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Surface modified gold nanowires for mammalian cell transfection

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Abstract
Aminothiol modified gold nanowires have been used as vectors for the delivery of plasmid DNA into two different types of mammalian cells: 3T3 and HeLa. It was measured that positively charged gold nanowires with a diameter of 200 nm and a length around 5 μm were capable of carrying 1 pg of plasmid DNA per nanowire into cells. Compared with other transfection reagents, the gold nanowires exhibited the highest transfection efficiency while almost no cytotoxicity was observed. In addition, it has been shown that individual nanowires can be visualized with sub-micrometer resolution, which may allow the use of functionalized multi-segment nanowires as local probes for the investigation of the microenvironment inside cells.

S Supplementary data are available from stacks.iop.org/Nano/19/025103

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1. Introduction

There have been increasing research activities in developing metallic nanoparticles for probing biological systems because of the potential applications of nanoparticles in drug and gene delivery [1–7], biosensing [8–10], bioseparation [11, 12] and bioimaging [13–15]. Among these metallic nanoparticles, gold nanoparticles have been widely studied because of their easy preparation process, chemical stability and possibility for surface modifications [16, 17]. In addition, it was shown that the spherical gold nanoparticles were inherently nontoxic to human cells and could be easily internalized by the cells while the surface modified gold nanoparticles exhibited some degree of toxicity, which depended on the surface charges of the gold nanoparticles [18–21]. Taking advantage of the low cytotoxicity and easy surface modification process, the spherical gold nanoparticles have been engineered to carry genetic materials into the cells with very high efficiency [1, 3]. When the oligonucleotide modified gold nanoparticles were used as intracellular gene regulation reagents, it was demonstrated that the knockdown of gene could be tuned by tailoring the density of DNA on the surfaces of gold nanoparticles [22]. Despite these fruitful successes in using spherical gold nanoparticles for various biological applications, it remains difficult to engineer spherical gold nanoparticles with multiple functionalities due to their single chemical composition. In an ideal nanoparticle based biological assay, it is desirable to decorate the surface of nanoparticles with multiple functionalities such as optical labeling for tracing the particles, magnetic properties for manipulation and chemical or biological functionalities for specific target recognition [23–25].

One simple approach to engineer particles with multiple functionalities is to utilize the nanorods or nanowires produced by template electrodeposition [26]. Since different types of materials can be deposited into the templates sequentially, it is possible to obtain multi-segment nanorods or nanowires [27]. Using different coordination chemistry, each segment of the nanorods or nanowires could be selectively modified [28]. Such multi-segment multifunctional nanorods and nanowires have been demonstrated to be capable of carrying plasmid DNA on one segment while the other segment

was modified with targeting ligands to enhance the uptake of the nanorods [29]. However, most of the studies on nanorods or nanowires have been limited to nanometer length; micrometer length nanowires have been less explored. Micrometer long multi-segment nanowires have been used as barcodes for biological multiplexing, which could be easily visualized with an optical microscope [30, 31]. Recently, it has been shown that micrometer long nickel nanowires could be internalized by cells allowing the manipulation of live cells through a magnetic field [32, 33]. However, it is not known whether micrometer long nanowires are capable of delivering DNA molecules or drugs into the cells without damaging the cells, which is an important issue for the development of nanowire based live cell probing systems.

To investigate the possibility of using nanowires for DNA delivery, it is necessary to engineer the surface of the nanowires in such a way that the DNA molecules can bind to the nanowires in a reversible manner, which can protect the DNA molecules from the digestion of enzymes in the extracellular environment while the DNA molecules can be released inside the cells for effective transfection. Since the DNA molecules are negatively charged, a common practice is to engineer the surface of the nanowires with positive charges. Therefore, the DNA molecules can bind to the surface of the nanowires through electrostatic interaction. Because of the low cytotoxicity and easy surface modification process of gold nanowires, we have investigated the interactions between the nanowires and the cells through studies of the DNA transfection efficiency using single segment surface modified gold nanowires.

