Silicon-Nanotube-Mediated Intracellular Delivery Enables Ex Vivo Gene Editing

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Cellular disruptive nanotechnology is a rapidly expanding interdisciplinary field that interfaces programmable nanomaterials with living cells.[1,2] With advancements in nanofabrication techniques, the role of vertical configuration of nanostructures on cell behavior and function has been explored using nanotopographies including, 1D nanomaterial formats, from nanostraws with hollow cores to high-aspect-ratio porous needles.[3–5] The key advantage of tunable vertically configured nanostructures lies in their enhanced interaction at the interface between cells and nanomaterials due to their nanoscale dimensions.[6] In particular, intracellular delivery of bioactive materials is a valuable paradigm that plays a critical role in advanced ex vivo gene and cell-based therapies, as well as facilitating fundamental cell research.[7]

Highly efficient silicon nanowire (SiNW)-mediated intracellular delivery has employed arrays of either solid[8–10] or porous[11–13] SiNWs, preloaded with diverse biomolecule cargoes of interest. The Engineered nano–bio cellular interfaces driven by vertical nanostructured materials are set to spur transformative progress in modulating cellular processes and interrogations. In particular, the intracellular delivery—a core concept in fundamental and translational biomedical research—holds great promise for developing novel cell therapies based on gene modification. This study demonstrates the development of a mechanotransfection platform comprising vertically aligned silicon nanotube (VA-SiNT) arrays for ex vivo gene editing. The internal hollow structure of SiNTs allows effective loading of various biomolecule cargoes; and SiNTs mediate delivery of those cargoes into GPE86 mouse embryonic fibroblasts without compromising their viability. Focused ion beam scanning electron microscopy (FIB-SEM) and confocal microscopy results demonstrate localized membrane invaginations and accumulation of caveolin-1 at the cell–NT interface, suggesting the presence of endocytic pits. Small-molecule inhibition of endocytosis suggests that active endocytic process plays a role in the intracellular delivery of cargo from SiNTs. SiNT-mediated siRNA intracellular delivery shows the capability to reduce expression levels of F-actin binding protein (Triobp) and alter the cellular morphology of GPE86. Finally, the successful delivery of Cas9 ribonucleoprotein (RNP) to specifically target mouse Hprt gene is achieved. This NT-enhanced molecular delivery platform has strong potential to support gene editing technologies.

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Melosh group demonstrated for the first time the development of a delivery platform based on nanostraws (hollow nanowires) integrated into a microfluidic device.\(^\text{[14]}\) By piercing the cell membrane, nanostraws created a permanent fluidic pipeline into the cytosol and achieved delivery of membrane-impermeable species (e.g., fluorescent dyes, ions, and GFP plasmids) into HeLa and Chinese hamster ovary cells. Following this, the same research group incorporated the nanostraw platform into an electroporation system.\(^\text{[15]}\) Carbon nanotube (CNT) arrays integrated into a microfluidic device were also reported to achieve high-throughput gene transfection into HEK 293 and rat L6 myocyte cells with low toxicity.\(^\text{[16]}\) Here, biomolecular transport was enabled through the nanotube lumen. The CNT-based platform achieved delivery of dye and plasmid DNA with 99% and 84% efficiency respectively as tens of thousands of cells from both HEK293 and rat L6 myocyte cell lines.\(^\text{[16]}\) But the use of nanoporous membranes as a template for fabrication results in stochastic positioning of the nanostraw/CNT elements, which does not allow for quantitative delivery studies. A more recent study overcomes this issue and reports a multimodal electrophoretic platform based on 3D plasmonic hollow nanotubes, which enables the controlled delivery of single gold nanorods in single NIH 3T3 mouse embryonic fibroblast cells. The microfluidic embedded platform performs as nanoelectrodes for cell electroporation, nanochannels for electrophoretic delivery, as well as plasmonic antennae.\(^\text{[17]}\)

