NANO LETTERS

2008
Vol. 8, No. 8
2150-2154

Cellular Uptake and Cytotoxicity of Silica Nanotubes

Anjan Nan,[†,‡,#] Xia Bai,[§,#] Sang Jun Son,[§,||] Sang Bok Lee,[*,†,§,⊥] and Hamidreza Ghandehari[*,¶,†,‡,⊥]

Center for Nanomedicine and Cellular Delivery, Department of Pharmaceutical Sciences, University of Maryland Baltimore, Maryland, Department of Chemistry and Biochemistry, Maryland NanoCenter, University of Maryland College Park, Maryland, and Gachon BioNano Research Institute, Kyungwon University, Republic of Korea

Received January 28, 2008; Revised Manuscript Received April 16, 2008

ABSTRACT

"Template synthesized" silica nanotubes (SNTs) provide unique features such as end functionalization to control drug release, inner voids for loading biomolecules, and distinctive inner and outer surfaces that can be differentially functionalized for targeting and biocompatibility. Very limited information is available about their biological interactions. This work evaluates the influence of size and surface charge of SNTs on cellular toxicity and uptake. Results additionally indicate endocytosis to be one possible mechanism of internalization of SNTs.

Multifunctional nanocarriers used to date such as liposomes, linear polymers, nanoparticles, and dendrimers suffer from inherent limitations such as instability or polydispersity, often resulting in nonspecific accumulation and toxicity in normal organs.[1] Several studies have suggested that physicochemical properties of nanocarriers such as size, shape, and surface functionalization influence their biodistribution (i.e., passing through endothelial and epithelial barriers), targetability (either passively by size control or actively by attaching targeting moieties to the surface of the carrier), and bioactive agent release (by control of pore size within the carrier).[2] Development of advanced delivery systems where size, size distribution, functionalization, and loading capacity is precisely tuned may allow a higher degree of control over their biological fate. Such control could potentially enhance therapeutic efficacy and reduce nonspecific toxicity associated with conventional carriers.

Silica nanotubes (SNTs) are a novel class of inorganic structures with potential in bioseparation, drug delivery, imaging, and other biomedical applications.[3] They can be fabricated by "template synthesis" and differentially functionalized on their inner and outer surfaces.[4] This unique strategy provides monodisperse nanotube dimensions (size and diameter) with features useful as drug carriers. Their inner voids can be used to load large amounts of bioactive agents (e.g., small molecule drugs, proteins, genes) to protect the payloads from enzymatic degradation in the body until SNTs reach the target site. Their open ends can also be used as a gate to control drug release.[5,6] Differential functionalization can allow selective attachment of moieties to the inside (such as drugs and imaging agents) and outside (targeting moieties, antifouling agents). Lee and co-workers have recently developed magnetic nanotubes (MNTs) (i.e., silica nanotubes with iron oxide nanoparticles deposited inside) with superparamagnetic properties which can be potentially used for a variety of applications such as image-guided magnetically controlled bioseparation and biointeraction.[3] Combining the attractive tubular structure with magnetic properties, SNTs can be an ideal multifunctional platform for magnetic resonance image (MRI) guided delivery.[7] SNTs are mechanically robust with no swelling or porosity changes under physiological conditions.[8] By loading biomolecules inside the tubes, the outer surface can be kept biocompatible which can prevent aggregation and nonspecific adsorption as commonly seen with nanoparticles where hydrophobic drugs are attached to the surface.

Despite the unique advantages of SNTs in biomedical applications, exploration of their interactions with biological systems remains at a very early stage. To effectively develop these systems for application, it is necessary to systematically delineate the structural and functional properties that influ-

* To whom correspondence should be addressed. E-mail: (H.G.) hamid.ghandehari@pharm.utah.edu; (S.B.L.) slee@umd.edu.

† Center for Nanomedicine and Cellular Delivery, University of Maryland Baltimore.

‡ Department of Pharmaceutical Sciences, University of Maryland Baltimore.

§ Department of Chemistry and Biochemistry, University of Maryland College Park.

|| Kyungwon University.

⊥ Maryland NanoCenter, University of Maryland College Park.

¶ Present address: University of Utah Departments of Pharmaceutics and Pharmaceutical Chemistry and Bioengineering, 383 Colorow Way, Salt Lake City, Utah 84108.

