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Functionalized tetrapod-like ZnO nanostructures for plasmid DNA purification, polymerase chain reaction and delivery

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Abstract
Functionalized tetrapodal ZnO nanostructures are tested in plasmid DNA experiments (1) as a solid-phase adsorbent for plasmid DNA purification, (2) as improving reagents in a polymerase chain reaction (PCR) and (3) as novel carriers for gene delivery. The amino-modification, the tetrapod-like shape of the nanostructure and its high biocompatibility all contribute to measurements showing promise for applications. A sol–gel method is used for silica coating and amino-modification. Plasmid DNA is purified through reversible conjugations of amino-modified ZnO tetrapods with DNA. Also, as additional reagents, functionalized tetrapods are shown to improve the amount of PCR product. For transfection, ZnO tetrapods provide some protection against deoxyribonuclease cleavage of plasmid DNA and deliver plasmid DNA into cells with little cytotoxicity.

1. Introduction

Isolation, amplification and transfection of exogenous DNA are central to today's biomedical research. Consequently, improving methods for versatile and efficient DNA experiments is of great interest. In recent years, due to their electrical, optical and magnetic properties, materials less than 100 nm (the same scale as biomolecules) have become very promising for biomedical applications [1–3]. Recently, nanomaterials such as nanoparticles and nanotubes have been tried in biological experiments, with various advantages [4–6]. However, applications of ZnO nanostructures for DNA-related experiments are still rare. Here we report functionalized tetrapod-like ZnO nanostructures (1) as a solid-phase adsorbent for plasmid DNA purification, (2) as improving reagents for polymerase chain reaction (PCR) and (3) as novel carriers for mammalian cell transfections. The reasons for choosing ZnO nanostructures were as follows. First, it is easy to prepare large quantities of ZnO nanostructures, such as nanoparticles, nanorods and tetrapods [7, 8]. Second, the bio-modification of ZnO nanostructures is versatile. Coated with silica, the nanostructures can be modified easily with various functional groups. Finally, the cytotoxicity of silica-coated ZnO nanostructures is slight.

In this work, silica-coated and amino-modified tetrapod-like nanostructures were prepared. Without toxic chemicals, plasmid DNA was purified through reversible conjugation of amino-modified ZnO tetrapods with DNA. As additional reagents, functionalized tetrapods can improve the amount of PCR product. In PCR, the products mediated by amino-modified tetrapods were produced in greater quantities than those by amino-free tetrapods. For transfections, ZnO tetrapods provided some protection against deoxyribonuclease (DNase) cleavage of plasmid DNA. Just as for gene delivery phages stand on a cell with six legs, ZnO nanostructures stand on cells for DNA delivery with three needle-shaped legs as a result of their tetrapodal shape. In addition, the geometry of the tetrapods implies a much larger steric hindrance which makes it difficult for tetrapods to wholly pass through cell membranes, hence contributing to reduced cytotoxicity. These results demonstrate novel applications of tetrapod-like nanostructures in DNA experiments.

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2. Experimental details

The process of silica coating and amino-modification of tetrapod-like ZnO nanostructures has been described in our previous publication [9]. We added 26 mg of tetrapod-like ZnO nanostructures into the mixture solution of 2-propanol (20 ml) and ethanol (40 ml). Then 0–5 ml deionized water and 1.5 ml ammonia solution (25 wt%) were consecutively added. Next aminopropyltriethoxysilane (APTES; amino-modification reagent) and tetraethylorthosilicate (TEOS; precursor of silica) in different ratios (0:1, 1:10, 1:6, 1:4 and 1:2, total 400 μl) were added and the solution was stirred at room temperature for ∼8 h. Then, the core–shell nanostructures were separated from the reaction medium by centrifuging at ∼3000 rpm, and dispersed into ethanol. The separation procedure was repeated several times. Dried in air, the samples were observed by scanning electron microscopy (SEM).

