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Fabrication of Sub-cell Size "Spiky" Nanoparticles and Their Interfaces with Biological Cells

Xi Xie[1,2], *Nicholas A. Melosh*[1,*]

[1]Department of Materials Science and Engineering, Stanford University, Stanford, California 94305, USA

[2]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139; Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Abstract

Chip-based arrays of vertical nanowires (NWs) have attracted biomedical research interest for their one dimensional architecture and cell-interface properties, yet delivery of these devices in solution is not possible due to the inherent attachment of NWs to a planar substrate. To overcome this structural limitation, we report synthesis of hierarchical nanoparticles covered with stiff NWs, namely "spiky particles" which combine the advantages of supported vertical nanowires and aqueous delivery of suspended nanoparticles. ZnO NWs were grown onto SiO_2 nanoparticles using a solution-based synthesis to avoid dispersing particles grown on solid substrates, and increase the particle quantity. These spiky particles can be fabricated with tunable particle and nanowire dimensions. The cell membrane interface with these hierarchical nanostructures were examined using scanning electron microscopy to determine the extent of engulfment and interaction.

KEYWORDS: Nanobiotechnology, Nanowires, Nanoparticles, Cell penetration, biointerfaces

One dimensional nanowires (NWs) have demonstrated unique high aspect-ratio architectures and extraordinary properties (e.g. optical,[1] electrical,[2-4] mechanical,[5] plasmonic,[6] and magnetic properties[7,8]), which have significantly advanced a broad range of research areas including electronics, energy convention devices, and photonics devices. While these have been well developed in the semiconductor field,[1-4] NWs are just beginning to be applied to biological

problems, and have achieved recent success for drug delivery,[9-11] electrical recording,[12,13] biochemical sensing,[14,15] and cell manipulations.[7,16] For example, vertical nanowires have been proposed as a physical tool to penetrate cell membrane by local stress concentration at the sharp needle like tip.[9,17-19] This physical membrane penetration would side step the endocytosis or phagocytosis pathway so that delicate biomolecules can be directly introduced to the cytoplasma from the NWs, avoiding the endosomal degradation routes.[20,21]

However, the further advances of high aspect-ratio NWs for biomedical applications still present several challenges. For example, planar NW arrays must be attached to a surface, and are not amenable for solution-based delivery, such as for cells in suspension or in vivo. Therefore developing hierachical nanoparticles covered with NWs, namely "spiky particles", that utilize the aquesous delivery mechanism of suspended nanoparticles and high aspect-ratio NWs' cell penetration capability may posses potential biological applications such as direct introduction of drug molecules into cells (Figure 1a). In addition, nanoparticles with nanoscale topography on the surfaces have recently showed anomalous dispersion behaviour,[22] and abstracting biological applications such as increased tissue adhesion for local drug delivery,[23] or enhanced uptake into biological cells.[24] Characterizing the interfaces between cells and hierarchical nanostructures is important for understanding these interactions to guide design of new particle architectures. Although cell-NW interfaces have been studied recently,[17,25,26] the interaction between biological cells and more complicated nano-objects is still an emerging area.

Previous NW research for other applications has shown that it is possible to grow NWs from microbead surfaces using vapor-phase synthesis, yet these structures are either difficult to suspend in solution, or not stiff enough for cell penetration. For example, Fischer et al. demonstrated Si NW-coated microbeads (30-50 μm diameter) that greatly enhanced tissue adhesion to the beads for mucosal drug delivery systems,[23,27] yet the large microparticle size was designed for tissue-level adhesion, and the curved Si NWs are neither stiff enough nor radially directed from the surface for cell penetration purposes. Other particles include hierarchical nanostructures or particles with Si, Ge, or ZnO NWs grown on them for solar cells or other energy applications.[28-32] These NW are generally synthesized under high temperature conditions [e.g. chemical vapor deposition (CVD)] using heterogeneous nucleation on the particle surface. However, our efforts using this approach rapidly found that particle removal was a significant barrier, and the high temperatures and orthogonal etching chemistry required severely limited the

range of possible sacrificial layers. NW entanglement between particles on the surface lead to particle aggregates rather than individual particles, with a significant fraction of the NWs destroyed after sonication or other dispersion processes (Supporting Information S1). Of equal importance was the relatively low numbers of particles created through this process, about 10^8-10^9/cm^2, compared to ~10^{12}/mL for a 10 wt% solution of 500 nm diameter nanoparticles.

