J|A|C|S
COMMUNICATIONS

Published on Web 09/08/2004

Charge-Reversal Amphiphiles for Gene Delivery

Carla A. H. Prata,[†] Yuxing Zhao,[†] Philippe Barthelemy,[‡] Yougen Li,[§] Dan Luo,[§] Thomas J. McIntosh,[||] Stephen J. Lee,[⊥] and Mark W. Grinstaff*,[†]

Departments of Biomedical Engineering and Chemistry, Boston University, Boston, Massachusetts 02215, Departement de Chimie, Université d'Avignon, F-84000 Avignon, France, Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710, Department of Biological and Environmental Engineering, Cornell University, Ithaca, New York 14853, and Army Research Office, RTP, North Carolina 27709

Received April 29, 2004; E-mail: mgrin@bu.edu

Gene therapy offers the potential to cure a wide range of diseases by delivering a missing gene or a functional substitute of a defective gene.[1−6] The two most common methods for gene delivery use either viral[7,8] or synthetic vectors.[1−4,9,10] Viruses are efficient carriers of genes, but there are risks associated with their clinical application.[8] Consequently, there is intense activity in developing and evaluating synthetic nonviral vectors, including cationic amphiphiles,[10−16] linear polymers,[17−19] and dendrimers.[20−22] Since the pioneering research of Felgner, MacDonald, and Magee, cationic amphiphiles have been investigated because of their low toxicity, nonimmunogenicity, and ease of synthesis; today, these amphiphile vectors are in clinical trials. Yet with these amphiphiles, the gene transfection activity is low, reflecting inefficiencies in the overall transfection pathway that includes DNA−synthetic vector complexation, endocytosis, endosomal escape, nuclear entry, and finally, expression. Our research effort is focused on improving the release of DNA from the DNA−amphiphile supramolecular complex. Herein we describe and characterize a functional amphiphile for gene delivery that undergoes an electrostatic transition intracellularly from cationic to anionic and shows enhanced gene transfection efficiency.

This charge-reversal amphiphile performs two roles: first, it binds and then releases DNA, and second, as an anionic multicharged amphiphile, it destabilizes bilayers. Thus, the amphiphile undergoes the following reactions: it complexes plasmid DNA and forms a supramolecular DNA−cationic amphiphile assembly; upon entering the cell (e.g., via endocytosis) esterases hydrolyze the terminal ester linkages to afford anionic amphiphiles; and finally, the anionic amphiphiles repel DNA and disrupt the lipid bilayer of the supramolecular complex releasing the plasmid DNA for subsequent transcription (Figure 1). This approach, which benefits from a change in electrostatic forces to release DNA, departs from previous functional vectors.[23−32] The aforementioned sequential steps require a cationic amphiphile possessing three distinct structural components: a cationic headgroup, hydrophobic chains, and terminal ester linkages. The prototype amphiphile, **1**, has a cationic ammonium headgroup to bind DNA, lipophilic acyl chains to form a bilayer, and benzyl esters at the terminus of the acyl chains for enzymatic hydrolysis (Figure 2). To assess the role of each structural component, we prepared compounds **2**−**4**. Compounds **1** through **4** were synthesized as described in the Supporting Information.

To determine whether the amphiphiles bind DNA, we performed a standard ethidium bromide−DNA fluorescence quenching exclusion assay. DNA binding is observed for **1**, **3**, and 1,2-dioleoyloxy-3-(trimethylammonio)-propane (DOTAP, **5**) as the fluorescence intensity decreases rapidly, but not for **2** or **4** (Figure 3A).

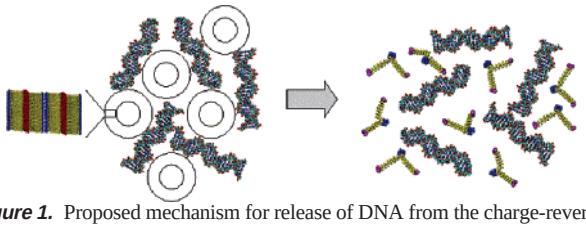

Figure 1. Proposed mechanism for release of DNA from the charge-reversal amphiphiles. A supramolecular assembly is formed between the DNA and the multiwalled vesicles of the amphiphile. Upon enzymatic hydrolysis of the terminal esters of this amphiphile, the DNA is released from the assembly by the newly formed anionic amphiphiles. Not drawn to scale.

