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Synthetic Nanoscale Elements for Delivery of Materials Into Viable Cells

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Summary

Arrays of vertically aligned carbon nanofibers (VACNFs) provide structures that are well suited for the direct integration and manipulation of molecular-scale phenomena within intact, live cells. VACNFs are fabricated via a combination of microfabrication techniques and catalytic plasma-enhanced chemical vapor deposition. In this chapter, we discuss the synthesis of VACNFs and detail the methods for introducing these arrays into the intracellular domain of mammalian cells for the purpose of delivering large macromolecules, specifically plasmid DNA, on a massively parallel basis.

Key Words

Carbon nanofibers; gene delivery; material delivery; cellular interfacing; tethered genes.

1. Introduction

Because they reside at the same size scale as the biomolecular machines of cells, engineered nanoscale devices may provide the means to construct new tools for monitoring and manipulating cellular processes. The ideal tools would interface directly with subcellular, biomolecular processes, allowing the control of these processes while also providing transduction of responses with both spatial and temporal resolution. Ultimately, this interfacing might be performed without adversely affecting cell viability or functionality. Investigators have highlighted this interface of nanotechnology and biotechnology through the use of nonbleaching fluorescent nanocrystals in place of dyes as a means of monitoring cellular processes ***(1–3)***. Pulled-glass capillaries with nanoscale tips have been implemented for cellular and subcellular electrophysiological stimulus and monitoring ***(4)*** and for manipulating intracellular material using microinjection

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of membrane-impermeable molecules (e.g., proteins, DNA) ***(5)***. Conventionally, these devices have required manipulating cells one at a time while visualizing the process under a microscope and, thus, provide a serial interface to individual cells. Parallel embodiments of these devices have been fabricated using silicon microfabrication methods ***(6)***, but as with all micromachining techniques, there are limitations to the ultimate size scale and density of features (tip radii and spacing of the silicon needles) and to the choice of substrate materials (i.e., parallel embodiments not easily fabricated on transparent substrates convenient for cell culture). By contrast, recent advances in the synthesis of nanomaterials, including carbon nanofibers and carbon nanotubes, can avoid these limitations and provide the means to construct massively parallel, addressable functional nanoscale devices including chemically specific atomic force microscope probes ***(7,8)***, electrochemical probes ***(9)***, and electromechanical manipulators ***(10)***. Within this family of structures, carbon nanofibers are particularly well suited for the construction of intracellular devices because of the ability to control their synthesis exquisitely. Deterministic arrays of closely spaced (pitch ≥ 1 μm) vertically aligned carbon nanofibers (VACNFs) ***(11–13)*** may be grown on a wide variety of substrates (including quartz and glass slides) with wide bases that provide mechanical strength while still generating a small diameter tip (≥5-nm tip radius) appropriate for insertion directly into cells.

Here we describe the steps for fabricating functional VACNF devices and for implementing these devices in a first step toward molecular-scale integration with live cells. As a demonstration, these devices are used to introduce exogenous material (e.g., nanofiber-scaffolded plasmid DNA) into viable cells. While providing a novel and effective method for gene delivery, the functional insertion of nanofiber-scaffolded DNA has exciting potential for ultimately providing a nanoscale genomic interfacing platform (*see* **Note 1**).

2. Materials

2.1. Nanofiber Synthesis

1. Photoresist (Shipley).
2. Si wafers (Silicon Quest).
3. Ni slugs (Goodfellows).
4. Acetone and isopropanol.
5. Dry nitrogen.
6. Acetylene (Matheson gases).
7. Ammonia (Matheson gases).

2.2. Nanofiber-Mediated Material Delivery

1. Plasmid DNA: pd2EYFP-N1 (Clontech) or pgreenlantern-1.
2. 100 m*M* 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer adjusted to pH 4.5–4.8 with NaOH (Sigma-Aldrich).

3. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma-Aldrich).
4. Phosphate-buffered saline (PBS) or other serum-free and dye-free cell suspension buffer.
5. Eppendorf microcentrifuge tubes (1.5 mL) or equivalent.
6. Sylgard 184 two-part poly(dimethylsiloxane) (PDMS) kit (Dow Corning, Midland, MI).
7. Propidium iodide (Sigma-Aldrich).

3. Methods

3.1. Synthesis of VACNFs

VACNFs are synthesized deterministically using catalytically controlled, direct current (DC) plasma-enhanced chemical vapor deposition ***(11,12,14)***. VACNFs grow from catalyst particles (e.g., Ni) deposited onto a substrate at predefined locations when placed onto a cathode of a glow discharge system (**Fig. 1**) at elevated temperature (e.g., 700°C) in a flow of acetylene and ammonia. Carbon species decompose at the surface of the catalyst particle, and free carbon atoms diffuse through it and are incorporated into a growing nanostructure between the particle and the substrate ***(15)***. Such nanofibers, which have a catalyst particle at the tip, grow oriented along the electric field lines ***(13,16)***. Thus, the orientation can be determined by the direction of the field. The lateral dimensions of the nanofibers are determined by the size of the catalytic particle, and their length can be precisely controlled by the growth time. Growth conditions can be manipulated to control the shape of the nanofibers (e.g., conical vs cylindrical) ***(13)*** and to control the chemical composition of material deposited on the walls (e.g., amorphous carbon or silicon nitride) ***(17)***. We provide next the specific steps for the synthesis of VACNFs suitable for functional integration into viable cells. An example of such an array is shown in **Fig. 2**.

