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Nonviral Gene Delivery by Tetraamino Fullerene

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Abstract: A fullerene derivative bearing two diamino side chains binds to a plasmid vector DNA, either 4 or 40 kbp in size, delivers it to mammalian cells on incubation, and leads to expression of the encoded gene either transiently or stably. The initial physicochemical investigations upon DNA-binding and protective properties of various fullerene compounds against nuclease led us to identify the tetraamino fullerene as an ideal candidate to probe the new concept of fullerene-mediated gene delivery to mammalian cells. Studies on transient and stable transfection of COS-1 cells using green fluorescent protein and luciferase reporter genes revealed several useful properties of the fullerene transfection as compared with the conventional lipid-based transfection method, including much higher efficiency of stable transfection and ability to transfect confluent cells. Chemical and biological studies suggested that the cell uptake of the fullerene/DNA complex takes place by an endocytosis mechanism and that the DNA internalized by endosomes is protected by the fullerene against enzymatic digestion. The stiffness of the fullerene/DNA complex may play some role in the success of the fullerene method.

Keywords: Fullerene; stable transfection; DNA protection; endocytosis

Introduction

Delivery of an experimental DNA to mammalian cells (transfection), achieved with the aid of virus vectors or chemical (nonviral) vectors, is a fundamental tool in modern biology.[1,2] A class of popular chemical methods is the use of a cationic lipid designed to bind to the DNA, to deliver it through the cell membrane, and to allow the coded protein to be expressed in the target cells either transiently (transient transfection) or stably (stable transfection). The lipid-based transfection method is generically called lipofection, and a number of such cationic lipids (or their polymeric analogues) have been commercialized and used widely in biology laboratories (Lipofectin **1** is a mixture of two lipid molecules and a representative example of the widely used reagents).[3,4] The central concept of lipofection is to wrap the DNA with a lipid sheath taking advantage of ionic interaction between the DNA's phosphate anion and the cationic part of the lipid. The lipid sheath being structurally similar to the cell membrane, it assists the DNA molecules to pass through the membrane, but also may cause loss of DNA before reaching

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(4) Lipofectin is one of the most popular transfection reagents. Among 224 597 hits of transfection-related data in SciFinder database, lipofectin appears 1523 times and another popular polymer reagent, polyethylenimine, appears 1117 times.

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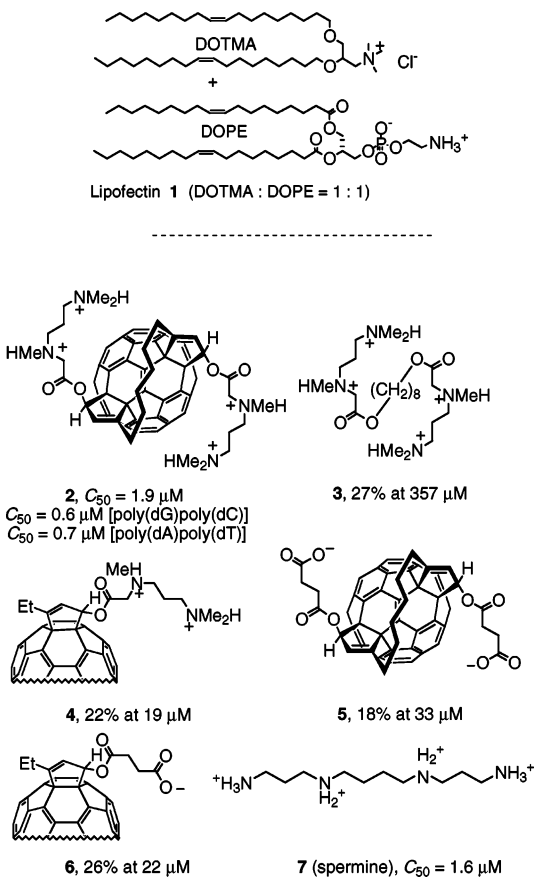

Figure 1. Lipofectin (**1**) and cationic two-handed fullerene **2** as well as reference compounds. Binding affinities (C_{50}) were measured for **2**−**7** by ethidium bromide displacement assay for calf thymus DNA (unless noted) and poly(dG)poly(dC) and poly(dA)poly(dT). For **3**−**6**, the reagent concentrations that effected maximum % displacement are shown.

the target cells through interaction with biological substances present in the medium of incubation (or in blood in the case of in vivo applications).[5]

During the course of our investigation of fullerene/DNA interactions since 1993,[6,7] an interesting observation was made: that is, organofullerene derivatives capable of tightly binding to DNA[8] cause formation of aggregates that resist elecrophoresis. Struck by this finding, we considered the possibility that a cationic fullerene such as **2** may bind to duplex DNA and deliver it to the cell nucleus. While the entire lack of structural resemblance between the lipid **1** and the fullerene **2** seemed to make such a conjecture useless, we expended some effort to synthesize **11**, which possesses two diamine side chains separated by 1.2 nm from each other, a distance complementary to that of the separation of the two phosphate backbones flanking the DNA major groove. The amine groups will be protonated in a pH 7 buffer to form in situ the cationic fullerene **2**, and interact with the parallel phosphate backbones. We found and communicated[9] that the cationic fullerene **2** binds to duplex DNA, delivers the fullerene/DNA complex into cytoplasm, and allows the DNA to be expressed transiently with an efficiency comparable to that achieved by a conventional lipofection reagent such as **1**. We subsequently found that the fullerene-mediated stable transfection is over an order of magnitude more efficient than that achieved by **1**. Supporting chemical and biochemical studies on the mechanism of the transfection suggested that the fullerene reagent forms a protective sheath around the DNA, makes longer the lifetime of the DNA in the endosome, and, as a result, increases the chances of chromosomal incorporation of the experimental DNA. The better efficiency of the fullerene-mediated gene delivery may also be related to the stiffness[10] and the cell adhesion property.[11] In the present article, we describe the full account of the synthesis of **2**, the DNA-binding and protecting properties, the transient and stable transfection capability, and the mechanism of the fullerene/DNA transport through the cell membrane.

Results and Discussion

Synthesis of Tetraamino Fullerene. We conceived an idea of the use of fullerene for transfection when we recognized the ability of a conjugate of fullerene and a 14-mer DNA sequence[8] to cause aggregation of DNA. Since this molecule is too complex to synthesize, we looked for alternatives that would be easier to synthesize but would still show a good DNA-binding ability. Without prior knowledge on the DNA-binding properties of fullerenes, we synthesized the amphiphilic fullerene **2** on the basis of the assumed structural complementality of the side chain to the phosphate backbone, a conjecture still requiring experimental verification.

