DNA delivery

DOI: 10.1002/smll.200500193

Three-Dimensional Functionalized Tetrapod-like ZnO Nanostructures for Plasmid DNA Delivery**

*Leng Nie, Lizeng Gao, Ping Feng, Jingyuan Zhang, Xingqiu Fu, Yonggang Liu, Xiyun Yan, and Taihong Wang**

Due to their special electrical, optical, and magnetic properties, materials less than 100 nm in size are very promising for biosensors, bio-separation, and drug delivery.[1–3] In recent years liposomes and polymers have been used as carriers for transfections.[4] Certain inorganic materials such as silica nanoparticles, carbon nanotubes, and silica nanotubes have been used as transporters with little toxicity in mammalian-cell transfections.[5–7] These zero-dimensional nanoparticles and one-dimensional nanotubes suggest that nanomaterials, if modified properly, can be used as carriers for transfections. However, the application of three-dimensional nanostructures as biomolecule carriers is less well-studied.

Here, we report three-dimensional functionalized tetrapod-like ZnO nanostructures as novel carriers for mammalian cell transfections. In this work, silica-coated amino-modifed nanostructures were prepared. Through electrostatic interactions, ZnO tetrapods could be bound to plasmid DNA. When mixed with cells, the tetrapods attached to cell membranes. Just as phages stand on cells with six legs suitable for gene delivery, ZnO nanostructures stand on the cells with three needle-shaped legs for DNA delivery as a result of their tetrapodal shape. With three tips located on the cell surfaces, the opportunity of internalization of the tips by cells should be increased. In addition, the geometry of the tetrapods imply a much larger steric hindrance, which makes it difficult for the tetrapods to pass wholly through the cell membranes. Just as phages insert genes into cells without entering them, tetrapods delivered plasmid DNA into the cells while standing on the cell membrane. This result is helpful in decreasing any cytotoxic effects. These results demonstrate a novel application of tetrapod-like nanostructures for gene delivery.

[*] L. Nie, P. Feng, J. Zhang, X. Fu, Y. Liu, Prof. T. Wang
Institute of Physics, Chinese Academy of Sciences
100080 Beijing (China)
Fax: (+86) 108-264-9531
E-mail: thwang@aphy.iphy.ac.cn
L. Gao, Prof. X. Yan
Institute of Biophysics, Chinese Academy of Sciences
100101 Beijing (China)

[**] This work was partly supported by the Chinese Ministry of Education (Grant No. 705040), the Special Funds for Major State Basic Research Project (No. G2001CB3095), and the National Natural Science Foundation of China (Nos. 69925410 and 60236010).

Three-dimensional ZnO nanostructures were synthesized by thermal evaporation at 900 °C.[8] The nanostructures consisted of four needle-shaped tetrahedrally arranged legs connected at the center, forming a tetrapod-like ZnO structure. The legs were single-crystalline and stable in air, with a mean diameter of ≈80 nm and a length of 5–10 μm. As shown in Figure 1A, one of the needle-shaped legs was perpendicular to the substrate while the other three legs remained in contact with the substrate. Utilizing their tetrapodal shape, the ZnO crystals were used as stable field emitters by our group or as atomic force microscopy probing tips by Tohda and co-workers.[9,10]