3.1.1. Patterning of Substrate

1. Spin a photoresist or an electron beam resist (depending on whether photolithography or electron beam lithography is used, respectively) on the surface of a silicon wafer and process according to the manufacturer's guidelines.
2. Expose and develop a pattern so that the resist is removed from the areas where the carbon nanofibers will be grown.

Specific details depend on the lithography tool and resist. For a general overview of the processes, readers are referred to **ref. *18***. For transfection of mammalian cells, such as Chinese hamster ovary (CHO) cells, the pattern is an array of dots that are 0.5 μm in diameter arranged on a square grid with 5-μm spacing ***(19)***. Additional grid lines every 100 μm and indexing numbers are helpful to track transfected cells ***(20)***.

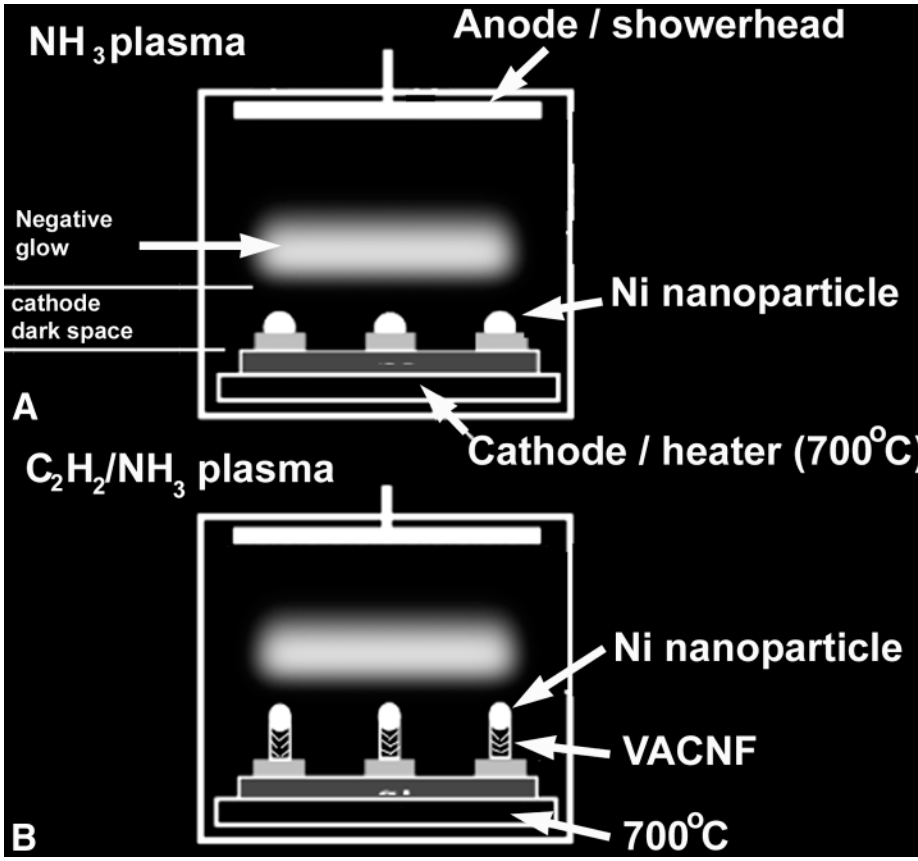

Fig. 1. Schematic representation of the PECVD process for growing vertically aligned carbon nanofibers. **(A)** Catalyst pretreatment/nanoparticle formation; **(B)** Growth of carbon nanofibers.

3.1.2. Deposition of Catalyst

1. Load patterned wafers into a physical vapor deposition chamber, and after an appropriate vacuum is achieved (10^{-7} torr), deposit 20 nm of Ni.

3.1.3. Lifting Off of Excess Metal

1. Place a metallized wafer in a glass dish filled with acetone, cover the dish with a looking-glass cover to prevent evaporation of acetone, and soak it for 30–60 min.
2. Ultrasonicate for 30 s.
3. Remove the wafer from the dish but do not let the acetone dry out, and while holding the wafer with tweezers above the dish immediately rinse the wafer with

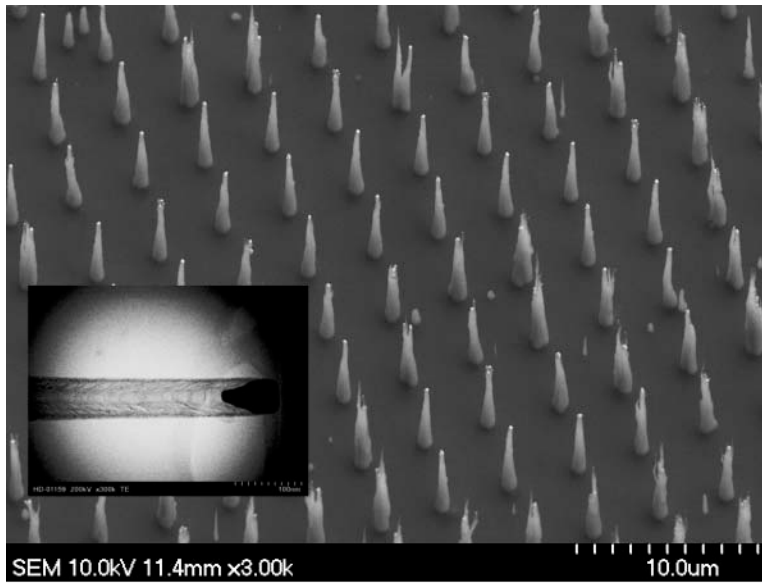

Fig. 2. Typical array of VACNFs suitable for integration with viable cells; **(inset)** transmission electron microscope image of nanofiber.