# These authors contributed equally to this work.

10.1021/nl0802741 CCC: $40.75 © 2008 American Chemical Society
Published on Web 07/12/2008

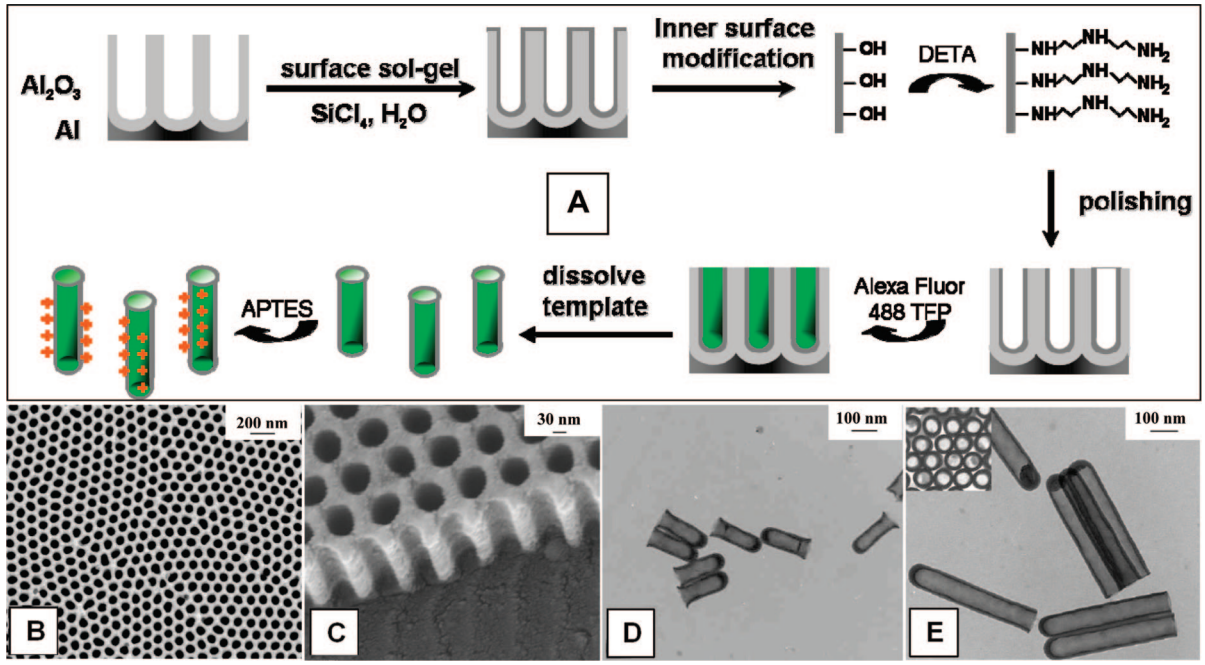

Figure 1. (A) Schematic representation of template synthesis of silica nanotubes, (B,C) scanning electron microscope images of porous alumina template for SNT (diameter 50 nm, length 200 nm), (B) top view, (C) cross section, (D,E) transmission electron microscope images of bare silica nanotubes (50 nm dia.) Length: (D) 200 nm, (E) 500 nm, with inset of TEM image for the cross section of the SNTs bundle. Images confirm the monodisperse, well-defined dimensions of SNTs.

ence biocompatibility and mechanisms of cellular uptake and interactions. In this paper, we describe the cytotoxicity and cellular uptake of SNTs with two different sizes and surface charges in primary (nonmalignant) and cancer cell models.

Using the "template synthesis" strategy (Figure 1A) combined with surface sol−gel chemistry, a series of 50 nm diameter SNTs were fabricated, namely, (1) 200 and 500 nm long, bare (nonfunctionalized) SNTs and (2) 200 and 500 nm long, SNTs functionalized with positively charged aminosilane group on the outer surface. The TEM images demonstrate discrete monodisperse dimensions (diameter and length) of the template (Figure 1B,C) and the fabricated nanotubes (Figure 1D,E). The dimensions of the nanotubes can be controlled by the pore diameter and thickness of the template.[9,10]