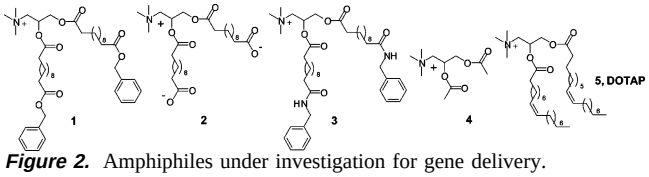

Figure 2. Amphiphiles under investigation for gene delivery.

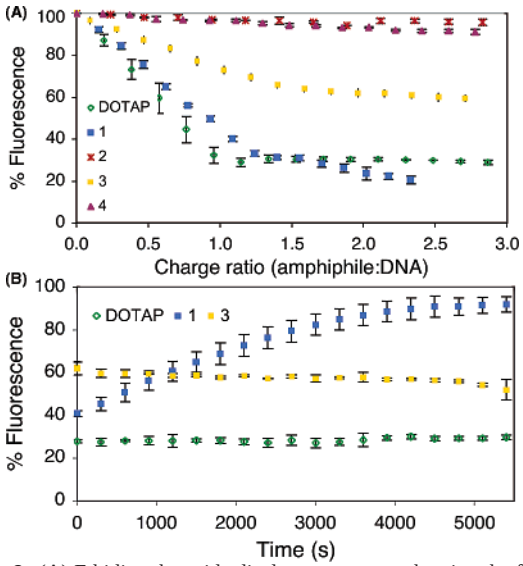

Figure 3. (A) Ethidium bromide displacement assay showing the fluorescence intensity as a function of synthetic vector/DNA charge ratio for compounds **1**−**4** and DOTAP. (B) Ethidium bromide displacement assay showing the fluorescence intensity as a function of time in the presence of a porcine liver esterase (300 units/mL).

Amphiphile **1** forms a 1:1 complex with a binding constant of $\sim 10^{-7}$ M,$^{-1}$ similar in magnitude to DOTAP.[33] Amphiphile **2** is anionic and does not bind DNA as a consequence of unfavorable electrostatic interactions. Compound **4** possesses a cationic charge but lacks the hydrophobic acyl chains also required for formation of a strong interaction with DNA. Next, the above DNA/EtBr/amphiphile (**1**, **3**, or DOTAP) solution was incubated with an esterase at pH 7.4 and 37 °C (100 mM NaCl, 100 mM Tris buffer).

[†] Boston University.
[‡] Université d'Avignon.
[§] Cornell University.
[||] Duke University Medical Center.
[⊥] Army Research Office.

10.1021/ja0474906 CCC: $27.50

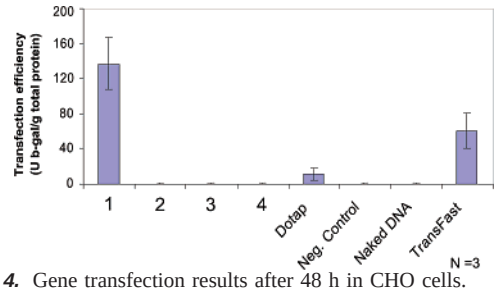

Figure 4. Gene transfection results after 48 h in CHO cells.

As shown in Figure 3B, an increase in fluorescence over time is seen for amphiphile **1**, consistent with hydrolysis of the terminal benzyl esters and disruption of the DNA−amphiphile supramolecular complex followed by re-intercalation of EtBr in DNA.[34] No increase in fluorescence over time is observed with amphiphiles **3** or DOTAP, which possess a terminal amide linkage or methyl group, respectively. The fluorescence data indicate that release of DNA from the supramolecular assembly does not occur with amphiphiles lacking a terminal hydrolyzable ester linkage and those linkages near the cationic headgroup are less accessible to enzymatic hydrolysis.

Given the polar headgroup and long hydrophobic acyl chains present in the structure of **1**, this amphiphile is likely to form bilayer vesicles in aqueous solution. Dispersion of **1** in water by sonication leads to vesicles in the presence and absence of DNA. Vesicles are not observed with the anionic amphiphile **2** (multicharged surfactant) or cationic compound **4**, which lacks the necessary hydrophobic chains necessary for bilayer formation. A differential scanning calorimetry (DSC) trace of hydrated amphiphile **1** shows a phase-transition temperature at approximately 55 °C.