To develop suspended spiky nanoparticles with suitable size for cellular applications, here we present an alternative strategy to synthesize sharp ZnO NWs on SiO_2 nanoparticles in aqueous solution using a hydrothermal growth approach, with tunable particle size, NW length and NW density. These spiky particles were employed to examine the interfaces between cells and hierarchical nanoparticles. The particle-cell interface as well as engulfment profiles were examined using scanning electron microscopy (SEM), and the NW-cell interfaces were compared to the profiles where cells were then cultured on top of the spiky particles. Our results demonstrate the successful release of NW array structure limitations from large planar substrate to suspended nanoparticles, and present an investigation on cell membrane engulfment conformation interfacing with hierarchical nanostructures.

Suspended spiky particles with ZnO NWs grown isotropically from the surface were synthesized in aqueous solution. The fabrication method and the corresponding SEM images of spiky particles are shown in Figure 1b. Briefly, SiO_2 nanospheres (600-800 nm in diameter) prepared by Tetraethyl Orthosilicate (TEOS) based sol-gel procedure were coated with 20 nm of ZnO by using diethylzinc (DEZ)/H_2O atomic layer deposition (ALD), and then re-dispersed in water and sonicated overnight. This ZnO thin layer served as the seed layer for ZnO NWs growth through the thermal decomposition (at 100 ^oC) of a zinc complex including 25 mM zinc nitrate hydrate [$Zn(NO_3)_2$•$6H_2O$], 25 mM hexamethylenetetramine ($C_6H_{12}N_4$, HMTA), and 7 mM polyethylenimine (PEI).[33-36] In order to achieve sharp NWs with high aspect ratio, polyethylenimine (PEI) was used in the solution to hinder the lateral growth of NWs.[33,34]

The dimensions of the spiky particles could be tuned by controlling reaction precursor composition, temperature, reaction time and number of growth cycles. Figure 2a shows the SEM images of typical spiky particles using 600-800 nm SiO_2 nanospheres as core for NW growth. This hydrothermal growth approach yielded spiky particles without generating unwanted ZnO precipitant by controlling the particle to precursor ratio (250 μmol $Zn(NO_3)_2$•$6H_2O$ for 100 mg ZnO-coated nanospheres). Typical NW tip diameters were ~10-20 nm (Figure 2b), and NW

length was ~300-700 nm for a single growth cycle of 30 min. While most of the nanospheres were uniformly covered by NWs after the growth process, a few particles remained plain without NWs. These were a result of an incomplete ALD coating process when multiple layers of SiO_2 nanospheres were drop-casted onto a glass substrate and then placed in the ALD chamber for ZnO coating. Depending on the number of layers, some nanospheres present in the deeper layers were not well coated with ZnO, and hence fewer ZnO NWs grew on these nanospheres (Supporting Information S2). This can be improved by reducing the number of the nanosphere stack layers, or by repeating the ZnO ALD step, but these methods tend to reduce particle dispersibility (Supporting Information S2). Coating the ZnO thin layer on silica beads could also be prepared with a sol-gel technique, however required extra purification and separation steps to remove unreacted chemicals and side-products. The thin film uniformity was also generally poor, thus ALD was chosen to form the thin ZnO film.

The final particles size is determined by the core particles and NW length. For example, by using larger SiO_2 particles (~2 μm) as the core, larger spiky particles can be produced (Figure 2c). For a growth time of 2 hours, the NW radius was 20-40 nm, and the NW length was ~1-1.5 μm (Figure 2e). When smaller nanospheres with 200 nm diameters were used, NWs can still be successfully grown on these nanospheres, but the morphology of each individual particle appears to be less "spiky", because the diameter of the grown NWs (~20 nm) is not significantly smaller than the size of the used SiO_2 nanospheres (200 nm).

