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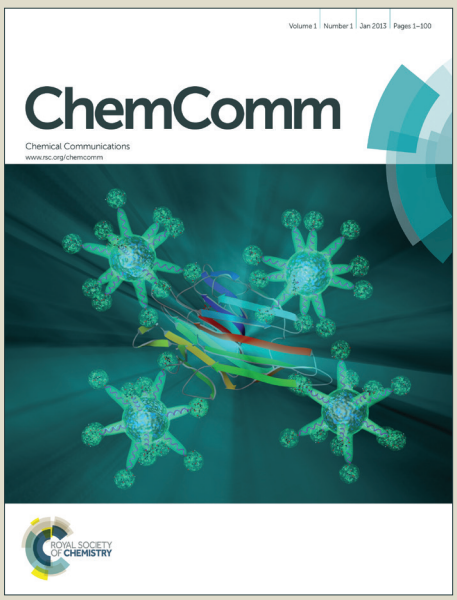

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A naphthalimide-based [12]aneN$_3$ compound as an effective and real-time fluorescence tracking non-virus gene vector

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A novel bifunctional naphthalimide-based [12]aneN$_3$ compound 1 was successfully applied as an effective non-virus gene vector in cancer cells, the fluorescent property of 1 clearly demonstrated the process of cellular uptake, DNA translocation and release based on real-time fluorescence tracking.

Gene therapy, as a promising approach for the treatment and prevention of genetic disorders, cancer, and AIDS, has gained wide attention over the past two decades.[1] Viral vectors such as retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAVs) have served as the favorities for several clinical applications due to their highly efficient intracellular DNA delivery.[2] However, the applications of these viral vectors are limited by their carcinogenesis, immunogenicity, broad tropism, limited DNA packaging capacity and difficulty of vector production. Therefore, non-viral vectors with flexibility in chemical design and safety have received increasing attention in recent years.[3] Non-viral gene transfer agents include lipids，cationic polymers, polypeptides, dendrimers, metal complexes and nano-particles.[4] Furthermore, exploring the transfection mechanism is also very important in the development of highly efficient non-viral gene vectors, which could help us to understand the critical steps in the transfection process. For tracking of gene delivery process, the most commonly used method is the fluorescent dye labeling of non-viral gene vectors,[5] which showed notable shortcomings such as poor water solubility, high toxicity and poor photo-stability. More recently, multifunctional nano-composites containing quantum dots and polynuclear ruthenium complexes have been developed as fluorescence tracking non-viral gene vectors.[6] However small organic molecules as tracking non-viral gene vectors have not been explored, although they would show more advantages such bio compatibility, structure variety, easy production etc.

In recent years, we have developed a series of bifunctional compounds containing fluorescent moieties such as carbazol /coumarin and macrocylic polyamine [12]aneN$_3$ units, which showed highly effective DNA condensation ability and potential gene delivery activity.[7] Through structure modification, these bifucntional molecules were expected to be developed into efficient non-viral gene vectors with fluorescent tracking ability, which would be particularly attractive because of their simplicity of preparation and easy of structural modification.

Based on the above considerations, we report here a bifunctional compound consisting [12]aneN$_3$ and naphthalimide moieties, **1** (Scheme 1), as the first example of a small organic chemical for the fluorescent tracking of non-viral gene vector. Macrocyclic polyamine [12]aneN$_3$ has often been proven to be a good positively charged units in constructing DNA condensation agents and non-viral gene vectors due to its ready protonation under physiological conditions.[7b, 8] As a hydrophobic tail, rigid naphthalimide moiety can easily form stable lamellar complexes with DNA,[9] and was expected to further improve the gene delivery efficiency. More importantly, naphthalimide moieties exhibit a strong yellow-green fluorescence and possess good photo-stability, and have been widely used as fluorescent dyes, fluorescent probes and bio-imaging makers.[10] Thus the presence of naphthalimide moiety in compound **1** will facilitate the monitoring of the intracellular DNA delivery process and afford information concerning the mechanism of gene delivery.

