Preparation of Fluorescent Silica Nanotubes and Their Application in Gene Delivery

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The application of nanomaterials such as nanoparticles, nanotubes, nanorods, and nanowires in biological systems has attracted great interest in the fields of materials science and biochemistry. Because of their dimensions, which make them suitable for application in biological systems, the potential of nanomaterials for biolabeling, biodetection, bioseparation, and biomolecule delivery has been explored. In particular, the use of some inorganic nanomaterials as biomolecule carriers has been shown; gold nanoparticles and nanorods, for example, have been employed in DNA delivery. Unlike nanoparticles or nanorods, nanotubes have a unique, hollow structure, which allows the modification of their inner surface and filling with specific biomolecules. In addition, the tube structure may act as a physical shield for the inserted biomolecules and provide advantages for biomolecule delivery. However, the applications of nanotubes as biomolecule carriers are still very rare. In this work, fluorescent silica nanotubes with an inner diameter of hundreds of nanometers are synthesized by a sol–gel reaction using an anodic aluminum oxide (AAO) membrane as a template. The green- and red-fluorescent silica nanotubes are obtained by incorporating CdSe/ZnS core–shell semiconductor nanocrystals with diameters of about 4 nm and 8 nm, respectively. The fluorescent nature of the nanotubes allowed us to visualize their localization in living cells. The inner surfaces of the nanotubes were coated with positive charges to provide efficient DNA loading. We found that nanotubes filled with the gene encoding green fluorescence protein (GFP) entered monkey kidney COS-7 cells and that these cells exhibited GFP expression. These results demonstrate a novel application of nanotubes in biomolecule delivery.

Figure 1 describes briefly the preparation of fluorescent silica nanotubes (fNTs) and their use for gene delivery. A commercial AAO membrane with an average pore diameter of about 200 nm was employed as the template for silica-nanotube preparation. A layer of silica was coated on the entire surface of the membrane by a sol–gel process using tetraethoxysilane as reactant. The silica layer was then passivated with a monolayer of 3-(aminopropyl)trimethoxysilane (APTMS) to generate a polycationic surface. The resulting membrane was placed in a CdSe/ZnS core–shell nanocrystal solution to form a nanocrystal layer on the silica surface due to the electrostatic forces. An additional silica layer was then coated onto the membrane to protect the nanocrystals from oxidation. In order for the nanotubes to be usable in DNA delivery, the silica surface was further passivated with a polycationic layer of APTMS to facilitate DNA loading. The membrane was then mechanically polished to remove the silica from the top and bottom surfaces of the membrane until the resulting membrane had a thickness of approximately 1–3 μm. The membrane was subsequently removed to release the nanotubes. The resulting silica nanotubes were well-dispersed and chemically stable in the water-based solution.

Figure 2 shows a typical transmission electron microscopy (TEM) image of individual silica nanotubes; they had a hollow structure with two open ends. The average outer diameters and length of the nanotubes were approximately 200 nm and 2 μm, respectively, and the wall thickness was about 20–30 nm. The outer diameter was similar to the channel diameter of the template membrane. The TEM analysis reveals that less than 1% of the nanotubes were longer than 5 μm, which shows that the nanotube length is controlled quite well by the mechanical-polishing method. The fNTs had ample internal space (approx. 10 3 nm 3) for subsequent DNA insertion.

Green- and red-fluorescent silica nanotubes were generated by the incorporation of CdSe/ZnS core–shell nanocrystals with diameters of about 4 nm and 8 nm, respectively, during nanotube preparation. The nanotubes clearly exhibited fluorescence under a confocal microscope (Fig. 2, inset). An energy-dispersive X-ray spectrum taken along with the TEM shows that the fNTs contained the major constituent elements of the nanocrystals. In addition, the emission peaks of green and red fNTs at about 520 nm and 630 nm, respectively, agree well with the values of solutions containing free nanocrystals. Taken together, these data show that the fluorescence of fNTs comes from the embedded core–shell nanocrystals. As the fluorescence of the nanocrystals was bright, easily distinguishable, and resistant to photobleaching, long-term observation of fNTs in cells became feasible.

To explore the potential application of these silica nanotubes as biomolecule carriers, we first investigated whether

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silica nanotubes can enter cultured mammalian cells by treating monkey kidney COS-7 cells with green-fluorescent fNTs. We found by confocal microscopy that the fNTs entered about 60–70% of the cells during the incubation, probably by endocytosis, and were mostly localized in the cytoplasm (Fig. 3). We also investigated the cytotoxicity of nanotubes in the cell under our experimental conditions by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Approximately 75–85% of the cells were still viable after the treatment with fNTs (200 μL, ca. 10^6 nanotubes μL^−1), which indicates that silica nanotubes are not especially toxic under our experimental conditions. No significant difference in cell viability was observed when the concentration of nanotubes in the culture was increased two- or threefold.

