

pubs.acs.org/Langmuir © 2009 American Chemical Society

Photo-Assisted Gene Delivery Using Light-Responsive Catanionic Vesicles

Yu-Chuan Liu,[†] Anne-Laure M. Le Ny,[†] Judith Schmidt,[‡] Yeshayahu Talmon,[‡] Bradley F. Chmelka,[§] and C. Ted Lee, Jr.^{*,†}

[†]Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, California 90089, *Department of Chemical Engineering, Technion—Israel Institute of Technology, Haifa 32000, Israel, and [§]Department of Chemical Engineering, University of California, Santa Barbara, California 93106

Received October 28, 2008. Revised Manuscript Received March 13, 2009

Photoresponsive catanionic vesicles have been developed as a novel gene delivery vector combining enhanced cellular uptake with phototriggered release of vesicle payload following entry into cells. Vesicles with diameters ranging from 50 to 200 nm [measured using cryo-transmission electron microscopy (TEM) and light-scattering techniques] form spontaneously, following mixing of positively charged azobenzene-containing surfactant and negatively charged alkyl surfactant species. Fluorescent probe measurements showed that the catanionic vesicles at a cation/anion ratio of 7:3 formed at surfactant concentrations as low as 10 μ M of the azobenzene surfactant under visible light (with the azobenzene surfactant species principally in the *trans* configuration), while $50-60 \,\mu\text{M}$ of the azobenzene surfactant is required to form vesicles under UV illumination (with the azobenzene surfactant species principally in the cis configuration). At intermediate surfactant concentrations (ca. $15-45 \,\mu\text{M}$) under visible light conditions, transport of DNA-vesicle complexes occurred past the cell membrane of murine fibroblast NIH 3T3 cells through endocytosis. Subsequent UV illumination induced rupture of the vesicles and release of uncomplexed DNA into the cell interiors, where it was capable of passing through the nuclear membrane and thereby contributing to enhanced expression. Singlemolecule fluorescent images of T4-DNA demonstrated that the formation of vesicles with a net positive charge led to compaction of DNA molecules via complex formation within a few seconds, while UV-induced disruption of the vesicle-DNA complexes led to DNA re-expansion to the elongated-coil state, also within a few seconds. Transfection experiments with eGFP DNA revealed that photoresponsive catanionic vesicles are more effectively taken up by cells compared to otherwise identical alkyl (i.e., nonazobenzene-containing and thus nonlight-responsive) catanionic vesicles, presumably because of $\pi - \pi$ stacking interactions that enhance bilayer rigidity in the photoresponsive vesicles. Subsequent UV illumination following endocytosis leads to further dramatic enhancements in the transfection efficiencies, demonstrating that vector unpacking and release of DNA from the carrier complex can be the limiting step in the overall process of gene delivery.

Introduction

Nonviral gene delivery has been a goal in the field of bionanotechnology for many years. Despite promising successes in this area,^{1,2} however, transfection efficiencies still generally remain below those of viral vector counterparts, as a result of two primary effects. First, viruses often interact with specific receptors on cell surfaces, providing for targeted delivery of genetic material. Second, viruses deliver DNA to cells in a state more readily accessible for expression, such as through direct injection of uncomplexed DNA into a cell or through fusion of the virus envelope with the cell membrane. In contrast, nonviral vectors by necessity must form complexes with DNA, which, once introduced into the cell, are often slowly liberated, thereby delaying or preventing potential nuclear uptake and subsequent expression. Consequently, viral vectors have largely remained the approach of choice, although their inherent associated immunological risks provide large incentives toward the development of nonviral vectors capable of targeted and triggered release of DNA.

Recent years have seen considerable interest in compacted DNA complexes as attractive candidates for nonviral gene delivery vectors. Complexing agents, such as cationic surfactants,³⁻⁹ lipids,^{10,11} and polyelectrolytes,¹²⁻¹⁵ as well as

- (7) Gorelov, A. V.; Kudryashov, E. D.; Jacquier, J.-C.; McLoughlin, D. M.; Dawson, K. A. Phys. A 1998, 249, 216-225.
- (8) Miguel, M. G.; Pais, A. A. C. C.; Dias, R. S.; Leal, C.; Rosa, M.; Lindman, B. Colloids Surf., A 2003, 228, 43-55.
- (9) Bathaie, S. Z.; Moosavi-Movahedi, A. A.; Saboury, A. A. Nucleic Acids Res. 1999, 27, 1001-1005.

- (11) Mahato, R. I.; Rolland, A.; Tomlinson, E. Pharm. Res. 1997, 14, 853-859. (12) Gershon, H.; Ghirlando, R.; Guttman, S. B.; Minsky, A. Biochemistry 1993, 32, 7132-7151.
- (13) Yoshikawa, K. Adv. Drug Delivery Rev. 2001, 52, 235-244.
- (14) Huang, W.; Zhang, Z.; Han, X.; Tang, J.; Wang, J.; Dong, S.; Wang, E. Bioelectrochem. Bioenerg. 2003, 59, 21–27.
 (15) Minnagawa, K.; Matsuzawa, Y.; Yoshikawa, K.; Khokhlov, A. R.; Doi,
- M. Biopolymers 1994, 34, 555-558.

^{*}To whom correspondence should be addressed: Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, CA 90089-1211. Telephone: 1-213-740-2066. Fax: 1-213-740-8053. E-mail: tedlee@usc.edu.

 ⁽¹⁾ Naldini, L.; Blomer, U.; Gallay, P.; Ory, D.; Mulligan, R.; Gage, F. H.;
 Verma, I. M.; Trono, D. *Science* 1996, *272*, 263–267.
 (2) Felgner, J. H.; Kumar, R.; Sridhar, C. N.; Wheeler, C. J.; Tsai, Y. J.; Border,

R.; Ramsey, P.; Martin, M.; Felgner, P. L. J. Biol. Chem. 1994, 269, 2550-2561.

⁽³⁾ Llères, D.; Clamme, J.-P.; Dauty, E.; Blessing, T.; Krishnamoorthy, G.; Duportail, G.; Mély, Y. Langmuir 2002, 18, 10340-10347

⁽⁴⁾ Guillot, S.; McLoughlin, D.; Jain, N.; Delsanti, M.; Langevin, D. J. Phys.: Condens. Matter 2003, 15, S219-S224.

⁽⁵⁾ Dias, R.; Mel'nikov, S.; Lindman, B.; Miguel, M. G. Langmuir 2000, 16, 9577-9583.

⁽⁶⁾ Mel'nikov, S. M.; Sergeyev, V. G.; Yoshikawa, K. J. Am. Chem. Soc. 1995, 117, 2401-2408.

⁽¹⁰⁾ Felgner, P. L.; Gadek, T.; Holm, M.; Roman, R.; Chan, H.; Wenz, M.; Northrop, J.; Ringold, G.; Danielson, M. Proc. Natl. Acad. Sci. U.S.A. 1987, 84, 7413-7417.

multivalent ions¹⁶⁻²¹ and alcohols,²²⁻²⁴ have been shown to condense DNA from extended, worm-like conformations into a variety of compact structures (e.g., toroids, spheroids, and rods in complexes, lipoplexes, or polyplexes^{17,25,26}) with sizes on the order of 100 nm, analogous to the confinement of DNA in virus heads. Neutralization and compaction are essential to protect DNA from nucleases¹⁷ and allow entry of DNA into cells (because both the cell surface and DNA are negatively charged), mainly by the endocytic pathway.²⁷ However, while complexing agents can increase cellular uptake relative to uncomplexed DNA, the tight binding of these same agents to DNA may also generally preclude or greatly reduce nuclear uptake, resulting in low overall transfection efficiencies compared to viral vectors.

Indeed, it has been shown that vector unpacking can be a limiting barrier to gene delivery.^{28,29} After endocytosis, DNA encapsulated in endocytic vesicles would be prevented from interacting with intracellular molecules, such as importin or transportin, which transport DNA into the nucleus,^{30,31} and would instead undergo degradation by hydrolysis. Thus, many systems have been designed for programmed release of DNA from the carrier complex, including vesicles made of biodegradable cationic polymers,³² as well as microparticles with biodegradable polymers, such as polysaccharides.33 poly[α -(4-aminobutyl)-L-glycolic acid],³⁴ polylactide-co-glyco-lide,³⁵ polyphosphazenes,³⁶ chitosan,³⁷ and polyethylenimine.³⁸ With traditional cationic polymers, however, DNA release rates are determined by polymer hydrolysis or biodegradation rates, which are generally too slow to avoid parallel DNA degradation, thus, yielding transfection efficiencies that are lower than viral vectors.³

