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FULL PAPER

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# Studies of Surface-Modified Gold Nanowires Inside Living Cells**

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The cytotoxicity of various surface-functionalized gold nanowires with different aspect ratios is investigated by (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) (MTT) assays for two cell lines, fibroblast and HeLa. It is found that functionalized gold nanowires with a diameter of 200 nm and lengths up to a few micrometers can be readily internalized by both types of cells regardless of the type of surface functionalization. However, the cytotoxicity of the gold nanowires is observed to depend on their surface modification. Serum-coated gold nanowires are the least toxic, whereas more than 50 % of the cells are damaged in the presence of mercapto-acid-modified gold nanowires even at very low concentrations ($10^3$ nanowires $mL^{-1}$). Nanowires with different aspect ratios exhibit the same cytotoxicity within limits of experimental error. However, the uptake efficiency is found to be higher for shorter nanowires as compared to their longer counterparts. Therefore, we conclude that internalized nanowires with high aspect ratios are more toxic to cells than nanowires with low aspect ratios. Positively charged aminothiol-modified gold nanowires are employed to deliver both plasmid DNA and probe molecules into cells without compromising the viability of the cells. The local environment of individual nanowires within the cells is studied by monitoring the fluorescence signal from probe molecules attached to the nanowires.

## 1. Introduction

With recent rapid advances in nanotechnology, various techniques have been developed to synthesize nanoparticles with novel optical, magnetic, electrical, mechanical, and catalytic properties.[1] By controlling the size, shape, and composition of the nanoparticles, their properties can be tailored to fit specific requirements, which permits great flexibility in designing new experiments and applications. Recently, there has been a lot of interest focused on the development of nanomaterials for solving complicated biological problems. For example, the use of nanoparticles such as quantum dots or metallic nanoparticles leads to significant improvements over conventional techniques for biosensing[2–5] and biolabeling.[6–9] However, the use of nanoparticles in studying living cells has not been extensively explored because of biocompatibility and cytotoxicity issues.[10–17]

Noble metals such as gold have been used in biological studies for a long time because of their stability and low toxicity. Lately, there has been renewed interest in developing metal-nanoparticle-based techniques for labeling,[18–20] drug delivery,[21–24] and gene regulation.[25] A common approach used in these applications is to chemically modify the nanoparticle surface to enable the recognition of a specific molecule or receptor on the cell surface. Alternatively, the nanoparticles can form complexes with drugs or genetic materials to obtain ingress into the cells. However, in more complex experiments, the nanoparticles may be required to possess several functionalities so that several tasks can be performed by a single particle. Towards this end, two peptides have been attached to the same gold nanoparticle to allow both receptor-mediated endocytosis and endosomal escape.[26] Multi-segment nanowires or nanorods are particularly useful for such types of applications owing to their symmetry. In a recent study, two types of molecules have been incorporated onto gold–nickel nanowires: the gold end has been used to bind plasmid DNA molecules through electrostatic interactions, whereas the nickel surface has been engineered to carry a specific polypeptide for site recognition.[22] However, most studies of nanorods or nanowires have been limited to particles with nanometer-sized dimensions. Micrometer-long nanowires are relatively less explored. Micrometer-long multi-segment nanowires have been used as barcodes for biological multiplexing; these barcodes can be readily visualized by an optical microscope.[27] Recently, it has been shown that micrometer-long nickel nanowires can be internalized by cells, enabling the manipulation of living cells by magnetic fields.[28,29] However, it is not known whether micrometer-long nanowires can be internalized by cells without in turn damaging them, which is an important issue for the development of nanowire-based probes of living cells. The internalization of the nanowires by living cells will allow us to directly observe the intracellular microenvironment around the

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individual nanowires through an optical microscope. We are primarily interested in developing nanowire-based probes of living cells. Towards this goal, we have attempted to modify the nanowire surfaces to carry plasmid DNA or probe molecules into cells, thus enabling the nanowires and probe molecules to be monitored by optical microscopy. Since molecules can be attached to the nanowire surfaces through electrostatic interactions, it is necessary to control the surface charge density. This is achieved by various surface modification schemes. To utilize surface-modified nanowires to study living cells, it is very important to investigate the cytotoxicity of these nanomaterials. The surface-modified nanowires can be used to explore the local environment inside living cells only if they can be internalized by the cells without damaging them. However, on some occasions, the surface-modified nanowires have been found to settle to the bottom of the cell culture dishes. Therefore, it is essential to compare the internalization process of nanowires for adherent and non-adherent cell types. Herein, we report a cytotoxicity study of the internalization process of functionalized gold nanowires possessing different surface charges for two different cell lines: National Institute of Health (NIH) 3T3 fibroblast cells from normal tissue, which is an adherent cell line, and HeLa S3 cells from neoplastic tissue, which can grow in suspension.