a spray of acetone to remove metal particulates. Wash the acetone by spraying the wafer with isopropanol.
4. Blow-dry with N_2.

3.1.4. Growth of Carbon Nanofibers

Growth parameters such as gas flows, pressure, and plasma current will vary depending on the particular chamber setup, type of substrate, chamber and substrate size, and catalyst pattern.

1. Mount a wafer on top of the heater-cathode of a DC plasma enhanced chemical vapor deposition (PECVD) system.
2. Evacuate the chamber until a vacuum below 0.1 torr is achieved.
3. Set the substrate temperature controller to 700°C.
4. Introduce ammonia by opening the automatic valve and set the flow to 80 standard cubic centimeters per minute (sscm) and pressure to 3 torr.
5. When the temperature reaches 700°C, introduce acetylene at 20 sccm.
6. Ten seconds after opening the acetylene valve, start DC plasma discharge by turning on the high voltage. Set the high-voltage power supply to constant current mode with the current set to 350 mA.
7. Continue growth for 60 min to produce 6-μm-long fibers.

8. Terminate growth by turning off the high voltage.
9. Turn off the gas flow, open the pressure control valve to pump down the chamber, turn off the heater, and wait until the wafer is cooled down to at least below 300°C before removing.

3.2. Spotting of Nanofiber Arrays With Plasmid DNA

For DNA delivery applications, VACNF arrays must be surface modified with DNA prior to cellular interfacing. VACNF arrays may be spotted with DNA or DNA may be covalently linked to the nanofibers ***(19,20)***. The former procedure relies on physiadsorption of DNA on the nanofiber surface during spotting, and release of this DNA once the nanofiber has been introduced via penetration into the intracellular domain. Nanofiber surface composition is an important factor that influences both adsorption and release of material. We have found that bare carbon nanofibers are poor DNA carriers. They are typically hydrophobic ***(21)*** and, thus, do not wet easily with aqueous DNA solutions. Furthermore, DNA is only weakly held on the surface of bare carbon nanofibers, apparently often being shed prior to introduction of the nanofiber within a cell. By contrast, nanofibers that are synthesized under conditions that redeposit silicon nitride on the sidewalls of the growing structure ***(26)*** are found to promote strong adsorption of DNA during spotting but release at least some of this material once the structure is introduced into a cell. The specific steps for spotting DNA on VACNF arrays are as follows:

1. To promote wetting of prepared nanofiber arrays, expose the array samples to a 30-s radio-frequency (RF) oxygen plasma etch process (RF power = 115 W, pressure = 350 torr, oxygen flow rate = 50 sccm).
2. In a chemical fume hood, cleave large samples into 5-mm^2 chips using a diamond scribe to make straight cleavage lines across the surface being broken. Snap at the scored line by placing the line directly over a rigid, straight edge and tapping the side to be broken at an edge, *not* on the fibered surface.
3. Following all cleavage procedures, rinse the samples in a spray of distilled water to eliminate debris.
4. Spot 5-mm^2 fiber arrays samples with 1 to 2 µL of plasmid DNA at concentrations of 10–500 ng/µL. Ideally, plasmid DNA should be suspended in water, as opposed to conventional buffering solutions, in order to avoid the formation of salt crystals during the subsequent drying step.
5. Air-dry each spotted sample in a sterile culture hood. Typically, this takes approx 10 min under normal laboratory humidity.

3.3. Covalent Modification of Fibers With Plasmid DNA

To provide more control over the fate of introduced genes than that provided by the physisorption/desorption mechanism of spotting, delivered material may be physically tethered to the nanofiber scaffold. Covalent attachment of plasmid

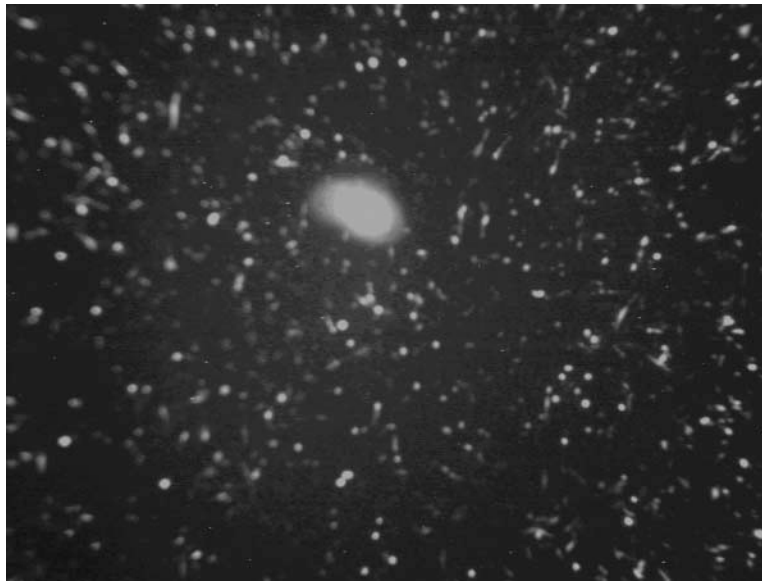

Fig. 3. Fluorescent micrograph of green fluorescent protein (GFP) expression in CHO cells 1 d after integration with fiber array covalently derivatized with pgreenlantern-1, a GFP reporter plasmid.

