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Gene Delivery by Aminofullerenes: Structural Requirements for Efficient Transfection

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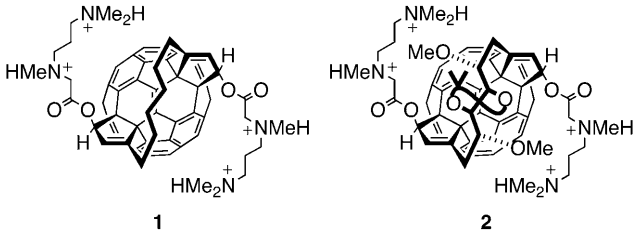
Abstract: A series of aminofullerenes that share a common structural motif have been synthesized and subjected to a systematic investigation of structure activity relationship regarding their ability for transient transfection and cytotoxicity. DNA-binding tests indicated that any water-soluble fullerene-bearing amino group would bind to double-stranded DNA. For these molecules to be effective transfection reagents, however, they require additional structural features. First, the molecule must be capable of producing submicrometer-sized fullerene/DNA aggregates that can be internalized into mammalian cells through endocytosis. Second, the molecule must be capable of releasing DNA as the aggregates are transferred into the cytoplasm. This can be achieved in at least two ways: by loss of the DNA-binding amino groups from the fullerene core, and by transformation of the amino groups to neutral groups such as amides. The screening experiments led us to identify the best reagent, a tetrapiperidinofullerene, that can be synthesized in two steps from fullerene, piperazine, and molecular oxygen, and that is more efficient at transfection than a commonly used lipid-based transfection reagent.

Keywords: aggregation · amphiphiles · drug delivery · fullerenes · gene expression

Introduction

The biological activity of fullerenes is attracting the interest of scientists both in a productive[1,2] and in a cautionary sense.[3] The activities of these molecules rest on the properties of both the fullerene core and its chemical modification. Hydrophobicity is a conspicuous property of the fullerene core, while the functional groups attached to the core add further complexity to the behavior of the functionalized fullerenes. It is this possibility of modular molecular design that has caught our interest in their use as DNA-binding and transgene expression (transfection) agents. We previously reported that cationic fullerene molecules such as the tetraammonium **1** (Scheme 1) not only bind to double-stranded DNA but condense it into globules.[4] Cooperation of the hydrophobic fullerene and the DNA-binding side chain is important, as is the case with DNA-binding cationic lipids.

Some but not all cationic lipids can bind to DNA, deliver the bound DNA through the cell membrane, and effect gene expression, and several factors that control the success of lipid-mediated transfection have been elucidated.[5–7] The cationic fullerene **1** effects transfection of the cells with an efficiency comparable to that achieved by commercially available cationic lipids.[8] However, understanding of the



Scheme 1. Transfection-active cationic fullerenes reported in previous studies.[8]

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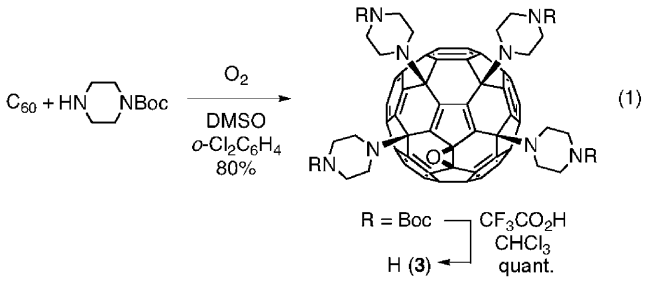
behavior of the fullerene reagent is minimal, and there is as yet no structure activity relationship (SAR) study available. Among known cationic fullerenes, **1** and its very closely related analogue **2** (Scheme 1) have thus far been the only cationic fullerenes that exhibit a practically useful level of transfection ability. Synthesis of these two compounds, however, needs laborious synthetic effort. To further examine cationic fullerenes for biological studies, it is necessary to find a new basic molecular scaffold that is synthetically available on a large scale and amenable to construction of a library of compounds from which suitable structures can be selected.

The process of transfection involves a series of complex events that are related to fundamental biological processes such as membrane permeation and gene expression.[9,10] To be an effective vector of double-stranded DNA, the cationic fullerene must first bind to the DNA to form a DNA/fullerene aggregate of a certain size (most likely <1 μm in diameter). The aggregate must survive in the incubation medium (or in blood in in vivo applications), pass through the cell membrane, and stay for some time in the cytoplasm before releasing DNA for gene expression. To gain insight into the biological behavior of fullerenes and to develop practically useful transfection agents, we performed an SAR study to identify the factors that control the DNA-binding and transfection capability of cationic fullerenes. We studied a library of 22 aminofullerenes and discovered a new type of cationic fullerenes that effect transfection with an efficiency as high as that achieved by common lipid-based transfection agents.

