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[23] TS can be considered an ellipsoid with a mean diameter (core plus ethoxy surface groups) of 17.5 Å; this estimation is based in the volume of the crystalline cluster, 5568 Å^3/cell (see ref [7b]). The approximate Ti–oxo core dimensions, taken as the maximum Ti···Ti distance along the major and minor axis, are 10.8 and 7 Å, respectively.
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Functionalized Fullerene as an Artificial Vector for Transfection

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Since our discovery that a photo-excited water-soluble fullerene oxidatively cuts the DNA duplex,[1] the interaction of organofullerenes with DNA has continued to attract the interest of chemists. Base-sequence-selective cutting has also been achieved with fullerene/DNA conjugates,[2] and a number of studies on structural variations of the reagent,[3] the mechanism of the photo cleavage,[4] and the applications to photodynamic therapy[5] have been reported. The lack of acute toxicity ($LD_{50} \gg 500\ mg\,kg^{-1}$) and the pharmacokinetics of oraganofullerene have been recorded.[6] We report herein an entirely new aspect of fullerene/DNA interaction, namely, the use of a functionalized fullerene as an agent for gene transfer (transfection). A newly designed "two-handed" fullerene (±)-**1** (see Figure 1) binds to duplex DNA, condenses it, and allows the complexed DNA to be delivered into and to be transiently expressed in the target cell.

The two-handed fullerene **1** is a unique amphiphile possessing [60]fullerene as the central core, on which a C_2-symmetric S-shape hydrocarbon structure, bearing two side chains, is attached. The basic C_2-core skeleton has been synthesized through the tether-directed regioselective double [3+2] cycloaddition by the route detailed in the Supporting Information.[7] The diamine side chains are separated by 1.2 nm from each other, they are protonated at pH 7,[8] and will exert strong electrostatic interactions with the parallel phosphate backbones of a DNA duplex (which, along the major groove, are also separated from each other by 1.2 nm). The phosphate charges on DNA will be neutralized at a reagent/base pair ratio (R) of 0.5. The central fullerene core provides a hydrophobic binding force for the condensation of DNA and probably also for the delivery of the fullerene/DNA complex to the interior of the target cell.

Various organic molecules have been developed for transfection,[9] and commercialized under a generic name of "lipofection" reagents such as Transfectam (**7**, also known as "DOGS", was used as the reference in these studies).[10] The central concept of lipofection is the conversion of a negatively charged DNA into a positively charged lipid-like entity through complexation with the reagent. Although these lipofection reagents have been extensively used in laboratories and in some cases in vivo,[11] the efficiency of transfection is by no means perfect and the reagents exhibit considerable cell toxicity, caused most likely through perturbation of the cell membrane by the lipophilic reagent. In designing a new strategy for gene delivery, we abandoned the idea of lipid mimicry and counted on the extremely high hydrophobicity of the fullerene core, with which the reagent/DNA complex may be delivered from aqueous medium into the cell.

For a given compound to act as a transfection vector, it must first bind tightly to DNA. In the absence of previous studies on a fullerene-based gene transfer approach, we needed to screen various molecules so that we could identify structural features necessary for tight DNA binding. To start the investigation, we synthesized the two-handed tetramine **1** as well as various related compounds **2**–**5** (Figure 1), each of which lacks some element of the structural features of **1**. The tetramine **2** lacks the fullerene core and the "one-handed" diamine **3** has only one cationic side chain. The dicarboxylic acid **4** is "two-handed", but is negatively charged in a neutral buffer solution. The affinities of these reagents for DNA duplexes were probed by several independent sets of experiments performed on calf thymus DNA, plasmid pBR322 DNA, and linear DNA fragments. The experiments were