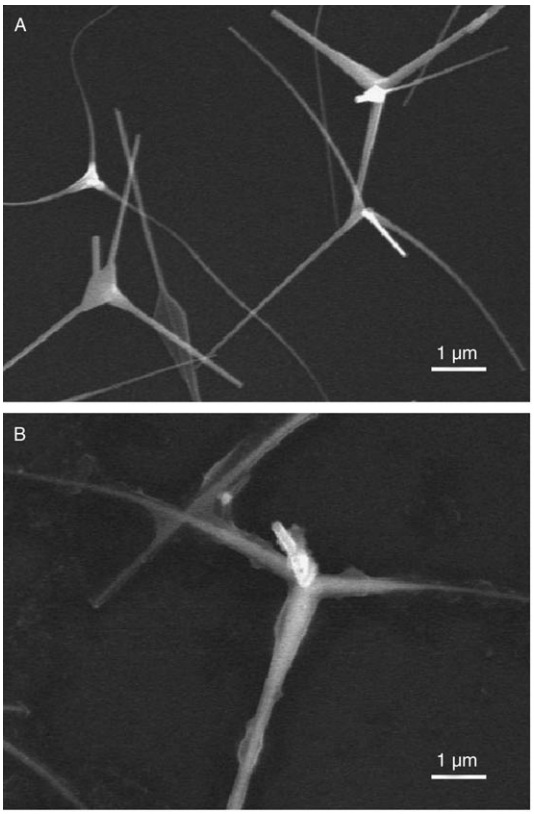

Figure 1. SEM images of tetrapod-like ZnO nanostructures, before (A) and after silica coating (B). After modification, the mean diameter of the nanostructures increased to ≈150 nm.

Inspired by the use of microneedles for transdermal drug delivery, it was considered to use needle-shaped ZnO legs to penetrate cell membranes for plasmid DNA delivery.[11,12] For binding plasmid DNA, tetrapods were coated with silica and amino groups by using a sol–gel process,[6,13] where both tetraethyl orthosilicate (TEOS, precursor of silica) and aminopropyltriethoxysilane (APTES, amino-modification reagent) were added to the reaction solution. The process was simple, and the amount of amino groups on the tetrapods could be controlled by using different ratios of APTES to TEOS. After the coating reaction, the core–shell nanostructures were separated from the reaction medium by centrifuging at ≈3000 rpm; they were then dispersed into ethanol. The separation procedure was carried

out several times to remove unwanted silica nanoparticles (produced during the coating procedure).

Since both the size and the weight of the tetrapod-like nanostructures were much higher than those of the silica nanoparticles, it was easy to separate the two species by centrifugation. As shown in Figure 1B, the diameter of the nanostructures increased ($\approx$150 nm) and their surfaces became more rough (amorphous silica) during the coating procedure; their tetrapodal shape was maintained. Energy dispersive X-ray anaylsis (EDX) indicated that after modification with both APTES and TEOS (in a ratio of 1:4), the weight percentage of nitrogen was over 7%. It is reasonable to assume that this nitrogen came from amino groups in APTES.

The quantity of amino groups on the tetrapods was measured by a Kaiser assay. As shown in Figure 2A, the number of amino groups on the tetrapods increased with an increase in the proportion of APTES used during the modification process. When the ratio of APTES to TEOS was 1:4, the concentration of amino groups on the tetrapods was $\approx$2.5 mmol g^{-1}, which suggested a high positive charge density on the surface in aqueous solutions.[14] Agarose gel electrophoresis was used to evaluate the conjugation of tetrapods with plasmid DNA. Amino-modified tetrapods (10 μg) were mixed with pEGFPN1 DNA (1 μL) for 3 min. After centrifugation, the supernatant was kept for electrophoresis. As shown in Figure 2B, plasmid DNA moved and dispersed in the gel under an electric field when no tetrapods were present (lane 1). When mixed with tetrapods modified only with TEOS, almost the same amount of DNA was found in the supernatant (lane 2). However, when APTES was used in the modification process, DNA in the supernatant decreased. When the ratio (APTES to TEOS) was equal to or over 1:4, DNA was not observed at all in the supernatant (lanes 5 and 6).