In the Kaiser assay 0.15 mg of the tetrapods, modified with different ratios of APTES to TEOS, were mixed with 200 μl acetate buffer (1 M, pH 4.5) and 200 μl ninhydrin buffer (150 mg of ninhydrin, 10 ml of isopropanol, 0.3 ml of acetic acid, diluted in water to 100 ml final volume). The mixtures were treated at 100 °C for 20 min. After centrifuging at 10 000 rpm for 10 min, the supernatant was analysed by spectrophotometry (565 nm). The number of amino groups on the tetrapods was calculated based on the standard curve of glycine.

In agarose gel electrophoresis of tetrapod–DNA complexes 30 μg of tetrapods, modified with different ratios of APTES to TEOS, was mixed with 1 μl pEGFPN1 DNA (∼1 mg ml^{-1}, a kind of plasmid DNA from Clontech Laboratories Inc.) in 30 μl water for 3 min at room temperature. After centrifuging at 3000 rpm, the supernatant was kept for electrophoresis. Then agarose gel electrophoresis (1% agarose) was used to examine the conjugations of tetrapods with DNA.

In plasmid DNA purification by amino-modified ZnO tetrapods plasmid DNA was usually produced in an *Escherichia Coli* host by fermentation. Next 1 ml of cells was lysed by the addition of 200 μl of 0.2 M NaOH, 1% sodium dodecyl sulfate (SDS). Then 0.2 ml of 2.55 M potassium acetate was added. The flocculent precipitate was removed by centrifugation. Then 2 ml ethanol was added into the supernatant for precipitating plasmids. After centrifuging at 12 000 rpm for 5 min, the supernatant was removed carefully and the precipitate (including 3–5 μg plasmid DNA) was dispersed into 30 μl deionized water. For purification, 2 μl of impure plasmid DNA solution was diluted into 50 μl deionized water containing 30 μg amino-modified tetrapods (the ratio of APTES to TEOS being 1:4). The solution was mixed for 1 min at room temperature to allow the binding of plasmid DNA. After centrifugation, the supernatant was removed and ZnO tetrapods were dispersed in 30 μl water. Then ZnO–DNA complexes were heated for 10 min at 25 °C, 50 °C and 75 °C, respectively. After centrifugation, the supernatant was kept for electrophoresis. For the control in electrophoresis, 2 μl of impure plasmid DNA was used with no purification process. The purity of the samples of plasmid DNA was evaluated by UV spectrophotometry.

In PCR mediated by ZnO tetrapods silica-coated tetrapods (without or with amino groups) were tested in the PCR. ZnO tetrapods were added into 25 μl PCR reaction solutions in amounts that varied in small increments between 0.01 and 2 μg. The solution contained 2.5 μl 10 × PCR buffer, 0.2 μl 2.5 mM deoxynucleosides (dNTPs), 0.8 μl 50 mM $MgCl_2$, 10 μM primers, 50 ng plasmid DNA, 0.5 μl Taq enzyme (5 U μl^{-1}) and 19 μl water. The PCR included four steps: (1) 94 °C denaturation for 10 min, (2) 55 °C renaturation for 1 min, (3) 72 °C for 1 min, (4) 94 °C for 1 min. From step (2) to step (4), the process was repeated 25 times. After the reactions, 10 μl of PCR products was used for electrophoresis. With no releasing process, ZnO–DNA complexes were tried directly in PCR. ZnO–DNA complexes were added into PCR solution in amounts of 0.01, 0.10, 0.25, 1 and 2 μg. The concentration of plasmid DNA on the tetrapods was ∼100 ng μg^{-1}. For a positive control, 50 ng free plasmid DNA was used. The PCR process was the same as described above.

To protect DNA against DNase cleavage 2 μg DNase I was mixed with 30 μg ZnO–DNA complexes (∼2 μg plasmid DNA) and incubated at 37 °C for 30 min or 60 min. After that, the solutions were heated at 75 °C for 10 min. After centrifugation, the supernatant was kept for electrophoresis. For the control, 2 μl free plasmid DNA (∼1 mg ml^{-1}) was used.

