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Synthesis and Complexation Ability of a Novel Bis-(guanidinium)-tetrakis-(β-cyclodextrin) Dendrimeric Tetrapod as a Potential Gene Delivery (DNA and siRNA) System. Study of Cellular siRNA Transfection

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The facile synthesis of a novel bis-(guanidinium)-tetrakis-(β-cyclodextrin) tetrapod, the first example of a new host family, was described, and the ability of the cyclodextrin CyD tetrapod to form molecular association with siRNA and DNA guest molecules was demonstrated. Affinity capillary electrophoresis was used to determine the binding constant with the evaluation of the shift in the electrophoretic mobility μ of injected siRNA when various CyD tetrapod concentrations were added to the run buffer. A significant association constant (K_a =16 000 M^{-1}) was obtained with borate buffer when double-stranded siRNA was primarily opened with the help of temperature. An efficient cellular transfection of siRNA into human embryonic lung fibroblasts was observed by fluorescence microscopy.

INTRODUCTION

Developing new, highly efficient, and nontoxic gene delivery carriers in cells remain an important challenge both in basic sciences and in clinical research fields. At the present time, viral vectors (clinical tests using adenoviruses, adeno-associate viruses, retroviruses, etc.) have been successfully applied, but their side effects such as severe pathogenicity limited further application (*1*). Besides, nonviral (cationic polymers, lipids, dendritic polymers, polyplexes, etc.) vectors have been studied as an alternative strategy for gene delivery because of their lower toxicity, non-immunogenicity, and convenient handling (*2*). However, the remaining difficulties with nonviral vectors remain with their relatively lower transfection efficiency than viral vectors. The poor performance was also due to polyplex aggregation as a consequence of decreasing water solubility induced by charge neutralization arising when electrostatic interaction between polyanionic DNA and polycationic vectors occurs (*3*).

Significant efforts for DNA delivery and more recently siRNA in cells have been conducted the past ten years through different nonviral approaches using macromolecular systems as carriers (*4*−*6*). Prosaically, two families of molecules are represented, on one hand nanoparticulate systems (*7*−*10*) and on the other hand polymeric systems (*11*−*15*).

Early in 1995, cyclodextrins (CyDs) and derivatives were introduced as potent carriers for phosphorothioate−ODN delivery. It was demonstated that cellular uptake was concentration and time dependent (*16*). Further, numerous contributions appear from that time in which either native and modified cyclodextrins were evaluated as ligands for nucleotides, oligonucleotides, and DNA (*17*−*19*).

Our first report on the subject suggested early in 1997 monothiogalactosyl and heptakis-thiogalactosyl-β-cyclodextrins as novel nonpolymeric efficient vectors for small antisense-ODN (*20*). Some other examples have been reported in the literature on complexes of CyD-DNA as, e.g., CyD−PAMAM dendrimeric conjugates (*21*) or bis-amidinium-CyDs polymers (*22, 23*) and their corresponding PEG derivatives, including monosaccharidyl inclusion polyplexes (*24*). The latter has been demonstrated further as totally biocompatible and efficient to transfect DNA in cells. In the same way, some liganded-CyDs cluster-organized, and dendrimers have been found able to form molecular associations with ODNs (*25, 26*). Furthermore recently, a family of sophisticated systems based on polymer−Cyd associations such as nanostructured multilayers using polylysine−CyD multilayers (*27*), polyelectrolyte multilayers (*28*), low weight or linear polyethyleneimine−CyD polymers (*29, 30*), or amphiphilic cationic CyDs (*31*) were successfully tested as *in vitro* nonviral gene delivery vectors. In parallel, the "molecular umbrella" tetrameric design and synthesis of nonviral noncyclodextrinyl vectors, notably based on the bottom-up construction of supramolecular systems, has been developed and has been shown to be able to cross the membrane of living cells and concentrate at the nucleus and therefore could be considered interesting molecular systems for nuclear drug targeting (*32*−*34*).

Here, we report in this way the synthesis of a novel bis-(guanidinium)-tetrakis-(β-cyclodextrin) dendrimeric tetrapod as a discrete molecular system, characterization of its self-assembly with a short interfering RNA (siRNA) oligonucleotide and single-stranded DNA, ability of the tetrapod to form molecular association with siRNA and DNA by affinity capillary electrophoresis, and siRNA *in vitro* transfection results.

