

# Enzyme-Coated Carbon Nanotubes as Single-Molecule Biosensors

Koen Besteman, Jeong-O Lee, Frank G. M. Wiertz, Hendrik A. Heering, and Cees Dekker\*

*Department of NanoSciences and DIMES, Delft University of Technology,  
Lorentzweg 1, 2628 CJ Delft, The Netherlands*

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## ABSTRACT

We demonstrate the use of individual semiconducting single-wall carbon nanotubes as versatile biosensors. Controlled attachment of the redox enzyme glucose oxidase (GOx) to the nanotube sidewall is achieved through a linking molecule and is found to induce a clear change of the conductance. The enzyme-coated tube is found to act as a pH sensor with large and reversible changes in conductance upon changes in pH. Upon addition of glucose, the substrate of GOx, a steplike response can be monitored in real time, indicating that our sensor is also capable of measuring enzymatic activity at the level of a single nanotube. This first demonstration of nanotube-based biosensors provides a new tool for enzymatic studies and opens the way to biomolecular diagnostics.

The unique electrical properties of single-wall carbon nanotubes (SWNTs) have generated a huge amount of research on nanoelectronic devices and nanosensors.<sup>1,2</sup> In 1998, Tans et al. demonstrated the possibility of using an individual semiconducting SWNT as a field-effect transistor.<sup>3</sup> Based on this transistor layout, several research groups started to construct SWNT nanosensors, where the solid-state gate is replaced by nearby molecules that modulate the tube conductance.<sup>4,5</sup> Because semiconducting SWNTs have a very high mobility and all their atoms are located at the surface, they are the ideal material for ultrasmall sensors.

Kong et al. were the first to build a SWNT chemical sensor for the detection of NO<sub>2</sub> and NH<sub>3</sub> gas.<sup>4</sup> Semiconducting SWNTs [or semiconducting silicon nanowires,<sup>6</sup> which are similar but have a diameter that is 1 order of magnitude larger than SWNTs] have been recognized as a very promising system for biosensors.<sup>7–12</sup> In a first step, Chen et al.<sup>9</sup> immobilized proteins on the sidewall of carbon nanotubes through a linking molecule, but they did not report the electrical characteristics. Proteins carry pH-dependent charged groups that can electrostatically gate a semiconducting SWNT, creating the possibility to construct a nanosize protein and/or pH sensor. Even more interesting, redox enzymes go through a catalytic reaction cycle where groups in the enzyme temporarily change their charge state and conformational changes occur in the enzyme. This enzymatic activity can potentially be monitored with a nanotube sensor.

Here, we demonstrate that SWNT transistors can indeed be developed into such biological sensors. As a case in point,

we study the redox enzyme glucose oxidase (GOx) that catalyses the oxidation of  $\beta$ -D-glucose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) to D-glucono-1,5-lactone (C<sub>6</sub>H<sub>10</sub>O<sub>6</sub>). Controlled immobilization of GOx onto the sidewall of a semiconducting SWNT is found to decrease the conductance of the tube. GOx-coated semiconducting SWNTs are found to act as reversible pH sensors and show an increase in conductance upon adding glucose, suggesting the use as a sensor for enzymatic activity.

Low-resistance high-mobility SWNT field-effect transistors are constructed from semiconducting SWNTs grown with chemical vapor deposition<sup>13</sup> (CVD) on degenerately doped silicon wafers with a 200 nm thermally grown oxide. Electrodes consisting of a 30 nm gold layer and a 5 nm titanium sticking layer are deposited on top of the semiconducting SWNTs with electron-beam lithography. The lowest device resistance that we observed was 25 k $\Omega$  and the highest mobility was about 3600 cm<sup>2</sup>/Vs. For testing the immobilization scheme, we also used laser-ablated tubes grown in the Smalley group at Rice University.<sup>14</sup>

All electrical measurements were done in aqueous solutions at room temperature. The liquid is used as a very efficient gate for the immersed semiconducting SWNT.<sup>15,16</sup> A liquid-gate voltage  $U_{lg}$  was applied between an Ag/AgCl 3M NaCl standard reference electrode immersed in the liquid and the semiconducting SWNT. The use of a standard reference electrode is important because its potential, and therefore the gate effect on the SWNT, does not depend on the liquid composition, enabling changes in the liquid composition without changing the liquid-gate voltage. With the use of a standard reference electrode, the applied gate voltage falls entirely over the tube–liquid interface. With a