For SEM analysis of tetrapods on cells A375 cells (human malignant melanoma cells) were routinely cultured directly on a glass cover slip in 2 ml Dulbecco's modified Eagle's medium (DMEM) culture containing 10% fetal bovine serum (FBS), endothelial cell growth supplement (ECGS; 0.03 mg ml^{-1}) and kanamycin (50 U ml^{-1}). When the cells had grown to confluence, 20 μg amino-modified ZnO nanostructures was added to the culture for 30 min. Then the cover slips were removed from the growth medium and washed several times with phosphate-buffered saline (PBS). After that, A375 cells were submitted to the fixation and sputter coating procedure before SEM observation.

In cell transfection of plasmid DNA A375 cells were cultured in 1 ml DMEM in a 24-well incubator. After the cells had grown to 60%–80% confluence, 10 μg amino-modified ZnO tetrapods (with a 1:4 ratio of APTES to TEOS) was mixed with 2 μl pEGFPN1 DNA (1 mg ml^{-1}) in 30 μl deionized water for 3 min. Then the tetrapod–DNA complexes were put into a cell culture medium. Amino-free ZnO tetrapods were used as negative controls and the process was the same as discussed above. For positive controls, 2 μl lipofectamine2000 (1 mg ml^{-1}) and 2 μl pEGFPN1 were dispersed in 50 μl DMEM for 10 min. Then the mixture was incubated with the cells in DMEM medium. A laser confocal microscope was used to record the images of transfections.

For MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay A375 cells were cultured in 96-well plates with DMEM with antibiotics. When the cells reached 80% confluence, amino-modified tetrapods (with a 1:4 ratio of APTES to TEOS) and lipofectamine2000 were added into the culture solution with the following final concentrations: 10, 20, 30, 40 and 50 μg ml^{-1}, respectively. After 12 h of incubation, MTT was added into the well with a final concentration of 1 mg ml^{-1} and incubated with the cells for 4 h at 37 °C. Then the cells were washed with PBS and incubated with 80 μl dimethlysulfoxide (DMSO) at room temperature for 3 min. Then the samples were evaluated by spectrophotometry at a wavelength of 595 nm.

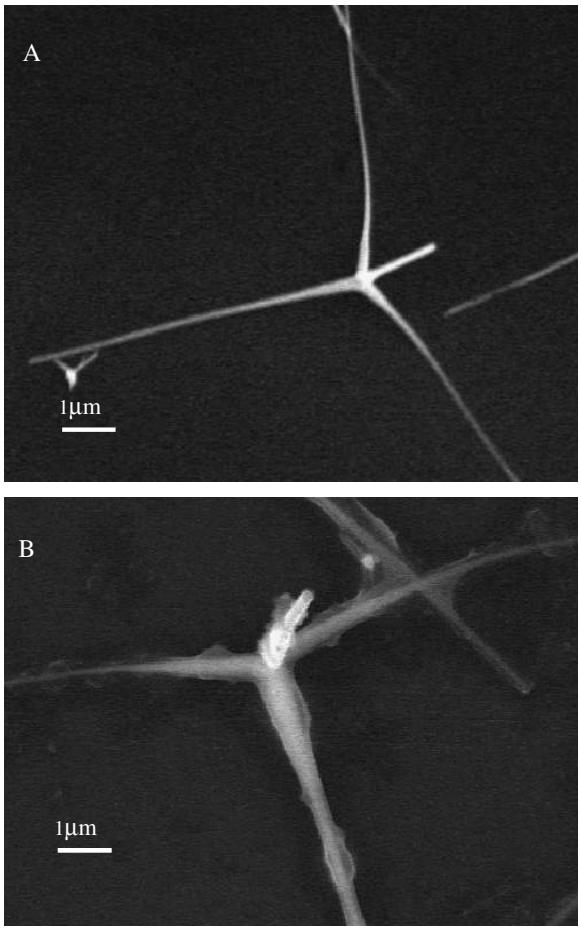

Figure 1. SEM image of tetrapod-like ZnO nanostructures, before (A) and after (B) silica coating and amino-modification.