EXPERIMENTAL SECTION

Materials. Chemicals for the synthesis, benzyl alcohol, and all other chemicals and solvents for electrophoresis experiments were of analytical grade and were purchased from Sigma-Aldrich. Ultrapure-grade water was obtained in a Milli-Q system from Millipore (Bedford, MA). MRC-5 cells (human pulmonary embryonic fibroblasts, ATCC CCL-171) were obtained from BioMerieux (France). The short interfering RNA (siRNA) oligonucleotide used is a 21 bp random duplex siRNA, purified and desalted, with no known homology to any mammalian

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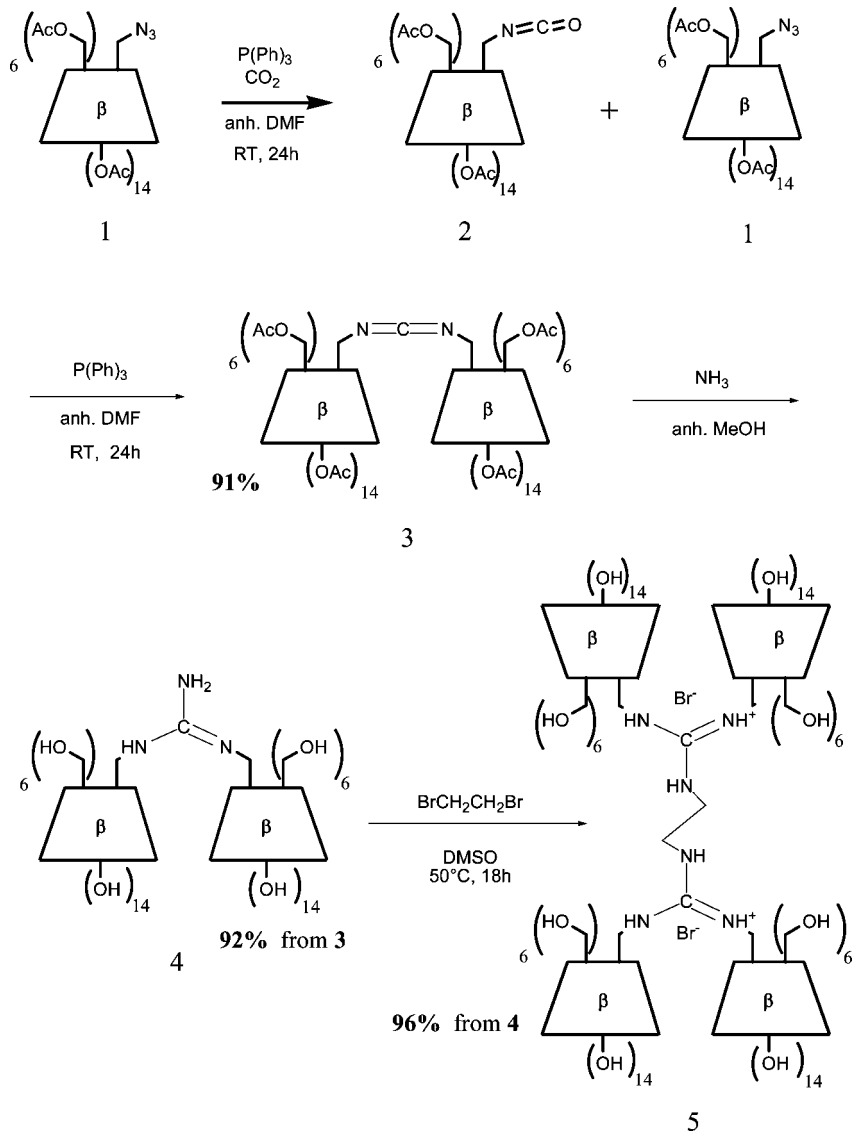
Scheme 1. Synthetic Scheme of CyD Tetrapod 5



sequence, labeled by Cy3 fluorochrome. siRNA stock solution was provided by Eurogentec (siRNA 633790−637791 SCB-1, Eurogentec, France) at a concentration of 100 μM. Modified essential medium (MEM, 41090, Invitrogen) supplemented with 10% decomplemented fetal bovine serum (FBS, 10270, Invitrogen, lot 40Q5150K).

