ATP Detection

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A Luciferase/Single-Walled Carbon Nanotube Conjugate for Near-Infrared Fluorescent Detection of Cellular ATP**

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All micro-organisms use adenosine 5′-triphosphate (ATP) as a universal energy storage molecule, and thus knowledge of its concentration is central to the detection of bacterial contamination[1] and the study of energetic processes in cell physiology from ion-channel regulation[2] to intercellular signaling cascades.[3] Additionally, ATP depletion is related to pathogenesis such as ischemia, Parkinson's disease, and hypoglycemia.[4–6] There remains a persistent need for more sensitive, higher-resolution, and more robust detection of ATP for, among other goals, the understanding of its spatial compartmentalization within living cells.[7–10] For this purpose, the conventional method of ATP assay within living cells is luciferase(Luc)-mediated bioluminescence,[11] whereby ATP reacts at the enzyme in the presence of D-luciferin (Lrin) and Mg^{2+} to produce oxyluciferin (oxyLrin) and a fluorescent emission.[9,12,13] However, this approach, which involves synthesis of Luc vectors and cell transfection is tedious, time-consuming, and has a low signal-to-noise ratio. The extension of this method to the modulation of quantum confined nanorods or nanotube fluorophores, such as single-walled carbon nanotubes (SWNT), has not been addressed to date, despite obvious benefits in sensitivity[14] and photobleaching resistance.[15]

Herein, we report a SWNT/Luc enzyme conjugate ($SWNT^{Luc}$) in which the bioluminescent reaction selectively recognizes ATP at luciferase. The SWNT near-infrared (NIR) fluorescence is ultimately quenched by a two-step reaction that involves detection of a target and generation of a redox quenching intermediate. This $SWNT^{Luc}$ sensor is very selective to ATP, but not to adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), cytidine 5′-triphosphate (CTP), and guanosine 5′-triphosphate (GTP), and is also able to detect ATP temporally and spatially in living HeLa cells. The approach, whereby an enzyme–nanotube complex creates a redox quenching intermediate from the target analyte, can be extended to a wide range of biologically important analytes.

We first constructed the Luc-conjugated SWNTs as shown in Figure 1 (see the Supporting Information). After immobilization of Luc on SWNTs functionalized with phospholipids

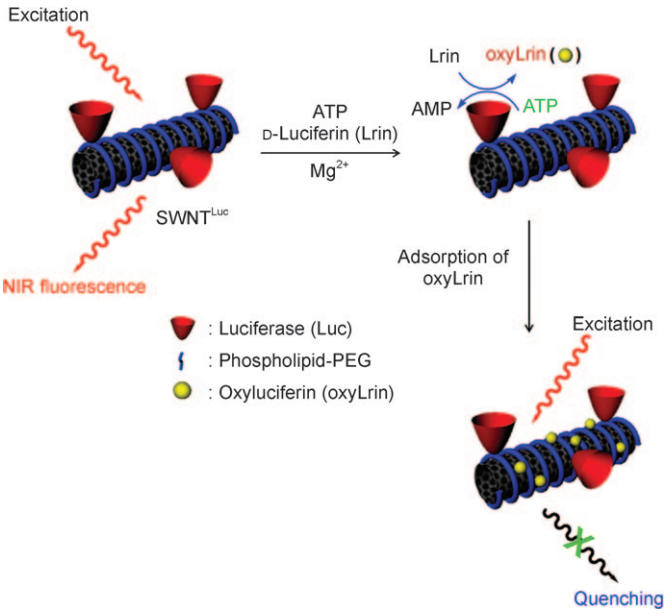

Figure 1. Illustration of the $SWNT^{Luc}$ sensor for ATP detection.

that bear carboxylated poly(ethylene glycol),[16] its colloidal stability in aqueous solution appears to remain constant, as shown in the image of $SWNT^{Luc}$ suspension (Figure S1a in the Supporting Information). In addition, Luc conjugation on SWNTs was confirmed by analysis of SDS-PAGE and atomic force microscopy (Figure S1b,c in the Supporting Information). $SWNT^{Luc}$ shows discrete NIR fluorescence and distinct absorption features without any spectral shift and diminution compared to those of SWNT before conjugation of Luc, as shown in the excitation/emission profile (Figure 2a) and fluorescence/absorption spectra (Figure S2 in the Supporting Information).

