



Short communication

Label-free detection of ATP release from living astrocytes with high temporal resolution using carbon nanotube network

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ABSTRACT

Owing to its unique combination of electrical, physiochemical, and one-dimension structural properties, single-walled carbon nanotube (SWNT) has recently emerged as a novel nanoelectronic biosensor for biomolecular detection with extraordinary sensitivity and simple detection scheme. All the realizations so far, however, are limited to static in vitro measurement. Dynamic detection of biomolecule release from living cells which may occur in millisecond timescale has yet to be demonstrated. In the present work, SWNT network was utilized to directly interface with living neuroglial astrocytes and label-freely detect the triggered release of adenosine triphosphate (ATP) from these cells with high temporal resolution. The secreted ATP molecules diffuse into the narrow interface gap between the SWNT-net and the astrocyte, and interact with the nanotubes. Highly charged ATP molecules electrostatically modulate the SWNT conductance leading to measurable current response. This technique provides a novel platform to study ATP release and signaling which play important roles in astrocyte–neuron crosstalk and other essential cellular functions.

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1. Introduction

Owing to its unique electrical, physiochemical, and one-dimension structural properties, single-walled carbon nanotube (SWNT), which is rolled-up carbon sheet of one-atom-thick, has recently emerged as a novel nanoelectronic biosensor with extraordinary sensitivity and simple detection scheme. As all the current carriers flow at the surface, the conductance of the nanotube is highly sensitive to the electrochemical perturbations at the nanotube surface or in its immediate vicinity. Taking advantage of this, SWNT devices have been successfully employed to detect various biomolecules, including DNAs (Dong et al., 2008; Star et al., 2006), proteins (Maehashi et al., 2007; Star et al., 2003) and glucose (Besteman et al., 2003). The success of these applications is attributable to the fact that SWNT with a diameter (~1 nm) comparable to the size of single biomolecule can interact intimately with the target analytes; in turn, these biomolecules modulate the nanotube conductance via electrostatic gating (Heller et al., 2008), doping effect (Gruner, 2006), reducing carrier mobility (Maroto et al., 2007), oxidation-induced electron transfer (Besteman et al., 2003), or Schottky barrier effect (Dong et al., 2008; Gruner, 2006).

All the realizations so far, however, are limited to static in vitro measurement. Dynamic detection of biomolecule release from living cells which may occur in millisecond timescale has yet to be demonstrated.

ATP is not only a universal energy currency, but also an important signaling molecule regulating many biological functions in various cells (Fields and Stevens, 2000). In particular, it serves as a neurotransmitter or neuromodulator in both central and peripheral nervous systems (Gourine et al., 2005). It has been recently recognized that ATP release from astrocytes plays key roles in astrocyte–neuron communication and consequently shaping synaptic neurotransmission (Haydon, 2001), adding a new evidence that glial cells, which were merely regarded as neuronal supporting cells, actually take active roles in the brain (Newman, 2003). In one scenario of the bidirectional astrocyte–neuron crosstalk, neurotransmitter glutamates secreted by neurons activate glutamate receptors on the membranes of the nearby astrocytes whereby Ca^{2+} efflux from endoplasmic reticulum (ER) is resulted and subsequently elevated cytosolic Ca^{2+} triggers ATP release from secretory vesicles (Montana et al., 2006). The released ATPs, in turn, after binding with ATP receptors on the adjacent astrocytes and neurons, lead to propagation of Ca^{2+} wave throughout the astrocyte–neuron network in the region (Guthrie et al., 1999). Such excitation potentiates further neurotransmitter release from neurons, acting like a positive feedback as a form of activity-dependent plasticity of neural network (Coco et al., 2003).