- (1) Biominica, V. A. Biopolymers D.Y., Phys. Lett.
 (18) Cárdenas, M.; Schillén, K.; Nylander, T.; Jansson, J.; Lindman, B. Phys. Chem. Chem. Phys. 2004, 6, 1603–1607.
- (19) Widom, J.; Baldwin, R. L. Biopolymers 1983, 22, 1621-1632.
- (20) Vijayanathan, V.; Thomas, T.; Shirahata, A.; Thomas, T. J. Biochemistry
- 2001, 40, 13644-13651. (21) Bustamante, C.; Housel, T. W.; Beach, D. A.; Maestre, M. F. J. Biomol.
- Struct. Dyn. 1990, 8, 643-655. (22) Mikhailenko, S. V.; Sergeyev, V. G.; Zinchenko, A. A.; Gallyamov, M. O.;
- Yaminsky, I. V.; Yoshikawa, K. Biomacromolecules 2000, 1, 597-603. (23) Roy, K. B.; Anthony, T.; Saxena, A.; Bohidar, H. B. J. Chem. Phys. B 1999,
- 103, 5117-5121. (24) Yoshikawa, K.; Yoshikawa, Y.; Koyama, Y.; Kanbe, T. J. Am. Chem. Soc.
- 1997, 119, 6473-6477. (25) Yoshikawa, Y.; Yoshikawa, K.; Kanbe, T. Langmuir 1999, 15, 4085-4088.
- (26) Arscott, P. G.; Ma, C.; Wenner, J. R.; Bloomfield, V. A. Biopolymers 1995, 36, 345-364.
- (27) Remy-Kristensen, A.; Clamme, J.-P.; Vuilleumier, C.; Duportail, G.; Khury, J.-G.; Mély, Y. Biochim. Biophys. Acta 2001, 1514, 21-32.
- (28) Schaffer, D. V.; Fidelman, N. A.; Dan, N.; Lauffenburger, D. A. Biotechnol. Bioeng. 2000, 67, 598-606.
- (29) Read, M. L.; Bremner, K. H.; Oupicky, D.; Green, N. K.; Searle, P. F.; Seymour, L. W. J. Gene Med. 2003, 5, 232-245.
- (30) Xu, Y.; Szoka, F. C.Jr. Biochemistry 1996, 35, 5616-5623
- (31) Lechardeur, D.; Lukacs, G. L. Curr. Gene Ther. 2002, 2, 183-194.
- (32) Koping-Hoggard, M.; Tubulekas, I.; Guan, H.; Edwards, K.; Nilsson, M.; Varum, K. M.; Artursson, P. Gene Ther. 2001, 8, 1108-1121.
- (33) Azzam, T.; Eliyahu, H.; Shapira, L.; Linial, M.; Barenholz, Y.; Domb, A. J. J. Med. Chem. 2002, 45, 1817-1824
- (34) Lee, M.; Koh, J. J.; Han, S.-O.; Ko, K. S.; Kim, S. W. Pharm. Res. 2002, 19, 246-249
- (35) Denis-Mize, K. S.; Dupuis, M.; MacKichan, M. L.; Singh, M.; Doe, B.; O'Hagan, D.; Ulmer, J. B.; Donnelly, J. J.; McDonald, D. M.; Ott, G. Gene Ther. 2000, 7, 2105-2112.
- (36) Luten, J.; van Steenis, J. H.; van Someren, R.; Kemmink, J.; Schuurmans-Nieuwenbroek, N. M. E.; Koning, G. A.; Crommelin, D. J. A.; van Nostrum, C.
- F.; Hennink, W. E. J. Controlled Release 2003, 89, 483-497. (37) Godbey, W. T.; Wu, K. K.; Mikos, A. G. Proc. Natl. Acad. Sci. U.S.A.
- 1999, 96, 5177-5181.
- (38) Lungwitz, U.; Breunig, M.; Blunk, T.; Goepferich, A. Eur. J. Pharm. Biopharm. 2005, 60, 247-266.
- (39) Antoine Kichler, C. L.; Coeytaux, E.; Danos, O. J. Gene Med. 2001, 3, 135-144.

In the present study, photoresponsive catanionic vesicles that can be formed and disrupted with simple light illumination are used to encapsulate and deliver DNA into cells, followed by light-induced vesicle rupture to trigger the release of DNA from the carrier vesicle complexes. Vesicle formation typically requires the input of energy into the system, which leads to an increase in interfacial area and, thus, free energy of the system. However, in aqueous mixtures of cationic and anionic surfactants (i.e., so-called "catanionic" systems $^{40-44}$), a combination of electrostatic attractions between the surfactant head groups and hydrophobic interactions between the surfactant tails can be sufficient to offset the entropic penalty associated with vesicle self-assembly. Using a "responsive" surfactant as one of the species in the catanionic pair, triggered vesicle disruption and spontaneous reformation can be achieved, as demonstrated with various light-responsive surfactants.^{45–49} In these previous studies, photoresponsive vesicles were examined over the concentration range of 0.1-1.0 wt % total surfactant. In the present study, however, catanionic vesicles at much lower total surfactant concentrations (0.0028 wt %) have been used for gene delivery under conditions that reduce toxicity effects on cells. The catanionic vesicles are formed using azobenzene-containing photoresponsive surfactant molecules as the cationic species, similar to surfactants used to photoreversibly control protein folding^{50–53} and DNA condensation.⁵⁴ The azobenzene surfactant undergoes photo-isomerization from the relatively hydrophobic *trans* isomer to the relatively hydrophilic *cis* isomer upon exposure to 350 nm UV light. Furthermore, the photoisomerization process is reversible, because the *cis* isomer can be converted back to the trans form with exposure to 436 nm visible light or by thermal relaxation. As a result, the trans conformation self-assembles into vesicles more readily than the cis form, allowing DNA release from the vesicle carriers to be rapidly initiated upon UV illumination and leading to dramatic enhancements in transfection efficiencies.

The use of light to induce DNA release for enhanced gene expression could potentially lead to in vivo treatments. In terms of the necessary light delivery, this process could be considered analogous to photodynamic therapy (PDT) for the treatment of cancer, where a photosensitizer drug, localized in tumors, is illuminated with light (typically 400-700 nm, although UVA is also used) to generate reaction products toxic to tumor cells. Following FDA approval in 1995, PDT has been applied to a variety of ailments, including cancers of the head and neck, brain, lung, pancreas,

- (40) Kaler, E. W.; Murthy, A. K.; Rodriguez, B. E.; Zasadzinski, J. A. Science 1989, 245, 1371-1374.
 - (41) Safran, S. A.; Pincus, P.; Andelman, D. Science 1990, 248, 354-356.
- (42) Jung, H. T.; Coldren, B.; Zasadzinski, J. A.; Iampietro, D. J.; Kaler, E. W. Proc. Natl. Acad. Sci. U.S.A. 2001, 98, 1353-1357
- (43) Talmon, Y.; Evans, D. F.; Ninham, B. W. Science 1983, 221, 1047-1048. (44) Hargreaves, W. R.; Deamer, D. W. Biochemistry 1978, 17, 3759-3768.
- (45) Sakai, H.; Matsumura, A.; Yokoyama, S.; Saji, T.; Abe, M. J. Phys. Chem.
- B 1999, 103, 10737-10740. (46) Eastoe, J.; Dominguez, M. S.; Wyatt, P.; Orr-Ewing, A. J.; Heenan, R. K.
- Langmuir 2004, 20, 6120-6126. (47) Eastoe, J.; Vesperinas, A.; Donnewirth, A.-C.; Wyatt, P.; Grillo, I.;
- Heenan, R. K.; Davis, S. Langmuir 2006, 22, 851-853.
- (48) Hubbard, F. P.; Santonicola, G.; Kaler, E. W.; Abbott, N. L. Langmuir 2005, 21, 6131-6136.
 - (49) Hubbard, F. P.Jr.; Abbott, N. L. Langmuir 2007, 23, 4819-4829
 - (50) Lee, C. T.Jr.; Smith, K. A.; Hatton, T. A. Biochemistry 2005, 44, 524-536.
- (51) Hamill, A. C.; Wang, S.-C.; Lee, C. T.Jr. Biochemistry 2005, 44, 15139-15149
- (52) Wang, S.-C.; Lee, C. T.Jr. J. Phys. Chem. B 2006, 110, 16117-16123.
- (53) Hamill, A.; Lee, C. T.Jr. Biochemistry 2007, 46, 7694.
- (54) Le Ny, A.-L. M.; Lee, C. T.Jr. J. Am. Chem. Soc. 2006, 128, 6400-6408.

⁽¹⁶⁾ He, S.; Arscott, P. G.; Bloomfield, V. A. Biopolymers 2000, 53, 329-341. (17) Bloomfield, V. A. Biopolymers 1997, 44, 269-282.

intraperitoneal cavity, breast, prostrate, and skin,^{55,56} with clinical trials underway.⁵⁷ With modern fiber optics, light can be delivered to "virtually any part of the body"⁵⁷ with penetration depths of ~1 mm.⁵⁸ The long-term rate of melanoma is low with UVA (although higher than the control).⁵⁹ Thus, it appears that the use of light to induce DNA release *in vivo* is technically feasible.

Materials and Methods

Surfactants. The photoresponsive azobenzene trimethylammonium bromide surfactant (azoTAB, $M_w = 420.4$ g/mol) of the form shown in Scheme 1a was synthesized according to published procedures.^{60,61} The surfactant undergoes reversible photo-isomerization upon exposure to the appropriate wavelength of light, with the *trans* isomer (436 nm visible light) exhibiting a lower dipole moment and, hence, greater hydrophobicity than the *cis* isomer (365 nm UV light).⁶⁰ The conventional alkyl surfactants, sodium dodecylbenzenesulfonate (SDBS, $M_w = 348.5$ g/mol, Scheme 1b), sodium dodecylsulfate (SDS, $M_w = 288.4$ g/mol, Scheme 1c), and dodecyltrimethylammonium bromide (DTAB, $M_w = 308.3$ g/mol), were purchased from Aldrich and used as received.