Next, we investigated the fluorescence response of the $SWNT^{Luc}$ sensor to ATP. After addition of ATP and Lrin (240 μM) to the $SWNT^{Luc}$ solution (50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 10 mM $MgCl_2$), NIR fluorescence spectra of $SWNT^{Luc}$ were measured in real

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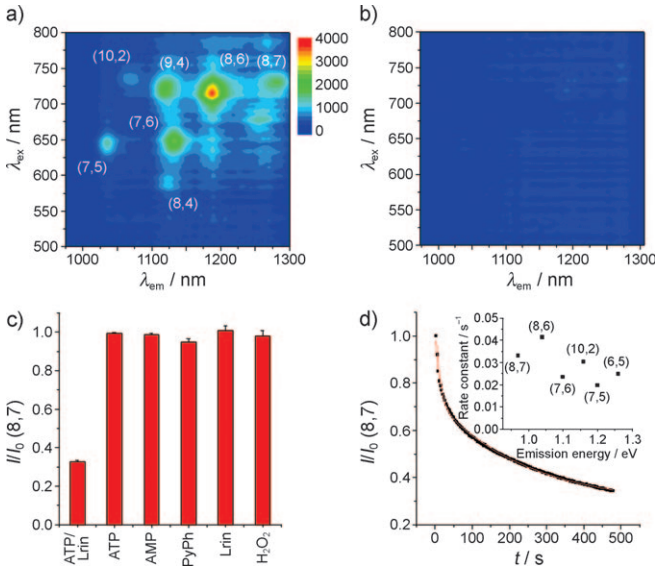

Figure 2. NIR fluorescence response of SWNT^{Luc} sensor to ATP. a) Excitation and emission profile of SWNT^{Luc} sensor showing distinct NIR fluorescence. b) Fluorescence quenching of SWNT^{Luc} sensor measured 10 min after addition of ATP and Lrin (240 μM). The color scale is the same as in (a). c) Fluorescence intensity changes (I/I_0, current intensity/initial intensity based on (8,7) SWNT) measured in real time for 10 min after addition of each analyte (60 μM). d) Fluorescence quenching rates as a function of emission energy of SWNT^{Luc} sensor during ATP detection. NIR fluorescence spectra were acquired for 1 s using 785 nm excitation (85 mW).