## 2. Results and Discussion

### 2.1. Characterization of Nanowires

Gold nanowires used in this experiment have been fabricated by electrochemical deposition utilizing commercial alumina membranes (200 nm) as templates. The details of the fabrication process and the experimental parameters are described in the Experimental. In order to investigate the influence of the aspect ratio (length-to-diameter) on the cytotoxicity, nanowires with four different aspect ratios have been prepared. Scanning electron microscopy (SEM) images and size distributions of the four different gold nanowire samples are shown in Figure 1. The lengths of the gold nanowires are $0.58 \pm 0.07$, $1.8 \pm 0.6$, $4.5 \pm 0.9$, and $8.6 \pm 1.4$ μm, as measured from the SEM images; the corresponding aspect ratios are 2.9, 9, 23, and 43, respectively. To study the influence of surface modification on cytotoxicity, the surfaces of the nanowires have been functionalized by a monolayer of thiols with amino, alkyl, or carboxyl end groups, or coated with serum, which is known to adsorb onto gold nanoparticles.[13] In a previous report,[30] the zeta potentials for self-assembled monolayers (SAMs) possessing amino, alkyl, and carboxyl end groups on the nanoparticle surfaces have been reported to be about + 10,

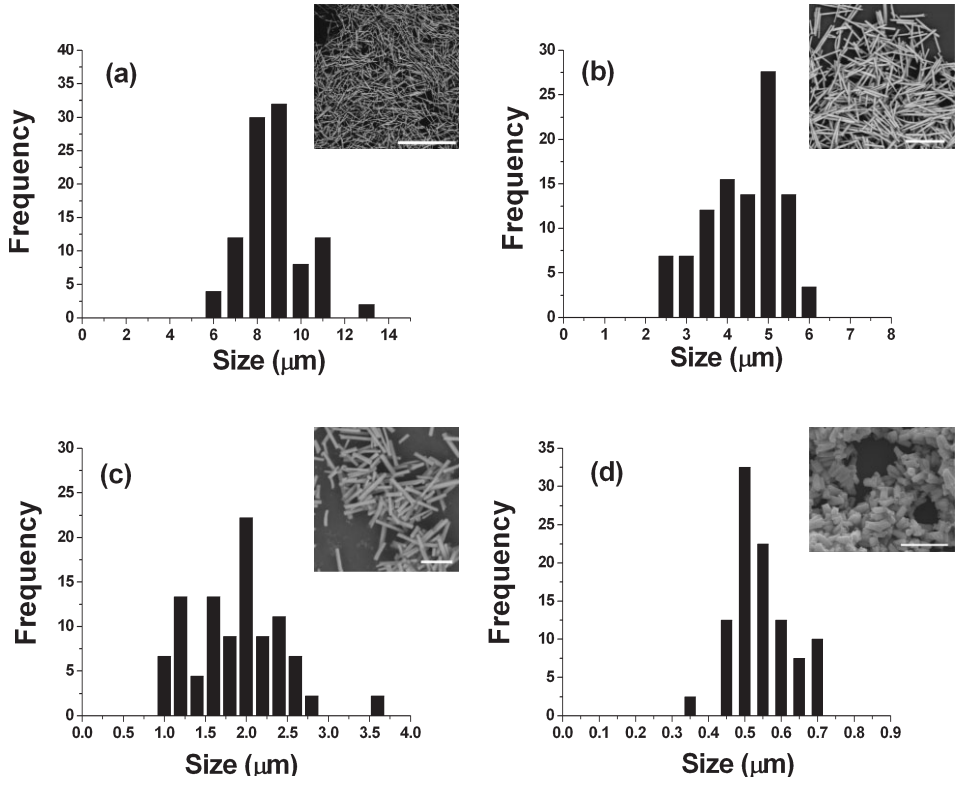


**Figure 1.** Size distributions of a) 8.6 μm gold nanowires. (the inset shows a SEM image, the scale bar represents 20 μm), b) 4.5 μm gold nanowires (the inset shows a SEM image, the scale bar represents 5 μm), c) 1.8 μm gold nanowires (the inset shows a SEM image, the scale bar represents 2 μm), and d) 0.58 μm gold nanowires (the inset shows a SEM image, the scale bar represents 1 μm).

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–30, and –50 mV, respectively, at pH 7. In this experiment, the zeta potential of unmodified gold nanowires has been measured to be –82 mV in doubly distilled water with a pH value of 6 in air (owing to the dissolution of $CO_2$). In contrast, the zeta potentials of nanowires modified with amino, alkyl, carboxyl, and serum moieties have been measured to be $+11.4 \pm 1.4$, $-7.2 \pm 0.6$, $-25.5 \pm 1.3$, and $-39.6 \pm 0.1$ mV, respectively, at pH 6. It is clear that amino-modified nanowires exhibit positive zeta potential, whereas a negatively charged surface is obtained for the mercapto-acid-modified nanowires. The nanowires functionalized with alkanethiols are slightly negatively charged, which may due to the presence of defects in the SAMs on the surfaces. The positively charged amino-modified nanowires have been used to bind negatively charged plasmid DNA molecules. The zeta potential for the nanowire/plasmid-DNA complex is about $-9.3 \pm 0.5$ mV in doubly distilled water at pH 6. However, when growth media are used to disperse the nanowires, the zeta potentials of nanowires capped with amino, alkyl, carboxyl, and serum moieties change to $-7.6 \pm 1.4$, $-6.9 \pm 1.2$, $-17.4 \pm 1.0$, and $-16.6 \pm 1.6$ mV, respectively, at pH 7.8. In the growth media, some of the surface-modified gold nanowires settle to the bottom of the cell culture dishes. However, the nanowires do not appear to aggregate up to nanowire concentrations of $10^6$ nanowires $mL^{-1}$.