Cell Culture. Both murine fibroblast NIH 3T3 cells and human embryonic kidney HEK 293T cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) were cultured in 35 mm diameter 6-well polystyrene cell culture dishes (Falcon, Franklin Lakes, NJ) at 37 °C and 5% CO₂ until the cell number reached 2×10^5 in each well. Each well contained 2 mL of growth media composed of 90% high glucose Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc., VA) and 10% fetal bovine serum (FBS, Gibco BRL). Cell numbers were determined with an inverted light microscope by measuring the cell concentration in 16.4 μ L growth media after the cells were suspended and distributed homogeneously in solution.

Cell Viability. To test the cytotoxicity of the surfactant, 2 mL of azoTAB-SDBS and azoTAB-SDS mixtures in serum-free DMEM were overlaid with NIH 3T3 cells in 6-well plates for 6 h, after which time the serum-free DMEM was refreshed with growth media and then incubated for an additional 18 h. Finally, the growth media was replaced with $1 \times$ ethylenediaminetetraacetic acid (EDTA) trypsin (Irvine Scientific, Santa Ana, CA) to suspend the surviving cells. Cell viability was calculated using a hemocytometer with the trypan-blue exclusion method and light microscopy, expressed relative to cell numbers without added surfactant. At the conditions of the transfection experiments (50 µM azoTAB), cell viabilities were 96 ± 0.5 and $97 \pm 1\%$ in the azoTAB/SDBS and azoTAB/SDS systems. For comparison, the cell viability measured with the same weight percent of PEI (25 kDa, Aldrich) over 6 h was 94 \pm 1%. This indicates a slight toxicity of these catanionic systems, consistent with previous observations for similar conventional catanionic vesicles.⁶² Indeed, the resulting cell viability was found to be a strong function of the surfactant concentration, decreasing to essentially 0% at only 500 μ M azoTAB. Further independent measurements indicated that cell viability was not affected by exposure (ca. 10 min) to 365 nm UV light. The extent Scheme 1. (a-c) Surfactants Used To Form Catanionic Pairs



azobenzene trimethylammonium bromide (azoTAB)

anionic:



of UV damage is determined by two factors: the wavelength of illumination and the light intensity, as measured by an "action spectrum" (i.e., reciprocal of the UV dose required to kill 50% of the cells). Typical UV sterilization uses high-intensity $(> 10\,000\,\mu\text{W/cm}^2)$ UV-C light at 264 nm, the peak in the action spectrum. From a typical action spectrum of Escherichia coli, for example,⁶³ exposure to 365 nm light with an intensity approximately 4 orders of magnitude higher that this typical UV-C dose would be required for a similar effect. However, the lamp used in the present study offers a relatively low UV-A dose of 1100 $\mu W/$ cm² at the peak intensity of 365 nm, as reported by the manufacturer. Thus, under the conditions used here, relatively little effect of the UV-A light is expected, as observed. With regard to potential long-term risks to DNA of UV-A exposure, the best comparative data come from the literature on PDT for the treatment of cancer, where a photosensitizer drug, localized in tumors, is illuminated with light to generate reaction products toxic to tumor cells (typically 400-700 nm, although UV-A is also used). The long-term rate of melanoma is low with UV-A (although higher than the control).⁶⁴

Transfection Measurements. After the cell concentration reached 2×10^5 cells in each well, the culture medium was replaced with 4 μ g of eGFP DNA (plRES2-EGFP vector, Clontech, Palo Alto, CA) encapsulated in photoresponsive catanionic vesicles suspended in fresh 2 mL of DMEM plus 10% FBS. The cells were then cultured at 37 °C in an incubator balanced with 5% CO2 for various "pre-incubation" times, followed by refreshing of the growth media to remove any DNA-catanionic complexes not internalized within cells. When desired, the cis-azoTAB samples were then exposed to a relatively low-intensity 84 W long wave UV lamp (365 nm, Spectroline, Model XX-15A) for 10 min in the incubator, with the lamp approximately 20 cm from the sample, such that heating was negligible. The measured absorbance of the cell culture medium at 365 nm was 0.1 at the depth of the medium used (0.2 cm); thus, this protocol was more than sufficient to convert the surfactant to the cis form (confirmed with UV-vis spectroscopy). After a total of 24 h, the cells were then examined by confocal microscopy (Zeiss LSM 510 META, Carl Zeiss, Inc.) after postincubation to determine expression efficiency, defined as the ratio of transfected cells exhibiting green fluorescence to the total number of viable cells measured under bright field. The reported results were averaged over three different spots for each cell culture, with an average number of 500 cells counted at each condition. For the control experiments, 4 μ g of pcDNA3.1-GFP (Invitrogen) was used in 2 mL of growth media and transfected with the catanionic pairs at various molar ratios (DTAB/SDBS = 7:3, azoTAB/SDBS = 7:3, and azo-TAB/SDBS = 3:7) with 50 μ M of the cationic surfactants. After 4 and 6 h of pre-incubation time, the growth media was refreshed

 ⁽⁵⁵⁾ Dougherty, T. J.; Gomer, C. J.; Henderson, B. W.; Jori, G.; Kessel, D.;
 Korbelik, M.; Moan, J.; Peng, Q. J. Natl. Cancer Inst. 1998, 90, 889–905.
 (56) Dolmans, D. E. J. G. J.; Fukumura, D.; Jain, R. K. Nat. Rev. Cancer 2003,

⁽⁵⁶⁾ Dolmans, D. E. J. G. J.; Fukumura, D.; Jain, K. K. *Nat. Rev. Cancer* 2003, 3, 380–387.

⁽⁵⁷⁾ Brown, S. B.; Brown, E. A.; Walker, I. Lancet Oncol. 2004, 5, 497–508.
(58) Moan, J.; Iani, V.; Ma, L. Proc. SPIE–Int. Soc. Opt. Eng. 1996, 2625, 544–549.

 ⁽⁵⁹⁾ Stern, R. S.; Nichols, K. T.; Vakeva, L. H. N. Engl. J. Med. 1997, 336, 1041–1045.

⁽⁶⁰⁾ Shang, T.; Smith, K. A.; Hatton, T. A. *Langmuir* 2003, *19*, 10764–10773.
(61) Hayashita, T.; Kurosawas, T.; Miyata, T.; Tanaka, K.; Igawa, M. *Colloid Polym. Sci.* 1994, *272*, 1611–1619.

Polym. Sci. 1994, 272, 1611–1619.
(62) Kuo, J.-H. S.; Jan, M.-S.; Chang, C.-H.; Chiu, H.-W.; Li, C.-T. Colloids Surf., B 2005, 41, 189–196.

⁽⁶³⁾ Smith, K. C.; Hanawalt, P. C. *Molecular Photobiology*; Academic: New York, 1969.

⁽⁶⁴⁾ Stern, R. S.; Nichols, K. T.; Vakeva, L. H. N. Engl. J. Med. 1997, 336, 1041–1045.

and the transfection efficiencies were determined by confocal microscopy, as above. Further control experiments demonstrated that UV light alone did not enhance the expression of uncomplexed DNA.

Flow Cytometry. Additional quantitative transfection efficiency measurements were conducted using a flow cytometer (Becton Dickinson, FACSort). The HEK293T cells were seeded in 6-well plates with 2×10^{5} cells/well in 2 mL of growth media. After an initial 24 h incubation period at 37 °C with 5% CO₂, the media was replaced with serum-free medium containing DNA (4 μ g of eGFP-coded DNA) plus carrier (either 15 μ M azoTAB/ $6.4 \,\mu\text{M}$ SDBS vesicles or $8 \,\mu\text{g}$ of PEI as a control). The cells were then incubated for 6 h, after which time UV illumination was applied to one of the azoTAB/SDBS samples for 10 min in the incubator. All culture media were then aspirated from each well and replaced with 2 mL of growth media, and the cells were additionally incubated for 18 h. The transfected cells were then washed with phosphate-buffered saline (PBS) solution and detached with 2 mL of trypsin. A total of 0.2 mL from each well was collected and then settled with centrifugation. After discarding the liquid phase, the cells were resuspended in 0.2 mL of PBS. The transfection efficiency was then calculated as the percentage of cells expressing green fluorescent protein (GFP), with the fluorescence from 20000 cells evaluated by flow cytometry.

Confocal Microscopy. To examine intracellular incorporation of DNA with catanionic vesicles, the plasmid DNA was covalently labeled with a LabelIT fluorescein labeling kit (Mirus). The HEK293T cells were seeded in two 35 mm glass base dishes (MatTek) with 2 mL of serum-free medium containing 15μ M azoTAB/6.4 μ M SDBS and 4μ g of DNA in each dish. After 6 h of incubation, one dish was exposed to UV illumination for 10 min. All samples were then fixed with 3.7% formaldehyde (Ted Pella, Inc.) and treated with 0.1% Triton X-100 (Shelton Scientific, Inc.) for 10 min. After that, mouse monoclonal antibody EEA1 (Abcam) and Texas Red goat anti-mouse IgG (Invitrogen) were used to label the early endosomes. Thus, confocal microscopy could independently determine the location of both DNA and endosomes. An argon/krypton laser with an excitation wavelength at 488 nm was used to induce emission from fluorescein of DNA and observed through a 500-530 nm band-pass filter, while the endosomes stained by Texas Red were observed using a HeNe laser with an excitation wavelength of 543 nm and a 565-615 nm band-pass filter.