The NW density could be tuned by changing the reaction conditions. For example, by modifying the reaction temperature to be 80 ^{o}C and the PEI concentration to be 9 mM in the reaction solution, spiky particles with sparse NWs can be produced, as shown in Figure 2f (based on 2 μm nanosphere) and 3g (based on 600-800 nm nanosphere). In addition, we found the fabrication process was fairly independent of the particle core. For example, by using a similar fabrication protocol, spiky particles can be synthesized based on Al_2O_3 nanospheres, suggesting flexibility of the material choice for the particle core (Figure 2f). This flexibility of particle core is advantageous in that nanoparticles with additional optical, magnetic, or plasmonic functionality can be easily incorporated with spiky particles for the designing of multi-functional nanoparticles.

To explore the interfaces between spiky particles and biological cells, the as-synthesized spiky particles were introduced to Chinese hamster ovary (CHO) cells. After overnight

incubation, the cell samples were fixed and prepared with critical point drying, and the particle engulfment profiles were studied with SEM (Figure 3). The SEM images showing that the spiky particles based on 800 nm nanospheres appeared to be either internalized, partially engulfed, or resting on the cell surfaces (Figure 3a-d). As shown in Figure 3b and 3c, cell membrane was observed to wrap around spiky particle, which may due to the natural occurring of particle endocytosis or phagocytosis, and a few particles appeared to be fully internalized by the cells. Note that rinsing during preparation of the SEM samples (>8 times) likely removed most adventitious particles, while particles in contact with the cells were still preserved. Therefore, although many particles appeared to passively rest on the cell surface (Figure 3d), there is likely still some adhesive binding or interaction between these particles and cells.

Larger spiky particles based on 2 μm nanospheres were also applied to the cells (Figure 3e and 3f), which were found to be contact on the cell surface and some of them were even engulfed by the cells (Supporting Information S3), but fewer were observed to be internalized. One interesting application inspired by these observations of spiky particle-cell interactions is utilizing the sharp NWs to induce localized stress leading to cell membrane penetration. This would allow the NWs to access to the intracellular environment, while most of the particles remain in the extracellular space. However, it should be noted that NW cell penetration is often indirectly supported by material delivery,[9,18,20,37] while conclusive visualization on penetration is still limited with existing techniques (Supporting Information S4).

In addition to the solution phase delivery, we tested the spiky particle-cell interfaces where cells were cultured on top of the spiky particles. Spiky particles were drop-casted onto a glass substrate, and cells were then seeded onto the particle-substrates. After 24 h, cell-particle interface were studied with SEM. As shown in Figure 4a and 4b, cells spread on top of the particle-substrate, and the spiky particles underneath the cell surface were closely engulfed by the cells. Interestingly, the cell membrane was not only deformed by the particles, but also wrapped around the smaller NWs (Figure 4c and 4d). This profile is illustrated in Figure 4e, which shows the cell membrane seems to response to individual NW structures, and was deformed with higher curvature. This is in contrast to the interface profiles of Figure 4f and 4g, where the spiky particles were engulfed by the top cell surface. The cell membrane on the top surface appears not to recognize the individual NW structure, and simply deformed into hemispherical pits around the particles (illustrated in Figure 4h). These results suggest the

bottom basal cell surface may be more apt to recognize and interact with smaller nanostructures. This is may be related to the gradually relaxation of the bottom surfaces cytoskeleton to release the bending energy, allowing the cell to deform and wrap around the NWs.