\* Corresponding author. E-mail: dekker@mb.tn.tudelft.nl

liquid gate, the capacitance per unit length  $C_t$  of the SWNT is given by

$$\frac{1}{C_t} = \frac{1}{C_q} + \frac{1}{C_d} \quad (1)$$

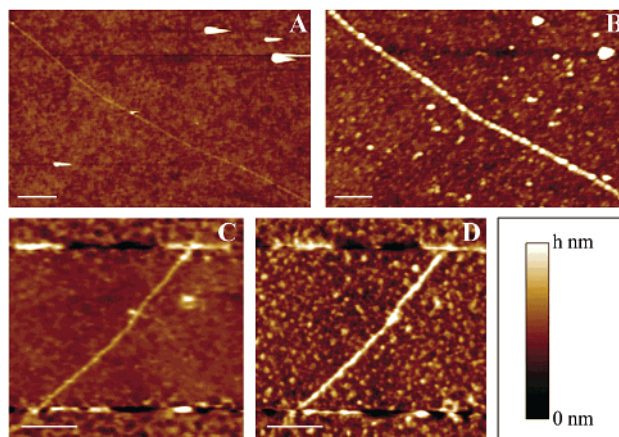
where  $C_q$  is its quantum capacitance and  $C_d$  is the gate capacitance, which is the electrochemical double-layer capacitance of the tube–liquid interface that can depend on the liquid composition near the electrochemical potential of zero charge.<sup>15,16</sup> Assuming diffusive transport, the conductance  $G$  of a semiconducting SWNT can be approximated by

$$G = \frac{C_t |U_{lg} - U_{go}| \mu}{L} \quad (2)$$

in the regime where the conductance changes as a function of the liquid-gate voltage. Here,  $U_{go}$  is the threshold voltage,  $\mu$  the mobility, and  $L$  the length of the tube. For a bare SWNT,  $C_q \ll C_d$ . Therefore,  $C_q$  is the dominant term in  $C_t$ , and the conductance of the tube does not depend significantly on  $C_d$  and thus on the liquid composition.<sup>15,16</sup>

We have succeeded to immobilize the enzyme glucose oxidase ( $\beta$ -D-glucose: oxygen 1-oxidoreductase, EC1.1.3.4, from *Aspergillus niger*) on carbon nanotubes by use of a linking molecule, which on one side binds to the SWNT through van der Waals coupling with a pyrene group and on the other side covalently binds the enzyme through an amide bond, following the scheme introduced by Chen et al.<sup>9</sup> SWNTs deposited on a silicon wafer were left in 2.3 mg/mL 1-pyrenebutanoic acid succinimidyl ester (Molecular Probes, Inc., USA) in dimethylformamide (DMF) for 2 h while stirring, washed with clean DMF, left in 10 mg/mL GOx (Sigma-Aldrich, Fluka)<sup>17</sup> in filtered de-ionized water (milli-Q water) for 18 h, and washed in milli-Q water for 6 h.

Figure 1 shows typical atomic force microscopy (AFM) images of the same SWNTs before (A, C) and after (B, D) the immobilization of GOx on the tube. Before the GOx immobilization, the height of the SWNT in (A) is slightly below one nanometer and constant over its entire length. After the immobilization of GOx (Figure 1B), closely spaced features with an apparent height of about 2–4 nanometers, as measured from the top of the SWNT, are observed at the tube. Similar heights are measured for the features on the tube in Figure 1D. The height of these features is in agreement with the height of dry GOx. From the AFM images we conclude that GOx is immobilized specifically on the SWNTs. We observe that on average roughly half of the SWNT surface is covered with GOx after immobilization, which corresponds to a density of about 1 GOx molecule each 12 nanometer. As a control experiment, we tried to immobilize GOx on laser-ablated and CVD-grown SWNTs without the linking molecule pyrenebutanoic acid succinimidyl ester. No specific binding of GOx to the SWNTs was observed in these experiments, showing that the linking molecule is essential for the immobilization of GOx onto