Several inorganic nanotubular structures such as carbon nanotubes,[11] gold nanorods,[12] etc. have emerged in the recent years as promising nanomaterials for biomedical utilization. However in most cases such novel structures have never been studied in the context of a pharmaceutical or clinical application. Research on the toxicity of nanostructures has just begun and the data are still fragmentary and subject to criticisms. Preliminary results highlight a number of parameters including structure, size, surface chemistry and surface charge, and agglomeration state to have considerable impact on the reactivity of nanotube structures.[13,14] There is a clear need for systematic studies to assess the toxicological and pharmacological profiles of nanotubes. In the present study, we attempted to delineate the physicochemical attributes of SNTs that affect their biological interactions which could potentially lead to toxicity.

The effect of varying size and surface functionalization of SNTs on cytotoxicity was evaluated against two different human cell models: an epithelial breast cancer cell line (MDA-MB-231) and a primary umbilical vein endothelial cell line (HUVEC). The two cell lines were chosen as representative models of the various cellular environments that SNTs are likely to interact with in vivo. In both the cell lines, the SNTs exhibited growth inhibition in a concentration dependent manner. At lower concentrations (0.05 and 0.005 μg/ml), the nanotubes did not inhibit the growth of either of the above cell lines. At higher concentrations (5.0 and 0.5 μg/ml) both the 200 and 500 nm SNTs caused up to 63% growth inhibition of breast cancer cells. At 5 μg/ml concentration, 200 nm positively charged SNTs were significantly more toxic ($p < 0.002$) than the corresponding bare nanotubes (Figure 2A). This higher toxicity suggests the possibility of increased cellular association and/or uptake of the nanotubes due to nonspecific interactions with the negatively charged cell surface. These observations are also in agreement with reports in the literature on other nanosized carrier molecules such as cationic polymers, amino- or polylysine-functionalized silica nanoparticles,[15,16] cationic PAMAM dendrimers, or several other positively charged nanostructures[17] whose inherent cytotoxicity and cellular uptake is shown to be primarily associated with cationic charge present on the surface.

The nanotubes showed a similar trend for concentration dependent cytotoxic effects against the primary HUVEC cells (Figure 2B). However compared to breast cancer cells at high concentrations (5.0 and 0.5 μg/ml) the effect of size and charge seemed to be more significant. Positively charged nanotubes were significantly more toxic than bare nanotubes even at 10-fold lower concentration (0.5 μg/ml) in HUVECs than in breast cancer cells. These results indicate that in addition to concentration and surface charge, cytotoxicity

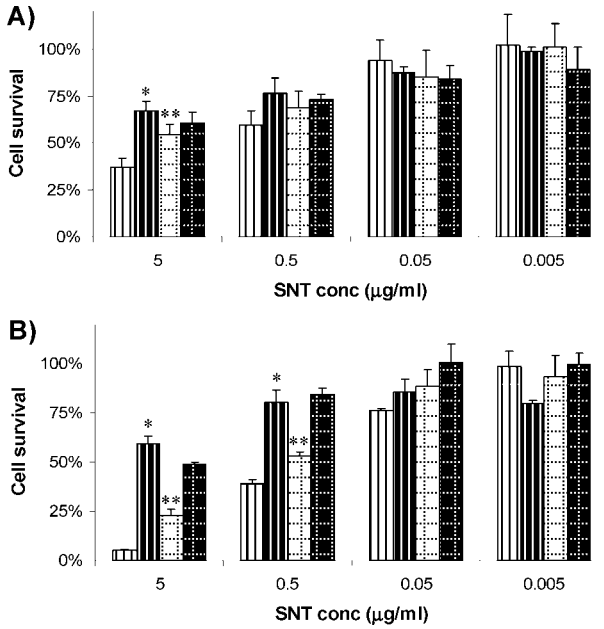

Figure 2. MTT cytotoxicity assay showing the effect of varying concentrations of nanotubes on growth inhibition of (A) MDA-MB-231 breast cancer cells and (B) human umbilical vein endothelial cells (HUVEC) cultured in vitro. Increase in nanotube concentration resulted in decreased cell viability. At higher concentrations, positively charged nanotubes were more toxic. At equal nanotube concentrations 200 nm nanotubes were more toxic than 500. Bars in order for each SNT concentration: 200 nm with positively charged APTS outer surface group (open vertical); 200 nm with no outer surface functionalization (solid vertical); 500 nm with positively charged APTS outer surface group (open hatch); 500 nm with no outer surface functionalization (solid hatch). Results are reported as mean ± SEM ($n = 3$). Statistically significant differences are indicated relative to 200 nm positively charged SNTs (*, $p < 0.002$; **, $p < 0.02$).