To obtain the target molecule **11** that will be protonated in neutral water to form **2**, we designed a series of doubly

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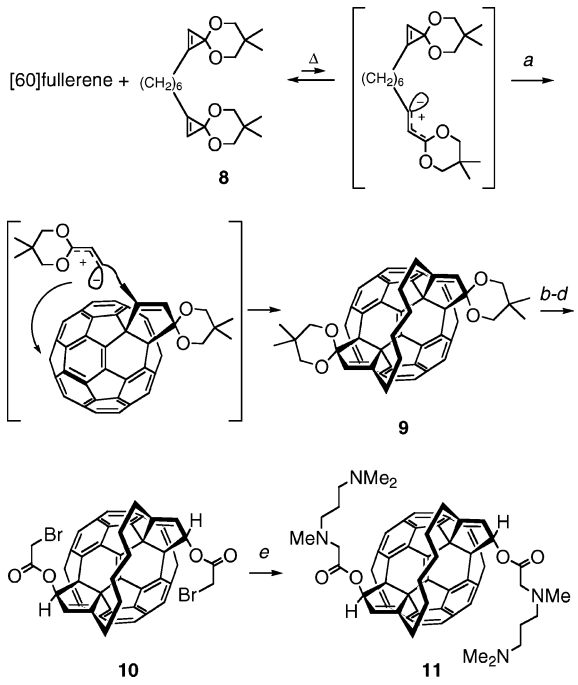
Scheme 1. Synthesis of C_2-Symmetric Fullerene **11** by Double Cycloaddition of a Vinylcarbene Intermediate Generated by Thermolysis of Cyclopropenonone Acetal[a]



[a] (a) 1,2-$Cl_2C_6H_4$, 150 °C, 3 days; (b) cat. H_2SO_4, H_2O/THF/PhCl, 50 °C, 10 h, 31% (2 steps); (c) DIBAL-H, PhCl, rt, 2 h; (d) bromoacetyl bromide, pyridine, PhCl, rt, 6 h, 50% (2 steps); (e) $HMeN(CH_2)_3NMe_2$, PhCl, rt, 1 h, 50%.

functional cyclopropene annulating agents and studied their regioselectivity of addition to [60]fullerene, and we found that a six-methylene compound **8** undergoes double [3 + 2] cycloaddition to give the bis-acetal **9** with complete regiocontrol (Scheme 1).[12] The regiocontrol crucially depends on the length of the methylene tether connecting the two cyclopropene units as revealed by theoretical and experimental studies. The acetal groups in **9** were deprotected, reduced, and converted to bis-bromoacetate **10**.[13] The bromide groups were displaced by two molecules of *N*,*N*,*N*′-trimethyl-1,3-diaminopropane to afford the two-handed fullerene **11** with an overall five-step yield of 8%. The other reference compounds **4**−**6** were synthesized in a similar manner.

DNA-Binding, Aggregation, and Protection Properties of 2. For a reagent to be useful for transfection, it needs to bind to DNA, to convert it to a certain form that can pass through the cell membrane, to protect it from enzymatic degradation, and to release it for delivery to the protein-producing machinery. To examine these issues, we first investigated physicochemical behavior of the tetracationic fullerene **2** toward interaction with short duplex DNAs and found that it binds to the DNAs, causes aggregation, and protects them against nucleases.

The DNA-binding property of **2** was examined first. We also studied the reference compounds **3**−**6**, which lack some important features present in **2**. The tetramine **3** lacks the fullerene core, and the one-handed diamine **4** has only one side chain. The dicarboxylic acid **5** has two carboxylic acid side chains to be charged negatively in a neutral buffer solution. The one-handed monocarboxylic acid **6** belongs to the class of compounds for which we previously reported the photodriven DNA nicking activity and HIV protease inhibition.[6,14] The affinity of these molecules to DNA duplexes was probed by several sets of experiments performed on calf thymus DNA and linear DNA fragments. The experiments were performed for a range of a reagent-to-base-pair (bp) ratio (R)[15] between 2.5×10^{-4} and 2.6. Spermine (**7**) was also examined as a DNA-binding reference compound.

The binding assay was performed by the standard ethidium bromide displacement assay[16] that showed a very clear structure/activity relationship as summarized in Figure 1. The two-handed fullerene **2** showed a C_{50} value as small as 1.9 μM (the smaller, the better binding). This value rivals the data of spermine (**7**; $C_{50} = 1.6$ μM) determined under the same conditions. The C_{50} values for poly(dG)poly(dC) and poly(dA)poly(dT) were equally small with 0.6 and 0.7 μM, respectively, indicating that **2** binds to DNA without recognition of specific bases. In contrast to **2**, other reference compounds **3**−**6** are unable to achieve 50% displacement even by addition of a large excess of the reagent.

The tetracationic fullerene **2** was found to cause sudden phase separation (e.g., precipitate formation) of a DNA solution as the R value was increased from 0.5 to 2.0 (Figure 2). No such phase separation took place with **3**−**7** even at a high R range (up to 2.6). For instance, experiments using a mixture of ^{32}P-labeled linear DNA fragments of 214 bp to 1418 bp size (lane 1) showed that addition of **2** ($R = 2.0$) converted DNA to material that stays at the origin of the electrophoresis gel (lane 2). The aggregation is reversible, and, when the mixture was extracted with chloroform in which **2** dissolves well, the duplex DNA was released into the aqueous phase without change of the ratio of the DNA molecules (lane 3). Similarly, **2** causes condensation of plasmid DNA intramolecularly and intermolecularly depending on the R value as reported elsewhere.[17] The comparison between **2** and **3**−**7** thus indicated that **2** condenses duplex DNA through synergistic effects of the large hydrophobic

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(15) The reagent-to-bp ratio (R) can be converted to the nitrogen-to-phosphorus (N/P) ratio by multiplying the R value by two.

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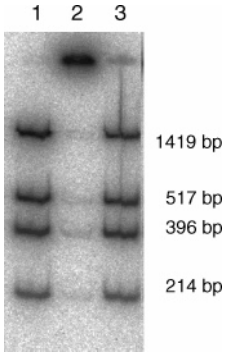

Figure 2. Formation of electrophoresis-resistant material caused by complexation of **2** with ^{32}P-labeled linear DNAs at $R = 2.0$. Addition of **2** to four linear DNAs of different size (control, lane 1) forms immobile material (lane 2). Chloroform extraction of **2** before loading to gel liberates soluble DNAs without change of their relative amounts (lane 3). Electrophoresis was performed on polyacrylamide gel in TBE buffer, and DNA was visualized and analyzed by imaging plate scanning.

