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Abstract
We developed integrated, carbon-based pipettes with nanoscale dimensions (CNP) that can probe cells with minimal intrusion, inject fluids into the cells, and concurrently carry out electrical measurements. Our manufacturing technique does not require cumbersome nanoassembly and is amenable to mass production. Using CNPs, we demonstrate the injection of reagents into cells with minimal intrusion and without inhibiting cell growth.

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Carbon Nanopipettes for Cell Probes and Intracellular Injection

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ABSTRACT

We developed integrated, carbon-based pipettes with nanoscale dimensions (CNP) that can probe cells with minimal intrusion, inject fluids into the cells, and concurrently carry out electrical measurements. Our manufacturing technique does not require cumbersome nanoassembly and is amenable to mass production. Using CNPs, we demonstrate the injection of reagents into cells with minimal intrusion and without inhibiting cell growth.

MANUSCRIPT TEXT

The most well-known and widely used tools for cell physiology and intracellular delivery are pulled glass micropipettes with tip diameters ranging from hundreds of nanometers to a few micrometers [1-5]. Although glass micropipettes are currently the

biologist's tool of choice, they suffer from a few drawbacks, such as fragility, a relatively large size compared to cells' dimensions, and inability to carry out electrical measurements concurrently with injection [1]. Often glass micropipettes cause irreparable damage to the cell membrane and cytoplasm [6, 7].

Probes made from carbon nanotubes (CNT) [8, 9] and nanoscale carbon pipes [10] offer an attractive alternative to glass micropipettes because of their small size, high mechanical strength, and high electrical conductivity. CNTs, decorated with macromolecules on their surface and attached to AFM tips, can deliver macromolecules into cells [8] without causing damage [9]. These probes, however, are difficult to assemble, require highly-specialized equipment to operate, and do not facilitate the transport of fluids.

We have developed a carbon nanopipette (CNP) to facilitate electrical measurements while delivering fluids into cells using standard cell physiology equipment. CNPs consist of a carbon film deposited inside quartz micropipettes to form a continuous hollow conductive carbon channel along its length. Figure 1a describes schematically the fabrication process. The inner bore of quartz capillaries (Q100-70-7.5, Sutter Co.) are filled with a catalyst solution (18mg Ferric Nitrate in 25ml IPA), allowed to air dry, and then pulled into fine-tipped micropipettes (P2000, Sutter Co.) (Figure 1a (i)). Carbon is selectively deposited on the catalyzed surface by chemical vapor deposition (CVD) [11-13] with Argon and Methane (300/200sccm, resp. at 900°C) (Figure 1a (ii)). The thickness of the carbon film is controlled by varying the CVD time (~30nm at 2hrs,

~80nm at 4hrs). The tip of the micropipette is then wet-etched (5:1 BHF at 20°C) to remove the quartz exterior and expose a short length of the interior carbon pipe (Figure 1a (iii)). The wet etching time and temperature dictate the length of the exposed carbon pipe. Further reduction in tip outer diameters can be achieved by etching the outer diameter of the carbon pipe by plasma oxidation, resulting in carbon pipes with outer diameters ranging from 10's to 100's of nanometers . The final product is a glass capillary lined with a continuous carbon film along its interior and a nanoscale carbon pipe extending from its end (Figure 1b). Hundreds of probes with consistent nanoscale dimensions are fabricated concurrently.

A unique property of CNPs is the existence of an electrically-conductive, hollow, interior carbon film running the entire length of the quartz pipette, lending itself capable of performing cell physiology measurements during cell injection. Making electrical connections to the tip and tail of CNPs having carbon wall thicknesses of ~80nm, we observe symmetric, ohmic resistance of ~15KΩ using linear scan voltammetry (± 1 V, HP 4145B parameter analyzer). In contrast, glass micropipettes with similar sized tips filled with electrolyte have resistances in the MΩs [14]. Taking into account the changing geometry of the CNP over its length, our estimated CNP resistivity (1070 μΩ-cm) falls between those of graphitic and amorphous carbon (800 - 4,000 μΩ-cm, reps.)[15], suggesting its varied carbon structure. Upon further inspection with transmission electron microscopy (TEM) (Figure 2) and selected-area electron diffraction (SAED), the existence of amorphous and graphitic structure was observed (Figure 2c (i) and (ii), resp.). Figure 2b demonstrates the electron-transparency of the carbon pipes

which facilitates measurement of their wall thickness as well as visualization of the tube's contents. In addition, CNPs are hydrophilic, as can be seen by observing a ~45° contact angle between water and the inner carbon wall after capillary filling. The structure of our carbon tips are similar to carbon pipes synthesized with alumina templates [16].