Results

Synthesis

Aminofullerenes examined in the present study (Scheme 2) were synthesized according to previously reported procedures with some necessary modifications[11–14] and characterized by ^{1}H and ^{13}C NMR, IR, and mass spectroscopy. The compounds **3**–**14** were prepared in one or two steps in high yield by a recently discovered procedure that can be carried out on a multigram scale.[11] For instance, **3** was synthesized in two steps with an overall yield of 80% [Eq. (1)].[15] This cost-effective two-step synthesis makes the transfection reagent readily available on a large scale (C_{60} is commonly available owing to its mass production in Japan). The other reference compounds **15**–**17** were prepared by other reported methods.[14]



(1)

Solubility

In the first stage of screening, we examined the solubility of the compounds in an acidic aqueous solution. All the aminofullerenes except **13** and **14**, which are insoluble, gave orange solutions at pH 2 (25 mM KCl/H_2O, adjusted to pH 2.0 by HCl).[16] The aminofullerenes dissolved in neutral to acidic aqueous solution in their protonated forms. As shown by the solubility of **15**, the presence of a single ammonium group is enough to enable the aminofullerene to dissolve in a polar medium. The cationic charge of each molecule is listed in Table 1.[17] The aminofullerene **14**, which bears amino groups directly attached to C_{60}, did not dissolve in the acidic aqueous solution (nor in a polar organic medium such as methanol, ethanol, or *N*,*N*-dimethylformamide (DMF)). This observation suggests that the nitrogen atoms are not protonated even at pH 2 because of the lack of basicity caused by the electron-withdrawing effect of the fullerene core.[18] Aminofullerene **13**, which bears carbamate groups, was insoluble in water, but gave an orange solution in dimethylsulfoxide (DMSO).

Abstract in Japanese:

さまざまな置換基をもつ一連のアミノフラーレンを合成し，一過性遺伝子導入および細胞毒性に関する構造活性相関研究を行った．DNA結合試験の結果からアミノ基をもつ水溶性フラーレンが二重鎖DNAに結合することが示唆された．しかし，遺伝子導入試薬として機能するためには，DNAに結合するだけ十分ではなく，次の要件を満たす必要があった．第一に，アミノフラーレンはDNAに結合し，さらに，その会合体のサイズを細胞内に取り込まれやすいサブマイクロメートル以下になるよう制御する必要がある．第二に，細胞内でDNAが放出されるために，アミノフラーレン上のアミノ基が除去される，あるいはアミド化により中性化される必要がある．構造活性相関研究の結果，[60]フラーレンから２段階で合成できるピペラジン付加体が，市販されている脂質類似試薬と同程度の高い遺伝子導入機能をもつことを見いだした．

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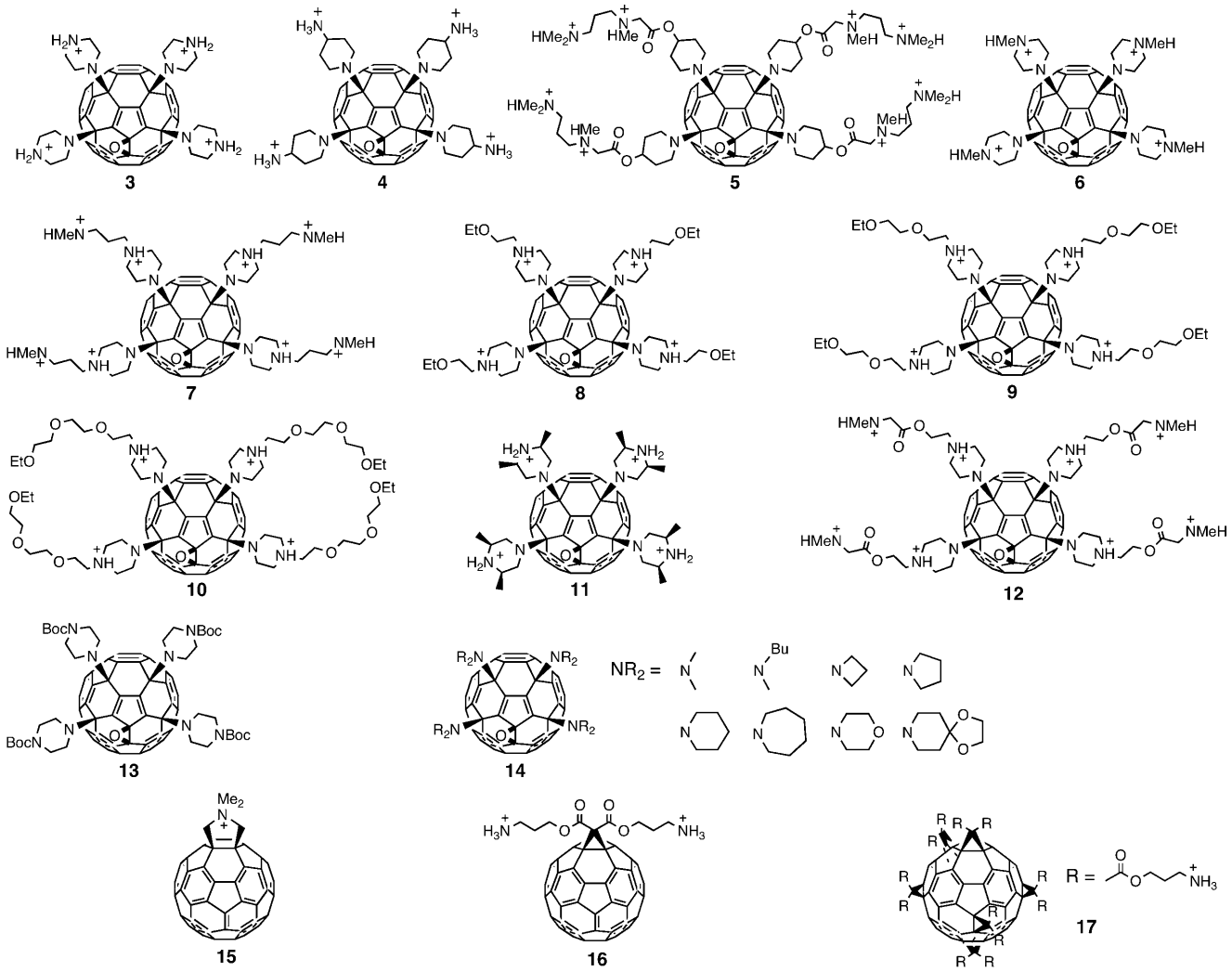