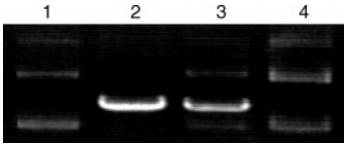

Figure 3. Nicking of plasmid DNA by endonuclease *Pst*I (1 h at 35 °C) in the presence and the absence of a transfection reagent. The test DNA contains three forms, catenated form (16%, the ratio relative to the total DNA on the gel), open circular form (34%), and supercoiled form (50%). Lane 1: Intact plasmid DNA containing no linear form. Lane 2: Plasmid DNA incubated with *Pst*I (all the plasmid in the linear form). Lane 3: Plasmid DNA incubated with *Pst*I in the presence of Lipofectin (catenated 9%, open circular 18%, linear 56%, and supercoiled 17%). Lane 4: Plasmid DNA incubated with *Pst*I in the presence of fullerene **2** (catenated 25%, open circular 38%, linear 0%, and supercoiled 37%).

core and the side chains that bear a suitable number of ammonium cations.

The cell-based experiments and long-term gene expression described in the following sections suggested that the DNA molecules encapsulated in the fullerene sheath resist enzymatic degradation. We performed chemical experiments to probe this hypothesis by allowing an endonuclease, *Pst*I, to attack a plasmid DNA composed of three forms (Figure 3, lane 1) either in the absence or in the presence of **2** and Lipofectin in Tris-buffered saline. In the control experiment, the enzyme cuts the plasmid DNA into a linear form after 1 h reaction time (lane 2). When the DNA was complexed with Lipofectin, the strand cleavage slowed and the conversion to the linear form occurred to the extent of less than 60% (lane 3). Finally, complexation with **2** resulted in complete blockage of the hydrolysis and gave back the DNA without change of the ratio of the three forms (lane 4).

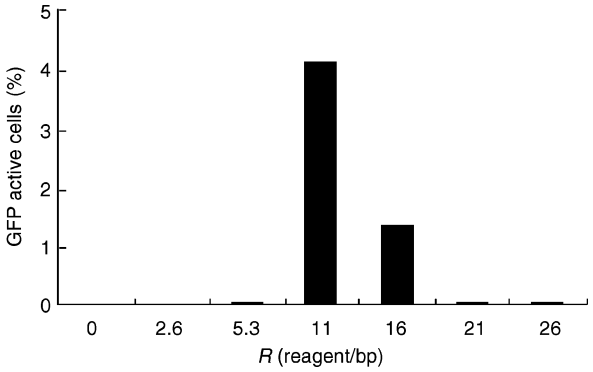

Figure 4. Dependency of transfection efficiency on the reagent/bp ratio (R) as examined for the GFP vector (COS-1 $= 1 \times 10^5$, DNA (pGreenLANTERN-1) $= 4$ μg, transfection time $= 6$ h, incubation time $= 2$ days). Effective transfection takes place only for a narrow range of the R value. Due to toxicity of DMF used to dissolve **2** in an aqueous solution, cell viability relative to the control experiment at $R > 21$ became so small (1−3%) that the data for $R > 21$ are rather unreliable.

Transient Transfection of Mammalian Cells. The physicochemical studies indicated that the tetraamino fullerene **2** has all properties necessary for a transfection reagent, while others (**4**−**6**) do not. In the first screening stage where we obtained qualitative data for a wide range of experimental parameters, we employed a 4 kbp plasmid DNA (pGreen LANTERN-1) that contains a green fluorescent protein (GFP) reporter gene that allows us to detect the gene expression with high sensitivity. We then reoptimized the conditions by using a luciferase reporter gene (pGL3-Control) suitable for quantitative determination of the efficiency. In this second stage, we carefully compared the fullerene vector **2** with that of the conventional lipid vector, Lipofectin.

In the first set of experiments, the R-dependency of the transfection efficiency was examined. The transfection efficiency is defined as the number of fluorescent GFP active cells relative to the total cells on the plate (see Experimental Section for details). We first fixed the time (denoted as transfection time) spent for initial treatment of the cells with the **2**/DNA complex in a buffer solution (i.e., without serum) to be 6 h, and the time (incubation time) to allow the cells to grow in the presence of serum (fetal bovine serum, FBS) to be 2 days. The efficiency increased toward $R = 11$ and dropped afterward (Figure 4). With the R value fixed at 11, the efficiency reached the maximum value of 4% by the use of 4 μg of DNA (Figure 5). The cell viability relative to the control experiment at the optimum transfection condition was 26%. The cell death was caused largely by DMF to dissolve **1** in aqueous solution.

The transfection and incubation times were then varied (Figures 6 and 7). The efficiency of the transfection increased

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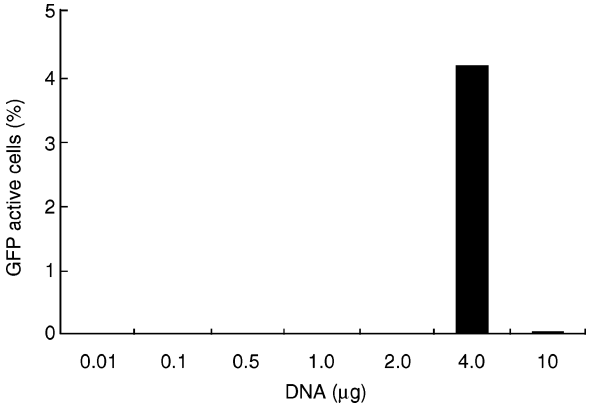

Figure 5. Dependency of transfection efficiency on the amount of the GFP vector (COS-1 = 1 × 10^5, R = 11, transfection time = 6 h, incubation time 2 days).

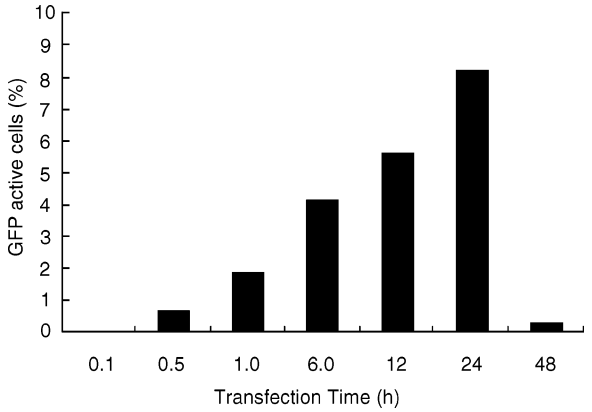

Figure 6. Dependency of transfection efficiency on the transfection time as examined for the GFP vector (COS-1 = 1 × 10^5, R = 11, DNA (pGreenLANTERN-1) = 4 μg, incubation time 2 days).

as the transfection time is made longer up to 24 h and drops after 48 h, when most cells are dead after standing for many hours in a buffer without serum. This continued increase of efficiency is interesting, since the cell viability drops during transfection as expected (data not shown). The incubation time dependency was also quite interesting. The amount of the expressed GFP continuously increased for 5 days, and, even after 12 days, some cells continued to produce the protein.[18] We suspect that such a long-term 12 day expression is partly due to the formation of stable transfectants (vide infra).