### 2.2. Internalization of the Nanowires

It has been shown that nanoparticles with diameters less than 100 nm can be internalized easily by cells via endocytosis;[13] therefore, relatively small gold nanoparticles have been used as carriers of genetic material bound through electrostatic or covalent interactions for gene therapy. These gold nanoparticles have been demonstrated to be very effective at gene therapy with a relatively low toxicity ($LD_{50}$ is about 750 $\mu g\,mL^{-1}$).[14,22,25] However, it is not known whether micrometer-long metallic nanowires can be internalized by cells without causing damage, which is important if the nanowires are to be utilized for probing the local environment inside a living cell. To observe the internalization process of nanowires, 4.5 μm long gold nanowires (200 nm in diameter) coated with serum have been added to a glass-bottomed culture dish containing HeLa cells. The dish is placed inside a $CO_2$ incubator on an inverted microscope. Phase contrast images of the cells and nanowires are recorded by a charge coupled device (CCD) camera every 10 min. Figure 2a shows the image of a gold nanowire sitting outside the cell. As the cell migrates around the glass surface, the nanowire is enclosed within the cell, as shown in Figure 2b–d. As a result of this enclosure, the nanowire is internalized by the cell; the entire process takes about 4 h to complete (movie M1 in Supporting Information).

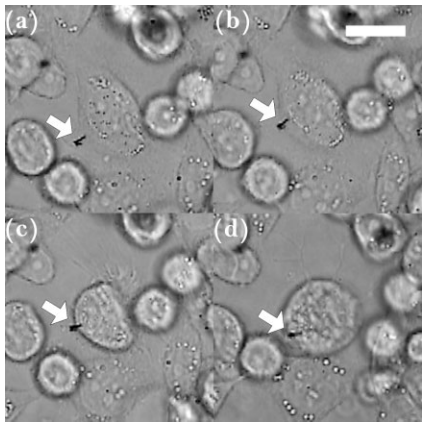


**Figure 2.** Phase contrast images of serum-coated gold nanowires entering a HeLa cell. The nanowire is indicated by an arrow. The scale bar represents 20 μm.

The above observations indicate that the nanowires can be internalized by the cells. However, the question still remains whether the surface-functionalized nanowires can be used as carriers to introduce foreign molecules into the cells. To investigate the ability of these nanowires to deliver DNA molecules into cells, 5 μm long nanowires have been first functionalized with aminothiols, which leads to the development of a positive charge on the nanowire surface.[31] Subsequently, negatively charged plasmid DNA molecules have been attached to the nanowires via electrostatic interactions. For visualization purposes, the plasmid DNA loaded onto the nanowire surfaces has been further labeled with YOYO-1, which emits a strong green fluorescence when bound to double-stranded DNA. The cells have been stained using an Image iT LIVE plasma membrane and nuclear labeling kit (Invitrogen). Figure 3 shows confocal images of HeLa cells, which have been incubated with the plasmid-coated nanowires for 24 h. As seen from the sectioning and stacked confocal images, the plasmid-coated nanowires have been internalized within the cell. The reconstructed

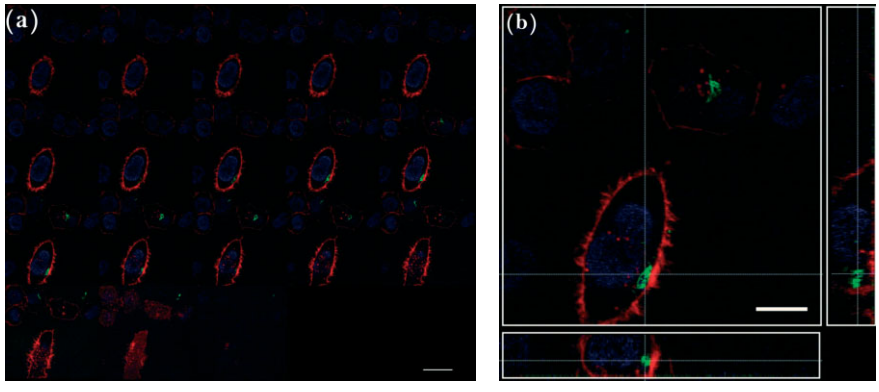


**Figure 3.** a) Sectioning images obtained from combined fluorescence images of aminothiol-functionalized gold nanowires coated with plasmids and labeled with YOYO-1 dye (green) in a HeLa cell. The nuclei have been stained blue in color, whereas the plasma membranes are stained red in color. The scale bar represents 20 μm. b) Stacked laser scanning confocal microscopy images of nanowires in a HeLa cell. The scale bar represents 10 μm.

3D image (Fig. M2) can be viewed in the Supporting Information. Two conclusions can be drawn from these observations: first, micrometer-long nanowires can be readily internalized by the cells despite their relative large size. Secondly, it is evident from the sectioning images that the surfaces of individual nanowires can be visualized with sub-micrometer-scale resolution, which may allow the use of functionalized multi-segment nanowires to probe the microenvironments within cells.

It is clear from the above results that surface-functionalized nanowires can carry plasmids into the cytoplasm. However, the functionality of the plasmids on the gold nanowires after entering the cell needs to be investigated. To study the functionality of the plasmids, a plasmid (pAcGFP1-Actin, BD) expressing green fluorescence protein (GFP) is coated onto gold nanowires modified by aminothiols and incubated with fibroblast cells for 24 h. Figure 4 shows a combined fluorescence and differential interference contrast (DIC) image of gold nanowires with GFP-expressing plasmids inside a fibroblast cell. It can be clearly seen that several gold nanowires reside inside the fibroblast cell after 24 h of incubation. The observation that the entire cell exhibits a strong green fluorescence signal indicates that the plasmids on the nanowires are still functional and capable of expressing GFP.[32] This experiment confirms that micrometer-long aminothiol-modified gold nanowires not only protect plasmid DNA molecules from degradation but also release plasmid DNA molecules inside the cells.