CNP tips are flexible and elastic, yet strong enough to readily spear cells (Figure 3). Unlike glass pipettes which would shatter, Figure 3a shows how CNPs buckle without breaking when pushed against a solid surface, then recover their original shape when the force is removed. Yet, Figure 3b shows the CNP is rigid enough to penetrate into smooth muscles cells (SMC, 15μm dia.) held in place at the tip of a microcapillary. CNPs have successfully penetrated various cells such as oral squamous carcinoma cells (OSCC) and neuron cells (not shown).

To show CNPs can be used as cell probes, we investigated the effect of CNP spearing on cells. First, we used the Trypan Blue dye (Cambrex) exclusion technique [17] to observe any effects on cell membrane integrity. OSCCs, maintained in a temperature-controlled culture dish (Bioptechs), were speared by micromanipulation and subsequently observed with an inverted optical microscope. The speared cells (as well as unspeared, control cells) excluded the membrane-diffusible dye, indicating that the pumping mechanism of the pierced cell membrane remained viable. Second, we monitored OSCC growth and proliferation subsequent to spearing. The experiment was performed by plating fewer than 5 OSCCs in a temperature-controlled culture dish. After spearing the

OSCCs with the CNP, we monitored their growth over two weeks (Figure 4). Figure 4a shows cell images immediately after spearing (day 0), after 8 days, and after 14 days. Figure 4b depicts the normalized number of cells as a function of time. The circles and squares correspond, respectively, to cells originating from speared and non-speared cells and show that cells continue to proliferate after spearing. Although the speared cells appear to proliferate at a higher rate than the normal cells, pending additional studies, we do not believe that the difference is statistically significant and conclude that CNP spearing does not hinder cell growth.

We demonstrated that CNPs can controllably inject single cells using standard microinjection equipment (Figure 5). CNPs were backloaded with fluorescent dye (Rhodamine 123, Sigma) and brought in contact with adherent OSCCs in a Petri dish. Figure 5a shows a CNP targeting the cytoplasm and fluorescent injected dye into the cells with pressure microinjection (Eppendorf Femtojet, 10-100 hPa, 2s duration). With the tip held in place, Figure 5b shows fluorescent dye being controllably injected several times, gradually increasing the fluorescent intensity inside the cell. Since we observed no noticeable cell expansion during injections, we estimate that the volume of dye injected was only a small fraction of the cell's volume. The cells were monitored for at least one hour subsequent to injection and appeared to maintain their shape and fluorescence. Furthermore, using our CNPs in other studies (not shown here), we have demonstrated that neuron cells injected with fluorescent dye continue to live at least one week afterwards. Hence, we conclude CNPs can be used to controllably inject cells without causing damage.

We described a fabrication process, amenable for mass production, to produce CNPs without any assembly. CNPs can penetrate through cell membranes and controllably inject minute quantities of material into the cells without hindering cell growth and without breaking. The hollow conductive carbon film allows one to concurrently use CNPs as injectors and electrodes. CNPs can be used as nanoscale biosensors by functionalizing their carbon surface with proteins and/or oligonucleotides. Since their carbon walls are transparent to electrons, we propose CNPs can be used as sample holders for electron microscopy. In future work, we envision measuring cell response such as changes in membrane potential during injection and developing massive CNP arrays that can concurrently interact with a large number of cells.