However, most NT delivery systems share a need for microfluidic integration, surface functionalization, and/or applied local electric fields, all of which may induce adverse effects on electrochemical-sensitive cells such as primary immune cells and hematopoietic stem cells.\(^\text{[18]}\) By contrast, our approach requires no functionalization, local electric fields, or complicated microfluidic integration, and hence appears to allow gentler perturbation to interfaced cells. Here, we use a top-down fabrication approach for the development of programmable vertically aligned silicon nanotube (VA-SiNT) arrays, demonstrating for the first time their power to trigger both efficient intracellular delivery as well as gene editing. Such SiNTs show effective loading across multiple types of biomolecules—immunoglobulin G (IgG) protein, mRNA, siRNA, Cas9 ribonucleoprotein (RNP)—without the need for prior surface modification. Loading of two fluorescence-tagged IgGs (IgG-AF647 and IgG-AF488) in SiNTs results in the simultaneous delivery of both cargoes into cells. Cellular membrane deformation induced by SiNTs promotes enhanced endocytosis pathways at the cell–SiNT interface, and in turn the active biomolecular uptake. Importantly, successful delivery of siRNAs (to silence Triobp) and Cas9 RNP (to knock out Hprt) demonstrates strong capacity to maximize the use of SiNTs as a platform for ex vivo gene editing. The ability to quantitatively deliver and codeliver biomolecule cargoes makes the proposed hollow-core SiNTs superior to the existing nanowires and nanoneedles, which are considered the mainstay for highly efficient membrane disruption-mediated delivery. The SiNT platform will have potential for future implementation in advanced cell therapeutics.

We fabricated programmable VA-SiNT arrays by a combination of direct e-beam lithography (EBL) and deep reactive ion etching (DRIE), yielding NT arrays with controlled geometry at predefined locations on the Si surface (Figure 1a). Critically, the spacing, height, and diameter are independently controllable. The fabrication method we used for SiNT arrays is precise, yet has a high level of flexibility in topographical design, overcoming the stochastic, unordered positioning of existing platforms such as polycarbonate-based nanostraws.\(^\text{[15]}\) Research that has used similar vertical nanostructures have shown promising results in delivering biomolecules into living cells; but many studies were restrained from addressing further biological

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**Figure 1.** Biomolecule cargo loading onto SiNT arrays. a) SEM images showing the SiNTs at tilted zoom-out (i) and zoom-in (ii), top (iii), and vertical (resin embedded) view (iv). Scale bars: 5 µm (i), 500 nm (ii), and 300 nm (iii,iv). b) Confocal images showing the top (i–iii) and 3D views (iv) of SiNTs loaded with IgG-AF488 (green; i,iv), IgG-AF647 (magenta; ii), and ssDNA-FAM (green; iii), respectively. Insets (i–iii) are 5× enlargements. Scale bars: 20 µm (i–iii) and 5 µm (iv).
questions, at least partially due to issues with reproducibility and consistency of nanotopography. [19] Our EBL–DRIE fabrication route offers high levels of reproducibility and flexibility of SiNT geometry. Such highly consistent nanostructures without need of surface functionalization enhance the repeatability of experimental results and allow more intricate biological questions to be addressed.

The current work focuses on SiNT arrays with dimensions of inner diameter of 300 nm, height of 2 µm, and pitch of 5 µm. Once fabricated, these SiNT arrays were first loaded by spotting a solution containing two types of fluorescently labeled biomolecules—IgG and ssDNA. Top and 3D views of confocal microscopy images (Figure 1b) demonstrate the homogenous loading distribution of IgG-AF488, IgG-AF647, and ssDNA-FAM across the whole SiNT array.

Using a serial dilution of IgGs and ssDNAs added onto SiNTs, we further explored and found the optimal payload concentration required to achieve maximum SiNT loading efficiency (Figure S1, Supporting Information). IgG-AF647 fluorescence intensity peaked at the loading concentration of 200 µg mL⁻¹, followed by slightly lower signal at 2000 µg mL⁻¹ and 20 µg mL⁻¹, while a concentration of 2 µg mL⁻¹ and below generated signal close to background noise (Figure S1a,c, Supporting Information). At concentrations above 200 µg mL⁻¹ aggregation of the IgG-AF647 was observed, potentially limiting the loading into the tubes. Unlike IgG-AF647, the fluorescence intensity of ssDNA-FAM on SiNTs steadily increased with loading concentrations from 1.6 µg to 1600 µg mL⁻¹ (Figure S1b,d, Supporting Information). The cargo was loaded in the SiNTs, with negligible release over 48 h in DPBS (Figure S1e, Supporting Information).