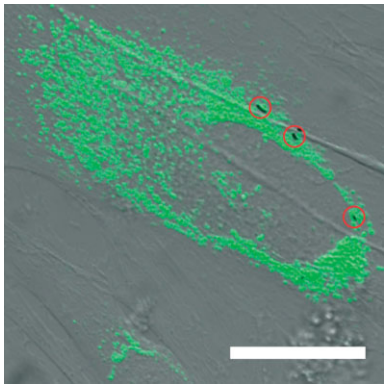

**Figure 4.** Combined differential interference contrast and fluorescence image of plasmid-coated nanowires and fibroblast cells expressing GFP. The nanowires are indicated by the red circles. The scale bar represents 20 μm.

### 2.3. Cytotoxicity of the Nanowires

It is clear from the previous sections that nanowires can carry plasmid DNA into the cells. The next step is to investigate the cytotoxicity of the surface-modified gold nanowires. Molecules are commonly carried on nanoparticle surfaces through electrostatic or covalent interactions. For metallic nanoparticles, it has been shown that positively charged surface capping agents such as aminothiols can be used to carry negatively charged DNA. However, it has also been found that a higher density of DNA molecules can be attached to mixed monolayer surfaces where alkanethiols are used to control the surface charge density.[23] To investigate the effect of these surface modifications on the cytotoxicity of the nanowires, we have performed a viability test using (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) (MTT) assays on three different types of SAM-modified surfaces. Aminothiols have been used to produce positively charged surfaces, mercapto acids have been used to generate negatively charged surfaces, and alkanethiols have been used as non-ionic surface modifiers. For comparison, nanowires coated with serum have also been tested. The viability tests for fibroblast 3T3 and HeLa cells are summarized in Figure 5.

In a typical viability test, 4.5 μm long gold nanowires with various surface modifications have been used. The density for both types of cells is about $10^5$ cells $mL^{-1}$, and the density of the nanowires is varied from $10^3$ to $10^6$ nanowires $mL^{-1}$. After incubating the cells for 24 h, the cytotoxicity of the nanowires

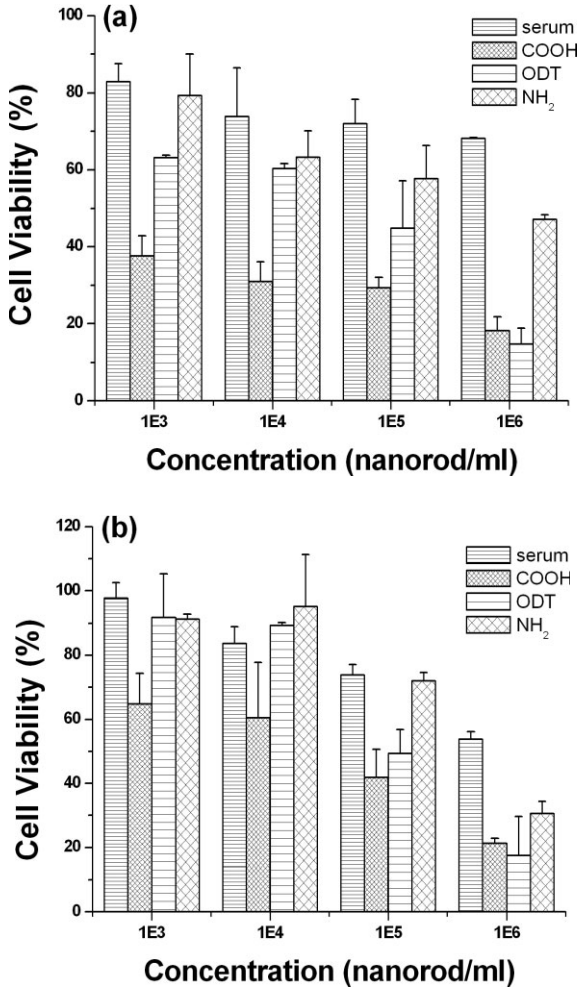


**Figure 5.** The cytotoxicity of gold nanowires has been measured for a) fibroblast and b) HeLa cells using MTT assays. The length of the nanowires is 4.5 μm and the incubation time is 24 h. The cell density is $10^5$ cells $mL^{-1}$.

ADVANCED FUNCTIONAL MATERIALS



is found to increase as the density of the nanowires increases; all the surface-modified nanowires except the serum-coated ones exhibit some degree of toxicity to both cell lines. At a low density of nanowires ($<10^4$ nanowires mL$^{-1}$), most of the cells appear to be unaffected by the addition of the nanowire solution. However, the carboxyl-presenting nanowires exhibit very strong cytotoxicity, even at very low nanowire concentrations ($10^3$ nanowires mL$^{-1}$). At higher nanowire concentrations, most of the cells are damaged by the addition of the nanowire solution except for the serum-coated samples. Extensive aggregation of nanowires is observed at a density of $10^7$ nanowires mL$^{-1}$, which makes it rather difficult to evaluate the number of nanowires. At nanowire densities higher than $10^8$ nanowires mL$^{-1}$, the bottom of the well is completely covered by nanowires. The $LD_{50}$ value of the serum-coated gold nanowires for 3T3 cells has been estimated to be $5\times10^7$ nanowires mL$^{-1}$ (ca. 150 μg mL$^{-1}$), which is lower than the value measured for small gold nanoparticles (ca. 750 μg mL$^{-1}$).[22] The origin of the lower $LD_{50}$ for the gold nanowires may perhaps be the larger size of the nanowires used in this experiment.