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But the dynamics and mechanisms of ATP release from astrocytes are not yet fully understood, due in part to the lack of methods to monitor this dynamic process in real-time. Given the important roles of ATP in cell physiology, development of methods to detect ATP signaling is of obvious significance. Currently, ATP detection mostly relies on bioluminescence assay (Allen et al., 2002), represented by a recent and widely adopted technique called firefly luciferase assay (Cruz-Aguado et al., 2004). Although these methods are sensitive, they can only report the amount of diffused ATP in the bulk extracellular medium resulting from the net activity of a cell population. Such measurements do not reflect the true local concentration of released ATP at the cell surface because of medium dilution and high ecto-ATPase activities. In addition, they cannot distinguish whether the extracellular ATP is resulted from nonspecific cytolysis or a particular release mechanism. Furthermore, ATP release is expected to be rapid and highly regulated. Apparently, current off-time measurements are not able to report the dynamic ATP flux at the cell membrane. In an effort to tackle this problem, firefly luciferase is attached to the cell surface to detect local ATP concentration (Nakamura et al., 2006). But this method requires sophisticated molecular engineering and is not real-time measurement. Alternatively, electrochemical approaches have been developed. Those techniques, however, relies on mediation by enzymes (Buckling et al., 2005) or ATP binding molecules (Zuo et al., 2007). In a so-called sniffer cell approach, a cell transfected with ATP receptors is used as the ATP sensor. ATP release from the target cell is relayed to the responding transmembrane current in the sensor cell which is continuously recorded by patch-clamp technique (Pangrsic et al., 2007). Such indirect detection involves tedious and complicated techniques and is poorly reproducible. In our approach, we use SWNT network to achieve localized real-time detection of ATP release from living astrocytes directly cultured on the SWNTs.

2. Materials and methods

2.1. Preparation of SWNT-net device

Carboxylated-SWNTs (Carbon Solution) were sonicated and dispersed in ddH₂O. Subsequently, pentane was added to form an organic layer above the SWNT suspension. Following injection of ethanol, SWNTs precipitated out and formed a visible thin layer sandwiched between the organic and the aqueous phases. And evaporation of pentane left the SWNT film floating on the top of the water. The SWNT thin film ($\sim 8 \text{ nm}^2$) was then transfer-printed onto a glass coverslip, followed by ddH₂O rinsing and blow-drying with nitrogen. Source and drain electrodes were then prepared across the SWNT-net using conductive silver paint. Finally, silicon rubber (Dow-corning) was used to insulate the electrodes and define the chamber ($2\text{--}3 \text{ cm}^2$) for cell culturing and recording.

2.2. Electrical measurements

The measurements were conducted using a semiconductor device analyzer (Agilent, B1500A). All measurements were carried out while the SWNT-net devices were biased at 400 mV and bathed in the physiological bath solution containing (pH 7.2, in mM): 10 HEPES, 140 NaCl, 1 MgCl₂, 5.5 KCl, and 2 CaCl₂. The gating voltage was applied via an Ag/AgCl electrode in the bath solution.

3. Results and discussion

In comparison with single SWNT devices usually used in biomolecular sensing, SWNT network devices, with compromise in sensitivity, have several advantages including larger detection area,

lower $1/f$ noise, relative simplicity in fabrication and high reproducibility. A schematic of SWNT-net preparation on glass coverslip is illustrated in Fig. 1a. As revealed by atomic force microscopy (AFM), an ultra-thin network comprising of small SWNT bundles (10–20 nm) was created on the coverslip (Fig. 1b).

The electrical properties of a SWNT-net device are demonstrated in Fig. 1c. The source–drain current biased at 400 mV was measured while varying the solution gate voltage. It is noted that SWNT-net manifests p-type field-effect characteristics, and its conductance is sensitive to voltage gating therefore desirable for electrical biosensing.

A bright-field image of astrocytes cultured on a SWNT-net is presented in Fig. 2a (supporting information). The cell type was confirmed by immunostaining using a specific astrocyte marker (supporting information) and subsequent confocal imaging (Fig. 2b). Cells grown on SWNT-net substrates showed no obvious differences in morphology and growth compared to those on glass coverslips.

An illustration of how ATP release from astrocyte is triggered and detected by a SWNT-net device is shown in Fig. 2c. Glutamate, one of the most common neurotransmitters in the brain, was used as the secretagogue to stimulate secretion of ATP from the astrocytes (Pangrsic et al., 2007), through a cascade of molecular events. The binding of glutamate to the metabotropic glutamate receptor first activates phospholipase C (PLC) which, in turn, hydrolyzes membrane lipid phosphatidylinositol bisphosphate (PIP₂) to inositol triphosphate (IP₃). IP₃ then opens IP₃ sensitivity Ca²⁺ channels on the internal Ca²⁺ stores (most notably, ER), leading to Ca²⁺ efflux into the cytosol and increase in cytosolic Ca²⁺ concentration. Finally, Ca²⁺ ions trigger actions of a set of secretory proteins, and consequently induce fusion of the secretory vesicles with the plasma membrane to release vesicular ATP molecules to the extracellular space. The discharged ATP molecules diffuse into the interface gap between the SWNT-net and the astrocyte, and interact with the nanotubes. Nucleobases of DNA are shown to bind nanotube sidewall via pi–pi stacking interaction (Johnson et al., 2008). Presumably, ATP which contains adenine nucleobase can similarly interact with nanotube non-covalently. The ATP molecules brought in close contact by such interaction exert strong influences on SWNT conductance and thus render measurable current response.