time for 10 min. The bioluminescence generated from the enzymatic oxidation of Lrin in the presence of ATP by SWNT^{Luc} was very intense. Simultaneously, the NIR fluorescence of SWNT^{Luc} was almost completely quenched (Figure 2b) during the Luc-mediated bioluminescent reaction that involves selective ATP consumption. In order to investigate the cause of the fluorescence quenching of SWNT^{Luc}, all substrates and the well-known by-products of the Luc-mediated reaction were evaluated. When only ATP or Lrin (60 μM) was added to the solution of the SWNT^{Luc} sensor, no fluorescence quenching was observed (Figure 2c). In addition, each byproduct such as AMP, pyrophosphate (PyPh), and H_2O_2 (60 μM) had no influence on the NIR fluorescence of SWNT^{Luc}. We found that the fluorescence quenching of SWNT^{Luc} is observed only as Luc-mediated bioluminescence occurs after addition of both ATP and Lrin, thus suggesting that the light-emitting luminescent product oxyLrin can quench the fluorescence of SWNTs. We also measured the absorption of the quenched SWNT^{Luc} sensor 10 min after addition of ATP and Lrin (60 μM). The visible and NIR absorption features of SWNT^{Luc} remain similar in intensity, although the NIR fluorescence is significantly quenched during the Luc-mediated bioluminescent reaction with ATP, Lrin as substrates, and Mg^{2+} ions as a cofactor (see Figure S3a in the Supporting Information). This result is consistent with a previously suggested mechanism of photoinduced excited-state electron transfer from the nanotube conduction band to the lowest unoccupied molecular orbital (LUMO) of an adsorbing molecule.[17,18] In addition, the stated quenching mechanism for electron transfer from SWNT^{Luc} to oxyLrin is thermodynamically favorable, since the reduction potential of oxyLrin is +0.24 V.[19] In addition to a redox discrimination between analytes, SWNT^{Luc} can also recognize specific molecules by the particular configuration of the adsorbed surfactant or polymer phase, as we have recently shown in the case of nitric oxide.[17] Moreover, the red shift (8 nm, 9 meV) is clearly observed in the absorption spectra (Figure S3a in the Supporting Information) after fluorescence quenching of SWNT^{Luc}, thus indicating a change of the local dielectric around SWNT^{Luc}. This red shift is not observed when only ATP or Lrin was added to the SWNT^{Luc} solution. The results clearly suggest that the product (oxyLrin) of the Luc-mediated bioluminescent reaction is responsible for the apparent quenching of NIR fluorescence of SWNT^{Luc} during ATP detection. We further investigated the fluorescence attenuation rate of SWNT^{Luc} as a function of emission energies for ATP detection. As shown in Figure 2d, the fluorescence of small-bandgap SWNT decays faster than that of the large-bandgap species, as reported previously for analogous systems.[17,20] The NIR photoluminescence from semiconducting SWNTs[21–23] has been effectively used to detect biologically important molecules.[24–29] However, the mechanism developed in this work is unique in its use of a conjugated enzyme to produce a quenching intermediate during recognition of the target molecule directly on the SWNTs.

We then evaluated the reversibility of fluorescence quenching of SWNT^{Luc} for ATP detection. After the NIR fluorescence of SWNT^{Luc} was quenched during ATP detection, the SWNT solution was dialyzed against Tris-HCl buffer for 24 h at 25 °C in order to remove oxyLrin from the SWNT^{Luc} solution, and then the NIR fluorescence was measured. The quenched fluorescence of SWNT^{Luc} was not restored after dialysis for 24 h (see Figure S4 in the Supporting Information) although SWNT^{Luc} is evenly suspended in the solution. After addition of β-nicotinamide adenine dinucleotide (NADH, reduced) to the quenched solution of SWNT^{Luc}, the restoration of the quenched fluorescence is not observed (Figure S4 in the Supporting Information). According to previous reports on Luc-mediated bioluminescence, oxyLrin is a competitive inhibitor of Luc, and blocks the active site of the enzyme with an affinity constant of $K_i = (0.5 \pm 0.03)$ μM.[30] This inhibition by oxyLrin of Luc on SWNT^{Luc} predicts a small desorption rate constant from the enzyme, and results in a practically irreversible response.

The selectivity and sensitivity of the SWNT^{Luc} sensor for ATP detection were investigated. Each potential interfering molecule (AMP, ADP, CTP, and GTP) was added with Lrin (240 μM) to the solution of SWNT^{Luc} in Tris-HCl buffer, and then the NIR fluorescence response was monitored in real time for 10 min at 25 °C. As shown in Figure 3a, the NIR fluorescence of SWNT^{Luc} is significantly attenuated only in the presence of ATP, thus allowing emission of Luc-mediated bioluminescence, but not for AMP, ADP, CTP, and GTP. This observation indicates that the SWNT^{Luc} sensor is able to selectively recognize ATP and is thus useful for cellular ATP detection. These selectivity results also suggest that oxyLrin, the Luc-mediated bioluminescent product, is responsible for