Conclusion

In summary, in this work, we demonstrated the synthesis of sub-cell size spiky nanoparticles, where suspended nanoparticles were covered with ZnO NWs, with tunable geometries and dimensions for cellular application. This work successfully demonstrates a new heterogeneous nanostructure, suspending spiky particles, to release the structure restriction of traditional NW arrays where NWs are attached to a planar substrate. The interfaces between spiky particles and biological cells were examined under the conditions where the particles were engulfed by the top cell surface or where the cells were cultured on top of the particles. These results provide an investigation on biological cells interacting with hierarchical nanostructures. The results suggest that the bottom cell surface has better recognition and deformability to the NW structure than the top cell surface, which contributes to the understanding of the complicated interfaces between cell and hierarchical nanostructures. In this work, ZnO was used as the NW materials, which has cytotoxic effects on biological cells. Although ZnO NWs can be employed for biological applications such as cancer cell destruction, the cytotoxicity limits their applications as safe materials for drug delivery or other therapeutic applications. Further development of spiky particles with more biocompatible materials and using new strategies to enhance NW cell penetration would enable spiky particles as novel cell membrane penetrant vehicles for biological applications such as intracellular drug delivery.

Materials and Methods

Synthesis of SiO_2 nanospheres: 1.3 ml Tetraethyl Orthosilicate (TEOS, 99.999% trace metals basis, Sigma) was added into 10 ml absolute ethanol (200 proof absolute ethanol, Fisher Scientific), and 9 ml ammonia hydroxide (28% NH_4OH) was added into another 10 ml absolute ethanol. The TEOS solution was then poured into the NH_4OH solution with vigorous stirring. The mixture solution was stirred at 600 rpm at room temperature for 4 h. After reaction, the SiO_2 spheres were cleaned with ethanol and DI water for totally 6 times (2× ethanol, 2× water, and 2× ethanol again, 10 min for each, centrifuge at 7000 rpm). SiO_2 nanospheres with 600-800 nm

diameter were produced with this protocol. SiO_2 nanospheres with 2 μm or 200 nm size were purchased from Corpuscular Company.

To improve the SiO_2 nanospheres dispersibility, the nanoparticles were re-dispersed in dry toluene, and 3-aminopropyltrimethoxysilane (APTMS, Sigma) was added into it at final concentration of 2% (v/v). The solution was stirred at room temperature for 4 h, followed by particles rinsing with ethanol for totally 3 times. By taking part of the particle solution and evaporating the solvent, the weight of the particle residue was measured, and the total weight of the particles in the whole solution was determined.

Synthesis of spiky particles: Solution containing SiO_2 nanospheres (in ethanol) was drop-casted onto a glass slide (cleaned with O_2 plasma) and dried. The whole slide was coated with ZnO layer via atomic layer deposition (ALD, Cambridge Nanotech) using the diethylzinc (DEZ)/H_2O ALD process. The ALD was performed with 0.015 s pulse time, and 8 s waiting time (time that precursors were retained in the ALD chamber) for both DEZ and H_2O pulses, with totally 200 pulse cycles at 200 ^oC. The glass slide was wrapped by Al foil (punched with holes) to prevent particles from blowing away in the ALD chamber. A blank Si wafer was placed in the ALD chamber for determining the thickness of the deposited ZnO film using Ellipsometry (Automatic c-slide on Ellipsometry, Gaertner Scientific Corporation). Normally 200 pulse cycles give 20-nm-thick ZnO film. After the ALD process, the whole slide with Al foil was immerged in water, vigorously spun and then sonicated overnight to collect the ZnO-coated nanospheres.

In the next step, 10 ml aqueous solution containing 100 mg ZnO-coated nanospheres, 25 mM zinc nitrate hydrate [$Zn(NO_3)_2$•$6H_2O$], 25 mM hexamethylenetetramine ($C_6H_{12}N_4$, HMTA), and 7 mM polyethylenimine (PEI, branched, M_w~800, Sigma) was stirred at 1000 rpm. Reaction was performed at 100 ^oC for 30 min. After reaction, the particles were rinsed with ethanol and centrifuged at 5000 rpm. For producing spiky particles with sparse NWs, SiO_2 nanoparticles were drop-casted on a glass slide (~1 cm^2) and coated with ZnO using ALD. The whole slide supporting the particles was then immersed into 10 ml aqueous solution containing 25 mM $Zn(NO_3)_2$•$6H_2O$, 25 mM HMTA, and 9 mM PEI. Reaction was performed at 80 ^oC for 2 h without stirring. The particles on the glass substrate can be further dispersed in solution by vigorously rinsing off from the glass substrate.

