

## Peroxidase activity of enzymes bound to the ends of single-wall carbon nanotube forest electrodes

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### Abstract

This communication reports the first example, to our knowledge, of enzymes covalently attached onto the ends of vertically oriented single-wall carbon nanotube (SWNT) forest arrays used as electrodes. Quasi-reversible Fe<sup>III</sup>/Fe<sup>II</sup> voltammetry was observed for the iron heme enzymes myoglobin and horseradish peroxidase coupled to carboxylated ends of the nanotube forests by amide linkages. Results suggest that the “trees” in the nanotube forest behaved electrically similar to a metal, conducting electrons from the external circuit to the redox sites of the enzymes. Electrochemically manifested peroxidase activity of myoglobin and horseradish peroxidase attached to the SWNT forests was demonstrated, with detection limits for hydrogen peroxide in buffer solutions of ~100 nM. These prototype SWNT-forest biosensors are easy to prepare, and enzyme layers were stable for weeks. © 2003 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

Arrays of highly conducting single-wall carbon nanotubes (SWNTs) could be uniquely suited to bioanalytical applications by coupling sensing biomolecules to the carboxyl-terminated ends [1–3] of the nanotubes. We recently reported a unique methodology for assembling *SWNT forests*, i.e., dense orthogonally oriented arrays of shortened SWNTs, onto various solid substrates [4]. The present communication demonstrates that the untethered ends of these stable SWNT forests can be linked to electrochemically active heme proteins through traditional bioconjugate chemistry to give electrochemical signatures of enzyme activity. We show herein that myoglobin and horseradish peroxidase covalently linked

to the SWNT forests exhibit quasi-reversible Fe<sup>III</sup>/Fe<sup>II</sup> voltammetry and sensitive responses to H<sub>2</sub>O<sub>2</sub>.

The unidimensional nature, high conductivity, and structural robustness of carbon nanotubes [5] promise important future applications for nanosensors. Unique electrochemistry of soluble molecules and adsorbed proteins on flat mat-like layers of single or multi-walled carbon nanotubes has been demonstrated [6–13]. It has been speculated that the similarity between size scales of enzymes and chemically shortened [14], nanoscale diameter SWNTs [15–17] might promote the likelihood of SWNTs being able to intimately interact and be within electron tunneling distance of enzyme redox sites [12]. To our knowledge, only one report has thus far appeared describing upright SWNT forests as electrodes, and dealt only with small soluble electroactive ions [11].

Peroxide-based detection has wide application in immunoassays and DNA hybridization assays where peroxidase enzymes are used as tags [18–22]. We set out in this work to attach peroxidases to the ends of SWNT

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forests, and to demonstrate a response to hydrogen peroxide.

## 2. Experimental

Single-wall carbon nanotubes (HiPco, from Tubes@rice) were carboxyl functionalized and shortened by sonication in a mixture of  $\text{HNO}_3$  and  $\text{H}_2\text{SO}_4$  for 4 h at 70 °C [1,4]. Monolayers of vertically aligned, shortened SWNTs were assembled on ordinary pyrolytic graphite (PG) electrodes from DMF dispersions onto an underlying composite bed of Nafion ionomer and  $\text{Fe}^{3+}$ -precipitated hydroxides, as described previously [4]. SWNTs on the Nafion- $\text{Fe}(\text{OH})_3$  layer on PG were dried in vacuo for 18 h. Cyclic voltammetry and amperometry were done as previously described [23]. Atomic force microscopy (AFM) was done with a Nanoscope IV scanning probe microscope.

Iron heme proteins horse heart myoglobin (Mb, Sigma, >90%) and horseradish peroxidase (HRP, Sigma, 250–330 unit  $\text{mg}^{-1}$ ) were attached onto the ends of the SWNTs in the forest by using the water-soluble carbodiimide, (1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, EDC), to promote amide linkages between the carboxyl-terminated nanotubes and the lysine residue of the proteins. This was done by placing a 20  $\mu\text{l}$  droplet of freshly prepared 24 mM EDC in water onto the SWNT forest, immediately followed by adding 30  $\mu\text{l}$  of 3  $\text{mg ml}^{-1}$  Mb or HRP. EDC and protein solutions were mixed carefully using a syringe needle, reacted for 4 h, then washed extensively with water.

## 3. Results and discussion

Cyclic voltammograms (CVs) of SWNT forests (Fig. 1(a)) showed a oxidation–reduction peak pair centered at 0.2 V vs. SCE that has been assigned to carboxylate in

non-oriented SWNT films [24]. The additional peak at  $-0.8$  V is in the potential range for reduction of adsorbed oxygen. Addition of 0.2 mM  $\text{H}_2\text{O}_2$  to the buffer resulted in little change in current at the 0.2 V peak, but a small increase in the peak at  $-0.8$  V, possibly due to the direct reduction of peroxide.