In a previous study,[13] it has been reported that the cellular uptake of gold nanoparticles depends on both the size and shape of the nanoparticles. For nanoparticles smaller than 100 nm, it has been found that the uptake efficiency of nanoparticles peaks at a size of 50 nm. It is also believed that the uptake efficiency decreases with increasing aspect ratio. Since the cellular uptake depends on the size of the nanoparticles, the cytotoxicity will also vary with the particle size. Therefore, we have investigated the cytotoxicity of different sizes of nanowires with aspect ratios up to ca. 43. In this study, the concentration of mercapto-acid-modified nanowires is set at $10^5$ nanowires mL$^{-1}$ for all aspect ratios. Figure 6 shows the cytotoxicity results for 3T3 and HeLa cells. Surprisingly, the cytotoxicity of the micrometer-long nanowires exhibits little dependence on their aspect ratios for both cell lines. However, in a separate experiment, the cytotoxicity of 250 nm sized spherical gold nanoparticles (BBInternational) modified with mercapto acid has been tested. The viability for both cell lines has been measured to be larger than 80 % for nanoparticle concentrations up to $10^7$ nanoparticles mL$^{-1}$. Considering that the mass concentration of $10^7$ nanoparticles mL$^{-1}$ 250 nm spherical gold nanoparticles is equivalent to that of 10 μm long nanowires with a diameter of 200 nm at a concentration of $1.6\times10^5$ nanowires mL$^{-1}$, the nanowires are more toxic than the spherical nanoparticles, which is probably because of the geometry of the nanowires. However, the exact origin of the cytotoxicity of the nanowires requires further investigation.

### 2.4. Uptake of Nanowires

To further understand the internalization process of the nanowires, we have studied the uptake kinetics of aminothiol-modified gold nanowires for both cell lines. In these experiments, $10^6$ gold nanowires have been incubated with both cell lines in 96 well dishes containing growth media. After 2, 4, 6, 8, 10, and 12 h of incubation with aminothiol-modified gold nanowires, the cells in each well are rinsed several times with phosphate-buffered saline (PBS) to remove the excess nanowires in the media. Subsequently, the cells are detached from the dish using the enzyme trypsin. The internalized nanowires are recovered by lysing the cells with alcohol, and are counted using a hemacytometer. The results of these experiments for nanowires with varying aspect ratios are plotted in Figure 7. The uptake kinetics for all sizes of nanowires are very similar in both HeLa and 3T3 cells. Both types of cells exhibit maximum uptake after 8 h of incubation with the nanowires; the uptake decreases after reaching a maximum. The maximum internalization efficiency is higher than 50 % for both cell lines. The reason for the eventual decrease in uptake is the increased cell death at high concentrations of nanowires, as indicated by the viability test (Fig. 5).

Another trend observed in this experiment is that the uptake efficiency of longer nanowires is lower than for the shorter ones except for the 0.58 μm long gold nanowires, which are too short for the accurate determination of the number of nanowires. This trend agrees well with a previous measurement using smaller nanorods.[13] Therefore, given that nanowires with different aspect ratios exhibit the same cytotoxicity and that the uptake efficiency of the nanowires decreases as the aspect ratio is increased, we conclude that internalized nanowires with higher aspect ratios are more toxic to both cell lines. This conclusion is consistent with the observations that gold nanowires are more toxic than spherical (250 nm) gold nanoparticles. Furthermore, these results also explain why the $LD_{50}$ of the nanowires is lower than that of nanorods with smaller aspect ratios. The origin of the toxicity of nanowires with relatively high aspect ratios may be their larger surface areas.

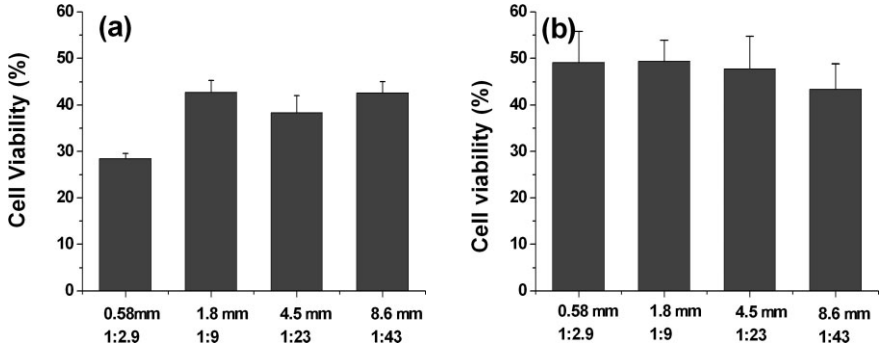


**Figure 6.** The cytotoxicity of gold nanowires with various aspect ratios (1:2.9, 1:9, 1:23, and 1:43) for a) fibroblast and b) HeLa cells measured using MTT assays. The incubation time is 24 h. The cell density is $10^5$ cells mL$^{-1}$.