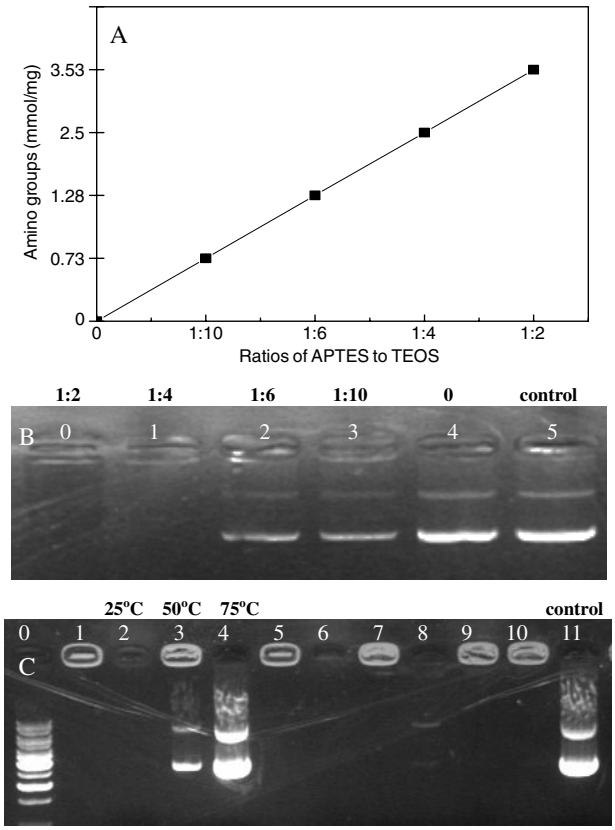

Figure 2. (A) Kaiser assay of amino-modified ZnO tetrapods with different ratios of APTES to TEOS. (B) Electrophoresis of the centrifuging supernatant separated from mixture solutions of tetrapods and pEGFPN1 DNA. Plasmid DNA without nanostructures (lanes 5), plasmid DNA mixed with tetrapods modified with only TEOS (lane 4) or with different ratios of APTES to TEOS, 1:10 (lane 3), 1:6 (lane 2), 1:4 (lane 1), 1:2 (lane 0). (C) Electrophoresis of ZnO–DNA complexes heated for 10 min at room temperature (lane 2), 50 °C (lane 3) and 75 °C (lane 4). Lane 11 was the impure sample and lane 0 was a DNA marker.

3. Results and discussion

Tetrapodal ZnO nanostructures were synthesized by thermal evaporation at 900 °C [7]. As shown in figure 1(A), the nanostructures consisted of four needle-shaped tetrahedrally arranged legs connected at the centre, forming a tetrapod-like structure. Measuring ∼80 nm in mean diameter and 5–10 μm in length, one of needle-shaped tetrahedrally arranged legs was perpendicular to the substrate while the other three legs touched the substrate. For binding plasmid DNA, tetrapods were coated with silica and modified with amino groups [4, 10]. Using a sol–gel method, a process for both silica coating and amino-modification has been accomplished when TEOS (precursor of silica) and APTES (amino-modification reagent) were added to the reaction solution together. As shown in figure 1(B), after silica coating the shape of the nanostructures was maintained, the diameter of the legs became greater (∼150 nm) and the surfaces of the nanostructures became rougher (silica shells and ZnO cores). The process was simple and the number of amino groups on the tetrapods was controllable through varying the ratio of APTES to TEOS.