Fluorescence Spectroscopy. A Ouanta-Master spectrofluorometer (Photon Technology International, Monmouth Junction, NJ, model QM-4) was used to collect the fluorescent spectra of the hydrophobic probe Nile Red. Experiments were performed at 37 °C using a cuvette with a working volume of 3.5 mL and a path length of 10 mm (Nova Biotech, El Cajon, CA). The excitation wavelength was 590 nm with excitation and emission slit widths of 4 nm. The spectrofluorometer was loaded with various concentrations of azoTAB-anionic surfactant mixtures and 0.3 μ M Nile Red in phosphate buffer (pH 7.4), followed by stirring for 10 min to reach steady state. UV-vis spectra were taken after each fluorescence measurement to determine the precise concentration of azoTAB, using an extinction coefficient of 22 000 M⁻¹ cm⁻¹ at 350 nm. For measurements using trans-azoTAB, 2 mL of a 0.3 µM Nile Red solution were successively mixed with 5.7 μ L of both a stock azoTAB solution (3.5 mM azoTAB and 0.3 µM Nile Red) and stock SDBS (1.5 mM SDBS and 0.3 μ M Nile Red) or SDS (1.5 mM SDS and 0.3 μ M Nile Red) solution to maintain an azoTAB/ anion molar ratio of 7:3. For measurements with cis-azoTAB, independent 1.98 mL azoTAB/anion (7:3 molar ratio) solutions were prepared at each desired concentration and pre-exposed to long wavelength 365 nm UV light (Spectroline, 120 V, 60 Hz, 0.7 A), followed by quickly mixing with 0.02 mL of a $30 \,\mu$ M Nile Red solution in the dark. In this manner, potential Nile Red photodegradation with UV exposure was avoided, although in some instances, not entirely prevented.⁵¹ In some cases, the fluorescence intensity was observed to decrease slightly with an increasing surfactant concentration (particularly below the critical aggregate concentration), an apparent result of Nile Red photobleaching during the 10 min allotted between adding surfactant and taking the measurement. Furthermore, the UV-illuminated samples possessed similar but not identical Nile Red concentrations, giving rise to some scatter in the measured emission intensities. As a result, the wavelengths of maximum emission, expected to be insensitive to Nile Red photobleaching and overall concentration, were chosen as the best reporters of micelle formation. The wavelength of maximum absorbance (λ_{max}) for each sample was determined by fitting the measured spectrum to a fifth-order polynomial near each peak.

For the time-resolved measurements, 2 mL of a 50 μ M/ 21.4 μ M azoTAB/SDBS solution with 1 μ M Nile Red were repeatedly exposed at 25 °C to a 200 W mercury arc lamp (Oriel, model 6283) equipped with either a 320 nm band-pass filter (Oriel, model 59800) or 400 nm long-pass filter (Oriel, model 59472) combined with a 300-500 nm short-pass filter (Oriel, model 57374) to isolate the 365 or 436 nm lines from the mercury lamp, respectively. The excitation and emission wavelengths were set at 590 and 650 nm, respectively. The emission spectra were recorded using a 15% neutral density filter just prior to the photomultiplier tube to prevent saturation of the detector with light from the mercury arc lamp (seen as an immediate jump or drop in the fluorescent intensity upon switching to 436 nm visible or 365 nm UV light). This effect results in different fluorescent intensity scales in the steady-state and time-resolved measurements.

Dynamic Light Scattering. Dynamic light scattering measurements were performed at 37 °C on a BI-200SM instrument equipped with a BI-9000AT digital correlator (Brookhaven Instrument Corp., Holtsville, NY). A 35 mW helium-neon (632.8 nm) laser (Melles Griot, model 05-LHP-928) was used for the measurements, which did not affect the isomeric state of azoTAB species. Pure azoTAB and SDBS aqueous solutions were separately passed through 200 nm Anatop filters and then mixed at the appropriate ratios, followed by vortexing for 1 min. Control experiments on separate surfactant solutions under the same conditions revealed no scattering in excess of the solvent, indicating that the results obtained upon mixing are due to selfassembly products within the catanionic mixture. The transient light-scattering measurements were repeated twice independently on each sample to ensure reproducibility of the timedependent behaviors. Hydrodynamic diameters were obtained from the measured diffusion coefficients using the Stokes-Einstein equation.⁵⁴ The intensity-weighted relative variances of the diffusion coefficient distributions were relatively low ranging from 0.05-0.12, indicating relatively monodisperse distributions.

Cryo-Transmission Electron Microscopy (TEM). Vitrified specimens for cryo-TEM were prepared in a controlled environment vitrification chamber (CEVS) and quenched into liquid ethane at its freezing point.⁶⁵ Specimens, kept below -178 °C, were examined in a FEI T12 G² transmission electron microscope, operated at 120 kV, using a Gatan 626 cryo-holder system. Images were recorded digitally in the minimal electrondose mode by a Gatan US1000 high-resolution cooled CCD camera with the DigitalMicrograph software package. AzoTAB isomerization from the *trans* to *cis* form was achieved through 2 min of exposure of the surfactant solution to UV light from a spectral calibration mercury (argon) lamp (Oriel, model 6035) equipped with a long-wave filter (Oriel, model 6047).

Fluorescence Microscopy. T4-phage DNA (165.6 kb pairs, 1.076×10^8 Da) was purchased from Wako Chemical (catalog

⁽⁶⁵⁾ Talmon, Y. Cryogenic temperature transmission electron microscopy in the study of surfactant systems. In *Modern Characterization Methods of Surfactant Systems*; Binks, B. P., Ed.; Marcel Dekker: New York, 1999; p 147.



Figure 1. Cryo-TEM images of aqueous 31:69 (molar) azoTAB/SDS catanionic vesicles formed at 0.2 wt % overall surfactant concentration and at 38 °C under (a) visible light and (b) following 2 min of UV illumination. The white arrowheads point to flat bilayer patches imaged face on; the black arrowhead points to a bilayer patch imaged edge on; the white arrow indicates an opening in a ruptured vesicle; and the black arrow indicates a partially folded bilayer patch.

number 318-03971), visualized with the fluorescent dye Yoyo-3 $(\lambda_{\rm ex}/\lambda_{\rm em} = 612/631$ nm) using an Olympus IX71 inverted fluorescence microscope, and recorded with a Hamamatsu digital CCD camera (model C4742-95), as previously described.⁵⁴ The concentrated DNA solution was diluted to $2.9 \,\mu\text{M}$ (base pair) in TE buffer (10 mM Tris and 1 mM EDTA) and gently mixed with the dye solution to a ratio of 15 bp per dye molecule. Approximately $250 \,\mu L$ of the DNA-dye solution was then placed in the custom-made slide and kept open to air. Concentrated stock solutions of first azoTAB and then SDBS, both in TE buffer, were then added to final concentrations of 48 and 19 μ m, respectively (a 7.2/2.8 ratio), with homogeneity ensured by swirling prior to optical observation. The DNAcatanionic surfactant mixture was then alternately exposed to UV and visible light using a 200 W mercury arc lamp (Oriel, model 6283) equipped with a liquid light guide (Oriel, model 77557), a fiber-bundle focusing assembly (Oriel, model 77800), and either a 320 nm band-pass filter (Oriel, model 59800) or a 435.8 nm band-pass filter (Oriel, model 56551) filter, respectively. A heat-absorbing filter (Oriel, model 59060) was placed in the beam path to absorb IR light produced by the lamp.⁶

Results and Discussion

The photoresponsive azobenzene-trimethylammonium bromide (azoTAB) surfactant shown in Scheme 1a undergoes reversible photo-isomerization upon exposure to visible or UV light, associated with absorption by the trans and cis isomers at ca. 365 and 436 nm for both pure azoTAB solutions and azoTAB-DNA mixtures.⁵⁴ For example, illuminating a 31 μ M azoTAB solution with 436 nm visible light from a mercury lamp results in a 75:25 *trans/cis* photostationary distribution within a few seconds, whereas UV illumination at 365 nm results in a 20:80 trans/cis photostationary distribution again within a few seconds.⁵⁴ These photostationary states develop when the rates of trans-cis and cis-trans conversions become equal, with the resulting compositions controlled by the relative absorption coefficients at the respective illumination wavelengths, as well as the efficiencies of the two isomerization reactions. In addition, both of these photostationary states thermally relax to the thermodynamically favored 100% *trans* state⁵⁴ under dark conditions in \sim 24 h. The smaller dipole moment of the trans azo moiety (0.5 D) compared to that of the *cis* moiety (3.1 D)⁶⁰ contributes to the *trans* state being more hydrophobic.⁶¹ Consequently, alternate exposure to visible and UV light causes photoreversible changes in the hydrophobicity/hydrophilicity of the surfactant species that can be used to control various surfactant properties,⁶⁷ including photocontrol of protein folding^{50–53} and DNA condensation.⁵⁴