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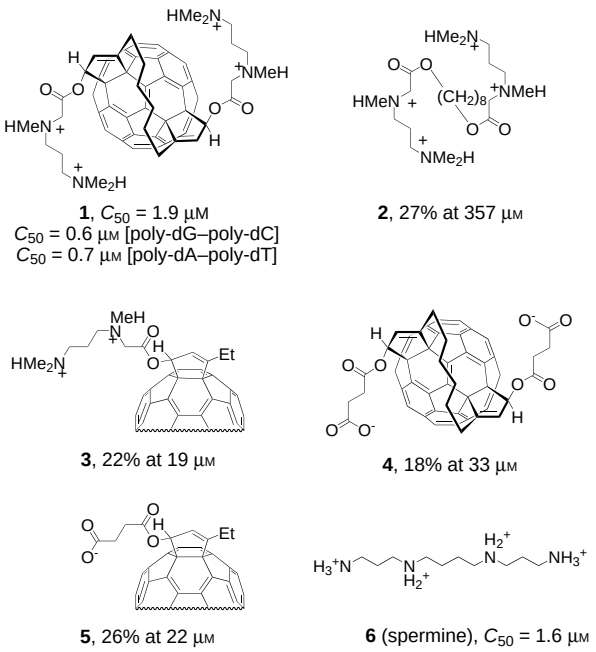


Figure 1. Tetramine **1** and reference compounds. The binding affinities (C_{50}) were measured by ethidium bromide displacement assay for calf thymus DNA (unless noted) and poly-dG – poly-dC and poly-dA – poly-dT. For **2** – **5**, the drug concentrations that effected maximum % displacement are shown.

performed in the range $R = 2.5 \times 10^{-4} - 2.6$. Spermine was also examined as a DNA binding reference compound.

The screening with the ethidium bromide displacement assay demonstrated a very clear structure/activity relationship for the compounds as shown in Figure 1. The two-handed fullerene tetramine **1** showed a C_{50}[12] as small as 1.9 μM. This value rivals the data of spermine ($C_{50} = 1.6$ 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, which indicates that **1** binds to DNA without recognition of specific bases. In contrast to **1**, the other reference compounds **2** – **5** are unable to achieve 50% displacement even when added in a large excess.

In addition to the difference in the binding assay, we also found a marked difference between **1** and **2** – **6** in their ability to induce phase separation of the reagent/DNA mixture at a higher R value range. Thus, when we increased the amount of **1** from $R = 0.5$ to 2.6, the amount of DNA in the aqueous phase decreased precipitously with visibly observable phase separation. No such phase separation took place with **2** – **6** even at a high R range (up to 2.6). For instance, experiments using a mixture of four ^{32}P labeled linear DNA fragments of different GC contents (44 – 57% GC) showed that an excess amount of **1** ($R = 2.0$) converted DNA into the material that stays at the origin of electrophoresis gel (Figure 2, lane 2). The formation of the insoluble material is reversible. Thus, when the mixture was extracted with chloroform, in which **1** is soluble, the duplex DNA was released into the aqueous phase without a change in the ratio between the fragments (Figure 2, lane 3). The above comparison between **1** and **2** – **6** demonstrate the strong binding power of **1** through the synergetic effects of the large hydrophobic core and the cationic side chains.

The experiments described above indicated that the two-handed fullerene tetramine **1** is unique among other fullerenes in its ability to bind to duplex DNA in a reversible manner (cf. chloroform extraction experiment). With such data in hand, we started our studies on transfection.

To optimize various parameters we initially followed the protocol used for lipofection agents (transfection for 6 h without serum, incubation for 2 d with serum). The reagent/base pair ratio (R), the amount of DNA, the transfection time, and the incubation time were the parameters examined extensively. Plasmid pGreen LANTERN-1 containing a green fluoresent protein (GFP) reporter gene was used for the determination of the transfection efficiency (TE), which is defined as the number of fluorescent cells relative to the total number of cells transfected on the plate.[13] In the first set of experiments, the R-dependency of the transfection efficiency was examined. The experiments with transfection and incubation times fixed for 6 h and 2 d, respectively (Figure 3), show that the efficiency

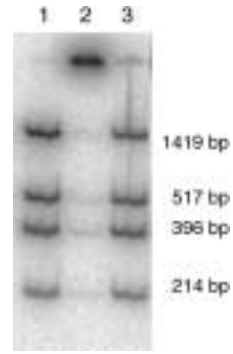


Figure 2. Precipitation of linear DNAs caused by **1** at $R = 2.0$. Addition of **1** to mixtures of four linear DNA fragments with different numbers of base pairs (bp; control, lane 1) forms immobile material (lane 2). Chloroform extraction of **1** before loading to gel liberates soluble DNAs without a change in their relative amounts (lane 3).