Synthesis. The pure tetrapod **5** was obtained in fairly good yield (96%) and in two steps from a carbodiimide-β-cyclodextrin dimer **3**, which is afforded by the "phosphine imide" strategy from the monoazido-CyD (Scheme 1). Compounds **1**, **2**, and **3** have been synthesized by known procedures in the literature (*35, 36*).

Synthesis of 6-Monoguanidino-(bis-β-cyclodextrin) Dimer 4. Carbodiimide **3** (1.40 g; 0.615 mmol) (*35*) was dissolved in dichloromethane/methanol (5:1 by volume, 60 mL) and the solution was stirred at 25 °C under NH_3 for 18 h. After evaporation of the solvent, the residue was dissolved in methanol (5 mL) and precipitated with diethyl ether, filtered, and evaporated to dryness to give pure **4** (92%). FTIR (KBr): ν = 3374.5 cm^{-1} (OH);1736−1663 cm^{-1} (N═C−(NH_2)−NH). ESMS: 2292 [M + H^+]. ^{1}H NMR (400 MHz, D_2O, 25 °C, TMS): δ = 5.07 (m, 14H; H1), 3.95 (m, 14H; H3), 3.90−3.80 (m, 26H; H6a; H5), 3.71−3.62 (m, 16H; H6b; H2), 3.51 (m, 14H; H4). ^{13}C NMR (100 MHz, D_2O, 25 °C, TMS): δ = 175.1 (N═C−(NH_2)−NH), 102.2 (C1), 81.5 (C4), 73.4 (C2), 72.3 (C3), 72.2 (C6), 60.6 (C5).

Synthesis of the Bis-guanidinium-tetrakis-β-cyclodextrin Tetrapod 5. 6-Monoguanidino-(bis-β-cyclodextrin) dimer **4** (0.622 g; 0.271 mmol) and 1,2-dibromoethane (0.025 g; 0.135 mmol; 0.5 equiv) was dissolved in DMSO (25 mL), and the solution was stirred at 50 °C for 18 h under argon. The reaction was stopped by precipitation with diethyl ether (100 mL) at 25 °C and filtered. The residue was dissolved in methanol/water (1:2 by volume, 53 mL). This solution was concentrated to dryness, and the residue was dissolved in water (100 mL). After lyophilization, this solution gave pure **5** (96%). Found: C, 33.02; H, 6.53; N, 1.58; S, 5.31. $C_{127}H_{286}Br_2N_6O_{136}$, 10DMSO, 19$H_2O$ requires C, 32.96; H, 7.23; N, 1.57; S, 5.99. FTIR (KBr): ν = 3359.7 cm^{-1} (OH);1737 cm^{-1}; ($^+$NH═*C*−(NH_2)−NH, Br^-) 1639 cm^{-1}. ESMS: 3662 [M − (β-CyD−NH)$^+$]. MALDI-TOF: 4591 [M − 18]$^+$, 2292 [dipode **4** + H]$^+$. ^{1}H NMR (400 MHz, D_2O, 25 °C, TMS): δ = 5.10 (m, 28H; H1), 3.99 (m, 28H; H3), 3.95−3.81 (m, 52H; H6a; H5), 3.74−3.65 (m, 32H; H6b; H2), 3.50 (m, 28H; H4), 3.17 (m, 4H; CH_2). ^{13}C NMR (100 MHz, D_2O, 25 °C, TMS): δ = 174.0 (NHBr═*C*−(NH_2)−NH), 102.3 (C1), 81.6 (C4), 73.6 (C2), 72.4 (C3), 72.3 (C6), 60.7 (C5), 22.3 (CH_2).