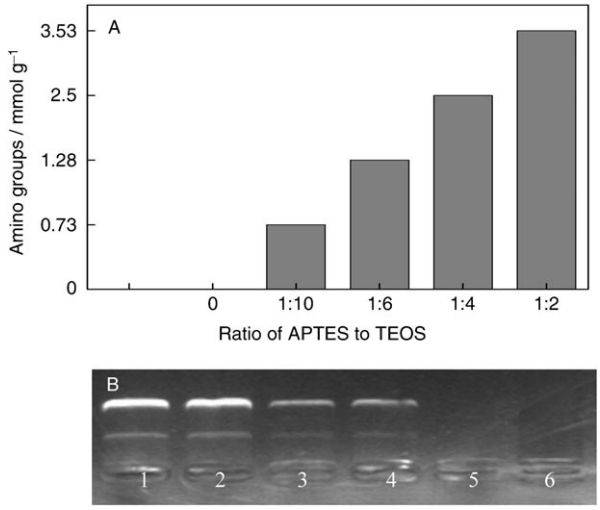

Figure 2. Agarose gel electrophoresis of the supernatant separated from the mixture of tetrapods and pEGFPN1 DNA: Plasmid DNA without tetrapods (lane 1), plasmid DNA mixed with tetrapods modified only with TEOS (lane 2), and plasmid DNA mixed with tetrapods modified with different ratios of APTES and TEOS; 1:10 (lane 3), 1:6 (lane 4), 1:4 (lane 5), 1:2 (lane 6). When the ratio was equal to or over 1:4, DNA was not observed in the supernatant (lanes 5 and 6).

Amino-modified tetrapods bound to pEGFPN1 DNA through electrostatic interactions between positively charged amino groups on the tetrapods and the negatively charged phosphate groups of DNA.[14,15] The ratio of phosphate to nitrogen was $\approx$1:1 when 1 μL of pEGFPN1 DNA and 10 μg of tetrapods (modified with both APTES and TEOS, ratio 1:4) were mixed together. Since they can effectively bind plasmid DNA, tetrapods modified with both APTES and TEOS (with the 1:4 ratio) were used for DNA delivery.

Tetrapods on cells were visualized with scanning electron microscopy (SEM). Either without tetrapods or with amino-modified tetrapods, A375 cells (a human melanoma cell line) were cultured on a glass cover slip in Dulbecco's modified eagle's medium (DMEM) culture. Without adding tetrapods, A375 cells attached to the cover slip (Figure 3A). When amino-modified tetrapods were added in the culture, tetrapods were found on the cell surface although the cover slip was washed several times with phosphate-buffered

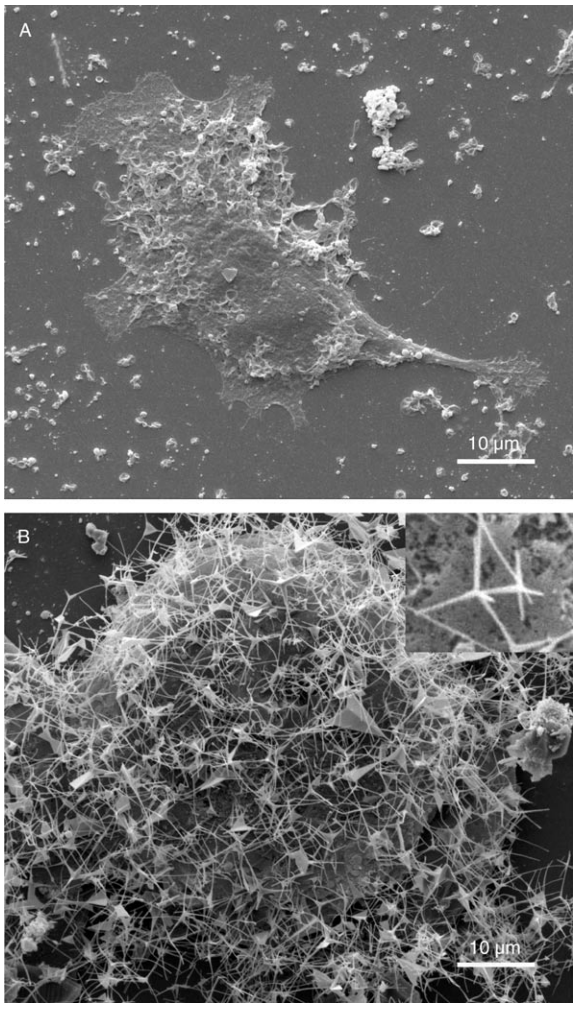

Figure 3. A) SEM image of an A375 cell on a glass cover slip before the addition of tetrapods. B) SEM image of an A375 cell cultured with amino-modified tetrapods. Although the cell was rinsed several times, tetrapods were still found on the cell membrane.