Fig. 2 shows AFM images of a SWNT forest on smooth silicon compared to a SWNT forest after treatment with EDC and Mb. The width of the main features on the SWNT forest surface is about 20 nm, significantly larger than the diameter of a single 1 nm diameter SWNT. This is due to the aggregation of SWNT in the assembly process and the tip-induced broadening artifact of the AFM [25]. The height of the SWNTs is tens of nanometers, in agreement with that reported previously [11]. After Mb was coupled onto the SWNTs using EDC, the spiky nanotube forest disappeared, and a globular coating reminiscent of polyion aggregates [26] can be seen. Additionally, there was a  $\sim 70\%$  increase in the domain height and an 80% increase in domain width after enzyme functionalization, consistent with a thin layer of Mb built on top of the nanotube forest. Similar results were found for HRP layers.

Cyclic voltammograms (Fig. 1(b)) of the protein–SWNT films in buffers showed oxidation–reduction peak pairs near  $-0.21$  V vs. SCE for Mb, and  $-0.25$  V for HRP. These potentials are characteristic of the  $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$  redox couples of Mb and HRP in thin films [23]. Furthermore, CV peaks of the SWNTs attributed to carboxylate disappeared from the CVs, suggesting that carboxyl groups on the SWNTs reacted during protein coupling. Separations between protein reduction and oxidation peaks were nearly zero. The peak current of the film increased linearly with increasing scan rate. All these results are typical of quasi-reversible voltammetry of a thin surface-bound layer of redox protein [27].

Electroactive protein surface concentrations were estimated by integrating the CV reduction peaks at low

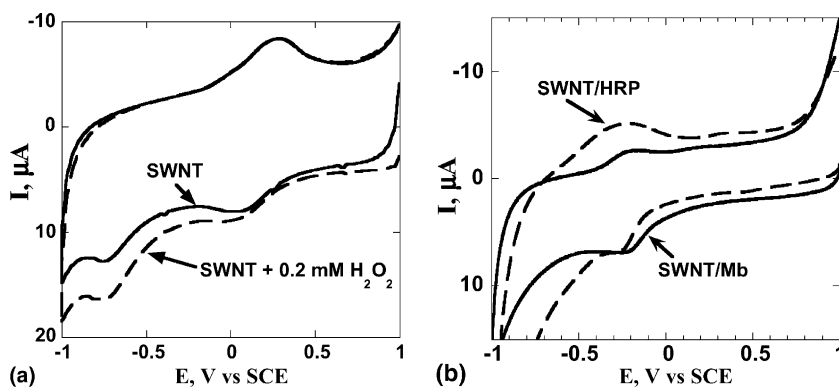


Fig. 1. Cyclic voltammograms at 300  $\text{mV s}^{-1}$  of: (a) underivatized SWNT forest on PG in pH 5.5 buffer with and without 0.2 mM  $\text{H}_2\text{O}_2$  and (b) SWNT/Mb (solid line) in pH 5.5 buffer and SWNT/HRP (dashed line) in pH 6.0 buffer (reduction is downward, oxidation is upward).

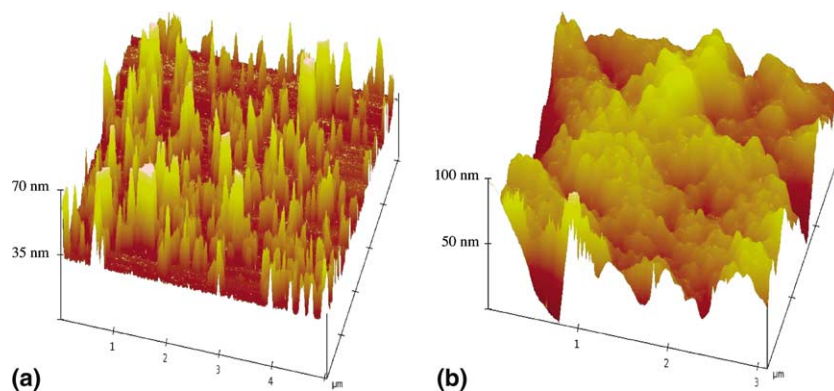


Fig. 2. Tapping mode atomic force microscopy (AFM) images of: (a) SWNT forest on smooth silicon and (b) myoglobin-functionalized SWNT forest on silicon.