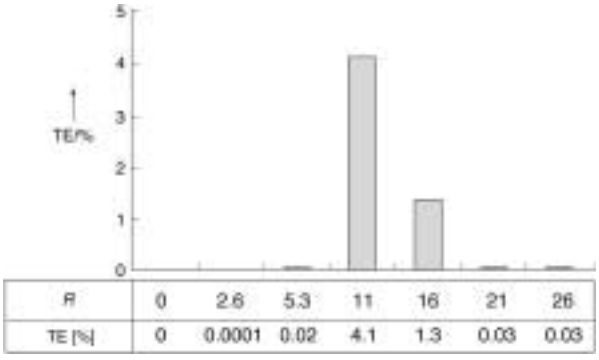
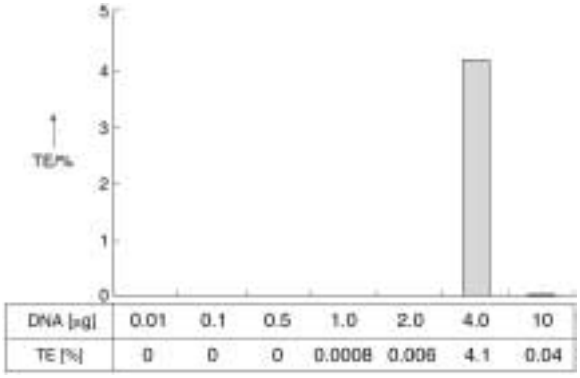


Figure 3. Dependency of transfection efficiency (TE) on R as examined for the GFP vector (COS-1 cells $= 1 \times 10^5$, DNA = 4 μg, transfection time = 6 h, incubation time = 2 d). Effective transfection takes place only within a narrow range of R values. See text for full details.

increased toward $R = 11$ and then dropped with further excess of **1**. A similar R-dependent profile has been reported for other lipofection reagents. With the R value fixed at 11, the efficiency reached the maximum value of 4.1% at the DNA amount of 4 μg (Figure 4). The cell viability relative to the control experiment at the optimum transfection condition was 26%, which is comparable to a 33% value with Transfectam (**7**). The cell death was caused at least partly by the dimethylformamide (DMF) used to dissolve **1** in aqueous solution (see below).

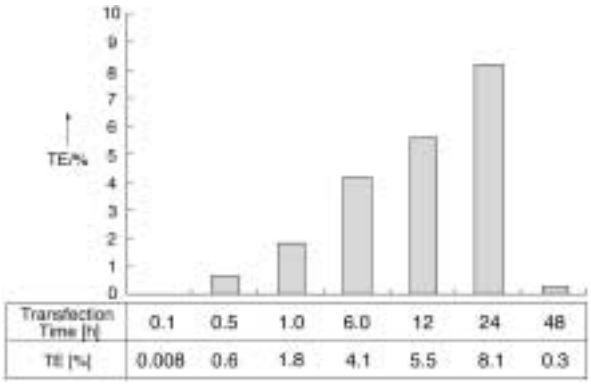


DNA [μg]	0.01	0.1	0.5	1.0	2.0	4.0	10
TE [%]	0	0	0	0.0008	0.006	4.1	0.04

Figure 4. Dependency of transfection efficiency (TE) on the amount of the GFP vector (COS-1 cells $= 1 \times 10^5$, $R = 11$, transfection time = 6 h, incubation time 2 d). Efficient transfection takes place with a relatively large amount of the DNA.