saline (PBS) buffer (Figure 3B). As they were not completely soluble, some tetrapods were deposited onto the cells by precipitation; the interaction between the positive charges of amino-modified tetrapods and the negative charges of cell membranes may have been helpful for the attachment of tetrapods onto the cells. Just as phages stand on the cells with six legs for gene delivery, some nanostructures stood on the cells with three legs. As a result of their tetrapodal shapes, one leg was, in some cases, perpendicular to the cell surface while the other three legs remained in contact with the cell membrane.

As amino-modified tetrapods can bind DNA and can attach to cell surfaces, they were used in an attempt to deliver plasmid DNA. ZnO tetrapods (10 μg) modified with both APTES and TEOS (ratio 1:4) were mixed with 2 μL of pEGFPN1 (containing GFP as a reporter gene) for 3 min. The tetrapod–DNA complexes were then introduced into cell culture solutions. The solution was further incubated for 48 h to allow GFP gene expression in the transfected cells. ZnO tetrapods without amino groups (modified with only TEOS) were used as controls. The number of cells emitting green fluorescence was counted under an inverted fluorescence microscope. Laser confocal microscopy was used to record images of the transfections. As shown in Figure 4B, green fluorescence was observed from some cells cultured with amino-modified ZnO tetrapods. The result indicated that green fluorescence proteins were successfully expressed in these cells and that there were exogenous GFP genes within the cells. However, no green fluorescence was found for cells cultured with tetrapods without amino groups (data not shown). Therefore, amino-modified tetrapod-like nanostructrues, as transfection carriers, can deliver plasmid DNA into mammalian cells.