Mouse embryonic fibroblast cells (MEFs) are an important model for in vitro biological studies. Although viral methods achieve high gene-delivery efficiency, challenges with cell mutagenesis and safety among others have prompted the quest for efficient nonviral gene-delivery methods in MEF cells. [20–22] However, primary MEFs are challenging to transfect with commonly used nonviral methods; [22] even immortalized MEF cells (NIH 3T3) show relatively low transfection efficiencies, with ≈22% using Lipofectamine, ≈25% using Magnefect-nano, and ≈27% using Attractene. [23,24]

Given the successful loading of biomolecules without significant leakage from SiNTs into cell culture media, we investigated whether SiNTs can provide alternative route for nonviral intracellular delivery into MEFs. GPE86 cells were seeded onto flat silicon (flat Si) and SiNT substrates that were preloaded with IgG-AF647 or ssDNA-FAM. To achieve rapid and optimal forceful cell–NT interfaces, external active force was applied via centrifugation at 250 × g, 32 °C, for 15 min. After 6 h incubation, cells were fixed on substrates and stained with Hoechst 33342 (Hoechst) and phalloidin-AF568 (phalloidin) to represent nucleus and actin cytoskeleton, respectively. Fluorescence confocal microscopy images show the accumulation of ssDNA-FAM in the cytosol, even the nucleus, of GPE86 cells (Figure 2aii); whereas GPE86 cells cultured on control ssDNA-coated flat Si gave undetectable FAM signal (Figure 2ai). The side view image and line profile shows that SiNTs that had intimate contact with cells have decreased FAM signal, while
those that did not interact with cells retained FAM fluorescence (Figure 2aiv,aiii, respectively). This provides additional evidence that ssDNA-FAM was released from SiNTs into the cells during the intimate interfacing; such molecular release could be attributed to passive diffusion through disrupted membrane[14] and/or active cellular uptake triggered by the local deformation induced by SiNTs.[13] Statistic quantification indicates that ssDNA-FAM were delivered into 77.6 ± 1.5% of GPE86 cells on SiNT substrates, compared with 6.9 ± 3.7% of that on flat Si (Figure 2b). Similar results were found for GPE86 cells seeded flat Si and SiNTs preloaded with IgG-AF647; 81.4 ± 6.1% of cells on SiNT arrays showed IgG-AF647 inserted into the cytosol, whereas only 0.8 ± 0.4% of cells on flat Si exhibited AF647 signals (Figure S2, Supporting Information).

To determine whether biomolecules delivered by SiNTs have entered the cell, or remained trapped at the outer cell membrane, we employed a fluorescent internalization probe (FIP-CF568): FIPs that are not internalized can be quenched instantly and permanently by Q2V1 quencher, so that only FIPs that are inside the cell will emit fluorescence.[23] Confocal images show that Q2V1 quenched effectively FIP-CF568 loaded in SiNTs (Figure S3b, Supporting Information), and CF568 fluorescence can be detected in GPE86 cells incubated on SiNTs with and without subsequent treatment with Q2V1 (Figure S3a,b, Supporting Information). In addition, we detached the cells incubated on FIP-CF568 loaded flat Si and SiNTs after 6 h incubation and used flow cytometry to analyze the CF568 fluorescence intensity within cells treated with or without Q2V1. The flow cytometry data (Figure S3c,d, Supporting Information) showed that cells from SiNT substrates had significantly higher CF568 signal (GMFI =78.3) than that from flat Si substrates (GMFI =35.1). When treated with Q2V1 (group SiNT + Q2V1), the CF568 intensity dropped from ≈78.3 to ≈69.6; this corresponds to >80% of the FIP-CF568 being internalized into the cells. Both confocal and flow cytometry findings indicate that SiNTs mediate the successful internalization of FIPs into GPE86 cells.

Moreover, to investigate whether SiNT-mediated internalization preserves the biological functionality of delivered cargoes, we used Cy5-tagged mRNA that encodes GFP reporter (Cy5-mRNA-GFP). Detection of Cy5 signal inside the cells would indicate mRNA internalization, while GFP expression would suggest the translational potency of mRNAs. GPE86 cells were added onto flat Si and SiNTs preloaded with Cy5-mRNA-GFP. After spin and 6 h incubation, cells were stained with Hoechst for confocal imaging. It was observed that Cy5 accumulated inside GPE86 cells on SiNTs, and these cells started to express GFP (Figure S4b, Supporting Information), indicating the internalization of intact mRNAs and their translation into protein product by ribosomes. In contrast, cells on flat Si exhibited negligible Cy5 and GFP signal (Figure S4a, Supporting Information). Similar results were found by flow cytometry, where >50% of cells detached from mRNA-coated SiNTs contained Cy5 and exhibited higher GFP intensity compared with cells from flat Si (Figure S4c,d, Supporting Information). These findings confirmed that SiNTs can mediate efficient cargo delivery while maintaining their biological functionality. Delivery of mRNA by SiNTs may open new opportunities for transient/nonintegrating transfection methods,[24] reducing the risk of mutation and tumor development associated with viral vectors, which is extremely important in clinical studies.