2. Experimental details

2.1. Preparation of gold nanowires

The nanowires used in this experiment were fabricated by electrodeposition using 200 nm alumina membranes (Andisc, Whatman) as templates. To perform electrodeposition, one side of the membrane was coated with a 200 nm thick silver film for conducting purposes and a commercial gold plating solution (Technic) was used to produce gold nanowires. Gold nanowires with different lengths could be produced by adjusting the electrodeposition time. After the electrodeposition process, the silver film was removed in 3 M nitric acid and the gold nanowires were released by dissolving alumina membranes in a 3 M NaOH solution. The nanowires were then washed by DI water and ethanol several times. Since the length of the nanowires was in the micrometer region, the concentration of the nanowires was determined by counting the number of nanowires using a hemacytometer on an inverted microscope (Olympus, IX 71).

2.2. Surface functionalization of nanowires

In order to bind the negatively charged plasmid DNA molecules through the electrostatic interaction, it is necessary to engineer the surface of the gold nanowires with positive charges. To modify the surface of nanowires, the suspended gold nanowire (1×10^8 nanowire ml^{-1}) solutions were mixed with an ethanol solution of 1 mM of 11-amino-1-undecanethiol (Dojindo). After 24 h of incubation, the nanowires were cleaned by DI water and the excess thiols were removed by dialysis for 24 h using a 3.5 kD cut off dialysis membrane with DI water. The zeta-potentials of the surface modified nanowires around at pH 7.4 were measured by a Zeta Potential Analyzer ZetaPALS (Brookhaven Instruments Corp.) at a field of 8–16 V cm^{-1}.

2.3. Plasmid preparation

A pDsRed-Monomer-golgi (4.9 kb) vector encoding the red fluorescent protein (DsRed-monomer) with golgi location signal was purchased from Clontech (BD Biosciences). These plasmid DNA molecules were propagated in DH 5α host strains and isolated and purified by using an EndoFree Plasmid Maxi kit (Qiagen GmbH, Hilden, Germany). The concentration of plasmid DNA molecules was determined by measuring the absorbance at 260 nm.

2.4. Gel electrophoresis

To measure the amount of plasmid DNA that can be carried by the nanowires, gel electrophoresis was used. Gels were prepared with 1%(w/v) agarose and ran for 40 min at 50 V. The buffer solutions used in this experiment were TBE buffer (90 mM Tris-acetate and 2mMEDTA, pH 8.3, Sigma) and the plasmids were stained with SYBR green (Invitrogen).

2.5. Cell culture and transfection

Fibroblast NIH 3T3 and HeLa S3 (BCRC) were cultured in T75 flasks with growth media, which were Dubbeco's modified Eagle's medium (DMEM, Gibco) for fibroblast cells, and minimum essential medium (MEM, Gibco) for HeLa cells, at 37 °C in a 5% CO_2 atmosphere. 10% fetal bovine serum (FBS, PAA Laboratories) and PEN–STREP–AMPHO solution (Biological Industries) were added to both media. In a typical transfection experiment, 3T3 and HeLa cells were seeded in six-well plates at a density of 1×10^5 cells. To compare the transfection efficiency of the nanowires with other transfection reagents, we have tested two types of commonly used transfection reagents, PolyFect (Qiagen) and calcium phosphate, and bared plasmid DNA molecules were used as controls.

The protocol for using the aminothiol modified nanowires as transfection reagents is described as follows: for 3T3 cells, 50 ng of DNA (pDsRed-Monomer-golgi) were mixed 10 μl of aminothiol modified gold nanowires (10^7 ml^{-1}) suspended in the sterile water. The nanowire solution was incubated with 89 μl of growth medium without serum for 10 min and then 600 μl of growth medium was added into the solution. To conduct the transfection experiment, the medium in the cell culture was removed and the cells were washed with PBS solution. After pipetting the nanowire solution into the cell culture, an additional 1 ml of fresh medium was added.