Compound **1** was synthesized based on the four steps shown in Scheme 1. Compound **2** was refluxed with 1,10-diaminodecane in 2-methoxyethanol to give compound **3**, which was then acylated with compound **4** to afford compound **5**. Further reaction of **5** with N-propargyl [12]-aneN$_3$ (**6**) through a copper(I) mediated click cycloaddition yielded key intermediate **7**. De-protectionof **7** under acidic condition afforded target compound **1**. The detailed procedures and characterization of all new compounds are described in the electronic supplementary information (ESI).

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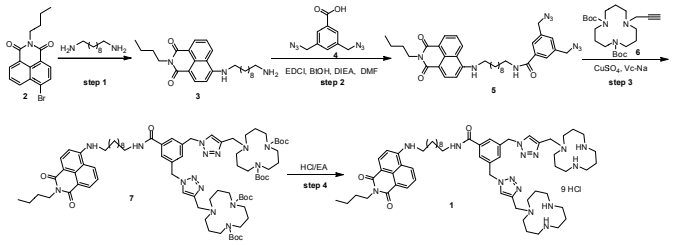
Electronic Supplementary Information (ESI) available: details of synthesis and characterization of all new compounds and properties studies. See DOI: 10.1039/x0xx00000x



Scheme 1 Synthesis of cationic lipid **1**

Efficient condensation of negatively charged DNA into nano-sized particles is of great importance for effective gene carriers. To test the DNA condensation ability of compound **1**, agarose gel electrophoresis was performed (Fig. 1A). It was observed that the electrophoretic mobility of DNA was completely inhibited at 10 μM of **1** (N/P = 3.6) in 5 minutes at 37 °C. The concentration needed to cause complete condensation of DNA is much lower than most previously reported lipids including the bifunctional compounds we developed.[11] The effective condensation ability can be attributed to the strong electrostatic interactions between the two triazole-[12]aneN$_3$ units and DNA as well as the hydrophobic interations from the rigid naphthalimide moiety and the aliphatic chain. The effect of dioleoylphosphatidyl ethanolamine (DOPE), a common helper lipid that enhances gene transfection efficiency, was also investigated in the condensation process. It was found that the liposome formed by mixing compound **1** with DOPE in a molar ratio of 1:2 clearly brought further improvement to the condensation ability. As shown in Fig. 1B, liposome of **1** could completely condense DNA at 5μM (N/P = 1.8). Thus, the following experiments were carried out by using this liposome.

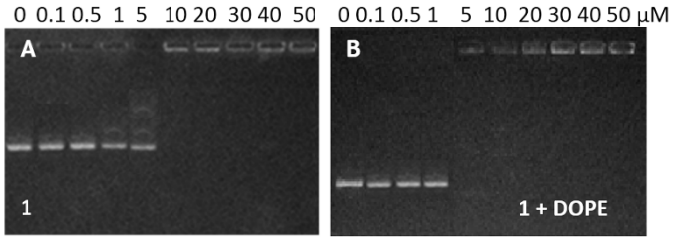


Fig. 1 Agarose gel electrophoresis assay to investigate the pUC18DNA condensation induced by different concentrations of **1** (A) and **1** with DOPE (B) in 50 mM HEPES buffer (pH = 7.4). The molar ratio of lipid **1**/DOPE was 1:2, [DNA] = 9 μg/mL, 37 °C incubate 5 minutes.

Dynamic light scattering (DLS) was useful in determining the size of the DNA condensates. The measurements were performed with 9 μg/mL of pUC18-DNA in HEPES buffer (50 mM, pH 7.4) at 25 °C (Fig. 1S). The results indicated that the liposome of **1** could bind and compact DNA into nano-particles with diameters in the range of 100-200 nm, which was suitable for cell endocytosis and further gene transfection.[3d, 3g] It is obvious that the size of condensed DNA particles increased with increasing concentrations of compound **1**, which is consistent with known reports.[12]

The morphology of DNA condensates was further characterized with scanning electron microscope(SEM) at different concentrations of **1** in ultrapure water. As shown in Fig. 2, compound **1** can effectively condense DNA into homogeneous spherical nano-particles at concentrations ranging from 5 μM to 30 μM. These results once again demonstrated that compound had effective DNA condensation capability.