Capillary Electrophoresis. All experiments were performed on a Beckman P/ACE 5500 model (Beckman, Fullerton, USA) equipped with a diode array detector (190−600 nm). Capillary temperature was controlled by coolant at 25.0 °C. An untreated

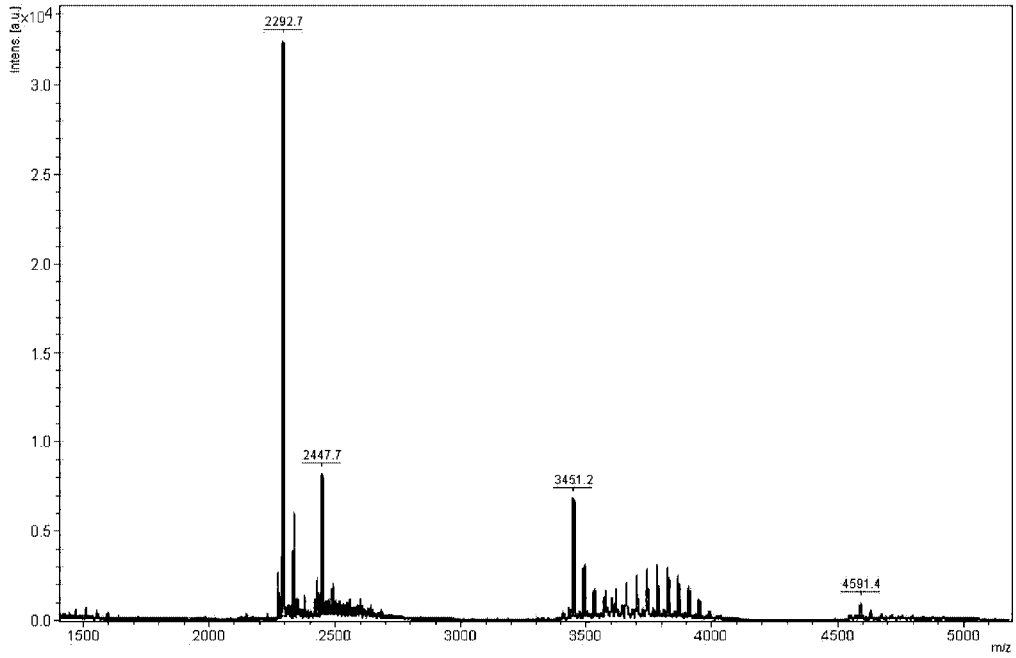


Figure 1. MALDI-TOF MS of Tetrapod **5**.

fused-silica capillary 75 μm i.d. with a total length of 47 cm (effective length 40 cm) was employed. All samples were injected in the hydrodynamic mode (10 s under pressure) at 20 kV. The experimental setup, data acquisition, and processing were governed using Beckman P/ACE Station software. The background electrolyte (BGE) was prepared by dissolving 2 μmol to 50 μmol of CyD tetrapod **5** in the appropriate borate buffer (25 mM, pH 9.3). Everyday, at the beginning of a run batch, the capillary was rinsed with 0.1 M sodium hydroxide (20 min) and ultrapure water (20 min). Before each injection, the capillary was successively rinsed with 0.1 M sodium hydroxide (2 min), ultrapure water (2 min), and BGE (2 min). All solutions introduced in the capillary were first percolated through an Alltech (Tampleuve, France) regenerated cellulose filter (0.45 μm pore size).

Electrophoresis Sample Preparation. Sample solutions were obtained by dissolving biological material in ultrapure water at the final concentration of 2 μM. Double-stranded siRNA was opened with temperature (94 °C) in the presence of CyD tetrapod **5** and diluted in run buffer at 0.2 μM before injection.

Cell Line, Cell Culture. The MRC5 cell line was cultured in Modified Essential Medium without antibiotics at 37 °C, 5% CO_2, under a humid atmosphere. Cell viability was determined by the trypan blue dye exclusion method. MRC-5 cells were plated at 5×10^5 cells/slide flask (170920, Nunc). For all experiments, cells were allowed to adhere and grow for 24 h in culture medium prior to siRNA transfection.

Transfection Complex (CyD Tetrapod/siRNA). First, we prepared an aqueous solution of tetrapod **5** at a final concentration of 2.5 mg/mL. The solution was sterilized through a 0.22 μm filter (SLGP033RS, Millipore). Second, we added 57.2 μL of this aqueous solution of CyD **5** to 12 μL of siRNA stock solution. After that, the solution was heated at 94 °C during 3 min, and then we controlled the cooling (less 0.8 °C per 5 s) until a final temperature of 2 °C was reached. This step is supposed to permit siRNA and CyD tetrapod **5** to complex together. Finally, we added this solution to 12 mL of MEM supplemented with 2% decomplemented fetal bovine serum in order to obtain our transfection solution.