of SNTs is cell-type dependent. Reports in the literature on similar studies with silica nanoparticles suggest[18] that cancer cells are more resistant to nanoparticle-mediated toxicity than normal nonmalignant primary cells of the body. Such resistance is attributed to the fact that normal primary cells of the body are more susceptible to nanoparticle-mediated cellular membrane injury leading to more rapid onset of apoptotic or necrotic cell death.[18–21] Others more informatively relate cytotoxicity data of different cell lines to their metabolic activity or their rate of proliferation.[18,22] The cell population doubling times of normal human endothelial cells were about 30 h compared to 20 h doubling time of the MDA-MB-231 breast cancer cells. However further mechanistic toxicity studies are needed to confirm these observations in the context of the SNTs.

At higher concentrations (5.0 and 0.5 μg/ml) positively charged SNTs of smaller length (200 nm) were significantly more toxic to the HUVECs than their longer (500 nm) counterparts. This was also observed at 5 μg/ml concentration for breast cancer cells. The results indicate that in addition to charge, size of nanotubes may also determine the extent of interaction with cells and toxicity. Reports in the literature[23–25] generally suggest that smaller sized particles allow increased cellular interactions and therefore may possess enhanced intrinsic toxicity. Further when comparing equivalent concentrations, the number of 200 nm SNTs is about 2.5 times of that of 500 nm given they have the same mass. Therefore smaller particle size along with increased number of particles may be responsible for the increased toxicity observed with the 200 nm length SNTs. Given the control over nanotube dimensions and functionalization using the template synthetic strategy one can conceive the ability to predict the influence of discrete size and charge on cytotoxicity and uptake.

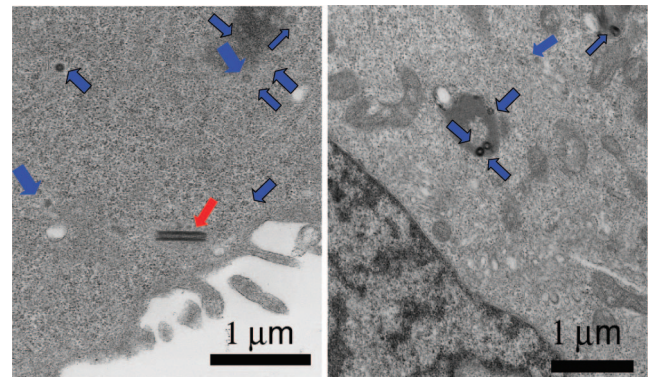

Figure 3. Transmission electron microscope images showing uptake of 500 nm positively charged SNTs in MDA-MB-231 cells. Blue arrows indicate cross sectional view and red arrows horizontal view of nanotubes.

Although growth inhibitory effects were observed for both bare and positively charged SNTs, these effects were primarily seen at very high concentrations. At 0.05 μg/ml concentration ($\sim 10^8$ SNTs/ml) and lower all SNTs irrespective of their surface characteristics were nontoxic. This indicates the suitability of these nanotubes for drug delivery applications. Additionally appropriate surface modification of SNTs such as with biocompatible polymers could potentially enhance their safety profile but such studies need to be performed.

We further evaluated the internalization of SNTs by transmission electron microscopy (TEM). As shown in Figure 3, intact nanotubes are primarily seen vertically embedded (in the cross-sectional view, marked by blue arrows) as confirmed by measurement of their diameters. Few intact nanotubes are seen in a horizontal alignment (lengthwise view Figure 3 left panel marked with a red arrow). These results provide visual evidence for the first time of cellular uptake of SNTs.