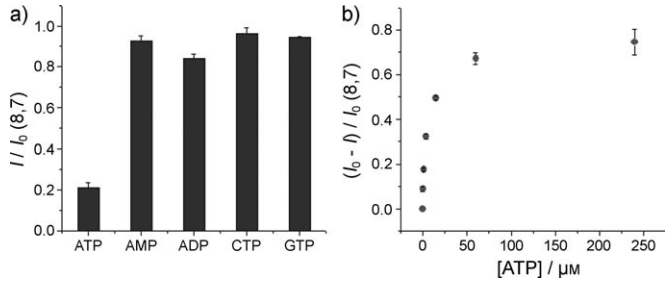

Figure 3. Selectivity and sensitivity of SWNT^{Luc} sensor for ATP. a) Selectivity of SWNT^{Luc} sensor based on fluorescence intensity changes (I/I_0 based on (8,7) SWNT). For investigation of selectivity, each analyte (240 μM) was added to SWNT^{Luc} sensor with Lrin (240 μM), and then the NIR fluorescence response was monitored in real time for 10 min. b) Sensitivity of SWNT^{Luc} sensor for ATP detection based on the fluorescence quenching of (8,7) SWNT measured in real time after addition of each ATP solution. NIR fluorescence spectra were acquired for 1 s using 785 nm excitation (85 mW).

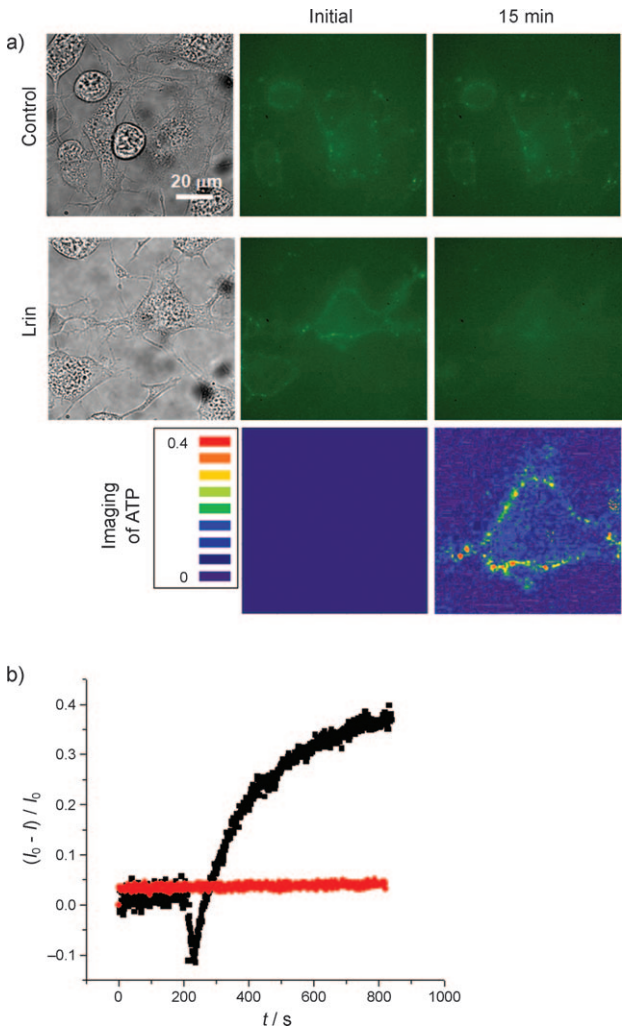

Figure 4. Cellular ATP detection with SWNT^{Luc} sensor. a) NIR fluorescence images of HeLa cells containing SWNT^{Luc} sensor (2 μg mL^{-1}) with and without addition of Lrin, showing that NIR fluorescence in HeLa cells is quenched after addition of Lrin (240 μM). In addition, the spatial imaging for ATP detection is generated by normalizing each pixel by its corresponding intensity at the start of the experiment. Fluorescence images were obtained in real time for 15 min. b) Real-time and quantitative tracking of NIR fluorescence response of SWNT^{Luc} sensor to ATP in HeLa cells after addition of Lrin (240 μM; black trace), showing quenching of NIR fluorescence for ATP detection. Without addition of Lrin (control; red trace), the NIR fluorescence intensity remains constant. Lrin was added around 200 s. All fluorescence images were obtained with 1 s acquisition using 658 nm excitation (35 mW).