While biasing the source and drain at 400 mV, the current (I_{ds}) passing through the SWNT-net was continuously monitored. A typical experiment is presented in Fig. 3a. As shown, many upward current spikes were detected with about 100-s delay after introduction of 300 μM glutamate into the recording chamber where the culture medium was replaced by the bath solution to incubate living astrocytes. Single events of the transient current signals are depicted in an expanded timescale. A spike exhibits two distinct kinetic phases, i.e., a initial rapid rising phase in a timescale of a few milliseconds followed by a long-lasting decaying phase in a timescale of a few hundred milliseconds to a few seconds. Each spike likely corresponds to burst release of ATP molecules from a single astrocyte for several reasons. First of all, biphasic response with similar timescales from single astrocyte was also observed in a study using sniffer cell approach (Pangrsic et al., 2007). Secondly, it is unlikely caused by single vesicle release which only lasts for a few milliseconds. Finally, it is also unlikely that individually dispersed cells react in groups with millisecond synchronization to produce spikes with rapid rising phase.

The delay following the stimulation is rather long comparing to Ca²⁺ dependent secretion of neurotransmitters from neurons or hormones from neuroendocrine cells which usually takes off within seconds after triggering. The complicated molecular reactions involved in this secretion process partly account for such long delay (Fig. 2c). It is also conceivable that, unlike neurons and neuroendocrine cells which have many readily releasable vesicles

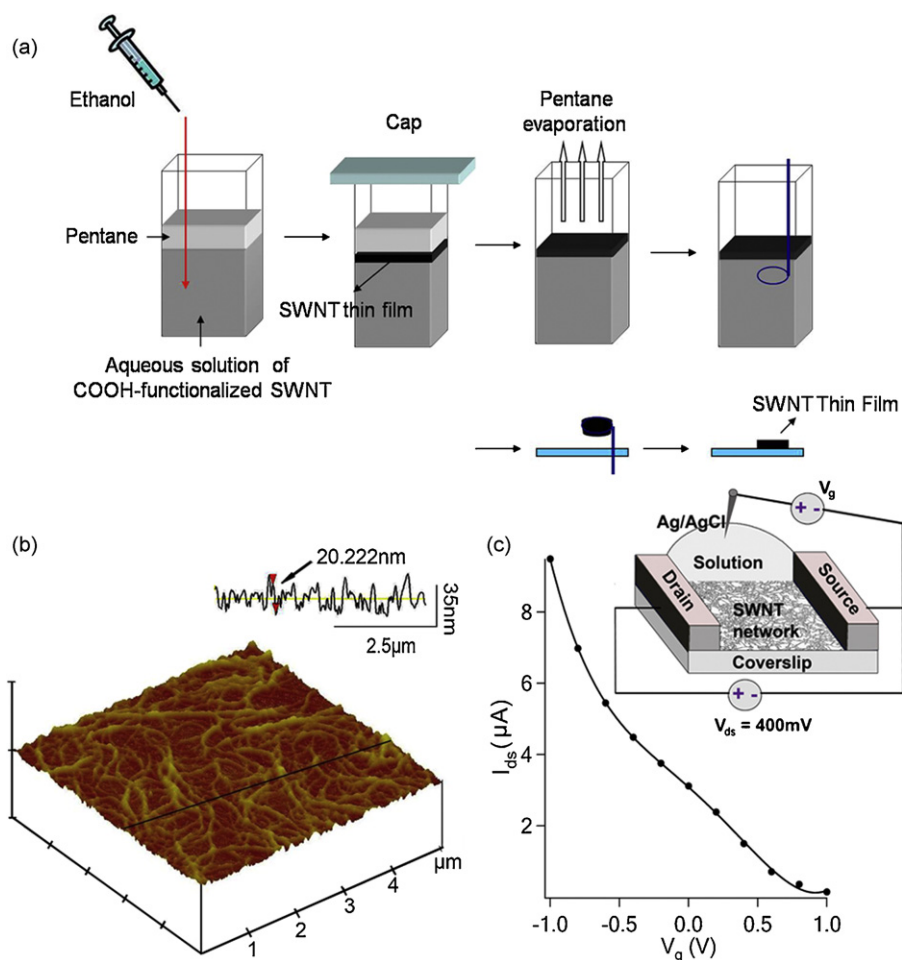


Fig. 1. (a) Schematic of SWNT-net preparation. (b) AFM image of a SWNT-net with a cross-section profile. (c) Conductance of SWNT-net is sensitive to the solution gate voltage (V_g).