Cell culture. CHO or Hela cells were cultured in cell medium containing 89% DMEM, 10% fetal bovine serum and 1% antibiotics at 37° C and 5% CO_2, with a density of ~10000-50000 cells/well (96-wells plates). Normally cells were pre-cultured for 12-24 h before assays.

Cell-particles interface experiments: Cells were plated onto a glass slide for 12 hours, then incubated with spiky particles overnight. Samples were then fixed with 2% glutaraldehyde solution for 5 min, and labeled with Osmium Tetroxide for 10 min to improve contrast. Extended exposure of the samples to the fixation solution may affect the spiky particle morphology. The cell samples were gradually rinsed with ethanol of increasing concentrations (0%, 30%, 50%, 70%, 95%, and 100%), and then prepared by critical point drying (CPD). Samples were finally sputter-coated with gold-palladium for SEM imaging.

Cells cultured on top of spiky particles: Spiky particles in ethanol were uniformly deposited onto a poly-L-lysine coated glass slide (~0.5 cm^2) with a density of ~150 mg/cm^2. This was performed by placing a PDMS well on top of the glass slide, and then adding the particles solution into the well and letting the ethanol dried. The PDMS well was then removed. This resulted in homogenous deposition of spiky particles in a local area on the substrate. Cells were seeded onto the particle-substrates. After 24 h, cell-particle interface was studied with SEM.

Figures:

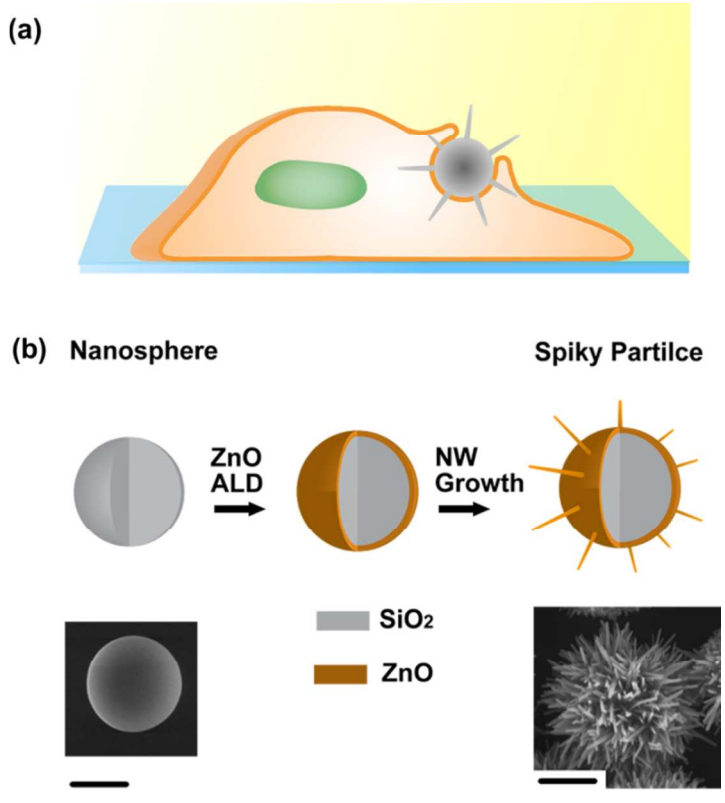

Figure 1. (a) Illustration of cell interfaces with spiky particle. (b) Illustration of spiky particle fabrication process. SiO_2 nanospheres were used as core particles. They were coated with 20-nm ZnO by ALD, and then ZnO NWs were grown on them to produce spiky particles. Scale bar: 500 nm.