the NIR fluorescence quenching of the SWNT^{Luc} sensor during ATP detection. In order to determine the sensitivity of the SWNT^{Luc} sensor for ATP, it was treated with various concentrations of ATP. As shown in Figure 3b, SWNT^{Luc} is able to detect ATP at a concentration of 240 nM. However, further optimization of the sensor, including an increase in the available area for oxyLrin adsorption and the use of optimal (n,m) SWNTs in place of a mixture, could potentially lower the detection limit. We also note that our recent efforts to extend sensitivities of related systems to the single-molecule region,[14] inspired by the recent success of Cognet et al.[31] may result in single-molecule detection of ATP, even in live cells and bacterial culture.

Finally, we evaluated the capability of the SWNT^{Luc} sensor to spatially and temporally detect ATP in living cells. After HeLa cells were incubated with SWNT^{Luc} (2 μg mL^{-1}) for 2 h at 37 °C, the cells were washed several times with phosphate-buffered saline (PBS) and a growth medium. Then, the NIR fluorescence response of SWNT^{Luc} in HeLa cells was monitored in real time for 15 min using a NIR fluorescence microscope. As shown in Figure 4, the NIR fluorescence of SWNT^{Luc} in the cells is very photostable and consistent without addition of Lrin during whole measurement (Figure 4a, top). However, after addition of Lrin (240 μM) to the medium, the fluorescence of SWNT^{Luc} in the cells is quenched, as shown in the NIR fluorescence images (Figure 4a, center), thus indicating that the SWNT^{Luc} sensor is able to detect ATP in the living cells. Although SWNT^{Luc} is a turn-off sensor based on fluorescence quenching, the photostability and small diffusion constant of SWNT enable a turn-on analysis for ATP imaging that is not possible with existing organic fluorescence probes. We normalized each pixel by its corresponding intensity at the start of the experiment. As shown in Figure 4a (bottom), the imaging for ATP detection based on the pixel-by-pixel ratio in the cells proves that the SWNT^{Luc} sensor can provide the spatial information of ATP. When the fluorescence response at three separate regions in a single cell (four pixels per region) are compared, the degree of quenching is different for each region (Figure S5 in the Supporting Information). The region $(x,y=119,65)$ near the membrane is quenched by 30%, while another part $(x,y=160,110)$ of the same cell is quenched by 10% (Figure S5b in the Supporting Information), which suggests that the SWNT^{Luc} sensor is able to resolve ATP compartmentalization in living cells. In addition, this fluorescence quenching for ATP detection in the cells is more easily observed in the quantitative and real-time tracking of NIR fluorescence (Figure 4b). These results suggest that the SWNT^{Luc} sensor is also capable of temporal detection of ATP in the living cells. Hence, we conclude that the SWNT^{Luc} sensor is able to

provide spatial and temporal information of ATP in living cells.

In summary, the principal contribution of this work is the demonstration of a new optical sensing mechanism: enzymatic generation of a fluorescence quencher on the SWNT using a precursor that serves as the analyte. In this specific case, the NIR fluorescence sensor was readily prepared by conjugating Luc on SWNT for selective ATP detection in living cells. The $SWNT^{Luc}$ sensor shows very intense and distinct NIR fluorescence that is selectively quenched by the product of Luc-mediated bioluminescent reaction during ATP detection, but not for AMP, ADP, CTP, and GTP. In addition, the $SWNT^{Luc}$ is successfully applied to the detection of cellular ATP in living cells, which can provide spatial and temporal information. $SWNT^{Luc}$ is the first SWNT-based optical sensor for the detection of ATP in living cells.

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