We found that the cytotoxicity of the fullerene **2** is negligible either in the presence or in the absence of ambient light. Thus, essentially the same results (transfection efficiency and cell viability) were obtained in the experiments performed under black light and ambient light. A separate tetrazolium assay[19] indicated that the cytotoxicity of **2** in DMF (1.8% v/v) or DMSO (2.5% v/v) was largely due to

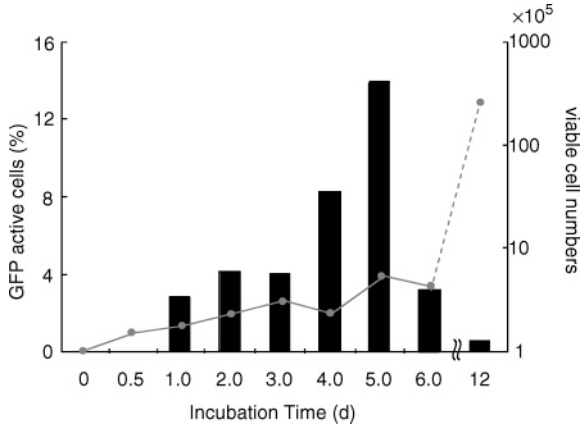

Figure 7. Dependency of transfection efficiency as examined for the GFP vector (left vertical axis, bar graph) and the number of viable cells (right vertical axis, line graph) on the incubation time (COS-1 = 1 × 10^5, R = 11, DNA (pGreenLANTERN-1) = 4 μg, transfection time 6 h). After 4 days of incubation, the cells were approximately 100% confluent and replated in a 35 mm plate and incubated again in 10% FBS/DMEM (this operation caused deviation from exponential cell growth).

the toxicity of the solvent. Further toxicological studies of **2** will be a subject of future study.

It has been reported that the lipid-mediated transfection is less efficient when the initial cell treatment is performed in the presence of serum than when it is done without serum.[5] For instance, when the COS-1 cells are treated with DNA/Lipofectin in the presence of 10% FBS, the efficiency was reduced to half (Figure 8, bar c vs d; 2.2 × 10^8 to 1.2 × 10^8 relative light unit (RLU)/mg of protein). The adverse effect of serum has been ascribed to the disturbance of the structural integrity of the lipid/DNA complex by lipophilic biomaterials in the serum.[1,5,19] In contrast, serum was found to increase the efficiency of the fullerene-mediated transfection. Thus, the presence of 10% FBS increases 7.8 times the efficiency of the transfection with **1** (bar b vs a; 5.0 × 10^9 vs 6.4× 10^8 RLU/mg of protein), making it 2.5 times more effective than the lipofection experiment.

A more subtle medium effect was found (Figure 8). The efficiency also depends on the buffer used for complexation of the fullerene **2** with the plasmid DNA. The efficiency was the highest when TBS was used (bar b) and became lower when HEPES-buffered saline (HBS; 72% of TBS, bar e), phosphate-buffered saline (PBS; 48%, bar f), and Dulbecco's modified Eagle's medium (DMEM; 15%, bar g) alone were used. The effect of the serum and the buffer appears to be related to the particle morphology as discussed later.

Another interesting feature of fullerene-mediated transfection is its ability to transfect cells (COS-1) of 100% confluency, at a level (6.6 × 10^8 RLU/mg of protein) twice better than the Lipofectin data (3.4 × 10^8 RLU/mg of

(18) Our vector plasmid (pGreen LANTERN-1) does not contain an SV40 origin for replication, and the long GFP-expression is not due to the ability of COS-1 cells to produce the SV40 large tumor antigen. Gluzman, Y. *Cell* **1981**, *23*, 175−182.

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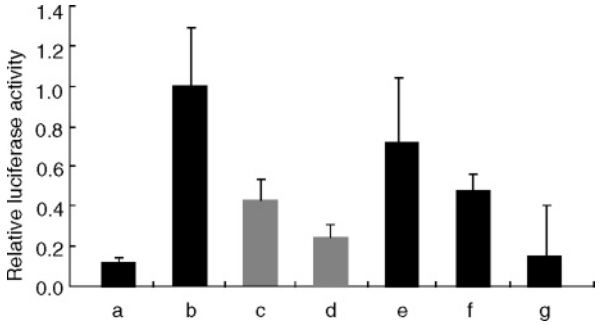

Figure 8. Effects of serum and buffer on the fullerene transfection as examined for the luciferase vector (COS-1 = 1×10^5, $R = 11$, DNA (pGL3-Control) = 4 μg, incubation time 2 days). Transient transfection by fullerene **1** (black) or Lipofectin (gray). (a) Cells were transfected in DMEM without FBS using DNA/fullerene complex prepared in TBS. (b) Cells were transfected in 10% FBS/DMEM using DNA/fullerene complex prepared in TBS. (c) Cells were transfected in DMEM without FBS using DNA/Lipofectin complex. (d) Cells were transfected in 10% FBS/DMEM using DNA/Lipofectin complex. (e) Cells were transfected in 10% FBS/DMEM using DNA/fullerene complex prepared in HBS. (f) Cells were transfected in 10% FBS/DMEM using DNA/fullerene complex prepared in PBS. (g) Cells were transfected in 10% FBS/DMEM using DNA/fullerene complex prepared in DMEM. Luciferase activities were measured in relative light units (RLU) per mg of total protein and shown relative to the standard data ($b = 3-7 \times 10^9$ RLU/mg of protein) obtained in the presence of serum.

protein). The reason for this favorable result is unclear at this time. Brief investigation of cell lines other than COS-1 showed that the applicability of the fullerene transfection roughly parallels that of Lipofectin. The following luciferase activity data (unoptimized) were obtained: 1.9×10^8 RLU/mg of protein with CHO, 2.5×10^7 RLU/mg of protein with BHK-21, 2.0×10^6 RLU/mg of protein with HeLa, 1.2×10^6 RLU/mg of protein with NIH3T3,[20] and 4.4×10^5 RLU/mg of protein with NRK.