The capability of simultaneous delivery of multiple biomolecules into a single cell would advance cellular manipulation technology such as stem cell reprogramming, gene editing, and cancer therapy.[26] Given the observed SiNT-mediated loading and intracellular delivery of IgGs and ssDNAs into GPE86 cells, we further examined whether such SiNT arrays can be used as a codelivery platform. First, we mixed IgG-AF647 and IgG-AF488, both at 200 µg mL⁻¹ concentration but with different v/v ratios, and loaded the IgG mixture onto SiNTs. After 1 h incubation in dark at room temperature, excess payload was rinsed off. Confocal microscopy images and statistical analysis demonstrated that with decreasing AF647/AF488 input ratio (v/v), the measured intensity of AF647 decreased whereas AF488 increased (Figure S5a,b, Supporting Information). The total fluorescence signal remained ≥200 (a.u.) across all ratios, and was maximal at AF647/AF488 ratios of 6:4, 5:5, and 4:6 (Figure S5c, Supporting Information), where the concentration of each IgG (AF647 or AF488) was close to 100 µg mL⁻¹. The results further confirm that the optimal concentration for IgG loading is within 100–200 µg mL⁻¹, and indicate that SiNTs enable cargo codelivery.

Next, we seeded GPE86 cells on SiNTs coloaded with IgG-AF647 and IgG-AF488 (v/v, 10:0, 5:5, 0:10). After 6 h culture, samples were divided into two sets—one set had cells that remained on SiNTs for confocal microscopy imaging, the other had cells harvested from substrates by trypsinization and was analyzed by flow cytometry. The maximum intensity projection of confocal z-stack images demonstrated that the combined total signal of AF647/AF488 was reduced in SiNTs that were interacting with GPE86 cells compared with those did not have cell contact (Figure S6a–c, Supporting Information). Flow cytometry results showed that >80% of cells recovered from SiNTs with an IgG-AF647/IgG-AF488 ratio of 0:10 became AF488⁺, and 27.3 ± 7.9% of cells contained AF647 with an IgG-AF647/IgG-AF488 ratio of 0:10 (Figure S6d,e, Supporting Information). Importantly, in the case where SiNTs were loaded with equal amounts of IgG-AF647 and IgG-AF488 (v/v, 5:5), the combined AF647 and AF488 fluorescence signal was detected inside the cells, with a 37.9 ± 14.0% AF647⁺/AF488⁺ double positive population (Figure S6d,e, Supporting Information). It is not clear why the uptake efficiency of IgG-AF488 is significantly higher than of IgG-AF647. One possible scenario could be that the origin of IgG-AF647 (goat antimouse) tends to trigger the protein degradation machinery such as ubiquitin-proteasome system[27,28] in mouse GPE86 fibroblasts, leading to the acute and rapid degradation of IgG-AF647; while IgG-AF488 (chicken antirabbit) remains unaffected. Nevertheless, these results confirm the capability of SiNTs in codelivering multiple biomolecules into single cells.