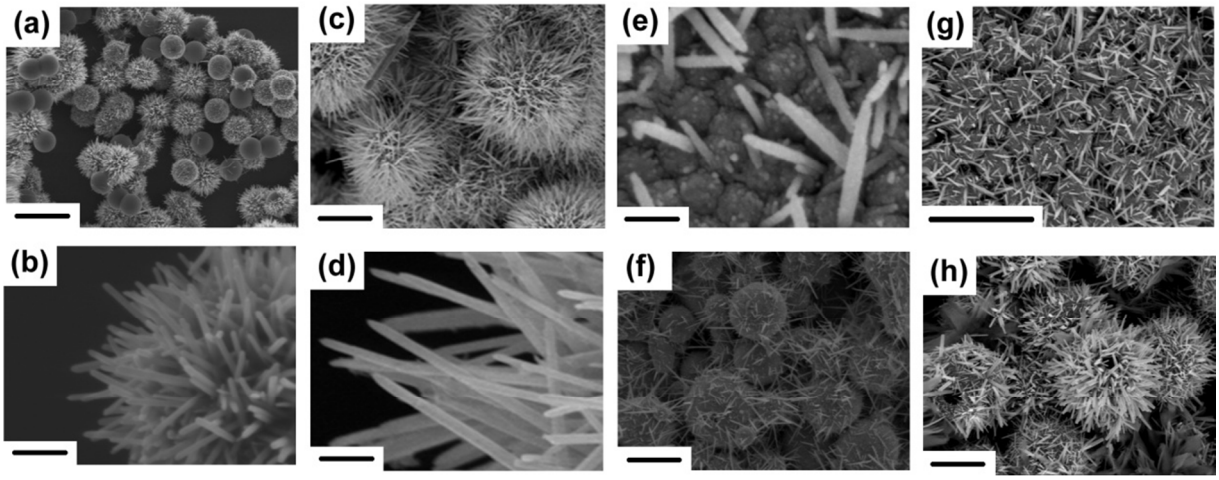

Figure 2. Synthesis of spiky particles. (a) Spiky particles based on 800 nm SiO_2 nanoparticles, and (b) the zoom in image showing NWs. (c) Spiky particles based on 2 μm SiO_2 particles, and (d) the zoom in images showing NWs. (e) Spiky particles based on 200 nm SiO_2 particles. (f) and (g) Spiky particles with spare NWs. (h) Spiky particles based on 2 μm Al_2O_3 particles. Scale bar: 2 μm in (a), (c), (f) (g) and (h), and 200 nm in (b) and (d) and (e).