Scheme 2. Structures of aminofullerenes examined in this study.

Table 1. Cationic charges and C_{50} of aminofullerenes **1**–**17** and standard polyamines **18**–**22**.

Comp.	Cationic charge at pH 7	C_{50} [μM]	Comp.	Cationic charge at pH 7	C_{50} [μM]
13	0	n.d.[a]	**4**	4	0.56
14	0	n.d.[a]	**9**	4	0.55
15	1	2.8	**12**	8	0.84
16	2	2.3	**4**	8	0.80
1	4	1.9	**7**	8	0.20
2	4	1.6	**17**	12	0.30
8	4	1.7	**18**	1	28000[b]
10	4	1.4	**19**	2	2300[b]
6	4	1.2	**20**	3	41[b]
3	4	1.0	**21**	4	1.6[b]
11	4	0.68	**22**	6	0.15[b]

[a] Not determined; **13** and **14** were insoluble in water. [b] See reference [21].

Water-soluble fullerenes congregate in aqueous solution and form vesicles with diameters of the order of 10^{-8} m.[19] To investigate the solution behavior of cationic fullerenes, we prepared a solution of **3** (pH 2.0) and examined the pH-dependency of the ζ potential and the particle size (Figure 1). At low pH, **3** forms a stable solution that contains aggregates with an average diameter of 65 nm, the surface of which is positively charged. As the pH is raised, the positive surface charge decreases, and at pH 9, when the charge is nearly neutralized, the aggregate solution suddenly

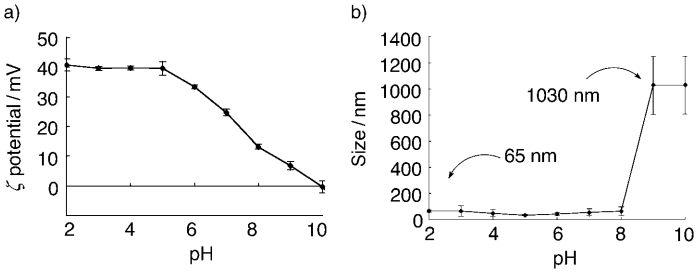


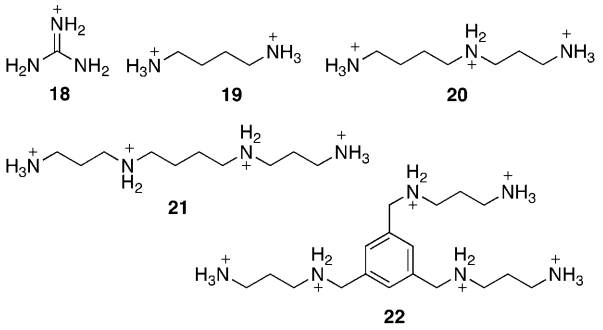
Figure 1. a) ζ potential and b) size of **3** in aqueous phase plotted against pH. The ζ potential and the size of **3** were determined by DLS measurements (repeated more than three times).

becomes unstable and forms micrometer-sized particles. The aggregation behavior of the fullerenes in the biological experiments in the presence of buffer and serum used for incubation would admittedly be more complex.

DNA-Binding Study

The DNA-binding ability of the aminofullerenes was examined by competitive-binding assay with ethidium bromide, as in our previous study on the DNA-binding affinity of **1**.[8,20] The binding ability was examined for calf thymus DNA (73.6 μM base pairs (bp) in 2 mM HEPES, 10 μM EDTA, 9.4 mM NaCl) with 1.26 μM ethidium bromide and was quantified for the ability to displace the intercalated ethidium bromide (C_{50} values defined as the drug concentration at which the fluorescence intensity of the intercalated complex becomes half the initial intensity; the smaller the number, the stronger the binding).