The number of amino groups on tetrapods was quantified by a Kaiser assay. As shown in figure 2(A), with an increasing proportion of APTES in the modification process, the number of amino groups on the nanostructure increased linearly. When the ratio of APTES to TEOS was 1:4, the concentration of amino groups on tetrapods was ∼2.5 mmol g^{-1}. The concentration was higher than that of amino-modified carbon nanotubes (∼0.55 mmol g^{-1}), suggesting a high positive charge density on the surface in aqueous solutions [11].

The conjugation of tetrapods with plasmid DNA was evaluated by agarose gel electrophoresis. 30 μg amino-modified tetrapods was mixed with 1 μl plasmid DNA (∼1 mg ml^{-1}) for 3 min. After centrifuging, the supernatant was kept for electrophoresis. As shown in figure 2(B), if not mixed with amino-modified tetrapods, plasmid DNA moved and dispersed in gel under an electric field (lane 5). When mixed with tetrapods modified with only TEOS, almost the same amount of DNA was found in the supernatant (lane 4) as appeared in lane 5. However, with increasing APTES, DNA in the supernatant decreased. When the ratio was equal to or over 1:4, no DNA was observed in the supernatant (lanes 1, 0). Through electrostatic interactions between positively charged amino groups on tetrapods and negatively charged phosphate groups of plasmid DNA, amino-modified tetrapods bound DNA [4]. With increasing APTES, amino groups on

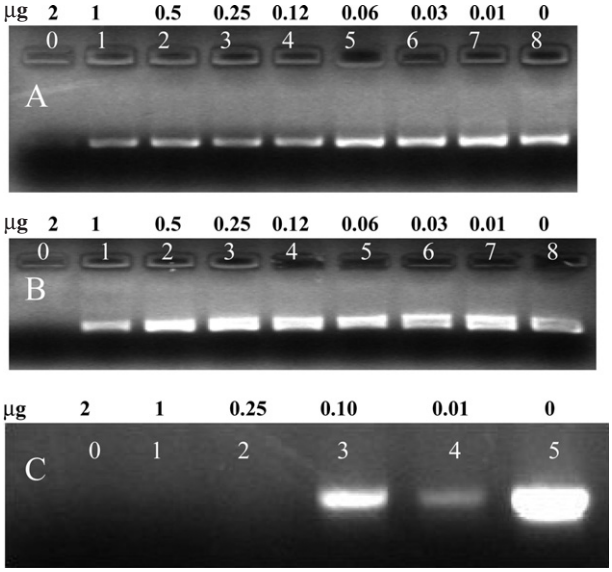

Figure 3. (A) Electrophoresis of PCR mediated by silica-coated ZnO tetrapods without amino groups. Lane 8 is the control (without tetrapods). (B) Electrophoresis of PCR mediated by silica-coated ZnO tetrapods with amino groups. Lane 8 is the control (without tetrapods). (C) Electrophoresis of ZnO–DNA complex PCR. Without adding another template, the amount of plasmid DNA on tetrapods increased (lanes 3, 4). Lane 5 was the PCR product of free plasmid DNA.

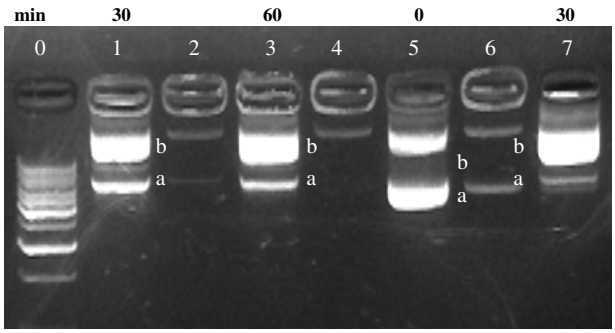

Figure 4. Electrophoresis of plasmid DNA released from ZnO–DNA complexes after DNase I cleavage for 30 min (lane 1) and 60 min (lane 3). Free plasmid DNA was used as the controls, without DNase I cleavage (lane 5) or with DNase I cleavage (lane 7).

the nanostructures increased, which suggested more positive charges on the surfaces of nanostructures in aqueous solutions. Therefore, the binding efficiency increased. Since they can effectively bind plasmid DNA, amino-modified tetrapods, on which the ratio of APTES to TEOS was 1:4, were used for DNA delivery.