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FIGURE CAPTIONS

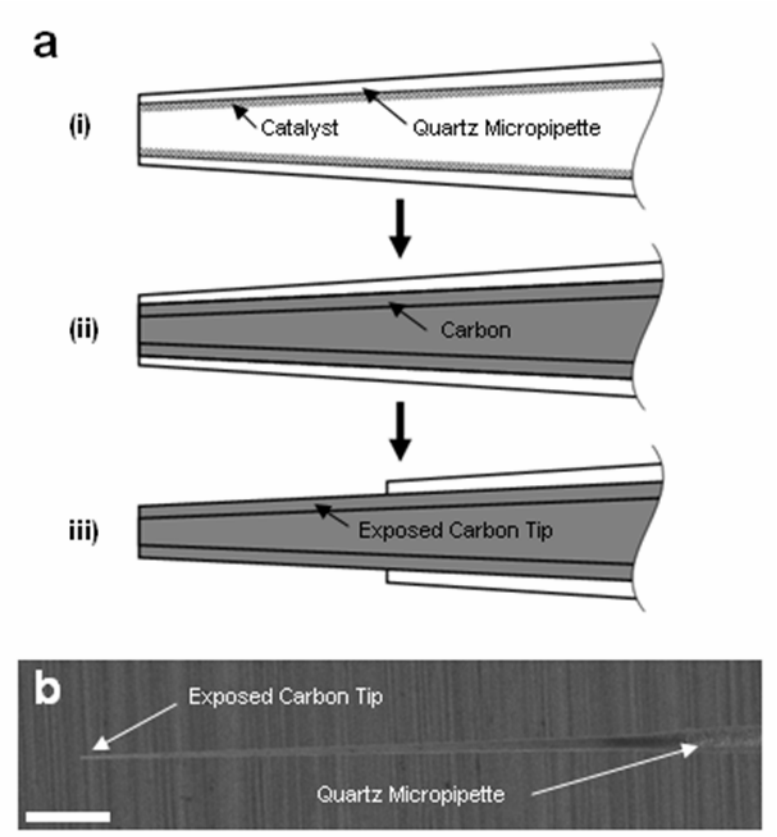

Figure 1. (a) CNP fabrication schematic showing (i) internal deposition of catalyst before micropipette pulling, (ii) deposition of carbon by CVD, and (iii) exposing carbon tip by wet-etching. (b) SEM micrograph of a carbon tip integrated into a quartz micropipette. Scale bar, 5 μm.

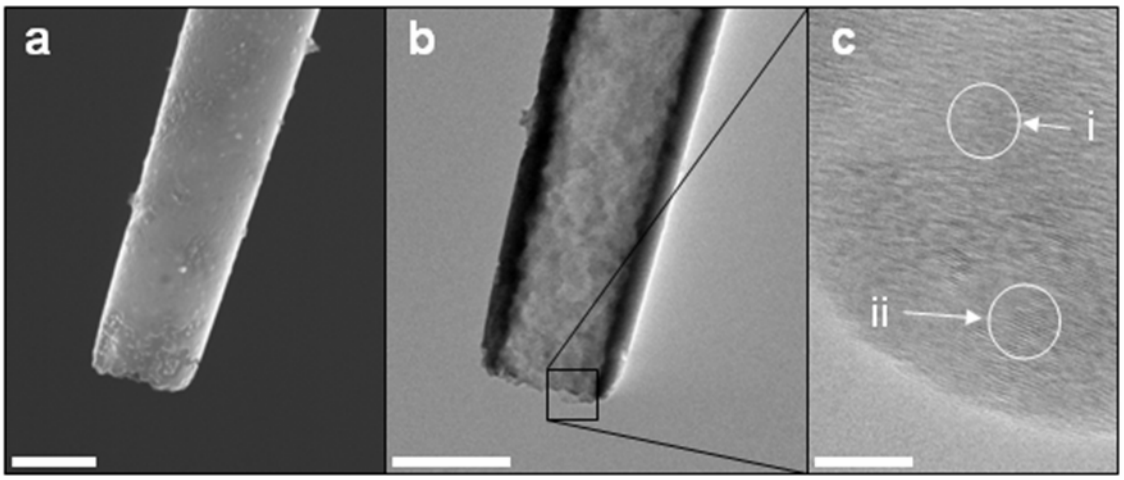

Figure 2. (a) SEM micrograph at 15 KV of a CNP tip. Scale bar, 500 nm. (b) TEM micrograph at 200 KV of the same CNP tip. Scale bar, 500 nm. (c) Amorphous (i) and graphitic (ii) carbon is observed with TEM. Scale bar, 10 nm.