The C_{50} values and the amount of cationic charge are summarized in Table 1. The insoluble aminofullerenes **13** and **14** were not examined further. Previously reported data (for **1**, **2**, **6**, **15**)[8] and those for nonfullerene polyamines[21] (Scheme 3) are included as references. Notably, the monocationic fullerene **15** exhibited much higher DNA-binding ability (C_{50} = 2.8 μM) than the corresponding monocationic amine **18** (C_{50} = 28000 μM). The binding ability of the dicationic fullerene **16** (C_{50} = 2.3 μM) was very similar to that of **15**, while that of the corresponding alkyldiamine **19** (C_{50} = 2300 μM) was ten times that of **18**. For tetracationic fullerenes, (**1**–**4**, **6**, **8**–**11**), the C_{50} values (1.9, 1.6, 1.0, 0.56, 1.2, 1.7, 0.55, 1.4, and 0.68 μM, respectively) improved only slightly over the dicationic fullerene **16**; similarly so for the octacationic fullerenes (**5**, **7**, and **12**; C_{50} = 0.80, 0.20, and 0.84 μM, respectively). The C_{50} value of **17** (C_{50} = 0.30 μM), which has 12 cationic nitrogen atoms, the largest number examined in this study, was not much smaller than the less cationic ones. The DNA-binding ability of the cationic fullerene derivatives were generally higher than the cationic lipids, and were less sensitive to the amount of cationic charge. On the other hand, the effect of cationic charge on the binding affinity is very large for cationic lipids; that is, increasing the charge by one may decrease the C_{50} value by a factor of 10 (see Discussion).



Scheme 3. Structures of standard polyamines.

Transient-Transfection Study

Effect of Medium on Fullerene/DNA Complexation

To achieve transfection of mammalian cells with aminofullerenes, DNA binding is required but not sufficient. Some preliminary data largely based on a green fluorescent protein assay was available at the outset of the present study: the transfection efficiency of **1** is comparable to that of a commercial lipid reagent. Fullerene **2** has a comparable transfection capability to **1**, but **15** did not show any transfection capability.[8,22] Compounds **12**, **16**, and **17** were entirely inactive. We found that **6**[8] has a very weak transfection capability (less than 10% of **1**).

For a more precise estimation of the efficiency of transient transfection, we used a bioassay that relies on the expression of a luciferase reporter gene; the results are summarized in Figure 2. The experiments were performed on COS-1 cells in the presence of 10% fetal bovine serum (FBS). The efficiency of transfection is defined as the amount of luciferase production relative to the total protein production during incubation, and the highest efficiency shown in Figure 2 I-a was used as the standard (also in Figure 4). This data is comparable to that obtained in experiments with the common lipid-based transfection agent lipofectin (Figure 2 I, column a vs. e).[23]

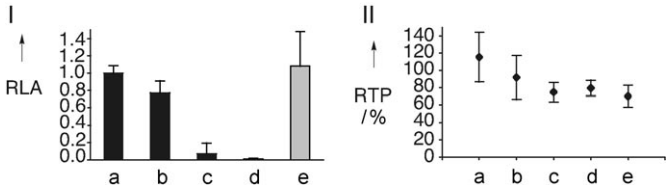


Figure 2. Transient transfection of luciferase gene into COS-1 cells by **3** (a–d) or lipofectin (e) in the presence of 10% FBS. The *R* value for **3** is 5.0. Figure 2 I shows the transfection efficiency (RLA = relative luciferase activity), and Figure 2 II shows the cell viabilities (RTP = relative total protein). The cells were transfected with the **3**/DNA complex prepared in TBS (a), HBS (b), PBS (c), and DMEM (d). RLA (transfection efficiency) was measured in relative light units (RLU) per milligram of total protein relative to the standard data (a: 2.8×10^8 RLU mg^{-1} protein) obtained. RTP (total amount of protein relative to control cells) represents the viability of the cells. The data show the mean value with the standard deviation ($n \geq 3$).

Given our recent finding that the fullerene-mediated transfection takes place by endocytosis,[8c] we suspected that the size of the fullerene/DNA complexes would be an important factor, as cell uptake of particles by endocytosis is most effective when the particles are a few hundred nanometers in diameter. It is fortuitous that the fullerene/DNA complexes are deeply colored and can observed in situ by an optical microscope during complex preparation and incubation, a convenient feature that is unavailable for optically transparent lipofection reagents. We thus found that the medium for the preparation of the fullerene/DNA complexes affects the particle size and hence the efficiency of transfection.

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Taking into account the optimum fullerene/DNA ratio discussed in the next section, we examined the effect of buffer used in the complexation of **3** with DNA (Figure 2). Complexation in tris-buffered saline (TBS) gave the highest transfection efficiency; the efficiency dropped slightly with HEPES-buffered saline (HBS), and decreased several fold with phosphate-buffered saline (PBS) or Dulbecco's modified Eagle's medium (DMEM). The cell viability also decreased in this order (Figure 2 I). Overall, **3** is comparable to lipofectin in terms of the transfection efficiency, but superior with respect to cell viability (Figure 2 I and II, columns a vs. e). In the same experiments, we found that the efficiency of **3** is about 90 % that of **1** (data not shown), which suggests that **3** is as good as **1**.

Transfection efficiency seems to be related to particle size. The mixture of **3** and DNA in TBS or HBS appeared homogeneous under the microscope, whereas that in PBS or DMEM appeared heterogeneous. This difference persisted even after the mixture was added to the incubation plate with roughly the same amount of medium used for sample preparation and the DMEM-containing incubation medium. Figure 3 shows the micrograph images taken after incubation for 6 h of COS-1 cells with the **3**/DNA complexes prepared in various media. When the complexation was performed in TBS or HBS, the medium was essentially homogenous (Figure 3 a and b), but it was entirely heterogeneous and contained dark-colored particles as large as 10 μm when PBS or DMEM was used for the complexation. Dynamic light scattering (DLS) analysis showed that the size of **3**/DNA particles is about 100 nm in TBS and HBS and over 1000 nm in PBS and DMEM.[24] The particles in PBS and DMEM must be too large to be internalized into cells by any spontaneous membrane permeation process.