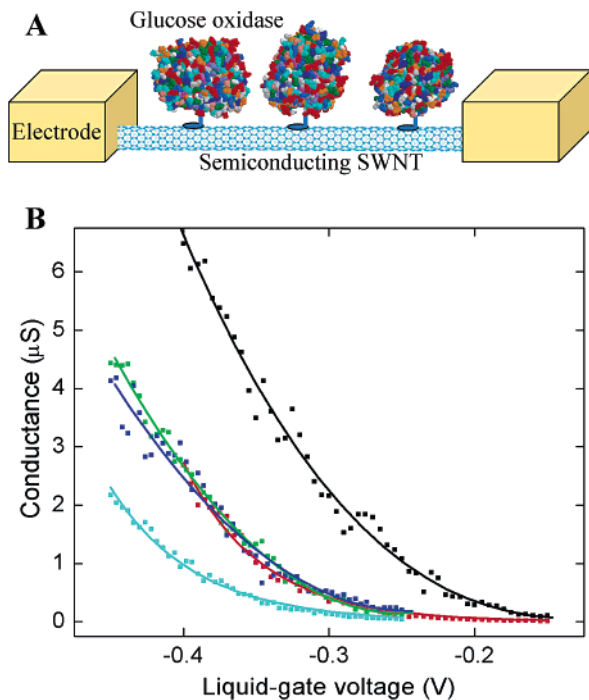


**Figure 1.** AFM height images of the same SWNTs before (A, C) and after (B, D) immobilization of the redox enzyme GOx. Specific attachment of GOx to the SWNT is observed in (B) and (D). Scale bars are 200 nm. The height scale bar applies to all images, with  $h = 5$  nm for images (A) and (B) and  $h = 8$  nm for (C) and (D). The SWNTs were grown by laser ablation (A, B) or CVD (C, D). The SWNT in (C, D) is a semiconducting tube between two electrodes (at top and bottom) on a silicon/siliconoxide substrate. Imaging was done by tapping-mode in air.

the tubes. As is apparent from Figure 1, GOx immobilization on SWNTs works well for both laser-ablated and CVD-grown tubes. We now turn to the electrical properties of individual semiconducting SWNTs coated with GOx (cf. Figure 2A).

GOx immobilization appears to have a strong effect on the conductance of a semiconducting SWNT. The two-probe conductance of the same semiconducting SWNT was measured as a function of the liquid-gate voltage in milli-Q water for a number of conditions (see Figure 2B): before modifying the tube, after the tube was in DMF for 2 h, after the tube was in DMF for 4 h, after the tube was in the solution of 2.3 mg/mL 1-pyrenebutanoic acid succinimidyl ester in DMF for 2 h, and after finishing the GOx immobilization. Data were collected for six different semiconducting SWNTs, and all gave similar results. Typical data are shown in Figure 2B. The data first show that DMF decreases the conductance of a semiconducting SWNT, which may be the result of the electron-donating power of DMF upon adsorption to the tube. Surprisingly, attachment of the linking molecules to the SWNT by  $\pi$ -stacking<sup>9</sup> appears to have no effect on the conductance. Most importantly, the measurements show that attachment of only about 50 molecules of GOx (tube length is on average 600 nm) significantly decreases the conductance of a semiconducting SWNT (difference between the dark and light blue lines in Figure 2B). This demonstrates the sensor potential for measuring the presence of GOx proteins.

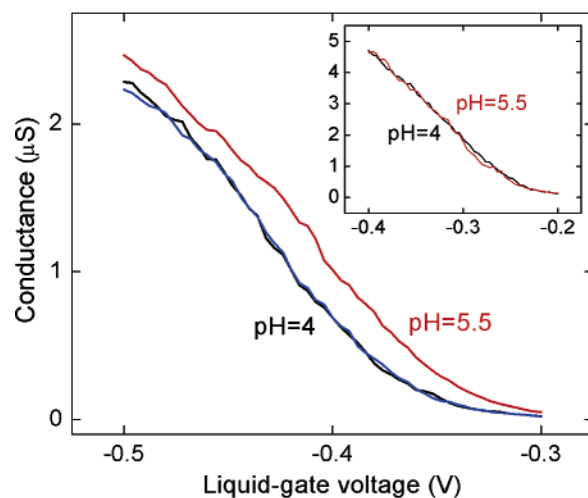
The decrease in conductance of the SWNTs upon GOx attachment cannot simply be an effect of electrostatic gating by GOx. Glucose oxidase has an isoelectric point of about 4.2 and therefore carries a net negative charge in milli-Q water ( $\text{pH} \approx 5.5$ ), while we observe the effect of an apparent positive charge. Alternatively, we propose that the conductance decrease is the result of a change in the capacitance of the tube that occurs when GOx is immobilized on it: GOx



**Figure 2.** (A) Schematic picture of two electrodes connecting a semiconducting SWNT with GOx enzymes immobilized on its surface. (B) Conductance of a semiconducting SWNT as a function of the liquid-gate voltage in milli-Q water. Data are for the bare SWNT (black), after 2 h in DMF (red), after 4 h in DMF (green), after 2 h in DMF with the linking molecule (dark blue), and after GOx immobilization (light blue). Lines are guides to the eye. We observe that attachment of GOx significantly decreases  $G(U_{lg})$ . The source-drain voltage was kept constant at 9.1 mV for all measurements.