***In Vitro* Transfection.** To start the transfection reaction, culture medium was replaced by 2 mL of this preparation (assay). Negative controls were transfected with either (i) CyD tetrapod **5** in the absence of any siRNA or (ii) siRNA in the absence of any CyD tetrapod **5**. Then, slides were incubated 37 °C, 5% CO_2, under a humid atmosphere, until appropriate times.

Table 1. Linear Regression Obtained with Equation 1 and Corresponding Binding Constants *K* for Single Stranded (ss) DNA in Phosphate Conditions (phos.) and for Single-Stranded DNA and Double- (ds) and Single-Stranded siRNA in Borate Conditions (bor.)

analyte	slope	intercept	corr. coef.	$K \pm$ s.d. M^{-1}
ss DNA phos.	1.818	7730.294	0.9996	4250 ± 750
ss DNA bor.	1.513	9189.418	0.9927	6050 ± 1550
ds siRNA bor.	~0	–	–	–
ss siRNA bor.	0.630	10187.151	0.9992	16150 ± 3700

^{1}H NMR and ^{13}C NMR Spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker DRX-400 (400 MHz and 100 MHz, respectively).

Fluorescence Microscopy. In order to perform fluorescent microscopy analysis, cells were fixed with a 4% formaldehyde solution (in PBS) for 1 min and subsequently washed 3 times with PBS. Then, cells were counterstained with Hoechst 33342 for 1 min and washed 3 times in PBS. Cells were mounted in PBS and examined by fluorescence microscopy with appropriate filters (microscope Axioskop SIP 48843, Zeiss, and camera AxioCam MRC, Zeiss). Observations were made after 6, 12, and 24 h of exposure with a 400× magnification.

RESULTS AND DISCUSSION

Synthesis and Characterization of CyD Tetrapod 5. Tetrapod **5** was synthesized according to our previous report (*37*) using the "phosphine imide" efficient strategy (also named Staudinger-Aza-Wittig tandem reaction) developed by our team in four steps from the monoazido-β-CyD (*35*). Scheme 1 shows the entire synthetic procedure of the tetrapod **5** available in a large-scale batch. The structure of **5** was confirmed by FTIR, ^{1}H and ^{13}C NMR, MALDI-TOF, and elemental analysis. Figure 1 shows the MALDI-TOF MS of **5**.

Self-Assemblies of CyD Tetrapod 5 and siRNA or DNA. Numerous analytical methods have been developed and applied in the study of the interactions between small ligands and biomacromolecules. Among them, some chromatographic

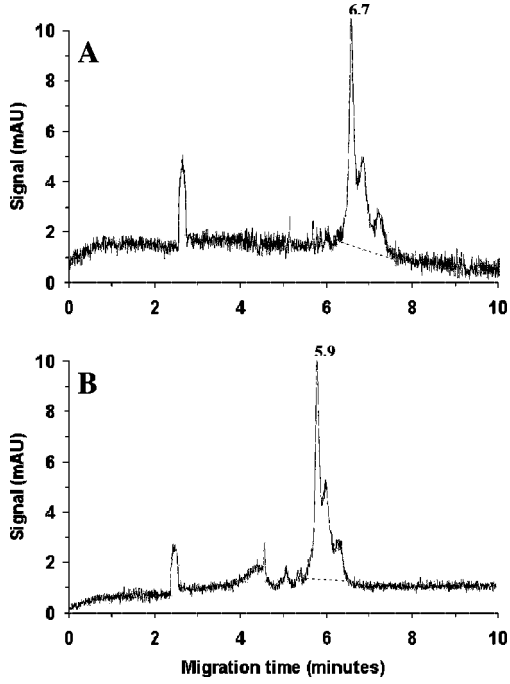


Figure 2. Electrophoregrams of single-stranded siRNA with background electrolyte containing 0 (A) and 25 μM of CyD (B). (Electrophoretic conditions were described in the Experimental Section).

methodologies, such as affinity chromatography or size exclusion, have also been employed to determine binding parameters (*38, 39*). Capillary electrophoresis (CE) is an attractive method for such determination. Low sample consumption, rapid analysis speed, and high efficiency are advantages that make this technology a major tool of association constant measurement. Several CE modes are available for such measurement, such as affinity capillary electrophoresis (ACE), Hummel-Dreyer method (HD), vacancy affinity capillary electrophoresis (VACE), vacancy peak method (VP), frontal analysis (FA), or frontal analysis continuous capillary eletrophoresis (FACCE) (*40*−*43*). Measurement in the ACE mode is based on the change in electrophoretic mobility of the biomolecule due to complexation to the ligand added at various concentrations in the background electrolyte. Both DNA and siRNA were used in this work to demonstrate the ability of **5** to form stable complexes with oligonucleotides.