To form photoresponsive catanionic vesicles, an aqueous solution of the cationic azoTAB surfactant species was mixed with an aqueous solution of either of two conventional anionic surfactants, sodium dodecylbenzenesulfonate (SDBS, Scheme 1b) or sodium dodecylsulfate (SDS, Scheme 1c). Representative cryo-TEM images of the catanionic vesicles under visible and UV illumination are shown in Figure 1 for the azoTAB-SDS system (31:69 molar ratio, 0.2 wt % solution) at 38 °C. Under visible light conditions (Figure 1a), spherical vesicles are present, with sizes generally ranging from 50-200 nm, consistent with separate lightscattering measurements (see below). Illumination of the same sample with UV light for 2 min, however, results in significant changes to the vesicle ensemble. The cryo-TEM image in Figure 1b shows that a number of the catanionic vesicles have ruptured, because of the *trans-cis* isomerization induced in the azoTAB surfactant by the UV light. Arrows in Figure 1b show bilayer patches that have separated from the rest of the vesicle but have not dissolved or formed micelles. This suggests that $\pi - \pi$ stacking interactions, which are likely prevalent in the bilayers formed from the planar trans-azoTAB surfactant species, are not completely disrupted upon conversion to the bent *cis* form. While the light-dependent vesicle structures are expected to be complicated, the cryo-TEM images show that the catanionic vesicles are clearly present under visible light conditions. Furthermore, some of these vesicles are disrupted upon UV irradiation.

Whether and when catanionic vesicles form depend upon numerous factors, including head group and chain interactions among the cationic and anionic surfactant pairs, their relative composition ratios, the total surfactant concentration, and temperature. The formation of photoresponsive catanionic vesicles can be readily monitored using the sensitivity of probe molecules to their local environments. For example, the lipophilic probe molecule Nile Red incorporates readily into self-assembled vesicle bilayers and yields distinct fluorescence spectra, according to whether it is in polar or nonpolar environments. Whereas, in water, Nile Red exhibits relatively low fluorescence, upon solubilization in a nonpolar medium (e.g., lipid or vesicle bilayers), the fluorescence intensity increases and the emission spectrum blue

⁽⁶⁶⁾ Lee, C. T.Jr.; Smith, K. A.; Hatton, T. A. *Macromolecules* **2004**, *37*, 5397–5405.

⁽⁶⁷⁾ Eastoe, J.; Vesperinas, A. Soft Matter 2005, 1, 338-347.



Figure 2. Wavelength at maximum fluorescence emission of 0.1 μ M Nile Red ($\lambda^{ex} = 590$ nm) as a function of the azoTAB surfactant concentration in aqueous catanionic solutions of (a) azoTAB/SDBS (molar ratio of 7:3) and (b) azoTAB/SDS (molar ratio of 7:3) under visible and UV illumination. Arrows denote the approximate cac values. T = 37 °C.

shifts to lower wavelengths.^{68,69} Nile Red, thus, represents a convenient polarity indicator that assists in establishing the onset of bilayer formation associated with catanionic vesicle formation.

Figure 2 displays the wavelengths of maximum fluorescence emission (λ_{max}) collected for several isotropic solutions of azo-TAB-SDBS and azoTAB-SDS catanionic surfactant mixtures containing a small (0.1 µM) amount of Nile Red and different azoTAB concentrations (the complete emission spectra can be found in the Supporting Information). A fixed cation/anion molar ratio of 7:3 was selected to yield catanionic vesicles with net positive charges capable of complexing with DNA (see below). In pure water, the wavelength corresponding to maximum emission occurs at approximately $\lambda_{max} = 657$ nm in both parts a and b of Figure 2, similar to values for Nile Red expected from the literature.⁶⁸ As shown in Figure 2a for the azoTAB-SDBS system, increasing the surfactant concentration under visible light with \sim 75% of azoTAB in the *trans* state leads to a characteristic decrease in λ_{max} at concentrations above 10 μ M azoTAB (Figure 2a), indicating that bilayer formation and the onset of vesicle formation [i.e., the *critical aggregation concentration* (cac)] occurs at approximately 10 μ M azoTAB surfactant. Similar to phenomena observed for other catanionic mixtures,⁷⁰ this value is far below the critical micelle concentration (cmc) of separate (noncatanionic) aqueous solutions of cationic azoTAB under visible light (5.0 mM)⁶⁶ or anionic SDBS (2.2 mM).⁷¹ A gradual decrease (as opposed to a step change) in the emission maximum of Nile Red is observed beyond the cac as a result of Nile Red preferentially partitioning from the water phase into the growing hydrophobic bilayer phase.

Under UV illumination with ~80% of azoTAB in the *cis* state, a similar effect is observed, although the decrease in λ_{max} does not occur until ca. 60 μ M (Figure 2a), corresponding to a significantly larger cac value. This increase in the cac may be expected on the basis of the relative hydrophilicity of the *cis*

isomer compared to the *trans* state, as well as potential steric hindrances for bilayer formation because of the bent *cis* conformation. Indeed, from neutron and light-scattering data, Hubbard and Abbott concluded that the *trans* and *cis* isomers of a similar azobenzene-containing photosurfactant segregate into separate microstructures, with vesicles being rich in the *trans* isomer.⁴⁹ Interestingly, at the respective cac values in Figure 2a, the resulting concentrations of azoTAB in the *trans* conformation would be similar based on the aforementioned photostationary states (i.e., 75% for 10 μ M and 20% for 60 μ M). This implies that between 7.5 and 12 μ M of *trans*azoTAB is required for vesicle formation under either visible or UV illumination and, in general, agrees with the argument of Hubbard and Abbott that primarily *trans* isomers participate in bilayer formation.

Similar results for the light-dependent formation of vesicles are found for the azoTAB/SDS catanionic system, as shown in Figure 2b. Under visible light, λ_{max} is observed to undergo a similar sudden blue shift, as the azoTAB surfactant concentration is increased above 10 μ M, consistent with the onset of vesicle formation. In contrast, under UV illumination, vesicles do not form until 50 μ M azoTAB, again, corresponding to a substantially higher cac, because of the greater hydrophilicity of the *cis*azoTAB isomer and its diminished tendency to form vesicles. The somewhat lower cac of the cis-azoTAB/SDS solution (50 µM azoTAB, Figure 2b) compared to that of cis-azoTAB/SDBS (60 μ M azoTAB, Figure 2a) appears to be due to differences in the trans-azoTAB populations of the two systems under UV light conditions. Control experiments revealed a shift in the UVilluminated photostationary state of azoTAB in the presence of SDS to favor a greater proportion of trans-azoTAB isomers (27%) than in the presence of SDBS (21%). Bonini et al. similarly reported that the presence of SDS in catanionic mixtures with an azobenzene-containing cationic surfactant can influence the photostationary state.⁷² The higher proportion of vesicle-promoting trans-azoTAB isomers in the presence of SDS is consistent with its lower cac compared to SDBS.

These cac measurements are useful for two important reasons. First, it is noteworthy that previous studies of photoresponsive catanionic vesicles have all used surfactant concentrations ranging from 0.1 to 1.0 wt % surfactant.45-49 In contrast, the transfection experiments below are performed at 50 μ M azoTAB (corresponding to 0.0028 wt % total surfactant), between the measured cac values of azoTAB in the trans and cis forms. The use of surfactant concentrations nearly 2 orders of magnitude lower than in previous studies significantly mitigates the toxicity effects that low-molecular-weight surfactants can have on target cells. Toxicity measurements (see the Materials and Methods) show that cell viability is dramatically improved at such low azoTAB surfactant concentrations, increasing to $\sim 97\%$ viability in the presence of 50 μ M azoTAB from ~0% viability for 500 μ M azoTAB. Determining and controlling the lowest possible concentration(s) at which photoresponsive catanionic vesicles form is crucial to establishing conditions and compositions required for efficient and safe incorporation of DNA into cells.

Second, the cac measurements demonstrate that both the azoTAB–SDBS and azoTAB–SDS catanionic systems exhibit large differences in their respective cac values under visible and UV light conditions, which can be exploited to control their self-assembly behaviors. In particular, operating at a surfactant concentration between the respective UV and visible cac values

⁽⁶⁸⁾ Sackett, D. L.; Wolff, J. Anal. Biochem. 1987, 167, 228-234.

⁽⁶⁹⁾ Deye, J. F.; Berger, T. A.; Anderson, A. G. Anal. Chem. 1990, 62, 615-622.

 ⁽⁷⁰⁾ Wang, G.-J.; Engberts, J. B. F. N. Langmuir 1994, 10, 2583–2587.
 (71) Singh, C. D.; Shibata, Y.; Ogita, M. Sens. Actuators, B 2003, B96, 130–132.

⁽⁷²⁾ Bonini, M.; Berti, D.; Di Meglio, J. M.; Almgren, M.; Teixeira, J.; Baglioni, P. Soft Matter 2005, 1, 444–454.