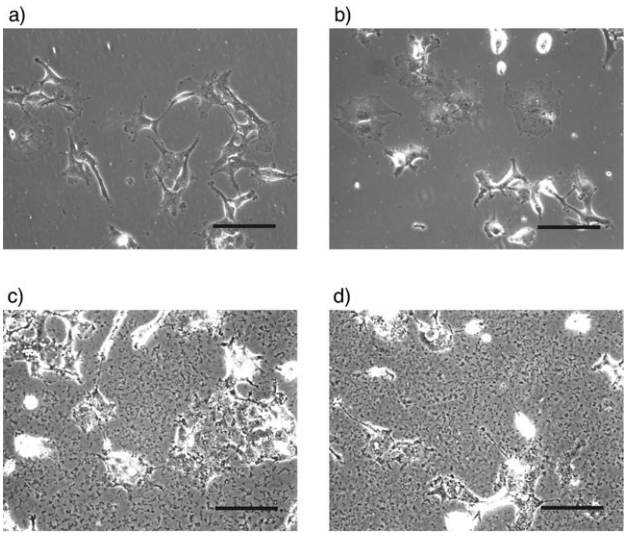

Figure 3. Optical microscopic images of **3**/DNA complexes prepared in a) TBS, b) HBS, c) PBS, and d) DMEM and the COS-1 cells after 6 h incubation. Scale bar shows 500 μm. The medium is essentially homogeneous in a) and b), but heterogeneous in c) and d) due to formation of micrometer-sized aggregates of the **3**/DNA complex.

Transfection and Cell Viability

We then examined the transfection efficiency and the cell viability of the aminofullerenes in TBS buffer against the variation of the molar ratio of the fullerene/DNA base pair (R)[25] (Figure 4). The optimum R value differs among the fullerenes; hence it was optimized for each case. For the most efficient fullerene **3**, the efficiency was best at $R=5$ (Figure 4 I-a). This value was also optimum for the second-best fullerene **4**, the transfection efficiency of which was 32 % of **3** (Figure 4 I-b). For the third-best fullerene **5**, the optimum $R=20$, and the efficiency was 13 % of **3** (Figure 4 I-c). Other fullerenes **6**–**13** showed much lower efficiency (< 10 %), and the optimum R values (2–20) could not be defined with certainty (Figure 4 I-d–i). Through examination of 24 diverse cationic fullerenes, only three (**1**, **2**, and **3**) were found to be efficient reagents for transient transfection.

High cell viability was observed for all fullerenes (70–100 % at the optimum R value), thus indicating that fullerenes are not toxic to COS-1 cells (the experiments were done under ambient light). The fullerene **3** ($R=5$) caused no cell morphological changes either.

Discussion

Since the first successful transfection with lipofectin by Felgner et al.,[5] a number of cationic lipid reagents as well as nonlipid chemical vectors based on polymers,[26] dendrimers,[27] peptides,[28] cyclodextrins,[29] silica nanoparticles,[30] gluconanoparticles[31] and nanotubes[32] have been reported. The process of transfection by these reagents is considered to involve the following steps: 1) DNA binding, 2) entry of the DNA/fullerene complex to the cell, 3) release of the bound DNA from the fullerene complex, 4) entry of the DNA into the nucleus, and 5) production of protein coded on the DNA. We previously reported that the same process is involved in the fullerene-mediated transfection. Among the DNA complexes of 24 diverse aminofullerenes described in this paper, however, only the complexes of **1**, **2** and **3** successfully went through these processes to effect efficiently the desired transfection; the complexes of **4** and **5** did so moderately, but the remaining 19 aminofullerenes failed in at least one of these checkpoints. The observed SAR raised two issues: 1) the transfection capability has little to do with the total cationic charge, and 2) it depends heavily on seemingly small modifications of the structure. We discuss DNA binding first followed by transfection.

It is known that the DNA-binding ability of cationic lipids depends both on the hydrophobicity of the lipid hydrocarbon chain and on the cationic charge. The binding becomes stronger as the molecule becomes more hydrophobic and more cationic. Furthermore, the generally observed instability of the DNA/lipid complexes in vivo and in a serum-containing medium[32] appears to be partly due to disturbance of the lipid–lipid interaction within the DNA/lipid complex