Stable Transfection of Mammalian Cells with Short and Long Vectors. Production of stable transformants through chromosomal incorporation of extracellular gene is not an easy task for chemical transfection methods.[1,2,21] The foregoing information (e.g., nuclease resistance, favorable serum effect, and long-term GFP production) suggested that the fullerene transfection may be useful for stable transfection, and we found that it is indeed the case. We used a plasmid DNA (pHygEGFP) carrying a gene for production of hygromycin B phosphotransferase so that the transfected cells acquire resistance to the antibiotic hygromycin B. COS-1 cells were transfected by the serum-containing protocol and grown for 2 weeks under the antibiotic selection. The number of surviving cells/control without anitibiotic

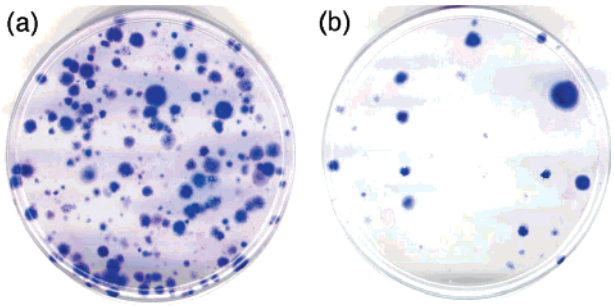

Figure 9. Colonies of stable transformants (COS-1) after two weeks of antibiotic selection. (a) Cells transfected with pHygEGFP by fullerene **2**. The transfection was carried out in DMEM containing 10% FBS, and the efficiency of stable transformation (percent of the average number of surviving colonies against the average number of colonies that grew in nonselective growth medium) was 0.73%. The same experiment but in the absence of the serum at the time of transfection showed an efficiency of 0.08%. (b) Cells transfected with pHygEGFP by Lipofectin in the presence of the serum in DMEM. The efficiency of stable transformation was 0.039%. The same experiment in the absence of serum showed an efficiency of 0.10%.

selection were compared with the Lipofectin reference. As shown in Figure 9, we observed much better efficiency in fullerene-mediated transfection (0.73%) than that in lipofection (0.039%). The favorable effects of the serum during fullerene transfection as well as its adverse influence in lipofection were observed here too in the stable transfection experiments (Figure 9 caption).

The general merit of chemical vectors is their ability to deliver long DNA sequences, which cannot be achieved by virus vectors because of the small size of the virus capsid. We found that the fullerene method is useful also for a 40 kbp plasmid DNA. The vector was prepared by inserting a GFP gene into the multiple cloning site of ZAP Express vector and contained both a GFP reporter gene and a resistant gene for an antibiotic, G418. The transient expression of the GFP gene in COS-1 cells was observed by the fluorescent microscope analysis, and the formation of stable transformants was detected by the selective growth over 2 weeks. The efficiency of the GFP expression was qualitatively comparable to that of the fullerene transfection of the 4 kbp plasmid DNA (data not shown). The level of the stable transfection (2.2×10^{-3}% against nonselection control) was lower than that with the shorter vector, but is suitable for further consideration.[22]

Transport through Cell Membrane: Microscopic and Biochemical Analyses. Unlike other vectors, the fullerene **2** is dark brown in color and therefore can be easily detected by optical microscope. Microscopic analysis of the **2**/DNA complex in an incubation medium suggested what could be a reason for the favorable effect of serum (FBS) in the transfection. Figure 10a shows the **2**/DNA aggregates

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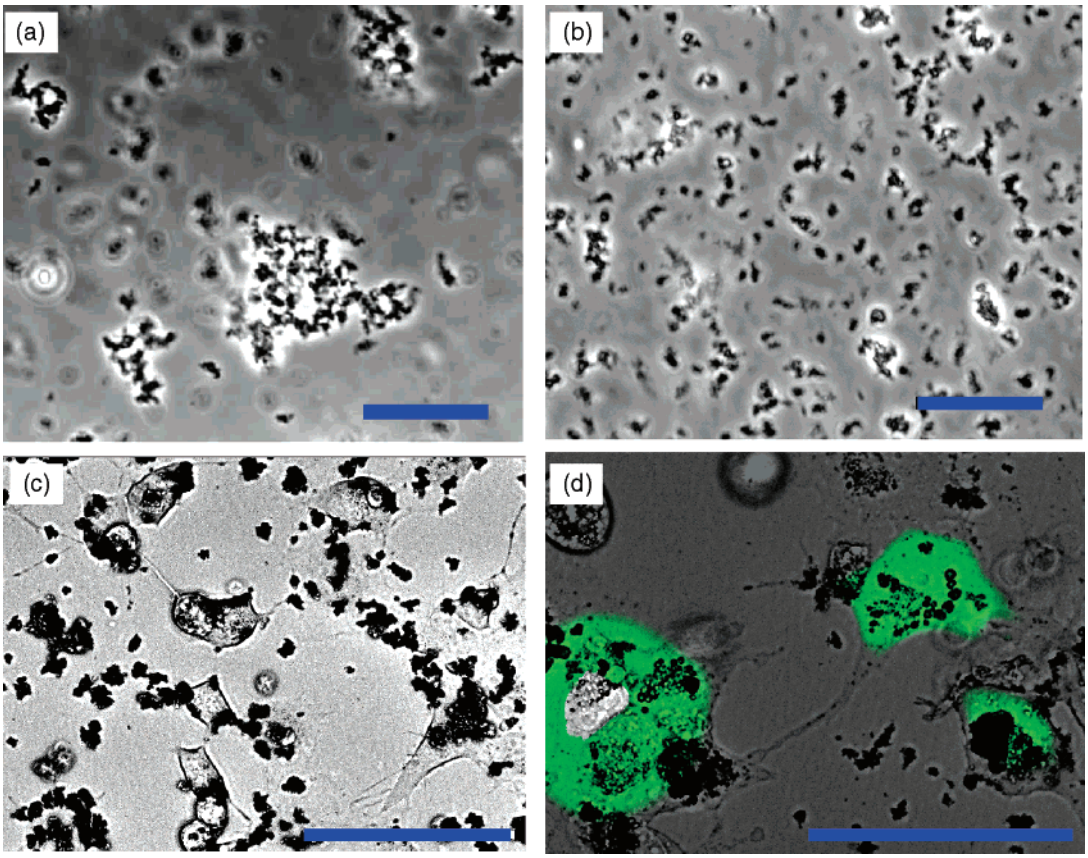