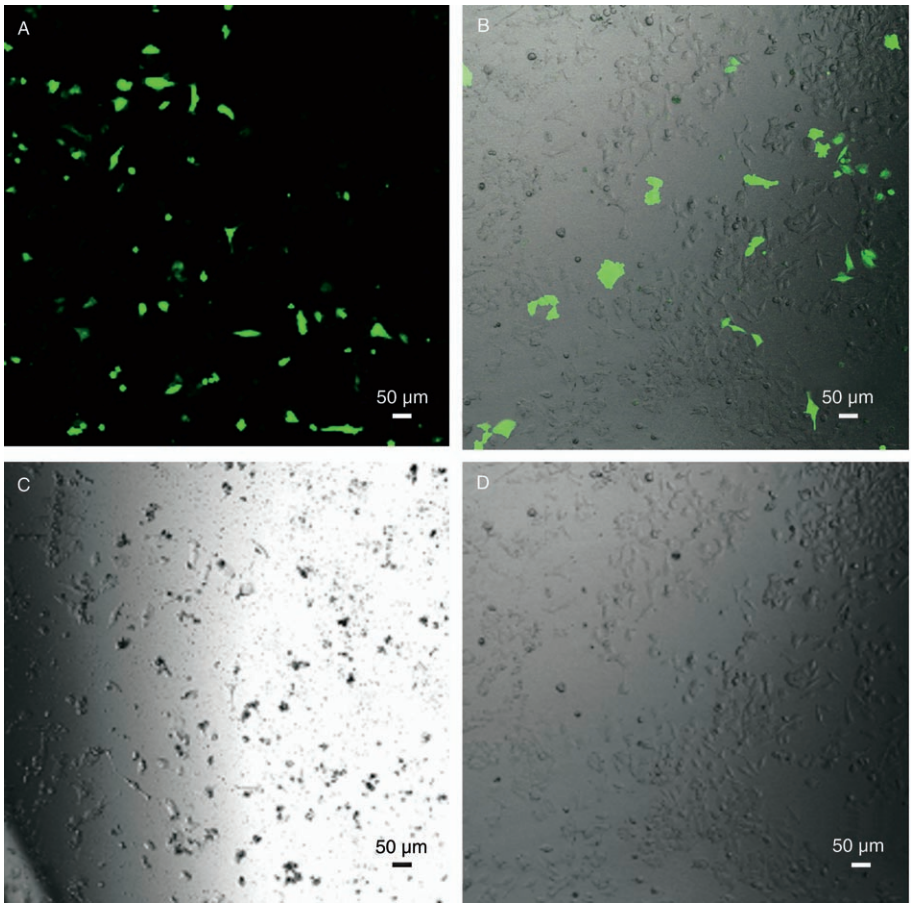

Figure 4. Confocal images of cell transfection: A) Fluorescence image of A375 cells cultured with lipofectamine 2000–DNA complexes. Green fluorescence was observed from some cells. B) Combined optical and fluorescence image of A375 cells cultured with tetrapod–DNA complexes. Green fluorescence was observed from some cells. C) Optical image of A375 cells cultured with lipofectamine 2000–DNA complexes. Several black spots (dead cells) were observed. D) Optical image of cells cultured with tetrapod–DNA complexes. Fewer black spots were found.

In addition, lipofectamine 2000–DNA complexes, zero-dimensional amino-modified ZnO nanoparticles, and one-dimensional amino-modified ZnO nanorods were used as positive controls. In experiments, ZnO nanorods and ZnO nanoparticles were coated with silica and loaded with DNA in the same way as for the ZnO tetrapods. ZnO nanorods (≈100 nm in diameter and ≈2 μm in length) were synthesized from aqueous solutions.[16] Using APTES and TEOS (in a ratio of 1:4), the nanorods and nanoparticles (≈20 nm in diameter) were coated. After modification, the diameter of the ZnO nanopariticles was ≈50 nm and the diameter of ZnO nanorods was ≈110 nm. Under an inverted fluorescence microscope, transfection efficiency was evaluated by the number of cells emitting green fluorescence (Figure 5A). The transfection efficiency was measured as follows: nanorods < tetrapods < nanoparticles < lipofectamine 2000.

It is well known that transfection efficiency depends on the size of carriers.[17] For nanoparticles with a small diameter (≈50 nm), cellular uptake was easy and transfection efficiency was high (Figure 5A). However, the transfection efficiency of tetrapods was better than that of nanorods, despite the fact that the size of the tetrapods (5–10 μm in length, ≈150 nm in diameter) was larger than that of the nanorods (≈2 μm in length, 110 nm in diameter). Similar to phages standing on cell membranes, with needle-shaped tips on the cell surfaces, three legs of the tetrapods stood obliquely (Figure 3B), thus the opportunity for internalization of the tips within the cells was increased, which may lead to an increase in transfection efficiency. Therefore, the tetrapod-like shape was helpful in transfecting DNA into mammalian cells.