To investigate the possibility of using silica nanotubes as gene carriers, the inner surfaces of the transparent silica nanotubes (tNT; they do not contain nanocrystals) were coated with APTMS ligands to facilitate the subsequent loading of negatively charged DNA by electrostatic interactions. Plasmid DNA carrying the GFP gene was labeled with the green DNA stain SYTO-11 in order to monitor DNA localization, and subsequently loaded onto the tNTs to form fluorescent DNA (fDNA)/tNT complexes. Figure 4 shows a representative microscopy image of the fDNA/tNT complex under a confo-

**Figure 1.** Schematic illustration of fNT preparation and its application in gene delivery: 1) The AAO membrane coated with a silica layer is modified with APTMS to form a polycationic surface. 2) Core–shell nanocrystals are incorporated onto the polycationic surface. An additional silica layer and a subsequent APTM coating are layered over the nanocrystals (see text). 3) The fNTs with polycationic inner surfaces are generated by removal of the AAO membrane. 4) The plasmid DNA is inserted into the nanotubes to form a DNA/fNT complex. 5) The DNA/fNT complex enters the cell. The DNA may be released from the complex and subsequently transcribed in the nucleus.

**Figure 2.** TEM and fluorescent images of fNTs. The TEM microscopy image of fNTs shows a hollow structure with two open ends. The variation in the diameters of the nanotubes is likely caused by the size differences of the AAO-membrane pores. The green fNTs exhibited a rod-like structure under a confocal microscope (inset).

**Figure 3.** The microscopy image of cytoplasmic localization of fNTs in living cells. COS-7 cells incubated with green fNTs show the cytoplasmic localization of fNTs under a confocal microscope. The nuclear DNA (red) was stained with propidium iodide. A strong green fluorescence is also observed in both orthogonal sections of the cell under a confocal microscope.

**Figure 4.** The confocal microscopy image of the fDNA/tNT complex, which displays green fluorescence due to the DNA stained with SYTO-11 under a confocal microscope.
cal microscope, where the green fluorescence emitted by the DNA can clearly be seen. The IDNA appears to be co-localized with the nanotubes under the microscope. That the DNA was located inside the nanotubes was further supported by the observation that no IDNA was detected in nNTs whose inner surface was not coated with APTMS ligands.

To test whether the nanotubes may provide some protection for the loaded DNA, the IDNA/nNT complex and free IDNA were independently treated with DNase and monitored under a fluorescence microscope. The qualitative observations showed that the fluorescence of IDNA disappeared much faster than that of the IDNA/nNT complex. This result suggests that the nanotube wall may act as a physical shield that protects the loaded materials from potential environmental damage.

To explore the application of nanotubes in gene delivery, red-fluorescing nNTs were loaded with the GFP plasmid (without the SYTO-11 label) to generate the DNA/nNT complex, and the complex was added to COS-7 cells. As a control, two samples, one containing the free GFP plasmid and the other red-fluorescing nNTs, were each added to COS-7 cells under the same experimental conditions as the DNA/nNT complex. We found that cells treated with the DNA/nNT complex showed cytoplasmic GFP expression whereas the cells treated with free DNA or red nNTs did not (Fig. 5). Approximately 10–20% of the cells treated with the DNA/nNT complex expressed GFP. In addition, all GFP-expressing cells were found to also contain red nNTs. This observation suggests that the DNA delivered by the DNA/nNT complex is mediated by the nanotubes. Taken together, our results demonstrate that the GFP gene can be loaded in and delivered to cells by silica nanotubes.

Gene delivery has been achieved previously by several methods, including viral systems.[10] As viral systems are likely to cause unexpected cytotoxicity and immunogenicity after delivery, nonviral delivery systems such as lipids, dendrimers, and poly(ethyleneamine) have been developed as gene carriers.[11] Recently, inorganic nanomaterials such as gold nanoparticles and nanorods have also been employed for gene delivery.[12] In this work, we have shown a novel way of filling nanotubes with DNA by electrostatic forces and have demonstrated its application in gene delivery to living cells.

The efficiency of our nanotube-mediated transfection (ca. 10–20%) is less than that of conventional calcium phosphate transfection (ca. 60–70%). However, the cargo molecules carried by the nanotubes will not be limited to DNA in the future. Instead, RNA, proteins, and other biomolecules may also be loaded into the nanotubes. In addition, the size of the nanotubes can be adjusted as required according to the size and amount of cargo molecules, and a smaller nanotube vehicle might increase the transfection/delivery efficiency. Modification of the outer surface of silica nanotubes with chemical and biological molecules might allow their specific intracellular/tissue targeting, which will broaden and strengthen their biological applications.

**Experimental**

**Preparation of Fluorescent Silica Nanotubes (fNTs):** A silica sol was prepared from a mixture of tetraethyl orthosilicate (52 mL, 98%, Acros), ethanol (230 mL), H₂O (18 mL), and HCl (0.27 mL). After aging the sol at room temperature for 15 days, a highly ordered nanochannel array of anodic aluminum oxide (AAO, Whatman) was dipped into the sol–gel for 1 min at 4°C. The AAO membrane was then dried at 90°C under vacuum. After this coating procedure, the AAO membrane was rinsed with deionized water and methanol. The membrane was then placed in a solution of 10% 3-(aminopropyl)triethoxysilane (APTS, 97%, Aldrich) in methanol for 12 h and washed with methanol upon removal. The membrane was finally dried at 90°C for 1 h under vacuum.