DNA is achieved using a condensation reaction between primary amines of DNA bases and carboxylic acid sites on the nanofiber surface. Using this method, the DNA may attach at multiple locations, and the sites of attachment within the DNA cannot be specified. As such, it is likely that much of the plasmid DNA will be rendered transcriptionally inactive, because attachment at sites within the active coding region of the plasmid will interfere with polymerase access and function. Nonetheless, this technique has provided transcriptionally active, bound plasmid using a 5081-bp plasmid with a 30% active coding region (pgreenlantern-1; **Fig. 3**).

For highest yield, it is recommended that plasmid DNA be suspended only in water. Both Tris and EDTA of TE DNA buffers contain reactive groups that will interfere with the EDC condensation reaction of DNA onto the carboxylic acid sites of fibers. The specific steps for covalent attachment of DNA to VACNF arrays are as follows:

1. Provide or increase the number of carboxylic acid sites on the fiber surfaces by exposing array samples to a 5-min RF oxygen reactive ion etch (RIE). A typical oxygen RIE recipe for a Trion etcher is a pressure of 350 mt, an RIE power of 115 W, and an oxygen flow of 50 sccm. This step may be conducted on either discrete samples or large (wafer-scale) nanofiber arrays.

2. In a chemical fume hood, cleave large samples into 5-mm^2 chips using a diamond scribe to make straight cleavage lines across the surface being broken. Snap at the scored line by placing the line directly over a rigid, straight edge and tapping the side to be broken at an edge, *not* on the fibered surface.
3. Following all cleavage procedures, rinse the samples in a spray of distilled water to eliminate debris.
4. Place individual 5-mm^2 array samples into 1.5-mL Eppendorf tubes, taking care to handle the samples by the edges. The curve of the Eppendorf tube will protect the fibered surface from contact with the tube walls.
5. Dispense 500 µL of 0.1 *M* MES, pH 4.5 buffer containing 10 mg of EDC to cover each fibered sample in its reaction tube. Ensure that the fibered surface is wetted and remains submerged and does not harbor trapped air bubbles.
6. If a control sample is desired in order to evaluate the effects of covalently attached vs nonspecifically adsorbed DNA on fibers, dispense 500 µL of 0.1 *M* MES, pH 4.5 buffer containing noO EDC to cover the control samples in their reaction tubes. Ensure that the fibered surfaces are wetted and remain submerged and do not harbor trapped air bubbles.
7. Add 1 µg of plasmid DNA in water to each sample, triturating the dispensed fluid to disperse into the solution.
8. Agitate these reaction tubes on an orbital shaker for at least 2 h at room temperature, ideally overnight, to allow the reaction to run to completion.
9. Aspirate each reaction mixture with a Pasteur pipet, taking care not to touch the fibered surface.
10. Rinse each reaction tube in two, 1-mL aliquots of PBS and then soak in 1 mL of PBS for 1 h at 37°C.
11. Rinse each reaction tube in two, 1-mL aliquots of deionized water.
12. Dry each sample prior to use.

3.4. Preparation of Microcentrifuge Spin Tubes

For some cell types, fiber penetration into a cell may be achieved by centrifuging the cells down onto the vertical array of nanofibers. A modified Eppendorf tube may be implemented for rapid cell-fiber interfacing with a benchtop microcentrifuge. Because microcentrifuges typically employ rotors that hold tubes at a 45° angle, the modified Eppendorf tube is designed to feature a 45° slanted surface such that a small (approx 5 × 5 mm) VACNF array chip may be positioned on the slant normal to centrifugal force. The specific steps for constructing a modified Eppendorf tube are as follows:

1. Wearing appropriate chemical protection gloves and glasses, prepare approx 10 mL of PDMS by mixing 10 g of component A (Sylgard 184; Dow Corning) with 1 g of Sylgard 184 curing agent. Avoid generating excessive bubbles during mixing by using a gentle folding motion with a flat, stainless steel weigh spatula.
2. Place the mixed PDMS solution into a 10-mL syringe to allow convenient dispensing of aliquots of the PDMS solution.