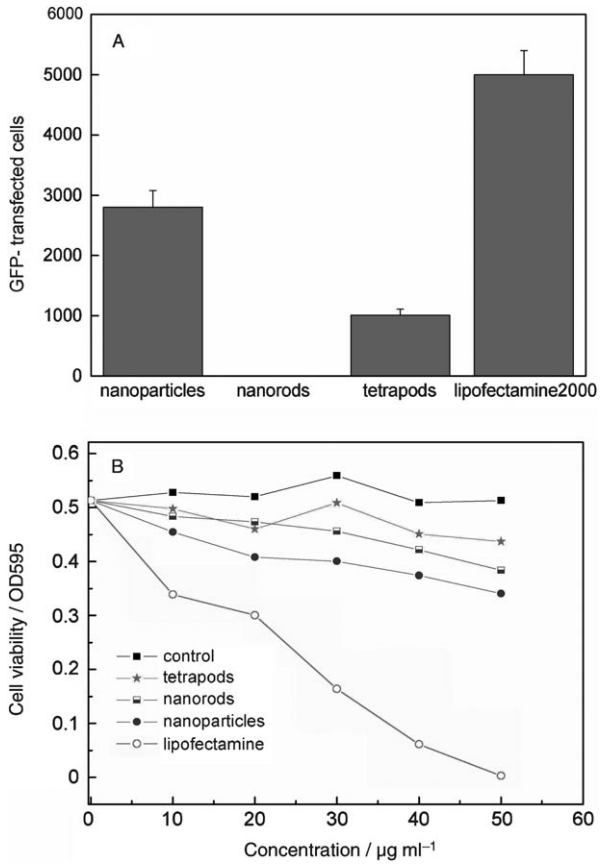

Figure 5. A) Transfection efficiency of different carriers. The transfection efficiency of tetrapods was higher than that of nanorods. B) MTT assay of carriers at different concentrations. The cytotoxicity of tetrapods was less than that of nanorods.

Cytotoxicity was also examined for this system. Figure 4C and D show optical confocal images of cells cultured with lipofectamine 2000–DNA complexes and tetrapod–DNA complexes, respectively. Dead cells appeared as black spots. Several black spots were observed in Figure 4C whereas fewer black spots were found in Figure 4D. These images indicated that the cytotoxicity of the tetrapodal nanostructures was less than that of liposome. In addition, an MTT assay (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used for further investigation. As shown in Figure 5B, tetrapods do not alter cell growth enormously, even at a high concentration (50 $\mu g\,mL^{-1}$). However, lipofectamine 2000 affected cell growth at a concentration of 10 $\mu g\,mL^{-1}$. The data was consistent with the results obtained through microscopic methods (Figure 4C and D). The degree of cytotoxicity was measured as follows: tetrapods < nanorods < nanoparticles < lipofectamine 2000.

ZnO nanomaterials coated with silica (a well-known biocompatible material) exhibited better biocompatible surfaces than liposome. Compared with nanoparticles, tetrapodal nanostructures showed less cytotoxicity and less efficiency. Compared with nanorods, tetrapods had lower cytotoxicity, but with better transfection efficiency. The geometry of the tetrapods implied a much larger steric hindrance, which makes it difficult for nanostructures to wholly pass through the cell membranes. Just as phages insert genes into cells without entering them, tetrapods delivered plasmid DNA into cells while standing on the cell membrane. Thus, tetrapod-shaped nanostructures were shown to reduce cytotoxicity.

In conclusion, we have reported the preparation of silica-coated amino-modified tetrapod-like ZnO nanostructures. The functionalized tetrapods can bind plasmid DNA through electrostatic interactions. When mixed with cells, the tetrapods attach to cell membranes and can stand on the cells using three needle-shaped legs for DNA delivery. The legs can stand obliquely on the cell surface, so that internalization of their tips within cells can be maximized. In addition, the geometry of the tetrapods makes it more difficult for these nanostructures to pass through the cell membranes, which reduces their cytotoxicity. The charge concentration, tetrapod-like shape, and biocompatibility of these functionalized nanostructures provide a basis for mammalian-cell transfection carriers.

Experimental Section

Silica coating and amino modification of tetrapod-like ZnO nanostructures: Tetrapod-like ZnO nanostructures (26 mg) were mixed with 2-propanol (20 mL) and ethanol (40 mL). Then deionized water (5 mL) and ammonia solution (1.5 mL, 25 wt%) were ten added into the reaction mixture. Under continuous mechanical stirring, APTES and TEOS in various ratios (0, 1:10, 1:6, 1:4, and 1:2; total 400 μL) were added into the reaction solutions. The reaction was allowed to proceed at room temperature for 8 h. After the coating reaction, the core–shell nanostructures obtained were separated from the reaction medium by centrifugation at ≈3000 rpm for 10 min. They were then dispersed into ethanol. The separation procedure was carried out several times to remove unwanted silica nanoparticles that were produced during the coating process. Dried in air, the samples were analyzed by SEM and EDX.