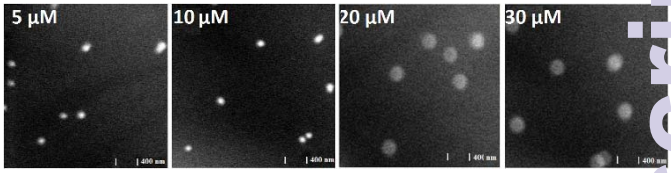


Fig. 2 SEM Images of pUC18 DNA (9 μg/mL) and its condensation induced by complexes of **1** with DOPE (1:2) at different concentrations in HEPES buffer (50 mM, pH 7.4)

The cytotoxicity of the prepared liposome of **1** was examined at different concentrations in cancer (A549, HeLa and SKOV-3) and normal cell lines (CCC-HPF-1) by MTT assay. As shown in Fig. 3, the cytotoxicity of the liposome was slightly higher than that of the commercially available transfection agent Lipofectamine 2000. The percentage of viable cells decreased with increasing concentrations of **1**. However, about 80% cells were still viabe in the concentration range in which the effective condensation happened.

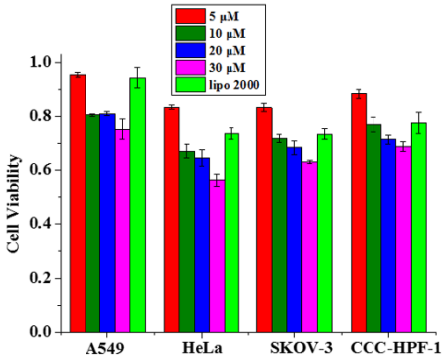


Fig. 3 Cytotoxicity of liposome **1**/DOPE (1:2) with DNA at different concentrations toward different cell lines, with lipofectamine 2000 as the control.

To examine the possibility of using compound **1** as non-viral vectors for gene delivery, we performed cellular uptake studies with A549 cells using Cy5-labeled dsDNA. The labeled DNA emitted red light under fluorescence microscopy condition, while the condensation agent **1** gave rise to a strong green fluorescence. The

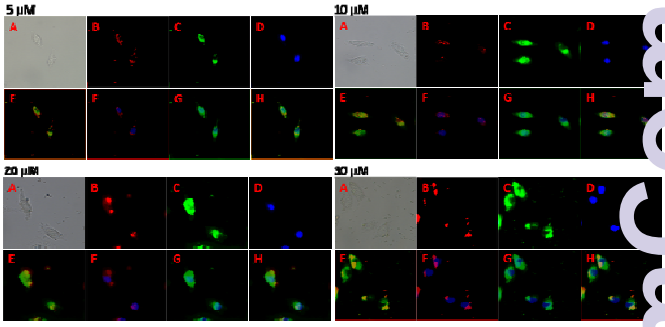


Fig. 4 CLSM images of A549 cells transfected with Cy5-labaled DNA (9 μg/mL) by **1**/DOPE (1:2) at different concentrations. A: BF; B: Cy5-labeled DNA (red); C: cell stained by **1** (green); D: cell nuclei stained by DAPI (blue); E: the merged images of B and C; F: the merged images of B and D; G: the merged images of C and D; H: the merged images of B, C and D.

locate the DNA condensates, we stained the nuclei of cells with DAPI, a nucleus-specific blue fluorescence dye. As shown in Fig. 4, almost all of the cells were stained by compound **1** (green) and Cy5-labeled dsDNA (red), which clearly indicates that cellular uptake of the condensed DNA was successful. It was obvious that Cy5-labeled dsDNA indeed reached the nucleus (blue), even partly transferred into it. The particle size of the lipoplexes formed by the DNA and the liposome of **1**/DOPE gradually increased with increasing concentrations of **1**/DOPE, which is in accordance with DLS and SEM experiments.