3. Dispense 0.5 mL of the PDMS solution into each 1.5-mL Eppendorf tube to be modified. Cap each tube after filling.
4. Place the filled Eppendorf tubes in a microcentrifuge, carefully rotating each tube so that each tube's lid hinge is positioned pointing away from the centrifuge rotor's center. This positioning will be used each time the spin tube is implemented in order to orient the tube properly.
5. Spin the centrifuge for 12 h at 2000*g* and ambient temperature. If the centrifuge features a heater, cure time may be reduced to 30 min at 65°C. During this time, the PDMS in each tube will slant and cure, forming a semirigid, planar surface normal to the centrifuge's radial vector.
6. Open each tube, place the tubes in a beaker, cap the beaker with aluminum foil and autoclave indicator tape, and sterilize the lot in an autoclave.
7. Following sterilization, dry any excess moisture by placing in a drying oven (temperature not to exceed 95°C).

3.5. Interfacing of Cells Onto Carbon Nanofiber Arrays

Fiber penetration and material delivery into a cell appears to be a multistep procedure. For small dye molecules, simply centrifuging the cell onto fibers for brief intervals (minutes) at high pelleting forces (approx 1000*g*) can provide cell loading. For DNA delivery, centrifugation and a subsequent press step is much more effective than centrifugation alone, perhaps providing improved penetration of nanofibers across the nuclear membrane barrier (**Fig. 4**). Although some success at cell-fiber interfacing has been achieved simply by allowing cells to settle out of suspension onto fibers and then performing a press, interfacing effectiveness and material delivery to the nucleus is improved by first centrifuging cells onto the fibers. Centrifuge parameters will likely vary for different cell types, and these should be adjusted and optimized appropriately. For CHO cells, pelleting forces of 600*g* for 30 s to 1 min are effective if followed by a subsequent press step.

Following centrifugation of cells onto fibers, a subsequent press step dramatically improves fiber penetration into the intracellular domain. This press step should be performed on a relatively flat surface (<1 μm surface roughness) that is compatible with the cells being studied. Ideally, to increase the effectiveness of the press step for the entire chip surface, the surface should be somewhat flexible and compliant. An autoclavable dish that has been partially filled with PDMS, as described next, works well as a stamping pad.

3.5.1. Preparation of Sterile, Compliant Pressing Surface

1. Wearing appropriate chemical protection gloves and glasses, prepare approx 2 mL of PDMS for each stamping pad to be used by mixing 2 g of component A (Sylgard 184; Dow Corning) with 0.2 g of Sylgard 184 curing agent. Avoid

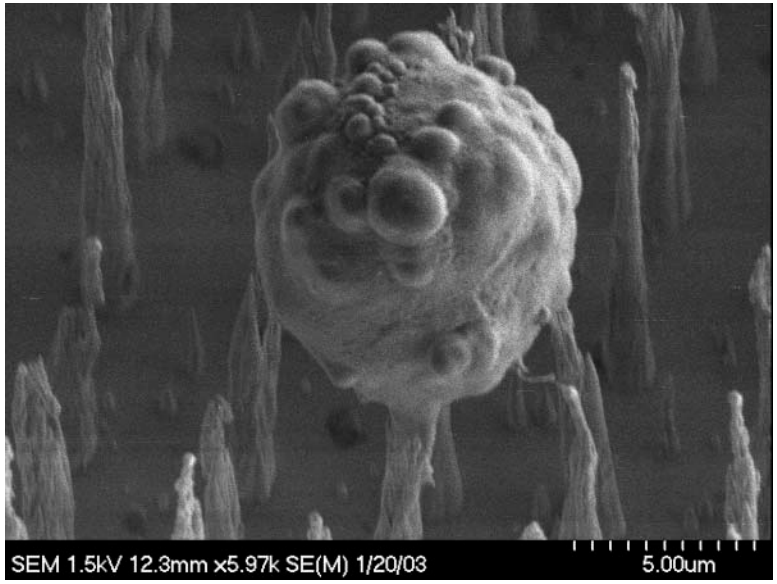

Fig. 4. Mouse myeloma cell (SP2/O-AG12) cultured for 3 d after centrifuging and pressing onto a 5-µm spaced nanofiber forest. This cell grows in suspension culture and does not attach and stretch out onto the fibers or fiber substrate.

generating excessive bubbles during mixing by using a gentle folding motion with a flat, stainless steel weigh spatula. If excessive bubbles are formed during mixing, place the mixture in a centrifuge tube and spin at more than 100*g* for 5 min. This will effectively remove air bubbles from the mix.
2. Pour the PDMS mixture into an autoclavable dish (more than 35 mm in diameter) such that at least 1 mm of PDMS solution covers the entire bottom of the dish.
3. Cure the PDMS by placing the dish on a flat surface at 65°C for at least 30 min.
4. Autoclave the stamping dish to sterilize.

3.5.2. Optimization of Centrifugation Parameters

Prior to interfacing cells to nanofiber arrays, cells must be suspended in serum- and dye-free buffer solutions. Medium constituents, and particularly serum, can have mitogenic effects on cells if administered directly to the intracellular domain. Thus, for all cell-interfacing procedures, cells should be washed of their medium and resuspended in PBS, or other buffers appropriate to the cell being studied.