on the surface of a SWNT inhibits ions in the liquid to come close to the tube, thereby decreasing  $C_d$ .<sup>18</sup> As a result,  $C_t$  decreases (eq 1) and the conductance of the semiconducting SWNT decreases (eq 2). Numerical estimates show that the decrease in conductance predicted by this model is in agreement with the measurements.<sup>19</sup>

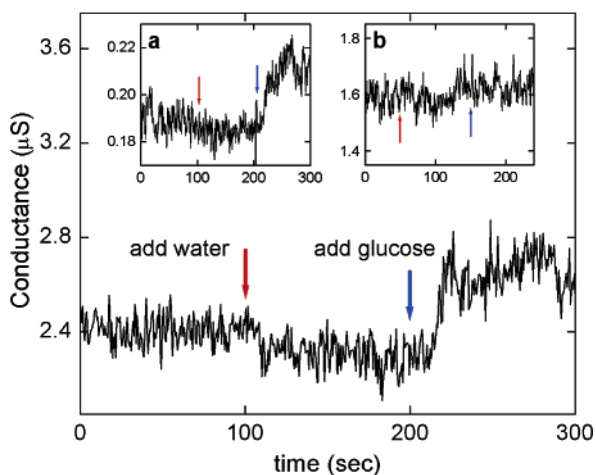
GOx-coated semiconducting SWNTs show a strong pH dependence, see Figure 3. The inset of Figure 3 shows that, before immobilizing GOx on the semiconducting SWNT, its conductance does not depend on pH in the range of 4 to 5.5. After the immobilization of GOx, however, the conductance of the same tube is much higher at  $\text{pH} \approx 5.5$  (0.1 mM KCl in milli-Q water) than at  $\text{pH} \approx 4$  (0.1 mM HCl in milli-Q water). The conductance changes are found to be reversible, as is evident from a comparison of the black and blue curves in Figure 3. Upon changing the pH from 4 to 5.5, the effective gate voltage changes by 20 mV, which leads to a change in the conductance of the tube by about  $0.3 \mu\text{S}$ . The data suggest that it is possible to use these nanotube sensors to measure pH changes down to 0.1. The semiconducting SWNT coated with GOx thus appears to serve as an excellent nanosize pH sensor. We attribute the pH sensitivity to charged groups on the GOx that become more negative with increasing pH. This decreases the electrostatic gate voltage, thereby increasing the conductance of the semiconducting SWNT.<sup>20</sup> We measured the pH dependence of four different semiconducting SWNTs after GOx im-



**Figure 3.** pH sensitivity of the nanotube sensor. Conductance of a GOx-coated semiconducting SWNT as a function of the liquid-gate voltage measured at  $\text{pH} = 4.0 \pm 0.2$  (black line), then at  $\text{pH} = 5.5 \pm 0.2$  (red line), and finally again at  $\text{pH} = 4.0 \pm 0.2$  (blue line). The inset shows a measurement on the same tube before GOx is immobilized, at  $\text{pH} = 4.0 \pm 0.2$  (black line) and at  $\text{pH} = 5.5 \pm 0.2$  (red line). The pH was set by using 0.1 mM HCl in milli-Q water (pH 4) and 0.1 mM KCl in milli-Q water (pH 5.5). For all measurements the source-drain voltage was kept at 9.1 mV. It is seen that the conductance increases with increasing pH and that pH changes induce a reversible change in the conductance.

mobilization, and all tubes gave similar results. These measurements show that it is possible to clearly measure charge changes of about 50 protein molecules attached to a  $\sim 600$  nm long semiconducting SWNT.

GOx-coated semiconducting SWNTs also appear to be sensitive to glucose, the substrate of GOx. Figure 4 shows real-time measurements where the conductance of a GOx-coated semiconducting SWNT in milli-Q water has been recorded as a function of time. When milli-Q water was added to the liquid (red arrow in Figure 4), no significant change in conductance was observed. When 0.1 M glucose in milli-Q water was added to the liquid (blue arrow), however, the conductance of the tube increased by about 10%. As shown in inset (a) of Figure 4, a similar 10% conductance change was observed for another device, which had a factor 10 lower conductance. With yet another semiconducting SWNT we measured the influence of glucose on the conductance before and after immobilizing GOx on the tube. Glucose did not change the conductance of the bare SWNT but did increase the conductance after GOx was immobilized. Inset (b) of Figure 4 shows such measurement on a bare semiconducting SWNT.<sup>21</sup> To ensure that the conductance increase is not due to pH changes upon liquid addition, measurements were also repeated on yet another device in a buffer solution at pH 6 (10 mM 2-(N-morpholino)ethanesulfonic acid buffer). Again, the conductance increase upon glucose addition was only observed after GOx was immobilized.<sup>22</sup> Testing the quantitative reproducibility of the effect was hampered by significant low-frequency noise in  $G(t)$ ; a conclusive step-like response was observed in four low-noise devices out of a total of twelve devices.