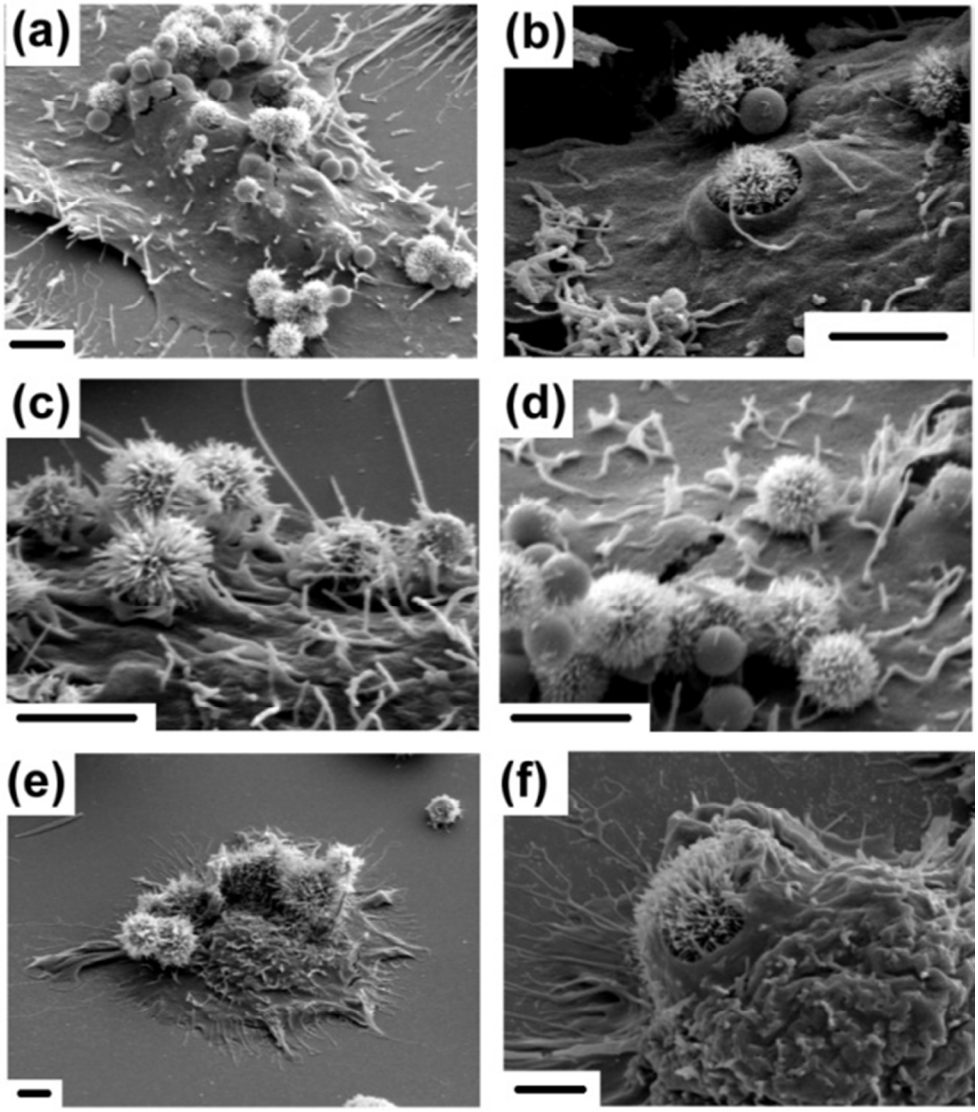

Figure 3. SEM images of spiky particle-cell interface. (a)-(d) Spiky particles were fabricated using 800 nm SiO_2 nanospheres as the core. The spiky particles appear to be either internalized, partially engulfed, or resting on the cell surfaces. (e) and (f) Spiky particles were fabricated using larger SiO_2 particle (2 μm) as the core. The spiky particles were engulfed by the cells. Scale bar: 2 μm.