The next set of experiments aimed at gaining a more thorough understanding of whether and how SiNT–cell interfacial interactions affect cell behavior and function. Scanning electron microscopy (SEM) images in Figure 3a depict the tilted and top view of GPE86 cells interfaced with SiNT arrays. The majority of GPE86 cells spread and generate focal adhesions on SiNTs, with membranes reaching out to attach to the SiNTs. Long filopodial and short lamellipodia protrusions produced by GPE86 cells were also observed, which can further strengthen and promote SiNT–cell adhesion forces.[29]
To examine if such tight interfacing would impair cell viability, we performed a subsequent live/dead assay. Fluorescein diacetate (FDA) and propidium iodide (PI) were used to stain GPE86 cells after 6 h culture on control flat Si and SiNTs with or without IgG-AF647 loading. FDA staining indicates live GPE86 cells, while the staining of impermeable dye PI indicates dead cells and damaged plasma membrane. We observed that >90% of cells remained viable (FDA+) with no PI staining, on both SiNTs and flat Si; and this is regardless of the loading of biomolecule cargo IgG-AF647 (Figure 3c; Figure S7a–c, Supporting Information). Similar viability (>90%) was observed for the cells detached from flat Si and SiNT substrates after 6 h incubation (Figure S7e, Supporting Information). Furthermore, to track the proliferation of the harvested cells, we stained GPE86 cells with CellTrace Violet (CTV) dye before seeding onto flat Si and SiNT substrates. After 6 h incubation, cells were detached from both substrates and divided into four groups. The first group of cells were analyzed immediately with flow cytometry to detect their CTV expression. The other three groups of cells were cultured back in fresh media and analyzed by flow cytometry after 24, 48, and 72 h, respectively. It was evident that >40% of cells underwent proliferation after 24 h and >90% after 48 h; no significant difference was observed on the proliferation rate of cells detached from flat Si and SiNT substrates (Figure S7d,f, Supporting Information). Therefore, SiNTs do not induce significant cell death and have minimal impact on cell proliferation after harvesting.

Focused ion beam (FIB)-SEM imaging data lend further support to this view. Figure 3b and Video S1 (Supporting Information) show that SiNTs induced prominent cellular and nuclear deformations of GPE86 cells, but the plasma membrane remained intact, wrapping around the SiNT elements. This type of cellular and nuclear membrane remodeling has been shown in recent studies, probing the interface between adherent cells and vertically configured nanostructures.[17,30,31] Figure S8 (Supporting Information) shows the presence of membrane invaginations and vesicles at the cell–NT interface, suggesting the presence of endocytic pits. There is increasing evidence that the programmable surface nanotopographies on which a cell resides can trigger endocytosis pathways, which facilitate active biomolecular uptake without requiring direct penetration.[11,32] To determine whether endocytic pathways can be stimulated by SiNTs, we probed the distribution of clathrin heavy chain (CHC), which is key component required for clathrin mediated endocytosis. We also probed the localization of caveolin-1 (CAV-1), a protein that works with cavins and other accessory proteins to form bulb-shaped pits on the plasma membrane known as caveolae. Confocal microscopy images show that the distribution of CHC was largely homogenous on both flat Si and SiNTs (Figure 4c,d,g,h). In contrast, CAV-1 accumulated at the SiNT sites, appearing periodic corresponding to the SiNT pattern (Figure 4b,f); such CAV-1 pattern was not found in cells on flat Si (Figure 4a,e). When seeding GPE86 cells on SiNTs preloaded with ssDNA-FAM, we observed the colocalization of ssDNA-FAM with CAV-1 (Figure S9, Supporting Information).
indicated by white triangles), but less so with CHC. These findings suggest that SiNTs may directly activate endocytic processes. Since caveolae play a key role in accommodating the membrane tension induced to the cell membrane by cell–substrate interactions,[33] it is possible that CAV-1 is recruited to the cell membrane as part of a stress response.

To further investigate the role of endocytosis in facilitating SiNT-mediated delivery, we performed an endocytosis inhibition study. Cells were treated with two inhibitors: 1) nystatin, which binds cholesterol and inhibits the formation of lipid rafts, cholesterol enriched domains and caveolae; or 2) chlorpromazine, which inhibits clathrin mediated endocytosis, but can also interfere with fast endophilin-mediated endocytosis.[34–37] After 6 h incubation on Cy5-mRNA-GFP coated SiNTs, cells were stained with Hoechst and imaged by confocal microscopy. We found that nystatin-treated cells exhibited the lowest Cy5 signal and undetectable GFP, compared with untreated and chlorpromazine-treated cells (Figure 5a–f; Figure S10, Supporting Information). Similar results were obtained by flow cytometry analysis: 57.3 ± 2.8% of cells detached from Cy5-mRNA-GFP coated SiNTs expressed Cy5 and GFP, followed by 29.5 ± 9.7% of that treated with chlorpromazine (Figure 5g,h); cells treated with nystatin exhibited the lowest Cy5 and GFP signal, with only 3.1 ± 0.6% which is close to that from control flat Si.