Similar protocols for the dendrimer based commercial agent PolyFect (Qiagen) and calcium phosphate were used. 50 ng of DNA were dissolved in 89 μl medium without serum

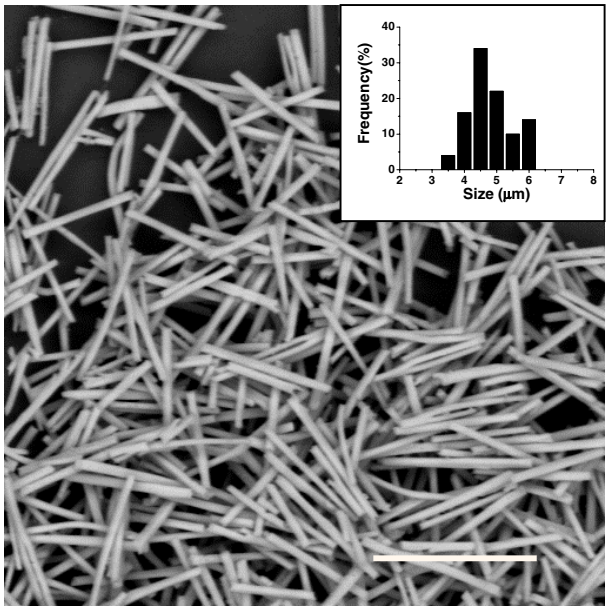

Figure 1. An SEM image of gold nanowires. Bar: 5 μm. Inset: the size distribution of the nanowires. The length of the nanowires was measured to be 4.78 ± 0.7 μm.

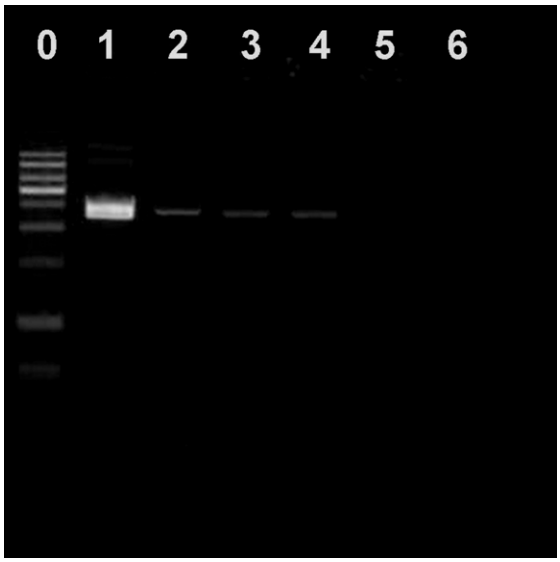

Figure 2. Agarose gel electrophoresis of the aminothiol modified gold nanowires and plasmid. Lane 0 is the DNA marker. Lane 1 is the 50 ng plasmid DNA without nanowires. Lanes 2–6 are a mixture of 50 ng plasmid DNA with increasing numbers of gold nanowires (5×10^3, 2.5×10^4, 5×10^4, 2.5×10^5, 5×10^5).

and then 10 μl of PolyFect transfection reagent were added to the medium. After 10 min of incubation, 600 μl growth medium was added to the transfection mixture. When the calcium phosphate was used as the transfection reagent, 50 ng of DNA were mixed with 10 μl of 2 M $CaCl_2$ solution with phosphate buffer. The mixture solutions were added to 89 μl of medium without serum and incubated for 10 min. The final solution was prepared by adding 600 μl of growth medium. Both PolyFect and calcium phosphate mixture solutions were added to the washed cell culture together with 1 ml fresh growth medium. The naked DNA solution contained 50 ng of DNA and 99 μl of medium without serum. After 10 min of incubation, the final naked DNA solution was prepared by adding 600 μl of growth medium. Similar protocols were used for the HeLa cells. The only difference was that the amount of plasmid DNA used in each experiment was increased to 500 ng.

After 4 h of incubation with the transfection reagents, the cells were washed with PBS solution and 1.5 ml of fresh medium was added to each well. The transfection efficiency was measured by counting the number of cells that exhibited red fluorescence and the total number of cells on an inverted microscope (Olympus IX 71) equipped with a color CCD (Olympus, DP70). To measure the location of the nanowires and the expressed DsRed-monomer, a confocal microscope (Leica TCS SP5) was used where the transfection experiments were conducted on 3 mm glass-bottomed dishes coated with collagen (Bioptechs, Inc.).