Centrifugation parameters can be optimized by using a membrane-impermeant DNA intercalating stain to monitor membrane rupture (an indicator for fiber penetration) and membrane resealing (required for cell recovery). Ideal centrifugation parameters will result in a high probability of

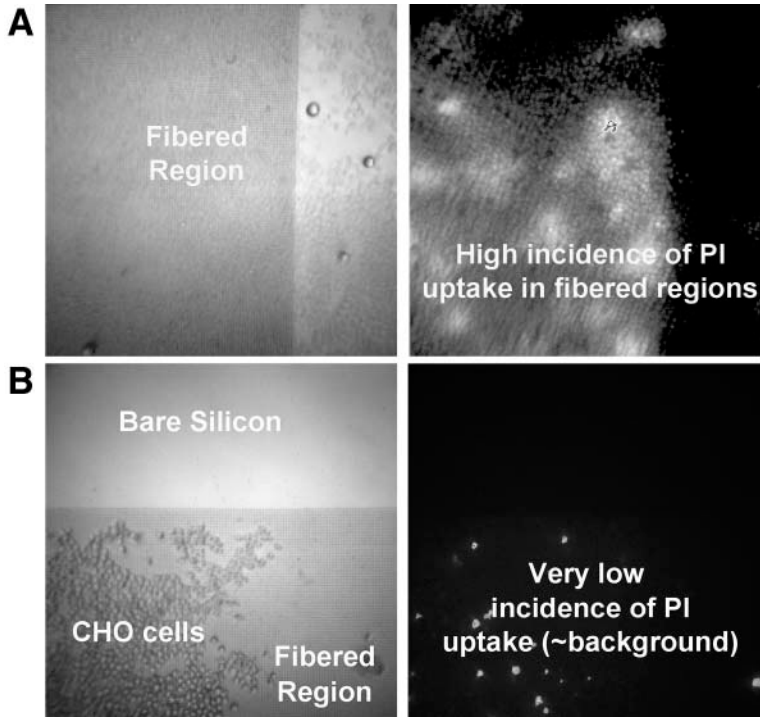

Fig. 5. PI uptake in cells centrifuged onto nanofiber arrays. **(A)** When cells are centrifuged onto fibers in the presence of impermeant PI, the dye passes the plasma membrane and stains DNA/RNA within the cell. **(B)** If PI is added to the system 5 min after the centrifugation procedure, it is excluded from the intracellular domain, apparently owing to resealing of the plasma membrane following fiber penetration.

membrane rupture, but also a high probability of membrane resealing, such that manipulated cells remain viable after the interfacing procedure. These tests can be performed using centrifugation trials with a membrane-impermeant stain in solution during centrifugation (to evaluate membrane rupture), and with this stain added to solution approx 5 min after centrifugation (to evaluate membrane resealing or general cell recovery). Propidium iodide (PI) and ethidium homodimer are both effective impermeant dyes (**Fig. 5**). Both, however, are suspected mutagens and should be handled with caution. The specific steps for optimizing centrifugation parameters are as follows:

1. Using appropriate laboratory safety procedures, prepare a 1 m*M* stock solution of either PI or ethidium homodimer.
2. Prepare cells for interfacing by suspending adherent cell types (scraping or trypsinization as appropriate) and washing all types free of medium, dyes, and

serum using pelleting and resuspension. Resuspend in a cell-specific buffer, such as PBS, at a dilution that will provide the desired monolayer coverage for a 1-cm^2 surface area using 0.5 mL of cells. Approximately 30 mL of cells will allow 10 different centrifugation trials.

3. In the following steps, work with only one set of samples at a time for each spin setting to be evaluated. Each set will consist of three samples with dye in the solution during the spin, and three samples with dye added to the solution after the spin. Label these P1, P2, and P3 for "penetration," and R1, R2, and R3 for "resealing."
4. Place the fibered samples into the PDMS slant of modified Eppendorf tubes such that the unfibered back side of the substrate is flat on the PDMS surface in the center of the tube.
5. Triturate the cell suspension to resuspend the cells before each trial, and place 0.5 mL of the cell suspension in each sample tube.
6. Add 5 µL of the 1 m*M* solution of dye to three of the samples labeled P1, P2, and P3. Triturate to mix.
7. Close all the samples and load into a microcentrifuge. Ensure that the samples are loaded opposite to one another in order to balance the microcentrifuge. Also ensure that the lid hinge of each tube is positioned pointing outward from the center of the centrifuge (as they were positioned during fabrication) such that the slant of the PDMS is oriented appropriately. The slant of the PDMS should be oriented straight up and down when loaded correctly.
8. Spin for the desired force and time. Trial 1 should be set at a time and force typical for gentle pelleting of the cell line studied. Subsequent trials will increase either or both of these parameters.
9. Following the spin, wait 5 min.
10. Carefully aspirate the solution from the P1, P2, and P3 samples and carefully add 0.5 mL of neat PBS.
11. Add 5 µL of the 1 m*M* solution of dye to the three samples labeled R1, R2, and R3.
12. Incubate for 5 min plus the centrifugation time, in order to incubate the R samples for the same amount of time the P samples were incubated with dye.
13. Carefully aspirate the solution from the R1, R2, and R3 samples and carefully add 0.5 mL of neat PBS.
14. Remove the samples from all six modified Eppendorf tubes, and observe the surfaces of each sample with an epifluorescent microscope equipped with a TRITC filter set (535/610).
15. Repeat **steps 4–14** until an optimized centrifugation protocol is achieved in which P samples result in high numbers of dyed cells (indicating potential penetration by fibers into cells), but R samples maintain very low numbers of dyed cells (indicating resealing of the membrane following puncture). Note that depending on the cell line, culture conditions, and harvesting techniques, membrane penetration and resealing may not be mutually obtained. Ultimately, to

achieve puncture merely using centrifugation, pelleting conditions may be too extreme to allow cell resealing and recovery. In this case, a more gentle spin protocol should be combined with a subsequent press step to provide potential DNA delivery.