The binding constants were estimated by the changes in the migration time of the analytes based on the different additions of **5** (2 to 50 μmol) in running buffers. For 1:1 association, the change in solute mobility with changing ligand concentration is related to the following equation (double reciprocal plot) (*44*).

$$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)K}\frac{1}{[\mathrm{CyD}]} + \frac{1}{(\mu_c - \mu_f)} \qquad (1)$$

where μ_i is the experimentally measured electrophoretic mobility of the solute, μ_f is the mobility of the free (uncomplexed) solute, μ_c is the electrophoretic mobility of the solute−ligand complex, K is the binding constant, and [CyD] is the equilibrium ligand concentration. Binding constants can be estimated by varying the ligand concentration at constant solute concentrations and fitting the data by linear regression to eq 1. To develop and optimize the electrophoresis methodology, a single-stranded DNA primer was primarily used. It is well-known that capillary electrophoresis of biological material such as DNA with BGE containing borates could lead to inappropriate complexation study. In a first approach, the single-stranded DNA under evaluation was used with two electrolyte conditions: borate 25 mM pH 9.3 and phosphate 50 mM pH 9.0. As illustrated in Table 1 lines 1 and 2, both conditions lead to equivalent binding constants (similar order) indicating that borates have no influence on the association process with the CyD tetrapod **5**.

SiRNA was first used without temperature treatment (double strand not open), and no binding constant could be determined as no modification of the analyte migration time was observed (Table 1, line 3). When siRNA was opened (single-stranded configuration), the addition of CyD to the background electrolyte led to a significant decrease of the electrophoretic mobility, as