3. Results and discussion

3.1. Characterization of nanowires

The anodic aluminum oxide membranes (200 nm) were used as the templates for electrodeposition. Following the procedure described in the literature, it was possible to obtain multi-segment nanowires (see supporting materials (available at stacks.iop.org/Nano/19/025103)). One important issue in developing a multifunctional biological probing system is the capability of delivering external materials such as drugs or DNA through the multifunctional probes. To simplify this problem, we have concentrated on the study of the transfection efficacy using single segment gold nanowires. The detailed fabrication procedures and experimental parameters for gold nanowires are described in section 2. Shown in figure 1 are the scanning electrom microscope (SEM) image and the size distribution of the 5 μm long gold nanowires. The size of the nanowires was measured to be 4.78 ± 0.7 μm and the zeta potential measured for the newly synthesized gold nanowires was about -83 ± 1.7 mV. To bind the negatively charged DNA molecules, the surfaces of the nanowires were modified with aminothiol to exhibit positive surface potential, which was measured to be $+11.4 \pm 1.4$ mV.

3.2. Binding efficiency of nanowire

Once the positively charged gold nanowires were prepared, the next step was to bind the negatively charged plasmid to the nanowires through the electrostatic interaction. To find out the optimal binding efficiency of the plasmid to the nanowires, we have tested five different concentrations of 5 μm long aminothiol modified gold nanowires (5×10^3, 2.5×10^4, 5×10^4, 2.5×10^5, 5×10^5 ml^{-1}), which were incubated with 50 ng of the DsRed-Monomer-golgi vector for 24 h. These solutions were then mixed with XYBR green and loaded in an agarose gel. The result is shown in figure 2 where the plasmid bands in lanes 5 and 6 were missing, indicating that at these concentrations all the plasmids were bound to the nanowires. Therefore, we estimated that the binding efficacy

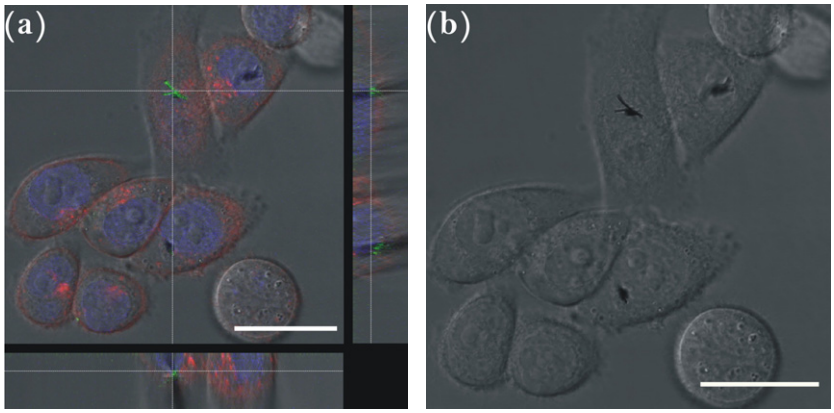

Figure 3. (a) The stacked laser scanning confocal microscope images of the aminothiol functionalized gold nanowires coated with plasmid and labeled with YOYO-1 dye (green) in a HeLa cell. The nuclei were stained with blue color and the plasma membranes were stained with red color. Bar: 25 μm. (b) The DIC image of gold nanowires inside the HeLa cells. Bar 25 μm.