waiting for final Ca^{2+} triggering, secretory vesicles in astrocytes may need to go through a sequence of steps to be readily releasable including, transport to the plasma membrane, docking, and priming. Despite of the long delay, the release of ATP molecules from a cell seems to be highly synchronized and very fast, which reaches the peak in a few milliseconds. Such burst release may be necessary for synergetic and timely reactions of the downstream molecular events. The decay in the current signal presumably is due to diffuse-away of ATP molecules and ATP break-down by ecto-ATPases which are believed to be co-localized with the vesicle fusion sites (Joseph et al., 2003).

A voltage pulse (400–800 mV in amplitude on top of the biasing voltage, 5–100 ms in duration) to the SWNT-net, which is expected to depolarize the cell membrane and open the voltage-dependent Ca^{2+} channels (Gheith et al., 2006), failed to elicit any responses. It implies that the triggered ATP release is not derived from the Ca^{2+} influx through these Ca^{2+} channels. On the other hand, the application of calcimycin, a calcium ionophore that inserts into cell membrane and selectively transports Ca^{2+} ions from the extracellular to the intracellular space, produced similar responses as glutamate, suggesting that the stimulated ATP release from astrocytes is indeed Ca^{2+} -dependent.

After recordings, the same SWNT-net device used in Fig. 3a was treated with 0.125% trypsin to completely remove all the cells, and thoroughly rinsed with the bath solution. Then, a quartz nanopipette with a diameter of ~ 200 nm positioned ~ 1 μm above SWNT-net was used to locally puff in pulses of tiny amount of 1 mM

ATP containing bath solution onto the SWNT-net sensor. Each puff gave rise to a transient current signal with amplitude comparable to the recordings from the astrocytes (Fig. 3b). This experiment highlights that small ATP flux locally discharged onto SWNT-net can give similar signals as from astrocytes. But would the acidic vesicular fluids ($\sim \text{pH}$ 5.0) or glutamate that arguably may also be contained in the vesicles be responsible for the recorded responses? These possibilities are ruled out by the observations that nanopipette administration of bath solution of pH 5.0 or with 1 mM glutamate did not elicit any appreciable responses from the SWNTs. In another experiment using a different device (Fig. 3c), SWNT-net was pre-incubated with the culture medium for 2 days and immersed in the bath solution prior to the testing. Once again, this cell-free device was responsive to the bulk bath solution containing ATP (0.5–10 mM), but silent to acidic (pH 5.0) or glutamate containing (1 mM) bath solution introduced into the recording chamber. These control experiments and the fact that SWNT-net responds with characteristic kinetics anticipated from single cell release of ATP only after application of the ATP secretagogue (glutamate) establish that our observations are resulted from triggered ATP release. Moreover, the spiky responses were never observed when SWNT-net was immersed in the bath solution or the culture medium without cells. The ions, proteins or other molecules in the solution may only affect the base-line current of SWNT-net. In fact, not all biomolecules are able to directly interact with nanotubes and affect their conductance. Lastly, the observed transient spikes are not originated from constitutively secreted molecules (e.g., metabolites) from the