Unlike covalent bonding, these electrostatic interactions were weak and the conjugation of ZnO tetrapods with DNA was reversible. When ZnO–DNA complexes were heated at 75 °C for 10 min, plasmid DNA released from the complexes and dispersed into the solution again. As shown in figure 2(C), at room temperature no plasmid DNA was found in supernatant (lane 2); when heated at 50 °C, some plasmid DNA was observed in solution (lane 3); when heated at 75 °C, almost the same amount of DNA was found in supernatant (lane 4) as was found in the control. Since the conjugations of amino-modified tetrapods with DNA are reversible, the tetrapods can be used for purification of plasmid DNA in cell lysates. The purity of plasmid DNA was evaluated by UV spectrophotometry. Before purification, the ratio of OD_{260}/OD_{280} (OD_{260}, optical density at wavelength of 260 nm and 1 OD_{260} unit $\approx$ 50 μg ml^{-1} for double-stranded DNA) was below 1.5, whereas after purification the average OD_{260}/OD_{280} ratio was $\sim$1.9, suggesting a high purity with negligible protein contamination [12]. Plasmid DNA can be purified easily and efficiently with no toxic chemicals such as phenol and chloroform.

Recently gold nanoparticles and carbon nanotubes have been used as additional reagents to improve the performance of PCR [5, 6]. Here, silica-coated tetrapods (without or with amino groups) were added into PCR solution as additional reagents. As shown in figure 3(A), when tetrapods (without amino groups) were less than or equal to 0.06 μg, compared to the control (lane 8, without tetrapods), the amount of PCR products was not changed much (lane 5, 6, 7). If tetrapods were more than or equal to 0.12 μg, PCR products decreased (lanes 1–4). In figure 3(B), when tetrapods (with amino groups) were from 0.01 to 0.50 μg, compared to the control (lane 8, without tetrapods), PCR products increased (lanes 2–7), whereas with higher numbers of tetrapods, no increase in PCR products was observed (lanes 0, 1). Comparing figures 3(A) with (B), we can see that more products were mediated by amino-modified tetrapods than were mediated by amino-free tetrapods. The reasons for such results are not clear at the present time. Usually DNA should release from a solid-phase adsorbent before PCR. Here, with no releasing process, ZnO–DNA complexes were added into PCR solution. As shown in figure 3(C), plasmid DNA on tetrapods was increased (lanes 3, 4). At 72 °C (the PCR extension temperature), plasmid DNA was released from ZnO–DNA complexes and acted as a template for PCR.

It has been reported that amino-modified silica nanoparticles are efficient in providing protection against enzymatic cleavage of DNA [4]. To investigate the protection of amino-modified ZnO against DNase, ZnO–DNA complexes were incubated with DNase I. As shown in figure 4, after a 30 min reaction, super-coils (band a) of free plasmid DNA were almost degraded (lane 7), whereas in the same digestion process, some super-coils on ZnO–DNA complexes were well preserved (lane 1). After a 60 min reaction, compared to lane 1, super-coils on ZnO–DNA complexes decreased (lane 3). Therefore amino-modified ZnO tetrapods provided some protection against enzymatic cleavage. ZnO–DNA complexes may result in conformational changes, which would protect DNA from cleavage [4].