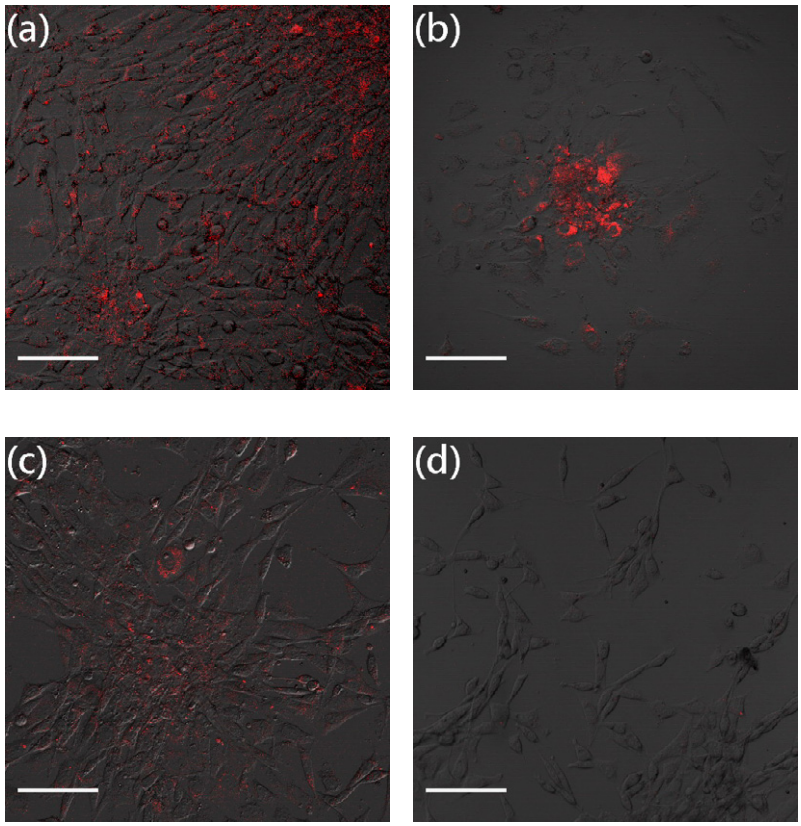

Figure 4. The combined DIC and confocal images of 3T3 cells 24 h after using (a) gold nanowires, (b) PolyFect, (c) calcium phosphate and (d) naked DNA as the transfection reagents. Bar: 100 μm.

for the plasmid DNA molecules on the 5 μm long aminothiol modified nanowires was about 1 pg/nanowire. The zeta potential of the plasmid bonded gold nanowires was measured to be -9.3 ± 0.5 mV, which clearly indicated that DNA bonded to gold nanowires to form a complex. The DNA gold nanowire complex could protect the DNA molecules from attack by the DNA nucleases.

3.3. Transfection efficiency of nanowires

One important issue for the nanowires to deliver plasmid DNA molecules into cells is that the DNA nanowire complex should be internalized by the cells. To investigate the internalization of the DNA nanowire complex, 5 μm long nanowires coated with plasmid DNA were used. For visualization purposes, the plasmid DNA on the nanowire surfaces was labeled with YOYO-1, which emitted a strong green fluorescence when bound to a double strain DNA, and the cells were stained with Image iT LIVE Plasma Membrane and Nuclear Labeling Kit (Invitrogen). Shown in figure 3(a) are the stacked confocal images of the HeLa cells, which were incubated with the plasmid coated nanowires for 24 h. As seen from the stacked confocal images, the plasmid coated nanowires were inside the