To directly visualize the processes of cellular uptake, DNA translocation and release from the lipoplex formed by compound **1**, DOPE, and Cy5-labeled dsDNA, time-dependent fluorescence microscopy assay was carried out. Excessive DNA was used to make the liposome of **1** to completely form the lipoplex. As shown in Fig. 5, the particle size of lipoplexes became smaller, which is helpful to trace the process of gene delivery. When the lipolexes of **1** were cultured for 0.5 h at 37 °C, DNA was condensed into small particles and showed clear affinity to the cell membrane. The DNA-containing particles subsequently penetrated into the cells through endocytosis.[6j,6k] Finally, DNA was released from the complexes and imported into the nucleus. However, most of the liposomes of **1** still remained outside the nucleus. To the best of our knowledge, this is the first time that the a gene uptake process was clearly demonstrated with a small organic molecule.

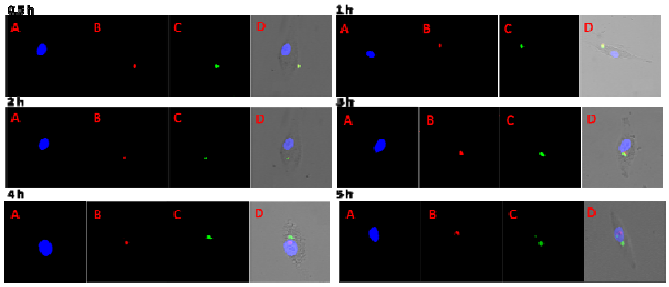


Fig. 5 CLSM images of A549 cells transfected with Cy5-labaled DNA by **1**/DOPE (1:2) (**1**: 20 μM, DNA: 100 μg/mL). A: cell nuclei stained by DAPI (blue); B: Cy5-labeled DNA (red); C: cell stained by **1** (green); D: merged images.

To further investigate the potential of the liposome's ability to serve as a non-viral vector, we conducted a luciferase assay to quantify the transfection efficiency of compound **1** in A549 cells, with **1**/DOPE mole ratio of 1:2, and lipofectamine 2000 as a positive control. As shown in Fig. 6A, liposome of **1** was able to deliver plasmid DNA successfully into A549 cells for efficient transfection, The early reported bifunctional compounds containing coumarin units showed very poor transfection efficiency under the same conditions. The best transfection efficiency of the liposome with **1** was obtained at 25 μM (N/P = 9), which was comparable to commercially available lipofectamine 2000. Expression of the luciferase gene transferred by the complex with **1** was also studied at the concentration of 25μM toward other cell lines (Fig. 6B). The obtained results indicated that the transfection efficiencies in cancer cells (A549, HeLa and SKOV-3) were higher than those in normal cells (CCC-HPF-1). Among the three types of cancer cells examined, the lipoplex of **1** was particular efficiency for gene transfer in A549 cells compared to HeLa and SKOV-3 cells.

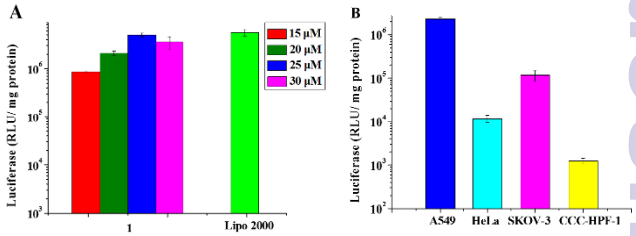


Fig. 6 Luciferase gene expression transfected by DNA complex of **1**/DOPE (1:2) (A) at different concentrations in A549 cells; (B) in A549, HeLa, CCC-HPF-1 and SKOV-3 cells at the concentration of 25μM.

In conclusion, the bifunctional naphthalimide-based [12]aneN$_3$ compound **1** was synthesized and high transfection efficiency in A549 cells with the nearly same cytotoxicity as Lipofectamine 2000. It was successfully applied for the in-situ monitoring of cellular uptake, DNA transportation and release through non-invasive fluorescence imaging, which is valuable for the mechanistic study of gene delivery and the development of new non-virus gene vectors. As the first example of a small organic chemical for the fluorescent tracking cellular gene delivery processes, the present work provides new insight towards the development of highly efficient non-viral gene vectors as well as bio-imaging agent for understanding critical step in the transfection process. Further studies, including the structural modification of the bifunctional compounds to improve the transfection efficiency and the elucidation of their structure-activity relationship, are in progress.

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