### 2.5. Behavior of Nanowires Inside Cells

To investigate the behavior of surface-modified nanowires inside cells, DIC images of living cells have been acquired in an incubator on an Olympus Fluoview 300 confocal microscope. Figure 8a shows an

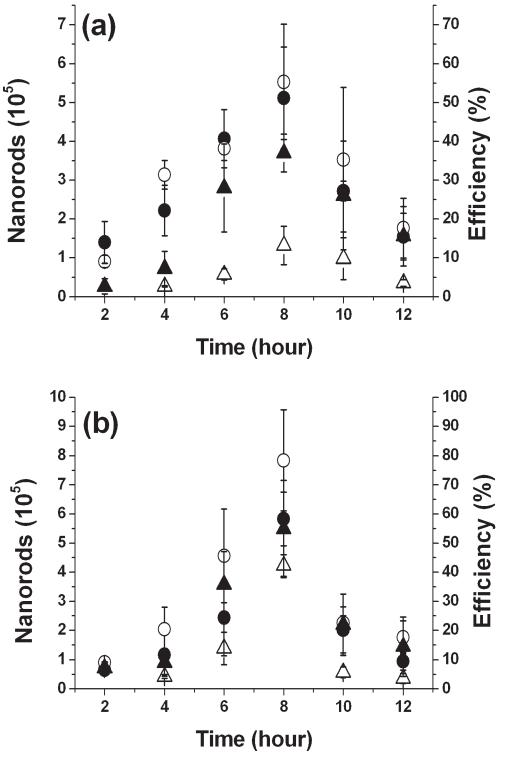


**Figure 7.** The uptake of aminothiol-modified gold nanowires with four different lengths, 0.58 μm (solid circles), 1.8 μm (open circles), 4.8 μm (solid triangles), and 8.6 μm (open triangles) for a) fibroblast and b) HeLa cells.

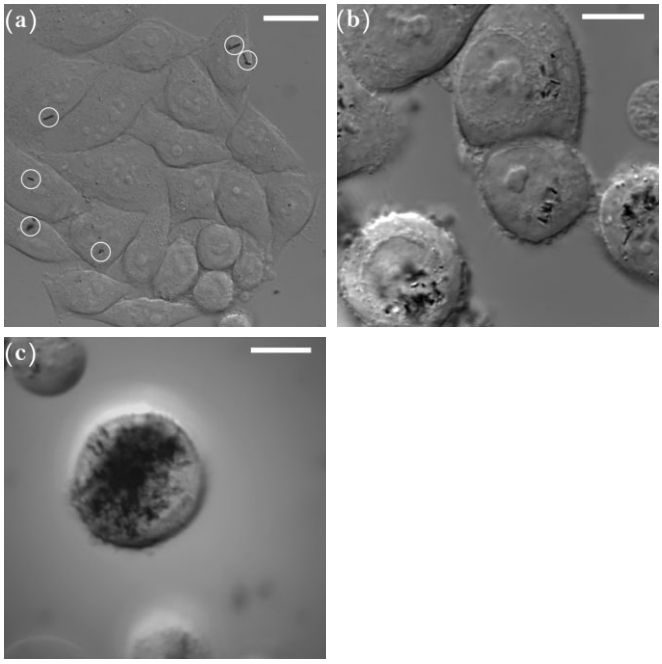


**Figure 8.** DIC images of alkanethiol-modified gold nanowires in HeLa cells at different nanowire concentrations. a) $5 \times 10^4$ nanowires $mL^{-1}$. The scale bar represents 20 μm. b) $5 \times 10^5$ nanowires $mL^{-1}$. The scale bar represents 10 μm. c) Aggregation of nanowires in a single cell. The scale bar represents 10 μm. The nanowires are indicated by the circles.

image of 5 μm long alkanethiol-modified gold nanowires internalized by the HeLa cells after incubation for 24 h. The nanowire density is about $5 \times 10^4$ nanowires $mL^{-1}$ and the cell density is about $10^5$ cells $mL^{-1}$. From the DIC image, it can be seen that the nanowires are unevenly internalized among the cells. For a solution of 2 μm long nanowires at a concentration of $5 \times 10^5$ nanowires $mL^{-1}$, several gold nanowires are found inside most of the HeLa cells, as shown in Figure 8b. Occasionally, the nanowires are seen to aggregate within a single cell, as shown in Figure 8c. At this concentration, more than 50 % of the HeLa cells are not viable, as indicated by a decrease in the cell density. This observation also agrees well with the results of the uptake measurements in Figure 7b showing that shorter nanowires exhibit a higher uptake efficiency. Upon the addition of a serum-coated gold nanowire solution with a concentration of $2 \times 10^6$ nanowires $mL^{-1}$ to a culture dish containing 3T3 cells, most cells are found to be attached to the surfaces. At this concentration, more than 70 % of the cells are still viable, and on average, each cell internalizes about 10 nanowires, as shown in Figure 9. This result also agrees well with the uptake measurements for the 3T3 cells shown in Figure 7a.

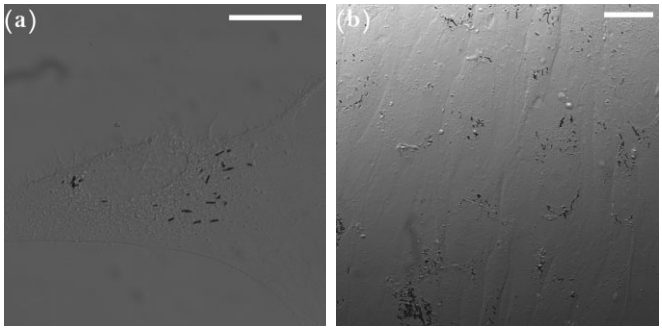


**Figure 9.** DIC images of serum-coated gold nanowires in fibroblast cells at a nanowire concentration of $2 \times 10^6$ nanowires $mL^{-1}$. a) Nanowires in a single cell. b) Nanowires among several cells. The scale bar represents 20 μm.