3.5.3. Increasing of Fiber/Cell Integration by Pressing

If the press step is employed, it must be implemented as quickly as possible following centrifugation in order to minimize additional trauma to the cells that may result owing to membrane attachment to the fiber surface and subsequent shear of these attached domains during the press step. The specific press steps are as follows:

1. Immediately following centrifugation, carefully remove the chip from the spin tube with fine-nosed tweezers. Grasp the chip by its edges with the tweezers. Do not clasp the fibered surface.
2. Gently place the chip face down on the PDMS stamping pad, and gently press the back surface of the chip using a force approximately equivalent to writing with a pencil. Minimize lateral movement of the chip on the PDMS surface so as not to shear fibers and cells from the substrate.
3. Immediately remove the chip from the PDMS surface and place face up in a culture dish.
4. Place sufficient buffer solution in the culture dish to submerge the chip.
5. Allow the cells to recover in buffer solution for at least 15 min.
6. Aspirate the buffer solution from the culture dish and replace with growth medium.
7. Incubate under appropriate growth conditions for the cell line being studied.

4. Notes

1. The procedures discussed describe the steps for delivery of material, in this case plasmid DNA, into cells. There are many other potential applications of functionally integrated nanostructures into viable cells that can be realized if more extensive postgrowth processing of the VACNFs is performed. In essence, the VACNF is a high-aspect-ratio, mechanically and chemically robust conductor of electrons that can be deterministically produced on any substrate compatible with the PECVD growth process. Use of large-scale growth reactors created the opportunity to synthesize high-quality VACNFs in precisely defined locations on substrates compatible with microelectronic device manufacturing equipment (e.g., 100-mm-diameter round Si and quartz wafers). Consequently, it was discovered that VACNFs are compatible with many of the standard microfabrication techniques used in the production of integrated circuits and microelectromechanical systems ***(9,22–26)***. Although a comprehensive description of microfabrication techniques for VACNF substrates is beyond the scope of this discussion, we briefly describe next some of the more useful processing steps.

a. Perhaps the most fundamental operation is the deposition of thin films on VACNF arrays. We have examined the effect of SiO_2 and amorphous Si deposition onto VACNF using rf PECVD. We found that these layers could be uniformly deposited onto the fibers, resulting in a conformal coating. Physical vapor deposition techniques including sputtering and electron beam evaporation have also been used successfully for this purpose. This provided us with a mechanism to modify the surface of the VACNF. Of particular interest was the coating of dielectric layers onto the VACNF that could be selectively removed from regions of the fiber with subsequent microfabrication processes. This provides the ability to control the amount of the fiber body capable of participating in electron transport independent of the aspect ratio or geometry of the fiber.
b. Once material has been deposited onto a substrate, typically some sort of patterning is performed. Photolithography has long been established as the standard workhorse technique used in the microelectronics industry for this purpose. This process involves the patterning of ultraviolet–sensitive polymer layers (photoresists). Photoresists are typically spin cast onto substrates at speeds ranging from 1000 to 6000 rpm. Simple experiments involving the deposition of photoresist layers with a thickness between 200 nm and 2 μm demonstrated that even high-aspect-ratio VACNFs survive this processing. Not only can a photoresist be applied to substrates with VACNF on them, but it can be exposed and developed using well-established techniques. Moreover, a photoresist can be stripped from substrates containing fibers using a combination of organic solvents and ultrasonic agitation with no damage to the structural integrity of the VACNF.
c. Once a pattern has been exposed in a layer of photoresist and developed, it is transferred into the substrate by either the addition or removal of material, referred to as additive or subtractive pattern transfer, respectively. In the case of subtractive pattern transfer, some form of etching is used to remove the desired layer or layers from the patterned area. These processes include various forms of plasma-based etching along with wet chemical etching. Material deposited onto a VACNF can be removed using several combinations of these techniques without significantly damaging the fiber. The fact that the VACNF is composed of graphitic carbon provides it with a robust body that can withstand bombardment of ions during plasma-based processes and any sort of chemical reaction with the exception of those designed to attack carbonaceous materials.
d. The processing techniques just described have resulted in the fabrication of several microscale structures that exploit the unique nanoscale properties of the VACNF ***(9,22–26)***. A process for passivating the body of the fibers with an insulating thin film while leaving the tips electrically and electrochemically active was developed for the fabrication of electrochemical probes with high spatial resolution (**Fig. 6**). This technology was then combined with a process for creating individually electrically addressable VACNFs on an insulating substrate to produce arrays of high-aspect-ratio electrochemical probes ***(9)***.