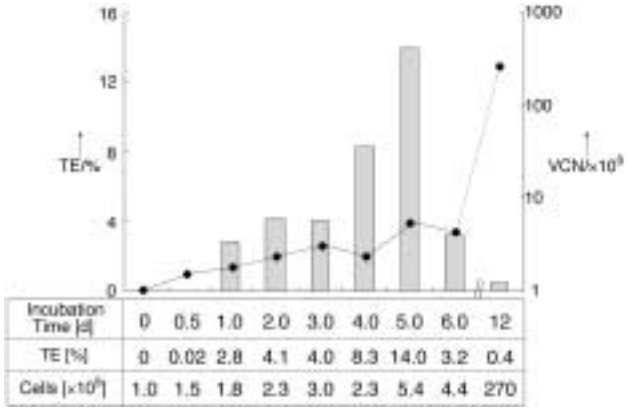
The effects of transfection time and incubation time were examined, the TE increased up to the transfection time of 24 h (Figure 5), but after 6 h the relative cell viability fell down precipitously to ~10% (data not shown). The TE also depends upon the incubation time. The TE was very low on day 1 and increased rapidly until day 5 (Figure 6) when the maximum TE of 14% was achieved; fluorescence was detected in 0.4% of cells even after incubation for 12 days. In contrast, lipofection with Transfectam (**7**) under similar conditions (transfection time = 6 h, DNA = 4 μg, $R = 4.0$) showed the maximum efficiency of 29% after incubation for 2 days, and the efficiency dropped to 11% at day 5. Since our vector plasmid (pGreen LANTERN-1) has no ability to self-replicate (no SV40 origin contained),[14] the five-day-long persistent increase in the number of GFP-expressing cells is attributable to the continuous release of the DNA from the **1**/DNA complex taken up by the cell and its delivery into the nuclei (see above). The presence of fluorescent cells even after 12 days incubation suggests the occurrence of stable transfection.

Inspite of the photoreactivity of (weakly DNA binding) fullerenes,[1] no differences were noted when the transfection experiments with **1** were carried out under ambient light or black light. The cytotoxicity of **1** was found to be quite small, cells grew exponentially in the presence of **1** (Figure 6). In addition, tetrazolium assay indicated that the cytotoxicity of **1** in DMF or DMSO is largely caused by the solvent toxicity (IC_{50} (median growth inhibitory concentration) = 1.8% (v/v) and 2.5% (v/v), respectively), since the IC_{50} values of the same order of magnitude were obtained for the solution of Transfectam (**7**) in these solvents. Due to the toxicity of the DMF used to dissolve **1** in aqueous solution, cell viability relative to the control experiment at $R > 21$ became so small (1–3%) that the data for $R > 21$ is rather unreliable.



Transfection Time [h]	0.1	0.5	1.0	6.0	12	24	48
TE [%]	0.008	0.6	1.8	4.1	5.5	8.1	0.3

Figure 5. Dependency of transfection efficiency (TE) on the transfection time (COS-1 cells = 1×10^5, $R = 10.6$, DNA = 4 μg, incubation time 2 d).



Incubation Time [d]	0	0.5	1.0	2.0	3.0	4.0	5.0	6.0	12
TE [%]	0	0.02	2.8	4.1	4.0	8.3	14.0	3.2	0.4
Cells [$\times 10^5$]	1.0	1.5	1.8	2.3	3.0	2.3	5.4	4.4	270

Figure 6. Dependency of transfection efficiency (TE; bar graph) and the number of viable cells (VCN = viable cell number; line graph) on the incubation time (COS-1 = 1×10^5, $R = 11$, DNA = 4 μg, transfection time 6 h). After 4 days incubation, cells were approximately 100% confluent and re-plated in a 35-mm plate and incubated again in 10% FBS/DMEM (this operation caused deviation from exponential cell growth). FBS = fetal bovine serum, DMEM = Dulbecco modified Eagle medium.