These results indicated that the inhibition of lipid rafts and cholesterol enriched domains severely impaired the cellular uptake of cargoes from SiNTs. Conversely, chlorpromazine did not significantly impact GFP expression, suggesting that clathrin mediated endocytosis does not play a prominent role in uptake of the mRNA.

siRNA-mediated gene silencing and CRISPR gene deletion/insertion have been used as powerful tools in genome-editing technologies.[38] We studied whether SiNTs can be used to achieve functional gene editing via the delivery of siRNAs and Cas9 RNP into GPE86 cells via SiNTs.

siRNAs were customized to specifically target Triobp, an F-actin bundling protein that is crucial for actin skeleton reorganization and cell migration.[39] Knockdown of Triobp can severely impact filopodial formation and cell motility.[40] We
seeded GPE86 cells on SiNT arrays containing anti-Triobp and control scramble (Neg) siRNAs. After 48 h incubation, cells were fixed and stained with anti-Triobp, wheat germ agglutinin (WGA, stain for membrane), phalloidin, and Hoechst. The large-scan fluorescence images demonstrate the presence of even distribution and larger coverage of GPE86 cells on SiNT substrates preloaded with Neg siRNAs, whereas SiNTs that were loaded with anti-Triobp siRNAs showed more than four times lower cell attachment (Figure S11a,b, Supporting Information).

Confocal images at higher magnification (Figure 6a) provide more detailed information of nuclear morphology, Triobp expression and localization, and actin cytoskeleton of GPE86 cells on the two SiNT substrates (Neg and anti-Triobp). It is evident that cells treated with anti-Triobp siRNAs reduce dramatically the expression of Triobp; therefore, they cannot support the actin meshwork and filopodial formation. These phenotypic changes can significantly impact the focal adhesion and migration[41,42] of GPE86 cells on SiNTs, which, at least to some extent, explain the lower cell count on anti-Triobp SiNTs. The results suggest that SiNT platforms are applicable for siRNA-mediated gene silencing.

CRISPR-Cas9 technology is having a tremendous impact in fundamental and translational biomedical research,[43] providing a programmable platform for precision gene targeting.[44] Despite of the advantages of this relatively new technology, severe limitations remain such as difficulties in delivery, off-target effects, and raising safety concerns.[45,46] One strategy to reduce off-target-effects is to exchange the intracellular delivery of Cas9/sgRNA-encoding plasmids for in vitro-assembled Cas9 RNP complexes, which have shown higher efficiency in genome editing, decreased off-target effects, and less cellular toxicity in mammalian cells.[47,48] However, the effective, safe, and cell-specific intracellular delivery of Cas9 RNPs remains an important challenge in the field.[49]

Here, we investigated whether SiNT arrays can be used to deliver Cas9 RNPs for gene editing. Negative control CRISPR
RNA (crRNA) was annealed with ATTO 550-tagged trans-activating crRNA (tracrRNA-ATTO 550). The crRNA mixture was then incubated with high-fidelity Streptococcus pyogenes Cas9 nuclease V3 to generate Cas9:crRNA/tracr-ATTO complex (Cas9-ATTO RNP). We incubated GPE86 cells with SiNT arrays loaded with Cas9-ATTO RNP (Figure S12c, Supporting Information). After 6 h culture, cells were removed from the substrates; half of the cells were analyzed by flow cytometry immediately and the second half cultured in fresh media for a further 24 h before confocal imaging. The flow cytometry results show that 14.7 ± 1.9% of GPE86 cells were ATTO 550+, indicating the positive transfection of Cas9-ATTO RNP into this population, compared with the untreated control (Figure 6b,c). Confocal microscopy imaging at 24 h confirms the tracking of Cas9-ATTO RNP into the nucleus of GPE86 cells (Figure S11c, Supporting Information).

After validating the successful delivery of Cas9 RNP into cells, we next studied the gene-editing efficiency by delivering either a control (SiNT-Neg) or Hprt-targeted Cas9 RNP (SiNT-Hprt) into GPE86 cells using SiNTs; cells transfected with Hprt Cas9 RNP using Lipofectamine 2000 (Lipo-Hprt) served as the positive control. Using the T7 endonuclease 1 (T7E1) assay, where T7E1 cleaves DNAs at mismatched spots that can be generated by Cas9 cutting,[26] we observed the two cleavage bands at expected sizes (130 and 520 bp) for cells transfected with SiNT-Hprt and Lipo-Hprt. In contrast, SiNT-Neg cells and those without T7E1 digestion did not show these two cleavage bands, but the intact DNA strand at size 650 bp (Figure S11d, Supporting Information). By measuring the intensity of the two cleavage products, we calculated that the cleavage efficiency by SiNT-Hprt was ≈6% (Figure S11d,e, Supporting Information). Note that the gene cleavage efficiency by SiNT-Hprt is lower than the delivery efficiency of Cas9-ATTO RNP (≈14%), which might be related to suboptimal nuclear localization and target binding.