Liu et al.

is expected to allow vesicles formed under visible light (relatively low cac) to be completely disrupted upon UV illumination (relatively high cac), as opposed to just partial disruption at concentrations higher than the *cis* cac. This useful property of azobenzene-containing catanionic mixtures allows for photoreversible control of vesicle formation and rupture and suggests that DNA release from the carrier complex can be photo-initiated, as will be shown below.

Photo-induced Catanionic Vesicle Rupture and Reformation. To test for photo-initiated vesicle rupture, transient lightscattering and fluorescence measurements were used to measure the intensities of scattered and fluorescent light from a catanionic azoTAB-SDBS solution (7:3 molar ratio, 50 uM azoTAB, 21.4 µM SDBS, and 0.1 µM Nile Red) at 37 °C, as correlated with incident visible and UV light illumination. For this catanionic solution composition, vesicles are expected to form under visible light conditions but not under UV illumination (see Figure 2a). Figure 3a shows that, under visible light conditions, the azoTAB-SDBS solution scattered HeNe laser light extensively at a rate of $\sim 150 \times 10^3$ counts per second (cps) (separate and otherwise identical aqueous solutions of azoTAB and SDBS at twice the desired concentration were independently filtered and then mixed at equal volumes to the appropriate ratio to ensure that any scattering observed resulted from surfactant self-assembly into vesicles and not because of small micellar assemblies or impurities). By comparison, upon UV illumination of the same azoTAB-SDBS solution, however, the count rate rapidly decreased to $\sim 7 \times 10^3$ cps, on par with the count rate of the filtered solvent and the filtered separate surfactant solutions. These results are consistent with UV-induced vesicle dissolution that is essentially complete within \sim 30 s. Re-illumination with visible light some time later leads to restoration, within ~ 30 s, of the intensity of scattered HeNe light to a similar count rate ($\sim 150 \times 10^3$) as previously measured for the sample before exposure to UV light. Such restoration of scattered light intensity corresponds to reformation of the catanionic vesicles, indicating the reversibility of this process, as well as its triggerability. Furthermore, dynamic light-scattering measurements yielded essentially identical sizes for both the originally formed and the ruptured and reformed vesicles (185 ± 5 nm). The slight decrease in scattering intensity observed after the initial jump, following visible-light-induced vesicle reformation in Figure 3a, is likely due to a slow approach to the final equilibrium vesicle size. This overall process could be repeated practically indefinitely with essentially fully reversible "visible ↔ UV light" cycles. These observations are consistent with catanionic vesicles behaving as equilibrium structures, as opposed to non-equilibrium entities resulting from shear processes that are inevitably present during mixing of the cationic and anionic surfactant solutions.73

Time-resolved fluorescence emission measurements made on the same catanionic azoTAB–SDBS solution (7:3 molar ratio, $50 \,\mu$ M azoTAB, 21.4 μ M SDBS, and 0.1 μ M Nile Red) at 37 °C quantitatively corroborate reversible vesicle formation and disruption under visible and UV illumination. As shown in Figure 3b, fluorescence intensity changes rapidly from high values under visible light conditions to low values under UV, corresponding to Nile Red probe species in lipophilic bilayers and polar aqueous environments, respectively. The sharp changes in fluorescence intensity observed in Figure 3b correlate with light-induced vesicle rupture and reformation, both



Figure 3. (a) Light-scattering intensity and (b) fluorescence emission spectra from an aqueous catanionic surfactant solution at T = 37 °C containing 50 μ M azoTAB, 21.4 μ M SDBS, and 0.1 μ M Nile Red under alternating exposure to visible (436 nm) and UV (350 nm) light. The sharp changes correspond to photo-induced vesicle rupture and reformation, which are reversible.

occurring on time scales of \sim 30 s, as established independently by the light-scattering measurements in Figure 3a.

The dynamics of vesicle rupture/formation have been monitored in conventional catanionic surfactant mixtures [e.g., dodecyltrimethylammonium bromide (DTAB) and SDS] under conditions of rapid mixing.⁷⁴ In these systems, vesicle formation was found to be a complicated process, manifesting multi-exponential transient behavior that could be modeled as a progression through a series of intermediate states [e.g., small non-equilibrium aggregates (mixed micelles and elongated micelles) that eventually lead to vesicles]. Conversely, the DTAB-SDS vesicle rupture was found to be a kinetically simpler process, for which single-exponential behavior was observed with a characteristic time constant of ~ 10 s.⁷⁴ The gradual decrease in scattering intensity observed in Figure 3a after the initial rapid jump following visible-light-induced vesicle reformation is also consistent with a slow approach to equilibrium of the final vesicle size that would account for the multi-exponential dynamics of DTAB-SDS vesicle formation (although, in the azoTAB-SDBS case, the gradual decrease in light-scattering intensity suggests that the aggregates decrease in size over time, in contrast to the DTAB-SDS system). Scattered light intensity is expected to be more sensitive to subtle changes in the vesicle size distribution than Nile Red fluorescence, which reports mainly on hydrophobic bilayer formation. Thus, it is not surprising that this apparent multi-exponential behavior of vesicle formation is not detected in Figure 3b. Both the lightscattering and fluorescence results in Figure 3 similarly suggest that UV-light-induced vesicle disruption is a single-exponential process, akin to the DTAB-SDS system.

Fluorescence Microscopy. Cyclical visible–UV light illumination can be used to reversibly compact and release DNA molecules from vesicles that undergo photo-induced rupture and reformation. Fluorescence microscopy measurements were

⁽⁷³⁾ Horbaschek, K.; Hoffmann, H.; Hao, J. J. Phys. Chem. B 2000, 104, 2781–2784.

⁽⁷⁴⁾ O'Connor, A. J.; Hatton, T. A.; Bose, A. Langmuir 1997, 13, 6931–6940.

Article



5 μΜ

Figure 4. Fluorescent microscope images of conformational changes in T4–DNA molecules in (a) aqueous solution, upon the addition of (b) azoTAB and also (c) SDBS surfactants, as well as (d) under UV or (e) visible light illumination to disrupt and reform the catanionic vesicles, respectively. [DNA]_{bp} = $2.9 \,\mu$ M, [azoTAB] = $48.2 \,\mu$ M, and [SDBS] = $18.9 \,\mu$ M in TE buffer. Three representative molecules are displayed for each condition.

conducted to correlate DNA free volume with the presence of vesicles before and after illumination of a catanionic azoTAB-SDBS solution (48.2 μ M azoTAB, 18.9 μ M SDBS, and 2.9 μ M DNA). The catanionic surfactant solutions were similar to those reported above. The DNA concentration was the same as that used in the transfection experiments below, although the larger T4-DNA molecule was employed to provide better visual observation in the microscopy measurements. As shown in the images of Figure 4, 2.9 μ M T4–DNA in a buffered solution in the absence of cationic or anionic surfactant species yields DNA molecules in highly elongated states (Figure 4a). After the subsequent addition of 48.2 μM azoTAB to this solution (preserving buffering and other component concentrations) without the anionic counterpart, no evidence of DNA compaction was observed (Figure 4b) nor expected on the basis of our previous studies.⁵⁴ However, upon further addition of 18.9 μ M anionic SDBS surfactant (Figure 4c), DNA condensed from the elongated-coil to a compact-globular state within a few seconds. For the resulting cationic azoTAB/anionic SDBS molar ratio of 7.2:2.8, the catanionic vesicles have net positive charges and would thus be expected to interact strongly with and effectively compact anionic DNA, as suggested in the literature,⁷⁵ into likely lamellar or hexagonally packed assemblies.^{76,77}

By comparison, DNA in the presence of azoTAB/SDBS or azoTAB/SDS vesicles with net *negative* charges (e.g., with 50 μ M azoTAB and an azoTAB/SDBS molar ratio of 3:7, confirmed to form vesicles by light scattering and Nile Red fluorescence measurements) was not compactable using the protocol in Figure 4 under otherwise identical conditions. This suggests that encapsulation of DNA in catanionic vesicles is electrostatically driven, as reported in the literature for non-photoresponsive catanionic vesicles.⁷⁸ Thus, the fluorescence microscopy images in Figure 4 demonstrate that simple molecular binding of the positively charged azoTAB species to negatively charged DNA molecules at this relatively low azoTAB concentration (50 μ M) is not the cause of conformational changes in DNA; instead, only upon the formation of positively charged catanionic vesicles does DNA condensation appear to occur. Subsequent UV illumination to disrupt the vesicles (Figure 4d), as expected from the cac measurements in Figure 2 then lead to re-expansion of DNA molecules upon their release from the vesicle complexes. This phenomenon is completely photoreversible. Re-illumination with visible light reconstituted the vesicles (Figure 3), leading to photo-induced recompaction of DNA within ~ 2 s, similar to previous measurements.⁵⁴

DNA Delivery with Catanionic Vesicles for Cell Transfection. On the basis of the evidence above, photoresponsive catanionic vesicles are capable of forming reversible complexes with DNA that result in compacted DNA-containing vesicles with diameters less than about 250 nm. Such small vesicles are generally within the size range considered suitable for efficient cellular internalization via endocytosis.⁷⁹ However, it should be noted that, while reversible complex formation was demonstrated above in simple buffer, the situation in the extracellular environment is likely to be more complex. Thus, direct structure-function comparisons should be made with caution. Nevertheless, to test whether these systems might be used as novel gene delivery vectors, NIH 3T3 cells were exposed to eGFP-coded plasmid DNA that was encapsulated in various catanionic vesicles. Cells were incubated for 24 h total, with the growth media refreshed after pre-incubation times of 2, 4, 6, or 8 h to remove any external vesicles. The representative photomicrographs shown in Figure 5 were obtained following DNA delivery with azoTAB/SDBS (parts a and b of Figure 5) or azoTAB/SDS (parts c and d of Figure 5) vesicles both with and without UV-induced vesicle rupture, following a pre-incubation time of 8 h. Successful delivery of eGFP-DNA into a given cell was established by comparing the bright-field image of a cell with the fluorescence image, detecting the presence of the greenfluorescent protein (GFP) expression product within the cell.