Figure 10. Optical micrographic analysis of fullerene transfection. (a) Fullerene/DNA aggregates (black colored) in DMEM without FBS where the aggregates form clusters. (b) Fullerene/DNA aggregates in 10% FBS in DMEM where the aggregates are uniformly dispersed. (c) COS-1 cells and fullerene/DNA aggregates after 1 h transfection ($R = 11$, DNA amount = 4 μg). (d) COS-1 cells after 2 day incubation with fullerene/DNA aggregates in FBS/DMEM (6 h transfection time with 4 μg of GFP vector at $R = 11$). Superimposed photograph of a differential interference contrast micrograph (shown in gray) and fluorescence micrograph (shown in green; excitation = 450−490 nm, observation = 515−565 nm). Round black lumps of 0.5−3 μm observed in the fluorescent cells (e.g., in the center right) are endosomes containing **2**/DNA complex. Scale bars show 200 μm (a and b) and 100 μm (c and d).

prepared in a DMEM buffer, and Figure 10b shows the **2**/DNA aggregates prepared in a mixture of DMEM and FBS. In both pictures, the black particles are a few tens of micrometers in size and show irregular outlines, suggesting that these aggregates are composed of even smaller pieces that have been identified previously by atomic force microscope.[17] However, in Figure 10a, the aggregates gather together to form large clusters of several hundred micrometers, while they are uniformly dispersed in Figure 10b. It has been reported that a phagocytosis mechanism of uptake of extracellular materials (the fullerene transfection occurs by such a mechanism, vide infra) operates most efficiently for particles of a submicrometer size than for larger ones.[23] The tendency of the **2**/DNA complex in FBS/DMEM to form a smaller aggregate is certainly consistent with the higher transfection efficiency in this medium. The presence of nutritious serum during transfection is obviously desirable for cell growth and for active intake of the fullerene/DNA complex.[24] In addition, the size and morphology of the particles did not show any apparent change at all during the observation with a microscope (for a period of a few hours), indicating stiffness and rigidity of the fullerene/DNA aggregates.[25]

The micrograph taken 1 h after transfection in FBS/DMEM (Figure 10c) shows a largely empty plate surface, several cells, and the fullerene/DNA aggregates. The aggregates look similar to those in Figure 10b, and some appear to be sticking to the cell surface. Figure 10d is a superimposition of a fluorescence microscopic image and the normal image of the cells after 2 day incubation. The black lumps 2−10 μm

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(24) It is also reported for polymer-mediated transfection that the effect of serum on transfection may involve factors other than size effect. See for instance: Cherng, J.-Y.; van de Wetering, P.; Talsma, H.; Crommelin, D. J. A.; Hennink, W. E. *Pharm. Res.* **1996**, *13*, 1038−1042.

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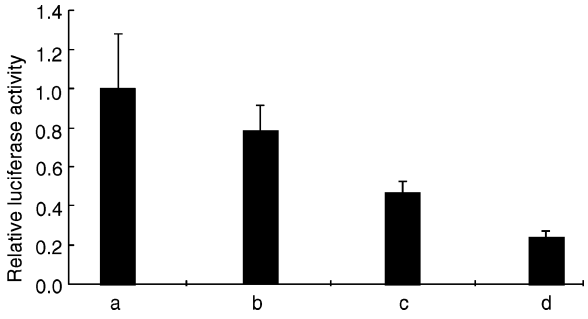

Figure 11. Reduction of transfection efficiency by cytochalasin B, as examined for the luciferase vector. (a) Transient transfection in the absence of cytochalasin B (COS-1 = 1 × 10^5, R = 11, DNA (pGL3-Control) = 4 μg, incubation time 2 days). (b−d) Transient transfection in the presence of cytochalasin B: (b) 3.3 μM, (c) 10 μM, (d) 33 μM. Luciferase activities were measured in relative light units (RLU) per mg of total protein and shown relative to the standard data [(a) 3−7 × 10^9 RLU/mg protein)] obtained in the absence of the inhibitor.

in diameter are seen in the cytoplasm of the fluorescent cells. The smooth surface of the aggregates in the GFP-expressing cells suggests that they are located inside of the cells as endosomes rather than sticking to the surface (vide infra).

Endocyctosis is a probable mechanism of the internalization of the aggregates in the form of endosomes (Figure 10d).[26,27] If it is true, it should be inhibited by cytochalasin B,[28] which is known to block endocytosis by inducing depolymerization of actin cytoskeleton.[29] In support of this conjecture, cytochalasin B caused a dose-dependent decrease of the efficiency of transient transfection from 100% (reference) to 73%, 50%, and 25% in the presence of 3.3, 10, and 33 μM cytochalasin B, respectively (Figure 11, bars b−d).

Several lines of information in the foregoing paragraphs indicated that the lifetime of the DNA delivered by **2** into the cytoplasm is quite long, longer than that delivered by Lipofectin. The chemical and cell experiments being taken together, we consider that the fullerene **2** retards lysosomal degradation of the complexed DNA in the endosomes. To examine if it is the case, we studied the effect of chloroquine on the transfection efficiency with **2**. Chloroquine retards lysosomal digestion of DNA in the Lipofectin/DNA complex,[1,30] and hence addition of chloroquine increases the Lipofectin-mediated transfection efficiency by 20 times (Supporting Information). This level of the efficiency was achieved by the fullerene **2** already without chloroquine, and chloroquine did not improve the efficiency. The observation indicates that the lysosomal digestion is not a major issue in the fullerene-mediated transfection, an observation consistent with an assumption that the fullerene protects the DNA in the endosomes.

Conclusion

We reported in 1993 that water-soluble fullerene exhibits a sign of interaction with DNA in water, and in 1995 that a fullerene derivative possessing a base-recognition DNA side chain undergoes base-specific binding. In the following years, studies on the structure/activity relationship,[31] on the mechanism of DNA photocleavage,[32] and on the applications to photodynamic therapy[33] as well as the (lack of) acute toxicity and the pharmacokinetics[34] were reported. On the basis of such information, we conceived an idea of developing a DNA-delivery reagent.