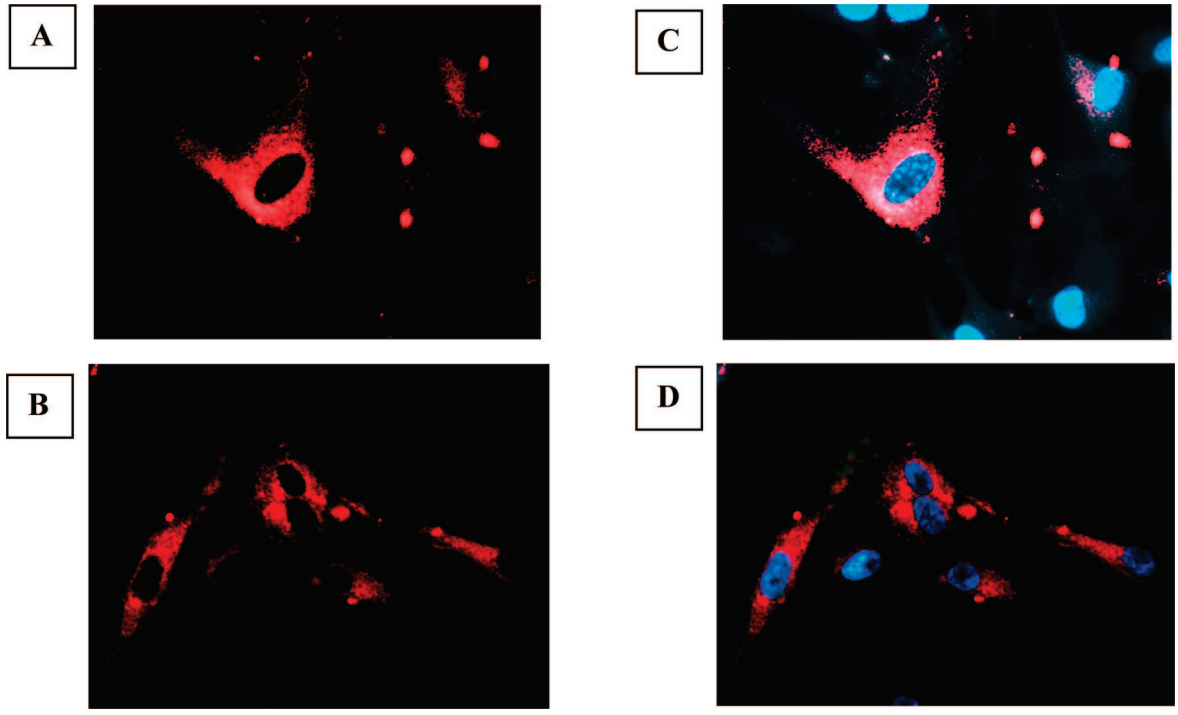


Figure 3. (A) Cy3-labeled siRNA at 6 h after injection; (B) after 12 h; (C) Cy3-labeled siRNA at 6 h after injection with Hoechst counterstained nuclei; (D) Cy3-labeled siRNA at 12 h after injection with Hoechst counterstained nuclei.

illustrated in Figure 2 for 0 and 25 μM of CyD added. The association constant obtained (16 150 M^{-1}, Table 1, line 4) indicates the ability of ligand **5** to form a strong association with such a biological molecule.

Toxicity Experiments. Toxicity experiments on tetrapod were performed *in vitro*, by measuring cellular viability (e.g., MTT assay) in the presence of increasing amounts of tetrapod. We have demonstrated that the tetrapod was poorly cytotoxic on MRC-5 cells, with a greater toxic effect for prolonged exposure times (e.g., 168 h), and concluded that it weakly affects MRC-5 cell viability (*37*).

Transfection Experiments. For transfection experiments, we used an siRNA labeled with Cy3 (see Experimental Section) to investigate the intracellular delivery and traffic of siRNA by the CyD tetrapod **5** in MRC-5 cells. Negative controls (see Experimental Section for details) showed no Cy3 labeling (data not shown), while numerous Cy3-stained cells, showing an intense red fluorescence, were detected in tetrapod-siRNA transfected MRC-5 cells, and thus until 6 h post-transfection (Figure 3A,C).

Hence, we demonstrated that a final concentration of 100 μM of scrambled siRNA is sufficient for an efficient cell transfection until 6 h of incubation with our tetrapod (Figure 3A). Nevertheless, the transfection reaction is clearly time-dependent, with a more intense fluorescence for 12 h transfected cells (Figure 3C). We observed the same staining at 24 h post-transfection. Figure 3B,D, showing parallel Hoechst 33342 (e.g., blue fluorescence) and Cy3 (e.g., red fluorescence) staining on the same field, at both 6 and 12 h, respectively. Hoechst labeling was used to counterstain nuclei of MRC-5 cells. Hence, we demonstrated a, siRNA transfection and/or localization in the cytoplasm (e.g., at the periphery of nucleus) of transfected MRC-5 cells.

We have also demonstrated that the efficiency of CyD tetrapod **5** is comparable to these of commercial compounds, i.e., PEI as previously described by Ikeda (*45*) and Ou (*46*); but it is noteworthy that CyD tetrapod **5** is also clearly less toxic than such compounds.

In summary, this novel tetrapod has high gene transfection efficiency, low cytotoxicity, and great potential for gene delivery *in vitro*. From the above data, bis(guanidinium)-tetrakis-(β-CD) **5** has a significantly high gene transfection in primary human lung fibroblast cell line (MRC-5), and it could be a promising gene carrier in many other primary cancer or stem cells. We actually attempted to validate the efficiency of delivery by silencing cellular "housekeeping" gene.

CONCLUSION

We synthesized a novel bis-(guanidinium)-tetrakis-(β-cyclodextrin) dendrimeric tetrapod for gene delivery systems. The compound presented here is the first example of a new molecular family including future CyD linear oligomeric analogues (penta-, hexapod, etc.) presently in progress. Tetrapod **5** could form 1:1 supramolecular water-soluble complexes with single-stranded DNA and siRNA as shown by capillary electrophoresis, and a better association with siRNA instead DNA was demonstrated. Elsewere, efficiency of siRNA and DNA transfection in cells is comparable to existing polyplex or polycationic systems. Toxicity experiments reveal that CyD tetrapod **5** is less cytotoxic than existing PEI cationic polymers. Finally, we can conclude that CyD tetrapod **5** is efficient not only for complexation of nucleic acids, but also for transfection of siRNA.

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