Overall transfection efficiencies were estimated from confocal microscope measurements that establish the percentage of cells exhibiting green fluorescence following incubation. Table 1 shows such transfection efficiencies obtained from a sufficient number of confocal images to observe 300-500 cells at each condition, from which representative comparisons were made. Even without light-induced vesicle rupture, the catanionic vesicles generally increase transfection efficiencies, compared to uncomplexed DNA (measured to be < 5%). This suggests that the positively charged catanionic vesicles (at a molar ratio of

⁽⁷⁵⁾ Gershon, H.; Ghirlando, R.; Guttman, S. B.; Minsky, A. *Biochemistry* 1993, 32, 7143–7151.

⁽⁷⁶⁾ Raeder, J. O.; Koltover, I.; Salditt, T.; Safinya, C. R. Science 1997, 275, 810–814.

⁽⁷⁷⁾ Koltover, I.; Salditt, T.; Rädler, J. O.; Safinya, C. R. *Science* **1998**, *281*, 78–81.

⁽⁷⁸⁾ Mel'nikov, S. M.; Dias, R.; Mel'nikova, Y. S.; Marques, E. F.; Miguel, M. G.; Lindman, B. *FEBS Lett.* **1999**, *453*, 113–118.

⁽⁷⁹⁾ Zauner, W.; Ogris, M.; Wagner, E. Adv. Drug Delivery Rev. 1998, 30, 97-113.



Figure 5. Fluorescent (top left), bright-field (top right), and combined (bottom left) photomicrographs of the same regions of NIH 3T3 cells expressing eGFP, following exposure to eGFP-coded DNA encapsulated in (a,b) azoTAB/SDBS or (c,d) azoTAB/SDS catanionic vesicles before and after rupture. The cells were immersed in solutions containing premixed DNA/catanionic vesicles for pre-incubation times of 8 h at 37 °C, after which the growth media was replaced to remove any complexes external to the cells. The images were then obtained after a total incubation time of 24 h. In all cases, [azoTAB] = 50 μ M at a cationic/anionic surfactant ratio of 7:3, while [DNA] = 2 μ g/mL. Scale bars denote 20 μ m.

Table 1. Percent Transfection Efficiencies of NIH 3T3 Cells with
$2 \mu g/mL$ eGFP-Coded DNA Encapsulated in Photoresponsive
Catanionic Vesicles (7:3 Molar Ratios) Both without and with
UV-Induced Vesicle Rupture Following Various
Pre-incubation Times

	azoTAB/SDBS		azoTAB/SDS		
pre-incubation time	no UV	UV rupture	no UV	UV rupture	
(h)	(%)	(%)	(%)	(%)	
2 4	7(±3)	12 (±3)	3 (±3)	6(±3)	
	16	35	4	8	
6	16	39	9	12	
8	20	37	12	13	

7:3) allow for enhanced uptake past the negatively charged cell membrane. For comparison, negatively charged catanionic vesicles at a molar ratio of 3:7 resulted in a transfection efficiency on par with uncomplexed DNA, as expected on the basis of the inability of these vesicles to effectively condense DNA (Figure 4) or transit the cell membrane. As shown in Table 1 for positively charged catanionic vesicles, longer pre-incubation times yielded greater transfection efficiencies, as expected because of a greater degree of DNA endocytosis over time. The transfection efficiencies eventually appear to level off with increasing preincubation time (within experimental uncertainty of $\pm 3\%$ based on averaging several measurements), as the rates of endocytosis and exocytosis become comparable. Interestingly, the azoTAB/ SDBS vesicles appear to offer higher nonphoto-assisted transfection efficiencies (~20%) than the azoTAB/SDS system $(\sim 12\%)$. This suggests that the enhanced stability of the DNA-carrier complexes (hypothesized to be due to $\pi - \pi$ interactions) within the vesicle bilayers leads to greater cellular uptake, an advantage also of azoTAB- as opposed to alkyl-containing catanionic vesicle components.

To test this hypothesis, transfection experiments were performed using otherwise identical solutions of the non-photoresponsive catanionic pair DTAB/SDBS. DTAB species were selected because of their identical head groups and similar tail lengths compared to azoTAB. The DTAB/SDBS system is also known to form catanionic vesicles,⁸⁰ which were confirmed here for 50 µM DTAB and a 7:3 cationic DTAB/anionic SDBS ratio by Nile Red fluorescence and light-scattering measurements. After 4 and 6 h of pre-incubation time and using the same protocol as Figure 5, very low transfection efficiencies of 2.7 and 4%, respectively, were measured by confocal microscopy. These low values are (within experimental uncertainty) the same as obtained for uncomplexed DNA, indicating that favorable interactions involving the azobenzene moieties contribute to improved delivery properties. Further measurements are underway to examine the molecular origins of such effects, which are thought to result from increased bilayer rigidity because of $\pi - \pi$ interactions among aromatic moieties of the azoTAB and SDBS surfactant species. Similar $\pi - \pi$ interactions were observed previously in mixtures of DNA with azoTAB.54 Furthermore, extensive azobenzene interactions (so-called *H* aggregates) have been used as a means to control bilayer permeability.81

⁽⁸⁰⁾ Soederman, O.; Herrington, K. L.; Kaler, E. W.; Miller, D. D. *Langmuir* **1997**, *13*, 5531–5538.

Article

Transfection experiments were also performed and compared to the cationic polyelectrolyte polyethylenimine (PEI), a standard delivery agent known to enhance DNA transfection.82 Using a pre-incubation time of 8 h, a transfection rate of 44.3% was obtained for the DNA/PEI complexes. PEI is thought to mediate endosome rupture through a "proton sponge" mechanism, whereby the low pH within endosomes results in protonated nitrogen atoms along the PEI backbone, leading to a charge gradient that causes an influx of Cl⁻ counterions.^{83,84} This in turn increases the osmolarity of the endosome, resulting in an influx of water and, hence, endosome swelling/rupturing. It is noteworthy that the transfection efficiencies shown in Table 1 for the non-photoruptured catanionic vesicles were below the PEI benchmark, which exhibits facilitated endosome release. This suggests that, in the absence of light-initiated vesicle rupture, the release of DNA from the catanionic vesicle complexes within endosomes may limit the rate of overall transfection.

Photo-assisted DNA Delivery. To investigate potential enhancements in gene delivery provided by photo-initiated release of DNA from photoresponsive catanionic vesicle complexes, such vesicles were allowed to enter the cells via endocvtosis and their efficacy was monitored and compared against control solutions. For identical catanionic azoTAB/ SDBS/DNA and azoTAB/SDS/DNA vesicle solutions as described above (50 µM azoTAB, 7:3 cationic/anionic surfactant molar ratios, and $2 \mu g/mL$ DNA), endocytosis was allowed to proceed for 2–8 h of pre-incubation time, followed by illumination with 365 nm UV light to promote vesicle rupture and a wash step to remove any non-internalized disrupted vesicle complexes. Using this strategy, transfection efficiencies of \sim 40% (Table 1) were achieved for the azoTAB-SDBS system, approaching the value obtained for PEI. For example, after 8 h of incubation, transfection efficiencies of 37.0, 36.5, and 38.4% were obtained following UV-induced rupture in independent measurements. The relatively low extent of transfection achieved after only 2 h prior to illumination in the azoTAB/ SDBS system suggests that 4 h are required for maximum cellular uptake through endocytosis, consistent with literature reports.85

In contrast, the azoTAB/SDS system results in little enhancement in UV-light-induced transfection efficiency (Table 1), compared to the azoTAB/SDBS system (within experimental uncertainty). Three factors could explain this effect. First, the transfection experiments were performed at 50 μ M azoTAB to provide the maximum amount of DNA delivery, while keeping the overall surfactant concentration low to avoid issues of cell toxicity, as discussed above. From the cac values measured under UV light for the azoTAB/SDBS (60 μ M) and azoTAB/SDS (50 μ M) systems (Figure 2), more complete rupture of catanionic vesicles would be expected in azoTAB/SDBS solutions, compared to the azoTAB/SDS system, following UV illumination. Second, the rate of cellular uptake is likely affected by vesicle size. DLS measurements indicate that the vesicles are on average larger and exhibit a greater size distribution in the azoTAB/SDS system (230 nm) compared to the azoTAB/SDBS system (185 nm), in agreement with the cryo-TEM images in Figure 1. Thus, a greater fraction



Figure 6. Flow cytometry results of the transfection rate of HEK 293T cells infected with GFP-encoded DNA. The cells were incubated with DNA and (a) PEI for 2 h, (b) PEI for 4 h, (c) PEI for 6 h, (d) no DNA, (e) azoTAB/SDBS for 6 h, and (f) azoTAB/SDBS for 6 h followed by 10 min of UV exposure. After this time period, the growth medium was refreshed and the sample was incubated for a total of 24 h.

of the azoTAB/SDS vesicles are expected to be above the endocytosis size limit of ca. 250 nm. Finally, the potential for $\pi-\pi$ stacking interactions is reduced in the azoTAB/SDS system compared to azoTAB/SDBS, which could lead to lower transfection efficiencies, as similarly observed and discussed above for the DTAB/SDBS system. Thus, the azoTAB/SDBS system appears to offer two desirable and often mutually exclusive properties: high stability to enhance cellular uptake combined with photo-activated release of DNA from the vesicle complexes.