**Figure 4.** Real time electronic response of the SWNT sensor to glucose, the substrate of GOx. The conductance of a semiconducting SWNT with immobilized GOx is measured as a function of time in 5  $\mu\text{L}$  milli-Q water. The source-drain and liquid-gate voltage were kept constant at 9.1 mV and  $-500$  mV, respectively. After 100 sec (red arrow) 2  $\mu\text{L}$  milli-Q water was added to the liquid, and after 200 s (blue arrow) 2  $\mu\text{L}$  0.1 mM glucose in milli-Q water was added to the liquid. The conductance of the GOx-coated SWNT is observed to increase upon addition of glucose to the liquid. Inset (a) shows the same measurement on a second device where the conductance was a factor of 10 lower. Inset (b) displays the same measurement on a semiconducting SWNT without GOx. As expected, no conductance increase is observed here.

These data indicate that the GOx activity is responsible for the measured increase in conductance upon glucose addition, thus rendering our nanodevice an enzymatic-activity sensor. In the catalytic reaction where glucose is converted to gluconolactone, GOx undergoes conformational changes, groups on the GOx change their charge state, and an oxygen molecule is used to form a hydrogen peroxide molecule. Further experiments are needed to unambiguously identify which of these processes is responsible for the measured conductance change.

Numerical estimates show that higher-mobility devices with a very short tube length ( $\sim 20$  nm) have the potential to measure the enzymatic activity of even a single redox enzyme, thereby creating a very powerful tool for enzymatic studies.<sup>19</sup> Due to their ultrasensitivity and nanoscale size, these new nanotube biosensors provide great potential for the real time electronic and therefore label-free detection of a wide range biomolecules in applications such as array-based screening.

In conclusion, we have demonstrated the first biosensor based on an individual SWNT. Immobilization of the enzyme GOx decreases the conductance, which can be attributed to a change in the total capacitance of the tube rather than a simple electrostatic interpretation. We have shown that GOx-coated semiconducting SWNTs act as sensitive pH sensors. Moreover, changes of conductance of GOx-coated semiconducting SWNTs upon addition of glucose indicate that an enzyme-activity sensor can be constructed at the single molecule level of an individual SWNT.

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- (17) With SDS-page gel electrophoresis the purity of GOx was verified. Furthermore, GOx was tested for activity in milli-Q water with glucose by monitoring oxygen depletion with a Clark electrode. We did not measure any significant loss of activity after storing the enzyme for 24 h in milli-Q water at ambient temperature.
- (18) The capacitance  $C_d$  now depends on the capacitance across the GOx as well as on the double-layer capacitance of the tube-liquid interface. Changes in the liquid composition that only change the tube-liquid double layer capacitance still have as little influence on the overall capacitance as for a bare tube, because the tube-liquid double-layer capacitance is much larger than both quantum capacitance and capacitance across the GOx.
- (19) Besteman, K. et al., unpublished.
- (20) Experimentally we also observe that the conductance change does not result from a change in the tube-liquid double-layer capacitance due to a change in liquid composition.<sup>18</sup> The conductance of semiconducting SWNTs measured in pure milli-Q water was found to be comparable to that measured in the 0.1 mM KCl in milli-Q water solution. Both solutions have a comparable pH of about 5.5. When a change in ion concentrations would have a significant influence on  $C_d$  through a change in the tube-liquid double-layer capacitance, one would expect the conductance of the SWNT to be much higher in the 0.1 mM KCl and HCl solutions than in pure milli-Q water because of the higher ionic concentration.
- (21) Occasionally, in one or two out of eleven devices, a bare semiconducting SWNT did show a similar glucose dependence as well. These one or two devices had a significantly higher noise than the devices shown in Figure 4.
- (22) One does not expect the observed conductance change to result from a change in tube-liquid double-layer capacitance upon addition of glucose.<sup>18</sup> Moreover, it is unlikely that the uncharged glucose changes the tube-liquid double-layer capacitance at all. The influence of glucose was separately measured on 3 mm<sup>2</sup> basal plane graphite electrodes. Glucose turned out not to increase the capacitance of this carbon electrode. This indicates that a change in the tube-liquid double-layer capacitance as a result of a change in the liquid composition is not the origin of the conductance increase.

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