For gold nanoparticles smaller than 100 nm, it is believed that the nanoparticles enter the cells via receptor-mediated endocytosis. However, it is not clear which mechanism dominates the cellular uptake behavior of micrometer-long nanowires. In this experiment, we have monitored the entire internationalization process of the nanowires under a confocal microscope. In most cases, we have found that the nanowires enter the cell with one end pointing towards the cell, as shown in Figure 2. It is reasonable that the small dimensions of the nanowires leads to a greater probability of uptake by the cells. Once one end of the nanowire is internalized by the cell, the rest of the nanowire is surrounded by the cell membrane, resulting eventually in the entire nanowire being consumed by the cell. As long as the nanowires stay within the cell, they are able to move around inside the cytoplasm. Most commonly, we have found that the nanowires maneuver alongside the edge of the cell

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(movie M3 in the Supporting Information). More studies are needed to understand the detailed mechanism of the endocytosis pathway for micrometer-long nanowires.

To demonstrate that it is possible to monitor the local environment of the nanowires, LysoSensor yellow/blue DND-160 (Invitrogen) has been incubated with 2 μm long amino-modified gold nanowires for 10 min. The nanowire solution is then incubated with 3T3 cells for 24 h. Figure 10 shows confocal images of the LysoSensor-coated nanowires inside the 3T3 cells. The LysoSensor yellow/blue exhibits pH-dependent dual emission spectra with emission in the 500–600 nm region (artificial red color in Fig. 10) in an acidic environment and fluorescence in 410–500 nm region (artificial green color in Fig. 10) in a less acidic environment. As a result, LysoSensor can be used to probe acidic organelles such as endosomes or lysosomes. From Figure 10, it can be clearly seen that both the LysoSensor and nanowires can be simultaneously visualized on a confocal microscope. The co-localization of the nanowires and the green fluorescence signals indicate that all the nanowires are located in less acidic compartments, presumably within the endosomes, as a result of the endocytosis process. By monitoring both the DIC image of the nanowires and the confocal image of the probe molecules as a function of time, the evolution of the local environment around the nanowires can be explored (movie M4 in Supporting Information). In general, this approach can be extended to other types of probe molecules or biological assays.

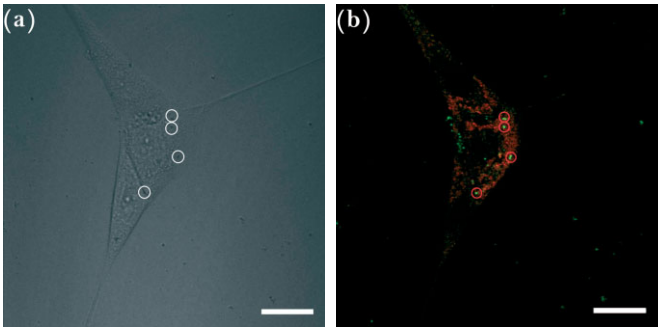


**Figure 10.** a) DIC image and b) combined confocal image of LysoSensor-coated gold nanowires inside 3T3 cells. The acidic organelles exhibit strong fluorescence in the 500–600 nm region (artificial red color has been used), whereas the less acidic compartments emit fluorescence in the 410–500 nm range (artificial green color). The locations of the nanowires are highlighted by circles. The scale bar represents 20 μm.

## 3. Conclusions

We have investigated the cytotoxicity of micrometer-long gold nanowires with four different aspect ratios and four different surface coatings. It has been found that serum-coated nanowires exhibit the least cytotoxicity with a $LD_{50}$ value of ca. 150 $\mu g\,mL^{-1}$, which is less than those measured for smaller gold nanoparticles. All other surface-functionalized nanowires possess some degree of toxicity, which is found to depend on the surface charge. Mercapto-acid-modified nanowires have been found to be the most toxic of all the samples tested here. For the same type of surface modification, HeLa cells, which can grow in suspension, have been found to be more resistant to the addition of the nanowire solution. As for nanowires with different aspect ratios, the cytotoxicity experiments indicate that nanowires with different aspect ratios exhibit almost the same degree of toxicity. However, the uptake efficiency for shorter nanowires has been measured to be higher than for their longer counterparts. Therefore, we conclude that internalized nanowires with higher aspect ratios are more toxic than shorter ones, which explains why the $LD_{50}$ values for these nanowires are lower than that for the low-aspect-ratio nanorods. This conclusion is also consistent with the cytotoxicity experiments for spherical nanoparticles where the nanowires have been found to be more toxic than the spherical nanoparticles.

To utilize the nanowires as probes for living cells, amino-modified gold nanowires have been used to deliver both GFP-expressing plasmids and pH-sensitive probe molecules into living cells without compromising the viability of the cells. The time evolution of individual nanowires as well as probe molecules in living cells has been recorded, and we conclude that it is possible to employ micrometer-long nanowires to explore the local environment inside living cells.

## 4. Experimental

*Synthesis of Gold Nanowires*: Commercial alumina membranes with a diameter of 200 nm (Whatman) were used as templates to produce the nanowires. One side of the membrane was sputter-coated with a 200 nm thick silver layer, which served as the electrode for electrochemical deposition. A 2 mm diameter platinum wire was used as the counter electrode. Electroplating was carried out using a Techni Gold 25E (Technic) electroplating solution, and a pulsed electrodeposition technique was used to deposit gold into the nanopores of the membrane. During the electroplating period, a negative voltage of –10 V was applied for 8 ms, followed by a short pulse of positive voltage at 3 V for 2 ms. Before the next application of the negative voltage, the system was maintained at 0 V for 990 ms. These pulse sequences were repeated for 80 min to produce 4.5 μm long gold nanowires. The length of the nanowires was controlled by monitoring the total charge passing through the electrochemical cells and the deposition time. Isolated nanowires were obtained by first removing the back-deposited silver film in a 3 M nitric acid solution, followed by dissolving the membrane in a 3 M NaOH solution to release the nanowires from the membrane. The nanowires were further cleaned by rinsing several times in doubly distilled water and centrifuging at 5000 rpm for 10 min. The concentration of the nanowire solution was measured by a hemacytometer and observed with a 20× objective. The length of the nanowires was determined from their SEM images, which were obtained using a Leo 1154 instrument at 5–20 kV.