Preparation and modification of ZnO nanorods and nanoparticles: ZnO nanorods were synthesized from aqueous solution.[16] An equimolar (0.1 M) aqueous solution of zinc nitrate and hexamethylene tetramine ($C_6H_{12}N_4$) was placed in a standard laboratory oven. The solution was heated at 95 °C for 6 h. After the reaction, ZnO nanorods were separated from the reaction medium by centrifugation at 3000 rpm and then thoroughly washed with water to remove residual salt. The ZnO nanorods were then dried in air at room temperature. After that, ZnO nanorods or ZnO nanoparticles (≈20 nm in diameter, China Xiamen Lulijia company) were modified with both APTES and TEOS in the same way as for the tetrapods (described above).

Kaiser assay: Tetrapods (0.15 mg) modified with different ratios of APTES and TEOS (0, 1:10, 1:6, 1:4, and 1:2) were mixed with acetate buffer (200 μL, 1 M, pH 4.5) and 200 μL of a ninhydrin buffer (ninhydrin (150 mg), isopropanol (10 mL), acetic acid (0.3 mL), diluted in water to a 100 mL final volume). The mixture was treated at 100 °C for 20 min. After centrifugation at 10000 rpm for 10 min, the supernatant was detected with a spectrophotometer ($\lambda = 565$ nm). The amount of amino groups

on tetrapods was calculated based on the standard curve of glycine.

Agarose gel electrophoresis of tetrapod–DNA complexes: Tetrapods (10 μg) modified with different ratios of APTES and TEOS (0, 1:10, 1:6, 1:4 and 1:2) were mixed with 1 μL pEGFPN1 DNA (≈10 mg mL^{-1}, Clontech, USA) in water (30 μL) for 3 min at room temperature. After centrifugation at 3000 rpm, the supernatant was collected for electrophoresis. Agarose gel electrophoresis (1 % agarose) was used to examine the conjugation of tetrapods with DNA.

SEM analysis of tetrapods on cells: A375 cells were cultured directly onto a glass cover slip in 2 mL DMEM culture containing 10 % FBS, ECGS (0.03 mg mL^{-1}) and kanamycin (50 units mL^{-1}). Once the cells had grown to confluence, 10 μL of amino-modified ZnO tetrapods (2 mg mL^{-1}) was added to the culture after ultrasonic treatment and incubation with cells for 30 min. The cover slip was then removed from the growth medium and placed onto microscope slides. The cover slip was washed several times with PBS. Afterwards, A375 cells were submitted to the fixation and sputter coating procedure before SEM observation. As controls, A375 cells without tetrapods were also observed by SEM.

Cell transfection of plasmid DNA: A375 cells were cultured in 10 mL DMEM in a 24-well incubator. After the cells had grown to 60–80 % confluence, ZnO tetrapods (10 μg) modified with both APTES and TEOS (in a ratio of 1:4) were mixed with 2 μL of pEGFPN1 DNA (10 mg mL^{-1}) in deionized water (30 μL) for 3 min. The tetrapod–DNA complexes were then placed into cell culture medium. For negative controls, ZnO tetrapods without amino groups (modified only with TEOS) were employed. For positive controls, 2 μL of lipofectamine 2000 (1 mg mL^{-1}) and 2 μL of pEGFPN1 were dispersed in 50 μL of DMEM for 10 min. Then the mixture was incubated with the cells in DMEM medium. In addition, amino-modified nanoparticles and amino-modified nanorods, coated with silica (1:4 ratio of APTES to TEOS) were used as positive controls. 10 μg of nanoparticles or nanorods were mixed with 2 μL of pEGFPN1 DNA (10 mg mL^{-1}) in deionized water (30 μL) for 3 min. The resulrtant nanoparticle–DNA or nanorod–DNA complexes were then placed into cell culture. The number of green fluorescent cells was counted under an inverted fluorescence microscope. At high resolution, the boundary between cells was clear and it was possible to judge which cells were fluorescent by comparing the optical image with the fluorescence image. Laser confocal microscopy was used to record images of the transfections.