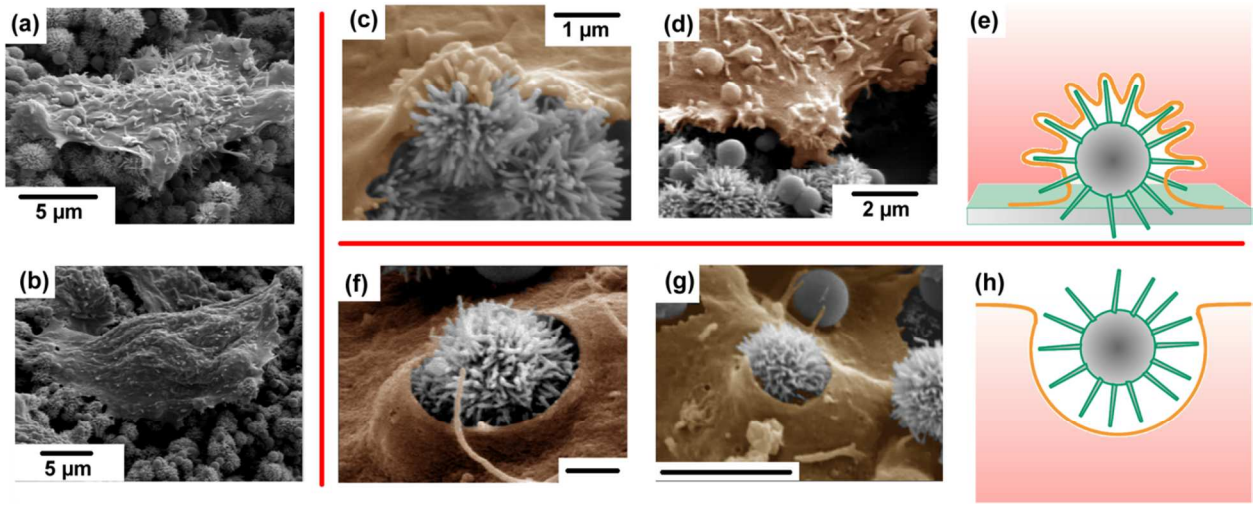

Figure 4. (a) and (b) SEM images showing cells cultured on top of spiky particles. (c) and (d) SEM images showing the interface between cells and spiky particles deposited on substrate. Cells were observed to spread on the spiky particles, and the cell membrane closely engulfed the NWs. (e) Illustration of cell engulfment on spiky particles when the cell was cultured on top of the spiky particles. The cell membrane deforms and wraps around the NWs. (f) and (g) SEM images showing the spiky particles were engulfed by the cells when the particles were added to the cell from top medium. Scale bar: 500 nm in (f) and 2 μm in (g). (h) The illustration of cell engulfment on spiky particles when the spiky particles were added to the cell from top medium. The cell membrane tends to deform as pits to engulf the particles, but not appears to wrap around the NWs. Cell surfaces were stained with brown color in (c), (d), (f) and (g).

ASSOCIATED CONTENT

AUTHOR INFORMATION

Corresponding author: Nicholas A. Melosh. Email: nmelosh@stanford.edu. Phone: (650) 724-3679. Fax: (650) 736-1984. Address: 476 Lomita Mall, McCullough Bldg. Stanford, CA 94305

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ABBREVIATIONS

NWs, nanowires; CHO cells, Chinese hamster ovary cell; SEM, *scanning electron microscope*; CVD, chemical vapor deposition; ALD, atomic layer deposition; PEI, polyethylenimine.

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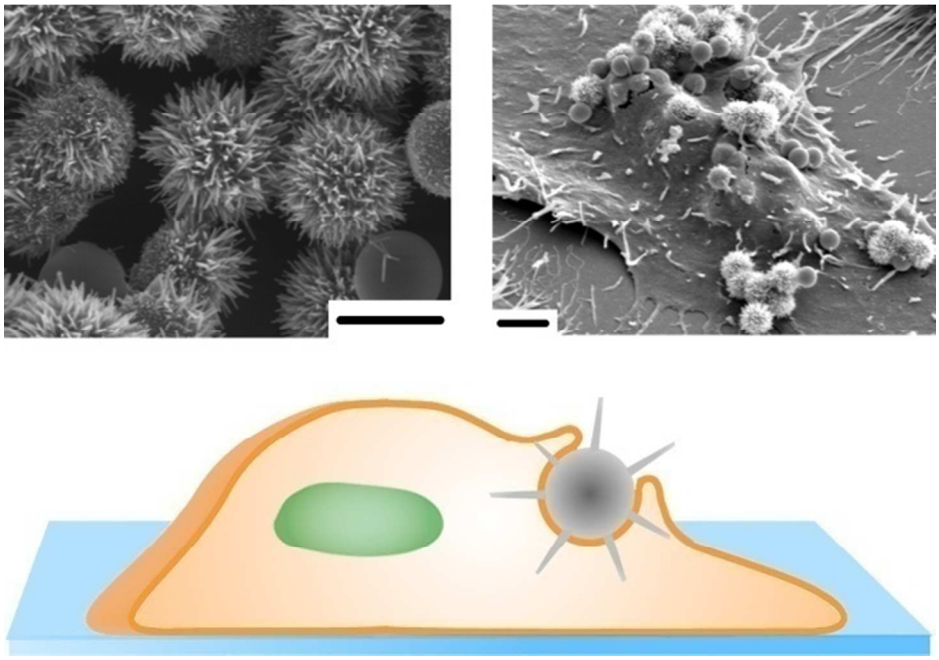
Graphical Abstract:



Synthesis of hierarchical “spiky” nanoparticles covered with stiff nanowires for biological cellular interface and engulfment.