Departing from the conventional concept of lipid-mimicking strategy for designing transfection reagents, we have synthesized a new class of transfection agent **11** (**2** is its protonated form) through tailor-made functionalization of [60]fullerene. The preliminary structure/activity relation study on the DNA-binding properties indicated that structural synergy in **2** plays important roles in binding: One structural feature that appears to be required for the DNA-binding fullerene is spatially separated multiple ammonium cation centers. The transfection using **2** was effective with mammalian cells, either dividing or confluent. Unlike Lipofectin, **2** is more effective for transfection in the presence of serum than in its absence. We consider that the serum is necessary

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not only for cell growth and the DNA uptake but also for making finely dispersed fullerene/DNA aggregates that are suitable for effective cell uptake. The fullerene method was found to be quite effective for transfecting confluent cells, and to achieve stable transfection with efficiency 1 order of magnitude higher than that obtained by Lipofectin. The results appear to owe much to the prolonged lifetime of the delivered DNA in the cytoplasm, and also to the stiffness of the DNA aggregates, which has recently been suggested to improve the cell adhesion properties and hence to improve transfection efficiency.[11]

The cell uptake occurs by the endocytosis mechanism, and the fullerene/DNA is internalized in the form of endosomes as supported by microscopic observation. Further comparison of the fullerene approach with the recently reported nanotube approach would be interesting.[35] The chemical and biological experiments suggest that the fullerene **2** protects the bound DNA from lysosomal degradation in the cell. We consider that this ability is related to the propensity of water soluble fullerene to form well-defined self-assembly in water.[10] It is highly likely that the phosphate backbone of DNA can act as a template for self-organization along the duplex DNA.[17,36]

If protected against enzymes in the endosome, when will the bound DNA be made available for the biological machinery of protein expression? One idea yet to be proved is that the cation-bearing ester linkage in **2** is slowly cleaved either chemically or biologically to release the DNA from the fullerene core. Verification of this idea needs a thorough structure/activity relation study on a library of fullerene derivatives. In this sense, the drawback of the prototype **2** is its structural complexity. Our effort to gain easy access to such compounds was recently rewarded by the discovery of a multigram scale synthesis of aminated fullerenes,[37] which will allow us to perform the structure/activity relation study. With the advent of basic understanding of the biology of carbon cluster/cell interaction as well as the development of supporting synthetic methodology, we expect that the concept of carbon cluster mediated drug and biomolecule delivery will prove to be generally useful in biology and medicinal applications.

Experimental Section

Materials. Lipofectin was purchased from Invitrogen, plasmid DNA pGL3-Control from Promega, plasmid DNA pHygEGFP from Clontech, ZAP-Express vector from Stratagene, fetal bovine serum (FBS) from Equitech-Bio, endonuclease *Pst*I and luciferase assay kit (including a picagene reagent) from Toyo Ink, reagents for Bradford assay from Bio Rad, and dimethyl formamide (DMF) from Kanto Kagaku. Other chemicals were purchased from Sigma. Plasmid DNA (pGL3-Control and pHygEGFP) was amplified from *Escherichia coli* and purified on a CsCl gradient.[38] Final concentration of plasmid was determined by absorbance at 260 nm. COS-1 cells (simian virus 40-transformed kidney cells of African green monkey) were purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS in a humidified atmosphere at 35 °C with 5% CO_2.

Synthesis of Two-Handed Fullerene. Two-handed fullerene **2** was prepared by the following synthetic sequence: regioselective double [3 + 2] cycloaddition reaction with [60]fullerene,[12] deprotection, reduction,[13] bromoacetylation, and amination. The synthetic procedures up to the reduction to diol have been reported previously, and the synthetic intermediate was prepared accordingly.[12,13] The crude diol was then converted to bromoacetate **10** by the reaction with bromoacetyl bromide. Thus, to a solution of diol (50.0 mg, 54.7 μmol) in chlorobenzene (50 mL) were added bromoacetyl bromide (23.7 μL, 274 μmol) and pyridine (22.1 μL, 274 μmol). The reaction was terminated after 6 h by addition of sodium bicarbonate. Aqueous extractive workup gave a crude product. Purification was performed by silica gel column chromatography to afford bromoacetate **10** (31.6 mg, 50% in 2 steps). Bromoacetate **10** was transformed to **11** by substitution reaction with trimethyldiaminopropane. Thus, to a degassed solution of dibromide **10** (23.1 mg, 20.0 μmol) in chlorobenzene (10 mL) was added *N*,*N*,*N*′-trimethyl-1,3-propanediamine (14.7 μL, 100 μmol), and the mixture was stirred for 1 h at ambient temperature. Aqueous extractive workup gave a crude product. Purification was performed by gel permeation chromatography (JAIGEL-1H 20 × 600 mm and JAIGEL-2H 20 × 600 mm GPC columns, 0.5% triethylamine/chloroform elution) to afford **11** (12.2 mg, 50%). Other derivatives (**4**−**6**) have been synthesized in a similar manner. All compounds are racemic. Two-handed fullerene **11**: R_f = 0.05 ($CHCl_3$/MeOH/AcOH, 85/10/5); IR (KBr) 2925, 2854, 1732, 1458, 1153, 750 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.40−1.54 (overlapped m, 8 H,), 1.50−1.75 (br m, 2 H), 1.75−2.00 (br m, 2 H), 2.08 (overlapped s, 16 H), 2.20 (s, 6 H), 2.36 (t, 4 H, J = 7.4 Hz), 2.60−2.74 (br m, 4 H), 3.15 (d, 2 H, J = 17.2 Hz), 3.28 (d, 2 H, J = 17.2 Hz), 6.08 (br s, 2 H), 7.35 (br s, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.53, 25.96, 26.91, 29.29, 42.09, 45.55, 54.63, 57.55, 58.30, 73.28, 74.08, 86.68, 125.86, 127.24, 132.76, 136.34, 136.40, 138.19, 139.49, 141.80, 141.83, 141.86, 142.08, 143.17, 144.65, 144.73, 144.97, 145.20, 145.44, 145.46, 145.65, 145.96, 145.99, 146.29, 147.52, 147.82, 148.71, 148.91, 148.96, 150.23, 151.21, 155.30, 170.68; FAB MS calcd for $C_{88}H_{50}N_4O_4$ (MH^+) m/z = 1227.39, found m/z = 1227.38.

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Ethidium Displacement Assay (C_{50}). We assayed the DNA-binding ability of **2**−**7** by displacement of ethidium bromide intercalated into calf thymus DNA.[16] The reagent concentrations to reduce the fluorescence intensity at 595 nm to 50% of the fully intercalated DNA is defined as C_{50} value. The displacement assay was carried out in a buffer containing 2 mM HEPES, 10 μM EDTA, and 9.4 mM NaCl (pH 7.0). Ethidium bromide was dissolved (1.26 μM) in the buffer, and a solution of DNA [calf thymus DNA (Sigma), poly(dG)poly(dC) or poly(dA)poly(dT) (Pharmacia)] was added to provide a base pair concentration of 1.26 μM. A solution of the reagent (0.1−1.0 mM) was added in microliter portions. Fluorescence emission was measured at 595 nm (slit width 0.75 nm) with excitation at 546 nm on a Hitachi 650−40 spectrometer at 25 °C.