Though with room to further improve both delivery and gene cleavage efficiency, SiNTs have demonstrated the proof-of-concept for a much simpler and safer way to conduct CRISPR gene editing.

Figure 6. SiNT-mediated siRNA gene knockdown and Cas9 RNP delivery. a) Confocal images showing GPE86 cells after 48 h culture on SiNTs that are preloaded with negative control scramble (Neg, top) and Triobp-targeting (anti-Triobp, bottom) siRNAs. Cells were stained with Hoechst (blue), Triobp antibody (green), phalloidin (red), and WGA (purple). Scale bars: 20 µm. b) Flow cytometry plots showing the gating of ATTO 550+ GPE86 cells detached from SiNTs loaded with Cas9-ATTO RNP. Untreated GPE86 cells serve as control (ctrl). c) Quantification of the percentage of ATTO 550+ GPE86 cells in both ctrl and Cas9-ATTO groups as in (b). *P < 0.0254 (Mann–Whitney’s U-tests).
functionalization or microfluidic integration. SiNTs with loading capacity ≈0.1 μm³ per NT demonstrated loading of five cargo types (in particular, IgG, ssDNA, FIP, siRNA, and Cas RNP); these cargoes remained within SiNTs for more than 48 h. Adherent GPE86 cells cultured on the preloaded SiNT substrates exhibited a relatively high transfection efficiency within 6 h of interfacing—in particular, 78% of GPE86 cells became FAM⁺ on SiNTs loaded with ssDNA-FAM, compared to 1% of their counterparts on control flat Si. Similar results were obtained for GPE86 cells seeded on flat Si and SiNTs preloaded with IgG-AF647. Both fluorescence-tagged cargoes, IgG-AF647 and IgG-AF488, were also loaded into SiNTs and simultaneously delivered to GPE86 cells. Using the unique FIP-CF568/quencher system to investigate the actual internalization of delivered molecules via SiNTs, we showed for the first time clear evidence of SiNT-facilitated biocargo internalization (>80% efficiency). Importantly, SiNT-interfacing did not affect cell viability, regardless of the biomolecule cargo loading. FIB-SEM imaging of the cell–SiNT interface revealed the absence of cell membrane penetration by the SiNTs, and the presence of membrane invaginations at the cell–SiNT interface. Confocal microscopy imaging highlighted the accumulation of caveolin-1 at SiNT sites. Both results taken together suggest the accumulation of endocytic pits at the cell–SiNT interface. Endocytosis inhibition by nystatin and chlorpromazine confirmed the involvement of an active endocytic pathway for biomolecular delivery. We achieved SiNT-mediated siRNA gene knockdown of Triobp (F-actin binding protein) in GPE86 cells, resulting in drastically altered cell morphology and more than four times lower cell attachment compared with that on SiNTs loaded with negative control siRNAs. Cas9-ATTO RNP were successfully (14.7% efficiency) delivered into GPE86 cells via SiNTs; 6% cleavage efficiency was obtained when using SiNTs to deliver Hprt targeting Cas9 RNP into GPE86 cells. These results indicate the potential of SiNTs in mediating ex vivo gene manipulation by transporting CRISPR into targeted cells.

While the platform still requires optimization and transfer to a more scalable fabrication method, we anticipate that our advances in the development of VA-SiNT-mediated intracellular delivery platforms will enable the use of this technology for gene editing as well as answering fundamental biological questions at the engineered nano-bio cellular interfaces.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
N.H.V. and R.E. developed the idea for the study and its scope and supervised the experimental team consisting of Y. C. who performed biological-related experiments and statistical data analysis and S.A. who performed SiNT fabrication and electron microscopy imaging. L.I.F. and A.P.R.J. provided the fluorescent internalization probes and protocols. G.G. contributed to the FIB-SEM sections. T.M., J.T., and S.S. assisted with DRIE processing. Y.M. and K.S. provided the DRIE equipment at ULVAC Inc. Y.C. and S.A. prepared the manuscript. N.H.V., R.E., and A.P.R.J. revised the manuscript.

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