The X-ray diffraction spectrum at 25 °C of the hydrated vesicle pellet of **1** shows three diffraction orders of a lamellar repeat period of 5.22 ± 0.03 nm with a sharp wide-angle spacing of 0.46 ± 0.01 nm, which is characteristic of an ordered bilayer phase. Upon addition of DNA, the lamellar repeat period ($d = 5.31 \pm 0.14$ nm) and wide-angle spacing (0.46 ± 0.01 nm) do not significantly change. This suggests a model, like that shown in Figure 1, where the DNA is entrapped at the surface or at the interface between multiwalled vesicles in solution. This is a different structural model than for complexes of DNA with either DOTAP[35] or cationic triesters of phosphatidylcholine,[16] where a smectic phase is formed with the DNA chains located between the adjacent lipid bilayers within the multilamellar liposome. Moreover, adding 1% w/w of the multicharge anionic amphiphile **2** to DOPE bilayers affords a broader phase-transition temperature compared to pure DOPE. This result is consistent with the anionic surfactant, **2**, formed in the hydrolysis reaction of **1**, destabilizing lipid bilayers.

Transfections experiments using the reporter gene, β-galactosidase (β-gal, pVax-LacZ1, invitrogen) were performed with chinese hamster ovarian (CHO) cells (see Supporting Information). As shown in Figure 4, cationic amphiphile **1** was the most effective vector for transfecting the β-galactosidase gene. Significantly, compounds **2** through **4** showed minimal transfection activity comparable to the negative control and naked DNA. DOTAP and TransFast reagent both transfect DNA, but at lower levels. The results observed with anionic amphiphile, **2**, and cationic compound, **4**, are consistent with the poor affinity of these compounds to bind DNA. The lack of transfection with amphiphile **3** conveys the important role the cleavable terminal ester linkages perform in these amphiphiles. Preliminary screening on additional cells lines showed that **1** can also facilitate the transport of DNA in human embryonic kidney (HEK293) and erythroleukemic (K562) cell lines.

In summary, a charge-reversal or charge-switchable amphiphile is shown to be an effective nonviral vector for gene delivery. These results also highlight the importance and sensitivity of noncovalent interactions in the formation and dissolution of supramolecular DNA−amphiphile assemblies. Current studies are focused on optimizing the structure of the charge-reversal amphiphile and the formulation conditions to further increase the gene transfection efficiency. Importantly, these amphiphiles represent a conceptual departure from the current cationic molecules under investigation, and these results are likely to facilitate the design, development, and evaluation of new synthetic nonviral vectors for the delivery of therapeutic DNA.

Acknowledgment. This work was supported by the Army Research Office (M.W.G.) and Grant GM27278 from the National Institutes of Health (T.J.M.). D.L. acknowledges Erin King for technical assistance and the USDA and Cornell Advanced Center for Biotechnology for financial support. We also thank Drs. Junlin Guan and Zara Melkoumian for providing the cell lines.