Precipitation of Linear DNA with Two-Handed Fullerene. A mixture of 5′-end labeled linear DNAs were prepared by digestion of plasmid pUC18 with *Hin*fI. The linear DNAs were labeled at the 5′-end using γ-^{32}P-ATP and T4 kinase. The mixture was incubated with **2** at *R* of 2 at 37 °C for 1 h. Gel electrophoresis was performed with 6% polyacrylamide gel and analyzed by imaging plate scanning.

Transient Transfection Experiments. Cultured cells were transfected either with a plasmid pGreenLANTERN-1 (a GFP reporter gene) or with a plasmid pGL3-Control (a firefly luciferase reporter gene).[19] Thus, approximately 2×10^4 cells were plated on a 24-well plate 24 h prior to transfection. The cells were to reach 25% confluency at the time of transfection and were washed with DMEM before the transfection. For each well, 0.8 μg of plasmid DNA was used. Fullerene **2** in DMF (6.4 μL, 2 mM) and plasmid DNA in Tris buffered saline (TBS) (320 μL, 2.5 μg/mL) were mixed at the molar ratio (fullerene **2**/DNA base pair) of 11, and the mixture was kept at room temperature for 30 min to give a solution of the fullerene−DNA complex. In some cases the buffer solution for **2** was changed to HBS, PBS, or DMEM. The solution of fullerene−DNA complex was diluted with medium (326 μL; with or without 10% FBS), and added to the cells in a well. The morphologies of the fullerene/DNA aggregate in the solution were analyzed with an optical microscope (Keyence, VH-8000) for the complex solution in the medium without cells. The cells were incubated in a humidified 5% CO_2 incubator at 35 °C with the transfection mixture for various time periods (transfection time). After the transfection period was over, the transfection medium was removed. Then 10% FBS/DMEM was added and the cells were incubated further at 35 °C for various time periods (incubation time). In experiments performed over 4 days, one-fourth of the cells were replated in a 35 mm plate after 4 day and 8 day incubation. Lipofectin was used as a reference transfection reagent, and lipofection was carried out following the manufacturer's procedure.[39]

Analysis of Transfection Efficiency Using a GFP Reporter Gene. When a plasmid pGreen LANTERN-1 containing a GFP reporter gene was transfected, the transfection efficiency was measured as the number of fluorescent GFP active cells relative to the total cells on the plate.[19] Both fluorescent and nonfluorescent cells were scored in 10 representative fields at 32× magnification using a Zeiss AIS microscope equipped with 50 W Hg lamp and filter set (excitation filter, 450−490 nm; emission filter, 515−565 nm). Cells were counted under phase-contrast microscopy, and the exponential cell growth was noted in the presence of **1**. The micrographs of the cells were taken with an Olympus AX70 microscope equipped with a 100 W Hg lamp, a filter set, and a CCD camera.

Analysis of Transfection Efficiency Using a Luciferase Reporter Gene. When a plasmid pGL3-Control containing a firefly luciferase reporter gene was transfected, the transfection efficiency was measured as the luciferase activity per mg of protein.[19,40] The transfected cells in each well were lysed with lysis buffer (25 mM Tris-HCl, 2 mM dithiothreitol, 10% glycerol, 0.1% Nonidet 40). Lysate was mixed with a picagene reagent containing luciferin, and the photon count of the lysate was measured using a luminometer (Lumat LB9507) to determine the luciferase activity in relative light units (RLU). The amount of total protein in the lysate was independently estimated by Bradford assay using Coomassie Brilliant Blue G-250,[41] and RLU per mg of protein were calculated. The experiments were carried out at least in duplicate, and the standard deviation from the mean value is shown as an error bar.

Digestion of Plasmid DNA with Endonuclease. In each digestion experiment, 80 ng of plasmid DNA containing supercoiled, open circular, and catenated forms was used.[42] The complexes of DNA with fullerene **2** (added as its neutral form) and Lipofectin were prepared as described above for the transient transfection. Intact plasmid DNA and the complexes with fullerene and Lipofectin were treated with 1 unit of restriction enzyme *Pst*I in 20 μL of buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 mM NaCl, pH 7.5) at 35 °C, respectively.[43] After 1 h of reaction at 35 °C, the mixture was diluted with loading buffer (20 mM Tris-HCl, 15 mM EDTA, 50% glycerol, 0.5% SDS, 0.1% Xylene Cyanol, 0.1% bromophenol blue, pH 7.5) and loaded onto a 1% agarose gel. After the electrophoresis, the gel was stained by ethidium bromide, and the amount of plasmid was measured by relative integrated optical density using the Image SXM program.[44,45]

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Transient Transfection in the Presence of Cytochalasin B. COS-1 cells were transfected with plasmid pGL3-Control in the growth medium (10% FBS in DMEM) containing cytochalasin B[28,29] at the concentrations of 3.3, 10, and 33 μM, respectively. The cells were grown for 6 h in the presence of cytochalasin B and for 48 h after the removal of cytochalasin B. Transient transfection efficiency was determined as described above and normalized to the data obtained in the absence of cytochalasin B.

Transient Transfection in the Presence of Chloroquine. COS-1 cells were transfected with plasmid pGL3-Control in the growth medium (10% FBS in DMEM) containing chloroquine[30] at the concentrations of 50, 100, and 200 μM, respectively. The cells were grown for 6 h in the presence of chloroquine and for 48 h after the removal of chloroquine. Transient transfection efficiency was determined as described above and normalized to the data obtained in the absence of chloroquine.

Stable Transfection Experiments. COS-1 cells (approximately 3×10^5 cells on a 6 cm plate) were transfected with plasmid pHygEGFP that contains a hygromycin B phosphotransferase gene[21] by the procedure described for transient transfection. After 48 h incubation, the cells were replated on a 10 cm plate (1×10^5 cells) and grown in the growth medium for 24 h before the selection experiment. Cells were then selected in medium (10% FBS in DMEM) containing hygromycin B (200 μg/mL) for 2 weeks. The resistant colonies of transformants were stained by Giemsa. The transfection efficiency, expressed as percent transformation, was calculated by dividing the average number of surviving colonies by the average number of colonies that grew in nonselective growth medium and multiplying the result by 100. Lipofection using Lipofectin was carried out as a reference standard. A 40 kbp vector containing a GFP reporter gene and a resistant gene for an antibiotic G418 was prepared by inserting a GFP gene into the multicloning site (*Not*I site) of the ZAP-Express vector and was propagated by the procedure provided by the manufacturer. COS-1 cells were transfected with the 40 kbp vector according to the procedure described for the short vectors.

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Supporting Information Available: Results of the transfection in the presence of chloroquine. This material is available free of charge via the Internet at http://pubs.acs.org.

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