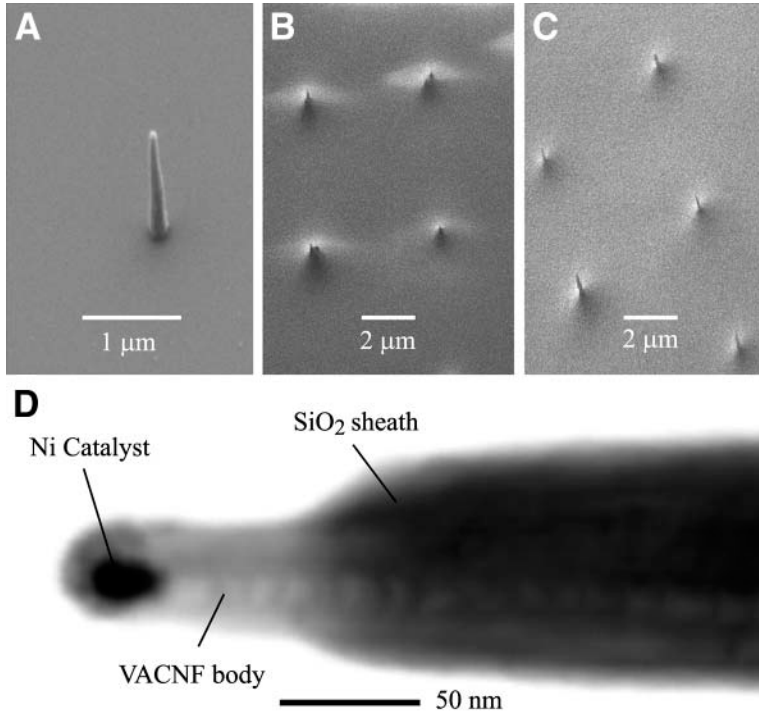

Fig. 6. **(A)** VACNF grown on Si passivated conformally with thin film of SiO_2; **(B)** array of passivated VACNF following spin coating of photoresist; **(C)** array after a brief RIE, liberating tip while leaving body passivated; **(D)** scanning transmission electron micrograph of passivated VACNF following tip release described in **(C)**. The VACNF tip extends beyond the oxide sheath, producing a nanometer-scale electrochemically active surface.

e. Chemical mechanical polishing is frequently used in microelectronic circuit manufacturing to planarize substrate morphology. This technique can also be applied to films deposited onto VACNF. Conformal layers of SiO_2 have been successfully planarized without damaging the VACNF. Continuation of this process has been shown to remove sections of the VACNF at the same rate as the SiO2, leaving the exposed fiber core coplanar with the surrounding oxide topography. This strategy has enabled the synthesis of coplanar electrode arrays for electrochemical applications in which many fibers perform transduction in a parallel fashion.
f. The VACNF can also be used as a sacrificial template for the creation of vertically oriented nanofluidic devices ***(24,26)***. In this process, arrays of VACNF are grown on either Si or Si_3N_4 membranes. The fibers are coated with a thin conformal film using PECVD or low pressure chemical vapor deposition

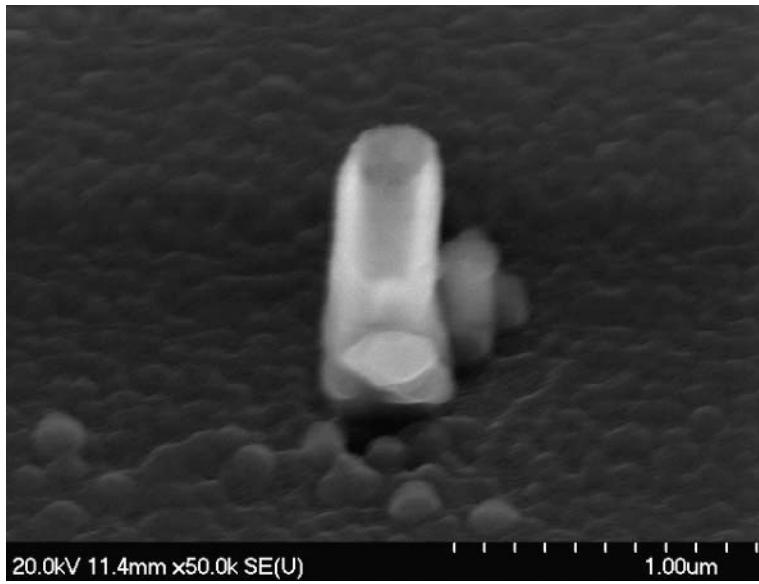

Fig. 7. SiO_2 nanopipe formed using VACNF as sacrificial template.

(LPCVD). The tips of the fibers are liberated using the process described in **Note 1.d.** for the individual electrochemical probes. Following removal of the remaining photoresist, the substrates are subjected to a brief etch in nitric acid to remove the Ni catalyst particle at the tip. An O_2 RIE is used to remove the body of the fiber from the thin film tube encasing it. Once the body of the fiber has been entirely removed, the bottom of the tube can be opened, creating a nanometer-sized pipe structure, or nanopipe (**Fig. 7**). These types of structures have been used in fluid transport experiments involving DNA and fluorescent intercalating dyes ***(26)***.

The combination of the growth, postgrowth processing, and interfacing techniques described in this chapter provides a set of unique tools for direct interaction with cellular processes at the molecular scale. There is a great deal of promise in the application of such high-volume, yet precisely engineered devices to problems of biological interest. Perhaps as these tools evolve further, they will eventually be as significant within biological fields as they have been for semiconductor electronics.

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