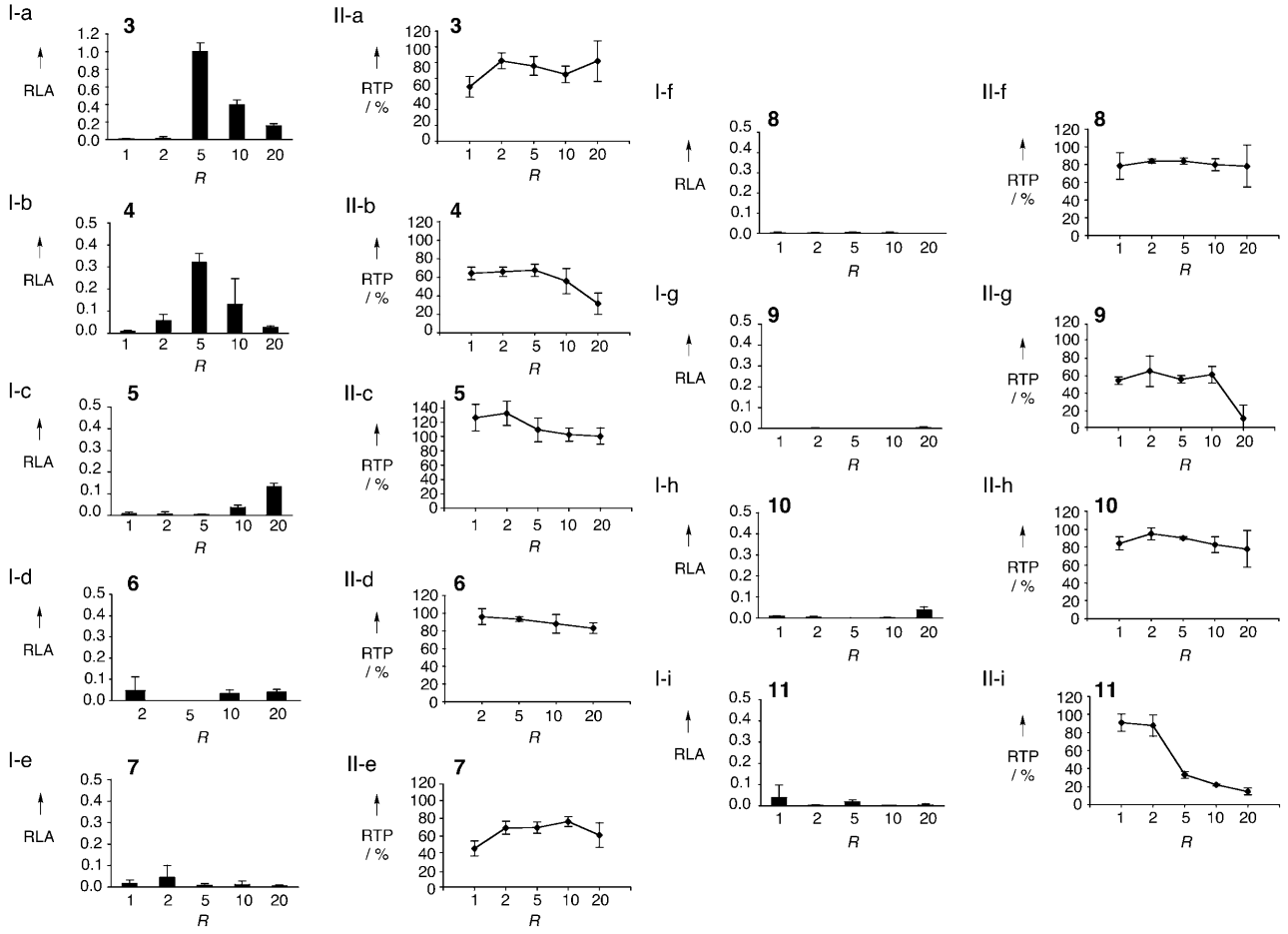

Figure 4. Transient transfection of COS-1 cells with luciferase gene by the tetracationic fullerenes **3**–**12** (a–k, respectively) in 10% FBS, and cell viability. The series I graphs report the transfection efficiency (RLA) relative to the best value (2.8×10^8 RLU mg^{-1} protein) obtained for **3** at $R=5$. The series II graphs report the cell viability (RTP). All data show the mean value together with the standard deviation ($n\geq3$).

by interaction of the lipid with biological substances such as protein and hydrophobic substances in serum and blood. The fluxional behavior of the hydrocarbon chains of lipid is well-known and is due to rather weak cohesive forces between the lipid side chains. Thus, the large cohesive forces of fullerene molecules and the ability of fullerene to form stable self-aggregated structures in water[2,4,19] must be considered when we discuss their DNA binding as well as DNA release.

The DNA binding of cationic lipids has been studied extensively in relation to their use for gene transfer.[34] Bloomfield reported recently the DNA-binding affinity of cationic lipids by isothermal titration calorimetry,[35] and showed that the thermodynamics of the binding depends both on the cationic charge and the hydrophobicity of the lipid moiety. Fullerene is a peculiar solvophobic molecule in that it does not dissolve well either in water or in hexane, but shows a high affinity to itself. This property makes the behavior of cationic fullerenes very different from that of cationic lipids.

It was reported for simple polyamines that the cationic charge and the C_{50} value are linearly correlated with a very steep slope, which indicates that complexation is favored as the charge is increased (Figure 5).[21] Similarly, the data

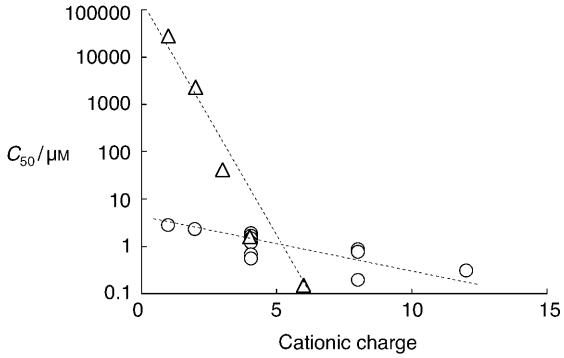

Figure 5. Relationship between C_{50} and the number of cationic amine groups. The data for standard polyamines are taken from reference [21] and those for fullerenes from Table 1. △ = standard polyamine, ○ = aminofullerene.