Supporting Information Available: Complete experimental details, characterization data, and cytotoxity experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Luo, D.; Saltzman, W. M. *Nat. Biotechnol.* **2000**, *18*, 33−37.
(2) Behr, J. P. *Acc. Chem. Res.* **1993**, *26*, 274−278.
(3) *Nonviral Vectors for Gene Therapy*; Huang, L., Hung, M.-C., Wagner, E., Eds.; Academic Press: New York, 1999; p 442.
(4) Davis, M. E. *Curr. Opin. Biotechnol.* **2002**, *13*, 128−131.
(5) *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial*; Kabanov, A. V., Felgner, P. L., Seymour, L. W., Eds.; Wiley & Sons: New York, 1998; p.
(6) *Gene Therapy Therapeutic Mechanisms and Strategies*; Templeton, N. S., Lasic, D. D., Eds.; Marcel Dekker: New York, 2000; p 584.
(7) Robbins, P. D.; Tahara, H.; Ghivizzani, S. C. *Trends Biotechnol.* **1998**, *16*, 35−40.
(8) Marshall, E. *Science* **2000**, *287*, 565−567.
(9) Felgner, P. L. *Adv. Drug Delivery Rev.* **1990**, *5*, 163−187.
(10) Miller, A. D. *Angew. Chem., Int. Ed.* **1998**, *37*, 1768−1785.
(11) Felgner, P. L.; Rhodes, G. *Nature* **1991**, *349*, 351−352.
(12) Remy, J.; Sirlin, C.; Vierling, P.; Behr, J. *Bioconjugate Chem.* **1994**, *5*, 647−654.
(13) Deshmukh, H. M.; Huang, L. *New. J. Chem.* **1997**, *21*, 113−124.
(14) Legendre, J. Y.; Szoka, F. C. J. *Pharm. Res.* **1992**, *9*, 1235−1242.
(15) Rui, Y.; Wang, S.; Low, P. S.; Thompson, D. H. *J. Am. Chem. Soc.* **1998**, *120*, 11213−11218.
(16) MacDonald, R. C.; Ashley, G. W.; Shida, M. M.; Rakhmanova, V. A.; Tarahovsky, Y. S.; Kennedy, M. T.; Pozharski, E. V.; Baker, K. A.; Jones, R. D.; Rosenzweig, H. S.; Choi, K. L.; Qiu, R.; McIntosh, T. J. *Biophys. J.* **1999**, *77*, 2612−2629.
(17) Kabanov, A. V.; Kabanov, V. A. *Bioconjugate Chem.* **1995**, *6*, 7−20.
(18) Lynn, D. M.; Anderson, D. G.; Putman, D.; Langer, R. *J. Am. Chem. Soc.* **2001**, *123*, 8155−8156.
(19) Choi, J. S.; Joo, D. K.; Kim, C. H.; Kim, K.; Park, J. S. *J. Am. Chem. Soc.* **2000**, *122*, 474−480.
(20) Tang, M. X.; Redemann, C. T.; Szoka, F. C. *Bioconjugate Chem.* **1996**, *7*, 703−714.
(21) Kukowska-Latallo, J. F.; Bielinska, A. U.; Johnson, J.; Spinder, R.; Tomalia, D. A.; Baker, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4897−4902.
(22) Luo, D.; Haverstick, K.; Belcheva, N.; Han, E.; Saltzman, W. M. *Macromolecules* **2002**, *35*, 3456−3462.
(23) Wang, J.; Guo, X.; Xu, Y.; Barron, L.; Szoka, F. C. *J. Med. Chem.* **1998**, *41*, 2207−2215.
(24) Akinc, A.; Lynn, D. M.; Anderson, D. G.; Langer, R. *J. Am. Chem. Soc.* **2003**, *125*, 5316−5323.
(25) Wang, C.; Ge, Q.; Ting, D.; Nguyen, D.; Shen, H. R.; Chen, J.; Eisen, H. N.; Heller, J.; Langer, R.; Putnam, D. *Nat. Mater.* **2004**, *3*, 190−196.
(26) Zhu, J.; Munn, R. J.; Nantz, M. H. *J. Am. Chem. Soc.* **2000**, *122*, 2645−2646.
(27) Jones, R. A.; Cheung, C. Y.; Black, F. E.; Zia, J. K.; Stayton, P. S.; Hoffman, A. S.; Wilson, M. R. *Biochem. J.* **2003**, *372*, 65−75.
(28) Miyata, K.; Kakizawa, Y.; Nishiyama, N.; Harada, A.; Yamasaki, Y.; Koyama, H.; Kataoka, K. *J. Am. Chem. Soc.* **2004**, *126*, 2355−2361.
(29) Lynn, D. M.; Langer, R. *J. Am. Chem. Soc.* **2000**, *122*, 10761−10768.
(30) Bell, P. C.; Bergsma, M.; Dolbnya, I. P.; Bras, W.; Stuart, M. C.; Rowan, A. E.; Feiters, M. C.; Engberts, J. B. F. N. *J. Am. Chem. Soc.* **2003**, *125*, 1551−1558.
(31) Wang, J.; Mao, H. Q.; Leong, K. W. *J. Am. Chem. Soc.* **2001**, *123*, 9480−9481.
(32) Tang, F.; Hughes, J. A. *Biochem. Biophys. Res. Commun.* **1998**, *242*, 141−145.
(33) Cain, B. F.; Baguley, B. C.; Demmy, M. W. *J. Med. Chem.* **1978**, *21*, 658−668.
(34) The mechanism of esterase attack is under investigation.
(35) Radler, J. O.; Koltover, I.; Salditt, T.; Safinya, C. R. *Science* **1997**, *275*, 810−814.

JA0474906