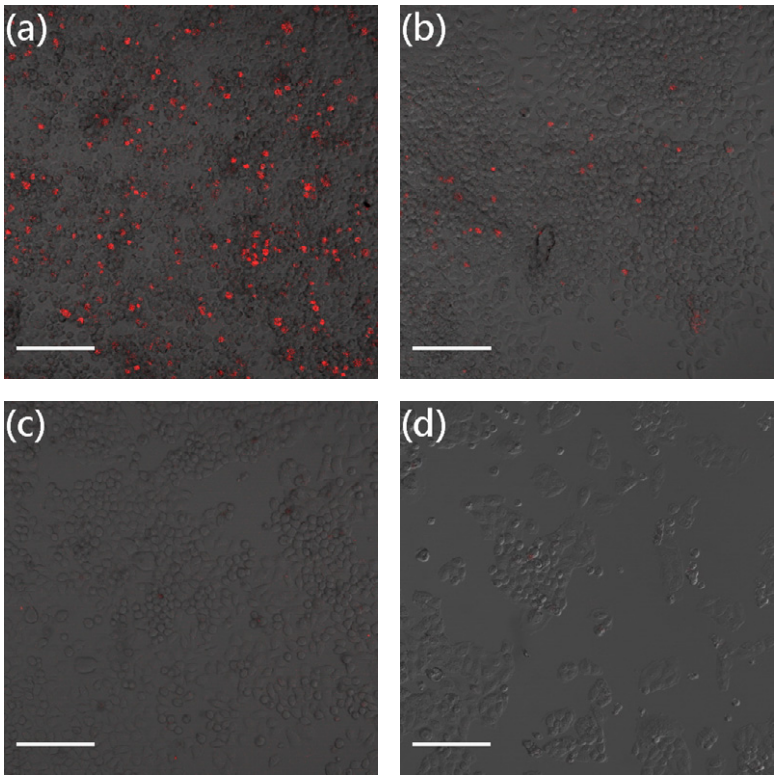

Figure 5. The combined DIC and confocal images of HeLa cells six days after using (a) gold nanowires, (b) PolyFect, (c) calcium phosphate and (d) naked DNA as the transfection reagents. Bar: 100 μm.

cell. Therefore, we concluded that the DNA nanowire complex can be internalized by the cells. In addition, the surface of individual nanowires could be visualized with sub-μm resolution. We note that the YOYO-1 dye was used to indicate the presence of the plasmid DNA molecules on the surface of nanowires whereas the behavior of the nanowires could be monitored by the DIC image without any labeling (figure 3(b)). A movie of the YOYO-1 labeled gold nanowires inside the HeLa cell during the transfection experiment can be found in the supporting material. When the multi-segment nanowires were used, additional functionality such as magnetic properties for manipulation could be engineered to the nanowires. The image of the two-segment silver-nickel nanowires inside the cells can also be found in the supporting material (available at stacks.iop.org/Nano/19/025103).

Knowing that the DNA nanowire complex could carry the plasmids into the cytoplasm, it was important to measure the transfection efficiency of the DNA nanowire complex. To test the transfection efficiency of the aminothiol modified gold nanowires, 5 μm long nanowires with concentration of 10^5 nanowire ml^{-1} were mixed with the 50 ng of DsRed-Monomer-golgi vector and incubated overnight. Two cell lines, NIH 3T3 and HeLa S3, were used in this experiment. The control experiments were conducted with dendrimer based commercial transfection agent (PolyFect) and calcium phosphate. The transfection efficiency for the naked plasmids was also measured. The viability of the cells incubated with the transfection reagents was checked separately by staining the cell with trypan blue 24 h after the transfections. The transfection efficiency was calculated by measuring the ratio of the number of cells exhibiting red fluorescence to the total number of the cells on the surfaces and normalized to the cell viability. Shown in figure 4 are the combined DIC and fluorescence images of 3T3 cells 24 h after transfection using four different transfection reagents. The viability of the 3T3 cells 24 h after transfection was measured to be 90%, 65%, 65% and 71% for gold nanowires, PolyFect, calcium phosphate and naked DNA, respectively. It is clear that aminothiol modified gold nanowires exhibited the highest transfection efficiency with very low toxicity for 3T3 cells. In one of our previous measurements [34], it was shown that more than 30% of the gold nanowires could be internalized by the 3T3 cells within 8 h (see supporting information (available at stacks.iop.org/Nano/19/025103)), which may explain the high transfection efficiency of the micrometer long gold nanowires.

As for the HeLa cells, the condition changed. Very few cells were transfected 24 h after incubation with all transfection reagents despite the fact that more DNA molecules were used. After a few days, the cells started to exhibit red fluorescence. Shown in figure 5 are the combined DIC and fluorescence images of the transfected HeLa cells six days after transfection. The viability of the HeLa cells 24 h after incubation with the transfection reagents was measured to be 95%, 87%, 86% and 90% for gold nanowires, PolyFect, calcium phosphate and naked DNA, respectively. HeLa cells seemed to resist the addition of the transfection reagents. Therefore, lower cytotoxicity as well as lower transfection efficiency was measured for the HeLa cell lines. The transfection efficiencies