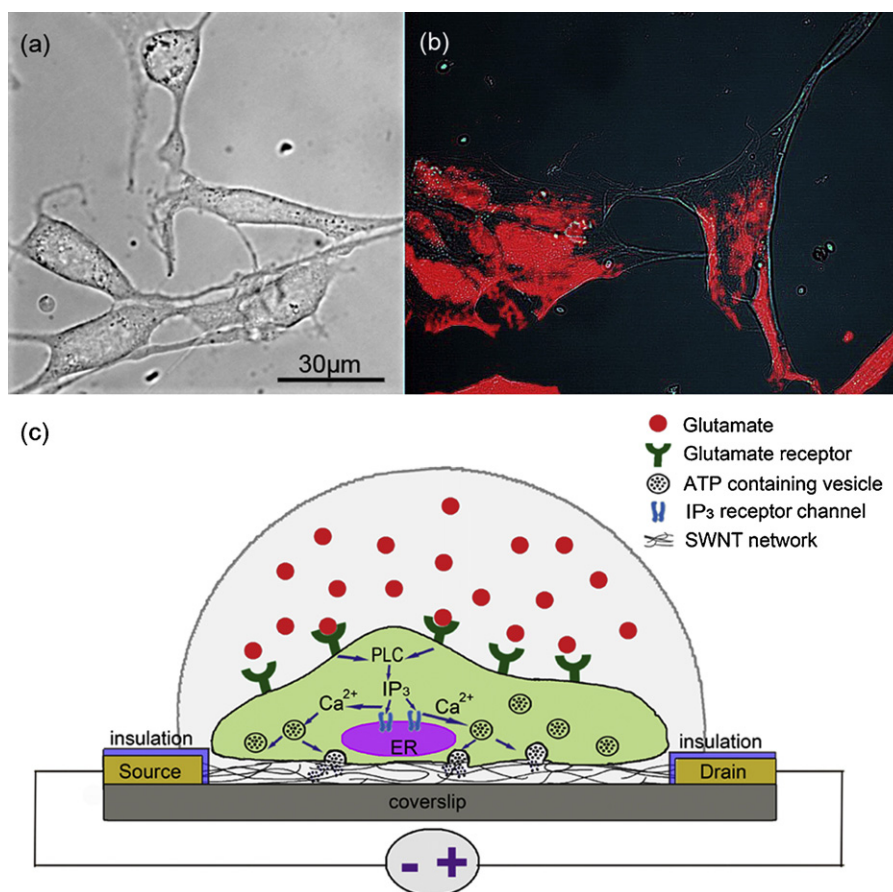


Fig. 2. (a) A bright-field image of astrocytes cultured on SWNT-net. (b) Confocal fluorescent image of immunostained astrocytes on SWNT-net. (c) Illustration of triggered ATP released and detection.

cells because, in contrast to triggered secretion, the constitutive secretion is a slow and constant process.

The increase in nanotube conductance induced by ATP can be explained by decrease of Schottky energy barrier at nanotube–

electrode contacts or doping of holes into nanotube or electrostatic gating. The first two mechanisms, nevertheless, can be excluded because the nanotube–electrode contacts are insulated and aromatic adenine base is unlikely to be hole-dopant. Therefore, it is plausible that highly negatively charged ATP molecules increase the hole concentration in the nanotube thus its conductance.

4. Conclusions

In summary, nanotechnology is bringing tremendous new opportunities to biology (Chen and Li, 2007; Kim et al., 2007). As another example, herein, we show that SWNT-net can be used as growth substrate for astrocytes and label-freely detects local ATP release from these cells. This technique, with high time resolution and simplicity, provides a novel platform to study ATP release and signaling, and, for the first time, demonstrates the possibility of using SWNT-based nanoelectronic devices to study dynamic biological processes in living cells.

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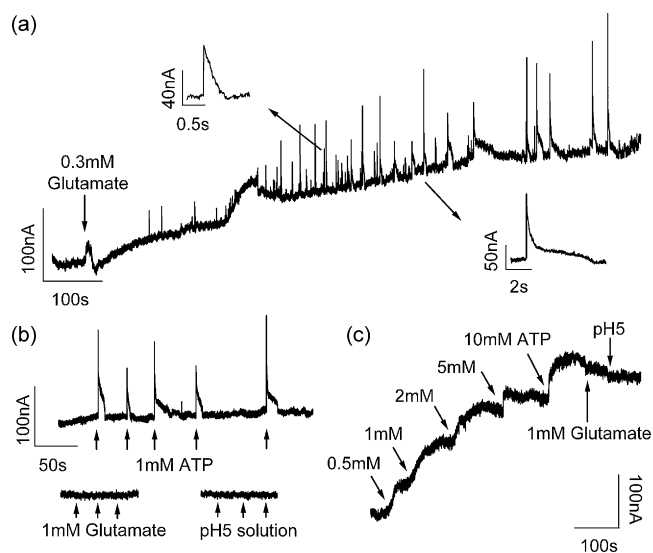


Fig. 3. (a) Real-time response of SWNT-net while astrocytes were stimulated by glutamate. (b) Responses of cell-free SWNT-net to locally delivered bath solution containing ATP or glutamate, or of low pH using a puffing quartz nanopipette. (c) SWNT-net current recorded in the bulk bath solution containing ATP or glutamate or of low pH. V_{ds} is 400 mV in (a), (b), and (c).

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