 nm) at w/w ratios ranging from 6 to 40. Interestingly, aromatic oligomers (except **TOHex**) gave distinctly smaller polyplexes, suggesting that the rigid aromatic group plays important role in DNA condensation. We speculate that the aromatic groups might stronger the interactions between the molecules via "face-to-face" π-π stacking, which leads to particles with smaller diameter.[21] Zeta-potentials of the polyplexes rose along with the increase of w/w ratio, and the surface charge was observed about +15~25 mV above the w/w of 6 (Fig. 3B). The size and zeta potential for all oligomers became stable from w/w of ~6, indicating that full condensation was achieved.

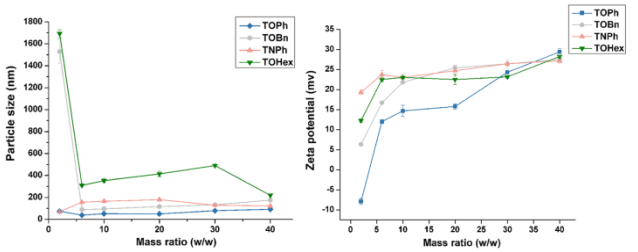

Fig. 3 Particle size (left) and zeta-potential (right) of polyplexes obtained at various weight ratios by DLS. Data represent mean ± SD (n = 3).

The stability of polyplexes is essential for gene delivery. A qualified vector should protect DNA from degradation caused by nuclease, and resist the competitive binding of negatively charged proteins. Agarose gel electrophoresis results (Fig. S1, ESI†) revealed that in the presence of heparin, **TOBn** and **TOHex** could no longer effectively condense DNA, and obvious DNA release was observed. However, DNA retardation and protection was retained for the polyplexes formed from **TOPh** and **TNPh**, and no DNA degredation was observed in the presence of DNase. Such difference should also be ascribed to the more rigid aromatic structures of **TOPh** and **TNPh**, and their consequent stronger interaction with DNA. By contrast, the more flexible PEI cannot maintain DNA condensation with the presence of heparin.

The *in vitro* gene transfection efficiency of the new polyplexes was initially tested in HEK 293 cells by using pGL-3 plasmid as a luciferase reporter gene. Fig. 4A shows the relative TE of these oligomers at various w/w ratios in comparison with PEI 25 KDa at its optimal w/w ratio of 1.4. Accordingly, oligomer with more rigid structure gave higher TE. At the optimal w/w ratio, **TOPh** and **TNPh** exhibited 35.6 and 4.7 times respectively higher TE than PEI. This advantage might be attributed to their higher DNA binding ability and the higher stability of relative polyplexes. On the other hand, **TOBn**, having only two more methylene group in each unit, gave dramatically lower TE than its analog **TOPh**. For the most flexible compound **TOHex**, poor TE was obtained. Green fluorescent protein (GFP) reporter gene transfection gave similar results, which are shown in Fig. 4B. The density of transfected cells by **TOPh** and **TNPh** (especially **TOPh**) was much higher than that obtained in the experiment involving PEI. Subsequently, these two superior materials were applied to other cell lines including HepG2 and HeLa cells. As showin in Fig. 4C, it was interesting to find that unlike the trasfection toward HEK 293 cells, **TNPh** gave much higher TE than **TOPh** in tumor cells, especially in HepG2 cells, in which near 100 times higher TE than PEI was obtained. Since different cell types possess varied transfection pathway and cell apoptosis feature,[22] the different TE of the title oligomers between various cell lines may be acceptable. By comparing the transfection performance of **TOPh** and **TNPh**, it could be confirmed that subtle structural change may have considerable influence on the TE, no matter what type of cell. The detailed transfection mechanisms leading to such difference are under further investigation.

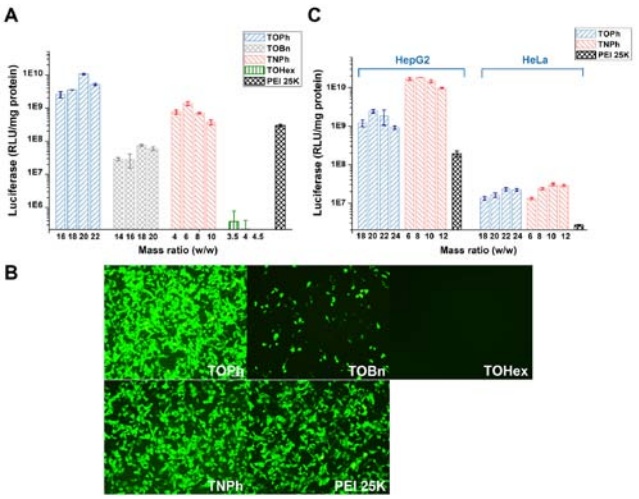

Fig. 4 (A) Luciferase gene expression transfected by polyplexes at different weight ratios in comparison with PEI (w/w = 1.4, N/P = 10) in HEK 293 cells in the absence of serum. Data represent mean ± SD (n = 3). (B) Fluorescence microscope image of pEGFP-transfected HEK 293 cells in the absence of serum at optimal weight ratio. (C) Transfection in HepG2 and HeLa cells.