MTT assay: A375 cells were cultured in 96-well plates with DMEM and antibiotics. When the cells reached 80 % confluence, lipofectamine, amino-modifed ZnO nanoparticles, amino-modified nanorods, and amino-modified tetrapods were added into the culture solution with the following final concentration: 10, 20, 30, 40, and 50 μg mL^{-1}, respectively. All of these ZnO nanomaterials were modified with both APTES and TEOS (in a ratio of 1:4). After incubation for 12 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the wells to give a final concentration of 1 mg mL^{-1}. The solution was then incubated with the cells for 4 h at 37 °C. The cells were washed with PBS, and then incubated with DMSO (80 μL) at room temperature for 3 min and detected at a wavelength of 595 nm. Cells without ZnO nanomaterials were cultured as controls.

Keywords:
DNA · silica · tetrapods · transfection · zinc oxide

[1] S. J. Park, T. A. Taton, C. A. Mirkin, *Science* **2002**, *295*, 1503 – 1506.
[2] W. C. W. Chan, S. Nie, *Science* **1998**, *281*, 2016 – 2018.
[3] J. Wang, G. Liu, B. Munge, L. Lin, Q. Zhu, *Angew. Chem.* **2004**, *116*, 2210 – 2213; *Angew. Chem. Int. Ed.* **2004**, *43*, 2158 – 2161.
[4] S. Zhang, Y. Xu, W. Qiao, D. Liu, Z. Li, *J. Controlled Release* **2004**, *100*, 165 – 180.
[5] C. C. Chen, Y. C. Liu, C. H. Wu, C. C. Yeh, M. T. Su, Y. C. Wu, *Adv. Mater.* **2005**, *17*, 1404 – 1407.
[6] D. Pantarotto, R. Singh, D. McCarthy, M. Erhardt, J. Briand, M. Prato, K. Kostarelos, A. Bianco, *Angew. Chem.* **2004**, *116*, 5354 – 5358; *Angew. Chem. Int. Ed.* **2004**, *43*, 5242 – 5246.
[7] N. W. S. Kam, T. C. Jessop, P. A. Wender, H. J. Dai, *J. Am. Chem. Soc.* **2004**, *126*, 6850 – 6851.
[8] Q. Wan, K. Yu, T. H. Wang, C. L. Lin, *Appl. Phys. Lett.* **2003**, *83*, 2253 – 2255.
[9] Q. H. Li, Q. Wan, Y. J. Chen, T. H. Wang, H. B. Jia, D. P. Yu, *Appl. Phys. Lett.* **2004**, *85*, 636 – 638.
[10] H. Kado, K. Yokoyama, T. Tohda, *Rev. Sci. Instrum.* **1992**, *63*, 3330 – 3332.
[11] M. Cormier, B. Johnson, M. Ameri, K. Nyam, L. Libiran, D. D. Zhang, P. Daddona, *J. Controlled Release* **2004**, *97*, 503 – 511.
[12] G. Cabodevila, B. Lepioufle, H. Fujita, *Proc. SPIE-Int. Soc. Opt. Eng.* **2002**, 4937, 90 – 97.
[13] Y. Lu, Y. Yin, B. T. Mayers, Y. Xia, *Nano Lett.* **2002**, *2*, 183 – 186.
[14] X. He, K. Wang, W. Tan, B. Liu, X. Lin, C. He, D. Li, S. Huang, J. Li, *J. Am. Chem. Soc.* **2003**, *125*, 7168 – 7169.
[15] X. X. He, K. M. Wang, W. H. Tan, C. M. He, S. S. Huang, B. Liu, X. Lin, X. H. Chen, *J. Dispersion Sci. Technol.* **2003**, *24*, 633 – 640.
[16] L. Vayssieres, *Adv. Mater.* **2003**, *15*, 464 – 466.
[17] K. Y. Win, S. S. Feng, *Biomaterials* **2005**, *26*, 2713 – 2722.

Received: June 13, 2005
Revised: October 28, 2005