taken from the polyaminofullerenes in Table 1 show linear correlation, but the slope is not as steep as that of the simple polyamines (Figure 5; the nitrogen atoms directly attached to the fullerene core are not basic enough to be protonated in water, and hence were omitted from the count). The binding ability of mono- and dicationic fullerenes is much higher than the corresponding cationic lipids, but

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there is not much gain when the cationic charge is increased further. It is rather remarkable that eight tetracationic as well as three octacationic fullerenes of diverse structures show essentially the same binding ability (see also Table 1). The DNA-binding propensity as assessed by the ethidium-displacement assay indicates that the extremely hydrophobic core of the cationic fullerenes contributes much more to the binding than the less hydrophobic alkyl side chains in the cationic lipids.

After the first DNA-binding process, the fullerene/DNA complex has four more steps to achieve production of the coded protein. We recently reported that the complex goes through the cell membrane by an endocytosis mechanism, and the DNA in the resulting endosome is protected by the fullerene sheath.[8c] As suggested previously, submicrometer-sized materials can go through cell membranes by endocytosis.[36] The 100-nm particle size of the **3**/ DNA complex in TBS and HBS (Figure 3) indicates that the present system satisfies the size requirement for efficient endocytosis.

Once the fullerene/DNA complex enters the cytoplasm, DNA must be released before it can enter the nucleus for production of the protein. Given that the cationic sites are important for DNA binding, loss of the cationic sites from the fullerenes in the cytoplasm must therefore be a critical process of the DNA release. The SAR analysis suggests two release mechanisms: the conversion of the protonated amine group to a neutral group so that it loses its DNA-binding ability, and the cleavage of the bond connecting the protonated amine groups and the fullerene. The former is discussed first. Among all the piperazine derivatives with secondary amines in Scheme 2, only **3** is an effective transfection reagent. We expect that these secondary amines would be easily acylated (or any equivalent reaction that causes the amine to become neutral) in cytoplasm and hence lose their DNA-binding activity. The hindered secondary amine **11** provides chemical support for this hypothesis, because its secondary-amine moieties are too hindered by the flanking methyl groups to be acylated (even by the most reactive chemical reagents). Therefore, **11** cannot release the bound DNA. The primary amine **4** provides further support, as the primary-amine moieties would also be readily acylated in the cytoplasm and lose their DNA-binding ability. Thus, the presence of primary or secondary amines that can be converted into neutral groups is a necessary feature of active transfection agents.

The second mechanism was revealed by another group of compounds. The ester groups in **1**, **2**, and **5** (all are active transfection reagents) connect the fullerene moiety and the amine groups, but are susceptible to chemical or enzymatic hydrolysis. The difference between **5** (active) and **7** (inactive) also supports this hypothesis. Thus, the presence of a linker that is cleavable under physiological conditions is an alternative, necessary feature of a successful transfection reagent.

The high levels of cell viability observed for all the aminofullerenes we studied suggests that these compounds have low toxicity or photocytotoxicity (under ambient light). In many runs, the cytotoxicity of the solvents DMSO and DMF (not shown in Figure 4) overrode the toxicity of the fullerenes. This lack of cytotoxicity stands in contrast to the photocytotoxity of a fullerene carboxylic acid that we previously reported (owing to photoexcitation of C_{60} to its triplet state),[1] and we tentatively ascribe this difference to the amine groups present, which can quench the triplet excited state of the fullerenes.[37]

Conclusions

We have synthesized a library of aminofullerenes and found that **3** is the best transfection reagent among them. It shows an efficiency of transient transfection that is comparable to that of the previously reported aminofullerene **1**. On the basis of our recent finding that **1** can protect DNA in the cytoplasm and is consequently 20 times more efficient at stable transfection than lipofectin,[8] we expect that the structurally related **3** is also useful for stable transfection. Compound **3** is readily available through two synthetic steps [Eq. (1)] that can be carried out on a large scale with minimal experimental effort (oxygen atmosphere without heating or precaution for excluding water). Such synthetic simplicity combined with the high transfection efficiency is a great merit over the commercialized lipofection reagents that are synthesized through longer methods.

The data summarized in Figure 5 suggests that, once made water-soluble by the attachment of amine groups, any fullerene compound can bind to DNA and condense it into aggregates at the nano- to micrometer scale. For the fullerene molecules to act as efficient transfection reagents, other requirements need to be fulfilled. First, the size of the aggregates must be controlled (subnanometer) so that they can pass through the cell membrane and be internalized. The size control may be achieved either by molecular design or by control of the complexation conditions. We consider that any such fullerene/DNA complexes would be delivered into mammalian cells, but that is not enough for the coded protein to be expressed in the cells. The second requirement is that the fullerene must release the DNA in the cytoplasm. This can be achieved either through loss of its amine groups or loss of the binding ability of the amines by transformation into neutral derivatives.