Finally we evaluated the possible mechanisms of cellular uptake of SNTs. Reports in the literature suggest that the uptake of nanoparticulate systems into cells typically can occur through several different processes such as nonspecific diffusion, phagocytosis and receptor mediated or fluid phase endocytosis.[26] As an initial attempt toward systematically delineating the mechanisms of uptake we investigated the involvement of fluid phase endocytosis. Confocal microscopy techniques were used in the absence and presence of known metabolic (ATP) inhibitors. Results demonstrated cellular uptake of fluorescently labeled SNTs (200nm) (Figure 4A). Internalization of SNTs was significantly inhibited in presence of sodium azide and sucrose (Figure 4B,C) suggesting the involvement of fluid phase endocytosis.

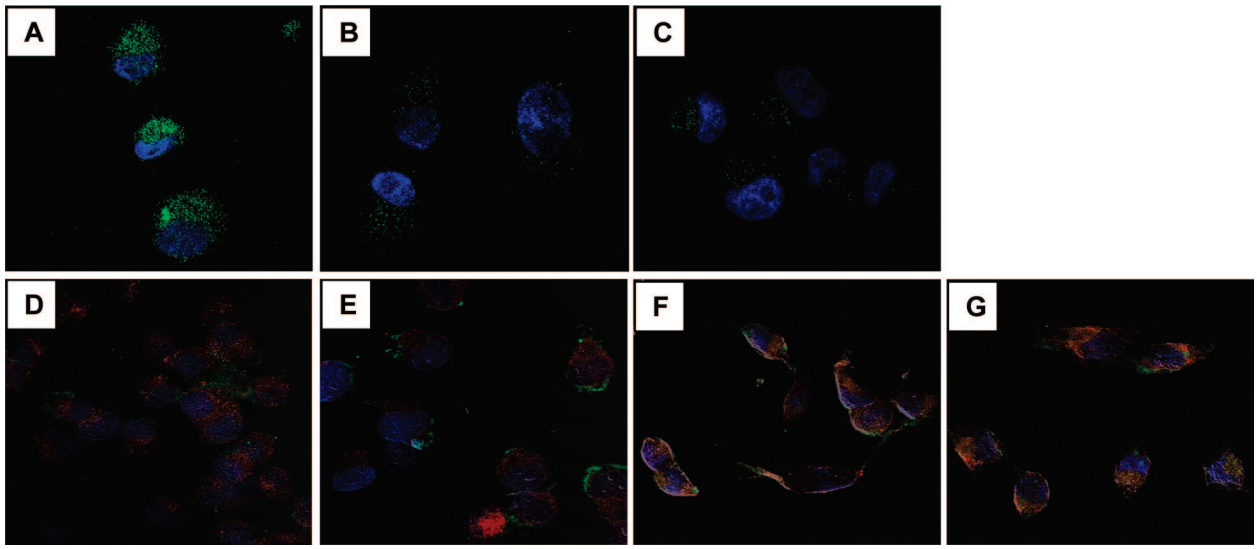

Figure 4. Confocal microscope images of uptake of fluorescent SNTs by MDA-MB231cells. To elucidate endocytosis, uptake studies were carried out with 200 nm positively charged SNTs at 37 °C (A), and in the presence of metabolic inhibitors of endocytosis namely (B) sodium azide and (C) sucrose. Colocalization studies were done with 200 nm SNTs with positively and negatively charged outer surfaces. (D,E) Colocalization of SNTs (green) with an early endosomal marker (Clathrin, red). (F,G) Colocalization with a lysosomal marker (Lysotracker, red). Positively charged nanotubes show higher degree of association and colocalization (represented by orange color) with cell membrane (E) as well as with the lysosomal compartments (G) than negatively charged nanotubes (panels D and F, respectively). Nuclei of cell stained with DAPI (blue). Concentration of SNTs is 0.01 μg/ml.

Endocytic uptake of the SNTs was further evaluated by colocalization with Clathrin (a marker of clathrin mediated early endosomal pathway) and Lysotracker (a marker for lysosomal uptake). Positively charged SNTs (labeled green) demonstrated a higher degree of association with the cell membrane as observed for both Clathrin (labeled red) and Lysotracker (labeled red) coincubation studies (Figure 4E,G) than the respective studies with bare SNTs (Figure 4D,F). This could be attributed to nonspecific charge interaction of cationic SNTs with the negatively charged cell surface. SNTs colocalized (labeled orange) to a larger extent with the Lysotracker than with Clathrin suggesting that by 30 min most of the internalized nanotubes accumulate in the lysosomal compartment. Together these results qualitatively indicate that positively charged SNTs interact with cells and accumulate intracellularly to a greater extent than unmodified bare SNTs. Such increased interaction and uptake of positively charged SNTs could therefore be attributed to the increased toxicity observed in growth inhibition studies at higher concentrations.