These results were confirmed for a much larger number of cells by using flow cytometry to examine the transfection rates of HEK293 cells in the presence of various carriers (e.g., PEI as well as azoTAB/SDBS vesicles with and without UV illumination), as shown in Figure 6. In this way, statistics were established on the basis of measurements of $\sim 20\,000$ cells, in addition to the 300-500 cells counted by hand and analyzed above. The transfection efficiency with PEI as a carrier after 6 h of transfection (32.2%) was found to be significantly higher than for the azoTAB/SDBS vesicles without UV illumination (19.9%). However, with the addition of a 10 min UV-illumination step to convert azoTAB to the *cis* form and disrupt the catanionic vesicles, the transfection rate improved markedly (29.8%), again approaching the value obtained for PEI. Moreover, the flow cytometry measurements additionally allowed cytotoxicity to be quantified over the much larger number of cells. In each of the cases, low cytotoxicities were determined for the transfection conditions discussed above: cell viabilities of 92.5, 92.2, and 92.3% were measured for the PEI, trans-azoTAB/SDBS, and cis-azoTAB/SDBS carriers, respectively. The flow cytometry results independently support the conclusions that UV illumination of azoTAB/ SDBS catanionic vesicles provides an effective, low-toxicity, and nonviral means of enhancing the delivery of DNA into cells.

Intracellular DNA Incorporation. One crucial mechanism remains to be explained. Exactly how would the *in vitro* release of DNA from catanionic vesicles demonstrated in Figure 4 give rise to the apparent release within cells suggested in Figures 5 and 6? The endocytic pathway results in encapsulation of the carrier in endosomes. It is the release of DNA from these endosomes and not the carrier per se that must be achieved for nuclear uptake and expression to occur. Transfection studies with

⁽⁸²⁾ Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J.-P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297–7301.

⁽⁸³⁾ Cho, Y. W.; Kim, J.-D.; Park, K. J. Pharm. Pharmacol. 2003, 55, 721–734.
(84) Brown, M. D.; Schatzlein, A. G.; Uchegbu, I. F. Int. J. Pharm. 2001, 229, 1–21.
(85) Godbey, W. T.; Wu, K. K.; Mikos, A. G. Proc. Natl. Acad. Sci. U.S.A.
1999, 96, 5177–5181.



Figure 7. Representative confocal microscopy images of fluorescein-labeled DNA transfected into HEK293T cells with azoTAB/SDBS catanionic vesicles and early endosomes labeled with Texas Red goat anti-mouse IgG. (a) Without UV illumination after 6 h of transfection with *trans*-azoTAB/SDBS vesicles; the majority of DNA molecules are observed to remain associated with endosomes (the arrows point to DNA not co-localized with an endosome). (b) With UV illumination, under otherwise identical conditions, to convert to *cis*-azoTAB, showing that the majority of DNA molecules are subsequently released from the endosomes.

conventional cationic lipids indicate that significant "lipid mixing" occurs between the carrier vesicles and the endosomes.^{86–88} Consequently, it is expected that the azoTAB surfactant species similarly experience such intracellular mixing, with the result that UV-induced endosome disruption may contribute to the photo-enhanced transfection efficiencies demonstrated in Figure 5. The mechanisms of light-triggered disruption of endosomes or catanionic vesicles are likely to be similar, both controlled by azoTAB hydrophobicity and molecular conformation. Nevertheless, such subtle and complex effects could have profound implications on the observed transfection efficiencies.

As a result, dual-labeling of DNA (fluorescein) and early endosomes (Texas Red) was used to study specifically UVinduced release of DNA from the endosomes. As shown in Figure 7a, without a UV-illumination step, the majority of the DNA molecules (green) are observed to be co-localized with the endosomes (red) after 6 h of incubation, resulting in yellow spots in the merged images. Counting ~ 100 DNA molecules from several images reveals that about 60-80% of the DNA molecules are associated with endosomes; for example, the image in Figure 7a shows only one non-endosome-associated DNA molecule. In contrast, after 6 h of incubation, the same samples when exposed to UV light for 10 min show that only $\sim 10\%$ of ~ 100 counted DNA molecules are co-localized with endosomes; for example, the image in Figure 7b shows all of the DNA molecules released from the endosomes following UV illumination.

Although Figure 7a does not prove that the azoTAB/SDBS vesicles are taken up into the cells by endocytosis, these images are at least consistent with this hypothesis and indicate that DNA is co-localized with (if not necessarily encapsulated within) endosomes. Regardless of whether the DNA/vesicles are encapsulated within endosomes or merely adhering to the surfaces of endosomes, UV illumination is seen to release DNA from the endosomes (from either the inside or surface of the endosome) in Figure 7. Significant photo-enhancement of the gene expression levels accompany this phenomenon, suggesting that endosome escape may be rate-limiting.

Although the transfection efficiencies were ultimately similar to that obtained with traditional PEI vector, the photoresponsive azobenzene-based carrier offers a number of potential advantages. First, phototriggered release of DNA from the endosomes is relatively rapid (i.e., within 10 min of UV illumination), which could offer benefits for the delivery of especially labile species (e.g., some RNAs that have hydrolysis half-lives on the order of 1 h or less). Furthermore, the azoTAB/SDBS system could potentially be engineered to further optimize the delivery and stability through changes in the surfactant ratio, architecture, and overall concentration.

Conclusions

Comparing the transfection efficiencies of DNA encapsulated in azoTAB/SBDS vesicles with and without UV illumination indicates that the release of DNA from the vesicle carriers and their associated endosomes appears to be the rate-limiting step. This points to the utility of using photoresponsive catanionic vesicles for the triggered release of DNA into cells. After 4 h, with the number of DNA–carrier complexes within the cells balanced by endocytosis and exocytosis, the transfection efficiencies reached maxima of around 15-20% without and 35-40% with UV-induced vesicle disruption. It is worth noting that these results were obtained using UV illumination of the entire sample compartment. With focused light used to target and rupture only internalized vesicles, substantially higher transfection efficiencies might be achieved, as the rate of exocytosis could be reduced, while non-internalized vesicles would continue to undergo endocytosis. Furthermore, strategies to deliver DNA to specific individual cells are expected to be possible and feasible by using focused light to target and illuminate cells selectively. The architectures and concentrations of photoresponsive cationic and anionic surfactant components are shown to be important to reversible formation of catanionic vesicles, their interactions with negatively charged DNA, and their interactions with the cell membranes to promote incorporation by endocytosis. By measuring and understanding the underlying physicochemical properties of the photoresponsive vesicle-DNA-cell systems, the compositions and conditions of these complicated mixtures can be selected to incorporate DNA into cells effectively, while maintaining low cytotoxicity. The identification and optimization of interactions among the various functional and cellular components yield important design criteria that are central to and expected to enable other nonviral approaches to gene delivery.

⁽⁸⁶⁾ Langner, M. Cell. Mol. Biol. Lett. 2000, 5, 295-313.

⁽⁸⁷⁾ Wasungu, L.; Hoekstra, D. J. Controlled Release 2006, 116, 255-264.

⁽⁸⁸⁾ Wiethoff, C. M.; Middaugh, C. R. J. Pharm. Sci. 2003, 92, 203–217.

Acknowledgment. This work was supported in part by the U.S. National Science Foundation under Grants DMR-0508535, CBET-0829182, and CBET-0829209 and the U.S. Army Research Office (USARO) through the UCSB Institute for Collaborative Biotechnologies. B.F.C. was a 2006 Joseph Meyerhoff Visiting Professor at the Weizmann Institute of Science, Rehovot, Israel. The cryo-TEM work was performed in the Hannah and George Krumholz Laboratory for Advanced Microscopy, part of the Technion Project on Complex Fluids, Microstructure, and Macromolecules, with partial support

from the Technion Russell Berrie Nanotechnology Institute (RBNI).

Supporting Information Available: Fluorescence emission spectra of 0.1 μ M Nile red as a function of the azoTAB concentration in azoTAB/SDBS (7:3) catanionic systems under (a) visible and (b) UV illumination and in azoTAB/SDS (7:3) solution under (c) visible and (d) UV illumination. This material is available free of charge via the Internet at http://pubs.acs.org.