Experimental Section

Spectral Measurement

NMR spectra were obtained with a JEOL ECX-400, ECA-500, or Bruker AV-500 spectrometer. IR spectra were recorded with a Jasco FT/IR-240 or an ASI Applied Systems REACT IR1000 spectrometer equipped with an attenuated total reflection (ATR) instrument and are reported in cm^{-1}. Mass spectra were acquired either with a Shimadzu QP-8000 (ionization: atmospheric pressure chemical ionization (APCI), detection: quadrupole) or a Waters ZQ-S spectrometer.

Materials

Aminofullerenes examined in the present study were synthesized by previously reported procedures with some necessary modifications.[8,11–14] The details of the syntheses are described in the Supporting Information. Plasmid DNA (pGL3-Control) was purchased from Promega, FBS from Equitech-Bio, luciferase assay kit (including a picagene reagent) from Toyo Ink, and reagents for Bradford assay from Bio Rad. Other chemicals were purchased from Sigma. Plasmid DNA (pGL3-Control) was amplified from *Escherichia coli* and purified on a CsCl gradient.[38] The final concentration of plasmid was determined by its UV/Vis absorption signal at 260 nm. COS-1 cells (simian virus 40-transformed kidney cells of the African green monkey)[39] were purchased from American Type Culture Collection and cultured in DMEM containing 10% FBS in a humidified atmosphere at 35 °C with 5% CO_2.

DLS Analysis and ζ Potential Study

The particle size of aminofullerene derivatives were determined by DLS analysis at 25 °C with a Nano ZS system that uses a 4-mW He–Ne laser (633 nm) as the incident beam by NIBS (Non-Invasive Back-Scatter) technology (Malvern Instrument Ltd., UK). For data analysis, the viscosity (0.8905) and refractive index (1.333) of pure water at 25 °C were used. The ζ potential of the particles of **3** was determined in disposable capillary cells with the same Nano ZS system. Aminofullerene **3** was dissolved (0.5–1 mM) in an acidic buffer (25 mM KCl, adjusted to pH 2.0 by HCl). The pH of the solution was increased from pH 2 to 10 by an autotitration system (MPT-2 Autotitrator, Malvern Instruments Ltd., UK).

DNA-Binding Assay[20]

Excitation of the buffer solution (3 mL in a 1-cm path length quartz cell) was performed at 546 nm, and fluorescence emission was measured at 595 nm (slit width 0.75 nm). Ethidium bromide (1.26 μmol) was dissolved in the buffer, and a solution of calf thymus DNA (38 μmol bp in 0.01 sodium-HEPES buffer) was added to provide a concentration of 1.26 μmol (ethidium bromide/DNA = 1:1), thus increasing the fluorescence reading of the ethidium solution from 15 to 100 units. The aminofullerenes were dissolved in DMSO (1–2 mM), and each solution (0.1–1.0 μmol, depending on the compound) was added in microliter portions to a portion of the ethidium solution. The C_{50} value was defined as the drug concentration required to reduce the fluorescence of the DNA–ethidium complex by 50%. We also obtained the C_{50} value with an aqueous solution of aminofullerene in acidic buffer (pH 2.0) and did not observe any difference from that obtained in DMSO solution.

Transfection Experiment

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 washed with DMEM before the event. For each well, 0.8 μg plasmid DNA was used. Aminofullerenes **3**–**12** in acidic buffer (25 mM KCl/H_2O, adjusted to pH 2.0 by HCl) and **13** in DMSO (0.15–12 μL, 0.5–2 mM) and plasmid DNA in TBS (320 μL, 2.5 μg mL^{-1}) were mixed, with *R* from 1 to 20, and the mixture was kept at room temperature for 30 min to give a solution of the fullerene/DNA complex. When the effect of buffer was to be analyzed, HBS, PBS, and DMEM were used instead of TBS. The solution of the fullerene/DNA complex was diluted with DMEM (320–342 μL), which generally contained 10% FBS, and added to the COS-1 cell dish. The transfection medium was changed to DMEM with 10% FBS after 6-h incubation in a humidified atmosphere at 35 °C with 5% CO_2. After incubation for 48 h, the medium was removed, and the 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 that contained luciferin, and the photon count of the lysate was measured with a luminometer (Lumat LB9507) to determine the luciferase activity in RLU.[40] The total amount of protein in the lysate was independently estimated by Bradford assay with Coomassie Brilliant Blue G-250,[41] and the luciferase activity per milligram of protein was calculated and used as the measure of transfection efficiency. Lipofectin was the reference transfection reagent, and lipofection was carried out according to the manufacturer's procedure.[5] Thus, lipofectin in DMEM (52.5 μL, 47.6 μg mL^{-1}) and plasmid DNA in DMEM (53.2 μL, 15 μg mL^{-1}) were mixed and kept at ambient temperature for 30 min. The mixture was then diluted with DMEM (534 μL) or 12% FBS/DMEM (534 μL) to give the growth medium for lipofection.

Microscopic Analysis of Fullerene/DNA Complexes

Fullerene/DNA complexes were observed with an optical microscope (Keyence, VH-8000). Phase-contrast micrographs were taken 6 h after the introduction and incubation of the transfection mixture with COS-1 cells, but before the change of medium mentioned in the transfection experiments. The micrographs show a plate surface on which there are several cells and many black spots corresponding to the fullerene/DNA complex (Figure 3).

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