In summary, SNTs of discrete length, diameter, and surface charge were fabricated. The nanotubes showed limited toxicity which was concentration-, surface charge-, and length-dependent and were internalized in cells at least in part by endocytosis. These initial studies pave the way for understanding the structural features of silica nanotubes influencing their behavior in cellular and tissue environments. In the long term these understandings can aid in designing silica nanotubes with specific biomedical applications including drug delivery.

Acknowledgment. This research was funded by National Institutes of Health (1R01DE019050) and a University System of Maryland Integrated Nanobio Seed Grant through the Maryland Department of Business and Economic Development.

Supporting Information Available: Template synthesis of SNTs; surface functionalization; growth inhibition assay; confocal microscopy; transmission electron microscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Juliano, R. Challenges to macromolecular drug delivery. *Biochem. Soc. Trans.* **2007**, *35* (Pt 1), 41–43.
(2) McNeil, S. E. Nanotechnology for the biologist. *J. Leukocyte Biol.* **2005**, *78* (3), 585–594.
(3) Son, S. J.; Reichel, J.; He, B.; Schuchman, M.; Lee, S. B. Magnetic nanotubes for magnetic-field-assisted bioseparation, biointeraction, and drug delivery. *J. Am. Chem. Soc.* **2005**, *127* (20), 7316–7317.
(4) Son, S. J.; Bai, X.; Nan, A.; Ghandehari, H.; Lee, S. B. Template synthesis of multifunctional nanotubes for controlled release. *J. Controlled Release* **2006**, *114* (2), 143–152.
(5) Hillebrenner, H.; Buyukserin, F.; Kang, M.; Mota, M. O.; Stewart, J. D.; Martin, C. R. Corking nano test tubes by chemical self-assembly. *J. Am. Chem. Soc.* **2006**, *128* (13), 4236–4237.
(6) Son, S. J.; Lee, S. B. Controlled gold nanoparticle diffusion in nanotubes: Platform of partial functionalization and gold capping. *J. Am. Chem. Soc.* **2006**, *128* (50), 15974–15975.
(7) Bai, X.; Son, S. J.; Zhang, S.; Liu, W.; Jordan, E. K.; Frank, J. A.; Venkatesan, T.; Lee, S. B. Synthesis of superparamagnetic nanotubes as MRI contrast agents and for cell labeling. *Nanomedicine* **2008**, *3* (2), 163–174.
(8) Weetall, H. H. Storage stability of water-insoluble enzymes covalently coupled to organic and inorganic carriers. *Biochim. Biophys. Acta* **1970**, *212* (1), 1–7.
(9) Lee, S. B.; Mitchell, D. T.; Trofin, L.; Nevanen, T. K.; Soderlund, H.; Martin, C. R. Antibody-based bio-nanotube membranes for enantiomeric drug separations. *Science* **2002**, *296* (5576), 2198–2200.
(10) Martin, C. R. Nanomaterials: A Membrane-Based Synthetic Approach. *Science* **1994**, *266* (5193), 1961–1966.