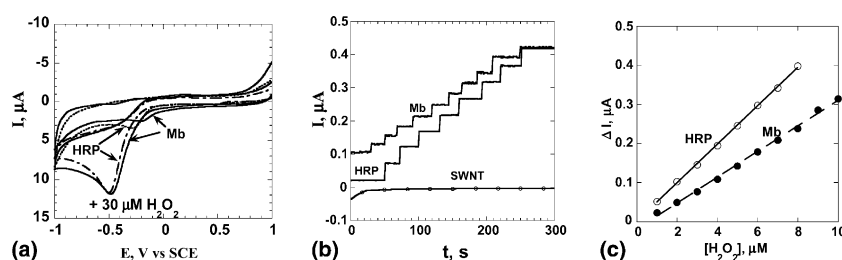


Fig. 3. Catalytic electrochemical signals for hydrogen peroxide on SWNT/enzyme electrodes: (a) CV at  $0.1 \text{ V s}^{-1}$  with and without  $30 \mu\text{M H}_2\text{O}_2$  and (b) rotating disk amperometry at  $0 \text{ V vs. SCE}$  and  $1000 \text{ rpm}$  of SWNT/Mb and SWNT/HRP electrodes showing stepwise increases in reduction current after  $\text{H}_2\text{O}_2$  injections into the buffer resulting in  $1 \mu\text{M}$  increases in concentration. The amperometric results also show that the oriented SWNT electrodes are stable under hydrodynamic conditions. (c) Calibration curves from rotating disk amperometry at  $0 \text{ V}$  for SWNT/Mb and SWNT/HRP electrodes.

scan rates ( $25\text{--}75 \text{ mV s}^{-1}$ ). These surface concentrations were  $0.17 \text{ nmol cm}^{-2}$  for HRP and  $0.09 \text{ nmol cm}^{-2}$  for Mb, based on PG area ( $0.2 \text{ cm}^2$ ).

Cyclic voltammogram peaks of the enzymes attached onto SWNT forests were stable, and did not decay during repetitive multiple scans. Electrodes could be stored for several weeks in buffer at  $4 \text{ }^\circ\text{C}$  with only  $\sim 20\%$  loss of peak current. This behavior is consistent with covalent attachment of the proteins to the carboxylate-bearing ends of the SWNTs. On the other hand, when the enzymes were placed onto SWNT forests without EDC coupling, CVs persisted for only a few repetitive scans. Additionally, when Nafion- $\text{Fe}(\text{OH})_3$  underlayers on PG were treated with EDC and enzyme, CV peaks observed for Mb and HRP decreased to background levels within 30 min or less, suggesting only transient binding of the positively charged proteins to the Nafion- $\text{Fe}(\text{OH})_3$  underlayer.

When Mb or HRP are coupled to an electrode, a complex catalytic cycle for the reduction of  $\text{H}_2\text{O}_2$  is set up that can be detected via catalytic reduction current [23]. Fig. 3(a) shows a large catalytic reduction peak when  $\text{H}_2\text{O}_2$  was added to buffers containing the SWNT/enzyme electrodes. Addition of  $30 \mu\text{M H}_2\text{O}_2$  increased the peak current 5-fold, and the oxidation peak disap-

peared. There was a linear increase in catalytic reduction peak with increasing  $\text{H}_2\text{O}_2$  concentration from 5 to  $40 \mu\text{M}$ .

Hydrodynamic amperometry provided better sensitivity of the SWNT/enzyme films for  $\text{H}_2\text{O}_2$  (Fig. 3(b)). The sensitivity to the change in the concentration of  $\text{H}_2\text{O}_2$  as the slope of the calibration curve below  $10 \mu\text{M}$  was  $0.049 \mu\text{A}/\mu\text{M}$  for SWNT/HRP, 50% better than the sensitivity of SWNT/Mb at  $0.033 \mu\text{A}/\mu\text{M}$  (Fig. 3(c)). This is consistent with the better peroxidase activity of HRP compared to Mb [23]. The estimated detection limits towards  $\text{H}_2\text{O}_2$  as three times the noise levels were  $\sim 70 \text{ nM}$  for SWNT/Mb and  $\sim 50 \text{ nM}$  for SWNT/HRP.

In summary, we demonstrated herein the first examples of using highly oriented SWNT forests in a biosensor configuration with enzyme linked to the ends of the nanotubes. The nanotube forest behaves electrically similar to a metal, conducting electrons from the external circuit to the enzymes. The preparation of these prototype SWNT-forest biosensors is easy, and enzyme layers are stable for weeks. The oriented SWNT forests also have the potential of being fabricated as ultramicroelectrodes on the nanometer scale, offering the future possibility of multielement nanobiosensor arrays.

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