The gene transfection involving **TOPh** and **TNPh** in the presence of 10% of serum was also studied. Results showed that

although the TE decreased in each case, both oligomers still exhibited higher TE than PEI in all three cell lines (Fig. S2, ESI†). The good stability of the polyplexes formed from these two oligomers containing rigid aromatic backbone may favour the resistance to serum-induced aggregation or premature DNA release.[23] It is worth mentioning that in all tested cell lines, **TOPh** gave higher TE than **TNPh**. Comparing the results obtained in the transfection experiments without serum, it could be drawn that **TOPh** has higher serum tolerance than **TNPh**. This might be attributed to its lower positive zeta-potential and cytotoxicity (Fig. S5, ESI†).

To obtain insight into the intracellular fate of the polyplexes, transfection experiments were performed using Cy5 labeled DNA in HEK 293 and HepG2 cells (Fig. 5). As expected, **TOPh** and **TNPh** showed considerably higher efficiency to transport pGL-3 plasmids into the cells than the flexible analogues and PEI. This enhanced cellular uptake was revealed by the larger red fluorescence intensity observed through confocal laser scanning microscopy (CLSM, Fig. 5A, B, F and G). In these cases, the red pGL-3 plasmids mainly located around the blue-labelled nuclei, which may facilitate nucleus importation and gene expression. The images obtained from the transfection in serum-containing medium further confirmed the superiority of these two vector materials in gene delivery (Fig. S3, ESI†). Furthermore, flow cytometry assays were also performed to verify the higher cellular uptake in the TOPh/TNPh-involved transfection (Fig. S4, ESI†). In HEK 293 cells, the percentages of Cy5-positive cells were found to be more than 90%, which was much higher than that obtained by using PEI. Similar results were obtained in HepG2 cells, in which slightly lower uptake was found.

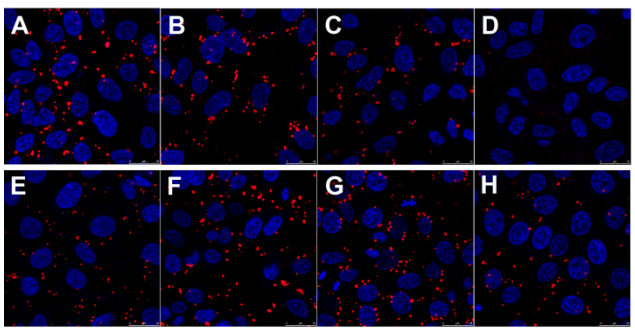

Fig. 5 CLSM images of HEK 293 (A~E) and HepG2 (F~H) cells transfected with Cy5-labeled DNA by polymers at optimal w/w ratio in absence of serum. A and F: **TOPh**; B and G: **TNPh**; C: **TOBn**; D: **TOHex**; E and H: PEI 25 KDa (w/w = 1.4). pGL-3 plasmids were stained red by Cy5 and the nuclei of cells were stained blue by DAPI.

The cytotoxicity of these polymers was estimated in three tested cell lines by MTT assays (Fig. S5, ESI†). Results showed that these materials have much lower cytotoxicity than PEI, especially **TOPh** at high concentration. This might be due to their lower molecular weights. It is noteworthy that **TNPh** showed distinctly higher cytotoxicity than **TOPh**, explaining that the optimal transfection w/w ratio for **TNPh** is much lower than the other one.

In summary, a series of linear oligomers with varied flexibility were synthesized. The oligomers with rigid aromatic backbone were first applied to gene delivery and showed better DNA binding ability than the flexible analogues. *In vitro* transfection experiments also revealed that the materials with more rigid backbone (**TOPh** and **TNPh**) exhibited considerably higher TE, which were much higher than "golden standard" 25 KDa branched PEI. Meanwhile, these materials displayed lower cytotoxicity than PEI, showing better biocompatibility. It was also shown that subtle structural diversity on the side chain of TACN may have large influence on the TE and cytotoxicity. These materials containing aromatic backbone were proved to be promising gene carriers in the further SAR study and *in vivo* application.

This work was financially supported by the National Program on Key Basic Research Project of China (973 Program, 2012CB720603), the National Science Foundation of China (Nos. 21232005, 51133004, J1310008 and J1103315), State Key Lab of Oral Diseases (Sichuan University) (SKLODOF2014OF01) and the Specialized Research Fund for the Doctoral Program of Higher Education (20120181130006) in China. J. Z. thanks the Program for New Century Excellent Talents in University (NCET-11-0354).

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† Electronic Supplementary Information (ESI) available: Experimental section, supplemental figures and compound spectra. See DOI: 10.1039/b000000x/

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