(11) Polizu, S.; Savadogo, O.; Poulin, P.; Yahia, L. Applications of carbon nanotubes-based biomaterials in biomedical nanotechnology. *J. Nanosci. Nanotechnol.* **2006**, *6* (7), 1883–1904.
(12) Huang, X.; El Sayed, I. H.; Qian, W.; El Sayed, M. A. Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods. *J. Am. Chem. Soc.* **2006**, *128* (6), 2115–2120.
(13) Kolosnjaj, J.; Szwarc, H.; Moussa, F. Toxicity studies of carbon nanotubes. *Adv. Exp. Med. Biol.* **2007**, *620*, 181–204.
(14) Hauck, T. S.; Ghazani, A. A.; Chan, W. C. Assessing the effect of surface chemistry on gold nanorod uptake, toxicity, and gene expression in mammalian cells. *Small* **2008**, *4* (1), 153–159.
(15) Kneuer, C.; Sameti, M.; Bakowsky, U.; Schiestel, T.; Schirra, H.; Schmidt, H.; Lehr, C. M. A nonviral DNA delivery system based on surface modified silica-nanoparticles can efficiently transfect cells in vitro. *Bioconjugate Chem.* **2000**, *11* (6), 926–932.
(16) Li, Z.; Zhu, S.; Gan, K.; Zhang, Q.; Zeng, Z.; Zhou, Y.; Liu, H.; Xiong, W.; Li, X.; Li, G. Poly-L-lysine-modified silica nanoparticles: a potential oral gene delivery system. *J. Nanosci. Nanotechnol.* **2005**, *5* (8), 1199–1203.
(17) Patri, A. K.; Dobrovolskaia, M. A.; Stern, S. T.; McNeil, S. E. Preclinical characterization of engineered nanoparticles intended for cancer therapeutics. In *Nanotechnology for Cancer Therapy*, 2007 ed.; Amiji, M., Ed.; Taylor and Francis: New York, 2008; pp 105−137..
(18) Chang, J. S.; Chang, K. L.; Hwang, D. F.; Kong, Z. L. In vitro cytotoxicity of silica nanoparticles at high concentrations strongly depends on the metabolic activity type of the cell line. *Environ. Sci. Technol.* **2007**, *41* (6), 2064–2068.
(19) Zhang, T.; Stilwell, J. L.; Gerion, D.; Ding, L.; Elboudwarej, O.; Cooke, P. A.; Gray, J. W.; Alivisatos, A. P.; Chen, F. F. Cellular effect of high doses of silica-coated quantum dot profiled with high throughput gene expression analysis and high content cellomics measurements. *Nano Lett.* **2006**, *6* (4), 800–808.
(20) Hardman, R. A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. *Environ. Health Perspect.* **2006**, *114* (2), 165–172.
(21) Lim, Y.; Kim, J. H.; Kim, K. A.; Chang, H. S.; Park, Y. M.; Ahn, B. Y.; Phee, Y. G. Silica-induced apoptosis in vitro and in vivo. *Toxicol. Lett.* **1999**, *108* (2−3), 335–339.
(22) Lin, W.; Huang, Y. W.; Zhou, X. D.; Ma, Y. In vitro toxicity of silica nanoparticles in human lung cancer cells. *Toxicol. Appl. Pharmacol.* **2006**, *217* (3), 252–259.
(23) Medina, C.; Santos-Martinez, M. J.; Radomski, A.; Corrigan, O. I.; Radomski, M. W. Nanoparticles: pharmacological and toxicological significance. *Br. J. Pharmacol.* **2007**, *150* (5), 552–558.
(24) Nel, A.; Xia, T.; Madler, L.; Li, N. Toxic potential of materials at the nanolevel. *Science* **2006**, *311* (5761), 622–627.
(25) Magrez, A.; Kasas, S.; Salicio, V.; Pasquier, N.; Seo, J. W.; Celio, M.; Catsicas, S.; Schwaller, B.; Forro, L. Cellular toxicity of carbon-based nanomaterials. *Nano Lett.* **2006**, *6* (6), 1121–1125.
(26) Chavanpatil, M. D.; Khdair, A.; Panyam, J. Nanoparticles for cellular drug delivery: mechanisms and factors influencing delivery. *J. Nanosci. Nanotechnol.* **2006**, *6* (9−10), 2651–2663.
(27) Kovtyukhova, N.; Nina, I.; Mallouk, T. E.; Mayer, T. S. Templated surface sol-gel synthesis of SiO_2 nanotubes and SiO_2-insulated metal nanowires. *Adv. Mater.* **2003**, *15*, 780–785.
(28) Torchilin, V. P.; Rammohan, R.; Weissig, V.; Levchenko, T. S. TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc. Natl. Acad. Sci. U.S.A* **2001**, *98* (15), 8786–8791.
(29) Nieland, T. J.; Ehrlich, M.; Krieger, M.; Kirchhausen, T. Endocytosis is not required for the selective lipid uptake mediated by murine SR-BI. *Biochim. Biophys. Acta* **2005**, *1734* (1), 44–51.

NL0802741