Inspired by micro-needles for transdermal drug delivery, we may use needle-shaped ZnO legs to penetrate cell membranes for plasmid DNA delivery [13, 14]. ZnO tetrapods on cells were viewed with SEM. With amino-modified tetrapods, A375 cells (human melanoma cells) were cultured on a glass cover slip in DMEM culture. As shown in figure 5, though the cover slip was washed several times with PBS, tetrapods were found on the cell surface. The interaction between positive charges of the amino-modified tetrapods and negative charges of the cell membrane might be one of the

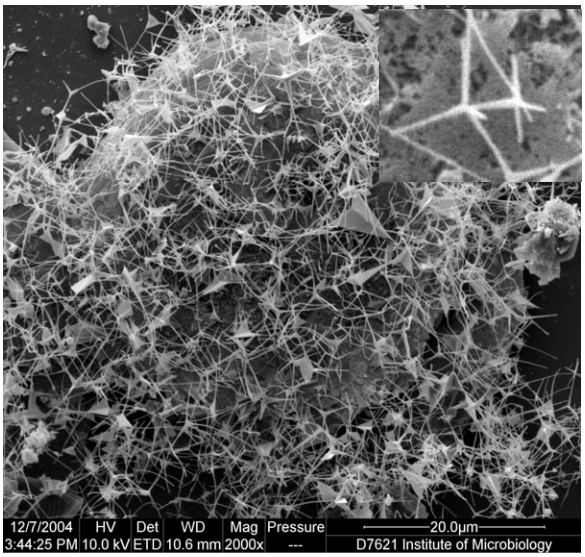

Figure 5. SEM image of A375 cell cultured with amino-modified tetrapods. A higher-resolution image of tetrapods on the cell was inserted.

reasons for the attachment of tetrapods onto cells. Just as phages stand on cells with six legs for gene delivery, some nanostructures stood on the cells with three legs. As a result of the tetrapodal shape, for some nanostructures on the cell surface one leg was perpendicular to the cell surface while the other three legs touched the cell membrane.

For gene delivery, ZnO–DNA complexes were cultured with cells. Here pEGFPN1 DNA, a kind of plasmid DNA containing the gene for green fluorescence protein (GFP), was used in the experiments. Lipofectamine2000, a commercial liposome (spherical, ∼100 nm in diameter), was used as a positive control [15]. The reasons for choosing lipofectamine2000 were as follows: (1) it is a commonly used carrier for gene delivery; (2) like amino-modified tetrapods, there are amino groups on the lipofectamine2000. As shown in figures 6(A) and (B), green fluorescence was observed from some cells cultured with lipofectamine2000–DNA complexes or ZnO–DNA complexes. This result indicated that green fluorescence protein was successfully expressed in these cells and there were exogenous GFP genes in these cells. Similar to the lipofectamine2000, there were amino groups on the tetrapods. Through electrostatic interactions between positively charged amino groups and negatively charged phosphate groups of DNA, tetrapods conjugated with plasmids such that they could be used for gene delivery. With its much smaller size, lipofectamine2000 (∼100 nm in diameter) had better transfection efficiency than amino-modified tetrapods (5–10 μm in length). However, no green fluorescence was found when the cells were cultured with silica-coated tetrapods without amino groups (data not shown). Therefore, amino-modified tetrapod-like nanostructures, as transfection carriers, indeed delivered plasmid DNA into mammalian cells. Just as phages stand on cells with six legs, tetrapods can also stand on cells with three needle-shaped legs. With their tips on the cell surfaces, three legs of the tetrapod stood obliquely (figure 5), so the possibility of internalization of the tips by cells should be increased. Together with the tips, plasmid DNA on tetrapod