The transfection process with the fullerene **1** (brown) could be directly observed with a microscope. Figure 7A is a differential interference contrast (DIC) micrograph taken 1 h after the addition of an aqueous DMF solution of the **1**/DNA complex to COS-1 cells from which most of the solvent

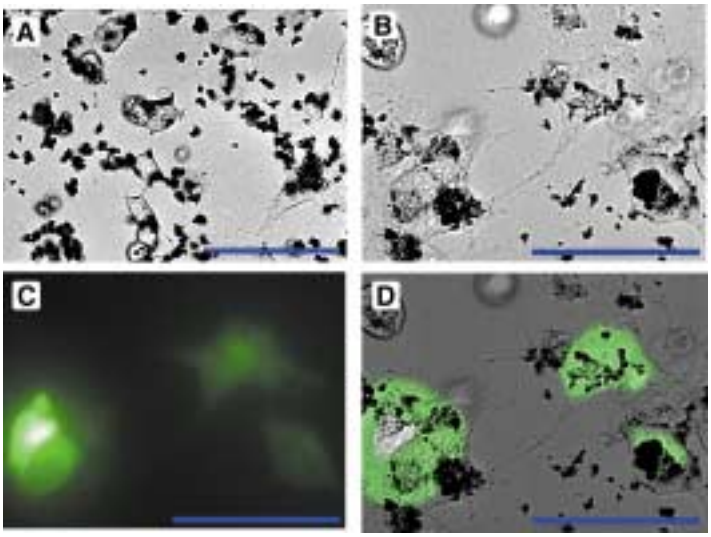


Figure 7. Transfection of the GFP plasmid into COS-1 cells by using **1**. Inset blue bars show 100 μm. A) DIC micrograph of COS-1 cells and small particles (2–10 μm) of the fullerene/DNA complex after 1 h transfection time; B) DIC micrograph of COS-1 cells and taken-up fullerene/DNA complex after 2 days incubation time; C) fluorescence micrograph of (B); D) superimposed photograph of (B) and (C), the round black clumps of 2–10 μm observed within the fluorescent cells (as in the one center right) contain fullerene material(s).

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has been removed (this experiment generated the data presented in Figure 5). The micrograph shows a plate surface on which there are several cells and many black spots of the fullerene/DNA complex. These black spots, of irregular outline, are more densely distributed on the cells than on the bare plate surface. Figures 7B and C are the DIC and fluorescence micrographs of the cells taken in the same series of experiments after 2 days incubation with **1**. As seen more clearly in Figure 7D, which is the superimposition of Figure 7B and 7C, the morphology and the distribution of the black objects in the fluorescent cells are different from those of the objects outside the cells. These black globules of 2–10 μm in diameter are located within the cytoplasm of the fluorescent cells, indicating that the fullerene/DNA complexes have been taken into the cells during incubation.

Departing from the conventional lipid-mimicking strategy for designing transfection reagents, we have synthesized an entirely new class of transfection agent (**1**) through tailor-made functionalization of [60]fullerene. The structure/activity relationship of the DNA binding properties indicate that the structural synergy in **1** plays an important role in binding. The efficiency of gene transfer by this first-generation fullerene-based transfection reagent is already comparable to that of commercial reagents. The morphology of large aggregates of the fullerene/DNA complex in the GFP-expressing cells indicates that the complex is taken up by cells through phagocytosis. The fullerene-based transfection reagent is unique among known transfection reagents in its ability to effect sustained protein expression for a prolonged period, suggesting that plasmid DNA in fullerene complex is stable inside phagosomes.

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Ternary Complexes Between DNA, Polyamine, and Cucurbituril: A Modular Approach to DNA-Binding Molecules**

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The design of a rational method for the delivery of a molecule to a target site of a biomolecule is an issue of general interest for chemists. Although a conventional targeting scheme relies on the covalent installation of a binding site on the molecule to be delivered (Scheme 1a), another intriguing approach is a modular strategy relying on a non-covalent many-body assembly of molecules as illustrated in Scheme 1b.[1] We report herein a chemical prototype of such supramolecular chemistry in the binding of a small molecule (**C**, that can not bind to DNA) to DNA (**A**) with the aid of a

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