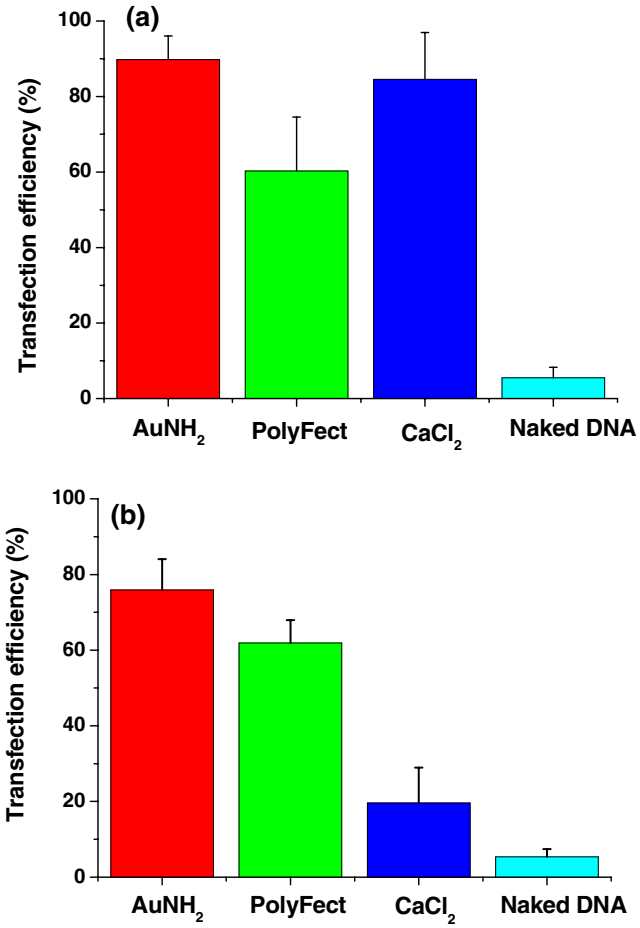

Figure 6. The transfection efficiency measured for different transfection reagents for (a) 3T3 cells and (b) HeLa cells.
(This figure is in colour only in the electronic version)

of four different reagents for both cell lines are summarized in figure 6. In both cases, the gold nanowires exhibited the highest transfection efficiency while very little cytotoxicity to both cells lines was measured. To achieve higher transfection efficiency for other transfection reagents, DNA loading in the μg region was needed [35]. However, higher DNA loading led to severe damage to the cells.

Learning that the micrometer long gold nanowires could be used to delivery DNA effectively into cells, it is also important to compare them with other newly developed nanomaterials for DNA delivery, such as nanotubes [36–39], nanorods [29] and nanoparticles [40, 41]. Since the transfection efficiency depended on many parameters such as cell types, promoters and expressed genes, only those systems expressing GFP proteins were compared. In this experiment, the maximum measured transfection efficiency was about 90%. A similar result was reported by using cationic Au/SiO_2 nanoparticles as the transfection reagents [40]. However, only 5% of HUVEC cells were found to express GFP proteins when amino modified carbon nanotubes were used and up to 10% transfection efficiency was measured for the HeLa cells using multi-shell calcium phosphate nanoparticles for DNA delivery. It seemed to us that the micrometer long gold nanowires exhibited relatively high transfection efficiency. In fact, the transfection efficiency for the 5 μm long gold nanowires was about 15 times higher than the naked DNA whereas only a fourfold increase in the GFP positive cells was observed when 200 nm gold–nickel nanorods were used. Therefore, we concluded that the micrometer long nanowires could effectively deliver plasmid DNA into both 3T3 and HeLa cells.

4. Conclusion

In summary, we have reported the studies of transfection efficiency for surface functionalized nanowires. It was found that the transfection efficiency of the aminothiol modified gold nanowires was the highest among the tested transfection reagents and other nanomaterials while almost no cytotoxicity was observed for gold nanowires under our experimental conditions. It was also shown that it was possible to trace the nanowires inside the cells with sub-micrometer resolution. Therefore, we concluded that the micrometer long multi-segment nanowires could be used for probing live cells with several advantages including easy fabrication and surface modification process, high transfection efficiency with very low cytotoxicity and easy observation with an optical microscope.

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