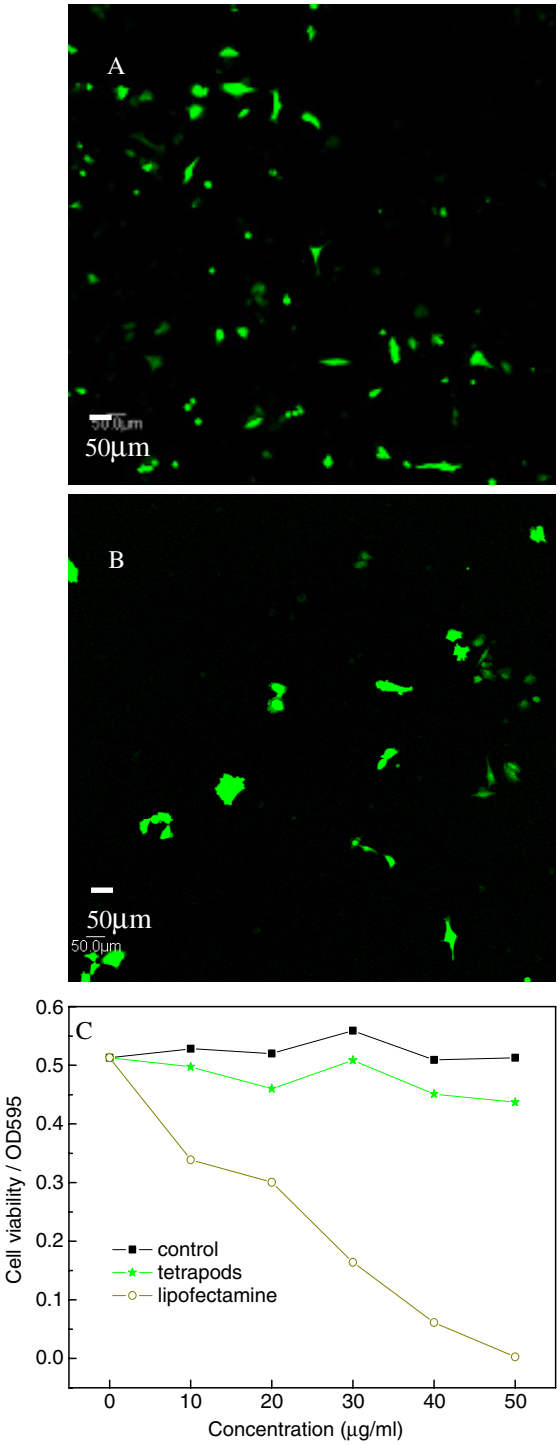

Figure 6. (A) Confocal fluorescence image of A375 cells cultured with lipofectamine2000–DNA complexes. (B) Confocal fluorescence image of A375 cells cultured with ZnO–DNA complexes. (C) MTT assay of carriers at different concentrations.
(This figure is in colour only in the electronic version)

tips can enter into cells easily and the amount of DNA in the cells for transfection would then be increased. Therefore, the tetrapod-like shape was helpful for transfecting DNA into mammalian cells.

Cytotoxicity was evaluated by MTT assay. As shown in figure 6(C), tetrapods have not changed cell growth very

much even at a high concentration (50 μg ml^{-1}). However, the cytotoxicity induced by lipofectamine2000 appeared at a concentration of 10 μg ml^{-1}. Coated with silica (a well-known biocompatible material), ZnO tetrapods had better biocompatible surfaces than the liposomes. In addition, the geometry of the tetrapods implied a much larger steric hindrance which made it difficult for nanostructures to wholly pass through cell membranes. Just as phages can insert genes into cells while they stay outside the cell membrane, tetrapods delivered plasmid DNA into cells while they stayed outside the cell. However, if silica nanoparticles were used for DNA delivery, it would be easy for them to completely enter into cells by cell internalization, which may introduce damage to cells [16]. Therefore, the tetrapod-like shape was helpful for reducing cytotoxicity.

4. Conclusions

In conclusion, we prepared silica-coated and amino-modified ZnO tetrapods and found these functionalized nanostructures can be used as a solid-phase adsorbent for plasmid DNA purification, as improving reagents for PCR and as novel carriers for mammalian cell transfections. The amino groups, the tetrapod-like shape and the biocompatibility provided the basis for DNA-related experiments.

Acknowledgments

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