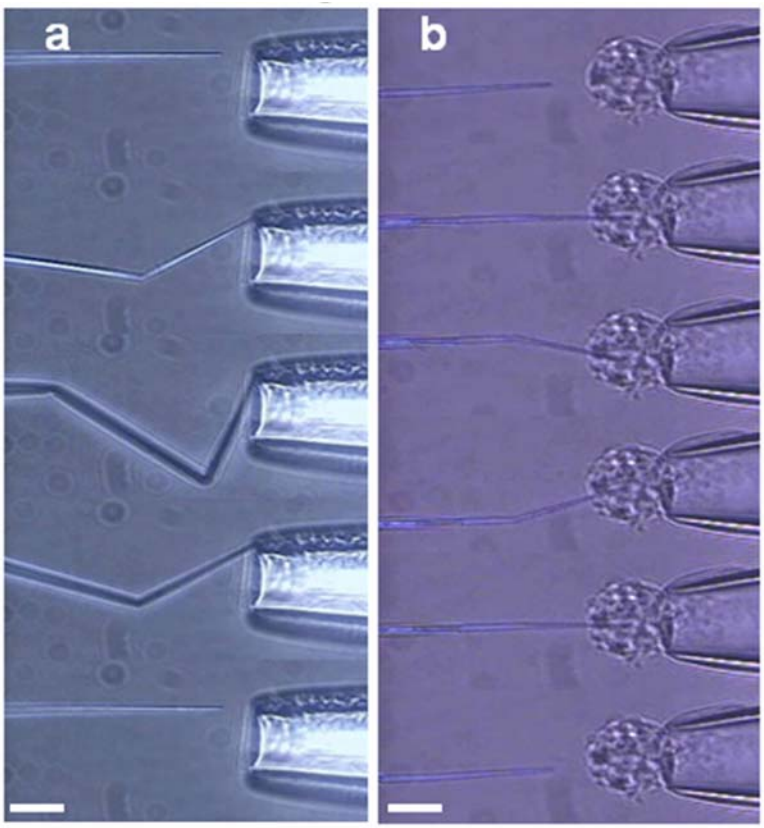

Figure 3. (a) From top to bottom: the carbon pipe tip of the CNP buckles when pushed against the wall of a glass pipette and recovers its initial shape once the force is removed. Scale bar, 15 μm. (b) From top to bottom: a CNP penetrates through the membrane of an SMC. The cell is held in place by glass micropipette aspiration. Scale bar, 15 μm.

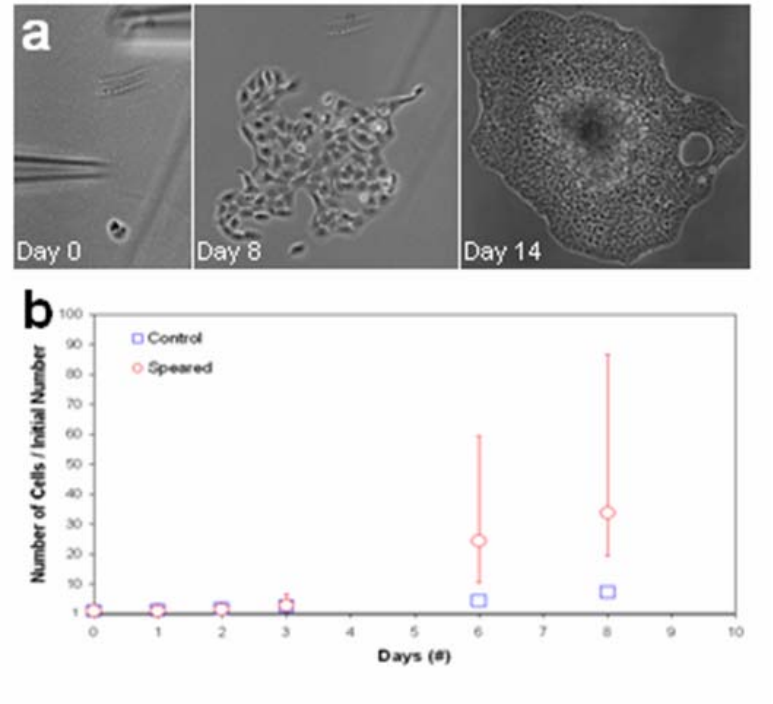

Figure 4. (a) Left to right: proliferation of speared OSCC observed over two weeks. (b) Average, normalized number of speared (circle) and un-speared (square) OSCC as a function of time.

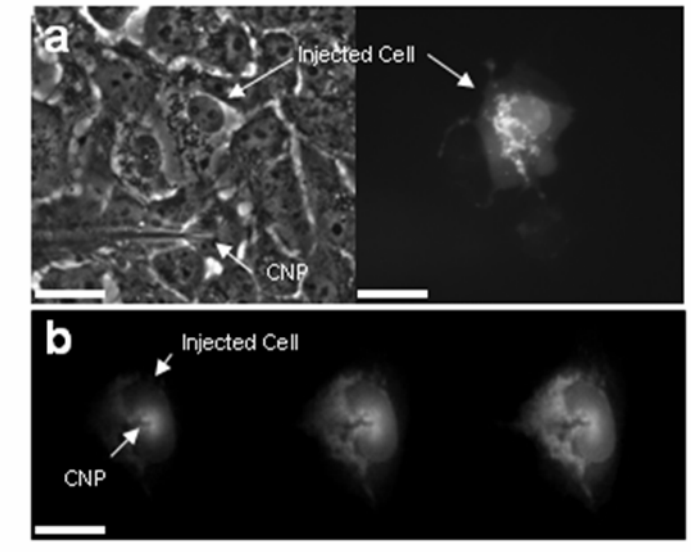

Figure 5. (a) Left to right: OSCC before and after fluorescent dye injection with a CNP. Scale bar, 10 μm. (b) Left to right: Fluorescent images show an OSCC being injected three times with a CNP. Scale bar, 10 μm.