CdSe/ZnS core–shell nanocrystals with diameters of about 4 nm and 8 nm were prepared according to previous reports [9]. The surface of the nanocrystals was exchanged with mercaptoproionic acid ligands (–S(CH₂)₃–COOH) to make them water-soluble. The resulting nanocrystals exhibited a narrow size distribution, as indicated by their sharp emission peak (decrease of inhomogeneous broadening) in the fluorescence spectrum. The AAO membrane with an APTMS-modified silica layer was placed in the nanocrystal solution for 12 h, and then rinsed with copious amounts of deionized water. The membrane was then washed repeatedly with methanol to remove free nanocrystals and dried at 50°C for 1 h under vacuum. Afterwards, the membrane was dipped into the silica sol–gel again at 4°C for 1 min and dried at 90°C for 1 h under vacuum. To prepare nanotubes with positively charged inner surfaces, the membrane was further soaked in the APTMS solution for 12 h. The resulting membrane was polished carefully on both the top and bottom sides with a commercial polishing machine used in the semiconductor industry (thickness precision: approx. 1 μm). Finally, the AAO membrane was dissolved entirely with HCl (1 mL, 5 M) and the residual nanotube product was washed with a large amount of water. The final nNTs were dispersed in water at pH 5 to form the nanotube solution for the further experiments described below. The concentration of the nanotube solution was about 10⁶ nanotubes/mL⁻¹, as estimated on the basis of the channel density of the AAO membrane (13 mm in diameter). The average

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**Figure 5.** Expression of GFP in cells incubated with the DNA-fNT complex. a) A bright-field image of cells incubated with the DNA-fNT complex. b) A confocal fluorescence image of the same cells showing GFP expression. c) A confocal fluorescence image of the same cells showing the red-fluorescing nNTs.
diameter and length of the nanotubes were obtained from about a hundred nanotubes in twenty transmission electron microscopy (TEM) images. The rNTs were characterized with a Jeol JEM-2010 TEM operating at 200 keV and a fluorescence spectrometer.

**Loading Plasmid DNA into the Silica Nanotubes:** The plasmid DNA encoding green fluorescence protein (GFP) (8 µg; Clontech) was added to the transparent nanotube (sNT) or rNT solution (200 µL) and mixed by rocking for 24 h at 4 °C. Unbound DNA was removed by washing with water several times. Finally, a CaCl₂ solution (2 µL, 2 M) was added to the DNA/nanotube complex solution, which was incubated for an additional 24 h. As a control, free DNA (8 µg) and rNTs without loaded DNA (200 µL) were treated independently with the CaCl₂ solution and incubated for 24 h under the same conditions as the DNA/nanotube complex.

**GFP-Transfection Experiments:** COS-7 cells were cultured in 10 cm dishes in Dulbecco’s Modified Eagle Medium (DMEM) with 10 % fetal calf serum (FCS) in the presence of 1 % penicillin-streptomycin. The cells were grown at 37 °C in a CO₂ incubator and passaged every 2–3 days. For transfection experiments, the cells were seeded on 3 cm dishes for 24 h. The serum-containing DMEM was then removed from the dishes and replaced by 200 µL of the DNA/rNT complex. After 6 h, the residual DNA/rNT complex in the solution was removed by washing with phosphate-buffered saline (PBS) and replaced with fresh serum-containing DMEM. The cells were incubated continuously for 48 h. For contrast experiments, the cells were treated with free plasmid DNA or rNTs under the same conditions. For observation, the cells were washed with PBS and observed with an epifluorescence or laser-scanning confocal microscope. The number of cells was counted by flow cytometry (Partec).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay: Different amounts of rNTs (from 100–400 µL in 50 µL intervals) were added to COS-7 cells. After 48 h incubation, the MTT assay (Acros) was performed as described by the manufacturer. In brief, the serum-containing medium was replaced by the MTT solution (200 µL). After incubation in the MTT solution for 4 h, the cells were collected by centrifugation and treated with dimethyl sulfoxide (500 µL). The optical density at 570 nm was measured with a spectrometer (Bio-TEK).

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Self-Assembly and Electronics of Dipolar Linear Acenes**

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Detailed below is an exploratory study into the synthesis, self-assembly, and electronics of new linear acenes (Fig. 1, compounds 1 and 2) that are end-functionalized with a 1,4-quinone moiety. These molecules are unique because they have a static dipole moment incorporated into the well-known organic semiconductor skeleton of pentacene. Organic transistors[1,2] are projected to be an integral portion of new lightweight, flexible, and inexpensive plastic electronics.[3] Here we show the utility of donor–acceptor-mediated self-assembly in thin-film transistors and emphasize how important it is to control the balance between the molecule–molecule and molecule–substrate interactions. The created acenes (Fig. 1a) form antiparallel co-facial stacks with aromatic planes closer than the π–π distance in graphite by approximately 0.1 Å. In thin films, atomic force microscopy (AFM) shows that the molecules assemble into lamellae with the molecular long axis upright. Tailoring the self-assembly of these lamellae on the gate-dielectric surface[4,5] provides long-lived devices for this.

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