*Surface Functionalization of Nanowires*: The nanowire surfaces were coated by a monolayer of thiols with amino, alkyl, or carboxyl end groups. To modify the nanowire surfaces, the suspended gold nanowire ($1 \times 10^8$ nanowires $mL^{-1}$) solutions were mixed with an ethanol solution of 1 mM 11-amino-1-undecanethiol (Dojindo), 1 mM of octadecanethiol (Aldrich), or 1 mM of 11-mercaptoundecanoic acid (Aldrich). After 24 h of incubation, the nanowires were cleaned using doubly distilled water and the excess thiols were removed by dialysis for 24 h using a 3.5 kD cut-off dialysis membrane with doubly distilled water. For comparison, the cytotoxicity of the serum-coated nanowires was also inves-

tigated. The serum-coated nanowires were obtained by incubating the gold nanowires in phosphate buffered saline (PBS) solution containing serum for 24 h. All the surface-modified nanowires could be suspended in aqueous solutions by strong vortexing. However, longer nanowires exhibited a higher sedimentation rate. The zeta potentials of the surface-modified nanowires were measured around neutral pH using a ZetaPALS (Brookhaven Instruments) zeta potential analyzer at fields of 8–16 V $cm^{-1}$.

*Cell Culture*: Fibroblast NIH 3T3 and HeLa S3 (BCRC, Bioresource Collection and Research Center) were used in the cell culture experiments. The medium used was Dulbbeco's modified Eagle's medium (DMEM, Gibco) for fibroblast cells, and minimum essential medium (MEM, Gibco) for HeLa cells. 10 % fetal bovine serum (FBS, PAA Laboratories), and PEN-STREP-AMPHO (penicillin-streptomycin-amphotericin) solution (Biological Industries) were added to both culture media. The fibroblast and HeLa cells were seeded in a T75 tissue culture flask containing 15 mL of the appropriate fresh growth medium. Subsequently, the cells were incubated at 37 °C in a 5 % $CO_2$ atmosphere.

*MTT Assays*: To determine the cytotoxicity of the gold nanowires, the cells were first seeded in 96 well plates at a density of $1 \times 10^5$ cells $mL^{-1}$ at 37 °C under a 5 % $CO_2$ atmosphere. After 24 h of culture, the wells were refilled with fresh media, and serial dilutions of nanowires with nanowire concentrations ranging from $10^3$ to $10^6$ nanowires $mL^{-1}$ were made. Subsequently, 90 μL of the gold nanowire solutions with different concentrations were added to each well. Control experiments were carried out with cells treated with an equivalent volume of serum medium without any nanowires. The cells were then incubated for 24 h at 37 °C. The cytotoxicity was measured using an MTT assay to measure the succinate dehydrogenase mitochondrial activity. PBS solutions containing 10 μL of the 5 mg $mL^{-1}$ MTT stain (in vitro toxicology kit, Sigma) were added into each well and incubated for 4 h. After mixing, 90 μL of the MTT solubilization solution was added to each well. The stain was then aspirated and the purple colored crystals were dissolved with acidic isopropyl alcohol. After 15 min, the absorbance in each well was measured at 570 nm using a microplate reader (μQuant, Biotek Instruments). The background absorbance was measured in PBS solution without the presence of cells and nanowires. All experiments were repeated three to nine times. The cell viability (%) relative to the control wells containing the cell culture medium without nanowires was calculated using $[A]_{test}/[A]_{control} \times 100$ where $[A]_{test}$ was the absorbance of the test sample and $[A]_{control}$ was the absorbance of the control sample. The cytotoxicity of the gold nanowires was also tested using media without serum. However, no significant difference was observed.

*Optical and Confocal Microscopy*: To study the internalization process of the nanowires, the HeLa cells were first seeded on a glass bottom culture dish (Bioptechs) at a density of $10^5$ cells $mL^{-1}$ and incubated with 4.5 μm long serum-coated gold nanowires in a $CO_2$ incubator (MIU-IBC-IF, Olympus) on an inverted microscope (IX71, Olympus). The phase-contrast images were recorded every 10 min using a CCD camera (DP 70, Olympus). To observe the nanowires inside the living cells, HeLa cells were plated onto 35 mm glass-bottomed culture dishes (Bioptechs) at a density of $10^5$ cells $mL^{-1}$. The cells were then incubated with nanowires in medium at 37 °C for 24 h. A laser scanning confocal microscope (Olympus, Fluoview 300) was used to obtain fluorescence and DIC images using an oil immersion objective (60 ×, numerical aperture (NA) of 1.35). To study of the internalization of the nanowires with the plasmid, 2 μm long gold nanowires modified with 11-amino-1-undecanethiol (Dojindo) were used. The GFP-expressing plasmid (pAcGFP1-Actin, BD) was bound to the nanowire surface through electrostatic interactions. For visualization purposes, a YOYO-1 dye (Invitrogen) was used to label the double-stranded DNA. The cross-sectional images of the nanowires inside the HeLa cell were taken using a laser scanning confocal microscope (SP5, Leica) with an oil immersion objective (63 ×, NA of 1.4).

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